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SPECIAL REPORT

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Reduction by Hoe 140, the B_2 kinin receptor antagonist, of antigen-induced nasal blockage

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In subjects with allergic rhinitis to house-dust mite (HDM), antigen challenge produced a significant increase in nasal blockage but had no effect on nasal vascular permeability. The B_2 kinin receptor antagonist, [D-Arg^0,Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin (Hoe 140), 200 μg administered by intranasal aerosol 2 min prior to challenge with HDM, 500 u significantly reduced nasal blockage induced by the antigen challenge. The data are compatible with a role for B_2 kinin receptors in the nasal response to challenge with antigen which is responsible for nasal blockage.

Keywords: Allergic rhinitis; bradykinin B_2 antagonist; acoustic rhinometry; allergen challenge; Hoe 140

Introduction Kinins have been implicated in the pathophysiology of allergic rhinitis. Bradykinin and kallidin appear in the nasal secretions of patients with allergic rhinitis (Svensson *et al.*, 1990), and intranasally applied bradykinin causes symptoms of rhinitis, including an increase in nasal blockage and an increase in nasal vascular permeability (Proud *et al.*, 1988). [D-Arg^0,Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin or Hoe 140 is a highly potent and long-acting bradykinin antagonist, effective in B_2 kinin receptor-containing preparations (Hock *et al.*, 1991). The increase in nasal airways resistance and vascular permeability in the human nasal airway induced by administration of exogenous bradykinin is inhibited by Hoe 140 (Austin & Foreman, 1994a).

The aim of this study was to investigate the effect of Hoe 140 on the nasal response to antigen challenge in subjects with allergy to house-dust mite (HDM), to determine whether kinin-mediated effects contribute to the pathology of allergic rhinitis.

Methods Fifteen subjects (19–70 year old) took part in the study. They were taking no medication at the time of, or in the 4 weeks prior to, the study. All had positive skin prick and nasal provocation tests to antigen from the house-dust mite, *Dermatophagoides pteronyssinus* (HDM). All subjects gave informed consent and the study was approved by the local Ethics Committee.

Minimum nasal cross-sectional area (A min) was determined by acoustic rhinometry as previously described (Austin & Foreman, 1994b).

Symptoms of nasal blockage, itching, sneezing and running were recorded on a 10 cm visual analogue scale (Aitken, 1969).

Vascular permeability was determined by measuring albumin in nasal lavage (Naclerio *et al.*, 1983), using a radial immunodiffusion assay (Behring Diagnostics, Milton Keynes, U.K.).

Compounds were delivered in a volume of 100 μl, by means of a nasal pump spray (Perfect-Valois, U.K., Ltd.). Both house-dust mite antigen (Allerayde, Nottingham, U.K.) and Hoe 140 (Bachem, Saffron Walden, Essex, U.K.) were dissolved in sterile saline (NaCl, 154 mM) at concentrations of 5000 u ml^{-1} and 2 mg ml^{-1} respectively.

Ten subjects were treated by intranasal aerosol in a single-blind, cross-over manner with: (a) saline (vehicle) or (b) Hoe 140, 200 μg. The dose of Hoe 140 was chosen on the basis of *in vitro* studies (Hock *et al.*, 1991). Two minutes after the treatment, aerosol nasal challenge with HDM, 500 u was performed. Subjects were randomly allocated to either of the two treatments. The cross-over treatment was conducted one week later.

At each attendance, subjects were first given an intranasal aerosol of sterile saline, followed by symptoms score collection and A min determination every 5 min over a 15 min period. The mean of these measurements formed the baseline. Subjects were then given either treatment (a) or (b) and A min values and symptom scores were collected 10 min after challenge with HDM.

The mean of the A min values for the left and the right nostrils was calculated for each measurement taken. The decrease in A min was expressed as % change from the pre-challenge, baseline values, in order to normalize the data.

The symptom scores for pre-challenge saline treatment and after each antigen challenge were obtained by measuring the lines on the visual analogue scale for each symptom.

For albumin measurements, 5 different subjects participated in a single-blind, cross-over design similar to that described for acoustic reflection measurements, and the two treatment groups were the same. At each attendance, subjects were given an intranasal aerosol of sterile saline and nasal lavage was performed 10 min later. Subjects were then randomly allocated to treatments (a) or (b) and 10 min after challenge with HDM, 500 u nasal lavage was performed. One week later the cross-over experiment was performed. Albumin levels (g l^{-1}) in the lavage were measured for the pre-challenge saline treatment and after the antigen challenges following treatment (a) or (b).

Data are presented as mean ± standard error of mean. Student's *t* test for paired data was used to evaluate statistically the differences between responses. A probability value of $P<0.05$ is considered significant.

Results Intranasal administration of Hoe 140, 200 μg alone had no effect on A min (data not shown). Figure 1 shows that intranasal challenge with house-dust mite, 500 u, induced a decrease in A min of 27.2 ± 5.8% ($P<0.001$, $n=10$).

The symptom scores for saline control were: nasal blockage, 1.4 ± 0.7; running, 1.0 ± 0.8, itching, 1.1 ± 0.8 and sneezing, 0.4 ± 0.4. Following antigen challenge, the symptom scores increased to: nasal blockage, 4.2 ± 1.2; running, 1.5 ± 0.7; itching, 1.5 ± 1.1 and sneezing, 0.5 ± 0.3. Only the change in symptom score for nasal blockage induced by HDM was significantly different from the control score ($P<0.05$).

Figure 1 shows that administration of Hoe 140, 200 μg prior to challenge with HDM, blocked the antigen-induced decrease in A min. The mean A min in the presence of

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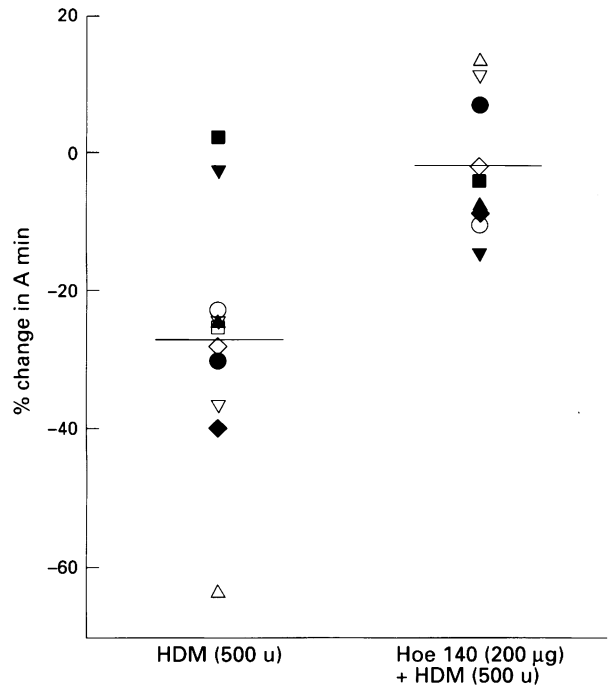


Figure 1 The effect of the bradykinin antagonist, [D-Arg^0,Hyp^3, Thi^5,D-Tic^7,Oic^8]-bradykinin (Hoe 140), 200 µg, given 2 min prior to challenge, on the response to intranasal challenge with house-dust mite, 500 u. The responses were measured 10 min after challenge and are expressed as % change from the baseline (saline) A min (minimal nasal cross sectional area) values which were 0.4 ± 0.1 cm^2 for the group challenged with house-dust mite and 0.4 ± 0.1 cm^2 for the group challenged with house-dust mite in the presence of antagonist, 200 µg. Each symbol represents a different individual. The horizontal bars represent the means from the 10 subjects.

Hoe 140 was significantly larger than the mean A min in the absence of Hoe 140 ($P<0.01$, $n = 10$). In addition, Hoe 140 reduced symptom scores to: nasal blockage, 1.1 ± 0.7; running, 0.2 ± 0.1; itching, 0.5 ± 0.4 and sneezing, 0.4 ± 0.3. The only significant reduction caused by Hoe 140 compared to antigen challenge alone was for nasal blockage ($P<0.05$).

Nasal challenge with HDM had no effect on the concentration of albumin measured in nasal lavage. Albumin levels after HDM were no different from control (saline) levels. The mean albumin concentration of nasal lavage prior to challenge with HDM was 0.038 ± 0.006 g l^{-1} and after HDM challenge, it was 0.029 ± 0.008 g l^{-1} ($n = 5$, $P<0.05$).

Discussion We have shown that Hoe 140 reduces the effect of antigen challenge in causing nasal blockage in subjects with perennial allergic rhinitis to house-dust mite antigen. The magnitude of the effect of Hoe 140 at the dose we used was large and the data imply that the nasal blockage which follows antigen challenge is mediated to a significant extent by kinin, formed in the nasal airway following antigen challenge.

Our data and those of others are consistent with the effect of bradykinin in the human nasal airway being mediated by a B_2 kinin receptor (Rajakulasingham *et al.*, 1991; Austin & Foreman, 1994a). Hoe 140 is a selective antagonist for B_2 receptors and so the fact that it inhibits antigen-induced nasal blockage indicates that a kinin is acting on a B_2 receptor to cause nasal blockage in perennial rhinitis to house-dust mite antigen. Although it is likely that Hoe 140 is acting at a B_2 receptor to inhibit the action of endogenous kinin generated by antigen challenge, we cannot exclude the possibility that Hoe 140 might reduce the formation of kinin, by inhibiting tissue kallikrein (Spragg *et al.*, 1988).

In contrast to our own data, we have recently learned that Hoe 140 does not block the symptoms of seasonal allergic rhinitis caused by exposure to pollen (Akbary & Bender, 1993). Interestingly, different types of rhinitis have been used as models in the two studies: perennial allergic rhinitis to house-dust mite and seasonal allergic rhinitis to pollens. Seasonal allergic rhinitis is characterized by sneezing, rhinorrhoea and nasal blockage, whereas the prominent feature of perennial allergic rhinitis is nasal blockage, with less marked rhinorrhoea and sneezing. The pathophysiology of the two types of allergic rhinitis may differ, with kinin being a major mediator of perennial but not seasonal rhinitis. Another reason for the difference in findings may be that the present study involved antigen provocation and objective measurement of responses in a laboratory, whilst the study of seasonal allergic rhinitis was based solely on symptom scores reported by patients during the pollen season.

Nasal blockage and an increase of nasal vascular permeability are detectable after aerosol administration of bradykinin (Proud *et al.*, 1988; Austin & Foreman, 1994a). However, in the present study, antigen provocation of subjects with perennial rhinitis to house-dust mite caused an increase in nasal blockage, but had no effect on nasal vascular permeability. We have previously shown that challenge of normal subjects with bradykinin causes nasal resistance and vascular permeability to increase, with similar dose-response curves and time-courses for the two effects (Austin & Foreman, 1994a). With antigen provocation, we have not detected increased vascular permeability. The antigen provocation may, in contrast to challenge with exogenous bradykinin, cause more local increases in kinins within the nasal airway, resulting in increased blood flow into nasal sinusoids, which may be more important in causing soft tissue swelling and nasal blockage than is swelling caused by plasma extravasation.

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References

AITKEN, R.C.B. (1969). Measurement of feelings using visual analogue scales. *Proc. R. Soc. Med.*, **62**, 989–993.

AKBARY, M.A. & BENDER, N. (1993). Efficacy and safety of Hoe 140 (a bradykinin antagonist) in patients with mild-to-moderate seasonal allergic rhinitis. In *Proceedings of Kinins 93*, Brazil, Abstract 22.02.

AUSTIN, C.E. & FOREMAN, J.C. (1994a). A study of the action of bradykinin and bradykinin analogues in the human nasal airway. *J. Physiol.*, (in press).

AUSTIN, C.E. & FOREMAN, J.C. (1994b). Acoustic rhinometry compared with posterior rhinomanometry in the measurement of histamine- and bradykinin-induced changes in nasal airway patency. *Br. J. Clin. Pharmacol.*, **37**, 33–37.

HOCK, F.J., WIRTH, K., ALBUS, U., LINZ, W., GERHARDS, H.J., WIEMER, G., HENKE, ST., BREIPOHL, G., KÖNIG, W., KNOLLE, J. & SCHÖLKENS, B.A. (1991). Hoe 140 a new potent and long acting bradykinin-antagonist: *in vitro* studies. *Br. J. Pharmacol.*, **102**, 769–773.

NACLERIO, R.M., MEIER, H.L., KAGEY-SOBOTKA, A., MEYERS, D., NORMAN, P.S. & LICHTENSTEIN, L.M. (1983). Mediator release after nasal airway challenge with allergen. *Am. Rev. Respir. Dis.*, **128,** 597–602.

PROUD, D., REYNOLDS, C.J., LACAPRA, S., KAGEY-SOBOTKA, A., LICHTENSTEIN, L.M. & NACLERIO, R.M. (1988). Nasal provocation with bradykinin induces symptoms of rhinitis and a sore throat. *Am. Rev. Respir. Dis.*, **137,** 613–616.

RAJAKULASINGHAM, K., POLOSA, R., HOLGATE, S.T. & HOWARTH, P.H. (1991). Comparative nasal effects of bradykinin, kallidin and [Des-Arg^9]-bradykinin in atopic rhinitic and normal volunteers. *J. Physiol.*, **437,** 577–587.

SPRAGG, J., VAVREK, R.J. & STEWART, J.M. (1988). The inhibition of glandular kallikrein by peptide analog antagonists of bradykinin. *Peptides*, **9,** 203–206.

SVENSSON, C., ANDERSSON, M., PERSSON, C.G.A., VENGE, P., ALKNER, U. & PIPKORN, U. (1990). Albumin, bradykinin and eosinophil cationic protein on the nasal mucosal surface in patients with hayfever during natural allergen exposure. *J. Allergy Clin. Immunol.*, **85,** 828–833.

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SPECIAL REPORT

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Augmentation by intracellular ATP of the delayed rectifier current independently of the glibenclamide-sensitive K-current in rabbit arterial myocytes

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Elevation of intracellular ATP levels by flash photolysis of caged ATP augmented the delayed rectifier K-current (I_{KDR}) in rabbit pulmonary artery myocytes. The percentage augmentation was unaffected when I_{KDR} was inactivated by 50% (holding potential −40 mV), although the magnitude of the ATP-induced current was substantially reduced. Inactivation of 90% I_{KDR} (holding potential −20 mV) virtually abolished the ATP-dependent augmentation. We conclude that modulation of I_{KDR} by ATP does not require conversion of the glibenclamide-sensitive K-current ($I_{K(ATP)}$).

Keywords: ATP; delayed rectifier; potassium channel; pulmonary artery; rabbit arterial myocytes

Introduction In smooth muscle cells, increases in a glibenclamide-sensitive K-current ($I_{K(ATP)}$) are often associated with concomitant decreases in the delayed rectifier current (I_{KDR}). For this reason it was suggested that $I_{K(ATP)}$ may be a voltage-insensitive form of I_{KDR} (Beech & Bolton, 1989). Recently, Edwards *et al.* (1993) proposed that channel phosphorylation converts $I_{K(ATP)}$ to I_{KDR} with dephosphorylation reversing the process. We present data that contradict this hypothesis and suggest that I_{KDR} is augmented by ATP, independently of any action on $I_{K(ATP)}$.

Methods Rabbit pulmonary artery myocytes were isolated and whole-cell voltage-clamp recordings made as previously described (Clapp & Gurney, 1991). Solutions contained (mM): Bath–Hank's balanced salt solution with $NaHCO_3$ 10, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, pH 7.4; Pipette–KCl 130, $MgCl_2$ 1, EGTA 1, HEPES 20, Na_3GTP 0.5, pH 7.2. 1-(2-Nitrophenyl)ethyl (caged) ATP (10 mM), Na_2ATP (1 mM) and glutathione (5 mM) were included in the pipette solution and dialysed into cells as required. A single light flash was estimated to generate millimolar intracellular ATP from caged ATP. Experiments were performed at room temperature (22–26°C).

Results Figure 1a shows the current-voltage relationship for sustained outward current elicited from a holding potential of −80 mV, in the presence and absence of 1 mM internal ATP. At positive potentials, ATP significantly enhanced the outward current. We previously suggested that this was due to augmentation of I_{KDR} (Evans *et al.*, 1993). I_{KDR} was studied at +40 mV, when the Ca^{2+}-activated K current and $I_{K(ATP)}$ were blocked with extracellular tetraethylammonium chloride (10 mM) and glibenclamide (10 μM), and the transient A-like current was inactivated by using 100 ms test pulses (Clapp & Gurney, 1991). Under these conditions, photolysis of caged ATP augmented I_{KDR} by 89 + 14% ($n = 5$; Figure 1b), in the absence of decreases associated with inhibition of $I_{K(ATP)}$ (Clapp & Gurney, 1992). This augmentation was unaffected in the presence of 5 mM intracellular glutathione (Figure 1c; 66 ± 17%, $n = 4$), but was not produced by a light flash alone or by photolysis of caged ATP in the presence of 20 mM intracellular ATP. Thus ATP appears to mediate the photolysis-induced augmentation of I_{KDR}.

Figure 2 a and b show the voltage- and time-dependence of I_{KDR} inactivation. I_{KDR} was inactivated by >50% at −40 mV, and almost completely at −20 mV (Figure 2a),

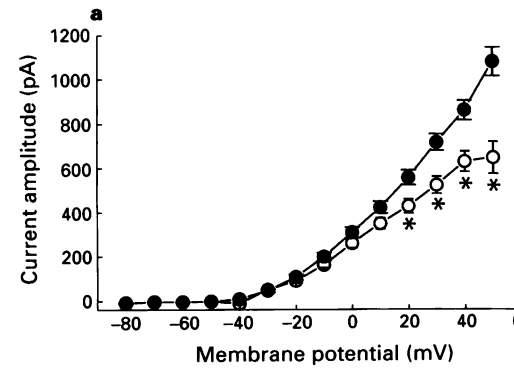


Figure 1 (a) Relationship between sustained outward current evoked from −80 mV and the test potential, with (●) and without (○) 1 mM intracellular ATP. Mean ± s.e.mean, >25 cells. *$P < 0.01$, two-tailed t test. Some experiments omitted pipette GTP. (b) Superimposed outward currents evoked by steps from −80 mV to +40 mV before (1), at photolysis (2; arrow), 5 s (3) and 25 s (4) after photolysis of intracellular caged ATP (10 mM). (c) Superimposed currents as in (b), with 5 mM intracellular glutathione, before (1) and 25 s after (4) photolysis. Extracellular tetraethylammonium (10 mM) and glibenclamide (10 μM) present in (b) and (c). Dotted lines indicate zero current.

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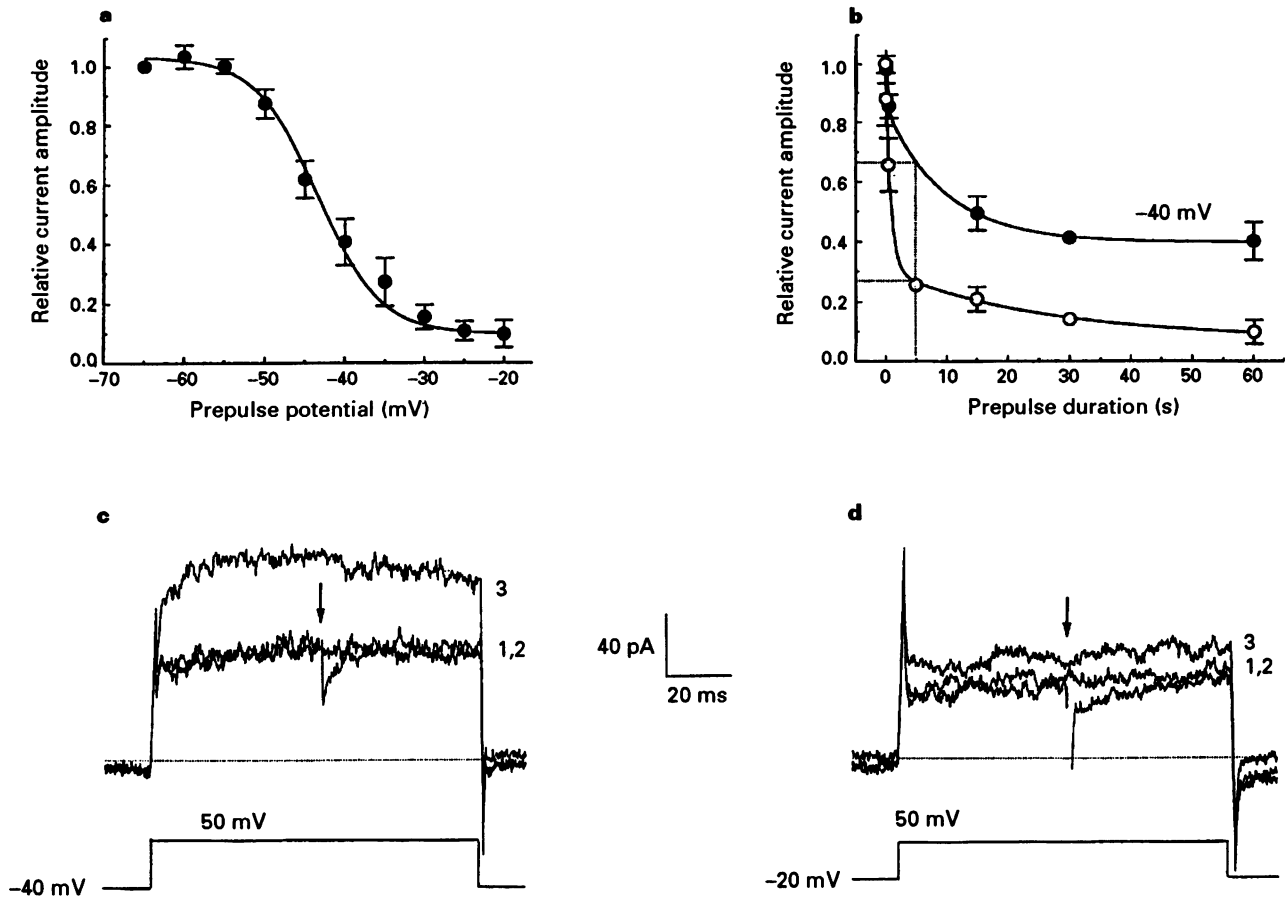


Figure 2 (a) Voltage-dependence of inactivation of I_{KDR} evoked by steps to + 40 mV or + 50 mV following 1 min pre-pulses to the potentials shown, measured relative to the maximum current activated with the same test pulse. Curve is best fit to $I = \{1 + \exp[(V-V_{0.5})/k]\}^{-1}$, with $V_{0.5} = -43.5$ mV, $k = 3.9$ mV, I the relative current and V the pre-pulse potential. Mean ± s.e.mean, 3 cells. (b) Development of inactivation at − 40 mV (●) and − 20 mV (○). Curves are bi-exponential fits, with time constants of 0.25 s and 9.6 s at − 40 mV and 0.84 s and 26.1 s at − 20 mV. Symbols represent means of 2–3 cells. Vertical bars show s.e.mean. (c) Current evoked by a step to + 50 mV after inducing steady-state inactivation by holding the cell at − 40 mV for > 60 s, before (1), at (2) and 5 s after (3) photolysis (arrow) of caged ATP (10 mM). (d) As (c) but holding potential − 20 mV. External tetraethylammonium (10 mM) and glibenclamide (10 μM) present. Dotted lines indicate zero current for (c) and (d).

with 60 s required to reach steady-state (Figure 2b). Measured 5 s after photo-release of ATP, the outward current at + 50 mV was increased by 38 ± 1 pA ($n = 3$) or 53 ± 5%, when cells were held at − 40 mV (Figure 2c). This compares with an increase of 159 ± 22 pA ($n = 5$) at 5 s, equivalent to 48 ± 6%, when cells were held at − 80 mV. When I_{KDR} was almost completely inactivated by holding cells at − 20 mV (Figure 2d), there was little time-dependent outward current evoked at + 50 mV and 5 s after photorelease of ATP the current was increased by only 8 ± 6 pA ($n = 3$).

Discussion We present the first demonstration of ATP-dependent augmentation of a delayed rectifier current in mammalian cells, and provide evidence that enhancement of I_{KDR} by ATP does not result from the modification of non-inactivating, glibenclamide-sensitive $I_{K(ATP)}$ channels.

Based on their findings in rat portal vein, Edwards and coworkers (1993) proposed that the channels underlying $I_{K(ATP)}$ are a dephosphorylated form of I_{KDR} channels. They suggested that when phosphorylated, $I_{K(ATP)}$ channels develop voltage-dependent gating and lose sensitivity to glibenclamide. Since the gating of $I_{K(ATP)}$ is voltage-independent, this hypothesis predicts that the number of channels converted to I_{KDR}, and hence the magnitude of the current induced by ATP, would not vary with the holding potential. On the contrary, the ATP-induced current was greatly reduced at a holding potential of − 40 mV and virtually abolished at − 20 mV. Rapid inactivation of newly converted channels cannot account for the reduced current at positive potentials. During the 5 s delay between photolysis and measurement of I_{KDR}, only 33% of the converted channels would have inactivated at − 40 mV and 73% at − 20 mV (Figure 2b), corresponding to an ATP-inducible current of 106 pA and 43 pA, respectively. The observed currents were substantially smaller than that. It is more likely that ATP modulated pre-existing I_{KDR} channels, and that inactivation reduced the number of channels available to respond at positive holding potentials. The similar percentage augmentation observed at − 80 mV and − 40 mV supports this view.

We conclude that in pulmonary artery myocytes, intracellular ATP modulates I_{KDR} independently of $I_{K(ATP)}$. Both phosphorylation-dependent (Edwards *et al.*, 1993) and independent (Evans *et al.*, 1993) mechanisms have been proposed to regulate these channels. We suggest that the effects of K-channel openers and dephosphorylating conditions on I_{KDR} and $I_{K(ATP)}$ (Edwards *et al.*, 1993) result from independent effects on each channel through inhibition of phosphorylation and/or ATP binding.

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References

BEECH, D.J. & BOLTON, T.B. (1989). Properties of the cromakalim induced potassium conductance in smooth muscle cells isolated from the rabbit portal vein. *Br. J. Pharmacol.*, **98,** 851–864.

CLAPP, L.H. & GURNEY, A.M. (1991). Outward currents in rabbit pulmonary artery cells dissociated with a new technique. *Exp. Physiol.*, **76,** 677–693.

CLAPP, L.H. & GURNEY, A.M. (1992). ATP-sensitive K^+ channels regulate the resting potential of pulmonary arterial smooth muscle cells. *Am. J. Physiol.*, **262,** H916–H920.

EDWARDS, G., IBBOTSON, T. & WESTON, A.H. (1993). Levcromakalim may induce a voltage-independent K-current in rat portal vein by modifying the gating properties of the delayed rectifier. *Br. J. Pharmacol.*, **110,** 1037–1048.

EVANS, A.M., CLAPP, L.H. & GURNEY, A.M. (1993). ATP augments the delayed rectifier in rabbit pulmonary artery myocytes: involvement of a phosphorylation-independent mechanism. *J. Physiol.*, (in press).

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SPECIAL REPORT

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Biochemical machinery involved in the release of ATP from sympathetic nerve terminals

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The biochemical mechanisms underlying the process of facilitation in sympathetic nerve terminals of the guinea-pig vas deferens have been investigated by intracellular and focal extracellular recording techniques. Activation of protein kinase C by the phorbol ester, phorbol 12,13-dibutyrate (PDBu) greatly increased the amplitude of excitatory junction potentials (e.j.ps) from the first stimulus in a train and altered the pattern of facilitation. The configuration of the extracellularly recorded nerve terminal impulse and the sensitivity of the postjunctional membrane to released ATP were unaffected. The specific protein kinase C inhibitor, Ro-31,8220, abolished facilitation and antagonized the effects of PDBu. These results suggest that protein kinase C plays a fundamental role in ATP release from sympathetic nerves and in particular in the mechanisms underlying facilitation.

Keywords: Protein kinase C; electrophysiology; ATP release; guinea-pig vas deferens

Introduction In recent years, electrophysiological techniques have proved invaluable in elucidating the fundamental mechanisms involved in the release of ATP from postganglionic sympathetic nerve terminals. When intracellularly recorded excitatory junction potentials (e.j.ps) are evoked by trains of stimuli at 1 Hz, there is a progressive increase in e.j.p. amplitude over the first 6–8 stimuli and this phenomenon is termed facilitation. Focal extracellular recording techniques have provided additional information about the mechanism of transmitter release at the level of the individual varicosity. Briefly, ATP release has been shown to be monoquantal and occur highly intermittently. The degree of intermittence is not related to failure of impulse propagation in varicose nerve terminals but is positively dependent on the frequency of nerve stimulation i.e. the number of quantal responses evoked per train of stimuli increases with stimulation frequency (0.1–4 Hz) (see Brock & Cunnane, 1992). Thus, the mechanisms underlying facilitation involve the recruitment of previously silent release sites. However, the molecular mechanisms underlying facilitation at the level of depolarization-secretion coupling remain obscure. We have investigated these mechanisms further by studying the effects of agents which modify the activity of protein kinase C (PKC).

Methods Male Duncan-Hartley guinea-pigs (200–350 g) were killed by a blow to the head and bled. The vasa deferentia and associated hypogastric nerve trunks were removed. Tissues were pinned to the base of a 3 ml organ bath and a small window was cut in the connective tissue sheath to expose the outer smooth muscle layer; electrophysiological recordings were made from this area. The tissues were superfused with Krebs solution of the following composition (mM): NaCl 118.4, $NaHCO_3$ 25.0, NaH_2PO_4 1.13, $CaCl_2$ 1.8, KCl 4.7, $MgCl_2$ 1.3 and glucose 11.0, gassed with a mixture of 95% O_2 and 5% CO_2 to pH 7.4 and maintained at 36–37°C. The hypogastric nerve trunk was stimulated with rectangular pulses applied either through ring electrodes or through a suction electrode (Ag-AgCl) into which the nerve trunk was drawn.

Intracellular recordings were made by conventional electrophysiological techniques. Extracellular electrical activity was recorded from the surface of the vas deferens using a small diameter bevelled glass electrode (tip diameter ~ 50 μm) perfused with Krebs solution as previously described (Brock *et al.*, 1990). Drugs were applied locally through the extracellular recording electrode via an internal perfusion system.

Phorbol 12,13-dibutyrate was obtained from Sigma and Ro-31,8220 was a gift from Roche Products Ltd.

Results The PKC activator phorbol 12,13-dibutyrate (PDBu) (0.3 μM) produced a large increase in the amplitude of the first e.j.p. in a train (440 ± 78%, $n = 6$). Subsequent e.j.ps were also increased in amplitude but to a lesser extent than the first (Figure 1a). The overall effect was to alter greatly the pattern of facilitation (Figure 1a). The increase in e.j.p. amplitude was concentration-dependent (0.03–0.3 μM) and was blocked by prior application of the N-type calcium channel blocker, ω-conotoxin (Wardell & Cunnane, 1991). The inactive phorbol ester, 4α-phorbol 12,13-didecanoate had no effect. When transmitter release was measured by focal extracellular recording techniques, PDBu (0.1 μM) increased the number of excitatory junction currents (e.j.cs) evoked per train of stimuli when locally applied inside the suction recording electrode (Figure 1b). PDBu had no effect on the configuration of the nerve terminal impulse (NTI) (Figure 1c) or on the amplitude distribution of spontaneous excitatory junction currents (s.e.j.cs) suggesting there is no marked change in the sensitivity of the postjunctional membrane.

The selective PKC inhibitor Ro-31,8220 (3–10 μM) markedly reduced or abolished facilitation of e.j.ps evoked at 1 Hz (Figure 2a); the effect of Ro-31,8220 could not be reversed, even after washout for up to 1 h. Furthermore, the potentiating effect of PDBu on e.j.ps and on e.j.cs was antagonized by Ro-31,8220 (Figure 2b). Similar effects were seen with the less selective PKC inhibitor, staurosporine (results not shown).

Discussion Our studies demonstrate that PKC powerfully increases the evoked quantal release of the co-transmitter ATP. There is a general belief that second messenger systems in nerve terminals are coupled through presynaptic receptors and serve to regulate neurotransmitter release on an impulse-to-impulse basis (Starke *et al.*, 1987). However, our results suggest that in sympathetic nerve terminals protein kinase C plays a fundamental role in the release of ATP *per se*. It seems probable that action potential-evoked calcium entry into sympathetic nerve terminals, through N-type calcium channels, leads to the activation of protein kinase C which in

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turn powerfully modifies the neurotransmitter release mechanism thus producing facilitation.

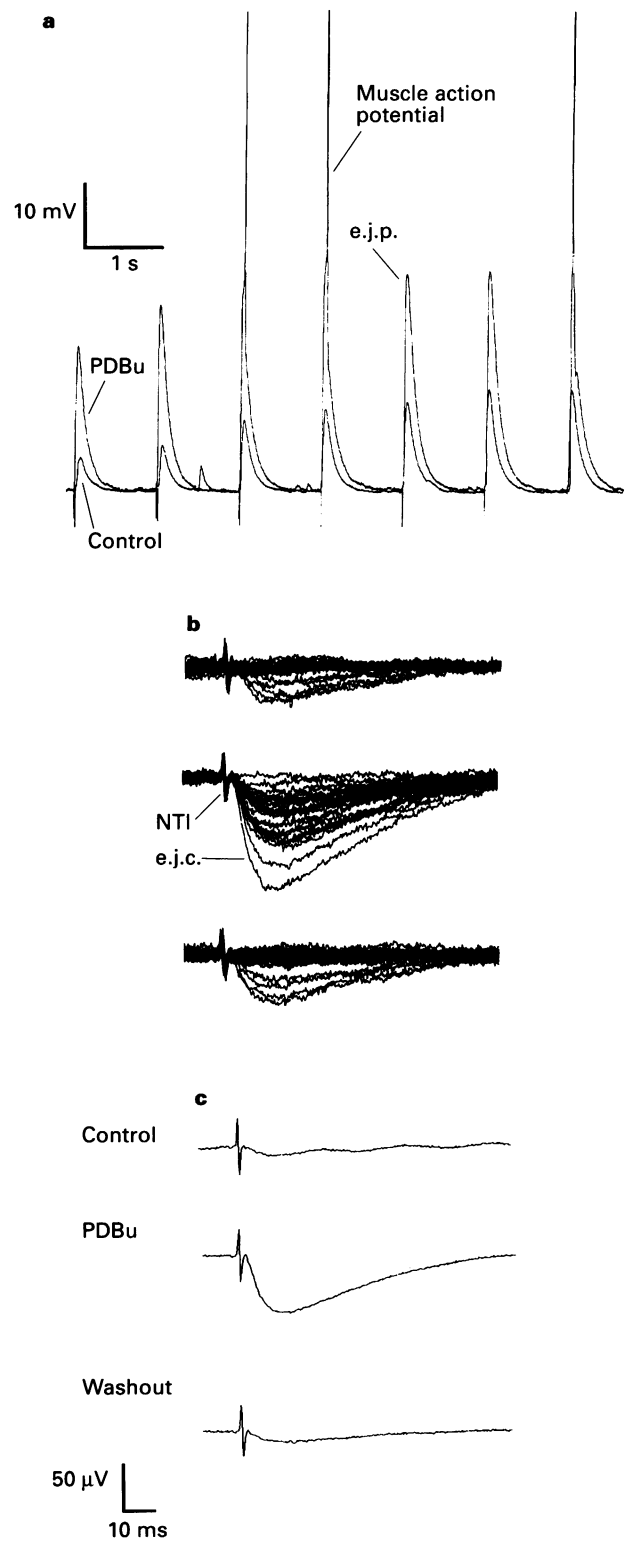


Figure 1 The effect of the protein kinase C (PKC) activator phorbol 12,13-dibutyrate (PDBu) on ATP release. (a) The effect of 0.3 μM PDBu on e.j.ps (train of 7 stimuli at 1 Hz). (b) Extracellular records showing the effect of local application of 0.1 μM PDBu on ATP release. Traces show superimposed records of nerve terminal impulses (NTI) and excitatory junction currents (e.j.cs) obtained following 25 stimuli at 1 Hz. (c) Averages of records shown in (b) showing that PDBu has no apparent effect on the nerve terminal impulse.

There are several ways in which activation of protein kinase C by PDBu might increase neurotransmitter release. Protein substrates for protein kinase C have been identified within nerve terminals, although most of these studies have been carried out on brain neurones. One or more of these proteins may be integral components of the membrane structures involved in the exocytosis of neurotransmitters (see, Walaas & Greengard, 1991). Alternatively, the PDBu-induced increase in neurotransmitter release may involve the direct activation of voltage-sensitive calcium channels, resulting in an increased Ca^{2+} entry into the nerve terminals. A third possibility is that activation of protein kinase C may increase the sensitivity of the secretory machinery to calcium (Knight & Scrutton, 1984).

These findings may have important implications for our understanding of brain function since ATP has recently been shown to act in the central nervous system as a fast neurotransmitter (Edwards *et al.*, 1992; Edwards & Gibb, 1993) and its release is also intermittent (F.A. Edwards, personal communication). The similarities between ATP release mechanisms in varicose sympathetic nerve terminals in the periphery and the brain suggests that protein kinase C may have an equally important role in synaptic transmission in the brain.

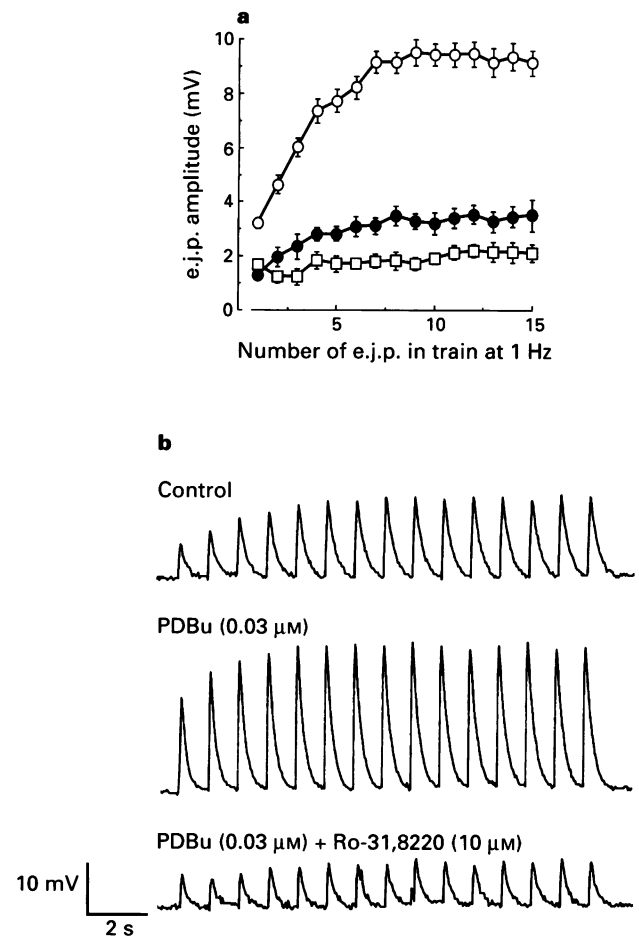


Figure 2 Effects of the protein kinase C inhibitor, Ro-31,8220, on facilitation of transmitter release in the guinea-pig vas deferens; (a) e.j.p. amplitudes recorded in control (○); 10 μM Ro-31,8220 (●) and following washout (□). Data represents the mean e.j.p. amplitude ± s.e.mean for $n = 6$ cells. (b) Antagonism by 10 μM Ro-31,8220 of the increase in e.j.p. amplitude produced by 0.03 μM phorbol 12,13-dibutyrate (PDBu): e.j.ps were evoked by nerve stimulation (15 pulses at 1 Hz) and representative traces during control and drug periods are shown.

References

BROCK, J.A. & CUNNANE, T.C. (1992). Electrophysiology of neuroeffector transmission in smooth muscle. In *Autonomic Neuroeffector Mechanisms*. ed. Burnstock, G. & Hoyle, C.H.V. pp. 121–213. Reading, Berks., UK: Harwood Academic Publishers.

BROCK, J.A., CUNNANE, T.C., STARKE, K. & WARDELL, C.F. (1990). α_2-adrenoceptor mediated autoinhibition of sympathetic transmitter release in guinea-pig vas deferens studied by intracellular and focal extracellular recording of junction potentials and currents. *Naunyn-Schmied. Arch. Pharmacol.*, **342**, 45–52.

EDWARDS, F.A. & GIBB, A.J. (1993). ATP – a fast transmitter. *FEBS Letts*, **325**, 86–89.

EDWARDS, F.A., GIBB, A.J. & COLQUHOUN, D. (1992). ATP receptor-mediated synaptic currents in the central nervous system. *Nature*, **359**, 144–147.

KNIGHT, D.E. & SCRUTTON, M.C. (1984). Cyclic nucleotides control a system which regulates Ca^{2+} sensitivity of platelet secretion. *Nature*, **309**, 66–68.

STARKE, K., GOTHERT, M. & KILBINGER, H. (1987). Modulation of neurotransmitter release by presynaptic autoreceptors. *Physiol. Revs.*, **107**, 73–146.

WALAAS, S.I. & GREENGARD, P. (1991). Protein phosphorylation and neuronal function. *Pharmacol. Revs.*, **43**, 299–349.

WARDELL, C.F. & CUNNANE, T.C. (1991). Protein kinase C, transmitter release and α_2-autoinhibition in the guinea-pig vas deferens. *J. Auton. Nerv. Sys.*, **33**, 174–176.

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SPECIAL REPORT

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Diosmin-induced increase in sensitivity to Ca^{2+} of the smooth muscle contractile apparatus in the rat isolated femoral vein

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The effect of diosmin, a flavone derivative, on the Ca^{2+} sensitivity of the venous contractile apparatus was investigated in chemically (β-escin) skinned strips from the rat isolated femoral vein. Diosmin (0.5–10 μM) shifted to the left the concentration-response curve to Ca^{2+} (0.05–5 μM). The maximal effect was observed in the presence of 1 μM diosmin which increased the contractile response evoked by 0.15 μM Ca^{2+} from 26.3% to 78.9% of the maximal Ca^{2+}-induced response. This work demonstrates that the venotonic action of diosmin involves an increase in the Ca^{2+} sensitivity of the contractile apparatus. Such a mechanism of action could represent a new and important means of therapeutic control of vasomotor activity.

Keywords: Diosmin; Ca^{2+}sensitivity; contractile apparatus; skinned fibres; β-escin; vascular smooth muscle; femoral vein

Introduction Modulation of vascular smooth muscle tone is clinically relevant in both systemic arterial and venous vascular beds. A currently prescribed drug in venous insufficiency is the hemisynthetic diosmin (Diovenor) which modifies the *in vitro* metabolism of noradrenaline by the varicose human saphenous vein (Araujo *et al.*, 1991). Diosmin ((7[6-deoxy-α-L-mannopyranosyl-β-D-glucocopyranosyl] oxyl]-5-hydroxy-2(3-hydroxy-4-methoxyphenyl)-4-*H*-1-benzopyran-4-one) is a flavone derivative exhibiting a high venoselectivity (Dacquet *et al.*, 1992). However, its mechanism of action remains largely unknown.

Recently, it has become clear that smooth muscle tone can be modulated at the level of the Ca^{2+} sensitivity of the contractile apparatus (Nishimura *et al.*, 1988; Kitazawa *et al.*, 1991). Whether or not drugs administered in vascular diseases act directly at the site of the contractile apparatus remains poorly investigated. *In vitro* studies have suggested the existence of an action produced by diosmin on calcium-dependent contractility (Dacquet & Finet, 1990; Dacquet *et al.*, 1992). We thus designed the present work to examine the effect of diosmin on the Ca^{2+} sensitivity of the smooth muscle contractile apparatus in β-escin skinned venous strips.

Methods Experiments were performed in the rat isolated femoral vein. Animals were killed with an overdose of pentobarbitone (40 mg kg^{-1}). Strips (200–300 μm in diameter, 4–5 mm in length) were cut transversely from veins in which connective tissue and endothelium were removed. Contraction was measured isometrically. Strips were chemically skinned with β-escin (80 μM for 30 min). Solutions as well as procedures used to skin smooth muscle strips and to impair the functioning of the sarcoplasmic reticulum have been described in detail elsewhere (Savineau *et al.*, 1993; Savineau & Marthan, 1994). Experiments were performed at 25°C. Concentration-response curves to Ca^{2+} (0.01–5 μM) were constructed in a cumulative manner in either the absence or the presence of diosmin (0.1–10 μM) applied 30 min before and throughout the $[Ca^{2+}]$-tension curve. The $[Ca^{2+}]$-tension relationship was fitted using the Hill equation: $T/T_{max} = K[Ca^{2+}]^h/1 + [Ca^{2+}]^h$ where T is a fraction of the maximal Ca^{2+}-activated force in the absence of diosmin (T_{max}); K is a constant related to the $[Ca^{2+}]$ value corresponding to 50% of T_{max} (EC_{50}); h is the Hill coefficient.

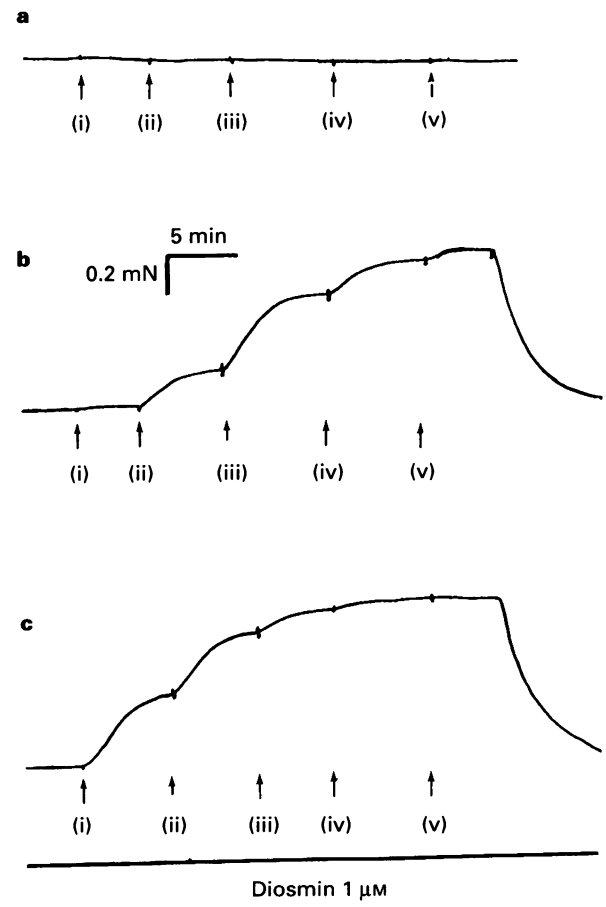


Figure 1 Effect of diosmin on Ca^{2+}-induced contractions in chemically skinned strips from the rat isolated femoral vein. (a) In the absence of β-escin treatment, Ca^{2+} concentrations of 0.08 (i); 0.15 (ii); 0.25 (iii); 0.76 (iv) and 1.75 μM (v) did not induce a contractile response. Prior to the application of Ca^{2+}, the strip was bathed for 10 min in the presence of a Ca^{2+}-free, 0.5 mM EGTA solution. (b) After β-escin treatment (80 μM for 30 min), the same Ca^{2+}-concentrations (i) to (v) induced concentration-dependent and maintained contractions. (c) In the presence of diosmin (1 μM), contractions induced by Ca^{2+} (0.08 to 0.76 μM) were increased. In (b) and (c) at completion of the Ca^{2+}-tension curve, relaxation was obtained by reperfusing the strip with a Ca^{2+}-free solution containing 10 mM EGTA.

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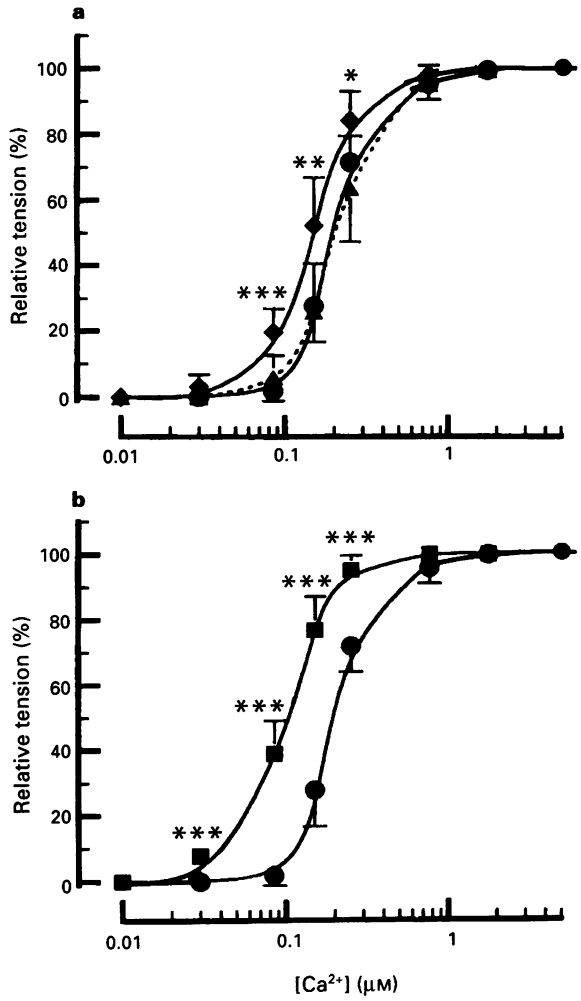


Figure 2 Effect of diosmin on the $[Ca^{2+}]$-tension relationship of the contractile apparatus in the rat isolated femoral vein. Concentration-response curves for the effect of Ca^{2+} were obtained in the absence (●, a, b) and in the presence of diosmin 0.1 μM (▲, a); 0.5 μM (◆, a) and 1 μM (■, b). The abscissae indicate the micromolar concentration of Ca^{2+} on a log scale. The ordinates indicate the tension expressed as a percentage of the maximal Ca^{2+}-induced contraction. Data points are means, $n = 10$ in the absence (●), and $n = 5$ in the presence (▲, ◆, ■) of diosmin. Vertical bars show s.d. $*P<0.05$; $**P<0.01$ and $***P<0.0001$ indicate a response in the presence of diosmin which is significantly different from the corresponding response in the control experiment.

The amplitude of contraction is expressed as mean ± s.d. for n, number of experiments. Significance was assessed by the Student's t test. A difference between means was considered significant when $P<0.05$.

Chemicals and drugs Chemicals were purchased from Sigma (St Quentin Fallavier, France). Hemisynthetic diosmin (Diovenor) was a kind gift of Laboratoire Innothéra (Arcueil, France). Diosmin was dissolved in dimethylsulphoxide (DMSO). The maximal concentration of DMSO in the solution was <0.1% and had no effect on the contractility of femoral venous strips.

Results Ca^{2+} (0.08–1.75 μM) did not produce a contraction in intact strips (Figure 1a) whereas, after β-escin treatment, Ca^{2+} induced concentration-dependent and maintained contractions (Figure 1b). Addition of 1 μM diosmin in skinned strips increased the amplitude of contractions induced by 0.02 to 0.76 μM Ca^{2+} (Figure 1c). For example, the contractile response evoked by 0.15 μM Ca^{2+} increased from 26.3% to 78.9% of the maximal Ca^{2+}-induced response. The $[Ca^{2+}]$-tension relationship of the skinned femoral vein in the absence of diosmin is shown in Figure 2a (●). EC_{50} and h values were respectively equal to 0.21 μM and 3.58. Diosmin (0.5–10 μM) shifted to the left the $[Ca^{2+}]$-tension relationship. The maximal effect was observed in the presence of 1 μM diosmin (Figure 2b). The EC_{50} value decreased from 0.21 in the absence of diosmin to 0.15 and to 0.10 μM in the presence of 0.5 and 1 μM diosmin, respectively. A higher concentration of diosmin had no further effect since the EC_{50} value was 0.16 μM in the presence of 10 μM diosmin (not shown). The Hill coefficient was only slightly reduced from 3.58 in the absence, to 2.83 in the presence of diosmin (1 μM) (Figure 2b).

Discussion To the best of our knowledge, this report is the first demonstration of a direct modulation of the Ca^{2+} sensitivity of the contractile apparatus by a venotonic drug. Diosmin increases the sensitivity to Ca^{2+} of the contractile apparatus from the femoral vein. This effect was particularly pronounced at low Ca^{2+} concentrations (0.02 to 0.76 μM, Figure 1) as previously reported in the rabbit depolarized saphenous vein (Dacquet *et al.*, 1992). The effect of diosmin at the site of the contractile apparatus could explain, at least in part, its venotonic action. A similar increase in the Ca^{2+} sensitivity of vascular smooth muscle has been observed in the presence of either phosphatase inhibitors (Gong *et al.*, 1992) or α-adrenoceptor agonists (Nishimura *et al.*, 1988). In the latter case, this increase appeared mediated by G-proteins and involved an enlarged phosphorylation of myosin light chains (Kitazawa *et al.*, 1991). Since both phosphatase and kinase activities control the tension state in vascular smooth muscle, precise investigation of the molecular target of diosmin require further biochemical investigations.

This work was supported by a grant from Laboratoire Innothéra (Arcueil, France).

References

ARAUJO, D., VIANA, F. & OSSWALD, W. (1991). Diosmin therapy alters the in vitro metabolism of noradrenaline by the varicose human saphenous vein. *Pharmacol. Res.*, **24**, 253–256.

DACQUET, C. & FINET, M. (1990). Effect of diosmin on the noradrenaline sensitive intracellular calcium store in isolated saphenous veins. *Eur. J. Pharmacol.*, **183**, 1248–1249.

DACQUET, C., ROUVREAU, C. & FINET, M. (1992). Comparison of hemisynthetic diosmin on $CaCl_2$ contractile response on rabbit saphenous vein and femoral artery. In *Phlébologie*, ed. Raymond-Martimbeau, P., Prescott, R. & Zummo, M., pp. 723–725. Paris: John Libbey Eurotext.

GONG, M.C., COHEN, P., KITAZAWA, T., IKEBE, M., MASUA, M., SOMLYO, A.P. & SOMLYO, A.V. (1992). Myosin light chain phosphatase activities and the effects of phosphatase inhibitors in tonic and phasic smooth muscle. *J. Biol. Chem.*, **267**, 14662–14668.

KITAZAWA, T., GAYLINN, B.D., DENNEY, G.H. & SOMLYO, A.P. (1991). G-protein-mediated Ca^{2+} sensitization of smooth muscle contraction through myosin light chain phosphorylation. *J. Biol. Chem.*, **266**, 1708–1715.

NISHIMURA, J., KOBLER, M. & VAN BREEMEN, C. (1988). Norepinephrine and GTP-γ-S increase myofilament Ca^{2+} sensitivity in α-toxin permeabilized arterial smooth muscle. *Biochem. Biophys. Res. Commun.*, **157**, 677–683.

SAVINEAU, J.P., GONZALEZ DE LA FUENTE, P. & MARTHAN, R. (1993). Effect of vascular smooth muscle relaxants on the protein kinase C-mediated contraction in the rat pulmonary artery. *Eur. J. Pharmacol.*, **249**, 191–198.

SAVINEAU, J.P. & MARTHAN, R. (1994). Activation properties of chemically skinned fibres from human isolated bronchial smooth muscle. *J. Physiol.*, **474**, 433–438.

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SPECIAL REPORT

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Lack of importance of NO in β-adrenoceptor-mediated relaxation of large epicardial canine coronary arteries

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This study was designed to test the role of endothelium and the L-arginine/NO pathway in the relaxation of canine large coronary arteries to the β-adrenoceptor agonist, isoprenaline. Relaxation of left circumflex (LCX) and left anterior descending (LAD) coronary arteries were measured in organ baths after contraction with the thromboxane analogue, U46619, either in absence or in presence of endothelium and in LCX arteries after pretreatment with N^G-nitro-L-arginine-methyl-ester (L-NAME). LAD arteries with and without endothelium relaxed identically to isoprenaline but their maximal relaxation was smaller than corresponding LCX arteries with endothelium. A slight but non significant difference was observed in LCX arteries with endothelium as compared to rings without endothelium or pretreated with L-NAME. No difference was observed in the relaxation of LCX arteries to forskolin in arteries with or without endothelium. These results suggest that endothelium is not essential and that NO is not directly involved in the relaxation of large coronary arteries induced by isoprenaline.

Keywords: Isoprenaline; dog coronary arteries; endothelium; nitric oxide

Introduction Conflicting results are found in the literature about the modulator role of endothelium in the β-adrenoceptor-mediated vasorelaxation and the possible involvement of the L-arginine/NO pathway in this response. In canine coronary artery it has been reported that *in vitro* relaxation to isoprenaline was either endothelium-independent (MacDonald *et al.*, 1987; O'Rourke & Vanhoutte, 1990) or endothelium-dependent (Rubanyi & Vanhoutte, 1985), although in the latter case the difference observed between vessels with and without endothelium was very slight. However, the direct involvement of NO was not investigated in these studies. *In vivo*, Parent *et al.* (1993) have reported that vasodilatation of canine coronary resistance arteries to isoprenaline was endothelium-dependent and mediated through NO release, but the response of large conductance arteries to isoprenaline was not investigated. Finally, Gray & Marshall (1992) have reported that β-adrenoceptor-mediated vasorelaxation was completely endothelium-dependent in rat thoracic aorta and suggested that the rise in adenosine 3':5'-cyclic monophosphate (cyclic AMP) could lead to the stimulation of NO synthase.

The aims of the present study were to investigate (a) whether endothelium is necessary for dilatation of large coronary arteries to isoprenaline, and (b) whether the L-arginine/NO pathway is involved in this phenomenon. We also compared the relaxation to isoprenaline of the left circumflex (LCX) and left anterior descending (LAD) coronary arteries, as both arteries were indifferently used as models of large coronary vessels and serve as controls for each other.

Methods Mongrel male dogs (15–20 kg) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.) and artificially ventilated after left thoracotomy. The heart was rapidly excised and both proximal epicardial LCX and LAD were dissected free (up to 3 cm from the origin). The rings were mounted in organ chambers for isometric tension recording as previously described (Drieu La Rochelle *et al.*, 1992). Endothelium removal was performed in a number of rings by rubbing the intimal layer with a forceps. All rings were precontracted with U46619 (1 μM), and isoprenaline (0.01–10 μM) or forskolin (0.1 μM) was added. Finally, some rings with endothelium were pretreated with L-NAME (100 μM). Integrity or absence of the endothelial layer was assessed by testing the relaxation to acetylcholine (1 μM).

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Chemicals All drugs used were obtained from Sigma Chimie (St-Quentin Fallavier, France), except forskolin which was a generous gift from Hoechst A.G. (Frankfurt, Germany).

Statistics Results are mean ± s.e.mean and n indicates the number of dogs. Comparison of the different dose-related curves was performed by a two-way ANOVA for repeated measures. Maximal relaxation to isoprenaline or forskolin and IC_{50} values (calculated as concentration giving half the maximal effect) were compared by Student's paired t test with Bonferroni's correction. A P value <0.05 was considered statistically significant.

Results Relaxations to isoprenaline of LCX and LAD arteries are shown in Figure 1a. Relaxation of LAD was similar in rings with and without endothelium. Relaxation of LCX with endothelium was slightly more pronounced than that of LCX without endothelium but the difference was not significant. A significant difference was observed between LCX with endothelium and LAD with or without endothelium for both the dose-related relaxation to isoprenaline ($P<0.01$) and the maximal relaxation achieved ($P<0.05$). However, IC_{50} values were not statistically different (0.07 ± 0.02 μM, 0.09 ± 0.02 μM, 0.12 ± 0.02 μM and 0.14 ± 0.04 μM for LCX with endothelium, LCX without endothelium, LAD with endothelium and LAD without endothelium, respectively; $n = 8$).

Relaxations to isoprenaline of LCX pretreated with L-NAME are shown in Figure 1b. Maximal relaxations were not statistically different as well as corresponding IC_{50} values (0.08 ± 0.02 μM, 0.12 ± 0.03 μM and 0.13 ± 0.04 μM, for LCX with endothelium, LCX without endothelium, and LCX pretreated with L-NAME, respectively, $n = 9$).

Relaxations to forskolin of LCX with endothelium, LCX without endothelium and LCX pretreated with L-NAME were not statistically different (75 ± 10%, 86 ± 11% and 74 ± 14%, respectively; $n = 7$).

Discussion The present results demonstrate that LAD coronary arteries do not exhibit identical relaxation to isoprenaline as corresponding LCX arteries, regardless of the presence or absence of an intact endothelium. Such a discrepancy between the two arteries originating from the same animals must be underlined, as LCX and LAD are often used as control vessels for each other. Although challenged

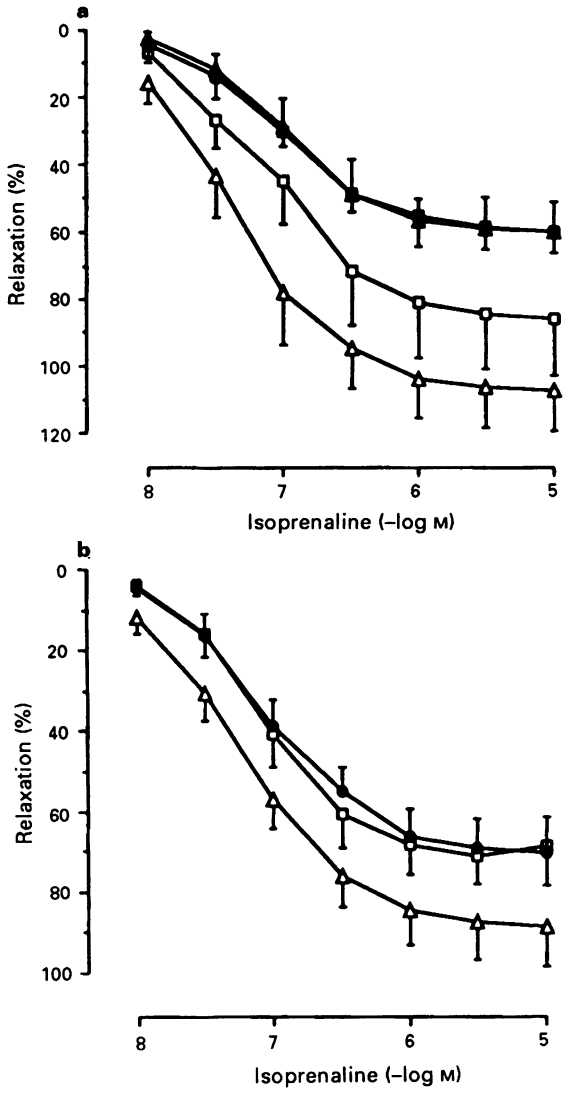


Figure 1 Relaxation of canine coronary arteries to isoprenaline: (a) relaxation to isoprenaline of left circumflex (LCX, open symbols) and left anterior descending (LAD, solid symbols) coronary arteries with (△, ▲) and without (□, ■) endothelium ($n = 8$); (b) relaxation to isoprenaline of LCX arteries with endothelium pretreated with N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM) (●) compared to LCX arteries with (△) or without (□) endothelium ($n = 9$).

LAD and LCX rings were obtained from artery segments dissected close to their common trunk, a difference in reactivity depending on the size of the vessel cannot be excluded to explain this discrepancy.

LAD arteries relaxation to isoprenaline was not at all affected by the absence of an intact endothelium whereas that of LCX was only slightly reduced after endothelium removal. In addition, blockade of the L-arginine/NO pathway did not prevent the relaxant effect of isoprenaline on LCX artery, suggesting that NO is not directly involved in β-adrenoceptor-mediated relaxation. This result is totally at variance with those of Gray *et al.* (1992) who found in rat thoracic aorta that relaxation to isoprenaline was completely abolished after endothelium removal. In the present experiments, the weak difference in isoprenaline-induced LCX relaxation observed in the absence or presence of endothelium could be attributed to the basal release of guanosine 3':5'-cyclic monophosphate (cyclic GMP) since blockade of the NO pathway by L-NAME resulted in the same slight decrease in maximal relaxation of the vessel. Indeed, synergistic cooperation between cyclic GMP and cyclic AMP has been previously suggested by Grace *et al.* (1988) in the relaxation of rat aorta to isoprenaline.

Stimulation of cyclic AMP formation by isoprenaline leading to a direct stimulation of NO synthase was also suggested by Gray & Marshall (1992). To test this hypothesis, we studied the effect of forskolin on LCX arteries in the absence or presence of an intact endothelium and after blockade of NO formation by L-NAME. Since relaxation of the vessel to forskolin was not affected by these procedures, it is clear that activation of the β-adrenoceptor/adenylate cyclase pathway does not directly stimulate NO synthase in canine coronary arteries.

We conclude from the present study that *in vitro*, β-adrenoceptor-mediated relaxation is not endothelium-dependent in canine coronary conductance arteries but the presence of a functional endothelium may facilitate this relaxation, probably through the basal release of NO and consequently the basal content of cyclic GMP in vascular smooth muscle.

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References

DRIEU LA ROCHELLE, C., RICHARD, V., DUBOIS-RANDÉ, J.L., ROUPIE, E., GIUDICELLI, J.F., HITTINGER, L. & BERDEAUX, A. (1992). Potassium channel openers dilate large epicardial coronary arteries in conscious dogs by an indirect, endothelium-dependent mechanism. *J. Pharmacol. Exp. Ther.*, **263**, 1091–1096.

GRACE, G.C., MACDONALD, P.S. & DUSTING, G.J. (1988). Cyclic nucleotide interactions involved in endothelium-dependent dilatation in rat aortic rings. *Eur. J. Pharmacol.*, **148**, 17–24.

GRAY, D.W. & MARSHALL, I. (1992). Novel signal transduction pathway mediating endothelium-dependent β-adrenoceptor vasorelaxation in rat thoracic aorta. *Br. J. Pharmacol.*, **107**, 684–690.

MACDONALD, P.S., DUBBIN, P.N. & DUSTING, G.J. (1987). β-Adrenoceptors on endothelial cells do not influence release of relaxing factor in dog coronary arteries. *Clin. Exp. Pharmacol. Physiol.*, **14**, 525–534.

O'ROURKE, S.T. & VANHOUTTE, P.M. (1990). Celiprolol has no direct or indirect relaxing effects in isolated arteries and veins. *J. Cardiovasc. Pharmacol.*, **15**, 831–835.

PARENT, R., AL-OBAIDI, M. & LAVALLÉE, M. (1993). Nitric oxide formation contributes to β-adrenergic dilation of resistance coronary vessels in conscious dogs. *Circ. Res.*, **73**, 241–251.

RUBANYI, G. & VANHOUTTE, P.M. (1985). Endothelium removal decreases relaxations of canine coronary arteries caused by β-adrenergic agonists and adenosine. *J. Cardiovasc. Pharmacol.*, **7**, 139–144.

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Production of PGE_2 by bovine cultured airway smooth muscle cells and its inhibition by cyclo-oxygenase inhibitors

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1 Prostaglandin E_2 (PGE_2) is thought to be an important inhibitory modulator of inflammatory processes in the airway. It inhibits inflammatory cell function and cholinergic neurotransmission *in vitro* and roles have been postulated *in vivo* in refractoriness and in the mechanism of action of the diuretic agent, frusemide.

2 The production of PGE_2 by bovine cultured airway smooth muscle cells has been studied under a range of conditions. The effects of cyclo-oxygenase inhibitors (flurbiprofen, indomethacin, acetyl salicylic acid) on serum-induced production of PGE_2 were assessed over a range of concentrations (10^{-7}–10^{-4} M).

3 Serum-stimulated production of PGE_2 in control wells ranged from 350 to 800 ng PGE_2 ml^{-1} in cells from different animals. All three cyclo-oxygenase inhibitors inhibited PGE_2 production with an order of potency, flurbiprofen > indomethacin > acetyl salicylic acid. Log IC_{50} values were − 6.24 for flurbiprofen, − 5.23 for indomethacin and − 3.50 for acetyl salicylic acid.

4 PGE_2 production was stimulated by arachidonic acid (10^{-5} M) or addition of the proinflammatory mediator, bradykinin (10^{-8}–10^{-5} M).

5 Incubation of cells for 24 h with 5 bromo deoxyuridine (BRDU) (10^{-4} M) to prevent DNA synthesis did not alter PGE_2 production in response to serum, suggesting that it was not a function of proliferation *per se*.

6 Our study suggests that airway smooth muscle may be an important source of PGE_2. Production of PGE_2 may be a novel feedback mechanism whereby airway smooth muscle cells can negatively modulate airways inflammation. The differing potencies of the cyclo-oxygenase inhibitors may explain the contrasting effect of these drugs in recent studies in asthma.

Keywords: PGE_2; airway smooth muscle; indomethacin; flurbiprofen; acetyl salicylic acid; bradykinin

Introduction

Prostaglandin E_2 (PGE_2) is thought to be an important inhibitory prostaglandin in airway tissue which has potent effects both *in vitro* and *in vivo*. *In vitro*, PGE_2 inhibits acetylcholine release from parasympathetic nerve endings in human airway smooth muscle (Ito *et al.*, 1990). It inhibits release of mediators from human lung mast cells (Peters *et al.*, 1982) and inhibits cellular responses in other inflammatory cells such as eosinophils (Giembycz *et al.*, 1990), macrophages (Christman & Christman, 1990) and T-lymphocytes (Minakuchi *et al.*, 1990). In asthmatic subjects *in vivo*, PGE_2 is a potent inhibitor of bronchoconstriction induced by metabisulphite (Pavord *et al.*, 1991), allergen (Pavord *et al.*, 1992a), exercise and ultrasonically nebulised distilled water (Pasargiklian *et al.*, 1976; 1977). A role for PGE_2 has also been postulated in the refractoriness to bronchoconstrictor challenges which occurs in asthma (Pavord *et al.*, 1992a) and in the effect of the diuretic drug, frusemide, which protects against several bronchoconstrictor challenges in asthma (Pavord *et al.*, 1992a).

It is not known which of the many types of airway cells are the most important in PGE_2 production as this has not been studied in detail with the exception of the epithelial cell. Airway smooth muscle has been relatively ignored as a possible site of production. A few studies in lung homogenates (Masumoto & Masuda, 1976) or whole airway preparations (Haye-Legrand *et al.*, 1986) have documented production of PGE_2, suggesting that airway smooth muscle is capable of PGE_2 synthesis but these preparations contain heterogenous populations of airway cells. As smooth muscle is an important source of prostaglandins in other organs (Chamley-Campbell *et al.*, 1979) it would seem likely that airway smooth muscle cells may also be an important source of PGE_2. In one preliminary report on dog trachealis smooth muscle strips, PGE_2 was produced in response to histamine, suggesting that airway smooth muscle is capable of PGE_2 synthesis (Anderson *et al.*, 1980). One method of studying PGE_2 production by airway smooth muscle more closely is to study cultured cells.

Cyclo-oxygenase inhibitors have been recently shown to have conflicting effects in asthma (Robuschi *et al.*, 1992; Bianco *et al.*, 1992; Pavord *et al.*, 1992b; Polosa *et al.*, 1992; O'Connor *et al.*, 1993). In view of this and the potential importance of PGE_2 in the airway, we studied the production of PGE_2 by cultures of bovine airway smooth muscle cells and its inhibition by flurbiprofen, indomethacin and acetyl salicylic acid.

Methods

Tissue preparation

Bovine trachea was obtained from the local abattoir and bathed immediately in ice cold Krebs-Henseleit solution. The trachealis muscle was then dissected free of epithelium and connective tissue. The muscle was minced with a McIlwain tissue chopper (Brinkmann Instruments, Westbury, New York, U.S.A.) and subjected to an enzymatic digestion procedure (Felbel *et al.*, 1988; Kamm *et al.*, 1989). The chopped tissue was incubated in 5 ml Krebs-Ringer-HEPES solution (KRH) without enzymes and shaken in a waterbath at 37°C for 30 min. The tissue was then incubated in 5 ml Krebs-Ringer-HEPES solution (KRH), containing 3.5 mg type IV collagenase, 5 mg soya bean trypsin inhibitor and 5 mM $CaCl_2$ at 37°C in a shaking water bath for 30 min. This

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procedure was then repeated twice more. The slurry was filtered, the cells were centrifuged, resuspended in Dulbecco's modified eagles medium (DMEM), counted and then plated out in several 175 cm^2 flasks. Cells were grown to confluence in DMEM + 10% fetal calf serum (FCS) containing penicillin/streptomycin/amphotericin B and L-glutamine (concentrations given below). Once confluent, cells were trypsinized with 0.25% trypsin, 0.02% EDTA and either passaged immediately or resuspended in 90% FCS + 10% dimethyl sulphoxide, frozen in liquid nitrogen and stored until required. Cells were thawed prior to use and plated at a density of 0.2×10^5 cells/well in 12 well plates containing DMEM + 10% fetal calf serum + penicillin/streptomycin/amphotericin B + L-glutamine.

Cell counting

Cells were removed from plates by incubation with 0.25% trypsin, 0.02% EDTA for 8–10 min. Cells were counted in a haemocytometer and viability assessed by trypan blue exclusion.

Radioimmunoassay for PGE_2

PGE_2 was measured by radioimmunoassay (Hawthorne *et al.*, 1991). Assays were performed in triplicate and expressed as pg ml^{-1}. Samples and standards were incubated overnight with assay buffer, [^{3}H]-PGE_2 (Amersham TRK 431) and rabbit PGE_2 antiserum (Sigma P5164). Free and bound PGE_2 were separated by incubation for 12 min with dextran-coated charcoal and centrifuged for 15 min at 3,500 r.p.m. (4°C). Supernatants were diluted in scintillation fluid and counted for 5 min. Doubling dilutions of PGE_2 standard (Sigma P5640) over the concentration-range 1000 to 7.5 pg 100 μl^{-1} were made up in ethanol and used to construct the standard curve. The antiserum has a low degree of cross reactivity with $PGF_{2\alpha}$ (3.4%) and $PGF_{1\alpha}$ (3.2%) but does not discriminate PGE_2 from PGE_1. The sensitivity of the assay, or the 90% intercept of the B/B_0 standard curve was 15 pg 100 μl^{-1}. Intra-assay variability was +/−6.8 pg 100 μl^{-1} ($n = 14$) and inter-assay variability +/−7.5 pg 100 μl^{-1} ($n = 45$). The assay results were comparable with those obtained with a sensitive ELISA assay (Cascade, UK).

Staining

Cells were grown on coverslips, washed with ice cold PBS (with 0.1% sodium azide) and fixed in methanol at − 20°C. Coverslips of cells were incubated in 1% goat serum in PBS for 45 min at room temperature. Cells were then incubated for 1 h at room temperature in monoclonal anti smooth muscle actin (Sigma A2547) antisera diluted in PBS. The optimum dilution was determined for each batch (between 1 in 100 and 1 in 400 dilution). The cells were then washed in PBS/azide and incubated for 1 h at room temperature in the dark with fluorescein isothiocyanate (FITC) conjugated anti-mouse antibody (Dako F313) (up to 1 in 8 dilution) before being washed and viewed by fluorescent microscopy.

Drugs

Indomethacin, acetyl salicylic acid and flurbiprofen were obtained from Sigma Chemicals, Poole, Dorset, UK. Stock solutions (10^{-2} M) were made up of each inhibitor. Indomethacin and acetyl salicylic acid were dissolved in the minimum amount of 1 M sodium hydroxide required and diluted in DMEM. Flurbiprofen was dissolved in water and 1 M NaOH in a 6:1 ratio and then diluted with DMEM.

[^{3}H]-PGE_2 was obtained from Amersham Interational, Amersham, U.K. Rabbit PGE_2 antiserum, PGE_2 standard, type IV collagenase, protease, DMEM, L-glutamine, penicillin/streptomycin, arachidonic acid, bradykinin, 5 bromo deoxyuridine, ascorbic acid, insulin, transferrin and soya bean trypsin inhibitor were obtained from Sigma Chemicals, Poole, U.K. Amphotericin was obtained from ICN Flow, High Wycombe, U.K. Bovine foetal calf serum was obtained from Seralab, Crawley Down, U.K.

Buffers: Krebs-Henseleit solution had the following composition (mmol l^{-1}): Na^+Cl^- 118, K^+Cl^- 4.7, $Mg^{2+}SO_4^{2-}$ 1.2, $Na^+H_2PO_4^-$ 1.2, $Ca^{2+}Cl_2^-$ 2.5, $Na^+HCO_3^-$ 25, glucose 11.1, pH 7.4 when gassed with 95% O_2, 5% CO_2.

Krebs-Ringer-HEPES was of the following composition (mmol l^{-1}):Na^+Cl^- 105, K^+Cl^- 5, $Ca^{2+}Cl_2^-$ 2, $K^+H_2PO_4^-$ 1, $Mg^{2+}SO_4^{2-}$ 1, glucose 14, HEPES 25 and phenol red 10 μg ml^{-1}, pH 7.4.

Culture medium

Penicillin/streptomycin solution (5000 u penicillin + 5 mg streptomycin ml^{-1}) 1 ml + 0.5 ml amphotericin (250 μg ml^{-1}) + 1 ml L-glutamine (200 mM) was added to 50 ml of DMEM before use.

Protocols

Cells were used on the first passage and plated at a concentration of 0.2×10^5 cells per well in 12 well culture plates containing DMEM + 10% foetal calf serum (FCS). Initial experiments were performed to determine the day in culture that PGE_2 production was maximal by counting cells and measuring PGE_2 production on days 4, 5, 6 and 7 of culture. PGE_2 production had plateaued by days 6 and 7 and subsequent experiments were performed to look at the effects of cyclo-oxygenase at this time. The time course of PGE_2 production in 10% FCS over 24 h was studied. For these experiments medium was changed on Day 6 and the fresh medium subsequently removed for measurement of PGE_2 at 2, 6 and 24 h from separate wells.

Experiments were performed to look at the effects of different cyclo-oxygenase inhibitors in tissue from four different animals, assays being performed in triplicate for each animal. Indomethacin, acetyl salicylic acid or flurbiprofen were added over a concentration range 10^{-7}–10^{-4} M on day 5 to separate wells of cells. On day 6, after 24 h incubation in each inhibitor, the supernatant from each well was collected, PGE_2 was measured and cells were trypsinized and counted.

Additional experiments were performed to determine the effects of bradykinin, arachidonic acid and foetal calf serum in quiescent cells. In these experiments cells were grown to confluence and growth arrested for 24 h by incubation in serum-free DMEM + insulin 6 μg ml^{-1} + transferrin 5 μg ml^{-1} + ascorbic acid 35 μg ml^{-1} prior to the addition of each agent. PGE_2 production was measured over 6 h.

In order to determine if production of PGE_2 was a function of cell proliferation we performed experiments where cells were incubated in 10^{-4} M 5-bromo deoxyuridine (BRDU) for 24 h prior to the addition of 10% FCS and measurement of PGE_2.

Results

Cells grown in the manner described reached confluence after 5–6 days, showed the characteristic appearance of smooth muscle cells in culture with a hill and valley appearance when confluent and stained positively for smooth muscle alpha actin in contrast to fibroblasts grown from connective tissue implants (Figure 1).

The initial experiments showed that airway smooth muscle cells cultured in 10% FCS produce large quantities of PGE_2 and that this was maximal after 6–7 days of culture (Table 1). PGE_2 production increased progressively over a 24 h period (Figure 2). Serum-stimulated PGE_2 production in control wells ranged from 350 to 800 ng per well. PGE_2 production was inhibited by all three cyclo-oxygenase inhibitors in a concentration-dependent manner. The concentrations used in

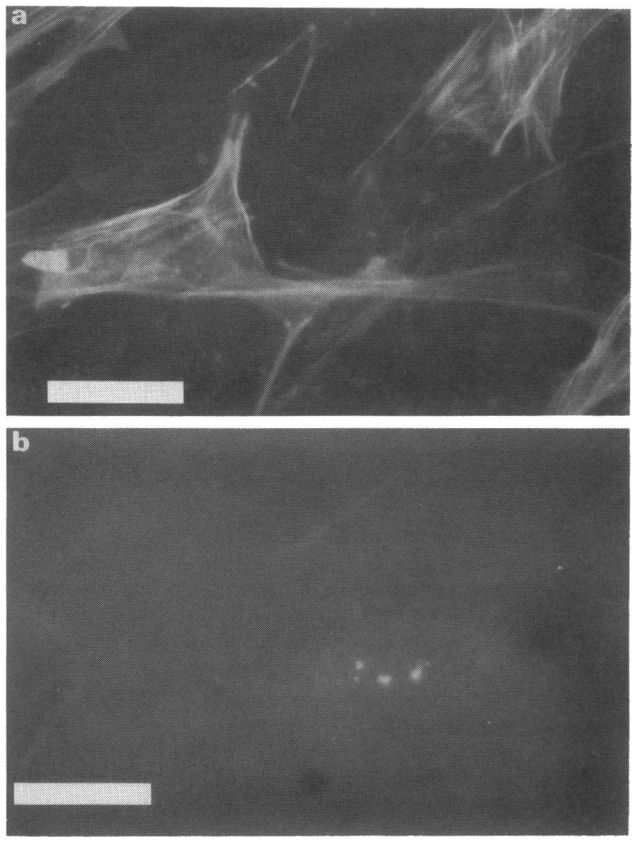


Figure 1 (a) Bovine airway smooth muscle cells in culture showing staining for smooth muscle actin in contrast to (b) fibroblasts grown from connective tissue explants. Cells were grown on coverslips and stained with monoclonal antismooth muscle actin antisera, labelled with fluorescein isothiocyanate antibody and veiwed by fluorescent microscopy. Scale bar = 50 μm.

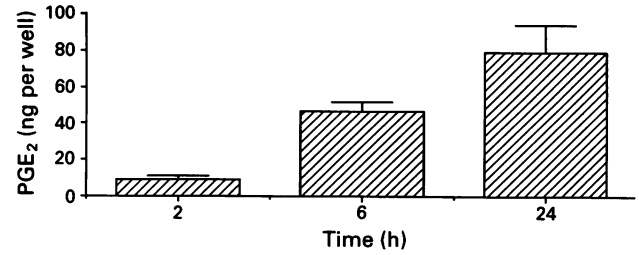


Figure 2 Time course of prostaglandin E_2 (PGE_2) production in cells grown to confluence in serum, medium changed at time 0 and PGE_2 measured over 24 h. Values shown are the mean ± s.e.mean of 4 experiments.

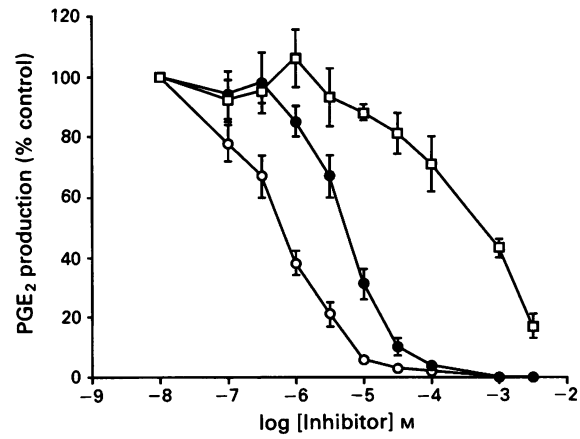


Figure 3 The effects of flurbiprofen (○), indomethacin (●) and acetyl salicylic acid (□) on serum induced production of prostaglandin E_2 (PGE_2) from confluent airway smooth muscle cells. Results are the mean ± s.e.mean values of 4 experiments performed in triplicate.

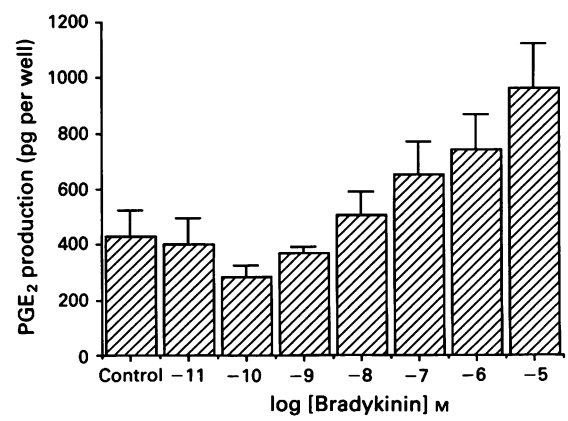


Figure 4 Effect of a range of concentrations of bradykinin on prostaglandin E_2 (PGE_2) production by growth arrested airway smooth muscle cells. Results are mean ± s.e.mean, $n = 4$.

the first experiment for acetyl salicylic acid (up to 10^{-4} M) did not inhibit PGE_2 production enough to obtain an IC_{50} value. Thus for the remaining experiments, acetyl salicylic acid was studied in concentrations up to 3×10^{-3} M. Figure 3 illustrates the mean (s.e.) production of PGE_2 in the presence of the three inhibitors shown as concentration-response curves. Flurbiprofen was ten times more potent than indomethacin and 500 times more potent than acetyl salicylic acid. Flurbiprofen at 3×10^{-5} M inhibited PGE_2 production by 97%, indomethacin at 10^{-4} M inhibited PGE_2 production by 96% and acetyl salicylic acid at 3×10^{-3} M inhibited PGE_2 production by 93%. Log IC_{50} values were -6.24 ± 0.1 for flurbiprofen, -5.23 ± 0.1 for indomethacin and -3.50 ± 0.3 for acetyl salicylic acid. None of the cyclo-oxygenase inhibitors altered cell counts significantly at any concentration.

Incubation of cells in serum-free medium for 24 h produced a lower basal level of PGE_2 production. This was stimulated over 6 h by 10^{-5} M arachidonic acid to 148 ± 17% of control wells ($P < 0.05$, $n = 4$ experiments each performed in triplicate). Addition of 10% FCS produced a marked increase in PGE_2 production over 6 h compared to serum-free alone ($P < 0.05$). PGE_2 production over 6 h was 10.4 ± 4 ng per well in serum-free medium and 190 ± 78 ng per well in 10% FCS ($n = 4$ experiments each performed in triplicate).

Arachidonic acid (10^{-5} M) increased PGE_2 production to 148 ± 17% of control values ($P < 0.05$, $n = 4$ experiments each performed in triplicate). Bradykinin caused a concentration-related increase in PGE_2 production. Bradykinin (10^{-6} M) increased PGE_2 production to 239 ± 80% of control values ($n = 4$ experiments each performed in triplicate) ($P <$ 0.05, Figure 4). Incubation of cells with 10^{-4} M BRDU for

Table 1 Cell counts and prostaglandin E_2 (PGE_2) production from bovine airway smooth muscle cells in culture, day 4–7

Day	*Mean cell count* ($\times 10^5$ cells ml^{-1})	*Mean PGE_2 prod^n* (ng ml^{-1})	*PGE_2 prod^n* (ng per 10^5 cells)
4	6.2 ± 0.5	174 ± 5.2	28.1
5	24.3 ± 2.7	327 ± 33.7	13.5
6	16.3 ± 0.9	747 ± 24.0	45.8
7	17.0 ± 0.4	837 ± 14.1	49.2

Values shown are mean ± s.e.mean, $n = 3$.

24 h prior to the addition of 10% FCS did not significantly alter PGE_2 production. PGE_2 produced was 313 ± 78 ng per well in control wells and 391 ± 71 ng per well in cells treated with 10^{-4} M BRDU ($P = 0.5$, $n = 4$).

Discussion

The aim of the study was to measure PGE_2 production by airway smooth muscle cells under different conditions and to study its inhibition by three cyclo-oxygenase inhibitors. We performed experiments on bovine cultured airway smooth muscle cells *in vitro*. The advantage of using cell culture for these studies is that lipid metabolism can be readily studied and it is easier to manipulate the system experimentally than in studies in tissue slices or whole animals. It also allows the study of individual cell types such as airway smooth muscle without confounding effects due to epithelial and inflammatory cells. We have been able to culture bovine airway smooth muscle cells readily from enzyme digested tissue strips and have confirmed that these cells stain positively for the smooth muscle contractile protein alpha actin unlike confluent cultures of fibroblasts grown from connective tissue explants.

We measured PGE_2 by radioimmunoassay. This is a convenient and reliable technique. The monoclonal antibody used, however, does not discriminate between PGE_1 and PGE_2. However, PGE_1 is produced in minute amounts relative to PGE_2 in airway tissue (Coleman *et al.*, 1990) and in preliminary experiments we showed that we were measuring PGE_2 rather than PGE_1 in our assay by comparing results from the radioimmunoassay with a more sensitive enzyme-linked immunosorbent assay (ELISA) which distinguishes PGE_1 from PGE_2. We did not measure other arachidonic acid metabolites as h.p.l.c. studies have shown that PGE_2 is by far the dominant arachidonic acid metabolite of airway smooth muscle (Tanaka *et al.*, 1993).

Ours is the first study to examine PGE_2 production in detail in cultured airway smooth muscle cells. There have, however, been studies measuring PGE_2 production in whole airway tissue preparations which have contained airway smooth muscle in addition to other cell types (Masumoto & Masuda, 1976; Haye-Legrand *et al.*, 1986) and one previous study measuring PGE_2 in canine trachealis strips (Anderson, 1980). However, studies in whole tissue preparations cannot identify the cell type responsible for prostaglandin production.

PGE_2 was stimulated over basal levels in our experiments by addition of arachidonic acid, the substrate for phospholipase A_2. As bradykinin has been shown to increase PGE_2 production in other cell types (White *et al.*, 1992) and is thought to be an important proinflammatory mediator in asthma, we studied the effect of bradykinin on PGE_2 production. Bradykinin caused a concentration-related increase in PGE_2. The finding that proinflammatory mediators such as bradykinin increase PGE_2 is important for several reasons. As PGE_2 is an inhibitory prostaglandin, the PGE_2 released may be involved in a negative feedback mechanism protecting the airway from excessive bronchoconstriction. Proinflammatory mediators such as histamine and bradykinin have also been shown to stimulate PGE_2 production from canine tracheal strips (Anderson *et al.*, 1980) and in human cultured tracheal epithelial cells (Churchill *et al.*, 1989) respectively. Any PGE_2 release by these mediators might feed back to inhibit inflammatory cell function (Giembycz *et al.*, 1990; Christman & Christman, 1990; Minakuchi *et al.*, 1990) and acetylcholine release from cholinergic nerve terminals (Ito *et al.*, 1990), thus suggesting a novel role for airway smooth muscle and airway epithelium in producing an anti-inflammatory mediator which may exert a braking effect on the inflammatory process. The protective effects of PGE_2 are likely to be due to its anti-inflammatory effects rather than effects on smooth muscle as its effects on airway smooth muscle are weak and variable *in vitro* (Sweatman & Collier, 1968). When PGE_2 is administered by nebuliser to normal and asthmatic subjects *in vivo* it has also been shown to cause variable effects on airway calibre with an initial transient bronchoconstriction being followed by bronchodilatation (Walters & Davies, 1982).

We were surprised by the large quantities of PGE_2 produced by bovine airway smooth muscle cells in our experiments where serum stimulated PGE_2 production was as high as 490 ng/10^6 cells in a 24 h period. This is large compared to PGE_2 production in other tissues. For example, a study in human tracheal epithelial cells grown in 5% fetal calf serum showed PGE_2 production of 1.5 ng/10^6 cells over a 24 h period. We considered whether differences in the concentration of fetal calf serum used might be responsible. The cells in our study were cultured in medium containing 10% fetal calf serum. Serum contains many growth factors including epidermal growth and platelet-derived growth factor and these have both been shown to stimulate PGE_2 production from other cell types by inducing enzymes in the cyclo-oxygenase pathway (Habenicht *et al.*, 1985; Casey *et al.*, 1988). The differences in PGE_2 production (300 fold) between our study and the study in tracheal epithelial cells (Churchill *et al.*, 1989) would seem to be too large, however, to be due solely to the differences in the concentration of fetal calf serum used (10% vs 5%). Even in serum-free conditions, production of PGE_2 by our airway smooth muscle cells was in excess of that reported in airway epithelium. This suggests that airway smooth muscle may be a more important site of PGE_2 production than epithelial cells. There was some variability in the amount of PGE_2 produced by different cultures in our studies. This probably reflected differences in the degree of confluence when PGE_2 production was studied.

As smooth muscle cells can change their phenotypic properties in culture we performed experiments where cells were incubated with BRDU to prevent DNA synthesis prior to stimulating PGE_2 production with serum. We found no inhibitory effect of BRDU on PGE_2 production suggesting that PGE_2 production is not a function of proliferation *per se*.

In addition to quantifying PGE_2 production by airway smooth muscle cells, we were interested in looking at the contrasting effects of three inhibitors of cyclo-oxygenase. PGE_2 production was inhibited by the three different cyclo-oxygenase inhibitors that we used in a concentration-dependent manner. We studied the effects of these inhibitors on PGE_2 production after 6 days in culture as initial standardization experiments showed that production per cell was large at that time. All three inhibitors, flurbiprofen, indomethacin and acetyl salicylic acid inhibited PGE_2 production. Flurbiprofen was ten times more potent than indomethacin and 500 times more potent than acetyl salicylic acid. Several previous studies have examined the effect of these three inhibitors in other tissues and shown a similar order of potency. In a study of guinea-pig lung homogenates *in vitro* (Masumoto & Masuda, 1976), flurbiprofen was found to be five times more potent than indomethacin and 100 times more potent than acetyl salicylic acid. In other tissues, and species however, these agents have been shown to have different potencies. In a cell-free homogenate of rabbit renal medulla, flurbiprofen was equipotent with indomethacin and 300 times more potent than aspirin (Crook *et al.*, 1976). In platelets the three agents were almost equipotent at inhibiting prostaglandin synthesis (Cockbill *et al.*, 1979). The varying results of the above studies indicate that the three inhibitors can have differential effects in different tissues with the IC_{50} values varying according to tissue type as well as the species studied. This may be due to the existence of different isoenzymes of cyclo-oxygenase in different tissues (Crook *et al.*, 1976). There are two cyclo-oxygenase (Cox) isoenzymes, Cox 1 and Cox 2 (Meade *et al.*, 1993). We were probably looking at Cox 1 in our studies.

We have considered whether the differences in potency of

the three prostaglandin synthesis inhibitors might explain their contrasting effects in asthma. It has been known for years that some asthmatic subjects respond to orally administered acetyl salicylic acid and similar drugs by bronchoconstriction (Szczeklik, 1976). However, recently Bianco and colleagues have shown that inhaled acetyl salicylic acid has protective effects in asthma and that its effects are additive with the diuretic agent frusemide. (Robuschi *et al.*, 1992; Bianco *et al.*, 1992). In contrast, oral indomethacin inhibits the protective effect of frusemide on exercise-induced bronchoconstriction (Pavord *et al.*, 1992a) giving rise to the hypothesis that frusemide may be acting by producing PGE_2. Oral flurbiprofen did not inhibit the protective effect of frusemide on exercise-induced bronchoconstriction in asthma (O'Connor *et al.*, 1993) but did inhibit the protective effect of frusemide on methacholine-induced bronchoconstriction in normal subjects (Polosa *et al.*, 1992). In our study acetyl salicylic acid was a relatively poor inhibitor of prostaglandin synthesis and it is therefore unlikely that the beneficial reported effects of inhaled aspirin in asthma are due to inhibition of prostaglandin synthesis. However, the potent effects of indomethacin on prostaglandin synthesis in airway smooth muscle cells *in vitro* would be consistent with its inhibitory effects on the action of frusemide *in vivo* if frusemide were acting through prostanoid mediated pathways.

The other implications of our study may be in aspirin-induced asthma which occurs in a small proportion of asthma patients. Several theories have been put forward to explain this, including an imbalance of constrictor and dilator prostaglandins or shunting of arachidonic acid metabolism or leukotriene synthesis (Szczeklik, 1976). The low potency of acetyl salicylic acid at inhibiting PGE_2 production would be consistent with the lack of effect of oral aspirin in the majority of patients. It may be that aspirin-sensitive asthmatic subjects are more sensitive to the effects of aspirin on prostaglandin synthesis.

In conclusion we have demonstrated that bovine airway smooth muscle cells produce large quantities of PGE_2 and that this can be inhibited by cyclo-oxygenase inhibitors. PGE_2 produced by airway smooth muscle may be an important negative modulator of inflammation in the airways.

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References

ANDERSON, W.H., KRAZANOWSKI, J.J., POLSON, J.B. & SZENTIVANYI, A. (1980). Prostaglandins as mediators of tachyphylaxis to histamine in canine tracheal smooth muscle. In *Advances in Prostaglandin and Thromboxane Research*. ed. Samuelsson, B., Ramwell, P.W. & Paoletti, R. pp. 995–1001. New York: Raven Press.

BIANCO, S., VAGHI, A., PIERONI, M.G., ROBUSCHI, M., REFINI, R.M. & SESTINI, P. (1992). Protective activity of inhaled non-steroid antiinflammatory drugs on bronchial responsiveness to ultrasonically nebulised water. *J. Allergy Clin. Immunol.*, **90**, 833–839.

CASEY, K.L., KARTE, K. & MACDONALD, P.C. (1988). EGF stimulation of PGE_2 biosynthesis in amnion cells. *J. Biol. Chem.*, **263**, 7846–7854.

CHAMLEY-CAMPBELL, J., CAMPBELL, G.R. & ROSS, R. (1979). The smooth muscle cell in culture. *Physiol. Rev.*, **59**, 1–61.

CHRISTMAN, B.W. & CHRISTMAN, J.W. (1990). PGE_2 decreases LTB_4 production in stimulated rat alveolar macrophages. *Am. Rev. Respir. Dis.*, **141**, A393.

CHURCHILL, L., CHILTON, F.H., RESAU, J.H., BASCOM, R., HUBBARD, W.C. & PROUD, D. (1989). Cyclo-oxygenase metabolism of endogenous arachidonic acid by cultured human tracheal epithelial cells. *Am. Rev. Respir. Dis.*, **140**, 449–459.

COCKBILL, S.R., HEPTINSTALL, S. & TAYLOR, P.M. (1979). Comparison of the abilities of acetylsalicylic acid, flurbiprofen and indomethacin to inhibit the release reaction and prostaglandin synthesis in human blood platelets. *Br. J. Pharmacol.*, **67**, 73–78.

COLEMAN, R.A., KENNEDY, I., HUMPHREY, P.P.A., BUNCE, K. & LUMLEY, P. (1990). *Prostanoids and their Receptors. Comprehensive Medicinal Chemistry*. Vol. 3. ed. Emmett, J.C. New York, Oxford: Pergamon Press.

CROOK, D., COLLINS, A.J. & ROSE, A.J. (1976). A comparison of the effect of flurbiprofen on prostaglandin synthetase from human rheumatoid synovium and enzymatically active animal tissues. *J. Pharm. Pharmacol.*, **28**, 535.

FELBEL, J., TROCKUR, B., ECKER, T., LANDGRAF, W. & HOFMANN, F. (1988). Regulation of cytosolic calcium by cAMP and cGMP in freshly isolated smooth muscle cells from bovine trachea. *J. Biol. Chem.*, **263**, 16764–16771.

GIEMBYCZ, M.A., KROEGEAL, C. & BARNES, P.J. (1990). PGE_2 inhibits platelet activating factor-induced eosinophil activation. *Am. Rev. Respir. Dis.*, **141**, A396.

HABENICHT, A.J.R., GOERIG, M., GRULICH, H., ROTHE, D., GRONWALD, R., LOTH, U., SCHETTLER, G., KOMMERELL, B. & ROSS, R. (1985). Human PDGF stimulates prostaglandin synthesis by activation and by rapid *de novo* synthesis of cyclo-oxygenase. *J. Clin. Invest.*, **75**, 1381–1387.

HAWTHORNE, A.B., MAHIDA, Y.R., COLE, A.T. & HAWKEY, C.J. (1991). Aspirin induced gastric mucosal damage: prevention by enteric coating and relation to prostaglandin synthesis. *Br. J. Clin. Pharmacol.*, **32**, 77–83.

HAYE-LEGRAND, I., CERRINA, J., RAFFESTIN, B., LABAT, C., BOULLET, C., BAYOL, A., BENVENISTE, J. & BRINK, C. (1986). Histamine contraction of isolated human airway muscle preparations: role of prostaglandins. *J. Pharmacol. Exp. Ther.*, **239**, 536–541.

ITO, I., SUZUKI, H., AIZAWA, H., HIROSE, T. & HAKODA, H. (1990). Pre-junctional inhibitory action of PGE_2 on excitatory neuroeffector transmission in the human bronchus. *Prostaglandins*, **39**, 639–655.

KAMM, K.E., HSU, L.-C., KUBOTA, Y. & STULL, J.T. (1989). Phosphorylation of smooth muscle myosine heavy and light chains. *J. Biol. Chem.*, **264**, 21223–21229.

MASUMOTO, S. & MASUDA, C. (1976). Inhibitory effect of 2-(2-fluoro-4-biphenyl) propionic acid (Flurbiprofen) on prostaglandin synthesis. *Folia Pharmacol Japonica*, **72**, 1025–1031.

MEADE, E.A., SMITH, W.L. & DE WITT, D.L. (1993). Differential inhibition of prostaglandin endoperoxide synthase (Cyclooxygenase) isozymes by aspirin and other non-steroidal anti-inflammatory drugs. *J. Biol. Chem.*, **268**, 6610–6614.

MINAKUCHI, R., WACHOLTZ, M.C., DAVIS, L.S. & LIPSKY, P.E. (1990). Delineation of the mechanism of inhibition of human T-cell activation by PGE_2. *J. Immunol.*, **145**, 2616–2625.

O'CONNOR, B.J., RIDGE, S.M., BARNES, P.J. & CHUNG, K.F. (1993). Inhibition of sodium metabisulphite-induced bronchoconstriction by frusemide in asthma: role of cyclooxyenase products. *Thorax*, (in press).

PASSARGIKLIAN, M., BIANCO, S. & ALLEGRA, L. (1976). Clinical, functional amd pathogenetic aspects of bronchial reactivity to prostaglandins $F_{2\alpha}$, E_1 and E_2. *Adv. Prostaglandin Thromb. Res.*, **1**, 461–475.

PASSARGIKLIAN, M., BIANCO, S., ALLEGRA, L., MOAVERO, N.E., PETRIGNI, G., ROBUSCHI, M. & GRUGNI, A. (1977). Aspects of bronchial reactivity to prostaglandins and aspirin in asthmatic patients. *Respiration*, **34**, 79–91.

PAVORD, I.D., WISNIEWSKI, A., MATHUR, R., WAHEDNA, I,. KNOX, A.J. & TATTERSFIELD, A.E. (1991). Effect of inhaled PGE_2 on bronchial reactivity to sodium metabisulphite and methacholine in patients with asthma. *Thorax*, **46**, 633–637.

PAVORD, I.D., WONG, C., WILLIAMS, J., WISNIEWSKI, A., SMYTH, E. & TATTERSFIELD, A.E. (1992a). Effect of inhaled prostaglandin E_2 on allergen-induced asthma. *Am. Rev. Respir. Dis.*, **145**, A54.

PAVORD, I.D., WISNIEWSKI, A. & TATTERSFIELD, A.E. (1992b). Inhaled frusemide and exercise induced asthma: evidence of a role for inhibitory prostanoids. *Thorax*, **47**, 797–800.

PETERS, S.P., SCHULMAN, E.S., MACGLASHAN, D.W., SCHLEIMER, R.P., NEWBALL, H.H. & LICHTENSTEIN, L.M. (1982). Pharmacological and biochemical studies of human lung mast cells. *J. Allergy Clin. Immunol.*, **68**, 150.

POLOSA, R., RAJAKULASINGRAM, K., PROSPERINI, G. & HOLGATE, S.T. (1992). Cyclo-oxygenase inhibition modulates changes in bronchial reactivity to methacholine with inhaled frusemide in normal subjects. *Thorax*, **47**, 853.

ROBUSCHI, M., SCURI, M., VAGHI, A., SPAGNOTTO, S., GAMBRARO, G., FAI, V., SESTINI, P. & BIANCO, S. (1992). Inhaled acetylsalicylic acid enhances the protective activity of frusemide against exercise-induced bronchoconstriction. *Am. Rev. Respir. Dis.*, **145**, A729.

SWEATMAN, W.J.F. & COLLIER, H.O.J. (1968). Effects of prostaglandins on human bronchial muscle. *Nature*, **217**, 69–70.

SZCZEKLIK, A., GRYGLEWSKI, R.J., CZERNIAWSKA-MYSIK, G. & ZIMUDA, A. (1976). Aspirin-induced asthma. *J. Allergy Clin. Immunol.*, **58**, 10–18.

TANAKA, H., WATANABE, K., TAMARU, N., NISHIDA, T., ISHIBASHI, M. & YOSHIDA, M. (1993). The correlation between intracellular calcium and PGE_2 production by porcine tracheal smooth muscle cells in culture. *Am. Rev. Respir. Dis.*, **147**, A846.

WALTERS, E.H. & DAVIES, B.H. (1982). Dual effect of PGE_2 on normal airway smooth muscle in vivo. *Thorax*, **37**, 918–922.

WHITE, S.R., SIGRIST, K.S. & SPAETHE, M. (1992). Topographic distribution of prostaglandin secretion caused by bradykinin in canine tracheal epithelial cells. *Am. J. Respir. Cell Mol. Biol.*, **6**, 375–381.

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Sensitivity of G-protein involved in endothelin-1-induced Ca^{2+} influx to pertussis toxin in porcine endothelial cells *in situ*

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1 We designed a new method to determine quantitatively the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in endothelial cells *in situ*, using front-surface fluorometry and fura-2-loaded porcine aortic valvular strips. Using this method, we investigated the characteristics of the G-protein involved in endothelin-1 (ET-1)-induced changes in $[Ca^{2+}]_i$ of endothelial cells *in situ*.

2 Endothelial cells were identified by specific uptake of acetylated-low density lipoprotein labelled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL). Double staining with DiI-Ac-LDL and fura-2 showed that the valvular strip was covered with a monolayer of endothelial cells and that the cellular component which contributed to the fura-2 fluorescence, $[Ca^{2+}]_i$ signal, was exclusively endothelial cells.

3 ET-1 (10^{-7} M) induced an elevation of $[Ca^{2+}]_i$ consisting of two components: the first was a rapid and transient elevation to reach a peak, followed by a second, sustained elevation (the second phase). The first phase was composed of extracellular Ca^{2+}-independent and -dependent components, while the second phase was exclusively extracellular Ca^{2+}-dependent. The extracellular Ca^{2+}-independent component of the first phase was due to the release of Ca^{2+} from intracellular storage sites. The second phase and part of the first phase of $[Ca^{2+}]_i$ elevation were attributed to the influx of extracellular Ca^{2+}. The Ca^{2+} influx component was completely inhibited by 10^{-3} M Ni^{2+} but was not affected by 10^{-5} M diltiazem.

4 Pertussis toxin (IAP) markedly inhibited the extracellular Ca^{2+}-dependent elevation of $[Ca^{2+}]_i$, but had no effect on the extracellular Ca^{2+}-independent elevation of $[Ca^{2+}]_i$ caused by ET-1 (10^{-7} M).

5 Bradykinin (10^{-7} M) or ATP (10^{-5} M) elevated $[Ca^{2+}]_i$ and these responses also consisted of extracellular Ca^{2+}-independent and extracellular Ca^{2+}-dependent components. IAP had no effect on either component of the $[Ca^{2+}]_i$ elevation induced by bradykinin or ATP.

6 From these findings we conclude that, in porcine endotheliel cells *in situ*, ET-1 elevates $[Ca^{2+}]_i$ as a result of a Ca^{2+} influx component from the extracellular space and release of intracelluarly stored Ca^{2+}. The Ca^{2+} influx is regulated by an IAP-sensitive G-protein, while the release of Ca^{2+} from the intracellular store is not.

Keywords: ATP; bradykinin; calcium; diltiazem; endothelial cells; endothelin; G-protein; nickel; pertussis toxin

Introduction

Endothelial cells play an important role in the regulation of tone of vascular smooth muscle by releasing several active substances, the production of which is modulated by changes in the cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$, in the endothelial cells (Newby & Henderson, 1990). Thus, it is essential to examine endothelial $[Ca^{2+}]_i$ when investigating the role of these cells in the regulation of vascular tone. However, in almost all studies relating to $[Ca^{2+}]_i$ transients, cultured endothelial cells have been used because of difficulties in measuring $[Ca^{2+}]_i$ in endothelial cells *in situ*. Since there is evidence of some alteration in protein composition, in surface structure (Børsum *et al.*, 1982) and in secretory function of endothelial cells during culture (Hennig *et al.*, 1989), it is important to devise a method to measure quantitatively $[Ca^{2+}]_i$ exclusively in mature *in situ* endothelial cells. We designed such a method using front-surface fluorometry (Kodama *et al.*, 1989) and fura-2-loaded valvular strips of the pig (Aoki *et al.*, 1991). We confirmed that the endothelial cells on the aortic valvular strip remained intact, both morphologically and functionally. Using this method, we investigated the mechanisms of changes in $[Ca^{2+}]_i$ induced by endothelin-1 (ET-1) in endothelial cells *in situ*.

Since the discovery of endothelin (Yanagisawa *et al.*, 1988) as a potent constrictor of vascular smooth muscle, a broad spectrum of the effects of these peptides was proposed. Several authors have reported that ET-1-induced elevation of inositol phosphates elevated $[Ca^{2+}]_i$ (Vigne *et al.*, 1990) and stimulated the secretion of endothelium-derived relaxing factor (EDRF) and prostaglandin I_2 (De Nucci *et al.*, 1988) in endothelial cells. As mRNA encoding only ET-1 but not ET-2 or ET-3 is expressed in endothelial cells (Vane, 1990), ET-1 may play an important role in the vascular system to regulate endothelial cells in an autocrine manner, in addition to the paracrine control of vascular smooth muscle tone (Ushio-Fukai *et al.*, 1992). Thus, it is of great interest to elucidate intracellular mechanisms, especially $[Ca^{2+}]_i$ transients activated by ET-1 in endothelial cells.

While involvement of a guanine nucleotide binding protein (G-protein) in the signal transduction pathway of ET-1 has been proposed, it is a matter of controversy whether or not this G-protein is pertussis toxin (IAP)-sensitive; results showing that it is IAP-sensitive (Kelly *et al.*, 1990; Thomas *et al.*, 1991; Marin *et al.*, 1991), and that it is not IAP-sensitive (Takuwa *et al.*, 1990; Lysko *et al.*, 1991) have been described. In the case of endothelial cells, the inhibitory effect of ET-1 on adenylate cyclase was found to be IAP-sensitive, thereby indicating the involvement of IAP-sensitive G-protein (Ladoux & Frelin, 1991). Using endothelial cells *in situ* we have obtained evidence favouring the idea that IAP-sensitive G-protein is coupled in the signal transduction pathway involved in the ET-1-induced influx of extracellular Ca^{2+} but not in ET-1-induced intracellular Ca^{2+} release.

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Methods

Preparation of valvular and vascular medial strips

Hearts and aortic roots from adult pigs of either sex were obtained from a local slaughterhouse immediately after the animals had been killed. These hearts and roots were placed in ice-cold normal physiological salt solution (PSS) and brought to the laboratory. The aortic valve leaflets were cut with special care not to touch their surface. The leaflets were cut in parallel with midline to make approximately 2 mm (width) × 5 mm (length) valvular strips. The centre of each leaflet, *corpus arantii* (Barry & Patten, 1960) was not used. The thickness of the strips was 0.18 ± 0.09 mm ($n = 14$). When indicated, these valvular strips were incubated for 1–18 h (37°C) in normal physiological salt solution (PSS) gassed with 5% CO_2 and 95% O_2 containing IAP (100–1000 ng ml^{-1}) before fura-2 loading.

To prepare medial strips, left circumflex coronary arteries were isolated and segments 2–3 cm from the origin were excised. To remove the endothelium, the luminal surface was rubbed with a cotton swab (Furchgott & Zawadzki, 1980). After removal of the adventitia, medial preparations were cut into approximately 1 mm (width) × 5 mm (length) × 0.1 mm (thickness) circular strips.

Fura-2 loading of valvular strips

The strips were incubated for 90 min (37°C) in gassed (5% CO_2, 95% O_2) Dulbecco's modified Eagles medium containing 5% fetal bovine serum, 10^{-3} M probenecid (Di Virgilio *et al.*, 1989) and 5×10^{-5} M acetoxymethyl ester of fura-2 (fura-2/AM). After loading with fura-2, the strips were washed at least 5 times by normal PSS and equilibrated for 1 h at room temperature. Each strip was mounted vertically in a quartz organ bath filled with normal PSS gassed with 5% CO_2 and 95% O_2. The experimental procedures were carried out at 25°C to prevent leakage of the fluorescence dye (Kobayashi *et al.*, 1986).

Identification of endothelial cells

Endothelial cells were identified by their specific uptake of acetylated-low density lipoprotein labelled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL) (Netland *et al.*, 1985). Valvular strips were incubated in gassed (5% CO_2, 95% O_2) Dulbecco's modified Eagles medium containing 10 μg ml^{-1} DiI-Ac-LDL for 2.5 h at 37°C. To this incubation medium, fura-2/AM dissolved in dimethyl sulphoxide (final concentration 5×10^{-5} M), fetal bovine serum (5%) and probenecid (10^{-3} M) were added, and the incubation was continued another 1.5 h. After double staining, the valvular strips were washed at least 5 times with normal PSS, and then, mounted on slide glass and examined under a fluorescence microscope (Axioskop; Zeiss, Germany) equipped with a water-immersion objective system (Plan-Neofluar 40, Zeiss) and an appropriate combination of filters. Fluorescence of DiI was observed through a 520 mm long-pass filter (LP 520, Zeiss) by illuminating the tissue with an excitation light from xenon light source through a 485 nm band-pass filter (BP 485, Zeiss). Fluorescence of fura-2 was observed through a 500–530 nm band-pass filter (BP 500–530, Zeiss) by illuminating the tissue with an excitation light through a 340 nm band-pass filter (BP 340, Zeiss).

Front-surface fluorometry

Changes in $[Ca^{2+}]_i$ were monitored with a front-surface fluorometer (CAM-OF-1; designed in collaboration with Japan Spectroscopic Co., Tokyo, Japan), as described in detail elsewhere (Hirano *et al.*, 1990). In brief, the aortic surface of the valvular strip was illuminated by guiding the alternating (400 Hz), 340 nm and 380 nm, excitation light from a xenon light source through quartz optic fibres arranged in a concentric inner circle (diameter: 3 mm). Surface fluorescence of the strip was collected by glass optic fibres arranged in an outer circle (diameter: 7 mm) and introduced through a 500 nm band-pass filter into a photon-counting photomultiplier. Fluorescence intensities (500 nm) at 340 nm (F340) and 380 nm (F380) excitation and its ratio (R = F340/F380) were continuously monitored. $[Ca^{2+}]_i$ was calculated according to the method described by Grynkiewicz *et al.* (1985). The apparent dissociation constant (K_d) of the fura-2:Ca^{2+} complex at 25°C was $1.62 \pm 0.08 \times 10^{-7}$ M, determined spectroscopically (Fluorescence Spectrophotometer 650-40; HITACHI, Japan). All data were collected using a computerized data acquisition system (MacLab; Analog Digital Instruments, Australia, Macintosh; Apple Computer, U.S.A.).

Normalization of fluorescence signals

The response of fluorescence ratio (F340/F380; $[Ca^{2+}]_i$) were normalized for those obtained with 10^{-5} M ATP, which was applied at least 15 min prior to each experimental measurement for 1 min, for the following reasons: (1) during repetitive applications of 10^{-5} M ATP for 1 min at 15 min intervals, each application induced a rapid and transient elevation of $[Ca^{2+}]_i$, to an equal extent. When the concentration exceeded 10^{-4} M, repetitive applications of ATP for 1 min at intervals of 30 min also induced repetitive increases in $[Ca^{2+}]_i$ with a progressive reduction of peak levels (data not shown). Thus, the application of 10^{-5} M ATP for 1 min had no effect on the elevation of $[Ca^{2+}]_i$ induced by similar stimulation applied 15 min later. (2) The peak level of the $[Ca^{2+}]_i$ elevation induced by ATP was concentration-

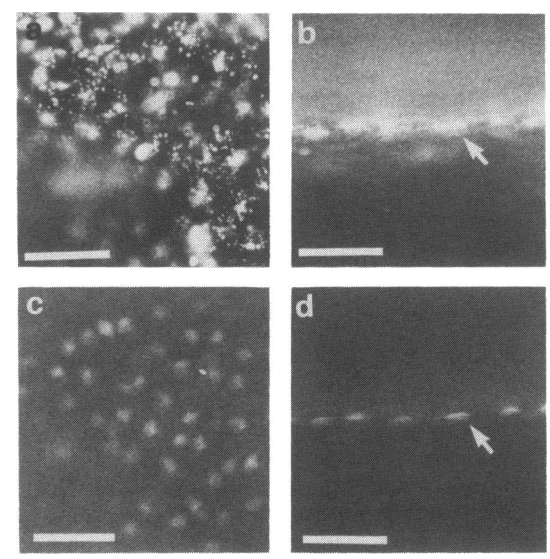


Figure 1 Fluorescence photomicrographs (× 400) of endothelial cells *in situ* doubly stained with DiI-Ac-LDL and fura-2. 1,1'-Dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyamine perchlorate (DiI-Ac-LDL) (a,b) and fura-2 (c,d) fluorescence were observed at (485 nm excitation, 520 nm < emission), and (340 nm excitation, 500–530 nm emission), respectively. (a) The surface aspect of the valvular strip had a typical cobble stone appearance with granular fluorescence of DiI-Ac-LDL, indicating that these were endothelial cells. The photograph is out of focus in part because of the folds on the valvular leaflet. (b) Trans-sectional observation of the valvular strip revealed that DiI-stained cells (arrow) are present only on the surface, in a single layer. (c) The surface aspect of characteristic fluorescence of fura-2 indicated that fura-2 was loaded only into the endothelial cells on the vulvular strip. (d) Trans-sectional view of fura-2 fluorescence indicated that it originated only from cells (arrow) on the surface of the valve. No other cells staining positively with fura-2 were observed under the endothelial lining. Each bar indicates 50 μm.

dependent (10^{-6}–3×10^{-4} M, $EC_{50} = 1 \times 10^{-5}$ M). (3) There were no such responses when the surface of the valvular strip had been rubbed with a cotton swab, thereby indicating that the fluorescence signal induced by ATP originated exclusively from endothelial cells on the surface of the valvular strips. (4) When the strips were treated with 300 ng ml^{-1} IAP for up to 18 h, neither the resting level nor the peak level of the ATP (10^{-5} M)-induced $[Ca^{2+}]_i$ transient was changed (data not shown). Accordingly, at least 15 min prior to each measurement, the response to ATP (10^{-5} M) was registered, the resting level and the peak level of the fluorescence ratio induced by ATP being designated 0% and 100%, respectively. Absolute values of $[Ca^{2+}]_i$ levels observed during 10^{-5} M ATP application were determined separately by the method of Grynkiewicz *et al.* (1985), and $[Ca^{2+}]_i$ levels at 0% and 100% were $6.53 \pm 0.75 \times 10^{-8}$ M and $18.59 \pm 2.08 \times 10^{-8}$ M, respectively ($n = 10$). Since a destructive and irreversible step was involved in the determination of absolute $[Ca^{2+}]_i$ levels in endothelial cells, that is, endothelial cells had to be permeabilized by ionomycin, which increased the chances of deviation of the data on $[Ca^{2+}]_i$, we expressed the response as a percentage of the response to 10^{-5} M ATP obtained under physiological conditions given in the text.

Experimental procedures

To avoid the possible induction of a $[Ca^{2+}]_i$ transient that could be caused by mechanical stimulation of the endothelial cells (Lansman *et al.*, 1987; Olesen *et al.*, 1988) during total exchange of the incubation solution, ET-1, bradykinin, adenosine triphosphate (ATP), and $CaCl_2$ in 60 µl were added and mixed in the organ bath (6 ml). When measuring the effects of ET-1, bovine serum albumin (BSA) was added to the stock solution (final concentration 0.002%) to maintain constant the concentration of the peptide in the solution. BSA did not affect fluorescence signals.

Solutions

The composition ($\times 10^{-3}$ M) of the normal PSS was: NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.25 and D-glucose 11.5. The Ca^{2+}-free PSS did not contain $CaCl_2$, and when indicated, contained 2×10^{-3} M ethyleneglycol-*bis*(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA).

Chemicals

Synthetic ET-1 and bradykinin were obtained from the Peptide Institute Co. Ltd. (Osaka, Japan), sodium salt of ATP was purchased from Boehringer Mannheim GmbH (Germany), fura-2/AM and EGTA were purchased from Dojindo (Kumamoto, Japan), probenecid and BSA were obtained from SIGMA (St. Louis, MO, U.S.A.), IAP was purchased from Seikagaku Co. (Tokyo, Japan). DiI-Ac-LDL was obtained from FUNAKOSHI (Tokyo, Japan). Diltiazem hydrochloride was kindly donated by Tanabe Seiyaku Co. (Osaka, Japan). All other chemicals were from Katayama Chemical (Osaka, Japan).

Statistical analysis

Values were expressed as means ± standard error. Student's *t* test was used to determine statistical significance and *P* values less than 0.05 were considered to be significant.

Results

Identification of endothelial cells

On the surface of the valvular strips there was positive staining of DiI fluorescence in cells with a cobble-stone lining (Figure 1a). Trans-sectional observation revealed that these cells with DiI fluorescence formed a monolayer on the strip (Figure 1b) and these cells with positive DiI staining also showed the typical fluorescence of fura-2 (Figure 1c and d). No cellular component other than endothelial cells showed fura-2 fluorescence.

We examined the functional integrity of the endothelial cells on the valvular strip by measuring the activity of endothelium-derived relaxing factor(s), using a bioassay technique (Furchgott & Zawadzki, 1980). The medial strip and the fura-2 loaded valvular strip were vertically and separately mounted on strip holders and brought into contact in a quartz organ bath containing gassed (95% O_2 and 5% CO_2 at 25°C) normal PSS. Application of 10^{-7} M bradykinin (BK) caused marked elevation of the fluorescence signal from the valvular strip and the precontracted medial strip relaxed (data not shown). These results confirmed the presence of morphologically and functionally intact endothelial cells in valvular strips prepared for this study.

Effect of IAP on ET-1-induced Ca^{2+} transients in normal PSS

As shown in Figure 2, in the presence of extracellular Ca^{2+}, 10^{-7} M ET-1 caused a rapid elevation of $[Ca^{2+}]_i$ which reached a peak level (221.7 ± 17.5%, $n = 7$) (the first phase) at 1 min. Then, $[Ca^{2+}]_i$ gradually declined to a sustained elevation (the second phase) and it was 39.7 ± 4.9% ($n = 7$) at 10 min. Pretreatment with IAP (300 ng ml^{-1}, for 3 h) markedly inhibited both the first and the second phases of elevation of $[Ca^{2+}]_i$ induced by 10^{-7} M ET-1. In IAP-treated samples, the level of $[Ca^{2+}]_i$ was 74.6 ± 17.1% at 1 min (the first phase, $P < 0.001$) and 5.5 ± 1.4% at 10 min (the second phase, $P < 0.05$), respectively ($n = 7$).

The inhibitory effect of IAP on the first phase of ET-1-induced $[Ca^{2+}]_i$ elevation was time- and concentration-

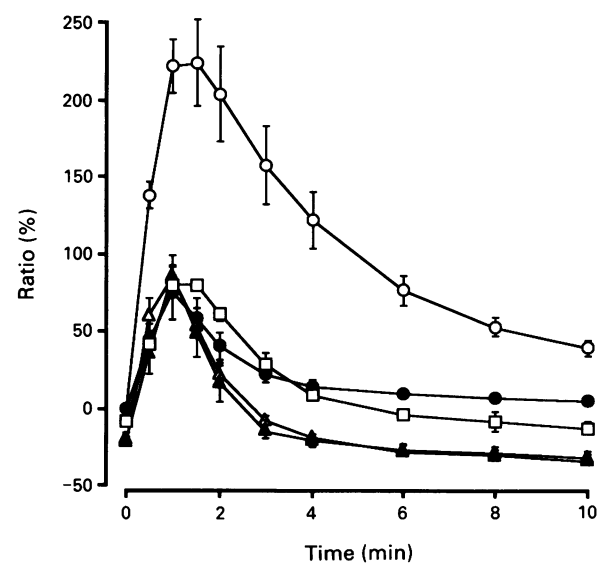


Figure 2 Effects of pertussis toxin (IAP) on the time course of 10^{-7} M endothelin-1 (ET-1)-induced changes in $[Ca^{2+}]_i$ in normal PSS and in Ca^{2+}-free PSS, and effects of Ni^{2+}. Symbols: without (○) and with (●) treatment with IAP in normal PSS, without (△) and with (▲) treatment with IAP in Ca^{2+}-free PSS, and without treatment with IAP in normal PSS containing 1×10^{-3} M Ni^{2+} (□). Ni^{2+} and Ca^{2+}-free PSS had nearly the same effects on the elevation of $[Ca^{2+}]_i$ induced by endothelin-1 (ET-1). IAP suppressed ET-1-induced changes in $[Ca^{2+}]_i$ in normal PSS, while no such effect was observed in Ca^{2+}-free PSS. The peak level of $[Ca^{2+}]_i$ induced by ET-1 in normal PSS with IAP treatment overlapped with those in Ca^{2+}-free PSS with or without IAP treatment and that in normal PSS containing Ni^{2+} without IAP treatment. Vertical bars show s.e. of means ($n = 6$–7).

dependent. The maximum effect was observed in strips treated with 300 ng ml^{-1} IAP for 3 h. No further effect was observed in samples treated with higher concentrations (up to 1000 ng ml^{-1}) of IAP or longer incubation time (data not shown). Thus all experiments in this study were carried out under this condition (IAP 300 ng ml^{-1}, 3 h).

Effect of IAP on ET-1-induced Ca^{2+} transients in Ca^{2+}-free PSS

Complete change of the medium, i.e. adding fresh medium after evacuating used medium, induced a small transient elevation of $[Ca^{2+}]_i$, as observed in the case of the change from normal PSS to Ca^{2+}-free PSS containing 2×10^{-3} M EGTA (Figures 3b, 4a–e). However, even a rapid mixing or stirring of the medium induced no elevation in $[Ca^{2+}]_i$. When the valvular strip was exposed to Ca^{2+}-free PSS containing 2×10^{-3} M EGTA after evacuation of normal PSS, after a transient rise, $[Ca^{2+}]_i$ gradually declined to reach a new steady level ($-21.6 \pm 2.8\%$) at 5 min. In this condition, the application of ET-1 (10^{-7} M) caused a rapid elevation of $[Ca^{2+}]_i$ to form a peak at 1 min but which was lower than that observed in normal PSS ($P < 0.01$). The second phase was not observed in Ca^{2+}-free PSS containing 2×10^{-3} M EGTA. When the valvular strip was exposed to normal PSS containing 10^{-3} M Ni^{2+}, an inorganic Ca^{2+} entry blocker, the

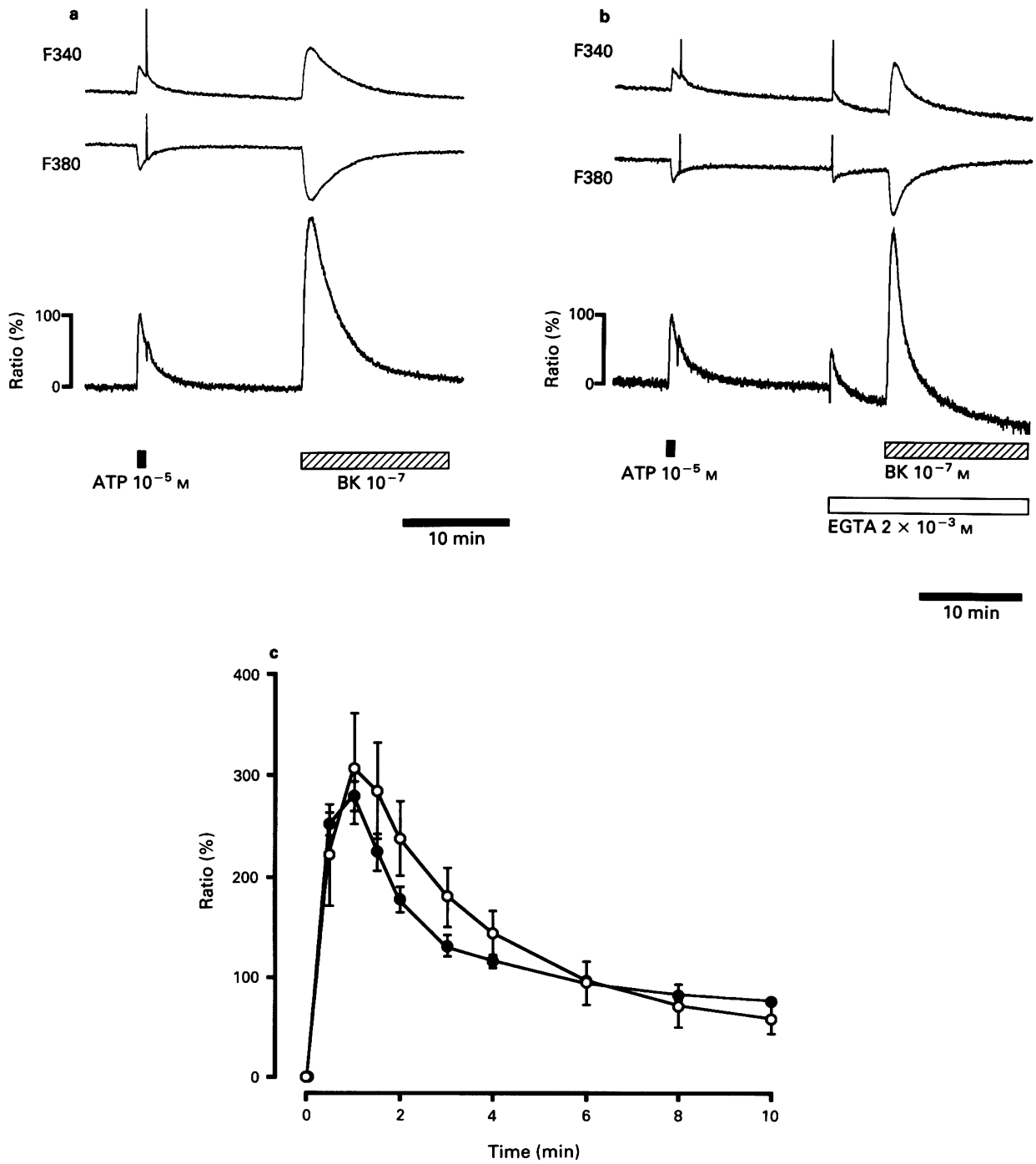


Figure 3 Representative recordings of the time course (of 3 similar results) of the changes in $[Ca^{2+}]_i$ induced by 10^{-7} M bradykinin (BK) (hatched bar) in normal PSS (a) and Ca^{2+}-free PSS (b), and the effect of pertussis toxin (IAP) on the time course of elevation of $[Ca^{2+}]_i$ induced by 10^{-7} M BK (c). (a) In normal PSS BK caused a rapid and transient elevation of $[Ca^{2+}]_i$ followed by a sustained elevation. (b) In Ca^{2+}-free PSS the sustained phase was not observed. (c) Valvular strips were incubated in PSS without (○) and with (●) 300 ng ml^{-1} IAP for 3 h. There was no apparent effect of IAP on BK-induced changes in $[Ca^{2+}]_i$. Vertical bars show s.e. of mean ($n = 3$).

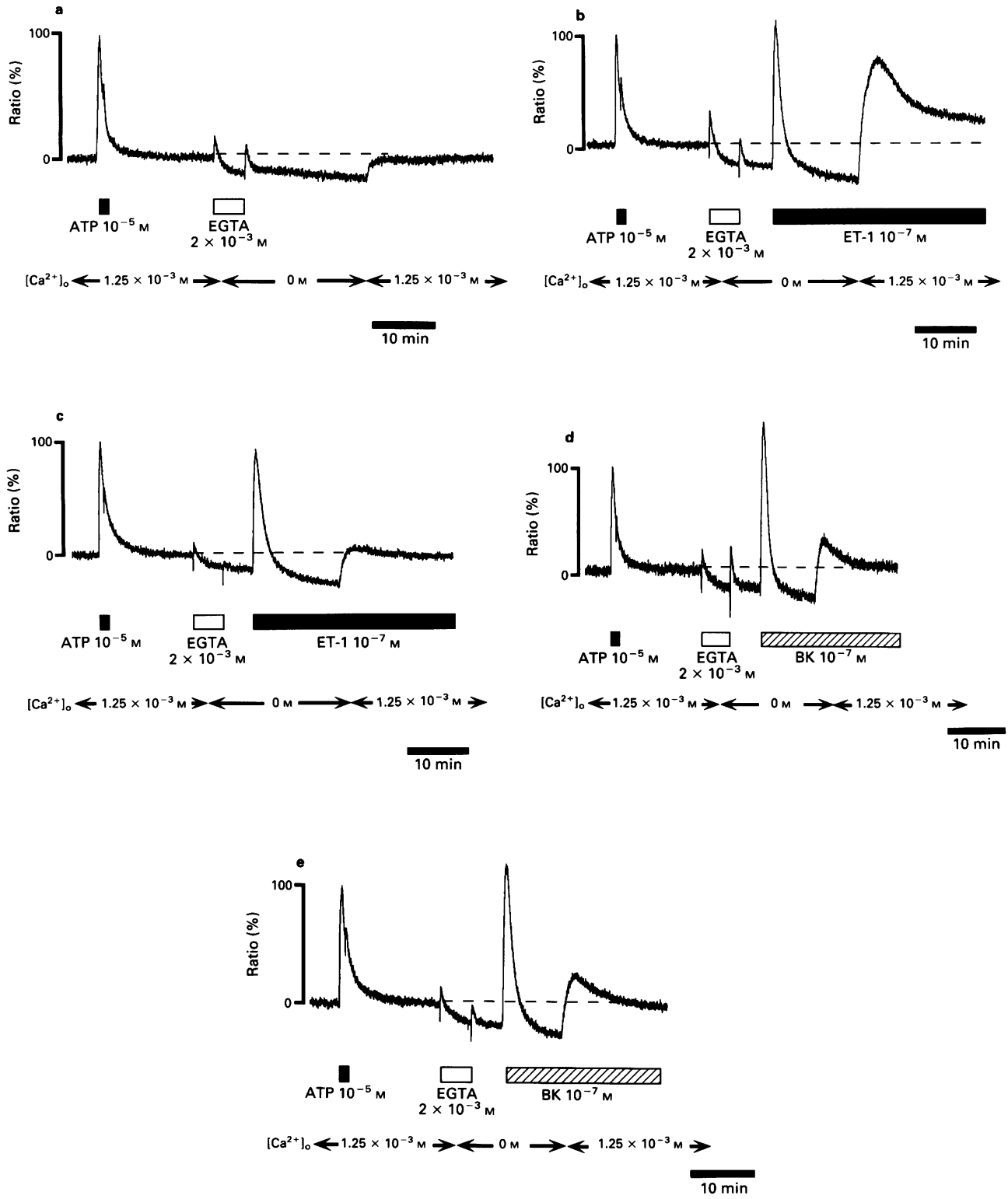


Figure 4 Representative recordings of the time courses (of 4 similar results) of $[Ca^{2+}]_i$ during restoration of extracellular Ca^{2+} after exposure of valvular strips to Ca^{2+}-free PSS in the absence of any agonist (a), in the presence of either 10^{-7} M endothelin-1 (ET-1) without (b) and with (c) pertussis toxin (IAP) pretreatment, or 10^{-7} M bradykinin (BK) without (d) and with (e) IAP pretreatment. (a) Extracellular Ca^{2+} was restored after exposure of the valvular strip to Ca^{2+}-free PSS. When extracellular Ca^{2+} was restored from 0 to 1.25×10^{-3} M, $[Ca^{2+}]_i$ gradually returned to the resting level. (b) Extracellular Ca^{2+} was restored after the application of 10^{-7} M ET-1 (stippled bar). ET-1 caused rapid, transient and extracellular Ca^{2+}-independent elevation of $[Ca^{2+}]_i$. Restoration of extracellular Ca^{2+} in the presence of 10^{-7} M ET-1 caused a relatively rapid elevation of $[Ca^{2+}]_i$ which was higher than the resting level. (c) Experimental procedure was similar to (b) except for pretreatment of the valvular strip with 300 ng ml^{-1} IAP for 3 h. Note that 10^{-7} M ET-1 caused an extracellular Ca^{2+}-independent elevation of $[Ca^{2+}]_i$ but failed to cause extracellular Ca^{2+}-dependent elevation observed in (b). (d) Extracellular Ca^{2+} was restored after the application of 10^{-7} M BK (hatched bar). BK caused a rapid, transient and extracellular Ca^{2+}-independent elevation of $[Ca^{2+}]_i$. Restoration of extracellular Ca^{2+} in the presence of 10^{-7} M BK caused a relatively rapid elevation of $[Ca^{2+}]_i$, which was higher than the resting level. (e) Experimental procedure was similar to (d) except for pretreatment of the valvular strip with 300 ng ml^{-1} IAP for 3 h. Pretreatment with IAP revealed no effect on extracellular Ca^{2+}-independent or dependent elevation of $[Ca^{2+}]_i$ caused by 10^{-7} M BK.

time course of changes in $[Ca^{2+}]_i$ induced by 10^{-7} M ET-1 was nearly the same as that observed in Ca^{2+}-free PSS containing 2×10^{-3} M EGTA ($P = 0.77$ at 1 min; Figure 2). On the other hand, 10^{-5} M diltiazem, an organic Ca^{2+} channel blocker, had no effect on the time course of the changes in $[Ca^{2+}]_i$ induced by ET-1 (data not shown). Figure 2 shows the effect of IAP treatment on the time course of $[Ca^{2+}]_i$ changes induced by 10^{-7} M ET-1 in the presence and absence of extracellular Ca^{2+}. When the valvular strip was treated with IAP, both the first and the second phase of $[Ca^{2+}]_i$ elevation induced by 10^{-7} M ET-1 in the presence of extracellular Ca^{2+} were suppressed. In particular, the second phase was inhibited almost completely. Moreover, the time course and the extent of the ET-1-induced Ca^{2+} transient in the presence of extracellular Ca^{2+} in strips treated with IAP ($74.6 \pm 17.1\%$, $n = 7$) overlapped with that in the absence of extracellular Ca^{2+}, which depended on the release of intracellularly stored Ca^{2+} and was not affected by treatment with IAP ($83.8 \pm 8.8\%$ ($n = 7$) without IAP-treatment ($P = 0.64$) and $85.6 \pm 12.9\%$ ($n = 6$) with IAP-treatment ($P = 0.63$)). Thus, $[Ca^{2+}]_i$ elevation, induced by ET-1 and being dependent on the influx of extracellular Ca^{2+}, was completely inhibited by IAP treatment.

Effect of IAP on bradykinin-induced Ca^{2+} transients

Bradykinin (BK), as well as ET-1, caused a biphasic elevation of $[Ca^{2+}]_i$, which consisted of two components in the presence of extracellular Ca^{2+} (Figure 3a). BK (10^{-7} M) caused an initial rapid elevation of $[Ca^{2+}]_i$ to reach a peak level ($307.1 \pm 54.7\%$, $n = 3$) at 1 min (the first phase), which was followed by a gradual decrease to reach a sustained elevation ($58.2 \pm 15.5\%$, $n = 3$) at 10 min (the second phase). In Ca^{2+}-free PSS containing 2×10^{-3} M EGTA, the first phase was slightly smaller than that observed in normal PSS, and no second phase was observed (Figure 3b). Although the pattern of BK-induced Ca^{2+} elevation was similar to that caused by ET-1, pretreatment with IAP (300 ng ml^{-1}, 3 h) had no effect either on the first phase ($278.9 \pm 14.4\%$, $n = 3$, $P = 0.28$) or on the second phase ($74.9 \pm 2.5\%$, $n = 3$, $P = 0.59$) of $[Ca^{2+}]_i$-transient induced by BK (Figure 3c).

Effect of restoration of extracellular Ca^{2+} to 1.25×10^{-3} M on the $[Ca^{2+}]_i$-transients induced by ET-1 or BK in Ca^{2+}-free PSS

As shown in Figure 4a, when the valvular strip was exposed to Ca^{2+}-free PSS (Ca^{2+}-free PSS containing 2×10^{-3} M EGTA for 5 min, and then, Ca^{2+}-free PSS), $[Ca^{2+}]_i$ gradually decreased to reach a new steady level at about 5 min. Subsequent restoration of extracellular Ca^{2+} to 1.25×10^{-3} M rapidly raised $[Ca^{2+}]_i$ followed by a reversion to the resting level and remained there. The application of 10^{-7} M ET-1 during exposure to Ca^{2+}-free PSS lead to a rapid elevation of $[Ca^{2+}]_i$ to form a peak at 1 min, then a decline to a level no higher than that observed before the application of ET-1 (Figure 4b). Subsequently, when extracellular Ca^{2+} was restored by adding 60 µl of 0.125 M $CaCl_2$ to the 6 ml bath (final concentration 1.25×10^{-3} M) in the presence of ET-1, $[Ca^{2+}]_i$ increased to form a peak much higher than the resting level and was followed by a sustained elevation. As this elevation higher than the resting level was not observed with the restoration of extracellular Ca^{2+} in the absence of ET-1 following exposure of strips to Ca^{2+}-free PSS (Figure 4a), this extracellular Ca^{2+}-dependent elevation was considered to be caused by ET-1-activated Ca^{2+} influx. Treating the valvular strip with 300 ng ml^{-1} IAP for 3 h almost completely abolished this extracellular Ca^{2+}-dependent elevation, while the initial extracellular Ca^{2+}-independent elevation of $[Ca^{2+}]_i$ induced by ET-1 was not affected (Figure 4c).

In the case of BK, restoration of extracellular Ca^{2+} in the presence of 10^{-7} M BK following exposure of the valvular strip to Ca^{2+}-free PSS led to an elevation of $[Ca^{2+}]_i$ higher than the resting level (Figure 4e). This extracellular Ca^{2+}- and BK-dependent elevation (i.e. BK-induced Ca^{2+} influx) was not affected by pretreatment with 300 ng ml^{-1} IAP for 3 h (Figure 4e).

Discussion

We have described in this report how to record $[Ca^{2+}]_i$ signals exclusively from endothelial cells *in situ*. We used aortic valvular strips and front-surface fluorometry of fura-2 to determine the changes in $[Ca^{2+}]_i$ of endothelial cells *in situ*. Double staining with DiI-Ac-LDL and fura-2 showed the presence of the cells positively stained with these dyes and forming a monolayer on the surface of the valvular strip. In trans-sectional observations of the doubly stained valvular strip, the fluorescence image of the cells obtained by DiI-fluorescence, which accumulates in lysosomal membrane in the cytosol of endothelial cells (Netland *et al.*, 1985), is similar to that obtained by fura-2-fluorescence. Under the monolayer of these endothelial cells, no other cellular components were observed, either with DiI or fura-2 fluorescence microscopy. Thus, the valvular preparation used in this study had intact endothelial cells and these cells were the only sources of fura-2 fluorescence. In addition, the vascular medial strip which was precontracted and brought into contact with this valvular strip showed relaxation in response to bradykinin, presumably via the production of endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980) or prostaglandin I_2 (Whorton *et al.*, 1982). Thus, endothelial cells of these valvular strips functioned normally, i.e. vasorelaxation and $[Ca^{2+}]_i$ signals could be obtained exclusively from the endothelial cells: it is well accepted that there is a cause and effect relationship between elevation of $[Ca^{2+}]_i$ and production of EDRF and/or PGI_2 in endothelial cells (Newby & Henderson, 1990).

We obtained evidence for the involvement of an IAP-sensitive G-protein in the signal transduction pathway activated by ET-1 in endothelial cells *in situ*. In normal PSS, ET-1 caused a rapid and transient elevation of $[Ca^{2+}]_i$ (the first phase) followed by a sustained elevation (the second phase). In Ca^{2+}-free PSS, the first phase was partially inhibited, while the second one was completely abolished. When the valvular strip was exposed to normal PSS containing 1×10^{-3} M Ni^{2+}, the first phase of the elevation of $[Ca^{2+}]_i$ induced by ET-1 was also partially inhibited and the second phase was abolished. The time course of the changes in $[Ca^{2+}]_i$ in the presence of the inorganic Ca^{2+} channel blocker was nearly the same as observed in Ca^{2+}-free PSS containing 2×10^{-3} M EGTA. Thus, part of the first phase was independent of extracellular Ca^{2+} and part of the first phase and all of the second phase were extracellular Ca^{2+}-dependent. An elevation of $[Ca^{2+}]_i$ which is independent of extracellular Ca^{2+} may be due to release of Ca^{2+} from intracellular storage sites (Newby & Henderson, 1990), presumably the endoplasmic reticulum, which was resistant to pretreatment with IAP. An increase in $[Ca^{2+}]_i$ which depends on extracellular Ca^{2+} may be due to the influx of Ca^{2+} via plasma membrane and was almost completely inhibited by IAP.

In various cell types ET-1 was found to mobilize Ca^{2+} from both intracellular and extracellular sources (Miasiro *et al.*, 1988; Takuwa *et al.*, 1990; Vigne *et al.*, 1990; Thomas *et al.*, 1991; Marin *et al.*, 1991). ET-1 binds to the specific receptor to activate phospholipase C (PLC), which catalyzes hydrolysis of phosphatidyl inositol bisphosphate into inositol trisphosphate ($InsP_3$) and diacylglycerol (Vigne *et al.*, 1990; Thomas *et al.*, 1991; Marin *et al.*, 1991). $InsP_3$ is believed to mediate the release of Ca^{2+} from intracellular storage site in endothelial cells (Newby & Henderson, 1990), presumably the endoplasmic reticulum. Simultaneously, ET-1 activates Ca^{2+} influx probably via Ca^{2+}-permeable channels (Goto *et al.*, 1989; Kodama *et al.*, 1989; Marin *et al.*, 1991). However, in endothelial cells, no L-type voltage-dependent Ca^{2+} chan-

nels were detectable in electrophysiological studies (Newby & Henderson, 1990). In the present study, an organic Ca^{2+} channel blocker, diltiazem had no effect on the ET-1-induced elevation of $[Ca^{2+}]_i$, thereby indicating that the L-type voltage-dependent Ca^{2+} channel is not involved in the ET-1-activated Ca^{2+} influx in endothelial cells *in situ* (Masaki *et al.*, 1991). Ca^{2+} may enter endothelial cells through non-selective cationic channels or 'receptor-operated' Ca^{2+} channels (Masaki *et al.*, 1991).

Recent studies indicate the involvement of a G-protein in ET-1-activated signal transduction pathways in various tissues. Using GTP or GDP analogues, other workers suggested the coupling of the ET receptor with G-protein(s) in vascular smooth muscle cells (Takuwa *et al.*, 1990), glomerular mesangial cells (Thomas *et al.*, 1991) and endothelial cells (Eguchi *et al.*, 1991). Moreover, the deduced amino-acid sequence of known endothelin receptors, ET_A (Arai *et al.*, 1990) and ET_B (Sakurai *et al.*, 1990) have seven putative transmembrane domains, which are common structures in the G-protein-coupled receptor superfamily. This finding also supports the notion of coupling between ET receptors and a G-protein. There is evidence indicating that a G-protein mediates coupling of the ET receptor and PLC (Takuwa *et al.*, 1990). G-proteins may also couple the ET receptor to the mechanism which mediates Ca^{2+} influx or may directly couple the receptor to ionic channels (Gilman, 1987). In the present study, IAP inhibited only the extracellular Ca^{2+}-dependent component of the Ca^{2+} transient induced by ET-1, a finding which suggests the coupling of the ET receptor to at least two separate G-proteins, in endothelial cells *in situ*. The G-protein that mediates the Ca^{2+} influx, presumably through non-selective cationic channels or other Ca^{2+} permeable channels, is IAP-sensitive. The G-protein that mediates Ca^{2+} release, presumably via the PLC/$InsP_3$ pathway, is IAP-insensitive. There is also the possibility that the ET receptor may directly couple to PLC without mediating G-protein (Ullrich & Schlessinger, 1990).

The involvement of IAP-sensitive G-protein(s) in the ET-1-activated signal transduction pathway remains controversial. In smooth muscle cells, IAP had no effect on the response to ET-1 such as breakdown of phosphatidylinositol (PI) and the changes in $[Ca^{2+}]_i$ (Mitsuhashi *et al.*, 1989; Takuwa *et al.*, 1990). In mouse striatal astrocytes (Marin *et al.*, 1991) and in rat mesangial cells (Thomas *et al.*, 1991), the responses to ET-1, such as elevation of $[Ca^{2+}]_i$ and PI turnover, are at least partially inhibited by IAP. In endothelial cells ETs inhibit adenylate cyclase via IAP-sensitive mechanisms (Ladoux & Frelin, 1991). Marin *et al.* (1991) reported the possible involvement of several G-proteins in the different actions of ET-1 in mouse striatal astrocytes. They concluded that Ca^{2+} influx is sensitive to IAP and PI turnover is insensitive, a proposal which agrees with our results, albeit with a different cell type. Thus, it is plausible that several G-proteins may be involved in the signal transduction of ET-1 in many different cell types. It was reported that ET-3 caused an elevation of $[Ca^{2+}]_i$, independent of extracellular Ca^{2+}, in cultured endothelial cells of bovine carotid arteries while ET-1 had no such effect, and that the ET-3 induced Ca^{2+} mobilization was completely blocked by IAP (Emori *et al.*, 1991). They concluded that ET-3 activated phosphoinositide breakdown via an IAP-sensitive G-protein. The apparent difference between their conclusion and ours may be due to species differences.

In the present study, BK (10^{-7} M), like ET-1, caused a biphasic elevation of $[Ca^{2+}]_i$ in endothelial cells *in situ*. As with ET-1, the first phase was partially reduced in Ca^{2+}-free PSS, while the second phase was completely abolished in Ca^{2+}-free PSS (Figure 3a and b). Thus, part of the first phase was mainly due to the release of stored Ca^{2+} and the second to Ca^{2+} influx. However, treatment with IAP had no effect on BK-induced Ca^{2+}-transients. In addition, the $[Ca^{2+}]_i$ transient induced by ATP was not affected by IAP treatment. These findings indicate that the inhibition of Ca^{2+} influx by IAP is specific to ET-1. As well as ET-1, BK and ATP activate G-protein-dependent pathways to cause phosphoinositide turnover in endothelial cells (Voyno-Yasenetskaya *et al.*, 1989; Cooper *et al.*, 1989). This study suggests that the G-proteins involved in BK- and ATP-activated pathway to elevate $[Ca^{2+}]_i$ are insensitive to IAP. These findings are consistent with the data reported by other investigators (Flavahan *et al.*, 1989).

In conclusion, we provide evidence for the involvement of an IAP-sensitive G-protein in the ET-1-activated Ca^{2+} influx mechanism in endothelial cells *in situ*. In contrast, the G-protein that mediates intracellular Ca^{2+} release, presumably via the PLC/$InsP_3$ cascade is IAP-insensitive. It remains to be determined whether the IAP-sensitive G-protein directly couples between the ET receptor and the Ca^{2+} permeable channel.

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References

AOKI, H., KOBAYASHI, S., NISHIMURA, J., YAMAMOTO, H. & KANAIDE, H. (1991). Endothelin induces the Ca^{2+}-transient in endothelial cells *in situ*. *Biochem. Biophys. Res. Commun.*, **181**, 1352–1357.

ARAI, H., HORI, S., ARAMORI, I., OHKUBO, H. & NAKANISHI, S. (1990). Cloning of expression of a DNA encoding an endothelin receptor. *Nature*, **348**, 730–732.

BARRY, A. & PATTEN, B.M. (1960). In *Pathology of the Heart*. ed. Gould, S.E. pp. 93–131. Springfield: Charles C. Thomas.

BØRSUM, T., HAGEN, I., HENRIKSEN, T. & CARLANDER, B. (1982). Alterations in the protein composition and surface structure of human endothelial cells during growth in primary culture. *Atherosclerosis*, **44**, 367–378.

COOPER, C.L., MORRIS, A.J. & HARDEN, T.K. (1989). Guanine nucleotide-sensitive interaction of a radiolabeled agonist with a phospholipase C-linked P_{2y}-purinergic receptor. *J. Biol. Chem.*, **264**, 6202–6206.

DE NUCCI, G., THOMAS, R., D'ORLEANS-JUSTE, P., ANTUNES, E., WALDER, C., WARNER, T.D. & VANE, J.R. (1988). Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 9797–9800.

DI VIRGILIO, F., STEINBERG, T.H. & SILVERSTEIN, S.C. (1989). Organic-anion transport inhibitors to facilitate measurement of cytosolic free Ca^{2+} with fura-2. In *Methods in Cell Biology*, ed. Tartakoff, A.M., pp. 453–462. San Diego: Academic Press.

EGUCHI, S., KOZUKA, M., HIROSE, S., ITOH, T., HIRATA, Y., MARUMO, F. & HAGIWARA, H. (1991). Identification of G protein-coupled endothelin receptors in cultured bovine endothelial cells. *Biochem. Biophys. Res. Commun.*, **174**, 1343–1346.

EMORI, T., HIRATA, Y., KANNO, K., OHTA, K., EGUCHI, S., IMAI, T., SHICHIRI, M. & MARUMO, F. (1991). Endothelin-3 stimulates production of endothelium-derived nitric oxide via phosphoinositide breakdown. *Biochem. Biophys. Res. Commun.*, **174**, 228–235.

FLAVAHAN, N.A., SHIMOKAWA, H. & VANHOUTTE, P.M. (1989). Pertussis toxin inhibits endothelium-dependent ralaxations to certain agonists in porcine coronary arteries. *J. Physiol.*, **408**, 549–560.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **299**, 373–376.

GILMAN, A.G. (1987). G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.*, **56**, 615–649.

GOTO, K., KASUYA, Y., MATSUKI, N., TAKUWA, Y., KURIHARA, H., ISHIKAWA, T., KIMURA, S., YANAGISAWA, M. & MASAKI, T. (1989). Endothelin activates the dihydropyridine-sensitive, voltage-dependent Ca^{2+} channel in vascular smooth muscle. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 3915–3918.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

HENNIG, B., BOISSONNEAULT, G.A. & GLAUERT, H.P. (1989). Effects of serum type on growth and permeability properties of cultured endothelial cells. *Exp. Cell Res.*, **181**, 589–596.

HIRANO, K., KANAIDE, H., ABE, S. & NAKAMURA, M. (1990). Effects of diltiazem on calcium concentrations in the cytosol and on force of contractions in porcine coronary arterial strips. *Br. J. Pharmacol.*, **101**, 273–280.

KELLY, R.A., EID, H., KRAMER, B.K., O'NEILL, M., LIANG, B.T., REERS, M. & SMITH, T.W. (1990). Endothelin enhances the contractile responsiveness of adult rat ventricular myocytes to calcium by a pertussis toxin-sensitive pathway. *J. Clin. Invest.*, **86**, 1164–1171.

KOBAYASHI, S., KANAIDE, H. & NAKAMURA, M. (1986). Complete overlap of caffeine- and K^+ depolarization-sensitive intracellular calcium storage site in cultured rat arterial smooth muscle cells. *J. Biol. Chem.*, **261**, 15709–15713.

KODAMA, M., KANAIDE, H., ABE, S., HIRANO, K., KAI, H. & NAKAMURA, M. (1989). Endothelin-induced Ca-independent contraction of the porcine coronary artery. *Biochem. Biophys. Res. Commun.*, **160**, 1302–1308.

LADOUX, A. & FRELIN, C. (1991). Endothelin inhibit adenylate cyclase in brain capillary endothelial cells. *Biochem. Biophys. Res. Commun.*, **180**, 169–173.

LANSMAN, J.B., HALLAM, T.J. & RINK, T.J. (1987). Single stretch-activated ion channels in vascular endothelial cells as mechanotransducers? *Nature*, **325**, 811–813.

LYSKO, P.G., FEUERSTEIN, G., PULLEN, M., WU, H.-L. & NAMBI, P. (1991). Identification of endothelin receptor in cultured cerebellar neurons. *Neuropeptides*, **18**, 83–86.

MARIN, P., DELUMEAU, J.C., DURIEU-TRAUTMANN, O., LE NGUYEN, D., PRÉMONT, J., STROSBERG, A.D. & COURAUD, P.O. (1991). Are several G proteins involved in the different effects of endothelin-1 in mouse striatal astrocytes? *J Neurochem.*, **56**, 1270–1275.

MASAKI, T., KIMURA, S., YANAGISAWA, M. & GOTO, K. (1991). Molecular and cellular mechanism of endothelin regulation. Implication for vascular function. *Circulation*, **84**, 1457–1468.

MIASIRO, N., YAMAMOTO, H., KANAIDE, H. & NAKAMURA, M. (1988). Does endothelin mobilize calcium from intracellular store sites in rat aortic vascular smooth muscle cells in primary cultures? *Biochem. Biophys. Res. Commun.*, **156**, 312–317.

MITSUHASHI, T., MORRIS, R.C. & IVES, H.E. (1989). Endothelin-induced increases in vascular smooth muscle Ca^{2+} do not depend on dihydropyridine-sensitive Ca^{2+} channels. *J. Clin. Invest.*, **84**, 635–639.

NETLAND, P.A., ZETTER, B.R., VIA, D.P. & VOYTA, J.C. (1985). *In situ* labeling of vascular endothelium with fluorescent acetylated low density lipoprotein. *Histochem. J.*, **17**, 1309–1320.

NEWBY, A.C. & HENDERSON, A.H. (1990). Stimulus-secretion coupling in vascular endothelial cells. *Annu. Rev. Physiol.*, **52**, 661–674.

OLESEN, S.-P., CLAPHAM, D.E. & DAVIES, P.F. (1988). Haemodynamic shear stress activates a K^+ current in vascular endothelial cells. *Nature*, **331**, 168–170.

SAKURAI, T., YANAGISAWA, M., TAKUWA, Y., MIYAZAKI, H., KIMURA, S., GOTO, K. & MASAKI, T. (1990). Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature*, **348**, 732–735.

TAKUWA, Y., KASUYA, Y., TAKUWA, N., KUDO, M., YANAGISAWA, M., GOTO, K., MASAKI, T. & YAMASHITA, K. (1990). Endothelin receptor is coupled to phospholipase C via a pertussis toxin-insensitive guanine nucleotide-binding regulatory protein in vascular smooth muscle cells. *J. Clin. Invest.*, **85**, 653–658.

THOMAS, C.P., KESTER, M. & DUNN, M.J. (1991). A pertussis toxin-sensitive GTP-binding protein couples endothelin to phospholipase C in rat mesangial cells. *Am. J. Physiol.*, **260**, F347–F352.

ULLRICH, A. & SCHLESSINGER, J. (1990). Signal transduction by receptors with tyrosine kinase activity. *Cell*, **61**, 203–212.

USHIO-FUKAI, M., NISHIMURA, J., AOKI, H., KOBAYASHI, S. & KANAIDE, H. (1992). Endothelin-1 inhibits and enhances contraction of porcine coronary arterial strips with an intact endothelium. *Biochem. Biophys. Res. Commun.*, **184**, 518–524.

VANE, J.R. (1990). Endothelins come home to roost. *Nature*, **348**, 673.

VIGNE, P., MARSAULT, R., BREITTMAYER, J.P. & FRELIN, C. (1990). Endothelin stimulates phosphatidylinositol hydrolysis and DNA synthesis in brain capillary endothelial cells. *Biochem. J.*, **266**, 415–420.

VOYNO-YASENETSKAYA, T.A., TKACHUK, V.A., CHEKNYOVA, E.G., PANCHENKO, M.P., GRIGORIAN, G.Y., VAVREK, R.J., STEWART, J.M. & RYAN, U.S. (1989). Guanine nucleotide-dependent, pertussis toxin-insensitive regulation of phosphoinositide turnover by bradykinin in bovine pulmonary artery endothelial cells. *FASEB J.*, **3**, 44–51.

WHORTON, A.R., YOUNG, S.L., DATA, J.L., BARCHOWSKY, A. & KENT, R.S. (1982). Mechanism of bradykinin-stimulated prostacyclin synthesis in porcine aortic endothelial cells. *Biochim. Biophys. Acta*, **712**, 79–87.

YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KOBAYASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **322**, 411–415.

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The effects of chronic imidazoline drug treatment on glial fibrillary acidic protein concentrations in rat brain

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1 The concentration of the astrocytic marker, glial fibrillary acidic protein (GFAP) was quantitated by immunoblotting (western blotting) in the rat brain after treatment with various imidazoline drugs and other agents.

2 Chronic (7 days) but not acute (1 day) treatment with the imidazoline drugs, cirazoline (1 mg kg^{-1}, i.p.) and idazoxan (10 mg kg^{-1}, i.p.), but not with the structurally related α_2-adrenoceptor antagonists, RX821002 (2-methoxy idazoxan) (10 mg kg^{-1}, i.p.) and efaroxan (10 mg kg^{-1}, i.p.), markedly increased (45%) GFAP immunoreactivity in the rat cerebral cortex. Chronic treatment (7 days) with yohimbine (10 mg kg^{-1}, i.p.), a non-imidazoline α_2-adrenoceptor antagonist, did not significantly modify GFAP immunoreactivity in the cerebral cortex.

3 Chronic treatment (7 days) with cirazoline and idazoxan did not alter the density of brain monoamine oxidase (MAO)-B sites labelled by [^{3}H]-Ro 19-6327 (lazabemide), another relevant astroglial marker. Moreover, these imidazoline drug treatments did not modify the levels of α-tubulin in the cerebral cortex. These negative results reinforced the specificity of the effects of imidazoline drugs on GFAP.

4 Irreversible inactivation of brain α_2-adrenoceptors (and other neurotransmitters receptors) after treatment with an optimal dose of the peptide-coupling agent EEDQ (1.6 mg kg^{-1}, i.p., for 6–24 h) did not alter GFAP immunoreactivity in the cerebral cortex. These results further disproved the involvement of these receptors on astroglial cells in the tonic control of GFAP levels.

5 The binding of [^{3}H]-idazoxan in the presence of 10^{-6} M (−)-adrenaline was used to quantitate in parallel I_2-imidazoline preferring sites in the rat brain after the same treatments. Chronic treatment (7 days) with cirazoline and idazoxan, but not with RX821002, efaroxan or yohimbine, significantly increased (25%) the density of I_2-sites in the cerebral cortex. The up-regulation of I_2-sites induced by cirazoline was not observed in the liver, a tissue that also expresses I_2-sites but lacks glial cells.

6 A strong correlation ($r = 0.97$) was found when the mean percentage changes in GFAP immunoreactivity were related to the mean percentage changes in I_2 imidazoline sites after the various drug treatments.

7 Together the results suggest a direct physiological function of glial I_2-imidazoline preferring sites in the regulation of GFAP levels.

Keywords: Glial fibrillary acidic protein; GFAP; astrocytes; rat brain; imidazoline binding sites; cirazoline; idazoxan

Introduction

The α_2-adrenoceptor imidazoline radioligands, [^{3}H]-clonidine and [^{3}H]-idazoxan, have been shown also to recognize with high affinity non-adrenoceptor sites which have been termed imidazoline binding sites or imidazoline-preferring receptors (Michel & Insel, 1989; Michel & Ernsberger, 1992). It is now believed that these sites exist in two principle subtypes (Michel & Insel, 1989; Ernsberger, 1992); the clonidine-preferring subtype, the I_1-imidazoline site (I_1-site), has been localised in the lower brainstem, which is involved in the regulation of blood pressure (Bousquet *et al.*, 1984; Ernsberger *et al.*, 1993), and also in the adrenal medulla (Ernsberger *et al.*, 1993; Molderings *et al.*, 1993). The idazoxan-preferring subtype, the I_2-imidazoline site (I_2-site), is expressed in several tissues (Langin & Lafontan, 1989; Coupry *et al.*, 1989; Zonnenschein *et al.*, 1990), including the brain (Mallard *et al.*, 1992; Olmos *et al.*, 1992; Miralles *et al.*, 1993a) and, at the subcellular level, is located on the mitochondrial outer membrane (Tesson & Parini, 1991; Tesson *et al.*, 1991; Regunathan *et al.*, 1993b). Recently, it has been shown that the density of I_2-sites increases with age in the human brain and that the regional distribution of these sites correlates well with that of the B isoform of the enzyme monoamine oxidase (MAO-B) (Sastre & Garcia-Sevilla, 1993), which is mainly located in 5-hydroxytryptaminergic and glial cells (Westlund *et al.*, 1988). Also in this context, it has been reported that cultured cortical astrocytes, but not cortical neurones, express I_2-sites (Regunathan *et al.*, 1993a). These facts suggest that the binding of [^{3}H]-idazoxan to non-adrenoceptor sites in the brain is preponderantly, if not exclusively, located on glial cells.

The glial fibrillary acidic protein (GFAP) is the main constituent of intermediate filaments of astrocytes (Eng *et al.*, 1971; Eng, 1985) which accumulates in response to brain injury (Herrera & Cuello, 1992; Kindy *et al.*, 1992; Norton *et al.*, 1992). The mechanisms governing the expression of this protein have not been elucidated (Masood *et al.*, 1993). In this sense, it has been shown that idazoxan induces a marked concentration-dependent (1–100 μM) increase in mRNA levels for GFAP in rat cultured cortical astrocytes, suggesting that occupancy of astrocytic I_2-sites may have some functional activity in the regulation of the expression of this protein (Regunathan *et al.*, 1993a). On the other hand, the density of rat brain I_2-sites has been found to be up-regulated after chronic imidazoline drug treatment including idazoxan (Olmos *et al.*, 1992). The present study was designed to assess whether chronic treatment with several imidazoline drugs,

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selective for the I_1- or I_2-sites, could modulate *in vivo* the density of GFAP in the rat brain, and also to relate possible changes in GFAP levels with parallel increases in the density of brain I_2-sites.

Methods

Animals and treatments

Male Sprague-Dawley rats (250–300 g) were used. The animals received a standard diet with water freely available and were housed at 20 ± 2°C with a 12 h light/dark cycle. In the chronic (7 days) treatments the animals received i.p. every 12 h either 0.9% saline vehicle, cirazoline (1 mg kg^{-1}), idazoxan (10 mg kg^{-1}), efaroxan (10 mg kg^{-1}), RX821002 (2-methoxy idazoxan) (10 mg kg^{-1}) and the non-imidazoline α_2-adrenoceptor antagonist, yohimbine (10 mg kg^{-1}). In another series of experiments, rats were treated with the peptide coupling agent EEDQ (dissolved in ethanol and then diluted with propylene glycol/water in a final volume ratio of 1:1:2) at 1.6 mg kg^{-1}, i.p. for 6–24 h. Because EEDQ irreversibly inactivates α_2-adrenoceptors and several other types of neurotransmitter receptors (Meller *et al.*, 1988), with a moderate effect on I_2-sites (20–45% inactivation) (Miralles *et al.*, 1993b), these experiments were designed to assess the basal influence of α_2-adrenoceptors on GFAP levels. In the acute (1 day) treatments, the rats received a single dose of cirazoline (1 mg kg^{-1}) or idazoxan (10 mg kg^{-1}). In the acute and chronic treatments the rats were killed 24 h after the last injection.

Immunoblot analysis of GFAP and α-tubulin and quantitation of specific immunoreactivity

Immunoblotting of GFAP was done as previously described (Chiu & Norton, 1982; Aquino *et al.*, 1988) with modifications. Animals were decapitated and brains rapidly removed under ice; 100–150 mg of brain parieto-occipital cortex was homogenized (30 s) with an Ultraturrax homogenizer in 50 volumes of 50 mM Tris buffer pH 6.8 containing 2 mM EDTA, 0.5% Triton X-100 and 2 mM phenylmethylsulphonyl fluoride (PMSF). The samples were then sonicated (20 s) and centrifuged at 14,600 *g* for 15 min at 4°C. The supernatant was recovered and diluted 1:20 in the previous homogenization buffer. The protein content in these samples was determined by the method of Bradford (1976), with bovine serum albumin used as the standard. An aliquot (200 µl) of the diluted supernatant was mixed with the same volume of electrophoresis loading buffer and boiled. After polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to nitrocellulose membranes (immunoblotting, western blotting), incubated with the primary antibody, rabbit anti-cow GFAP (1:5,000 dilution) (DAKO A/S, Denmark), and then with the secondary antibody, horseradish peroxidase-linked donkey anti-rabbit IgG, as previously described for other proteins (Escriba *et al.*, 1994). α-Tubulin was separated and quantitated similarly as described above using as the primary antibody a monoclonal anti-α-tubulin (clone DM 1A) diluted at 1:3,000. The secondary antibody, horseradish peroxidase-linked sheep antimouse IgG, was incubated at 1:5,000 dilution as above (Escriba *et al.*, 1994). Immunoreactivity was detected with the Enhanced Chemiluminescence (ECL) Western Blot Detection system (Amersham International plc, U.K.) followed by exposure to Kodak X-AR film (Figure 1). Omission of the primary antibody was used as a negative control; i.e. immunoreactivity was absent under these conditions. Films were scanned in the image analyser Bio Image (Millipore, Ann Arbor, MI, U.S.A.). After scanning, standard curves were constructed using samples from saline-treated rats. In these curves, the total protein loaded in, at least, four points of increasing protein content (2–8 ng) was plotted against the integrated optical density (IOD). A linear relationship between the amount of protein loaded in the gel and the IOD was found all over the range of protein content used. For the quantitation of GFAP samples from the saline-treated and drug-treated rats were loaded in the same gel as the standard curve and, for every sample, a theoretical amount of protein loaded in the gel (Pt) was obtained by intrapolation of its IOD in the standard curve. The percent of GFAP content of a given sample with respect to the standard (saline-treated) samples was calculated as [(Pt/Pr) × 100]; where Pr is the real amount of protein loaded in the gel well (Escriba *et al.*, 1994). This quantitative procedure was repeated at least 3 times for each sample in different gels. The mean interassay coefficient of variation was 9%.

[^{3}H]-idazoxan and [^{3}H]-RX821002 binding assays

Radioligand binding assays for [^{3}H]-idazoxan and [^{3}H]-RX821002 (2-methoxy idazoxan) and preparation of P_2 membrane fractions from brain parieto-occipital cortex were carried out as previously described (Miralles *et al.*, 1993a). Total [^{3}H]-idazoxan and [^{3}H]-RX821002 binding was measured in 1.1 ml-aliquots (50 mM Tris-HCl, 0.1% ascorbic acid, pH 7.5) of the membranes (0.8–1.2 mg protein) which were incubated with shaking for 30 min at 25°C. Binding of [^{3}H]-idazoxan to I_2-sites was always done in the presence of 10^{-6} M (−)-adrenaline to prevent the binding of the radioligand to α_2-adrenoceptors. Nonspecific binding was determined in the presence of 10^{-4} M naphazoline, as previously described (Olmos *et al.*, 1992). In the saturation studies, cortical membranes were incubated with eight concentrations of [^{3}H]-idazoxan (6×10^{-10} M to 5×10^{-8} M) as above. In the drug competition studies, cortical membranes were incubated as above with [^{3}H]-idazoxan (4×10^{-9} M) or [^{3}H]-RX821002 (10^{-9} M) and in the absence or presence of various concentrations of the competing drugs (10^{-10} M to 10^{-3} M; 15 concentrations). After incubation and dilution, bound ligand was separated from free ligand by vacuum filtration through glass fibre filters which were counted for radioactivity by liquid scintillation spectrometry at 50% efficiency (Packard, model

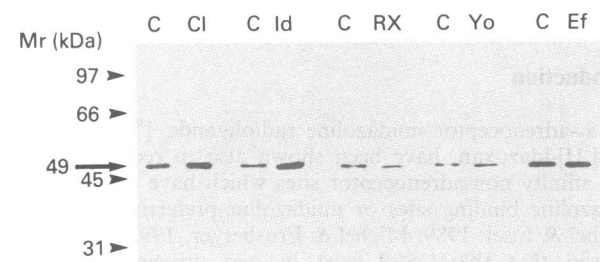


Figure 1 Representative immunoreactive bands using antisera against GFAP in the rat brain after saline (C) or chronic treatment (i.p., every 12 h for 7 days) with cirazoline (Cl) (1 mg kg^{-1}), idazoxan (Id) (10 mg kg^{-1}), RX821002 (2-methoxy idazoxan) (RX) (10 mg kg^{-1}), yohimbine (Yo) (10 mg kg^{-1}) or efaroxan (Ef) (10 mg kg^{-1}). Samples from the parieto-occipital cortex were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes (immunoblotting), incubated with the specific primary and secondary antibodies, and visualized by the Enhanced Chemiluminescence (ECL) method. Pairs of saline (C)-drug treatment samples were run in the same gel. The amount of total protein loaded per gel and well was as follows (in ng): 11.8 (C) – 9.5 (Cl); 2.8 (C) – 2.9 (Id); 3.1 (C) – 3.2 (RX); 3.1 (C) – 2.9 (Yo) and 4.2 (C) – 4.3 (Ef). Relative molecular masses (kDa) of marker proteins are given on the left. GFAP immunoreactivities (Mr = 49 kDa) (arrow) increased significantly after chronic treatment with cirazoline (+ 51%) and idazoxan (+ 41%), but not after chronic RX821002 (− 3%), yohimbine (+ 14%) or efaroxan (0%) treatments. See Table 1 for changes in mean percentage values of GFAP immunoreactivity after the various treatments and other details.

1900 CR). Protein content in these samples was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Analyses of individual saturation isotherms (K_d, dissociation constant; B_{max}, maximum density of binding sites) and competition experiments (K_i, inhibition constant) as well as the fitting of data to the appropriate binding models were performed using the EBDA-LIGAND programmes (Munson & Rodbard, 1980; McPherson, 1985). For other details of the computer analyses see Olmos *et al.* (1992) and Miralles *et al.* (1993a).

[^{3}H]-Ro 19-6327 binding assay

Binding of [^{3}H]-Ro 19-6327 (lazabemide) (4.5×10^{-9} M) was performed as described previously (Saura *et al.*, 1992; Sastre & García-Sevilla, 1993). This assay was similar to that described above for [^{3}H]-RX 821002 except in the buffer composition (50 mM Tris-HCl, 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA, pH 7.4) and the incubation conditions (90 min at 20°C). The specific binding of [^{3}H]-Ro 19-6327 to brain MAO-B defined with 10^{-4} M (−)-deprenyl, represented about 90% of total binding at this concentration of the radioligand. The density of MAO-B sites in rat cortical membranes was quantitated as described in Table 3.

Statistics

In GFAP and α-tubulin immunoblot quantitation experiments, results after treatments are expressed as a percentage of values of saline-treated rats, as described above, and represent the mean ± s.e.mean. In binding experiments, comparisons were done with the binding parameters (K_d, B_{max}) obtained in saturation curves and values of I_2-sites density were then expressed as mean ± s.e.mean percentage values over saline-treated rats. One-way analysis of variance (ANOVA), followed by Scheffé's test, was used for the statistical evaluations. Correlation coefficients were calculated by the method of least squares. The level of significance was chosen as $P = 0.05$.

Materials and drugs

Rabbit anti-cow GFAP, a purified immunoglobulin fraction of rabbit antiserum was purchased from DAKO A/S (Denmark). Anti-α-tubulin monoclonal antibody (clone DM 1A) was purchased from Sigma Chemical Co. (U.S.A.). Horseradish peroxidase-linked donkey anti-rabbit and sheep anti-mouse IgG were obtained from Amersham International plc (U.K.). [^{3}H]-idazoxan (specific activity, 42–46 Ci mmol^{-1}; batches 44–45) and [^{3}H]-RX821002 (2-methoxy idazoxan) (specific activity, 59 Ci mmol^{-1}; batch 10) were purchased from Amersham International plc (U.K.). [^{3}H]-Ro 19-6327 (lazabemide) (specific activity, 20.2 Ci mmol^{-1}) was a generous gift from Dr J.G. Richards and Dr J. Saura (F. Hoffman-La Roche Ltd., Switzerland). [^{3}H]-idazoxan and [^{3}H]-Ro 19-6327 were stored at − 30°C and [^{3}H]-RX821002 at 4°C. For the binding assays, appropriate amounts of the stock solutions were diluted with distilled and purified water (Milli-Q) containing 2.5 mM HCl and 6% ethanol. Other drugs (and their sources) included: cirazoline HCl (Synthélabo Recherche, France); efaroxan HCl (RBI, U.S.A.); EEDQ (N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline) (Sigma Chemical Co., U.S.A.); L-(−)-deprenyl (RBI, U.S.A.); idazoxan HCl (synthesized by Dr F. Geijo at S.A. Lasa Laboratories, Spain); RX821002 HCl (2-methoxy idazoxan) (synthesized by Dr F. Geijo at S.A. Lasa Laboratories) and yohimbine HCl (Sigma Chemical Co., U.S.A.). Other reagents were obtained from Sigma Chemical Co. (U.S.A.).

Results

Effects of imidazoline drug treatments on GFAP immunoreactivity in the rat cerebral cortex

Immunoreactive bands of GFAP were found in the rat cerebral cortex after immunoblotting (Figure 1); the relative molecular mass (Mr = 49 kDa) was in the range (47–51 kDa) previously described for the monomeric form of this cytoskeletal protein (Eng, 1985). Chronic treatment (7 days) with the imidazoline drugs, cirazoline (1 mg kg^{-1}) and idazoxan (10 mg kg^{-1}), consistently increased by about 45% GFAP immunoreactivity in the rat cerebral cortex (Figure 1 and Table 1). When tested *in vitro*, both drugs displayed high affinity for I_2-sites; cirazoline being more potent (4 times) and more selective (105 times) than idazoxan for these sites compared to α_2-adrenoceptors (Table 2). In contrast, chronic treatments with other α_2-adrenoceptor antagonists possessing the imidazoline structure, but with low affinity for I_2-sites, did not modify the levels of GFAP. Thus, chronic treatments with RX821002 (10 mg kg^{-1}), the 2-methoxy analogue of idazoxan, and efaroxan (10 mg kg^{-1}) did not increase the immunoreactivity of GFAP (Figure 1 and Table 1). These two drugs competed *in vitro* with very low affinities against [^{3}H]-idazoxan binding to I_2-sites (K_i values in the high micromolar range); but, as expected, they displayed high affinity (K_i values in the nanomolar range) competing against [^{3}H]-RX821002 binding to α_2-adrenoceptors (Table 2). In addition efaroxan is also a potent I_1-imidazoline drug *in vitro* (Ernsberger *et al.*, 1992). To prove further that the observed increase in GFAP immunoreactivity induced by idazoxan and cirazoline was not related to the α_2-adrenoceptor antagonism, the effect of yohimbine, a non-imidazoline α_2-adrenoceptor antagonist, was studied. Chronic treatment

Table 1 Effects of acute and/or chronic treatments with various imidazoline drugs and yohimbine on glial fibrillary acidic protein (GFAP) immunoreactivity in the rat cerebral cortex

Treatment	*Dose* (mg kg^{-1})	*GFAP immunoreactivity* (%)	n
Saline	–	100 ± 3	14
Cirazoline			
Acute	1	110 ± 7	6
Chronic	1	147 ± 9**	10
Idazoxan			
Acute	10	101 ± 8	3
Chronic	10	145 ± 12*	4
RX821002	10	97 ± 6	5
Yohimbine	10	112 ± 8	3
Efaroxan	10	105 ± 5	6

Every drug was administered i.p. every 12 h for 7 days or for only 24 h in the acute cirazoline and idazoxan treatments. The animals were killed 24 h after the last injection. Immunoreactive bands of GFAP were obtained in the parieto-occipital cortex after immunoblotting and the relative intensity was quantified as integrated areas of optical density (see Figure 1 for representative immunoreactive bands after the various treatments). Densitometric readings were normalized as percentages of values of samples from saline-treated rats, as described in methods. Each value represents the mean ± s.e.mean of n experiments per group with an animal per experiment. One-way ANOVA followed by a multiple comparison test detected a significant increase in GFAP immunoreactivity ($F[7,43] = 7.88$, $P = 0.0001$) after chronic treatments with cirazoline and idazoxan, but not after acute cirazoline and idazoxan or chronic RX821002, yohimbine or efaroxan treatments. *$P < 0.05$ and **$P < 0.01$ as compared with the saline-treated group (ANOVA followed by Scheffé's test).

with yohimbine (10 mg kg^{-1}) for 7 days did not significantly modify GFAP immunoreactivity in the rat cerebral cortex (Figure 1 and Table 1). *In vitro*, yohimbine displayed high affinity for α_2-adrenoceptors but very low affinity for I_2-sites (Table 2). Acute (1 day) treatments with cirazoline (1 mg kg^{-1}) and idazoxan (10 mg kg^{-1}), the most potent drugs on I_2-sites tested, failed to modify GFAP levels in the rat cerebral cortex (Table 1). On the other hand, irreversible inactivation of α_2-adrenoceptors after treatment with an optimal dose of EEDQ (1.6 mg kg^{-1}, i.p., for 6–24 h) did not alter the immunoreactivity of GFAP in the rat brain (saline-treated rats: 104 ± 3%, $n = 3$; EEDQ-treated rats: 112 ± 2%, $n = 9$; $P > 0.05$).

The specificity of imidazoline drug treatments on GFAP levels was investigated further with other astroglial markers, such as the MAO-B. For this purpose, the novel high-affinity ligand [^{3}H]-Ro 19-6327 (lazabemide), a reversible and highly selective inhibitor of MAO-B (Saura *et al.*, 1992) was used. Chronic treatment (7 days) of rats with cirazoline (1 mg kg^{-1}) and idazoxan (10 mg kg^{-1}) did not alter the density of MAO-B sites labelled by [^{3}H]-Ro 19-6327 (Table 3).

Table 2 Binding parameters of various imidazoline drugs and yohimbine to I_2-imidazoline sites and α_2-adrenoceptors in the rat cerebral cortex

Drug	*[^{3}H]-idazoxan* (I_2-imidazoline sites) K_i (nM)	*[^{3}H]-RX821002* (α_2-adrenoceptors) K_i (nM)
Cirazoline	3.4	307.0
Idazoxan	14.0	12.0
RX821002	45,000	1.4
Yohimbine	13,000	28.0
Efaroxan	58,000	6.0

Cortical membranes were incubated at 25°C for 30 min with [^{3}H]-idazoxan (4×10^{-9} M in the presence of 10^{-6} M (−)-adrenaline; I_2-imidazoline sites) or [^{3}H]-RX821002 (10^{-9} M; α_2-adrenoceptors) and in the absence or presence of the competing drugs (10^{-10} to 10^{-3} M; 15 concentrations). Binding parameters (K_i values) were determined directly by simultaneous analysis of three independent experiments for each drug using the EBDA-LIGAND programmes. Computer-assisted curve fitting demonstrated that for cirazoline competition curves aginst [^{3}H]-idazoxan binding a two-site fit was significantly better than a one-site binding model ($P < 0.001$, F test) ($K_{iH} = 3.4$ nM; $K_{iL} = 1{,}300$ nM; $R_H = 66\%$).

Table 3 Effects of chronic treatments with cirazoline and idazoxan on the specific binding of [^{3}H]-Ro 19-6327 (lazabemide) to monoamine oxidase-B (MAO-B) in the rat cerebral cortex

Treatment	Dose (mg kg^{-1})	*[^{3}H]-Ro 19-6327* B_{max} (fmol mg^{-1} protein)	n
Saline	–	415 ± 30	3
Cirazoline	1	376 ± 34	3
Idazoxan	10	488 ± 31	3

Every drug was administered i.p. every 12 h for 7 days. The animals were killed 24 h after the last injection. The specific binding of [^{3}H]-Ro 19-6327 (4.5×10^{-9} M, defined with 10^{-4} M (−)-deprenyl) to MAO-B was determined in cortical membranes. Because the binding of [^{3}H]-Ro 19-6327 to MAO-B was determined with a single concentration of the radioligand, close to the dissociation constant (K_d) (Saura *et al.*, 1992), B_{max} values were estimated according to the equation $B = B_{max}$ [F]/([F] + K_d), where B represents the amount of specific binding at the concentration of the radioligand (F) used. Each value represents the mean ± s.e.mean of n experiments per group with an animal per experiment. One-way ANOVA followed by a multiple comparison test (Scheffé's test) did not reveal any significant differences between groups ($P > 0.05$).

Moreover, chronic treatment (7 days) with cirazoline and idazoxan did not modify the levels of α-tubulin, a housekeeping control protein, in the rat cerebral cortex (98 ± 3%, $n = 3$, $P > 0.05$) compared to those obtained in saline-treated rats. These negative results discounted possible nonspecific effects of imidazoline drugs on GFAP levels.

Parallel modulation of I_2-sites density and GFAP levels by specific imidazoline drug treatments in the rat cerebral cortex

Since chronic administration of specific imidazoline drugs can modulate the density of I_2-sites in the rat brain (Olmos *et al.*, 1992), the possibility that this modulation could parallel the reported increases in GFAP immunoreactivities by specific drugs was investigated. In this way, the effects of the same chronic treatments were studied on the density and affinity of I_2-sites. Chronic treatment (7 days) with cirazoline (1 mg kg^{-1}) or idazoxan (10 mg kg^{-1}) significantly increased by about 25% the density of I_2-sites in the rat cerebral cortex without significantly changing the affinity (K_d) of [^{3}H]-idazoxan for these sites (Figure 2). Interestingly, the up-regulation of I_2-sites after chronic cirazoline treatment (1 mg kg^{-1}) was not observed in the rat liver, a tissue that also expresses I_2-sites but lacks glial cells ([^{3}H]-idazoxan binding, saline-treated rats: $K_d = 12.8 \pm 2.1$ nM, $B_{max} = 440 \pm 24$ fmol mg^{-1} protein, $n = 9$; cirazoline-treated rats: K_d =

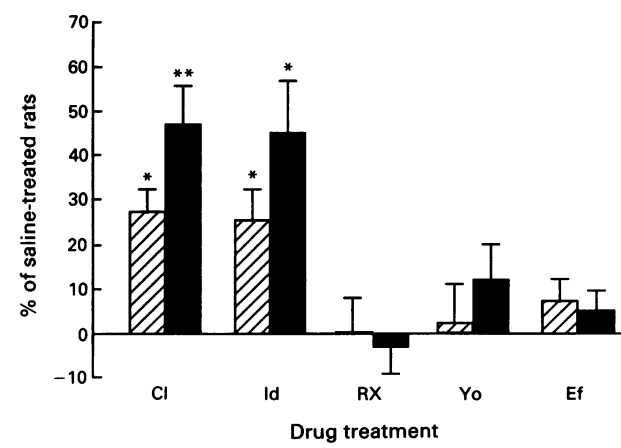


Figure 2 Effect of chronic treatment (i.p. every 12 h for 7 days) with cirazoline (Cl) (1 mg kg^{-1}), idazoxan (Id) (10 mg kg^{-1}), RX821002 (2-methoxy idazoxan) (RX) (10 mg kg^{-1}), yohimbine (Yo) (10 mg kg^{-1}) or efaroxan (Ef) (10 mg kg^{-1}) on the density (B_{max}) of I_2-sites (hatched columns) and GFAP immunoreactivity (solid columns) in rat cerebral cortex. Saline-treated rats were used as controls (data not shown). The specific binding of [^{3}H]-idazoxan to I_2-sites was determined in P_2 fractions of cortical membranes and was defined as the difference between binding in the presence of 10^{-6} M (−)-adrenaline (total non-adrenoceptor binding) and 10^{-4} M naphazoline (nonspecific binding). Binding parameters (K_d, B_{max}) were determined directly by computer-assisted nonlinear analysis from untransformed data using the EBDA-LIGAND programmes (mean values for saline-treated rats: $K_d = 14.8 \pm 1.3$ nM; $B_{max} = 55 \pm 2$ fmol mg^{-1} protein; $n = 12$). I_2-sites density (B_{max}) after treatments is expressed as percentage of that from saline-treated rats. One-way ANOVA followed by a multiple comparison test detected a significant increase in B_{max} for I_2-sites ($F[5,33] = 5.42$; $P = 0.0009$) after chronic treatment with cirazoline and idazoxan, but not after chronic RX821002, yohimbine or efaroxan treatments. No significant changes in K_d values were found after treatments (range of K_d values: 13.8 ± 2.2 nM–18.7 ± 1.5 nM). GFAP immunoreactivity data were taken from Table 1 for comparison. Columns represent the mean ± s.e.mean of 3 to 12 experiments with an animal per experiment. *$P < 0.05$ and **$P < 0.01$ as compared with saline-treated group (ANOVA followed by Scheffé's test). A strong correlation ($r = 0.97$; $P < 0.01$) exists between changes in the density of I_2-sites and GFAP immunoreactivity after the various treatments.

9.3 ± 0.8 nM, B_{max} = 398 ± 28 fmol mg^{-1} protein, n = 4). Chronic treatments (10 mg kg^{-1}, i.p., every 12 h for 7 days) with other α_2-adrenoceptor antagonists possessing (RX821002, efaroxan) or not (yohimbine) the imidazoline structure were not associated with changes in density of I_2-sites in the rat cerebral cortex (Figure 2). In this way, when the mean percentage changes in GFAP immunoreactivity (dependent variable) were related to the mean percentage changes in I_2-sites density (independent variable) after the various drug treatments, a very strong correlation was found (r = 0.97; P<0.01) (see Figure 2 for comparison of effects of drug treatments).

Discussion

This study demonstrates that chronic, but not acute, treatments with imidazoline drugs that possess marked affinity for the I_2-imidazoline site are associated with increased levels of the astrocyte marker GFAP and that this occurs in parallel with an up-regulation of the density of I_2-sites in the rat brain. These results extend to the *in vivo* system the recent observation of increased mRNA expression for GFAP after exposure to idazoxan of cultured rat cortical astrocytes (Regunathan *et al.*, 1993a). This effect of idazoxan was not due to nonspecific toxic effects because it was rapid, concentration-dependent and the drug did not affect the morphology of glial cells (Regunathan *et al.*, 1993a). Together the *in vitro* and *in vivo* results suggest a direct physiological function of glial I_2-imidazoline preferring sites in the regulation of the expression of GFAP.

In the present results, the increases in GFAP immunoreactivities after chronic cirazoline and idazoxan appear not to be attributable to α_2-adrenoceptor antagonism because chronic treatments with other α_2-adrenoceptor antagonists such as yohimbine, RX821002 (2-methoxy idaxozan) and efaroxan, which show very low affinity for I_2-sites (see Table 2), had no effects. Furthermore, irreversible inactivation of brain α_2-adrenoceptors and other neurotransmitter receptors (e.g. α_1-adrenoceptors, dopamine and 5-hydroxytryptamine receptors) with EEDQ (Meller *et al.*, 1988) did not modify the basal immunoreactivity of GFAP, suggesting that these functional receptors for classical neurotransmitter on astroglial cells (Murphy & Pearce, 1987; Salm & McCarthy, 1992) are not involved in the tonic control of GFAP expression. It also appears that the I_1-imidazoline sites are not involved because efaroxan, a highly selective drug for functional I_1-sites (Ernsberger *et al.*, 1992; Chan *et al.*, 1993), was unable to modify the levels of GFAP.

The increased GFAP levels after chronic treatment with cirazoline and idazoxan, both high affinity drugs for I_2-sites, were associated with a parallel up-regulation of these binding sites in the brain (see Figure 2). In contrast, no up-regulation of I_2-imidazoline sites was found when the effect of chronic cirazoline treatment was evaluated in the liver, a tissue also expressing I_2-sites but lacking glial cells. Interestingly, the simultaneous up-regulation of I_2-imidazoline sites and GFAP levels in the brain appears to be a specific parallel phenomenon since also during aging both increased GFAP expression (Goss *et al.*, 1991; O'Callaghan & Miller, 1991) and density of I_2-sites in the brain (Sastre & García-Sevilla, 1993) have been described. Also in this context, Alzheimer's disease is associated with higher GFAP and mRNA for GFAP levels (Duffy *et al.*, 1980; Le Prince *et al.*, 1993) and increased density of I_2-imidazoline sites in the human brain (Ruiz *et al.*, 1993). Therefore, it is tempting to conclude that the observed increases in the density of I_2-imidazoline sites and in GFAP expression in the rat brain after chronic imidazoline drug treatment reflect the well-known astrocytic hypertrophy associated with aging and Alzheimer's disease (Goss *et al.*, 1991; O'Callaghan & Miller, 1991; Frederickson, 1992).

The signal transduction mechanisms associated with the I_2-imidazoline sites as well as the factors that stimulate GFAP expression are not fully understood. Because these I_2-imidazoline preferring binding sites are mainly located on mitochondrial membranes (Tesson & Parini, 1991; Tesson *et al.*, 1991; Regunathan *et al.*, 1993b) and are associated with calcium mobilization (Regunathan *et al.*, 1991), it is possible that up-regulation of I_2-sites could be related to increased mobilization of mitochondrial calcium stores and this in turn may influence the pattern of GFAP gene expression (see also Regunathan *et al.*, 1993a). Astrocytic activation may function as a protective mechanism in the maintenance of neural function following brain injury by producing various neurotrophic and growth factors (Oderfeld-Nowak *et al.*, 1992). In this context, it is interesting to note that idazoxan and rilmenidine (an oxazoline structurally related to the imidazolines) can provide protection from global cerebral ischaemia (Maiese *et al.*, 1992). These findings suggest important roles for the new glial putative I_2-imidazoline preferring receptors.

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References

AQUINO, D.A., CHIU, F.-C., BROSNAN, C.F. & NORTON, W.T. (1988). Glial fibrillary acidic protein increases in the spinal cord of Lewis rats with acute experimental autoimmune encephalomyelitis. *J. Neurochem.*, **51**, 1085–1096.

BOUSQUET, P., FELDMAN, J. & SCHWARTZ, J. (1984). Central cardiovascular effects of alpha adrenergic drugs: differences between catecholamines and imidazolines. *J. Pharmacol. Exp. Ther.*, **230**, 232–236.

BRADFORD, M.M. (1976). A rapid sensitive method for the quantification of protein using the principle of protein dye binding. *Anal. Biochem.*, **72**, 248–252.

COUPRY, I., ATLAS, D., PODEVIN, R.-A., UZIELLI, I. & PARINI, A. (1989). Imidazoline-guanidinium receptive site in renal proximal tubule: asymetric distribution, regulation by cations and interaction with an endogenous clonidine displacing substance. *J. Pharmacol. Exp. Ther.*, **252**, 293–299.

CHAN, S.L.F., BROWN, C.A. & MORGAN, N.G. (1993). Stimulation of insulin secretion by the imidazoline α_2-adrenoceptor antagonist efaroxan is mediated by a novel, stereoselective, binding site. *Eur. J. Pharmacol.*, **230**, 375–378.

CHIU, F.-C. & NORTON, W.T. (1982). Bulk preparation of CNS cytoskeleton and the separation of individual neurofilament proteins by gel filtration: dye-binding characteristics and amino acid compositions. *J. Neurochem.*, **39**, 1252–1260.

DUFFY, P.E., RAPPORT, M. & GRAF, L. (1980). Glial fibrillary acid protein and Alzheimer-like senile dementia. *Neurology*, **30**, 778–782.

ENG, L.F. (1985). Glial fibrillary acidic protein (GFAP): the major protein of glial intermediate filaments in differentiated astrocytes. *J. Neuroimmunol.*, **8**, 203–214.

ENG, L.F., VANDERHAEGHEN, J.J., BIGNAMI, A. & GERSTL, B. (1971). An acidic protein isolated from fibrous astrocytes. *Brain Res.*, **28**, 351–354.

ERNSBERGER, P. (1992). Heterogeneity of imidazoline binding sites: proposed I_1 and I_2 subtypes. *Fundam. Clin. Pharmacol.*, **6**, 55s, P18.

ERNSBERGER, P.R., DAMON, T.H., GRAFF, L.M., SCHÄFFER, S.G. & ODILE CHRISTEN, M. (1993). Moxonidine, a centrally antihypertensive agent, is a selective ligand for I_1-imidazoline sites. *J. Pharmacol. Exp. Ther.*, **264**, 172–182.

ERNSBERGER, P.R., WESTBROOKS, K.L., ODILE CHRISTEN, M. & SCHÄFER, S.G. (1992). A second generation of centrally acting antihypertensive agents act on putative I_1-imidazoline receptors. *J. Cardiovasc. Pharmacol.*, **20** (Suppl. 4), S1–S10.

ESCRIBA, P.V., SASTRE, M. & GARCÍA-SEVILLA, J.A. (1994). Increased density of guanine nucleotide-binding (G) proteins in the postmortem brain of heroin addicts. *Arch. Gen. Psychiatry*, (in press).

FREDERICKSON, R.C.A. (1992). Astroglia in Alzheimer's disease. *Neurobiol. Aging*, **13**, 239–253.

GOSS, J.R., FINCH, C.E. & MORGAN, D.G. (1991). Age-related changes in glial fibrillary acidic protein mRNA in the mouse brain. *Neurobiol. Aging*, **12**, 165–170.

HERRERA, D.G. & CUELLO, A.C. (1992). Glial fibrillary acidic protein immunoreactivity following cortical devascularizing lesion. *Neuroscience*, **49**, 781–791.

KINDY, M.S., BHAT, A.N. & BHAT, N.R. (1992). Transient ischemia stimulates glial fibrillary acid protein and vimentin gene expression in the gerbil neocortex, striatum and hippocampus. *Mol. Brian Res.*, **13**, 199–206.

LANGIN, D. & LAFONTAN, M. (1989). [^{3}H]idazoxan binding at non-α_2-adrenoceptors in rabbit adipocyte membranes. *Eur. J. Pharmacol.*, **159**, 199–203.

LE PRINCE, G., DELAERE, P., FAGES, C., DUYCKAERTS, C., HAUW, J.-J. & TARDY, M. (1993). Alterations of glial fibrillary acidic protein mRNA level in the aging brain and in senile dementia of the Alzheimer type. *Neurosci. Lett.*, **151**, 71–73.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

MAIESE, K., PEK, L., BERGER, S.B. & REIS, D.J. (1992). Reduction in focal cerebral ischemia by agents acting at imidazole receptors. *J. Cereb. Blood Flow Metab.*, **12**, 53–63.

MALLARD, N.J., HUDSON, A.L. & NUTT, D.J. (1992). Characterization and autoradiographical localization of non-adrenoceptor binding sites in the rat brain. *Br. J. Pharmacol.*, **106**, 1019–1027.

MASOOD, K., BESNARD, F., SU, Y. & BRENNER, M. (1993). Analysis of a segment of the human glial fibrillary acidic protein gene that directs astrocyte-specific transcription. *J. Neurochem.*, **61**, 160–166.

MCPHERSON, G.A. (1985). Analysis of radioligand binding experiments: a collection of computer programs for IBM PC. *J. Pharmacol. Methods*, **14**, 213–228.

MELLER, E., GOLDSTEIN, M., FRIEDHOFF, A.J. & SCHWEITZER, J.W. (1988). N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ): a new tool to probe CNS receptor function. *Adv. Exp. Med. Biol.*, **235**, 121–136.

MICHEL, M.C. & ERNSBERGER, P. (1992). Keeping an eye on the I site: imidazoline-preferring receptors. *Trends Pharmacol. Sci.*, **13**, 369–370.

MICHEL, M.C. & INSEL, P.A. (1989). Are there multiple imidazoline binding sites? *Trends Pharmacol Sci.*, **10**, 342–344.

MIRALLES, A., OLMOS, G., SASTRE, M., BARTUREN, F., MARTIN, I. & GARCÍA-SEVILLA, J.A. (1993a). Discrimination and pharmacological characterization of I_2-imidazoline sites with [^{3}H]idazoxan and alpha-2 adrenoceptors with [^{3}H]RX821002 (2-methoxy idazoxan) in the human and rat brains. *J. Pharmacol. Exp. Ther.*, **264**, 1187–1197.

MIRALLES, A., RIBAS, C., OLMOS, G. & GARCÍA-SEVILLA, J.A. (1993b). Differential effects of the alkylating agent N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline on brain α_2-adrenoceptors and I_2-imidazoline sites in vitro and in vivo. *J. Neurochem.*, **61**, 1602–1610.

MOLDERINGS, G.J., MOURA, D., FINK, K., BÖNISCH, H. & GÖTHERT, M. (1993). Binding of [^{3}H]clonidine to I_1-imidazoline sites in bovine adrenal medullary membranes. *Naunyn-Schmied. Arch. Pharmacol.*, **348**, 70–76.

MUNSON, P.J. & RODBARD, D. (1980). Ligand: a versatile computerized approach for the characterization of ligand binding system. *Anal. Biochem.*, **107**, 220–239.

MURPHY, S. & PEARCE, B. (1987). Functional receptors for neurotransmitters on astroglial cells. *Neuroscience*, **22**, 381–394.

NORTON, W.T., AQUINO, D.A., HOZUMI, I., CHIU, F.-C. & BROSNAN, C.F. (1992). Quantitative aspects of reactive gliosis: a review. *Neurochem. Res.*, **17**, 877–885.

O'CALLAGHAN, J.P. & MILLER, D.B. (1991). The concentration of glial fibrillary acidic protein increases with age in the mouse and rat brain. *Neurobiol. Aging*, **12**, 171–174.

OLDERFELD-NOWAK, B., BACIA, A., GRADKOWSKA, M., FUSCO, M., VANTINI, G., LEON, A. & ALOE, L. (1992). In vivo activated brain astrocytes may produce and secrete nerve growth factor-like molecules. *Neurochem. Int.*, **21**, 455–461.

OLMOS, G., MIRALLES, A., BARTUREN, F. & GARCÍA-SEVILLA, J.A. (1992). Characterization of brain imidazoline receptors in normotensive and hypertensive rats: differential regulation by chronic imidazoline drug treatment. *J. Pharmacol. Exp. Ther.*, **260**, 1000–1007.

REGUNATHAN, S., EVINGER, M.J., MEELEY, M.P. & REIS, D.J. (1991). Effects of clonidine and other imidazole-receptor binding agents on second messenger systems and calcium influx in bovine adrenal chromaffin cells. *Biochem. Pharmacol.*, **42**, 2011–2018.

REGUNATHAN, S., FEINSTEIN, D.L. & REIS, D.J. (1993a). Expression of non-adrenergic imidazoline sites in rat cerebral cortical astrocytes. *J. Neurosci. Res.*, **34**, 681–688.

REGUNATHAN, S., MEELEY, M.P. & REIS, D.J. (1993b). Expression of non-adrenergic imidazoline sites in chromaffin cells and mitochondrial membranes of bovine adrenal medulla. *Biochem. Pharmacol.*, **45**, 1667–1675.

RUIZ, J., MARTÍN, I., CALLADO, L.F., MEANA, J.J., BARTUREN, F. & GARCÍA-SEVILLA, J.A. (1993). Non-adrenoceptor [^{3}H]idazoxan binding sites (I_2-imidazoline sites) are increased in postmortem brain from patients with Alzheimer's disease. *Neurosci. Lett.*, **160**, 109–112.

SALM, A.K. & MCCARTHY, K.D. (1992). The evidence for astrocytes as a target for central noradrenergic activity: Expression of adrenergic receptors. *Brain Res. Bull.*, **29**, 265–275.

SASTRE, M. & GARCÍA-SEVILLA, J.A. (1993). Opposite age-dependent changes of α_{2A}-adrenoceptors and non-adrenoceptor [^{3}H]idazoxan binding sites (I_2-imidazoline sites) in the human brain: strong correlation of I_2 with MAO-B sites. *J. Neurochem.*, **61**, 881–889.

SAURA, J., KETTLER, R. DA PRADA, M. & RICHARDS, J.J. (1992). Quantitative enzyme radioautography with [^{3}H]Ro 41-1049 and [^{3}H]Ro 19-6327 in vitro: localization and abundance of MAO-A and MAO-B in rat CNS, peripheral organs and human brain. *J. Neurosci.*, **12**, 1977–1999.

TESSON, F. & PARINI, A. (1991). Identification of an imidazoline-guanidinium receptive site in mitochondria from rabbit cerebral cortex. *Eur. J. Pharmacol.*, **208**, 81–83.

TESSON, F., PRIP-BUUS, C., LEMOINE, A., PEGORIER, J.-P. & PARINI, A. (1991). Subcellular distribution of imidazoline-guanidinium-receptive sites in human and rabbit liver. *J. Biol. Chem.*, **266**, 155–160.

WESTLUND, K.L., DENNEY, R.M., ROSE, R.M. & ABELL, C.W. (1988). Localization of distinct monoamine oxidase A and monoamine oxidase B populations in human brainstem. *Neuroscience*, **25**, 439–456.

ZONNENSCHEIN, R., DIAMANT, S. & ATLAS, D. (1990). Imidazoline receptors in rat liver cells: a novel receptor or a subtype of α_2-adrenoceptor? *Eur. J. Pharmacol.*, **190**, 203–215.

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Pharmacological properties of the cloned $\alpha_{1A/D}$-adrenoceptor subtype are consistent with the α_{1A}-adrenoceptor characterized in rat cerebral cortex and vas deferens

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1 The pharmacological characteristics of cloned mammalian $\alpha_{1A/D}$-, α_{1B}- and α_{1C}-adrenoceptor subtypes expressed in rat 1 fibroblasts were determined in comparison to the binding and functional properties of these subtypes in rat tissues.

2 Analysis of [^{3}H]-prazosin binding to membrane homogenates from rat 1 fibroblast cells expressing each of the α_1-subtypes indicated high affinity binding to a single population of binding sites. Binding affinities were similar for $\alpha_{1A/D}$-, α_{1B}- and α_{1C}-subtypes (K_ds: 0.13, 0.10 and 0.15 nM respectively) although a higher density of α_{1B}- and α_{1C}-receptors (B_{max}: 4068 and 10,323 fmol mg^{-1} protein respectively) were expressed in comparison to $\alpha_{1A/D}$ (838 fmol mg^{-1}).

3 Displacement of [^{3}H]-prazosin from membranes expressing cloned α_1-adrenoceptor subtypes revealed that 5-methyl-urapidil, WB 4101, benoxathian and phentolamine displayed high affinity and selectivity for $\alpha_{1A/D}$- over α_{1B}-subtypes. These compounds also had high affinity and selectivity for α_{1C}- over α_{1B}-subtypes. 5-Methyl-urapidil showed selectivity for α_{1C} (K_i 0.60 ± 0.16 nM) over both $\alpha_{1A/D}$ (K_i, 9.8 ± 2.8 nM) and α_{1B} (K_i 57.2 ± 12 nM) subtypes. Prazosin and doxazosin were not subtype selective.

4 In comparison to [^{3}H]-prazosin a similar pharmacological profile was obtained with [^{125}I]-HEAT using cloned $\alpha_{1A/D}$-, α_{1B}- and α_{1C}-adrenoceptors expressed in rat 1 fibroblasts.

5 The affinities of prazosin, WB 4101, 5-methyl-urapidil, phentolamine and benoxathian at cloned $\alpha_{1A/D}$-receptors were consistent with α_{1A} affinities determined with chlorethylclonidine-treated rat cortical membranes. Affinities at cloned α_{1B}-receptors were consistent with α_{1B} affinities determined with rat liver membranes.

6 Using the epididymal rat vas deferens as a functional measure of α_{1A} affinity, prazosin (pA_2 9.23 ± 0.28), WB 4101 (pA_2 9.58 ± 0.12), phentolamine (pK_B 7.90 ± 0.16), benoxathian (pK_B 9.21 ± 0.21) and 5-methyl-urapadil (pK_B 8.51 ± 0.16) were potent antagonists of noradrenaline-induced contractions.

7 At present, evidence from cloning studies suggests the existence of at least three α_1-adrenoceptor subtypes. In contrast to the recent proposal for α_1-adrenoceptor classification, the pharmacology of the cloned $\alpha_{1A/D}$ (or α_{1D})-adrenoceptor is more consistent with that of an α_{1A}-adrenoceptor characterized in rat cerebral cortex and vas deferens.

Keywords: α_1-Adrenoceptors; cloned receptors; rat vas deferens; rat cortex

Introduction

Heterogeneity amongst α_1-adrenoceptors has been evident for nearly a decade on the basis of functional responses in smooth muscle (see McGrath *et al.*, 1989). The first definitive classification of α_1-subtypes, established by use of radioligand binding, was proposed by Morrow & Creese (1986) based on the ability of certain antagonists such as WB 4101 and phentolamine to discriminate between high and low affinity binding sites in rat cerebral cortex. Further characterization with the alkylating agent chlorethylclonidine (CEC) indicated that the low affinity sites for WB 4101 were also sensitive to CEC and were designated α_{1B}, the high affinity sites being α_{1A} (Minneman *et al.*, 1988; Bylund, 1992). Both α_{1A}- and α_{1B}-subtypes have different anatomical distributions and appear to be differentially coupled to the mobilisation of extra and intracellular calcium respectively (Han *et al.*, 1987; Minneman *et al.*, 1988; Lomasney *et al.*, 1991). In addition to WB 4101, binding and functional studies have indicated that several compounds including 5-methyl urapidil and nigulpidine have selectivity for α_{1A}- over α_{1B}-subtypes (Hanft & Gross, 1989; Gross *et al.*, 1989; Minneman & Atkinson, 1991). Evidence for the existence of further α_1-subtypes has largely been substantiated by molecular cloning studies. The first subtype to be identified was the α_{1B}-adrenoceptor from the hamster DDT1-MF2 smooth muscle cell line (Cotecchia *et al.*, 1988), followed by the identification of a novel α_1-subtype from a bovine cDNA library which has been termed α_{1C} (Schwinn *et al.*, 1990; 1991). Both subtypes are pharmacologically distinct but share a common signalling mechanism in that both α_{1B} and α_{1C}-subtypes mobilize intracellular Ca^{2+} through the activation of phospholipase C via a pertussis toxin-insensitive G protein (Schwinn *et al.*, 1991). Using an oligonucleotide probe based on the cDNA of the hamster α_1-subtype, screening a rat cerebral cortex cDNA library then identified two further clones (Lomasney *et al.*, 1991). The first proved to be the rat homologue of the hamster α_{1B}-adrenoceptor whilst the second was claimed to represent the first identification of the putative α_{1A} subtype on the basis of insensitivity to CEC, high affinity for WB 4101 and phentolamine and tissue distribution in rat tissues: thus, Northern analysis with an α_{1A}-receptor cDNA probe detected high levels in rat vas deferens, hippocampus and cortex.

A putative fourth α_1-subtype, also identified from a rat brain cDNA library, was described by Perez *et al.* (1991). Initially this subtype was claimed to be distinct from the α_{1A} subtype and termed α_{1D} on the basis of its pharmacological

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profile and differential sensitivity to CEC. However, it is now believed that these clones code for the same α_1 subtype (differing by only two codons in sequence) and it has recently been proposed that this subtype be designated $\alpha_{1A/D}$ (Schwinn & Lomasney, 1992). This classification has been proposed because of the low affinities shown by several α_1 antagonists for the cloned $\alpha_{1A/D}$-subtype in comparison to the potency and pharmacological profile of these compounds at the α_{1A}-receptor defined in tissue binding and functional experiments (Schwinn & Lomasney, 1992). This therefore suggested the existence of a further α_1-subtype, yet to be identified in molecular terms, but corresponding to the tissue α_{1A}-subtype.

In this paper, we have characterized the pharmacological profile of a variety of compounds at cloned $\alpha_{1A/D}$, α_{1B} and α_{1C}-subtypes in conjunction with functional and binding studies. Our data indicate that the pharmacological properties of the cloned $\alpha_{1A/D}$-adrenoceptor are entirely consistent with the α_{1A}-subtype characterized in rat cerebral cortex and vas deferens.

Methods

Expression of cloned α_1-adrenoceptor subtypes

Construction and transfection of rat $\alpha_{1A/D}$-, hamster α_{1P}- and bovine α_{1C}-adrenoceptors was carried out at Duke University, Durham, N.C. U.S.A. by use of methods described by Allen *et al.* (1991). Cloned receptors expressed in rat 1 fibroblasts were subsequently obtained commercially through TULCO (Research Triangle Park, N.C., U.S.A.). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum and 300 $\mu g\ ml^{-1}$ G418 sulphate, a neomycin analogue. For subculturing, cell monolayers were washed with Hank's balanced salt solution and trypsinized briefly with 0.05% trypsin, 0.5 mM EDTA and split 1:5 to 1:20 every 3–4 days.

Radioligand binding assays

Radioligand binding experiments were performed with membranes prepared from rat 1 fibroblast cells expressing individual α_1-subtypes or from rat tissues as indicated. Scraped cells or dissected tissues were homogenized in ice cold 50 mM Tris buffer (pH 7.5) in a Polytron homogenizer (PT10, setting 6, 20 s). The membranes were washed three times by centrifugation (20 min at 20,000 *g*) and resuspended in fresh buffer before storage at − 70°C. For pretreatment with chlorethylclonidine, membranes were incubated at 37°C for 15 min in 50 mM Tris buffer (pH 7.5) in the presence of 10 μM CEC followed by three further washes in ice cold 50 mM Tris buffer before storage. Tissue membranes were stored at a concentration of 2.5 $mg\ ml^{-1}$ protein, cell membranes at 0.5 $mg\ ml^{-1}$ protein. Binding of [^{125}I]-HEAT was carried out as described by Schwinn & Lomasney (1992). Binding of [^{3}H]-prazosin was measured in 400 μl aliquots of diluted membranes (200 μg tissue protein or 10 μg fibroblast protein) in 50 mM Tris buffer (pH 7.5) in a final assay volume of 500 μl. Non specific binding was determined in the presence of 1 μM phentolamine. Construction of saturation isotherms was determined by sequential dilution from a final concentration of 8 nM [^{3}H]-prazosin. Assays were incubated at 25°C with [^{3}H]-prazosin for 30 min and for 45 min with [^{125}I]-HEAT. All reactions were terminated by the addition of ice cold Tris buffer and rapid cold filtration over Whatman GF/B filters under vacuum. Saturation and displacement binding data were analysed by iterative non-linear curve fitting programmes.

Epididymal rat vas deferens

Vas deferens were removed from male Charles River Caesarian derived rats (250–320 g). The epididymal portions were mounted in 15 ml organ baths containing Krebs buffer of the following composition (mM): NaCl 120, $NaHCO_3$ 25, glucose 11, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5 containing cocaine 10 μM and corticosterone 10 μM. The medium was maintained at 37°C, pH 7.4 and aerated with a 95% O_2/5% CO_2 mixture. A resting tension of 0.5 g was applied and changes in isometric tension measured via force-displacement transducers. The preparations were equilibrated for 60 min before any additions to the bath. Each tissue was exposed to a sensitizing dose of 100 μM noradrenaline (NA) for 1 min and then washed periodically for 30 min. Concentration-response curves (CRC) were constructed by non-cumulative addition of NA in 0.5 log increments from 0.01 μM to a maximum of 100 μM with a 10 min wash period between each dose. The preparations were then equilibrated with or without antagonist for 30 min before a second CRC to NA. Antagonist pA_2 values were obtained from a plot of log (agonist DR − 1) against log antagonist concentration where the slope was not different from unity (Arunlakshana & Schild, 1959). K_B (antagonist dissociation constant) was determined from the equation $K_B = [A]/(DR - 1)$ where the dose ratio (DR) was produced by a single concentration of antagonist [A].

Drugs used in the study

The following drugs (sources in parentheses) were used: [^{3}H]-prazosin and [^{125}I]-HEAT (2- {β-(4-hydroxy-3- [^{125}I]-iodophenyl)ethylaminomethyl}-tetralone) (Amersham, U.K.); noradrenaline, cocaine hydrochloride, and corticosterone (Sigma, U.K.); chlorethylclonidine, spiperone, benoxathian, 5-methyl-urapidil, WB 4101 (2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4-benzodioxane hydrochloride (Research Biochemicals Inc., Semat, U.K.); phentolamine hydrochloride (Ciba-Geigy, Basle, Switzerland); prazosin and doxazosin (Pfizer, Sandwich, U.K.). All other drugs and chemicals were obtained from Sigma (U.K.) or B.D.H. (U.K.) Drugs were dissolved in distilled H_2O or dimethyl sulphoxide (DMSO) at 1 mM and subsequent dilutions made in assay buffer.

Results

[^{3}H]-prazosin binding to cloned mammalian α_1-adrenoceptor subtypes

Analysis of [^{3}H]-prazosin binding to membranes prepared from rat 1 fibroblasts expressing each of the three α_1 subtypes indicated saturable, high affinity binding to a single population of receptor sites. The affinity of [^{3}H]-prazosin binding sites was similar for all subtypes, although a high density of α_{1B}- and α_{1C}-receptors was expressed in rat 1 fibroblasts in comparison to $\alpha_{1A/D}$ (Table 1). The non-selective antagonists, doxazosin and prazosin, displayed high and similar affinity at all three cloned α_1-subtypes and, as with all other compounds examined, displaced the ligand with Hill slopes not significantly different from unity, indicating an apparently competitive interaction at a single population of binding sites (Table 2). However, several compounds displayed differing affinities for the three subtypes having selectivity for one or more of the cloned α_1-subtypes. WB 4101, 5-methyl-urapidil, benoxathian and phentolamine showed high affinity and selectivity for cloned $\alpha_{1A/D}$-over α_{1B}-adrenoceptor subtypes. However, these agents also showed varying degrees of selectivity for α_{1C} over $\alpha_{1A/D}$ (Table 2) and in this respect, 5-methyl-urapidil was highly selective for α_{1C}- over both $\alpha_{1A/D}$- and α_{1B}-subtypes.

[^{3}H]-prazosin binding to rat liver and CEC-treated cortical membranes

In this series of experiments we considered it important to establish conditions with CEC which were appropriate and

Table 1 Saturation binding parameters for [^{3}H]-prazosin at cloned α_1-adrenoceptor subtypes

	$\alpha_{1A/D}$	α_{1B}	α_{1C}
K_d (nM)	0.13 ± 0.02	0.10 ± 0.01	0.15 ± 0.02
B_{max} (fmol mg^{-1})	838 ± 46	4068 ± 342	10323 ± 974

Saturation binding parameters were determined with [^{3}H]-prazosin (0.01–8.0 nM) using membrane homogenates from rat 1 fibroblast cells expressing each of the α_1-subtypes. Data represent the mean ± s.e.mean of 9–10 experiments carried out in duplicate.

Table 2 Comparative affinities of α_1-adrenoceptor antagonists for cloned $\alpha_{1A/D}$-, α_{1B}- and α_{1C}-adrenoceptor subtypes

	K_i (nM)		
Compound (n)	$\alpha_{1A/D}$	α_{1B}	α_{1C}
Prazosin (18)	0.32 ± 0.02	0.19 ± 0.02	0.27 ± 0.04
Doxazosin (6)	1.99 ± 0.05	0.80 ± 0.02	1.58 ± 0.10
WB 4101 (14)	0.67 ± 0.07	16.4 ± 2.0	0.29 ± 0.03
5-Methyl urapidil (7)	9.8 ± 2.8	57.2 ± 12	0.60 ± 0.16
Phentolamine (10)	6.1 ± 0.9	39.8 ± 4.3	2.8 ± 0.3
Benoxathian (6)	0.50 ± 0.02	17.2 ± 4.4	0.30 ± 0.05
Spiperone (4)	25.6 ± 3.2	6.8 ± 1.0	7.5 ± 0.9

Displacement of [^{3}H]-prazosin (0.2 nM) from rat 1 fibroblast membranes expressing each of the α_1-subtypes was determined with at least 10 concentrations of competing drug. Assay points were determined in duplicate. For all compounds examined Hill slopes (nH) were not significantly different from unity.

Table 3 The effect of chlorethylclonidine (CEC) on [^{3}H]-prazosin saturation binding to rat cortical and liver membranes

	Control		*+ CEC*	
Time	K_d	B_{max}	K_d	B_{max}
Rat cortex	0.20 ± 0.02	251 ± 27	0.22 ± 0.03	130 ± 19
Rat liver	0.32 ± 0.04	508 ± 31	0.28 ± 0.02	43 ± 9

Membranes were incubated with CEC (10 μM) for 15 min at 37°C. Saturation parameters K_d (nM) and B_{max} (fmol mg^{-1} protein) were determined by iterative curve fitting. Values represent the mean ± s.e.mean of 3–4 experiments.

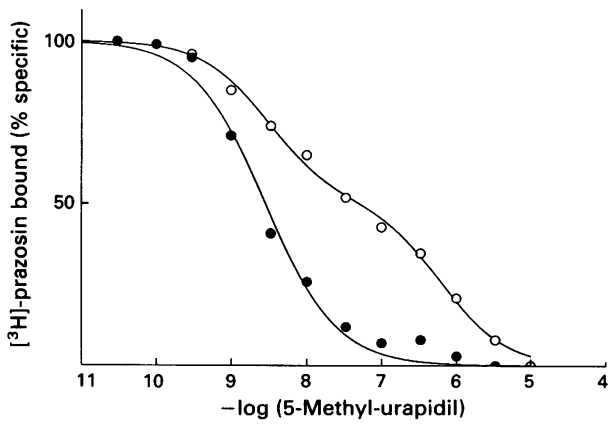


Figure 1 Inhibition of [^{3}H]-prazosin binding to rat cortical membranes by 5-methyl-urapidil using control membranes (○) or membranes pretreated with chlorethylclonidine (CEC) 10 μM for 15 min at 37°C (●). In control membranes the data were best described by a two site fit (site 1, 51.1%, IC_{50} = 632 nM and site 2, 48.9%, IC_{50} = 3 nM). Using CEC-treated membranes the data were best described by a one site fit (IC_{50} = 2.2 nM, nH = 0.92). Data from a representative experiment are shown, calculated mean K_i values are given in the text.

selective for the alkylation of a given population of adrenoceptors prior to the interpretation of [^{3}H]-prazosin data from CEC-treated cortical membranes containing a heterogeneous receptor population. Pretreatment of membrane homogenates with CEC (15 min at 37°C) resulted in alkylation of more than 90% of the sites in rat liver and 49% of the sites in rat cortex (Table 3).

In rat liver membranes, the affinity and selectivity of all compounds examined (prazosin > WB 4101 > benoxathian > phentolamine > 5-methyl-urapidil) was similar to that observed at cloned α_{1B}-subtypes (Table 4). Taken together with the sensitivity of the membrane preparations to CEC, these data indicated the presence of α_{1B}-receptors in rat liver. Under conditions with CEC which resulted in alkylation of 90% of the α_1 population in rat liver membranes (Table 3), we then examined the properties of the remaining α_1 population in rat brain membranes following a similar CEC protocol (Figure 1). Table 4 illustrates that under this protocol, the α_1 population remaining in rat brain following CEC treatment retained high affinity for all compounds examined. Furthermore, the affinities obtained were similar to those observed at cloned $\alpha_{1A/D}$-subtypes indicating a similar pharmacological profile of rat cortical and cloned $\alpha_{1A/D}$-adrenoceptors.

[^{125}I]-HEAT binding to cloned α_1-adrenoceptor subtypes

The pharmacological profile of cloned α_1-adrenoceptors labelled with [^{25}I]-HEAT was examined with a range of α_1-adrenoceptor antagonists (Table 5). Similar to the profile obtained with [^{3}H]-prazosin, WB 4101, benoxathian and 5-methyl-urapidil appeared to be selective for $\alpha_{1A/D}$ over α_{1B}. The rank order of compounds was similar to that observed with [^{3}H]-prazosin, except that phentolamine displayed slightly higher affinity for $\alpha_{1A/D}$ sites labelled by [^{3}H]-prazosin than [^{125}I]-HEAT. Similarly, at cloned α_{1C}-adrenoceptors, whilst the rank order of potency for compounds was similar with both ligands, slightly lower potency was observed against [^{125}I]-HEAT and thus spiperone and 5-methyl-urapidil appeared to be less selective for α_{1C} over $\alpha_{1A/D}$ than observed with [^{3}H]-prazosin.

Effects of α_1-adrenoceptor antagonists on contractile responses of epididymal rat vas deferens

Noradrenaline (0.01–100 μM) produced concentration-dependent contractions of epididymal rat vas deferens generating a maximal tension of 2.24 ± 0.12 g with an EC_{50} of 0.10 ± 0.65 μM (n = 10). Prazosin and WB 4101 antagonized NA-induced contractions and shifted control CRC to the right in parallel which yielded pA_2 values of 9.23 ± 0.28 (slope = 1.2) and 9.58 ± 0.12 (slope = 1.1) respectively (Figure 2). Estimated pK_B values (antagonists at 100 nM) were calculated to be 9.21 ± 0.21, 7.90 ± 0.11 and 8.51 ± 0.16 for benoxathian, phentolamine and 5-methyl-urapidil respectively.

Discussion

In this paper we have examined the pharmacological profile of cloned mammalian $\alpha_{1A/D}$-, α_{1B}- and α_{1C}-subtypes. We have compared these data with the profile obtained with these

Table 4 The affinity of compounds for adrenoceptor subtypes labelled by [^{3}H]-prazosin

Compound	Cloned $\alpha_{1A/D}$ K_i (nM)	Rat cortex + CEC K_i (nM)	Cloned α_{1B} K_i (nM)	Rat liver K_i (nM)
Prazosin	0.32 ± 0.02	0.18 ± 0.33	0.19 ± 0.02	0.31 ± 0.03
WB 4101	0.67 ± 0.07	0.57 ± 0.10	16.4 ± 2.0	28.1 ± 3.1
5-Methyl-urapidil	9.8 ± 2.8	1.93 ± 0.23	57.2 ± 12	189 ± 33
Phentolamine	6.1 ± 0.9	4.1 ± 0.8	39.9 ± 4.3	96.5 ± 13
Benoxathian	0.50 ± 0.02	1.3 ± 0.2	17.2 ± 4.4	85.0 ± 14

Affinities were determined from displacement of [^{3}H]-prazosin binding (0.2–0.3 nM) using membranes derived from rat 1 fibroblasts expressing $\alpha_{1A/D}$- and α_{1B}-subtypes in comparison to rat liver and CEC-treated cortical membranes. Values represent mean ± s.e.mean of at least 4 separate experiments. Hill slopes (nH) were not significantly different from unity.

Table 5 Comparative affinities of α_1-adrenoceptor antagonists for cloned α_1-adrenoceptor subtypes labelled by [^{125}I]-HEAT

Compound	K_i (nM) $\alpha_{1A/D}$	α_{1B}	α_{1C}
Prazosin	0.20 ± 0.01	0.15 ± 0.01	0.60 ± 0.07
WB 4101	0.71 ± 0.08	4.77 ± 0.05	0.55 ± 0.02
5-Methyl-urapidil	5.50 ± 0.4	144.6 ± 10.3	2.9 ± 0.4
Phentolamine	34.5 ± 2.1	88.2 ± 6.3	17.5 ± 1.4
Benoxathian	0.60 ± 00	9.60 ± 0.7	0.18 ± 0.01
Spiperone	40.5 ± 4.9	12.7 ± 1.1	18.0 ± 2.4

Displacement of [^{125}H]-HEAT (50–60 pM) from rat 1 fibroblast membranes expressing each of the α_1-subtypes was determined with at least 10 concentrations of competing drug. Values represent the mean ± s.e.mean of 3–4 experiments performed in duplicate. Hill slopes (nH) were not significantly different from unity for any compounds examined.

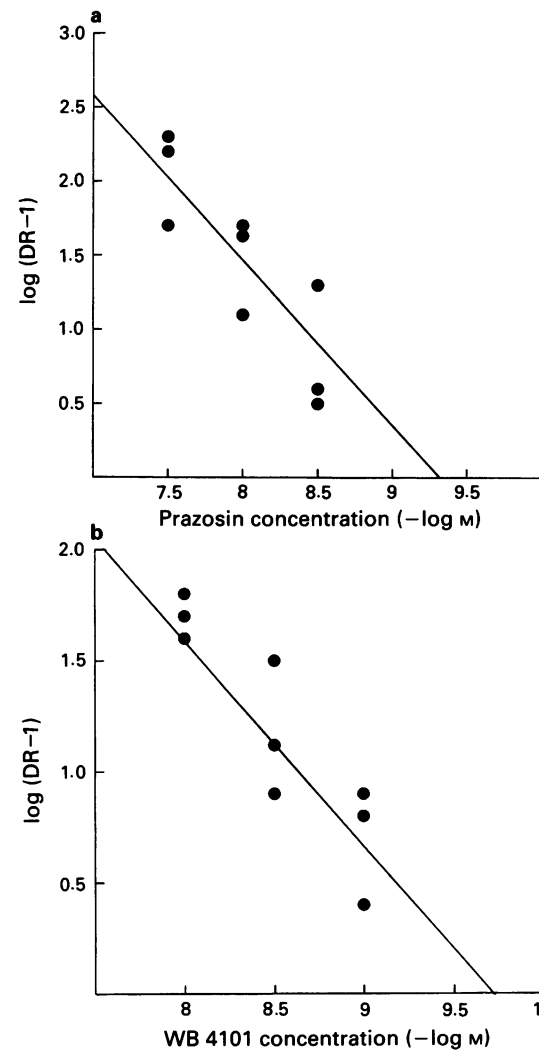


Figure 2 pA_2 determinations for (a) prazosin and (b) WB 4101 against contractions to noradrenaline in epididymal rat vas deferens. Dose-ratio determinations are shown for each antagonist concentration and a line of best fit to the data is shown. Mean pA_2 determinations from each set of experiments are given in the text.

receptors using tissue membranes and in functional studies to assess the relevance of the cloned data in terms of α_1-subtype distribution and implication for α_1-adrenoceptor subtype classification. Whilst heterogeneity amongst α_1-subtypes suggested by binding and functional experiments has been substantiated by molecular studies, the data obtained in the present study are not consistent with the reconciliation of cloning and pharmacological data obtained with the $\alpha_{1A/D}$-subtype described by Schwinn & Lomasney (1992). We have demonstrated that the high nM affinity of several α_1 antagonists at cloned $\alpha_{1A/D}$-adrenoceptors is retained at the α_{1A}-subtype in rat brain revealed in the presence of CEC. In contrast, Schwinn & Lomasney (1992) reported that several agents, in particular benoxathian ($pK_i = 5.1$), phentolamine ($pK_i = 7.0$) and 5-methyl-urapidil ($pK_i = 6.5$) possessed much lower affinity for cloned $\alpha_{1A/D}$-receptors than obtained in our study. The inconsistency of their affinities at cloned $\alpha_{1A/D}$-adrenoceptors in relation to functional and binding potency in appropriate tissues therefore suggested the existence of another α_{1A}-subtype, yet to be identified by molecular cloning. Our data are not consistent with this proposal. Several issues need to be resolved in the context of our findings. Firstly, the use of CEC to unmask the α_{1A} population in membrane preparations. Secondly, the discrepancy whereby we have obtained high affinity for phentolamine, benoxathian and 5-methyl-urapidil at cloned $\alpha_{1A/D}$-adrenoceptors. Thirdly, the significance of the affinity at cloned α_1-subtypes in relation to tissue distribution and subtype classification.

It is well established that receptor inactivation using CEC is dependent upon concentration, time of exposure, species and nature of the tissue preparation (Tian *et al.*, 1990; Perez *et al.*, 1991; Oriowo *et al.*, 1992). Under appropriate conditions the use of CEC to define α_{1B} populations is based on the finding that it is able to inactivate pure populations of α_{1B} sites, e.g. when the receptors are transiently expressed in COS-7 cells (Schwinn *et al.*, 1990). Additionally, compounds distinguishing between α_{1A}- and α_{1B}-subtypes are better described in terms of a one site fit in the presence of CEC (Han & Minneman, 1991). However, it should be noted that in some tissues, such as rat heart, CEC does not appear to inactivate all low affinity α_{1B} sites (see Han & Minneman, 1991). Additional resistance to CEC in some tissues could

also be explained by the additional presence of α_{1C}-adrenoceptors which have intermediate sensitivity to CEC (Schwinn *et al.*, 1990), although in rat tissues this is unlikely, given that this subtype appears to be absent or present in very low levels on the basis of Northern blot analysis (Schwinn *et al.*, 1990; 1991). In this study, using membrane preparations, we therefore used a CEC protocol inactivating most of the α_{1B} binding sites rat liver membranes before employing an identical protocol to inactivate these sites in rat cortical membranes. Using this protocol with cloned mammalian receptors, CEC caused a reduction of 15 ± 2%, 95 ± 5% and 85 ± 10% ($n = 3$) in the maximal number of [^{3}H]-prazosin sites at α_{1A}-, α_{1B}- and α_{1C}-subtypes respectively. We are confident that alkylation of the majority of α_{1B}-subtypes in rat cortex had occurred because we observed the well documented biphasic displacement curves to 5-methyl-urapidil, WB 4101 and phentolamine in control tissues, whereas in the presence of CEC all compounds examined had monophasic displacement isotherms with Hill slopes close to unity. In rat cortical membranes, the pharmacological profile of CEC-insensitive sites appeared to be indicative of α_{1A}-adrenoceptors since compounds displayed comparable potencies to that obtained with cloned $\alpha_{1A/D}$-receptors.

What then are the implications of these findings in the context of the currently proposed classification for α_1-adrenoceptor subtypes? In the present study the data obtained with cloned α_{1B}-adrenoceptors agree very well with previously reported values obtained under similar assay conditions (Perez *et al.*, 1991). It is well established that rat liver contains predominantly the α_{1B}-subtype (Han *et al.*, 1987; Torres-Marquez *et al.*, 1991) and based on CEC sensitivity and pharmacological profile this is consistent with our data. Thus, we obtained a similar binding profile using both cloned α_{1B} and rat liver membranes. Also consistent with previous findings (Schwinn *et al.*, 1991; Schwinn & Lomasney, 1992) we observed selectivity for α_{1C}- over α_{1B}-subtypes using WB 4101 and 5-methyl-urapidil, with this latter compound also showing selectivity for α_{1C}- over $\alpha_{1A/D}$-adrenoceptors.

The initial difference between the originally cloned α_{1A}-subtype and subequently cloned α_{1D}-subtype was based on pharmacological differences in a few compounds and sensitivity to CEC. However, these clones probably code for the same receptor in that they differ by only two amino acids and as discussed, the use of CEC is very much a function of tissue, concentration and time. In this respect, with the exception of phentolamine our data obtained with cloned $\alpha_{1A/D}$-receptors are similar to data previously obtained with [^{3}H]-prazosin at cloned α_{1D}-receptors (Perez *et al.*, 1991). The affinities we obtained with phentolamine at cloned $\alpha_{1A/D}$- and rat brain α_{1A}-receptors (K_is of 6.1 and 4.1 nM respectively) were also generally higher than that previously described on the basis of radioligand binding experiments in rat tissues (Johnson & Minneman, 1987; Han & Minneman, 1991). Nevertheless, our binding data to both cloned and rat brain $\alpha_{1A/D}$-adrenoceptors are consistent with phentolamine having selectivity for α_{1A}- over α_{1B}-subtypes (Hanft & Gross, 1989; Terman *et al.*, 1990). When we employed [^{125}I]-HEAT as a ligand to label cloned α_1-subtypes expressed in rat 1 fibroblast using previously described methods (Schwinn & Lomasney, 1992) a range of α_1 antagonists retained a broadly similar profile to that observed with [^{3}H]-prazosin. However, we obtained some differences in binding affinity, particularly with phentolamine and the selectivity between subtypes was less apparent for some agents. These differences may be a reflection of different experimental protocols since these subtypes were previously expressed transiently in COS-7 cells or stably in Hela cells (Schwinn & Lomasney, 1992), although in our studies benoxathian still retained high affinity for α_1-subtypes labelled by [^{125}I]-HEAT. In addition, benoxathian was a potent inhibitor of NA-stimulated inositol polyphosphate production in rat 1 fibroblasts expressing α_{1C}-receptors ($pK_B = 9.5$, unpublished observations) consistent with the binding affinity at this subtype using [^{3}H]-prazosin in this cell line. Indeed, benoxathian is a well established high affinity α_1-adrenoceptor antagonist and has been shown to have nM affinity at α_1-adrenoceptors in functional and binding experiments using rat aorta, kidney, cortex and vas deferens and guinea-pig liver (Eltze & Boer, 1992; Aboud *et al.*, 1993; Garcia-Sainz & Romero-Avila, 1993) which does not support the low μM affinity of this compound determined at all cloned α_1-subtypes described by Schwinn & Lomasney (1992). Since in our study we observed similar affinities for compounds examined at the cloned $\alpha_{1A/D}$-receptor and CEC-treated rat cortex, this raises the possibility that this subtype is indeed the functionally relevant α_{1A}-subtype typically located on tissues such as rat hippocampus, cerebral cortex and vas deferens.

Functional studies on rat vas deferens originally demonstrated α_{1A}-adrenoceptors to be the predominant subtype (Han *et al.*, 1987) and consistent with other studies (Aboud *et al.*, 1993) contractions of this tissue by NA were unaffected by CEC, indicative of α_{1A}-adrenoceptors. However, radioligand binding studies have revealed high and low affinity sites for [^{3}H]-prazosin on smooth muscle preparations (Muramatsu *et al.*, 1990a,b) including epididymal rat vas deferens (Ohmura *et al.*, 1992) in which it has been claimed that compounds such as WB 4101 and benoxathian have selectively for the high affinity component but inhibit agonist-induced contractions through a site corresponding to the low affinity component. Other functional evidence also suggests that a phasic, CEC-sensitive component can be demonstrated in response to single electrical pulses (Mallard *et al.*, 1992; Aboud *et al.*, 1993). Furthermore, the presence of α_{1B}-adrenoceptors in rat vas deferens has also been demonstrated using mRNA detection by Northern analysis (Lomasney *et al.*, 1991). Thus, whilst Minneman *et al.* (1988) were able to demonstrate a CEC-sensitive phosphatidylinositol phosphate response (α_{1B}-mediated), the contractile response to exogenous agonist remained CEC-resistant. The affinities obtained in our study with a range of α_1-adrenoceptor antagonists against NA-induced contractions of rat vas deferens are consistent with other reported values (Han *et al.*, 1987; Eltze & Boer, 1992; Couldwell *et al.*, 1993) which suggest the contractile element of this tissue in response to exogenous NA to be predominantly mediated via α_{1A}-adrenoceptors. Other studies have been able to demonstrate an excellent correlation between the affinity of antagonists against NA-induced contractions of rat vas deferens and the affinity for α_{1A} sites in rat cortex labelled by [^{3}H]-prazosin (Eltze *et al.*, 1991; Eltze & Boer, 1992). This is in agreement with our findings in relation to the nature of the cloned rat $\alpha_{1A/D}$-adrenoceptor which we have found to be characteristic of this subtype in cortex and vas deferens, consistent with Northern analysis of the distribution of this receptor using a rat α_{1A} cDNA probe (Lomasney *et al.*, 1991). Interestingly, recent data obtained with guinea-pig liver membranes have demonstrated the presence of an homogeneous population of α_1-adrenoceptors which possess pharmacological properties consistent with α_{1A}-adrenoceptors, although Northern analysis indicated the presence of $\alpha_{1A/D}$-adrenoceptors using an $\alpha_{1A/D}$ cDNA probe (Garcia-Sainz & Romero-Avila, 1993).

Whilst rat cortex and vas deferens appear to be pharmacologically characteristic of the cloned rat α_{1A}- or $\alpha_{1A/D}$-adrenoceptor, functional evidence indicates the presence of α_1 subtypes inconsistent with current classification. The very high affinity exhibited by prazosin in some tissues, and the different sensitivity of tissues to agents such as niguldipine and CEC is suggestive of further heterogeneity (Flavahan & Vanhoutte, 1986; Muramatsu *et al.*, 1990a; Ford *et al.*, 1993). Indeed, the characterization of subtypes controlling contractile responses in vascular preparations such as rat aorta relates poorly to subtypes defined by binding and molecular cloning (Mir & Fozard, 1989). Furthermore, the use of CEC to distinguish between subtypes may not be definitive since a recent report by Porter *et al.* (1993) has described a subtype on rat parotid acinar cells characteristic of an α_{1B}-adreno-

ceptor which appears to be totally insensitive to CEC. It remains to be established whether additional heterogeneity can be demonstrated by further cloning and expression of α_1-adrenoceptor subtypes.

In summary, this paper has demonstrated different pharmacological properties of three different cloned α_1-adrenoceptor subtypes which appear to be similar to the properties of these subtypes identified in appropriate tissues. In particular, functional and binding data indicate that the cloned rat $\alpha_{1A/D}$ is representative of pharmacologically defined α_{1A}-adrenoceptors in rat vas deferens and cerebral cortex. Therefore, at the present time, reference to this rat α_1-adrenoceptor subtype as α_{1A} appears to be more appropriate than $\alpha_{1A/D}$ or α_{1D}.

References

ABOUD, R., SHAFII, M. & DOCHERTY, J.R. (1993). Investigation of the subtypes of α_1-adrenoceptor mediating contractions of rat aorta, vas deferens and spleen. *Br. J. Pharmacol.*, **109**, 80–87.

ALLEN, L.F., LEFKOWITZ, R.J., CARON, M.G. & COTTECCHIA, S. (1991). G-protein coupled receptor genes as protooncogenes: constitutively activating mutation of the α_{1B} adrenergic receptor enhances mitogenesis and tumorigenicity. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 11354–11358.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–52.

BYLUND, D.B. (1992). Subtypes of α_1 and α_2 adrenergic receptors. *FASEB J.*, **6**, 832–840.

COTECCHIA, S., SCHWINN, D.A., RANDALL, R.R., LEFKOWITZ, R.J., CARON, M. & KOBLIKA, B.K. (1988). Molecular cloning and expression of the cDNA for the hamster α_1 adrenergic receptor. *Proc. Natl. Acad. U.S.A.*, **85**, 7159–7163.

COULDWELL, C.J., JACKSON, H.L., O'BRIEN, H.L. & CHESS-WILLIAMS, R. (1993). Alpha 1 adrenoceptor subtype mediating contractions of the rat prostate gland. *Br. J. Pharmacol.*, **108**, 163P.

ELTZE, M., BOER, R., SANDERS, K.H. & KOLASSA, N. (1991). Vasodilation elicited by $5HT_{1A}$ receptor agonists in constant pressure perfused kidney is mediated by blockade of α_{1A} adrenoceptors. *Eur. J. Pharmacol.*, **202**, 33–44.

ELTZE, M. & BOER, R. (1992). The adrenoceptor agonist, SDZ NVI 085, discriminates between α_{1A} and α_{2B} adrenoceptor subtypes in vas deferens, kidney and aorta of the rat. *Eur. J. Pharmacol.*, **224**, 125–136.

FLAVAHAN, N.A. & VANHOUTTE, P.M. (1986). α_1 adrenoceptor subclassification in vascular smooth muscle. *Trends Pharmacol. Sci.*, **7**, 347–349.

FORD, A.P.D.W., BERGE, N.V. & CLARKE, D.E. (1993). Characterisation of α_1-adrenoceptors in isolated anococcygeus muscle of rat. *Br. J. Pharmacol.*, **109**, 112P.

GARCIA-SAINZ, J.A. & ROMERO-AVILA, T. (1993). Characterization of the α_{1A} adrenoceptors of Guinea-Pig liver membranes: studies using 5-[^{3}H] methylurapidil. *Mol. Pharmacol.*, **44**, 589–594.

GROSS, G., HANFT, G. & MEHDORN, H.M. (1989). Demonstration of α_{1A} and α_{1B} adrenoceptor binding sites in human brain tissue. *Eur. J. Pharmacol.*, **169**, 325–328.

HAN, C., ABEL, P.W. & MINNEMAN, K.P. (1987). α_1 adrenoceptor subtypes linked to different mechanisms for increasing intracellular Ca^{2+} in smooth muscle. *Nature*, **329**, 333–335.

HAN, C. & MINNEMAN, K.P. (1991). Interaction of subtype selective antagonists with α_1 adrenergic receptor binding sites in rat tissues. *Mol. Pharmacol.*, **40**, 531–538.

HANFT, G. & GROSS, G. (1989). Subclassification of α_1-adrenoceptors recognition sites by urapidil derivatives and other selective antagonists. *Br. J. Pharmacol.*, **97**, 691–700.

JOHNSON, R.D. & MINNEMAN, K.P. (1987). Differentiation of α_1 adrenergic receptors linked to phosphatidylinositol turnover and cyclic AMP accumulation in rat brain. *Mol. Pharmacol.*, **31**, 239–246.

LOMASNEY, J.W., COTECCIA, S., LORENZ, W., LEUNG, W.Y., SCHWINN, D.A., YANG-FENG, T.L., BROWNSTEIN, M., LEFKOWITZ, R.J. & CARON, M.G. (1991). Molecular cloning and expression of the cDNA for the α_{1A} adrenergic receptor. *J. Biol. Chem.*, **266**, 6365–6369.

MALLARD, N.J., MARSHALL, R.W., SITHERS, A.J. & SPRIGGS, T.L.B. (1992). Separation of putative α_{1A}- and α_{1B}-adrenoceptor mediated components in the tension response of the rat vas deferens to electrical field stimulation. *Br. J. Pharmacol.*, **105**, 727–731.

MCGRATH, J.C., BROWN, C.M. & WILSON, V.G. (1989). Alpha adrenoceptors: a critical review. *Med. Res. Rev.*, **9**, 407–533.

MINNEMAN, K.P. & ATKINSON, B. (1991). Interaction of subtype selective antagonists with α_1 adrenergic receptor mediated second messenger responses in rat brain. *Mol. Pharmacol.*, **40**, 523–530.

MINNEMAN, K.P., HAN, C. & ABEL, P.W. (1988). Comparison of α_1 adrenergic subtypes distinguished by chlorethylclonidine and WB-4101. *Mol. Pharmacol.*, **33**, 509–514.

MIR, A.K. & FOZARD, J.R. (1989). A single α_1 adrenoceptor, distinct from the α_{1A} and α_{1B} subtypes, may mediate contraction of rat aorta to phenylephrine. *Br. J. Pharmacol.*, **98**, 651P.

MORROW, A.L. & CREESE, I. (1986). Characterisation of an α_1 adrenergic receptor subtypes in rat brain: a reevaluation of [^{3}H] WB 4101 and [^{3}H] prazosin binding. *Mol. Pharmacol.*, **29**, 321–330.

MURAMATSU, I., KIGOSHI, S. & OSHITA, M. (1990a). Two distinct α_1-adrenoceptor subtypes involved in noradrenaline contraction of the rabbit thoracic aorta. *Br. J. Pharmacol.*, **101**, 662–666.

MURAMATSU, I., OHMURA, T., KIGOSHI, S., HASHIMOTO, S. & OSHITA, M. (1990b). Pharmacological subclassification of α_1-adrenoceptors in vascular smooth muscle. *Br. J. Pharmacol.*, **99**, 197–201.

OHMURA, T., OSHITA, M., KIGOSHI, S. & MURAMATSU, I. (1992). Identification of α_1-adrenoceptor subtypes in the rat vas deferens: binding and functional studies. *Br. J. Pharmacol.*, **107**, 697–704.

ORIOWO, M.A., BEVAN, R.D. & BEVAN, J.A. (1992). Vascular α-adrenoceptor affinity variation is not due to varying populations of subtypes distinguished by WB-4101 and chloroethyclonidine. *Eur. J. Pharmacol.*, **216**, 415–420.

PEREZ, D.M., PIASCIK, M.T. & GRAHAM, R.M. (1991). Solution-phase library screening for the identification of rare clones: isolation of an α_{1D} adrenergic receptor cDNA. *Mol. Pharmacol.*, **40**, 876–883.

PORTER, J.E., DOWD, F.J. & ABEL, P.W. (1993). Atypical alpha I adrenergic receptors on the rat parotid gland acinar cell. *J. Pharmacol. Exp. Ther.*, **263**, 1062–1067.

SCHWINN, D.A. & LOMASNEY, J.W. (1992). Pharmacological characterization of cloned α_1 adrenoceptor subtypes: selective antagonists suggest the existence of a fourth subtype. *Eur. J. Pharmacol.*, **227**, 433–436.

SCHWINN, D.A., LOMASNEY, J.W., LORENZ, W., SZKLUT, P.J., FREMEAU, R.T., YANG-FENG, T.L., CARON, M., LEFKOWTIZ, R.J. & COTECCHIA, S. (1990). Molecular cloning and expression of the cDNA for a novel α_1 adrenergic subtype. *J. Biol. Chem.*, **265**, 8183–8189.

SCHWINN, D.A., PAGE, S.O., MIDDLETON, J.P., LORENZ, W., LIGGETT, S.B., YAMAMOTO, E.G., LAPETINA, E.G., CARON, M.G., LEFKOWITZ, R.J. & COTECCHIA, S. (1991). The α_{1C} adrenoceptor: characterisation of signal transduction pathways and mammalian tissue heterogeneity. *Mol. Pharmacol.*, **40**, 619–626.

TERMAN, B.I., RIEK, R.P., GRODSKI, H.J., HESS, H.J. & GRAHAM, R.M. (1990). Identification and structural characterization of α_1 adrenergic receptor subtypes. *Mol. Pharmacol.*, **37**, 526–534.

TIAN, W.N., GUPTA, S. & DETH, R.C. (1990). Species differences in chloroethylclonidine antagonism at vascular alpha 1 adrenergic receptors. *J. Pharmacol. Exp. Ther.*, **253**, 877–883.

TORRES-MARQUEZ, M.E., VILLALOBOS-MOLINA, R. & GARCIA-SAINZ, J.A. (1991). α_1 subtypes in aorta (α_{1A}) and liver (α_{1B}). *Eur. J. Pharmacol.*, **206**, 199–202.

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Characterization of ET_B receptors mediating contractions induced by endothelin-1 or IRL 1620 in guinea-pig isolated airways: effects of BQ-123, FR139317 or PD 145065

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1 We have characterized the receptors mediating contractions to endothelin-1 (ET-1) or IRL 1620, an ET_B receptor selective agonist, in isolated strips of tissue prepared from different parts of the guinea-pig airways. We used as antagonists BQ-123 and FR139317 (ET_A receptor-selective) and PD 145065 (ET_A/ET_B receptor non-selective).

2 ET-1 and IRL 1620 (10^{-10} M to 10^{-6} M) caused similar concentration-dependent contractions of strips of guinea-pig trachea and upper bronchus. In the guinea-pig trachea without epithelium or lung parenchyma, IRL 1620 was less potent than ET-1.

3 In the trachea, contraction to ET-1 ($<10^{-8}$ M) was preceded by a transient relaxation which was inhibited by BQ-123 (10^{-5} M) or FR 139317 (10^{-5} M) or by the removal of the epithelium. The concentration-response curve to ET-1 in the trachea was shifted to the right by PD 145065 (10^{-5} M to 10^{-4} M). PD 145065 (10^{-4} M) also inhibited the response to ET-1 (3×10^{-7} M) by 55%. Contractions induced by IRL 1620 were not affected by BQ-123 (10^{-6} M) or FR139317 (10^{-6} M) but were significantly attenuated by 10^{-5} M of either antagonist. PD 145065 at 10^{-6} M strongly attenuated and at 10^{-5} M abolished contractions induced by IRL 1620.

4 In the trachea, removal of the epithelium potentiated the effects of both agonists. BQ-123 (10^{-5} M) had no effect on contractions of the trachea without epithelium induced by ET-1, but FR139317 (10^{-5} M) caused a significant inhibition. PD 145065 (10^{-5} M to 10^{-4} M) caused a shift to the right of the ET-1 concentration-response curve without affecting the contractile effect at 3×10^{-7} M. All three antagonists inhibited contractions induced by IRL 1620.

5 In the upper bronchus, BQ-123 (10^{-5} M) did not affect contractions induced by ET-1, while FR139317 (10^{-5} M) attenuated (20–26%) only contractions induced by $1-3 \times 10^{-7}$ M ET-1. PD 145065 (10^{-5} M to 10^{-4} M) caused a shift to the right of the ET-1 concentration-response curve. The contractions induced by IRL 1620 were inhibited by BQ-123 or FR139317 (10^{-5} M to 10^{-4} M). PD 145065 (10^{-6} M) strongly inhibited contractions induced by IRL 1620 and PD 145065 (10^{-5} M) totally abolished them.

6 The contractile action of ET-1 in the lung parenchyma was significantly and similarly attenuated by BQ-123 (10^{-5} M) or indomethacin (10^{-5} M), while FR139317 (10^{-5} M) was less effective. PD 145065 (10^{-6} to 10^{-5} M) inhibited contractions to ET-1. IRL 1620, which is less potent than ET-1 in this preparation, was antagonized by PD 145065 (10^{-5} to 10^{-6} M) but unaffected by BQ-123 (10^{-6} M to 10^{-5} M) or FR139317 (10^{-6} M).

7 Thus, ET_B receptors mediate contractions to ET-1 in all four guinea-pig airway preparations. In addition, contractions to ET-1 in the trachea and lung parenchyma are mediated in part by ET_A receptors. In the latter tissue, these ET_A receptors mediate contraction through the release of cyclo-oxygenase metabolites. Similarly, ET_A receptors located on the epithelial cells also mediate the release of prostanoids in the trachea with epithelium but they are responsible for transient relaxations. Interestingly, contractions induced by IRL 1620 were more susceptible to inhibition by the different antagonists, most probably because it binds to the endothelin receptors in a reversible manner. High concentrations (10^{-5} M) of ET_A-selective antagonists also inhibit responses to IRL 1620, most probably by an effect at ET_B receptors in both the trachea and the upper bronchus.

Keywords: Endothelin-1; ET_A/ET_B non-selective agonist; IRL 1620; ET_B selective agonist; BQ-123; FR139317; ET_A-selective receptor antagonist; PD 145065; ET_A/ET_B non-selective receptor antagonist; endothelin receptor subtypes; ET_A receptors; ET_B receptors

Introduction

Endothelin-1 (ET-1) is a 21 amino acid peptide, first isolated from the culture medium of aortic endothelial cells (Yanagisawa *et al.*, 1988). ET-1, the related peptides (ET-2 and ET-3) and the sarafotoxins (SX6a, SX6b, SX6c, SX6d) cause vasoconstriction, vasodilatation, mitogenesis and various endocrine effects (see Rubanyi, 1992). These effects are mediated by at least two distinct endothelin receptors. The ET_A receptor is ET-1-selective (Arai *et al.*, 1990) whereas the ET_B receptor is equisensitive to all ET isopeptides (Sakurai *et al.*, 1990). ET_A and ET_B receptors have both been shown to mediate contractions of various vascular preparations induced by ET/SX peptides (Williams *et al.*, 1991; Bigaud & Pelton, 1992; Cristol *et al.*, 1993).

The endothelins are also potent constrictors of airways *in vitro* and *in vivo* (see Battistini *et al.*, 1992; Hay *et al.*, 1993). In guinea-pigs, these effects appear to be mediated by non-ET_A, presumably ET_B receptors. For instance, in anaesthetized guinea-pigs, ET-1, ET-2 or ET-3, given intravenously, cause similar bronchoconstrictions (Pons *et al.*, 1991) and in

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guinea-pig isolated airways, all three isopeptides are equipotent (Maggi et al., 1989a; Cardell et al., 1993). Similarly, SX6c and IRL 1620, two ET_B-selective agonists (Williams et al., 1991; Takai et al., 1992; Watakabe et al., 1992), cause comparable contractions of guinea-pig bronchi (SX6c; Hay, 1992) and trachea (IRL 1620; Takai et al., 1992). These data suggest mediation by predominantly ET_B receptors. However, others have suggested that ET-1 is more potent than ET-3 in the guinea-pig trachea (Ninomiya et al., 1992) and in the same preparation that SX6c is a much less effective agonist than ET-1 (Hay & Luttman, 1992). Thus, it appears that both ET_A and ET_B receptors may mediate these contractions. Data from experiments employing endothelin receptor antagonists are similarly contradictory. BQ-123 (Ihara et al., 1992b) or FR 139317 (Sogabe et al., 1993), two selective ET_A receptor antagonists, do not affect contractions induced by ET-1 in the isolated bronchus (BQ-123; Hay, 1992) or trachea (FR 139317; Cardell et al., 1993) or bronchoconstriction induced by ET-1 in anaesethetized guinea-pigs (BQ-123; Noguchi et al., 1993), suggesting mediation by ET_B receptors. Conversely, BQ-123 partially inhibits the contractions induced by ET-1 in the guinea-pig trachea (Hay, 1992), suggesting an involvement of ET_A receptors. Similarly, BQ-123 also inhibits 40% of the contraction induced by ET-1 in the rat trachea (Henry, 1993). These conflicting reports are indicative of the fact that the receptors mediating the effects of the ET/SX peptides in the guinea-pig airways have not been systematically characterized. Here, using as agonists ET-1 and IRL 1620 and as antagonists, BQ-123 or FR139317 (ET_A receptor selective) or PD 145065 (ET_A/ET_B receptor non-selective; Cody et al., 1993), we have characterized further the receptors mediating the contractile effects of the endothelins in the guinea-pig trachea (with or without epithelium), upper bronchus and lung parenchyma.

Methods

Tissue preparation

Male Hartley guinea-pigs weighing 350–450 g were killed by cervical dislocation and rapidly bled. The trachea, upper bronchus and lung parenchyma were excised, placed in Krebs-Henseleit buffer (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.18, $NaHCO_3$ 25, $MgSO_4.7H_2O$ 1.18 and glucose 5.6, pH 7.4) and trimmed of connective tissue and fat. The trachea and upper bronchus were cut into spirals. Some tracheal preparations were denuded of their epithelium by gentle rubbing with a wet cotton swab. Strips of peripheral parenchyma were prepared from the anterior surface of the lower lobe. The tissues were mounted in 10 ml organ baths and connected to auxotonic force-displacement transducers (Harvard Bioscience Apparatus) under tensions of 1.5 g. Tissues were allowed to stabilize for 1 h in warmed (37°C) and gassed (95% O_2:5% CO_2) Krebs-Henseleit solution with washing every 15 min. Changes in length were recorded continuously on a Linearcorder (Graphtec, model WR 3300, Japan).

Experimental protocol

Following an equilibration period (60 min) tissues were contracted three times with histamine (5×10^{-6} M) to induce 80% of the maximal contraction, with a 30 min washout period between contractions. Tissues were then incubated (20–30 min) with or without indomethacin (10^{-5} M), BQ-123 (10^{-6}, 10^{-5} M), FR139317 (10^{-6}, 10^{-5} M), PD 145065 (10^{-6}, 10^{-5}, 10^{-4} M) or vehicle before addition of cumulative concentrations of ET-1 (10^{-9} to 3×10^{-7} M) or IRL 1620 (10^{-10} to 10^{-6} M). Each preparation was used for only one concentration-response curve for ET-1 or IRL 1620. Responses are expressed as a percentage of the contraction evoked by 5×10^{-6} M histamine.

Drugs

Endothelin-1 (ET-1) and IRL 1620 (Suc-[Glu^9, $Ala^{11,15}$] ET-1 (8–21)) were synthesized at the INRS-Santé (Montreal, P.Q., Canada). IRL 1620 was also obtained as a gift from the International Research Laboratories, CIBA-GEIGY (Takarazuka, Japan). ET-1 and IRL 1620 were dissolved in distilled water and stored in aliquots of 5×10^{-5} M at − 20°C. Indomethacin (Sigma Chemicals Co., Poole, Dorset) was freshly dissolved each day in 5% w/v $NaHCO_3$. BQ-123 (cyclo (-D-Trp-D-Asp-L-Pro-D-Val-L-Leu-)), FR139317 ((R)2-[(R)-2-[(S)-2-[[1-(hexahydro-1H-azepinyl)] carbonyl] amino-4-methylpentanoyl] amino-3-[3-(1-methyl-1H-indoyl)] proprionyl] amino-3-(2-pyridyl) proprionic acid) and PD 145065 (Ac-D-Bhg-L-Leu-L-Asp-L-Ile-L-Ile-L-Trp; Bhg = 5H-dibenzyl [a,d]cycloheptene-10,11-dihydroglycine) were dissolved in distilled water and stored in aliquots of 10^{-3} M at − 20°C. All drugs were further diluted with Krebs-Henseleit solution when necessary. Histamine dihydrochloride was obtained from Sigma Chemical Co. (Poole, Dorset).

Statistical analysis

All values are expressed as mean ± s.e.mean. Statistical analysis of the data was performed by an unpaired, two-tailed, Mann-Whitney U test and $P < 0.05$ was considered significant.

Results

Endothelin-1 verus IRL 1620

Endothelin-1 or IRL 1620 caused strong concentration-dependent contractions of the four guinea-pig airway preparations (Table 1; Figure 1a-b). In the trachea with epithelium, ET-1 or IRL 1620 induced contractions with similar potencies, and produced similar responses at 3×10^{-7} M (Table 1; Figure 1a-b). At concentrations up to 10^{-8} M, ET-1, but not IRL 1620, induced transient relaxations prior to the contractile response. In the trachea without epithelium, the sensitivities to ET-1 and IRL 1620 were increased by 16 and 5 fold, respectively as compared to trachea whereas the contractions induced by 3×10^{-7} M of either peptide were not significantly affected (Table 1; Figure 1a-b). In the guinea-pig upper bronchus, ET-1 and IRL 1620 were equipotent (Table 1; Figure 1a-b). In the lung parenchyma, ET-1 was more potent than IRL 1620 (Table 1; Figure 1a-b).

Effect of antagonists on responses of the guinea-pig trachea to ET-1

In the trachea, BQ-123 (10^{-5} M) or FR139317 (10^{-5} M) completely inhibited the transient relaxations induced by ET-1. Contractions at 3×10^{-7} M were not affected by either antagonist although the sensitivities to ET-1 were slightly increased (Table 1; Figure 2a). PD 145065 (10^{-5} M and 10^{-4} M) also abolished the transient relaxations and shifted the concentration-response curve to ET-1 to the right by 1.5 and 5 fold, respectively. PD 145065 (10^{-5} M and 10^{-4} M) also significantly attenuated (− 35% and − 55%, respectively) contractions induced by 3×10^{-7} M ET-1 (Table 1; Figure 2b).

In the trachea with epithelium, PD 145065 antagonized contractions to ET-1 causing a shift to the right in the concentration-response curve of 7 fold (10^{-5} M) and 60 fold (10^{-4} M) (Table 1; Figure 2d) without causing any reduction in the responses at 3×10^{-7} M. BQ-123 (10^{-5} M) had no effect, whereas FR139317 (10^{-5} M) caused a significant inhibition (31%) of the contraction induced by 3×10^{-7} M ET-1, and shifted the ET-1 concentration-response curve to the right by 8 fold (Figure 2c).

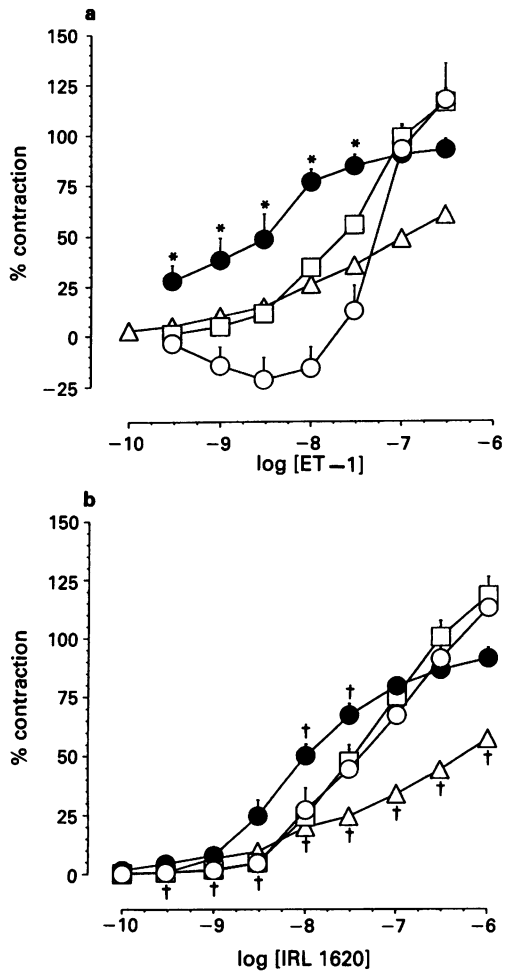


Figure 1 Effect of endothelin-1 (ET-1) (a) and IRL 1620 (b) on guinea-pig isolated trachea (○), trachea without epithelium (●), upper bronchus (□) and lung parenchyma (△); Results are expressed as a percentage of the response to histamine (5×10^{-6} M), and are means of 5–19 observations with s.e.mean. $*P<0.05$ between tracheal strips ± epithelium. $\dagger P<0.05$ between ET-1 and IRL 1620 (compared by an unpaired, two-tailed, Mann-Whitney U test).

Effect of antagonists on responses of the guinea-pig upper bronchus to ET-1

In the guinea-pig upper bronchus, PD 145065 (10^{-5} M) shifted the ET-1 concentration-response curve to the right by 2 fold while 10^{-4} M PD 145065 caused a further parallel shift of 32 fold (Figure 2f). The response to 3×10^{-7} M ET-1 in the presence of 10^{-4} M PD 145065 was also attenuated by 74% (Table 1; Figure 2f). In contrast, BQ-123 (10^{-5} M) or FR139317 (10^{-5} M) did not affect the ET-1 induced concentration-response curves to ET-1 (Figure 2e). The contraction to higher concentrations of ET-1 (10^{-7} to 3×10^{-7} M) was slightly but significantly attenuated (− 20%) by FR139317 (10^{-5} M; Table 1; Figure 2e).

Effect of antagonists on responses of the guinea-pig lung parenchyma to ET-1

In the guinea-pig lung parenchyma, BQ-123 (10^{-5} M) shifted the concentration-curve for ET-1 by 5 fold to the right (Table 1; Figure 2g) as did indomethacin (10^{-5} M; $n=9$; EC_{50}: 54.9 ± 14.0 nM (5 fold to the right); response at 3×10^{-7} M: 49.2 ± 3.2%; Figure 2g). Combination of BQ-123 (10^{-5} M) and indomethacin (10^{-5} M) did not further antagonize contractions induced by ET-1 ($n=4$; data not shown). FR139317 (10^{-5} M) produced a smaller inhibition (2 fold), especially at higher concentrations of ET-1 ($>3 \times 10^{-8}$ M). PD 145065 (10^{-6} M and 10^{-5} M) caused shifts of 3 and >50 fold in the concentration-response curve to ET-1. PD 145065 (10^{-6} M) did not affect the contractions induced by 3×10^{-7} M ET-1, while PD 145065 (10^{-5} M) caused a 62% inhibition (Table 1; Figure 2h). Combination of both indomethacin (10^{-5} M) and PD 145065 (10^{-5} M), completely abolished contractions induced by ET-1 ($n=4$; data not shown).

Effect of antagonists on responses of the guinea-pig trachea to IRL 1620

IRL 1620 caused only contractions of the trachea which were significantly inhibited by BQ-123 (10^{-5} M) or FR139317 (10^{-5} M) (Table 1; Figure 3a). For instance, the two antagonists attenuated contractions to 3×10^{-7} M IRL 1620 by 48% and 59%, respectively. PD 145065 (10^{-6} M) and (10^{-5} M) shifted the concentration-response curve for IRL 1620 to the right by 7 and >22 fold, respectively, and reduced the response to 3×10^{-7} M IRL 1620 by 52% and 79%, respectively (Table 1; Figure 3b).

In the trachea without epithelium, the concentration-response curve to IRL 1620, which was potentiated by the removal of the epithelium, was shifted to the right by more than 100 fold by PD 145065 (10^{-6} and 10^{-5} M) (Table 1; Figure 3d). The responses to 3×10^{-7} M IRL 1620 were attenuated by 73% and 77%, respectively, by the same concentrations of PD 145065 (Table 1; Figure 3d). BQ-123 (10^{-6} M) or FR139317 (10^{-6} M) had no effect. Either antagonist at 10^{-5} M caused a significant inhibition with FR139317 being more effective (Table 1; Figure 3c).

Effect of antagonists on responses of the guinea-pig upper bronchus to IRL 1620

In the upper bronchus, PD 145065 (10^{-6} M) shifted the concentration-response curve to IRL 1620 24 fold to the right, while PD 145065 (10^{-5} M) totally abolished contractions induced by IRL 1620 (Figure 3f). BQ-123 (10^{-6} M) or FR139317 (10^{-6} M) also significantly inhibited contractions to IRL 1620, causing shifts to the right of 7 and 8 fold in the concentration-response curve (Table 1; Figure 3e). Higher concentrations (10^{-5} M) of either antagonist further inhibited the responses to IRL 1620 causing 9 fold (BQ-123) and 25 fold (FR139317) shifts in the concentration-response curve (Table 1; Figure 3e).

Effects of antagonists on responses of the guinea-pig lung parenchyma to IRL 1620

IRL 1620 was significantly less potent than ET-1 in contracting the lung parenchyma. PD 145065 (10^{-6} M) attenuated IRL 1620-induced contractions, causing a 16 fold shift to the right in the concentration-response curve and an inhibition of 54%. PD 145065 (10^{-5} M) totally abolished contractions induced by IRL 1620 (Table 1; Figure 3h). BQ-123 (10^{-6} M to 10^{-5} M) or FR139317 (10^{-6} M) did not affect responses to concentrations of IRL 1620 greater than 10^{-8} M (Table 1; Figure 3g). For instance, FR139317 (10^{-5} M) caused a 7 fold shift to the right of the IRL 1620 concentration-response curve, but no inhibition of the response at 3×10^{-7} M (Table 1; Figure 3g).

BQ-123, FR139317 or PD 145065 (up to 10^{-4} M) had no effect on the basal tension of the tissues or on the responses of any of the four preparations to histamine.

Discussion

The present study demonstrates that contractile responses to ET-1 and IRL 1620 in guinea-pig isolated airways are mediated almost exclusively by ET_B receptors. PD 145065, an ET_A/

Table I Effects of PD 145065, BQ-123 or FR139317 on the contractions at 3×10^{-7} M and apparent EC_{50} values for ET-1 and IRL 1620 in the guinea-pig trachea (± epithelium), upper bronchus and lung parenchyma

	Control			+ PD145065 (10^{-6} M)			+ PD145065 (10^{-5} M)			+ PD145065 (10^{-4} M)		
	(n)	% C_{eff}	EC_{50}	(n)	% C_{eff}	EC_{50}	(n)	% C_{eff}	EC_{50}	(n)	% C_{eff}	EC_{50}
Trachea												
Endothelin-1	(9)	117 ± 18	45 ± 5		ND	ND	(6)	76 ± 16	64 ± 11	(3)	53 ± 12*	238 ± 115
IRL 1620	(5)	92 ± 8	45 ± 18	(3)	44 ± 14	316 ± 102	(4)	19 ± 3*	> 1000		ND	ND
Trachea without epithelium												
Endothelin-1	(7)	93 ± 5	3 ± 3		ND	ND	(8)	117 ± 12	19 ± 6	(4)	87 ± 28	166 ± 78
IRL 1620	(6)	87 ± 4	9 ± 2†	(6)	24 ± 4*	> 1000	(8)	20 ± 3*	> 1000		ND	ND
Upper bronchus												
Endothelin-1	(19)	116 ± 7	32 ± 3		ND	ND	(4)	104 ± 20	77 ± 22	(3)	30 ± 13*	> 1000
IRL 1620	(12)	101 ± 7	35 ± 4	(11)	18 ± 4*	817 ± 77	(6)	0.6 ± 0.3*	> 1000		ND	ND
Lung parenchyma												
Endothelin-1	(19)	60 ± 4	11 ± 2	(4)	61 ± 2	37 ± 11	(7)	23 ± 6	567 ± 145		ND	ND
IRL 1620	(8)	44 ± 3†	26 ± 7†	(8)	21 ± 4*	399 ± 14	(10)	1.8 ± 0.7*	> 1000		ND	ND

	+ BQ123 (10^{-6} M			+ BQ123 (10^{-5} M)			+ FR139317 (10^{-6} M)			+ FR139317 (10^{-5} M)		
	(n)	% C_{eff}	EC_{50}	(n)	% C_{eff}	EC_{50}	(n)	% C_{eff}	EC_{50}	(n)	% C_{eff}	EC_{50}
Trachea												
Endothelin-1		ND	ND	(4)	88 ± 10	18 ± 7		ND	ND	(8)	102 ± 15	13 ± 6
IRL 1620	(5)	108 ± 26	34 ± 9	(5)	47 ± 8*	435 ± 146	(5)	95 ± 17	92 ± 22	(4)	37 ± 10*	710 ± 191
Trachea without epithelium												
Endothelin-1		ND	ND	(4)	98 ± 22	6 ± 4		ND	ND	(4)	64 ± 6*	21 ± 12
IRL 1620	(4)	110 ± 16	9 ± 3	(5)	73 ± 10	93 ± 30	(4)	95 ± 16	8 ± 2	(4)	57 ± 4*	147 ± 59
Upper bronchus												
Endothelin-1		ND	ND	(11)	105 5	17 ± 8		ND	ND	(8)	93 ± 9	34 ± 15
IRL 1620	(5)	66 ± 9*	231 ± 109	(4)	56 ± 14*	316 ± 125	(4)	62 ± 10*	288 ± 148	(4)	29 ± 2*	855 ± 117
Lung parenchyma												
Endothelin-1		ND	ND	(5)	42 ± 6	54 ± 10		ND	ND	(13)	51 ± 2	26 ± 7
IRL 1620	(4)	56 ± 2	14 ± 3	(7)	51 ± 3	39 ± 6	(6)	55 ± 5	36 ± 11	(7)	40 ± 7	172 ± 69

Contractile effects (% C_{eff}) at 3×10^{-7} M of agonist are expressed as the percentage of the contraction to 5×10^{-6} M histamine. Effective concentrations (EC_{50}) values are the concentration ($\times 10^{-9}$ M) eliciting half of the response to 3×10^{-7} M of agonists in the paired control values. Values are mean ± s.e.mean. *Shows significance ($P < 0.05$) for the effects of antagonists and †shows significant difference ($P < 0.05$) between ET-1 and IRL 1630. ND: not determined.

ET_B non-selective endothelin receptor antagonist, shifted all the concentration-response curves in a concentration-related manner, whereas BQ-123 or FR139317 had little if any effect. This amplifies and agrees with data from experiments using both bronchial (Hay, 1992) and tracheal (Cardell *et al.*, 1993) preparations *in vitro*, and from studies examining the responses of guinea-pig airways *in vivo* (Noguchi *et al.*, 1993).

In our study, all three antagonists (at 10^{-5} M) abolished the transient relaxations of the trachea induced by low concentrations ($< 10^{-8}$ M) of ET-1. At the same time, our results show that BQ-123 or FR139317 (10^{-5} M) slightly potentiated contractions to low concentrations of ET-1 ($\leqslant 3 \times 10^{-7}$ M) without affecting those to higher concentrations of ET-1. These transient relaxations (as reported by Turner *et al.*, 1989; White *et al.*, 1991) are mediated via ET_A receptors, and counteract the direct constrictor effect of the endothelins. The receptors mediating this effect are most probably situated on the epithelial cells, for transient relaxations were not seen in preparations without epithelium. Furthermore, removal of the epithelium of the trachea potentiated the contractile effects of ET-1. The relaxation may well be secondary to an increase in production of prostaglandin E_2 (PGE_2), the predominant airway product of arachidonic acid in epithelial cells, which relaxes airway smooth muscle (Goodwin, 1989) and is released from feline tracheal epithelial cells by ET-1 (Wu *et al.*, 1993).

In intact tracheal strips, IRL 1620 induced similar contractions to ET-1 suggesting ET_B receptors mediate this response. Contractions induced by IRL 1620 were strongly inhibited or abolished by PD 145065. Similarly, the higher concentration of PD 145065 (10^{-5} M) slightly reduced the contractions to ET-1 at $\geqslant 10^{-7}$ M. The greater effectiveness of PD 145065 against IRL 1620 is not explained by the lower binding affinity of this selective agonist compared to ET-1, for competitive binding assays revealed almost identical displacement curves (Watakabe *et al.*, 1992). However, IRL 1620 binds to endothelin receptors in a reversible manner, whereas ET-1 only dissociates very slowly from the binding sites (Watakabe *et al.*, 1992). This different sensitivity of agonists to antagonists would agree with our previous observations that PD 142893, PD 145065 and Ro 46-2005 (Clozel *et al.*, 1993), which are all ET_A/ET_B non-selective receptor antagonists, more actively antagonize responses to SX6c or IRL 1620 than ET-1 at ET_B receptors (Warner *et al.*, 1993a,b; unpublished observations). This is of great interest for it suggests that the relative effectiveness of endothelin receptor antagonists varies with the agonist used. It also implies that great care should be taken in assessing the potency of endothelin receptor antagonists by competition experiments employing synthetic ligands.

Neither FR139317 (10^{-6} M) nor BQ-123 (10^{-6} M) had any significant effect on the contractions induced by IRL 1620. However, a concentration of 10^{-5} M of either ET_A-antagonist caused a significant inhibition of contractions induced by IRL 1620, shifting the IRL 1620 concentration-response curve to a similar extent to PD 145065 (10^{-6} M). Similarly, high concentrations of BQ-123 inhibit ET-1-induced bronchoconstriction *in vivo* (Noguchi *et al.*, 1993). Possibly this is because at these high concentrations (10^{-5} M), BQ-123 or FR139317 can displace ET-1 binding to ET_B receptors. Indeed, BQ-123 (IC_{50}: 18 μM) or FR139317 (IC_{50}: 4.6 μM) will displace the binding of ET-1 to ET_B receptors in brain tissue

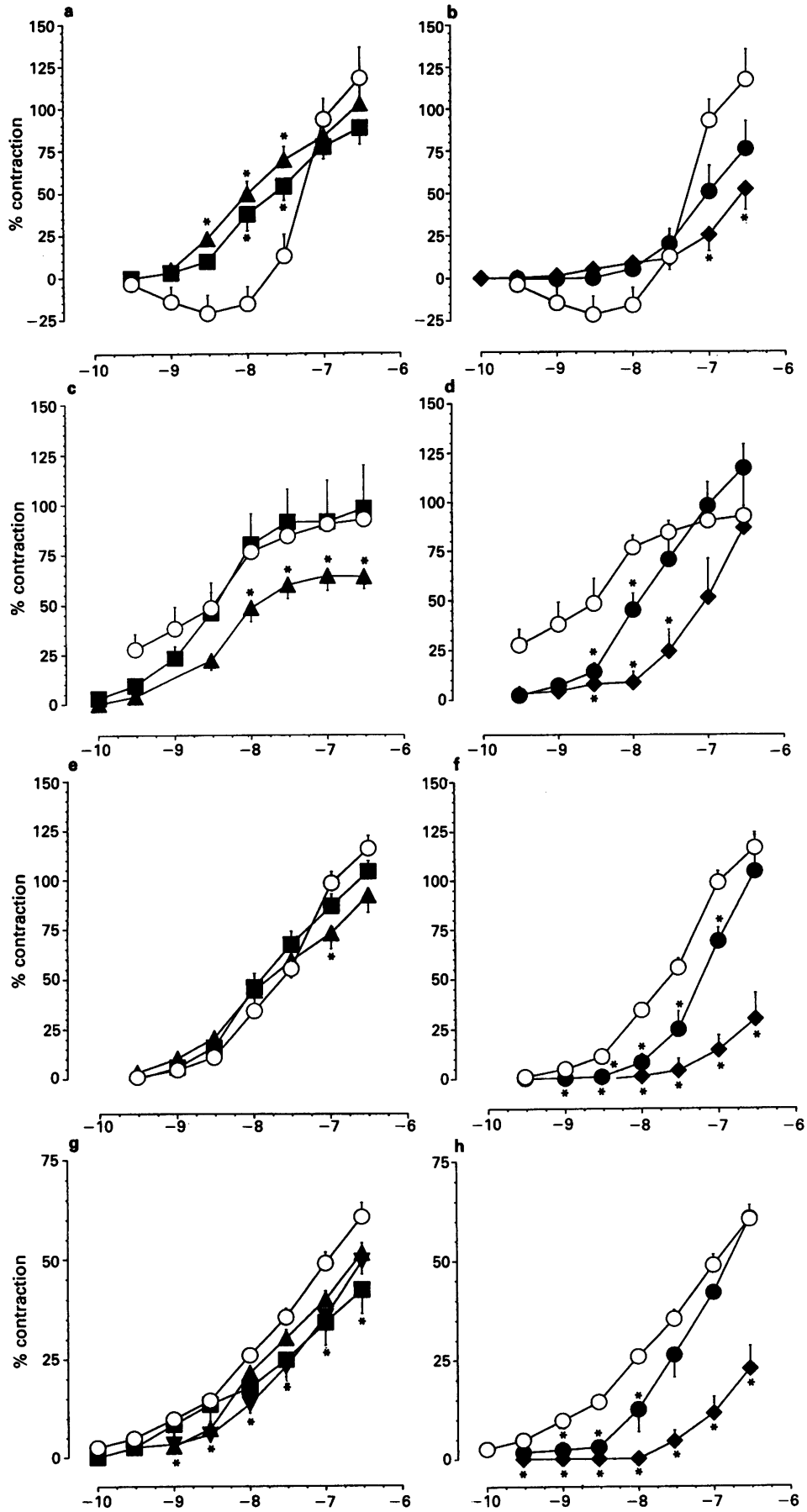


Figure 2 Effects of BQ-123 (10^{-5} M; ■), FR139317 (10^{-5} M, ▲) or PD 145065 (10^{-5} M, ● and 10^{-4} M, ◆) on contractions of guinea-pig isolated trachea (a-b), trachea without epithelium (c-d), upper bronchus (e-f) and lung parenchyma (g-h) induced by endothelin-1 (ET-1) (○). The concentrations of PD 145065 used in the guinea-pig lung parenchyma were 10 times less (10^{-6} M, ● and 10^{-5} M, ◆) than those used in other airway preparations. The effect of indomethacin (10^{-5} M, ▼) is also shown on contractions of lung parenchyma induced by ET-1 (g). Results are expressed as a percentage of the response to histamine (5×10^{-6} M), and are mean of 3–19 observations with s.e.mean. $^*P<0.05$ (compared to ET-1 by a Mann-Whitney U test).

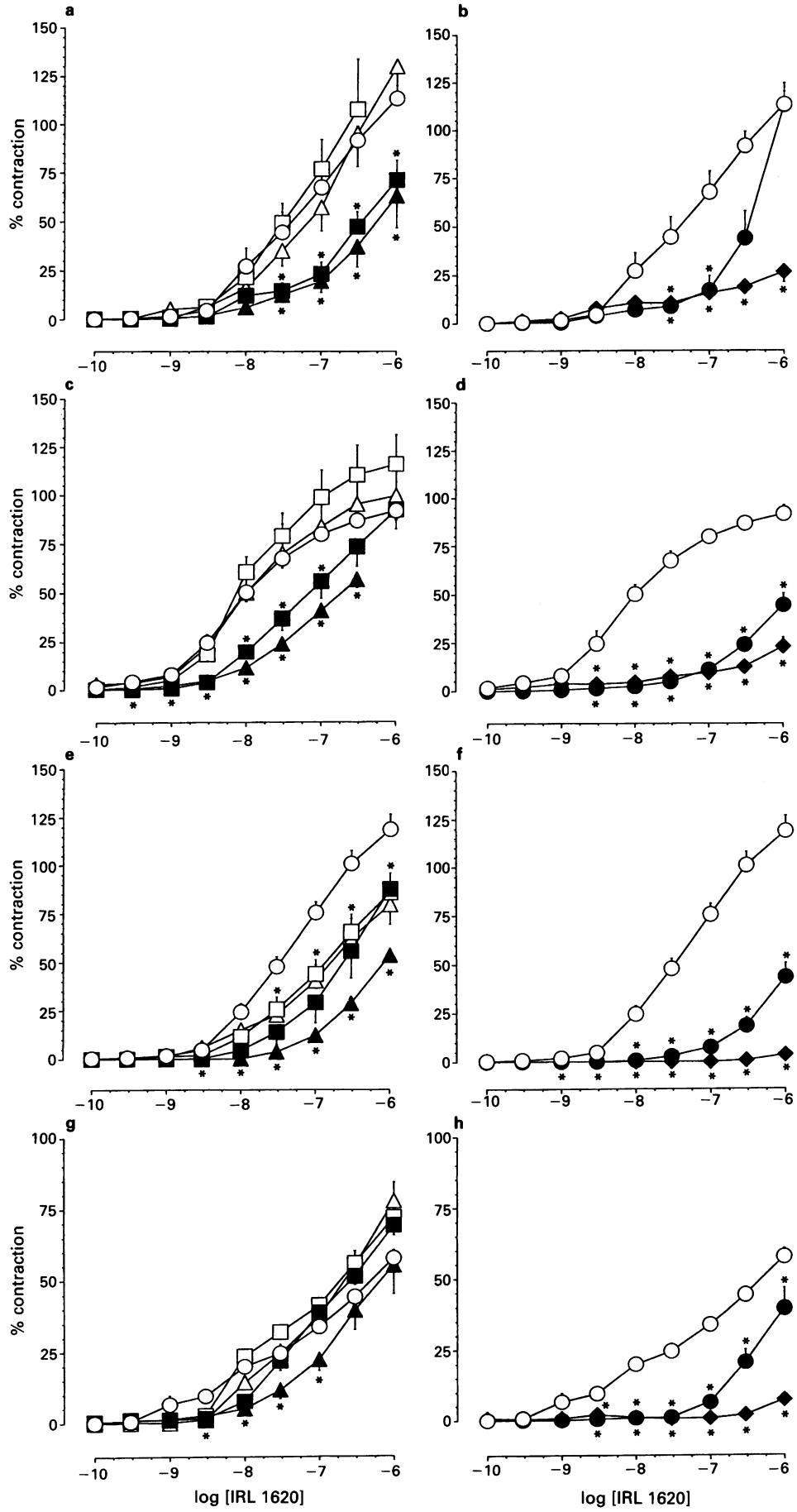


Figure 3 Effects of BQ-123 (10^{-6} M, □, 10^{-5} M ■), FR139317 (10^{-6} M △, 10^{-5} M ▲) or PD 145065 (10^{-6} M ●, 10^{-5} M ◆) on contractions of guinea-pig isolated trachea (a-b), trachea without epithelium (c-d), upper bronchus (e-f) and lung parenchyma (g-h) induced by IRL1620 (○). Results are expressed as a percentage of the response to histamine (5×10^{-6} M) and are mean of 3–12 observations with s.e.mean. $^*P < 0.05$ (compared to ET-1 by a Mann-Whitney U test).

(Ihara *et al.*, 1992; Sogabe *et al.*, 1993; Aramori *et al.*, 1993).

Removal of the epithelium in the trachea potentiated the contractile effects of both ET-1 (as shown by Maggi *et al.*, 1989b; Hay, 1989; 1990) and IRL 1620 (this study), as has been shown for other bronchoconstrictor agents such as histamine, acetylcholine, 5-hydroxytryptamine and leukotriene C_4 and D_4 (Barnes *et al.*, 1985; Holroyde, 1986; Goldie *et al.*, 1986; Hay *et al.*, 1987). Interestingly, while ET-1 and IRL 1620 were equipotent in contracting the intact trachea, ET-1 was three times more potent than IRL 1620 in contracting tracheal preparations without epithelium. This may be explained by the additional loss of the ET-1-induced relaxant response, which is epithelium-dependent (see above). In the trachea without epithelium, BQ-123 (10^{-5} M) had no effect on contractions induced by ET-1 while FR139317 (10^{-5} M) significantly inhibited them. This suggests that the trachea contains a mixed population of endothelin receptors. This would agree with previous reports comparing both agonist potencies and the effects of the endothelin receptor antagonists (Maggi *et al.*, 1989b; Ninomiya *et al.*, 1992; Hay & Luttman, 1992; Henry, 1993).

In the upper bronchus, ET-1 and IRL 1620 produced superimposable concentration-response curves suggesting mediation by a homogeneous ET_B receptor population. BQ-123 or FR139317 were ineffective against ET-1 but PD 145065 antagonized the effects of ET-1 or IRL 1620. As before, high concentrations of BQ-123 or FR139317 inhibited contractions induced by IRL 1620.

The present study supports the suggestion (Filep *et al.*, 1991) that the contractions induced by ET-1 in the lung parenchyma are partly mediated through the release of eicosanoids, particularly thromboxane A_2. BQ-123 may block the release of TxA_2 for it significantly inhibited part of the response curve induced by ET-1 and this inhibition was not additive to that of indomethacin. This agrees with experiments using rat perfused lungs where BQ-123 blocks the release of prostacyclin and/or thromboxane A_2 induced by ET-1 (D'Orleans-Juste *et al.*, 1992). It also provides a role for ET_A receptors, which account for 15% of ET-1 binding in the guinea-pig lung (Ihara *et al.*, 1992a).

Thus, our data indicate that ET-1 activates ET_A receptors on tracheal airway epithelial cells and cells in the lung parenchyma of the guinea-pig to induce the release of prostanoids. In the trachea, this causes a transient relaxation and in the lung parenchyma, contraction through the release of thromboxane A_2 (TxA_2). More importantly $\geqslant 85\%$ of the contractions induced by ET-1 in guinea-pig airways are mediated via ET_B receptors most probably present on the airway smooth muscle. As ET-1 may be an endogenous regulator of airway tone and is secreted from tracheal and bronchial epithelial cells (Black *et al.*, 1989; Mattoli *et al.*, 1990), endothelins may play an active role in diseases such as asthma, bronchitis and adult respiratory distress syndrome. These airway diseases are associated with vascular remodelling, reduction of airway calibre, increased microvascular permeability, ciliary function, ion transport and mucin release which are all affected by the endothelins (Wu *et al.*, 1990; Tamaoki *et al.*, 1991; Plews *et al.*, 1991; Peacock *et al.*, 1992; Helset *et al.*, 1993). The ability of PD 145065 to antagonize ET-1 in airway tissues therefore suggests it may be beneficial in disease states.

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References

ARAI, H., HORI, S., ARAMORI, I., OHKUBO, H. & NAKANISHI, S. (1990). Cloning and expression of a cDNA encoding an endothelin receptor. *Nature*, **348**, 730–732.

ARAMORI, I., NIREI, H., SHOUBO, M., SOGABE, K., NAKAMURA, K., KOJO, H., NOTSU, Y., ONO, T. & NAKANISHI, S. (1993). Subtype selectivity of a novel endothelin antagonist FR139317 for the two endothelian receptors in transfected Chinese hamster ovary cells. *Mol. Pharmacol.*, **43**, 127–131.

BARNES, P.J., CUSS, F.M. & PALMER, J.B. (1985). The effect of airway epithelium on smooth muscle contraction in bovine trachea. *Br. J. Pharmacol.*, **86**, 685–692.

BATTISTINI, B., D'ORLÉANS-JUSTE, P. & SIROIS, P. (1992). Les endothélines et le poumon. *Méd. Sci.*, **8**, 679–687.

BIGAUD, M. & PELTON, J.T. (1992). Discrimination between ET_A- and ET_B-receptor mediated effect of endothelin-1 and [$Ala^{1,3,11,15}$] endothelin-1 by BQ-123 in the anaesthetized rat. *Br. J. Pharmacol.*, **107**, 912–918.

BLACK, P.N., GHATEI, M.A., TAKAHASHI, K., BRETHERTON-WATT, D., KRAUSZ, T,. DOLLERY, C.T. & BLOOM, S.R. (1989). Formation of endothelin by cultured airway epithelial cells. *FEBS Lett,*. **255**, 129–132.

CARDELL, L.O., UDDMAN, R. & EDVINSSON, L. (1993). A novel ET_A-receptor antagonist, FR139317, inhibits endothelin-induced contractions of guinea-pig pulmonary arteries, but not trachea. *Br. J. Pharmacol.*, **108**, 448–452.

CLOZEL, M., BREU, V., BURRI, K., CASSAL, J.M., FISCHLI, W., GRAY, G.A., HIRTH, G., LOFFLER, B.M., MULLER, M., NEIDHART, W. & RAMUZ, H. (1993). Pathophysiological role of endothelin revealed by the first orally active endothelin receptor antagonist. *Nature*, **365**, 759–761.

CRISTOL, J.P., WARNER, T.D., THIEMERMANN, C. & VANE, J.R. (1993). Mediation via different receptors of the vasoconstrictor effects of endothelins and sarafotoxins in the systemic circulation and renal vasculature of the anaesthetized rat. *Br. J. Pharmacol.*, **108**, 776–779.

CODY, W.L., DOHERTY, A.M., HE, J.X., DEPUE, P.L., WAITE, L.A., TOPLISS, J.G., HALEEN, S.J., LADOUCEUR, K.E., FLYNN, M.A., HILL, D.M. & REYNOLDS, E.E. (1993). The rational design of a highly potent combined ET_A and ET_B receptor antagonist (PD 145065) and related analogues. *Med. Chem. Res.*, **3**, 154–162.

D'ORLEANS-JUSTE, P., TÉLÉMAQUE, S., CLAING, A., IHARA, M. & YANO, M. (1992). Human big-endothelin-1 and endothelin-1 release prostacyclin via the activation of ET_1 receptors in the rat perfused lung. *Br. J. Pharmacol.*, **105**, 773–775.

FILEP, J.G., BATTISTINI, B. & SIROIS, P. (1991). Pharmacological modulation of endothelin-induced contractions of guinea-pig isolated airways and thromboxane release. *Br. J. Pharmacol.*, **103**, 1633–1640.

GOLDIE, R.G., PAPADIMITRIOU, J.M., PATERSON, J.W., RIGBY, P.J., SELF, H.M. & SPINA, D. (1986). Influence of the epithelium on responsiveness of guinea-pig isolated trachea to contractile and relaxant agonists. *Br. J. Pharmacol.*, **87**, 5–14.

GOODWIN, J.S. (1989). Immunomodulation by eicosanoids and anti-inflammatory drugs. *Curr. Opin. Immunol.*, **2**, 264–268.

HAY, D.W.P. (1989). Guinea-pig tracheal epithelium and endothelin. *Eur. J. Pharmacol.*, **171**, 241–245.

HAY, D.W.P. (1990). Mechanism of endothelin-induced contraction in guinea-pig trachea: comparison with rat aorta. *Br. J. Pharmacol.*, **100**, 383–392.

HAY, D.W.P. (1992). Pharmacological evidence for distinct endothelin receptors in guinea-pig bronchus and aorta. *Br. J. Pharmacol.*, **106**, 759–761.

HAY, D.W.P., FARMER, S.G., RAEBURN, D., MUCCITELLI, R.M., WILSON, K.A. & FEDAN, J.S. (1987). Differential effects of epithelium removal on the responsiveness of guinea-pig tracheal smooth muscle to bronchoconstrictors. *Br. J. Pharmacol.*, **92**, 381–388.

HAY, D.W.P., HENRY, P.J. & GOLDIE, R.G. (1993). Endothelin and the respiratory system. *Trends Pharmacol. Sci.*, **14**, 29–32.

HAY, D.W.P. & LUTTMAN, M.A. (1992). Endothelin (ET) receptors mediating ET-1-induced contraction in guinea-pig and human pulmonary tissues. *Br. J. Pharmacol.*, **107**, 102P.

HELSET, E., KJAEVE, J. & HAUGE, A. (1993). Endothelin-1-induced increases in microvascular permeability in isolated perfused lungs requires leukocytes and plasma. *Circ. Shock*, **39**, 15–20.

HENRY, P.J. (1993). Endothelin-1 (ET-1)-induced contraction in rat isolated trachea: involvement of ET_A and ET_B receptors and multiple signal transduction systems. *Br. J. Pharmacol.*, **110**, 435–441.

HOLROYDE, M.C. (1986). The influence of epithelium on the responsiveness of guinea-pig trachea. *Br. J. Pharmacol.*, **87**, 501–507.

IHARA, M., ISHIKAWA, K., FUKURODA, T., SAEKI, T., FUNABASHI, K., FUKAMI, T., SUDA, H. & YANO, M. (1992a). *In vitro* biological profile of a highly potent novel endothelin (ET) antagonist BQ-123 selective for the ET_A receptor. *J. Cardiovasc. Pharmacol.*, **20**, S11–S14.

IHARA, M., NOGUCHI, K., SAEKI, T., FUKURODA, T., TSUCHIDA, S., KIMURA, S., FUKAMI, T., ISHIKAWA, K., NISHIKIBE, M. & YANO, M. (1992b). Biological profiles of highly potent novel endothelin antagonists selective for the ET_A receptor. *Life Sci.*, **50**, 247–255.

MAGGI, C.A., GIULIANI, S., PATACCHINI, R., ROVERO, P., GIACHETTI, A. & MELI, A. (1989a). The activity of peptides of the endothelin family in various mammalian smooth muscle preparations. *Eur. J. Pharmacol.*, **174**, 23–31.

MAGGI, C.A., PATACCHINI, R., GIULIANI, S. & MELI, A. (1989b). Potent contractile effect of endothelin on isolated guinea-pig airways. *Eur. J. Pharmacol.*, **160**, 179–182.

MATTOLI, S., MEZZETTI, M., RIVA, G., ALLEGRA, L. & FASOLI, A. (1990). Specific binding of endothelin on human bronchial smooth muscle cells in culture and secretion of endothelin-like material from bronchial epithelial cells. *Am. J. Resp. Cell. Mol. Biol.*, **3**, 145–151.

NINOMIYA, H., UCHIDA, Y., SAOTOME, M., NOMURA, A., OHSE, H., MATSUMOTO, H., HIRATA, F. & HASEGAWA, S. (1992). Endothelins constrict guinea-pig tracheas by multiple mechanisms. *J. Pharmacol. Exp. Ther.*, **262**, 570–576.

NOGUCHI, K., NOGUCHI, Y., HIROSE, H., NISHIKIBE, M., IHARA, M., ISHIKAWA, K. & YANO, M. (1993). Role of endothelin ET_B receptors in bronchoconstrictor and vasoconstrictor responses in guinea-pigs. *Eur. J. Pharmacol.*, **233**, 47–51.

PEACOCK, A.J., DAWES, K.E., SHOCK, A., GRAY, A.J., REEVES, J.T. & LAURENT, G.J. (1992). Endothelin-1 and endothelin-3 induce chemotaxis and replication of pulmonary artery fibroblasts. *Am. J. Resp. Cell Mol. Biol.*, **7**, 492–499.

PLEWS, P.I., ABDEL-MALEK, Z.A., DOUPNIK, C.A. & LEIKAUF, G.D. (1991). Endothelin stimulates chloride secretion across canine tracheal epithelium. *Am. J. Physiol.*, **261**, L188–L194.

PONS, F., LOQUET, I., TOUVAY, C,. ROUBERT, P., CHABRIER, P.E., MENCIA-HUERTA, J.M. & BRAQUET, P. (1991). Comparison of the bronchopulmonary and pressor activities of endothelin isoforms ET-1, ET-2, and ET-3 and characterization of their binding sites in guinea pig lung. *Am. Rev. Resp. Dis.*, **143**, 294–300.

RUBANYI, G.M. (1992). *Endothelin*. OUP U.S.A.: American Physiology Society.

SAKURAI, T., YANAGISAWA, M., TAKUMA, Y., MIYAZAKI, H, KIMURA, S., GOTO, K. & MASAKI, T. (1990). Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature*, **348**, 732–735.

SOGABE, K., NIREI, H., SHOUBO, M., NOMOTO, A., AO, S., NOTSU, Y. & ONO, T. (1993). Pharmacological profile of FR139317, a novel, potent endothelin ET_A receptor antagonist. *J. Pharmacol. Exp. Ther.*, **264**, 1040–1046.

TAKAI, M., UMEMURA, I., YAMASAKI, K., WATAKABE, T., FUJITANI, Y., ODA, K., URADE, Y., INUI, T., YAMAMURA, T. & OKADA, T. (1992). A potent and specific agonist, Suc-[Glu^9, $Ala^{11,15}$]-endothelin-1(8-21), IRL 1620, for the ET_B receptor. *Biochem. Biophys. Res. Commun.*, **184**, 953–959.

TAMAOKI, J., KANEMURA, T., SAKAI, N., ISONO, K., KOBAYASHI, K. & TAKISAWA, T. (1991). Endothelin stimulates ciliary beat frequency and chloride secretion in canine cultured tracheal epithelium. *Am. J. Resp. Cell. Mol. Biol.*, **4**, 426–431.

TURNER, N.C., DOLLERY, C.T. & WILLIAMS, A.J. (1989). Endothelin-1-induced contractions of vascular and tracheal smooth muscle: Effects of nicardipine and BRL 34915. *J. Cardiovasc. Pharmacol.*, **13**, S180–S182.

WARNER, T.D., ALLCOCK, G.H., CORDER, R. & VANE, J.R. (1993a). Use of the endothelin antagonists BQ-123 and PD 142893 to reveal three endothelin receptors mediating smooth muscle contraction and the release of EDRF. *Br. J. Pharmacol.*, **110**, 777–782.

WARNER, T.D., BATTISTINI, B., ALLCOCK, G.H. & VANE, J.R. (1993b). Endothelin ET_A and ET_B receptors mediate vasoconstriction and prostanoid release in the isolated kidney of the rat. *Eur. J. Pharmacol.*, (in press).

WATAKABE, T., URADE, Y., TAKAI, M., UMEMURA, I. & OKADA, T. (1992). A reversible radioligand specific for the ET_B receptor: [^{125}I] Tyr^{13}-Suc-[Glu^9,$Ala^{11,15}$]-endothelin-1 (8-21), [^{125}I] IRL 1620. *Biochem. Biophys. Res. Commun.*, **185**, 867–873.

WHITE, S.R., HATHAWAY, D.P., UMANS, J.G., TALLET, J., ABRAHAMS, C. & LEFF, A.R. (1991). Epithelial modulation of airway smooth muscle response to endothelin-1. *Am. Rev. Resp. Dis.*, **144**, 373–378.

WILLIAMS, D.L., JONES, K.L., PETTIBONE, D.J., LIS, E.V. & CLINE-SCHMIDT, B.V. (1991). Sarafotoxin S6c: An agonist which distinguishes between endothelin receptor subtypes. *Biochem. Biophys. Res. Commun.*, **175**, 556–561.

WU, T., MULLOL, J., RIEVES, R.D., LOGUN, C., HAUSFIELD, J., KALINER, A. & SHELHAMMER, J.H. (1990). Endothelin-1 stimulates eicosanoids production in cultured human nasal mucosa. *Am. J. Respir. Cell Mol. Biol.*, **6**, 168–174.

WU, T., RIEVES, .D., LARYVÉE, P., LOGUN, C., LAWRENCE, M.G. & SHELHAMMER, J.H. (1993). Production of eicosanoids in response to endothelin-1 binding sites in airways epithelial cells. *J. Resp. Cell Moll. Biol.*, **8**, 282–290.

YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KOBAYASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **332**, 411–415.

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Characterization of the capsaicin-sensitive component of cyclophosphamide-induced inflammation in the rat urinary bladder

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1 Cyclophosphamide (CYP) (150 mg kg^{-1}, i.p. 0.5–48 h before) caused a time-dependent plasma protein extravasation in the rat urinary bladder with the maximal extravasation occuring at between 2 and 4 h after administration of the drug.

2 Prior capsaicin desensitization of capsaicin-sensitive primary afferent neurones (CSPANs) (50 mg kg^{-1}, s.c., 4 days before) resulted in approximately 50% inhibition of the magnitude of the extravasation response at the 2 h time-point.

3 Intraperitoneal (i.p.) pretreatment with the tachykinin NK_1 receptor antagonist, RP 67,580 (0.44 mg kg^{-1}) or the bradykinin B_2 receptor antagonist, Hoe 140 (0.13 mg kg^{-1}) had significant inhibitory effects, giving responses of 56 ± 6% and 39 ± 4% of the control extravasation response to CYP treatment after 2 h. Pretreatment with the tachykinin NK_2 receptor antagonist, SR 48,968 (0.3 mg kg^{-1}, i.p.), the histamine H_1 receptor blocker, chlorpheniramine (10 mg kg^{-1}, i.p.), the 5-HT receptor blocker, methysergide (6 mg kg^{-1}, i.p.) or the cyclo-oxygenase inhibitor indomethacin (5 mg kg^{-1}, i.p.) had no significant effect upon the development of the extravasation response at this same time-point.

4 In rat isolated urinary bladder strips, the active metabolite of CYP, acrolein (1–300 μM) produced a concentration-dependent contraction that was significantly reduced by *in vitro* capsaicin desensitization (10 μM for 15 min) indicating direct stimulation of CSPANs. CYP was without appreciable effect.

5 The effect of acrolein *in vitro* was significantly reduced by pretreatment of the bladder with a combination of tachykinin NK_1 and NK_2 receptor antagonists, RP 67,580 (3 μM) and SR 48,968 (1 μM). The dose-response curve to acrolein was also significantly inhibited by treatment with indomethacin (10 μM) and slightly affected by Hoe 140 (1 μM).

6 These findings demonstrate the contribution of CSPANs to the development of CYP-induced cystitis. Plasma protein extravasation involves activation of tachykinin NK_1 and bradykinin B_2 receptors. Activation of CSPANs in the urinary bladder is likely to be due to the conversion of CYP into its active metabolite, acrolein, and not to a direct effect of CYP upon these nerve-endings.

Keywords: Cyclophosphamide; plasma protein extravasation; capsaicin-sensitive bladder afferents; rat urinary bladder; cystitis

Introduction

Cyclophosphamide (CYP) is an effective form of treatment for a variety of malignant and non-malignant disease states. Its activity is based upon conversion of CYP into its active oxidized metabolite of phosphamide mustard in the liver. CYP therapy has several toxicity complications (for review see Fraiser *et al.*, 1991) including bladder cystitis, which is due primarily to another CYP metabolite, acrolein. Acrolein has profound effects upon the urinary bladder causing painful voiding symptoms in the mildest of cases to haemorrhagic cystitis in the most severe. It has been shown that CYP-induced cystitis is due to the direct contact of acrolein with the urothelium (Phillips *et al.*, 1961; Cox, 1979); however, the exact mechanism of the ensuing inflammatory response is little understood.

Normal vesicourethral function is controlled by a complex interplay between the innervation provided by sympathetic, parasympathetic, somatic and sensory nerves. The latter includes a subpopulation of primary afferent neurones that are sensitive to the actions of capsaicin (Maggi *et al.*, 1986), the pungent ingredient from red peppers (for review of actions see Holzer, 1991). These capsaicin-sensitive primary afferent neurones (CSPANs) have been attributed a dual, sensory and efferent function (Maggi, 1990). The efferent activity of these neurones is achieved by the release of several neuropeptides (Maggi *et al.*, 1987; 1988a,b) many of which possess inflammatory activity, producing what is termed neurogenic inflammation (for reviews see Chahl, 1988; Maggi, 1991). In turn, various mediators of inflammation, such as bradykinin (Maggi *et al.*, 1989; 1993) and formyl-methionyl-leucyl phenylalanine (Giuliani *et al.*, 1991) have been shown to induce release of neuropeptides from sensory nerve endings in the urinary bladder. Further evidence for the inflammatory activity of sensory neuropeptides has been demonstrated by the fact that the acute administration of capsaicin itself will result in a plasma protein extravasation (PPE) (Maggi *et al.*, 1987) and that a variety of stimuli for sensory nerves in the bladder, such as xylene and hypertonic solutions, produce inflammatory effects that are dependent upon the existence of an intact sensory afferent system, the response being modified by capsaicin treatment (Maggi *et al.*, 1988b).

The possible pathophysiological role that activation of CSPANs has to play in CYP-induced cystitis has been investigated previously (Maggi *et al.*, 1992) in a rat model of CYP-induced cystitis described by Grinberg-Funes *et al.* (1990). This study demonstrated that, at 48 h after administration of CYP, extrinsic bladder denervation produced by bilateral removal of pelvic ganglia resulted in an attenuation of the plasma protein extravasation induced by CYP com-

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pared to animals with intact bladder innervation. However, prior depletion of sensory neurones with capsaicin potentiated the plasma protein extravasation 48 h after CYP administration. Maggi *et al.* (1992) suggested that CSPANs may provide some protective effect at this time point, but speculated that at an earlier time-point in the development of the inflammation these nerves may subserve a pro-inflammatory role. To investigate this possibility we have used the same model to determine the development of extravasation induced by CYP from between 0.5 and 48 h after its administration and the effect of capsaicin desensitization upon the development of this response. In this way any role that the sensory afferent system may have to play in CYP-induced plasma extravasation in the rat urinary bladder may be discerned. Secondly, we have addressed the question that CYP produces its effects via conversion to the metabolite, acrolein, by comparison of the effects of both CYP and acrolein upon smooth muscle activity of the rat bladder *in vitro* and the effects of capsaicin thereon.

Methods

In vivo

Plasma protein extravasation induced by CYP in the urinary bladder of male Wistar rats (Charles River, Italy) weighing 350–400 g, was measured by the Evan's blue dye leakage technique (Saria & Lundberg, 1983). Rats were anaesthetized with urethane (1.2 g kg^{-1}, s.c.) and the left jugular vein cannulated with PE 50 polyethylene tubing for i.v. administration. Following cannulation Evan's blue (50 mg kg^{-1}, i.v.) was administered and 15 min later the dye flushed out of the cardiovascular system by perfusion with warm (37°C) saline (25 ml min^{-1} for 2 min) via intracardiac puncture. The urinary bladder was then excised, the extraneous tissue removed, washed in saline and blotted dry before weighing. The Evan's blue content in each bladder was determined after extraction in formamide (24 h at 60°C) using a DB Beckman Spectrophotometer at 620 nm.

A time-course of the effect of CYP (150 mg kg^{-1}) was determined over a period spanning from 0.5 h to 48 h after CYP administration: the end point of the experiment was considered the start of the intracardiac perfusion procedure. To ascertain the involvement of activation of sensory nerves in the development of plasma extravasation in this system, animals were pretreated with a single s.c. injection of capsaicin (50 mg kg^{-1}) 4 days prior to the CYP treatment and the above experiment carried out alongside vehicle (10% ethanol, 10% Tween 80 in saline) controls. The capsaicin pretreatment has been repeatedly shown to produce >90% depletion of bladder content of sensory neuropeptides (Maggi *et al.*, 1988a,b). From these results those time-points at which modulation by capsaicin occurred were further studied to characterize that part of the plasma extravasation response involving sensory afferent activation.

In a second series of experiments, performed 2 h after CYP administration, the effect of various drugs known to block specific receptors or prevent formation of mediators of inflammation was assessed and compared with vehicle-treated control rats carried out on the same day. The drug treatments and their relevant actions are described in Table 1. The various *in vivo* treatments were selected from previous studies (referenced in Table 1) in which effective blockade of the corresponding targets and selectivity has been assessed.

In vitro

Male Wistar rats (Charles River, Italy) weighing 350–400 g were killed by cervical dislocation and the whole urinary bladder removed and cleared of extraneous tissue. Longitudinal strips of the bladder (approximately 2–3 mm wide) were mounted in 5 ml organ baths containing Krebs solution gassed with 5% CO_2 in O_2 maintained at 37°C and under a constant load of 0.5 g. The composition of the Krebs solution was as follows: (mM) NaCl 119, $NaHCO_3$ 25, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2 and glucose 11. The contractile activity of the bladder was measured with Basile isotonic transducers and recorded by means of a penrecorder (Unirecord 7050, Basile, Italy). An incubation period of 30 min was allowed before measurement of the first contractile response to neurokinin A (NKA, 0.1 μM). The preparation was considered fully equilibrated once constant responses to NKA, with 30 min washing intervals between responses, were obtained. Following this, dose-response curves to acrolein or CYP (1–300 μM) were constructed. To determine the involvement of CSPANs in the response to acrolein, capsaicin (10 μM) was added to the bathing medium and left in contact with the tissue for a period of 15 min to achieve full desensitization of sensory nerves prior to construction of the dose-response curve to acrolein.

The effects of RP 67,580 (3 μM), SR 48,968 (1 μM) and Hoe 140 (1 μM), all with 15 min contact times and indomethacin (10 μM, 30 min contact time) upon the dose-response curve to acrolein were determined.

Materials

Cyclophosphamide, capsaicin, indomethacin and chlorpheniramine maleate were purchased from Sigma Chemical Co, Italy. Evan's blue was obtained from EGA chemie (Steinheim, Germany). RP 67,580 ((3aR,7aR)-7,7-diphenyl-2-[1-imino-2-(2-methoxyphenyl)-ethyl]perhydroiso-indol-4-one) was a gift from Rhone-Poulenc (Vitry, France), Hoe 140 (D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]-bradykinin) was a gift from Hoechst (Frankfurt, Germany) and SR 48,968 ((S)-N-methyl-N [4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl) butyl]benzamide) was a gift from Sanofi (Montpellier, France). Methysergide maleate was a gift from Sandoz (Basel, Switzerland).

Cyclophosphamide, methysergide maleate, chlorpheniramine maleate were all made up freshly in saline on day of use. Indomethacin was dissolved in equimolar sodium hydroxide (0.1 N) and diluted in saline for *in vivo* pretreatment and in dimethylsulphoxide for *in vitro* experiments. Hoe 140 was dissolved in distilled water and stored as frozen aliquots at − 20°C and RP 67,580 was dissolved in hydrochloric acid and adjusted to pH 5 on day of use. Capsaicin was dissolved in 10% ethanol and 10% Tween 80 in saline for *in vivo* treatment and in ethanol for *in vitro* experiments. Ethanol (final concentration in the bath 0.1%) had no significant effect on the rat isolated urinary bladder.

Table 1 Different drug treatments used against cyclophosphamide-induced plasma protein extravasation in the urinary bladder of the rat *in vivo*

Drug	*Mode of action*	*Pretreatment*	*References*
RP 67,580	NK_1 receptor blocker	0.44 mg kg^{-1}, i.p., 5 min	Santicioli *et al.*, 1993
SR 48,968	NK_2 receptor blocker	0.3 mg kg^{-1}, i.p., 5 min	Unpublished data
Hoe 140	B_2 receptor blocker	0.13 mg kg^{-1}, i.p., 5 min	Maggi *et al.*, 1993
Indomethacin	Cyclo-oxygenase inhibitor	5 mg kg^{-1}, i.p., 5 min	Maggi *et al.*, 1988b
Chlorpheniramine	H_1 receptor blocker	10 mg kg^{-1}, i.p., 5 min	Eglezos *et al.*, 1992
Methysergide	5-HT receptor blocker	6 mg kg^{-1}, i.p., 15 min	Maggi *et al.*, 1988b

Data and statistical analysis

Plasma protein extravasation is expressed as the content of Evan's blue in nanogram per milligram of bladder weight. All of the results are expressed as mean ± s.e.mean. Effect of drug treatment upon the extravasation response is expressed as a percentage of the response obtained in vehicle control treated animals. Statistical significance was determined by Student's *t* test for unpaired data (two-tailed analysis). Contractile responses to CYP and acrolein in the absence or presence of drug pretreatment are expressed as a percentage of the control response to NKA. Analysis of variance, corrected for multiple comparisons with the Bonferroni test, was used to determine the levels of significance with a $P<0.05$ being considered as statistically significant.

Results

Plasma protein extravasation

The urinary bladder content of Evan's blue in control rats was 12 ± 2 ng mg^{-1} ($n = 9$) of tissue.

CYP (150 mg kg^{-1}) caused a time-dependent plasma extravasation ($n = 7-12$ for each time-point) which reached a maximal peak at between 2 and 4 h followed by a decline over the following 24 h (Figure 1). Haemorrhage was apparent from the 24 h timepoint onwards. Capsaicin pretreatment ($n = 7-14$ for each time-point) resulted in a significant inhibition of the plasma extravasation response presented at the 2 h time-point only ($P<0.01$) by approximately 45% ($n = 13$) as shown in Figure 1. The 2 h time-point was then used in all following *in vivo* experiments to characterize further the capsaicin-sensitive part of the inflammatory response to CYP. As shown in Figure 2, RP 67,580 (0.44 mg kg, $n = 11$), Hoe 140 (0.13 mg kg^{-1}, $n = 16$) and capsaicin pretreatment all significantly inhibited the plasma extravasation response whereas methysergide ($n = 7$), chlorpheniramine ($n = 7$), SR 48,968 ($n = 7$) and indomethacin ($n = 7$) had no significant effect. The combined administration of RP 67,580 and Hoe 140 failed to produce an inhibitory effect greater than that achieved by each antagonist alone (Figure 2).

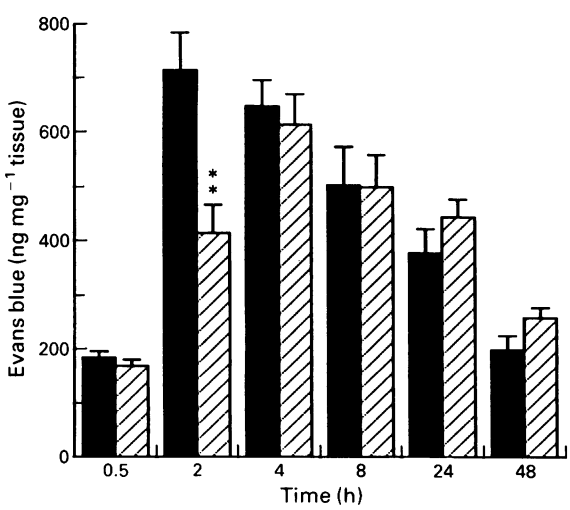


Figure 1 Plasma protein extravasation induced by a single administration of cyclophosphamide (CYP) (150 mg kg^{-1}, i.p.) in the urinary bladder of the rat at various times (0.5–48 h) after CYP administration as measured by assessment of Evan's blue content. Solid and hatched columns represent the responses to CYP in control (vehicle-treated) and in capsaicin-treated rats (50 mg kg^{-1}, 4 days prior to use) respectively. Each value is the mean with s.e.mean of 7–14 animals. **for $P<0.01$ significantly different from control value.

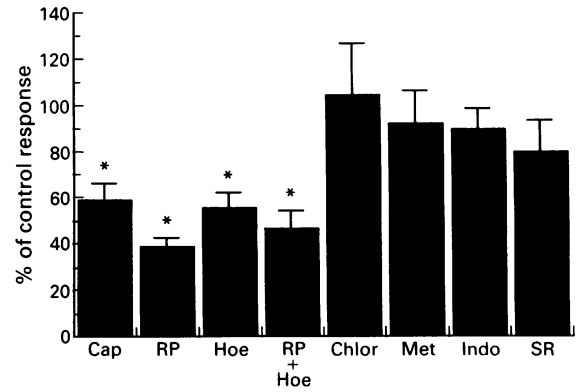


Figure 2 Effect of capsaicin pretreatment and a range of different drug treatments (see Table 1 for the concentrations and time of pretreatment) upon the plasma protein extravasation induced by cyclophosphamide (150 mg kg^{-1}, i.p., 2 h pretreatment) in the urinary bladder of the rat. Values are means with s.e.mean of the responses in 7–12 rats and are expressed as a percentage of the extravasation response in vehicle-treated control rats carried out on the same day. Cap = capsaicin (50 mg kg^{-1}, 4 days before experiment); RP = RP 67,580; Hoe = Hoe 140, Chlor = Chlorpheniramine; Met = methysergide; Indo = indomethacin; SR = SR 48,968. *$P<0.05$ significantly different from the corresponding control value.

Isolated urinary bladder strips

Acrolein (1–300 μM, $n = 7$) caused a concentration-related, slowly developing, tonic contraction of rat isolated urinary bladder strips, whereas CYP ($n = 9$) in the same concentration range had little or no effect as shown in Figures 3a and 4. Prior incubation of the preparation with capsaicin (10 μM) ($n = 7$) resulted in a suppression of the contractile effects of acrolein, significant differences being achieved at the upper part of the concentration-response curve (Figures 3a and 4). Administration of RP 67,580 (3 μM) or of SR 48,968 (1 μM), at concentrations producing effective blockade of NK_1 and NK_2 receptors respectively (Meini *et al.*, 1993), inhibited the response to 300 μM acrolein by $62 \pm 8\%$ and $66 \pm 5\%$ respectively ($n = 5$). Since muscle contraction of the rat isolated bladder to endogenous tachykinins is mediated through both NK_1 and NK_2 receptors (Maggi *et al.*, 1991), the two antagonists were combined to unravel a possible contribution of endogenous tachykinins in the response to acrolein. Co-administration of the two antagonists clearly induced a more pronounced inhibition on the response to 300 μM acrolein ($-80 \pm 7\%$, $n = 9$). For clarity, only the results showing the effect of the combined administration of RP 67,580 and SR 48,968 on acrolein (1–300 μM)-induced contraction are shown in Figure 3b.

Indomethacin (10 μM, $n = 6$) suppressed the concentration-response curve to acrolein in the same extent as the tachykinin antagonists mixture (Figure 3b). The inhibitory effect of indomethacin or RP 67,580 plus SR 48,968 on acrolein-induced contractions was specific since they did not significantly modify the contractile response to 10–80 mM KCl ($EC_{50} = 24$ mM (18–34 c.l.) and $E_{max} = 117 \pm 4\%$ of the response to 0.1 μM NKA, $n = 5-8$). Hoe 140 (1 μM, $n = 5$) (Figure 3b) significantly reduced the response to acrolein only at one point of the concentration-response curve (10 μM).

Discussion

CYP is an effective form of treatment for certain types of cancer; however, the toxicity complications raise problems when using this drug. One of the most pronounced of these complications is the development of cystitis. Previous studies

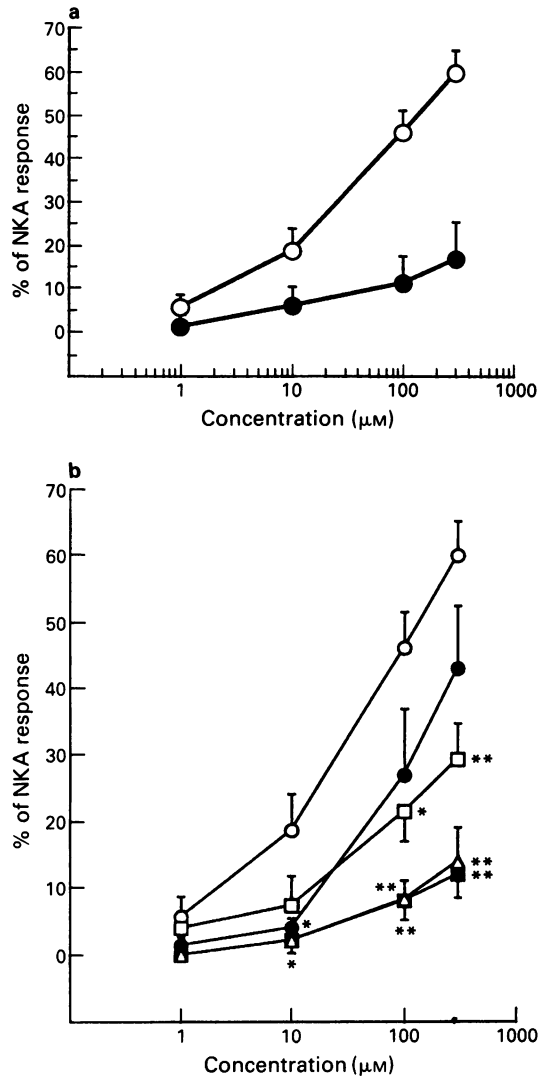


Figure 3 (a) Contractile concentration-response curves to both acrolein (○, 1–300 μM, $n = 7$) and cyclophosphamide (●, 1–300 μM, $n = 9$) on the isolated urinary bladder strip of the rat. Values are means ± s.e.mean. (b) Effect of several different drug treatments upon the contractile dose-response curve to acrolein (1–300 μM) on the isolated urinary bladder strip of the rat. Concentration-response curves to acrolein (○) are compared to those curves obtained in preparations pretreated with capsaicin (□, 10 μM, $n = 7$), RP 67,580 (3 μM) plus SR 48,968 (1 μM, $n = 9$) (■) and Hoe 140 (●, 1 μM, $n = 5$) all with a 15 min contact time prior to construction of the concentration-response curve to acrolein and indomethacin (Δ, 10 μM, $n = 6$) with a 30 min contact time. The contractile responses are expressed as % of the control response to neurokinin A (NKA, 0.1 μM) and values are means ± s.e.mean. $^*P<0.05$, $^{**}P<0.01$ statistically different from control values.

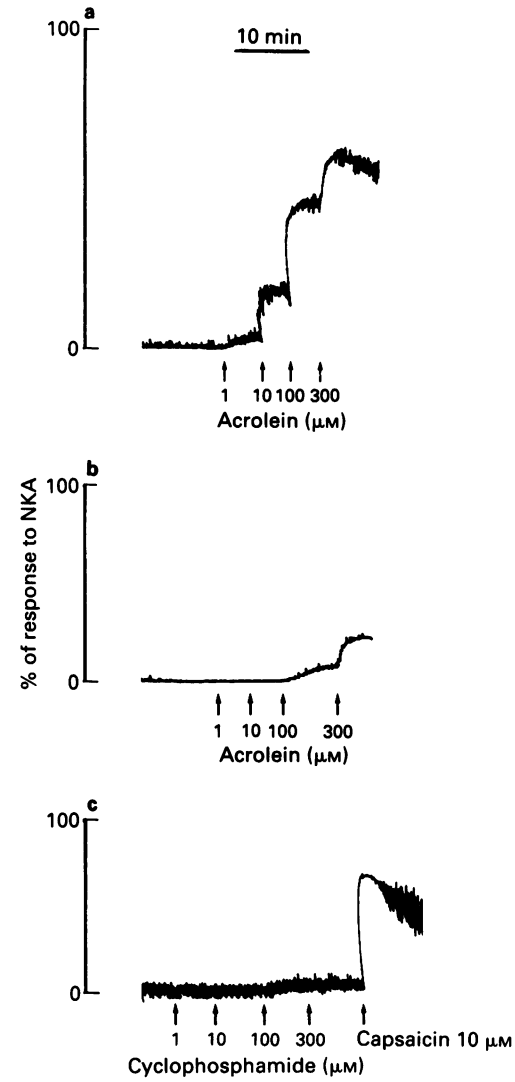


Figure 4 Typical tracings showing the concentration-dependent contractile effect of acrolein on isolated bladder from control rats (a) and from capsaicin-pretreated rats (b); (c) shows the negligible contractile effect of cyclophosphamide on isolated urinary bladder and the lack of blocking the contraction induced by capsaicin (10 μM).

in the airways (Lee *et al.*, 1992) and urinary bladder (Maggi *et al.*, 1992) suggested that activation of sensory afferent fibres may be involved in the irritant effects of CYP and that the metabolite of this agent, acrolein, could be responsible for the complications which occur from treatment with CYP. In agreement with these suggestions we have found that capsaicin pretreatment, and hence the removal of the possible CSPANs component to the response to CYP, resulted in a modulation of the extent of plasma protein extravasation at the 2 h time-point. At this time point CYP, most likely through acrolein generation, stimulates the release of pro-inflammatory neuropeptides from CSPANs which contribute to the formation of oedema. Thus neurogenic inflammation takes place during CYP-induced cystitis, although the major part of PPE produced following CYP administration seems to be independent of an active contribution of CSPANs.

In the present series of experiments we did not demonstrate any significant potentiation of CYP induced plasma protein extravasation by capsaicin pretreatment at the 48 h time-point (Maggi *et al.*, 1992). A possible explanation for this anomaly may be a different source of the animals used in each study, which itself can result in significant variability in the magnitude of plasma extravasation responses even in the same strain (Ahluwalia, unpublished observations).

With the use of the selective, non-peptide NK_1 receptor antagonist, RP 67,580 (Garret *et al.*, 1991), it would seem that at least part of the response to CYP at the 2 h time-point is mediated through activation of these receptors by endogenous tachykinins, the most probable candidate for this being substance P. In fact, studies investigating the inflammatory effects of direct application of capsaicin to the urinary bladder have demonstrated that the ensuing PPE is due to activation of tachykinin NK_1 receptors (Eglezos *et al.*, 1991). Pretreatment with Hoe 140, the selective, potent bradykinin B_2 receptor antagonist (Lembeck *et al.*, 1991; Wirth *et al.*, 1991), also resulted in a significant inhibition of the oedema response. This is congruent with findings in a

xylene-induced model of cystitis, where the PPE response was sensitive to both B_2-receptor blockade, by Hoe 140, and NK_1-receptor blockade (Giuliani *et al.*, 1993). However, contrary to the xylene-induced cystitis, the combination of both Hoe 140 and RP 67,580, did not produce an additive inhibitory effect on CYP-induced cystitis, suggesting that the CSPAN-sensitive part of this response is dependent upon the activity of bradykinin at B_2 receptors, rather than an unlinked involvement of these inflammatory mediators. In accordance with these results, it has been demonstrated, also using Hoe 140, that bradykinin is involved in the induction of detrusor hyperreflexia and increased micturition frequency during CYP-induced cystitis (Maggi *et al.*, 1993), events also known to be sensitive to capsaicin pretreatment.

As indicated earlier, CYP is believed to produce its toxic effects via conversion in the liver by P450 cytochrome enzymes into its active metabolite, acrolein. From the present results it would appear that CYP produces at least part of its inflammatory effects by activating CSPANs. Since it is known that capsaicin produces contractile effects upon urinary bladder through the release of tachykinins (Maggi *et al.*, 1988c) we used this *in vitro* preparation to investigate whether conversion to acrolein was in fact a prerequisite for the apparent activation of CSPANs by CYP. This was achieved by comparing the contractile effects of CYP and acrolein upon the smooth muscle activity of the rat urinary bladder *in vitro*. The present studies demonstrate quite clearly that CYP has no direct stimulatory effects upon CSPANs, whereas acrolein produced a concentration-dependent contractile response which was attenuated to similar extents by both capsaicin pretreatment and blockade of tachykinin receptors. These antagonists had no effect upon the response curve to potassium, suggesting that the inhibitory effects of these agents on the contractile responses to acrolein do not involve a non-specific effect on bladder smooth muscle contractility.

Contrary to the *in vivo* experiments, in which Hoe 140 blocked CYP-induced PPE, this bradykinin B_2 receptor antagonist was substantially ineffective towards acrolein on the rat isolated bladder. The latter finding could be explained by the fact that the necessary components of the kallikrein-kinin system are not available for *de novo* bradykinin formation *in vitro*. Therefore, while acrolein can activate CSPANs without inducing bradykinin generation *in vitro*, the *in vivo* findings, point to bradykinin generation as a necessary intermediate step in the activation of CSPANs following CYP-induced cystitis since combination of both RP 67,580 and Hoe 140 led to no greater inhibitory effect than when either was given alone, i.e. no summation. The acrolein-induced contraction of the rat isolated bladder was blocked by indomethacin, indicating that tachykinin release from CSPANs occurs via the generation of prostanoids. In contrast, indomethacin pretreatment had no effect on PPE induced by CYP administration. This second difference between the *in vivo* and *in vitro* findings may argue against the role of acrolein as activator of CSPANs during CYP-induced cystitis. At this stage we cannot clearly identify why this difference exists, however, one explanation for this anomaly could be that the amount of acrolein produced following conversion from CYP, *in vivo*, is much less than those concentrations used for the *in vitro* experiments, and that relatively low concentration of acrolein induce bradykinin generation. However, at present there exists no information concerning the ability of acrolein to induce bradykinin formation *in vivo*. Alternatively CYP itself or other metabolites may be capable of inducing bradykinin formation *in vivo*.

In conclusion, the present findings demonstrate an active role of CSPANs via tachykinin release in the early phase of the urinary bladder inflammation produced by CYP. Bradykinin generation seems an important intermediate step for CSPANs activation in *in vivo* conditions. Acrolein, a major metabolite of CYP, is capable of activating CSPANs in the isolated bladder; however, the mechanisms of action of acrolein *in vitro* appear to differ from those responsible for the *in vivo* inflammatory response induced by CYP.

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References

CHAHL, L.A. (1988). Antidromic vasodilatation and neurogenic inflammation. *Pharmacol. Ther.*, **37**, 275–300.

COX, P.J. (1979). Cyclophosphamide cystitis-identification of acrolein as the causative agent. *Biochem. Pharmacol.*, **28**, 2045–2049.

EGLEZOS, A., GIULIANI, S., VITI, G. & MAGGI, C.A. (1991). Direct evidence that capsaicin-induced plasma protein extravasation is mediated through tachykinin NK_1 receptors. *Eur. J. Pharmacol.*, **209**, 277–279.

EGLEZOS, A., LECCI, A., SANTICIOLI, P., GIULIANI, S., TRAMONTANA, M., DEL BIANCO, E. & MAGGI, C.A. (1992). Activation of capsaicin-sensitive primary afferents in the rat urinary bladder by compound 48/80: a direct action on sensory nerves? *Arch. Int. Pharmacodyn. Ther.*, **315**, 96–109.

FRAISER, L.H., SARATHCHANDRA, K. & KEHRER, J.P. (1991). Cyclophosphamide toxicity. *Drugs*, **42**, 781–795.

GARRET, C., CARRUETTE, A., FARDIN, V., MOUSSAOUI, S., PEYRONEL, J.F., BLANCHARD, J.C. & LADURON, P.M. (1991). Pharmacological properties of a potent and selective nonpeptide substance P antagonist. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 10208–10211.

GIULIANI, S., SANTICIOLI, P., LIPPE, I.TH., LECCI, A. & MAGGI, C.A. (1993). Effect of bradykinin and tachykinin receptor antagonist on xylene-induced cystitis in rats. *J. Urol.*, **150**, 1014–1017.

GIULIANI, S., SANTICIOLI, P., TRAMONTANA, M., GEPPETTI, P. & MAGGI, C.A. (1991). Peptide N-formyl-methionyl-leucyl-phenyl-alanine (FMLP) activates capsaicin-sensitive primary afferent nerves in guinea-pig atria and urinary bladder. *Br. J. Pharmacol.*, **102**, 730–734.

GRINBERG-FUNES, D.J., SHELDON, C. & WEISS, M. (1990). The use of prostaglandin F2 for the prophylaxis of cyclophosphamide induced cystitis in rats. *J. Urol.*, **144**, 1500–1504.

HOLZER, P. (1991). Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons. *Pharmacol. Rev.*, **43**, 143–201.

LEE, B.P., MORTON, R.F. & LEE, L.-Y. (1992). Acute effects of acrolein on breathing: role of vagal bronchopulmonary afferents. *J. Appl. Physiol.*, **72**, 1050–1056.

LEMBECK, F., GRIESBACHER, T., ECKHARDT, M., HENKE, S., BREIPOHL, G. & KNOLLE, J. (1991) New, long-acting, potent bradykinin antagonists. *Br. J. Pharmacol.*, **102**, 297–304.

MAGGI, C.A. (1990). The role of peptides in the regulation of the micturition reflex: an update. *Gen. Pharmacol.*, **22**, 1–24.

MAGGI, C.A. (1991). The pharmacology of the efferent function of sensory nerves. *J. Auton. Pharmacol.*, **11**, 173–208.

MAGGI, C.A., ABELLI, L., GIULIANI, S., SANTICIOLI, P., GEPPETTI, P., SOMMA, V., FRILLI, S. & MELI, A. (1988b). The contribution of sensory nerves to xylene-induced cystitis in rats. *Neurosci.*, **26**, 709–723.

MAGGI, C.A., GEPPETTI, P., SANTICIOLI, P., FRILLI, S., GIULIANI, S., FURIO, M., THEODORSSON, E., FUSCO, B. & MELI, A. (1988a). Tachykinin-like immunoreactivity in the mammalian urinary bladder: correlation with the functions of the capsaicin-sensitive sensory nerves. *Neurosci.*, **26**, 233–242.

MAGGI, C.A., LECCI, A., SANTICIOLI, P., DEL BIANCO, E. & GIULIANI, S. (1992). Cyclophosphamide cystitis in rats: involvement of capsaicin-sensitive primary afferents. *J. Auton. Nervous System*, **38**, 201–208.

MAGGI, C.A., PATACCHINI, R., SANTICIOLI, P., GEPPETTI, P., CECCONI, R., GIULIANI, S. & MELI, A. (1989). Multiple mechanisms in the motor responses of the guinea-pig isolated urinary bladder to bradykinin. *Br. J. Pharmacol.*, **98**, 619–629.

MAGGI, C.A., PATACCHINI, R., SANTICIOLI, P. & GIULIANI, S. (1991). Tachykinin antagonists and capsaicin-induced contraction of the rat isolated urinary bladder: evidence for tachykinin-mediated cotransmission. *Br. J. Pharmacol.*, **103**, 1535–1541.

MAGGI, C.A., SANTICIOLI, P., ABELLI, L., PARLANI, M., CAPASSO, M., CONTE, B., GIULIANI, S. & MELI, A. (1987). Regional differences in the effects of capsaicin and tachykinins on motor activity and vascular permeability of the rat lower urinary tract. *Naunyn-Schmied. Arch. Pharmacol.*, **335**, 636–645.

MAGGI, C.A., SANTICIOLI, P., BORSINI, F., GIULIANI, S. & MELI, A. (1986). The role of the capsaicin-sensitive innervation of the rat urinary bladder in the activation of micturition reflex. *Naunyn-Schmied. Arch. Pharmacol.*, **332**, 276–283.

MAGGI, C.A., SANTICIOLI, P., DEL BIANCO, E., LECCI, A. & GIULIANI, S. (1993). Evidence for the involvement of bradykinin in chemically-evoked cystitis in anaesthetised rats. *Naunyn-Schmied. Arch. Pharmacol.*, **347**, 432–437.

MAGGI, C.A., SANTICIOLI, P., PATACCHINI, R., GEPPETTI, P., GIULIANI, S., ASTOLFI, M., BALDI, E., PARLANI, M., THEODORSSON, E., FUSCO, B. & MELI, A. (1988c). Regional differences in the motor response to capsaicin in the guinea-pig urinary bladder: relative role of pre- and post-junctional factors related to neuropeptide-containing sensory nerves. *Neurosci.*, **27**, 675–688.

MEINI, S., PATACCHINI, R. & MAGGI, C.A. (1993). Tachykinin NK_1 receptor subtypes in the rat urinary bladder. *Br. J. Pharmacol.*, **108**, 177P.

PHILLIPS, F.S., STERNBERG, S.S., CRONIN, A.P. & VIDAL, P.M. (1961). Cyclophosphamide and urinary bladder toxicity. *Cancer Res.*, **21**, 1577–1589.

SANTICIOLI, P., GIULIANI, S. & MAGGI, C.A. (1993). Failure of L-nitroarginine, a nitric oxide sythase inhibitor, to affect hypotension and plasma protein extravasation produced by tachykinin NK-1 receptor activation in rats. *J. Auton. Pharmacol.*, **13**, 193–199.

SARIA, A. & LUNDBERG, J.M. (1983). Evans blue fluorescence: quantitative and morphological evaluation of vascular permeability in animal tissues. *J. Neurosci. Methods*, **8**, 41–49.

WIRTH, K., HOCK, F.J., ALBUS, U., LIINZ, W., ALPERMANN, H.G., ANAGNOSTOPOULOS, H., HENKE, S.T., BREIPOHL, G., KONIG, W., KNOLE, J. & SCHOLKENS, B.A. (1991). Hoe 140 a new potent and long acting bradykinin antagonist: *in vivo* studies. *Br. J. Pharmacol.*, **102**, 774–777.

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Routes of delivery of muscarinic acetylcholine receptors to the plasma membrane in NG108-15 cells

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1 We have examined the routes of delivery of muscarinic acetylcholine receptors to the plasma membrane in unstimulated and agonist-stimulated NG108-15 cells. Delivery of receptors to the plasma membrane was measured by irreversibly alkylating receptors already at the cell surface with propyl-benzilylcholine mustard (PrBCM) and then following the recovery of binding of the polar radioligand [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) in intact cells.

2 In unstimulated cells, recovery of [^{3}H]-NMS binding after 2 h of incubation at 37°C was 30% of binding in control cells. Binding affinity of [^{3}H]-NMS was unchanged. In cells that had been pre-exposed to carbachol (0.5 mM) for 30 min, initial [^{3}H]-NMS binding was reduced by 38% but recovery of binding was increased from 30% to 43% of control binding.

3 When the cells were pre-incubated for 1 h with the protein synthesis inhibitor cycloheximide (20 μg ml^{-1}), recovery of [^{3}H]-NMS binding was reduced by similar extents in unstimulated (30% to 9%) and carbachol-stimulated (43% to 19%) cells. Incubation of the cells at 20°C instead of 37°C reduced recovery of [^{3}H]-NMS binding from 30% to 9% in unstimulated cells and from 43% to 23% in carbachol-stimulated cells.

4 Depletion of cellular ATP by addition of antimycin (50 nM) and deoxyglucose (50 mM), reduced recovery of binding to 12% in unstimulated cells and to 6% in carbachol-stimulated cells.

5 These results indicate that in unstimulated NG108-15 cells, delivery of muscarinic receptors to the plasma membrane is almost exclusively through the synthetic pathway. In agonist-stimulated cells, receptor sequestration into an intracellular compartment (probably endosomes) occurs. Under these circumstances, delivery of receptors to the plasma membrane along the synthetic route is unaffected but an additional route of delivery (recycling) now operates.

Keywords: Muscarinic ACh receptor; NG108-15 cells; receptor recycling; sequestration

Introduction

It is well established that exposure of cells to muscarinic cholinoceptor agonists leads to a selective removal of muscarinic acetylcholine receptors from the plasma membrane (Harden *et al.*, 1985; Feigenbaum & El-Fakahany, 1985; Cioffi & El-Fakahany, 1986; Fisher, 1988; Baumgold *et al.*, 1989; Eva *et al.*, 1990; Thompson & Fisher, 1990; 1991; Thompson *et al.*, 1991). This process occurs in two phases: an initial sequestration, which is not associated with a reduction in the total number of receptors, followed by down-regulation, where receptors are degraded (Galper & Smith, 1980; Harden *et al.*, 1985; Ray *et al.*, 1989; Thompson & Fisher, 1990). Sequestration involves the movement of receptors to a light membrane fraction (Harden *et al.*, 1985; Baumgold *et al.*, 1989), which probably represents endosomes (Helenius *et al.*, 1983). From here, receptors can either be recycled to the plasma membrane on removal of the agonist, or, in the continued presence of agonist, delivered to another compartment (probably the lysosome), where they undergo proteolysis (Ray *et al.*, 1989).

Muscarinic receptors arriving at the plasma membrane, then, follow one of two routes – the 'synthetic' route, from the rough endoplasmic reticulum via the Golgi complex (Pfeffer & Rothman, 1987), and the 'recycling' route from the endosome (Helenius *et al.*, 1983). While the synthetic route is likely to be constitutive, the recycling route presumably requires a modification of the receptor in order to direct it into the endocytic pathway, and a reversal of this modification to signal its re-insertion into the plasma membrane. We are attempting to investigate the molecular characteristics of these two routes, and to identify the signals that operate on the recycling route. In the present study, we have quantitated and characterized the two routes in both unstimulated and agonist-stimulated NG108-15 cells. In this cell line the muscarinic receptor is of the M_4 sub-type, and agonist stimulation leads to an inhibition of adenylate cyclase (Evans *et al.*, 1984; Peralta *et al.*, 1987). Delivery of muscarinic receptors to the plasma membrane was measured by irreversibly alkylating receptors already at the membrane with propyl-benzilylcholine mustard (PrBCM; Young *et al.*, 1972) and then following the recovery of the binding of [^{3}H]-N-methylscopolamine, a polar, membrane-impermeant radioligand (Birdsall & Hulme, 1989). A preliminary account of this work has been presented to the British Pharmacological Society (Koenig & Edwardson, 1993).

Methods

Cell culture

NG108-15 (mouse neuroblastoma × rat glioma) cells were obtained from Prof. G. Henderson (Department of Pharmacology, University of Bristol) and grown in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum at 37°C in 5% CO_2, 95% humidified air. Cells were detached from the culture flasks for passaging by a brief (< 1 min) incubation with trypsin (0.5 mg ml^{-1}) and EDTA (0.2 mg ml^{-1}) in phosphate-buffered saline (PBS). Cells were passaged every 3–5 days and were used at passages 14–41.

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[^{3}H]-N-methylscopolamine binding

Cells were seeded into poly-L-lysine-treated (25 mg/well) 24-well plates at approximately 10^5 cells/well in 2 ml culture medium and used when almost confluent (2–3 days). Muscarinic receptors at the plasma membrane of the cells were quantitated through the binding of the polar radioligand [^{3}H]-N-methylscopolamine ([^{3}H]-NMS). Cells were washed with 1 ml PBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS-Ca,Mg), and [^{3}H]-NMS binding was determined by incubation of the cell monolayers with 0.25 ml/well of [^{3}H]-NMS in the same buffer for 90 min at 4°C. Non-specific binding was defined by N-methylatropine (1 μM). Incubations were terminated by two washes with 1 ml ice-cold PBS-Ca, Mg. The cells were then solubilized in 0.2 ml 0.5% (v/v) Triton X-100 and transferred to vials for liquid scintillation counting. Saturation data were analysed using the programme LIGAND (Munson & Rodbard, 1980). It has been shown previously (Eva *et al.*, 1990) that [^{3}H]-NMS binding to muscarinic receptors reaches equilibrium over 90 min at 4°C, and the linearity of Scatchard plots of the saturation data confirms this observation. At concentrations of [^{3}H]-NMS around the K_D, specific binding represented 90–95% of total binding. Cell density (2×10^5 cells/well) was determined by removing cells from the well with 0.2 ml PBS containing EGTA (1 mM) and counting in a Neubauer haemocytometer.

Treatment of cells with propyl-benzilylcholine mustard

Muscarinic receptors at the plasma membrane of the cells were irreversibly alkylated with propyl-benzilylcholine mustard (PrBCM). The pharmacologically-active species of PrBCM is the aziridinium ion, which was prepared by the method of Young *et al.* (1972). PrBCM (100 μM) was allowed to cyclize in PBS, pH 7.4, at room temperature for 1 h. The reaction was stopped by dilution to 10^{-8} M. This procedure gives a yield of approximately 91% (Young *et al.*, 1972). Cells were washed with 1 ml HEPES-buffered DMEM (DMEM-HEPES) containing 0.2% (w/v) bovine serum albumin (BSA), incubated with PrBCM (10^{-8} M) in PBS-Ca,Mg at 20°C, and then washed with 1 ml PBS-Ca,Mg to remove free PrBCM.

Delivery of muscarinic receptors to the plasma membrane

Following alkylation of muscarinic receptors at the plasma membrane of the cells by PrBCM treatment, delivery of receptors to the plasma membrane was assayed through the recovery of [^{3}H]-NMS binding and expressed as a percentage of binding in control cells (i.e. cells that had been incubated in PBS-Ca,Mg alone instead of buffer plus PrBCM). During recovery, cells were incubated in DMEM-HEPES containing 0.2% (w/v) BSA.

The aim of this study was to examine the effects of various manipulations on the delivery of muscarinic receptors to the plasma membrane. These manipulations included stimulation of the cells with the muscarinic agonist carbachol, preincubation of the cells with the protein synthesis inhibitor cycloheximide, reduction of the temperature during recovery from 37°C to 20°C and depletion of cellular ATP by treatment with antimycin plus deoxyglucose. Carbachol stimulation was at 0.5 mM in DMEM-HEPES containing 0.2% (w/v) BSA for 30 min. Carbachol was removed by two washes with 1 ml of the same buffer, and the cells were then treated immediately with PrBCM. Cycloheximide (20 μg ml^{-1}) in DMEM-HEPES containing 0.2% (w/v) BSA was added 1 h before PrBCM treatment (i.e. 30 min before carbachol stimulation, where appropriate) and was present in all buffers, including wash buffers, until determination of [^{3}H]-NMS binding. Pretreatment of cells with both carbachol and cycloheximide was carried out at 37°C. Washing prior to PrBCM treatment and PrBCM treatment itself was at 20°C. Recovery of [^{3}H]-NMS binding after PrBCM treatment was followed at either 37°C or 20°C. Antimycin and deoxyglucose were added, where appropriate, during washing of the cells before PrBCM treatment, and were present in all buffers up until the [^{3}H]-NMS binding assay.

Materials

[^{3}H]-NMS (specific activity 85 Ci $mmol^{-1}$) was from Amersham International plc, Amersham, Bucks. Cell culture medium was from ICN Biomedicals Ltd., High Wycombe, Bucks., and foetal calf serum was from Gibco, Uxbridge. PrBCM was a generous gift of Dr J.M. Young of the Department of Pharmacology, University of Cambridge. All other reagents were from Sigma, Poole, Dorset.

Statistical evaluation of data

Within each experiment, all values were determined in quadruplicate. Standard errors within quadruplicates were typically 5% of the mean. Except for the saturation binding data, all values are expressed as means ± s.e.mean of values from n separate experiments. The statistical significance of differences between groups of data was assessed by Student's t test (unpaired). A P value of <0.05 was considered to be significant.

Results

Binding of [^{3}H]-NMS to muscarinic receptors in NG108-15 cells

Muscarinic receptors at the plasma membrane of NG108-15 cells were quantitated through the binding of the polar radioligand [^{3}H]-NMS (Birdsall & Hulme, 1989). Specific [^{3}H]-NMS binding behaved as a single component of K_D 129 ± 13 pM and B_{max} 3.55 ± 0.22 fmol/well. Since cell density was 2×10^5/well, this value for B_{max} represents approximately 10^4 sites/cell. In this study [^{3}H]-NMS was routinely used at a concentration of 0.2–0.3 nM. At this concentration, specific binding was 90–95% of the total binding.

Alkylation of muscarinic receptors by propyl-benzilylcholine mustard

As has been shown previously in longitudinal strips of smooth muscle from the guinea-pig ileum (Young *et al.*, 1972), PrBCM, at a concentration of 10^{-8} M, rapidly and irreversibly alkylated muscarinic receptors at the plasma membrane (Figure 1). The half-time of loss of [^{3}H]-NMS binding was approximately 2 min. A 20 min incubation time was used routinely; at this time, no significant specific binding of [^{3}H]-NMS (at a concentration of 0.2 nM) was detectable.

Recovery of [^{3}H]-NMS binding in PrBCM-treated cells

When the PrBCM was removed and the cells were incubated at 37°C, [^{3}H]-NMS binding recovered (Figure 2), indicating that muscarinic receptors were being delivered to the plasma membrane. Over a 2 h period, the number of muscarinic receptors at the plasma membrane increased significantly from 2 ± 1% to 32 ± 2% ($n = 16$; $P<0.001$) of the number initially present in untreated cells; recovery was therefore 30 ± 2%. In some experiments, intermediate timepoints were included, which show that the recovery of binding was approximately linear with time over the first 2 h. It is reasonable to expect that recovery would eventually proceed to completion; the 2 h time-point was chosen simply for convenience in designing the experiments.

Analysis of [^{3}H]-NMS binding (Figure 3) showed that the receptors arriving at the plasma membrane after PrBCM treatment had a binding affinity for NMS (K_D 116 ± 10 pM) that was not significantly different from that in control cells (K_D 129 ± 13 pM). In the experiment illustrated, B_{max} after 2 h of recovery was 1.27 ± 0.07 fmol/well, that is, 35% of the value in control cells (B_{max} 3.55 ± 0.22 fmol/well). Since NMS binding affinity does not change after recovery, it is valid to use [^{3}H]-NMS binding at a single concentration as an index of delivery of receptors to the plasma membrane.

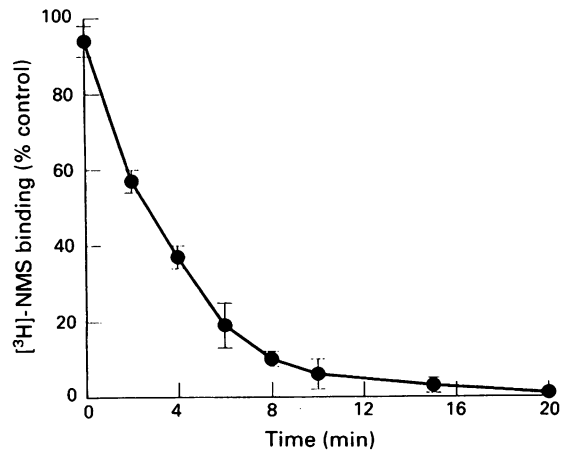


Figure 1 Time-course of alkylation of muscarinic receptors by propyl-benzilylcholine mustard (PrBCM). Cells on 24-well plates were incubated with PrBCM (10^{-8} M) for various times, and washed before determination of [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) binding. Cells were incubated with [^{3}H]-NMS (0.2 nM) for 90 min at 4°C, washed and then solubilized for liquid scintillation counting. Non-specific binding was defined by N-methylatropine (1 μM). Results are expressed as a percentage of [^{3}H]-NMS binding to cells that had not been treated with PrBCM. Values are means ± s.e.mean from three separate experiments, each performed in quadruplicate.

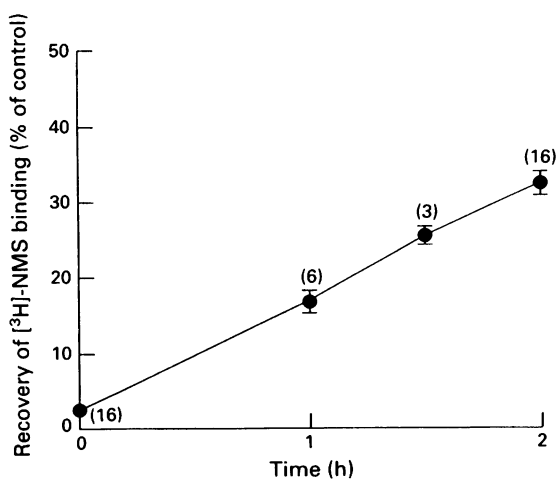


Figure 2 Time-course of recovery of [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) binding after incubation of unstimulated cells with propyl-benzilylcholine mustard (PrBCM, 10^{-8} M, 20 min). Cells were incubated for various times at 37°C, before determination of [^{3}H]-NMS binding (at a concentration of 0.3 nM). Recovery is expressed as a percentage of binding in cells that had not been incubated with PrBCM, but had otherwise been treated identically. Values are means ± s.e.mean from several separate experiments (numbers in parentheses above each point); each experiment was performed in quadruplicate.

Modulation of recovery of [^{3}H]-NMS binding

When the cells were exposed for 30 min to a concentration of carbachol sufficient to saturate the muscarinic receptors (0.5 mM), [^{3}H]-NMS binding was reduced to 62 ± 3% (n = 16) of the control value. This result is in close agreement with those reported by others for NG108-15 cells (Baumgold *et al.*, 1989), N1E-115 cells (Feigenbaum & El-Fakahany, 1985; Cioffi & El-Fakahany, 1986), 1321N1 cells (Harden *et al.*, 1985), SK-N-SH cells (Baumgold *et al.*, 1989; Thompson & Fisher, 1990; 1991; Thompson *et al.*, 1991) and corticostriatal cells (Eva *et al.*, 1990).

In carbachol-stimulated cells, recovery of [^{3}H]-NMS binding in the 2 h period following PrBCM treatment was increased significantly from 30 ± 2% (n = 16) to 43 ± 3% (n = 10; P<0.001, Figure 4). This increase in recovery is likely to be a consequence of recycling of receptors to the plasma membrane from an endosomal pool created by agonist-induced sequestration. In order to confirm this, we tested the effects on recovery of [^{3}H]-NMS binding in unstimulated and agonist-stimulated cells of two perturbations that should discriminate between delivery to the plasma membrane of newly-synthesized and recycled receptors. These perturbations were inhibition of protein synthesis and incubation at 20°C, which has been reported to block transport of proteins from the *trans*-Golgi network to the plasma membrane (Matlin & Simons, 1983) but not to affect recycling through endosomes (Helenius *et al.*, 1983).

Incubation of the cells with the protein synthesis inhibitor, cycloheximide (20 μg ml^{-1}) for 1 h before PrBCM treatment significantly reduced the subsequent recovery of [^{3}H]-NMS binding from 30 ± 2% to 9 ± 2% (n = 5; P<0.001) in unstimulated cells and by a similar amount (43 ± 3% to 19 ± 1%, n = 5; P<0.001) in carbachol-stimulated cells (Figure 4). When unstimulated cells were incubated at 20°C instead of at

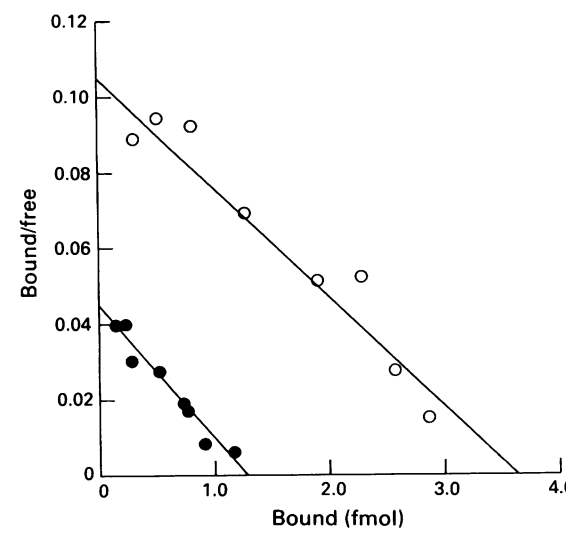


Figure 3 [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) binding characteristics before and after recovery from propyl-benzilylcholine mustard (PrBCM)-treatment. [^{3}H]-NMS binding was determined either in control cells (○) or in cells that had been treated with PrBCM (10^{-8} M for 20 min at 20°C), washed and incubated for 2 h at 37°C (●). Data were analysed using the programme LIGAND (Munson & Rodbard, 1980), and fitted to a single site with the binding parameters: K_D 129 ± 13 pM; B_{max} 3.55 ± 0.22 fmol/well (control cells), and K_D 116 ± 10 pM; B_{max} 1.27 ± 0.07 fmol/well (cells after recovery). Data are presented in the form of a Scatchard plot; the lines have been drawn using the parameters obtained from the LIGAND analysis. The result shown is typical of those from three separate experiments, each performed in quadruplicate. Values on the ordinate scale represent fmol bound [^{3}H]-NMS/fmol free [^{3}H]-NMS in 0.25 ml incubation volume.

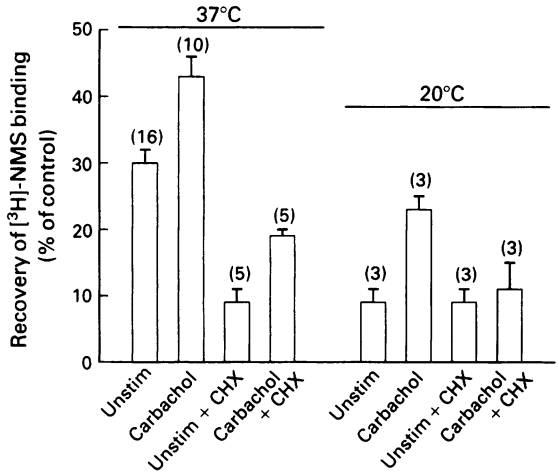


Figure 4 The effect of inhibition of protein synthesis and of incubation at 20°C on recovery of [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) binding following propyl-benzilylcholine mustard (PrBCM) treatment of unstimulated or carbachol-stimulated cells. Cycloheximide (CHX, 20 μg ml^{-1}) was added 1 h before PrBCM treatment and was present thereafter. Carbachol pre-incubation was for 30 min before PrBCM treatment. [^{3}H]-NMS binding (at a concentration of 0.3 nM) was determined after a 2 h incubation at either 37°C or 20°C. Recovery is expressed as a percentage of binding in control cells (i.e. cells that had not been treated with either cycloheximide or carbachol, and that had been incubated in PBS-Ca,Mg alone instead of buffer containing PrBCM). Values are corrected for the small amount of binding remaining after PrBCM treatment. Results shown are means ± s.e.mean from several separate experiments (numbers in parentheses); each experiment was performed in quadruplicate.

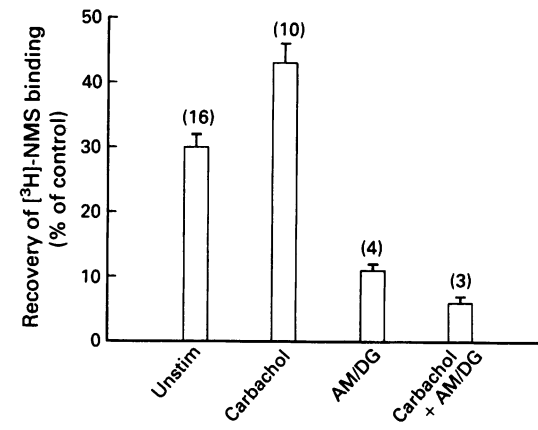


Figure 5 Effect of inhibition of protein synthesis and of ATP depletion on recovery of [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) binding following propyl-benzilylcholine mustard (PrBCM) treatment of unstimulated or carbachol-stimulated cells. Carbachol pre-incubation was for 30 min before PrBCM treatment. Antimycin (AM, 50 nM) and deoxyglucose (DG, 50 mM) were added immediately before PrBCM treatment and were present thereafter. [^{3}H]-NMS binding (at a concentration of 0.3 nM) was determined either immediately after PrBCM treatments or after 2 h at 37°C. Recovery is expressed as a percentage of binding in control cells (see caption to Figure 4), corrected for the small amount of binding remaining after PrBCM treatment. Values are means ± s.e.mean from several separate experiments (numbers in parentheses); each experiment was performed in quadruplicate.

37°C, recovery of [^{3}H]-NMS binding over 2 h was reduced from 30 ± 2% to 9 ± 2% ($n = 3$; $P < 0.001$). In carbachol-stimulated cells, recovery was reduced by a similar amount (43 ± 3% to 23 ± 2%, $n = 3$; $P < 0.01$). Recovery at 20°C in carbachol-stimulated cells was not significantly affected by pretreatment with cycloheximide (11 ± 4%; $n = 3$).

One potential drawback of the use of recovery of [^{3}H]-NMS binding following PrBCM treatment to measure delivery of muscarinic receptors to the plasma membrane is the possibility that receptors are arriving at the cell surface (and being alkylated) during incubation of the cells with PrBCM (20 min at 20°C). One would expect the delivery of newly-synthesized receptors to the plasma membrane to occur at a constant rate (see Figure 2), so that any delivery during alkylation would not interfere with the measurement of delivery over the subsequent 2 h incubation period. Recycling, on the other hand, involves a limited intracellular pool of receptors and would be expected to be an exponential process. Consequently, any delivery during alkylation would result in an underestimation of the extent of recycling. In order to establish whether this was a significant problem, we measured [^{3}H]-NMS binding immediately after treatment of the cells for 30 min at 37°C with carbachol (0.5 mM), and after a further 20 min incubation at 20°C in the absence of both carbachol and PrBCM. Cells were pretreated for 1 h with cycloheximide to block delivery of newly-synthesized receptors to the plasma membrane. The values obtained for [^{3}H]-NMS binding before and after the 20 min period at 20°C were respectively 49 ± 2% ($n = 4$) and 47 ± 2% ($n = 4$) of binding in untreated cells. Hence, no delivery of receptors to the plasma membrane will occur during treatment of the cells with PrBCM.

Figure 5 shows that incubation of cells with a combination of antimycin (50 nM) and deoxyglucose (50 mM) immediately before PrBCM treatment, in order to inhibit both mitochondrial and glycolytic generation of ATP (Hertel *et al.*, 1986), substantially reduced recovery of [^{3}H]-NMS binding in both unstimulated and carbachol-stimulated cells. Recovery fell significantly from 30 ± 2% ($n = 16$) to 12 ± 1% ($n = 4$; $P < 0.001$) in unstimulated cells and from 43 ± 3% ($n = 10$) to 6 ± 1% ($n = 3$; $P < 0.001$) in carbachol-stimulated cells. The residual recoveries were not significantly different from that seen after cycloheximide treatment of unstimulated cells (9 ± 2%, $n = 3$). This result indicates that delivery to the plasma membrane of both newly synthesized and recycled receptors requires the presence of ATP in the cell cytosol.

Discussion

Our aim was to dissect the two routes by which muscarinic receptors are delivered to the plasma membrane in NG108-15 cells – the 'synthetic' route and the 'recycling' route – and to examine how these routes are affected by agonist stimulation of the cells. In order to achieve this aim, we used two perturbations, cycloheximide treatment and 20°C incubation, that we hoped would block synthetic delivery but not recycling. In unstimulated cells the cycloheximide-sensitive component of recovery of [^{3}H]-NMS binding following PrBCM treatment was 21 ± 3%. The cycloheximide-resistant component was 9 ± 2%, indicating that most of the recovery in these cells is due to new synthesis of receptors. The residual recovery may represent delivery of receptors from a depleted synthetic pool, since it has been shown that the half-time of transport of a membrane protein along the synthetic pathway is typically of the order of 1 h (Daniels & Edwardson, 1988), so that a 1 h preincubation with cycloheximide will not completely deplete this pathway of receptors. In unstimulated cells, 20°C incubation caused a reduction in recovery (21 ± 3%) that was not significantly different from that caused by cycloheximide. It is likely, therefore, that the two perturbations are affecting the same population of receptors.

In carbachol-stimulated cells, the recovery at 37°C after PrBCM treatment was greater than that in unstimulated cells. This enhancement was entirely a consequence of an increased cycloheximide-insensitive component (from 9 ± 2% to 19 ± 1%; $P < 0.01$), which is likely to represent recycling

of receptors from endosomes. The cycloheximide-sensitive component of recovery, on the other hand (24 ± 3%), was not significantly different from that in unstimulated cells, indicating that short-term (30 min) agonist stimulation does not affect the synthetic pathway. It has been shown previously, in fact (Habecker & Nathanson, 1992), that long-term (up to 24 h) stimulation of cells with muscarinic agonists causes a progressive reduction in muscarinic receptor mRNA levels, most likely through a decrease in the rate of transcription. However, this effect is unlikely to feed through to a fall in the rate of receptor synthesis during the brief agonist exposure used here. As in unstimulated cells, reduction in temperature to 20°C caused the same fall in recovery as cycloheximide (20 ± 4%). Cycloheximide pretreatment did not cause a further significant reduction in recovery, supporting our argument that cycloheximide and low temperature are having similar effects on receptor traffic.

Is it possible, then, to use the data presented here to estimate the numbers of receptors moving along these various intracellular routes and quantitate the relative importance of the recycling and synthetic routes? The measurement of [^{3}H]-NMS binding after carbachol stimulation of the cells indicates that of 100 receptors initially at the plasma membrane, 38 are internalized during a 30 min treatment with agonist. The difference between the recoveries of [^{3}H]-NMS binding at 37°C in unstimulated and carbachol-stimulated cells (13%) suggests that, of these 38 receptors, 13 are recycled to the plasma membrane within 2 h. Since, it has been shown previously (Thompson & Fisher, 1990) that recycling occurs rapidly at 37°C ($t_{\frac{1}{2}}$ 7 min in SK-N-SH cells), the remaining 25 receptors must be degraded intracellularly. For every 100 receptors initially present at the plasma membrane, 30 are delivered to the plasma membrane over 2 h in unstimulated cells. Since the cycloheximide-resistant recovery of [^{3}H]-NMS binding at 20°C in unstimulated cells was 7%, an upper limit of 7 receptors can be placed on the contribution of recycling to this figure. At least 23 receptors must therefore be delivered along the synthetic pathway in 2 h. The true figure for recycling in unstimulated cells is likely to be lower than 7%, however, since a cycloheximide-resistant component of delivery along the synthetic route probably persists even at this reduced temperature.

It is unlikely that there is a significant amount of receptor recycling occurring during the PrBCM incubation at 20°C, since we have demonstrated that no recycling to the plasma membrane occurs during the first 20 min of incubation of the cells at 20°C after carbachol stimulation in the absence of PrBCM. This may indicate the presence of a lag period before delivery of receptors to the plasma membrane begins. This lag may represent the time required for receptors to be mobilised from an endosomal compartment and transported back to the cell surface, and may include a period for the removal of an 'internalization signal' from the receptors. The temperature-dependence of recovery of [^{3}H]-NMS binding following carbachol stimulation has been investigated previously in SK-N-SH cells (Thompson & Fisher, 1991), although in this study surface receptors were not pre-alkylated, so that recycling would have been occurring in parallel with internalization. Recovery was seen at 25°C but not at 15°C, which suggests that there might be a threshold temperature for recycling at around 20°C. The apparent lag in recovery might also be seen only at this reduced temperature, since there is no evidence for such a phenomenon at 37°C (Harden *et al.*, 1985; Thompson & Fisher, 1990).

Many steps in intracellular protein transport, including transport between the Golgi complex and the plasma membrane (Gravotta *et al.*, 1990; Miller & Moore, 1991) and recycling through endosomes (Goda & Pfeffer, 1988), are known to require ATP. The fact that treatment with a combination of antimycin and deoxyglucose substantially inhibited recovery of [^{3}H]-NMS binding in both unstimulated and agonist-stimulated cells, therefore, confirms our suggestion that both cycloheximide-sensitive and cycloheximide-insensitive components of delivery of receptors to the plasma membrane represent *bona fide* ATP-dependent intracellular protein transport processes.

The molecular signalling involved in the movement of receptors into the recycling route following agonist stimulation is poorly understood. In the case of the better-characterized β-adrenoceptor, agonist stimulation leads to phosphorylation of the receptor by both protein kinase A (PKA) and β-adrenoceptor kinase (βARK) (Lefkowitz *et al.*, 1990). Muscarinic receptors are thought to undergo similar processes (Kwatra *et al.*, 1989; Haga *et al.*, 1993; Tobin & Nahorski, 1993). Furthermore, a serine/threonine-rich domain of the third cytoplasmic loop of the m1 receptor has been shown to be required for sequestration (Lameh *et al.*, 1992; Moro *et al.*, 1993). It is tempting to speculate, therefore, that agonist-stimulated phosphorylation of this region of the receptor triggers sequestration. In support of this idea, there have been reports of a stimulation of sequestration/down-regulation by activation of protein kinase C (PKC; Liles *et al.*, 1986; Cioffi & Fisher, 1990). On the other hand, Hoover & Toews (1990) found that PKC activation inhibited sequestration/down-regulation in 1321N1 cells, and Thompson *et al.* (1991) have provided evidence that in SK-N-SH cells sequestration of the m3 sub-type of receptor can occur without the production of phosphoinositide-derived second messengers. The picture, therefore, is still confused, and it is reasonable to suppose that the molecular mechanisms of sequestration might be different in cell types expressing different sub-types of the receptor.

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References

BAUMGOLD, J., COOPERMAN, B.B. & WHITE, T.M. (1989). Relationship between desensitization and sequestration of muscarinic receptors in two neuronal cell lines. *Neuropharmacology*, **28**, 1253–1261.

BIRDSALL, N.J.M. & HULME, E.C. (1989). The binding properties of muscarinic receptors. In *The Muscarinic Receptors*. ed. Brown, J.H. pp. 31–92. New Jersey: Humana Press.

CIOFFI, C.L. & EL-FAKAHANY, E.S. (1986). Short-term desensitization of muscarinic cholinergic receptors in mouse neuroblastoma cells: Selective loss of agonist low-affinity and pirenzepine high-affinity binding sites. *J. Pharmacol. Exp. Ther.*, **238**, 916–923.

CIOFFI, C.L. & FISHER, S.K. (1990). Reduction of muscarinic receptor density and of guanine nucleotide-stimulated phosphoinositide hydrolysis in human SH-SY5Y neuroblastoma cells following long-term treatment with 12-*O*-tetradecanoylphorbol 13-acetate or mezerein. *J. Neurochem.*, **54**, 1725–1734.

DANIELS, P.U. & EDWARDSON, J.M. (1988). Intracellular processing and transport of influenza-virus envelope proteins in Madin-Darby canine kidney cells. Effects of the carboxylic ionophores monensin and nigericin. *Biochem. J.*, **252**, 693–700.

EVA, C., GAMALERO, S.R., GENAZZANI, E. & COSTA, E. (1990). Molecular mechanisms of homologous desensitization and internalization of muscarinic receptors in primary cultures of neonatal corticostriatal neurons. *J. Pharmacol. Exp. Ther.*, **253**, 257–265.

EVANS, T., SMITH, M.M., TANNER, L.I. & HARDEN, T.K. (1984). Muscarinic cholinergic receptors of two cell lines that regulate cyclic AMP metabolism by different molecular mechanisms. *Mol. Pharmacol.*, **26**, 395–404.

FEIGENBAUM., P. & EL-FAKAHANY, E.S. (1985). Regulation of muscarinic receptor density in neuroblastoma cells by brief exposure to agonist: Possible involvement in desensitization of receptor function. *J. Pharmacol. Exp. Ther.*, **233**, 134–141.

FISHER, S.K. (1988). Recognition of muscarinic cholinergic receptors in human SK-N-SH neuroblastoma cells by quaternary and tertiary ligands is dependent upon temperature, cell integrity, and the presence of agonists. *Mol. Pharmacol.*, **33**, 414–422.

GALPER, J.B. & SMITH, T.W. (1980). Agonist and guanine nucleotide modulation of muscarinic cholinergic receptors in cultured heart cells. *J. Biol. Chem.*, **255**, 9571–9579.

GODA, Y. & PFEFFER, S.Y. (1988). Selective recycling of the mannose 6-phosphate/IGF-II receptor to the *trans* Golgi network in vitro. *Cell*, **55**, 309–320.

GRAVOTTA, D., ADESNIK, M. & SABATINI, D.D. (1990). Transport of influenza HA from the *trans*-Golgi network to the apical surface of MDCK cells permeabilized in their basolateral plasma membranes: energy dependence and involvement of GTP-binding proteins. *J. Cell Biol.*, **111**, 2893–2908.

HABECKER, B.A. & NATHANSON, N.M. (1992). Regulation of muscarinic acetylcholine receptor mRNA expression by activation of homologous and heterologous receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 5035–5038.

HAGA, T., HAGA, K., KAMEYAMA, K. & NAKATA, H. (1993). Phosphorylation of muscarinic receptors: regulation by G proteins. *Life Sci.*, **52**, 421–428.

HARDEN, K.T., PETCH, L.A., TRAYNELIS, S.F. & WALDO, G.L. (1985). Agonist-induced alterations in the membrane form of muscarinic cholinergic receptors. *J. Biol. Chem.*, **260**, 13060–13066.

HELENIUS, A., MELLMAN, I., WALL, D. & HUBBARD, A. (1983). Endosomes. *Trends Biochem. Sci.*, **8**, 245–250.

HERTEL, C., COULTER, S.J. & PERKINS, J.P. (1986). The involvement of cellular ATP in receptor-mediated internalization of epidermal growth factor and hormone-induced internalization of β-adrenergic receptors. *J. Biol. Chem.*, **261**, 5974–5980.

HOOVER, R.K. & TOEWS, M.L. (1990). Activation of protein kinase C inhibits internalization and downregulation of muscarinic receptors in 1321N1 human astrocytoma cells. *J. Pharmacol. Exp. Ther.*, **253**, 185–191.

KOENIG, J.A. & EDWARDSON, J.M. (1993). Delivery of muscarinic ACh receptors to the plasma membrane in unstimulated and agonist-stimulated NG108-15 cells. *Br. J. Pharmacol.*, **110**, 70P.

KWATRA, M.M., BENOVIC, J.L., CARON, M.G., LEFKOWITZ, R.J. & HOSEY, M.M. (1989). Phosphorylation of chick heart muscarinic cholinergic receptors by the β-adrenergic receptor kinase. *Biochemistry*, **28**, 4543–4547.

LAMEH, J., PHILIP, M., SHARMA, Y.K., MORO, O., RAMACHANDRAN, J. & SADEE, W. (1992). Hml muscarinic cholinergic receptor internalization requires a domain in the third cytoplasmic loop. *J. Biol. Chem.*, **267**, 13406–13412.

LEFKOWITZ, R.J., HAUSDORFF, W.P. & CARON, M.G. (1990). Role of phosphorylation in desensitization of the β-adrenoceptor. *Trends Pharmacol. Sci.*, **11**, 190–194.

LILES, W.C., HUNTER, D.D., MEIER, K.E. & NATHANSON, N.M. (1986). Activation of protein kinase C induces rapid internalization and subsequent degradation of muscarinic acetylcholine receptors in neuroblastoma cells. *J. Biol. Chem.*, **261**, 5307–5313.

MATLIN, K.S. & SIMONS, K. (1983). Reduced temperature prevents transfer of a membrane glycoprotein to the cell surface but does not prevent terminal glycosylation. *Cell*, **34**, 233–243.

MILLER, S.G. & MOORE, H.-P.H. (1991). Reconstitution of constitutive secretion using semi-intact cells: regulation by GTP but not calcium. *J. Cell. Biol.*, **112**, 39–54.

MORO, O., LAMEH, J. & SADEE, W. (1993). Serine- and threonine-rich domain regulates internalization of muscarinic cholinergic receptors. *J. Biol. Chem.*, **268**, 6862–6865.

MUNSON, P.J. & RODBARD, D. (1980). Ligand: a versatile computerized approach for characterization of ligand binding systems. *Anal. Biochem.*, **107**, 220–239.

PERALTA, E.G., ASHKENAZI, A., WINSLOW, J.W., SMITH, D.H., RAMACHANDRAN, J. & CAPON. D.J. (1987). Distinct primary structure, ligand-binding properties and tissue-specific expression of four human muscarinic acetylcholine receptors. *EMBO J.*, **6**, 3923–3929.

PFEFFER, S.R. & ROTHMAN, J.E. (1987). Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Annu. Rev. Biochem.*, **56**, 829–852.

RAY, P., MIDDLETON, W. & BERMAN, J.D. (1989). Mechanism of agonist-induced down-regulation and subsequent recovery of muscarinic acetylcholine receptors in a clonal neuroblastoma × glioma hybrid cell line. *J. Neurochem.*, **52**, 402–409.

THOMPSON, A.K. & FISHER, S.K. (1990). Relationship between agonist-induced muscarinic receptor loss and desensitization of stimulated phosphoinositide turnover in two neuroblastomas: methodological considerations. *J. Pharmacol. Exp. Ther.*, **252**, 744–752.

THOMPSON, A.K. & FISHER, S.K. (1991). Preferential coupling of cell surface muscarinic receptors to phosphoinositide hydrolysis in human neuroblastoma cells. *J. Biol. Chem.*, **266**, 5004–5010.

THOMPSON, A.K., MOSTAFAPOUR, S.P., DENLINGER, L.C., BLEASDALE, J.E. & FISHER, S.K. (1991). The aminosteroid U-73122 inhibits muscarinic receptor sequestration and phosphoinositide hydrolysis in SK-N-SH neuroblastoma cells. *J. Biol. Chem.*, **266**, 23856–23862.

TOBIN, A.B. & NAHORSKI, S.R. (1993). Rapid agonist-mediated phosphorylation of m3-muscarinic receptors revealed by immunoprecipitation. *J. Biol. Chem.*, **268**, 9817–9823.

YOUNG, J.M., HILEY, C.R. & BURGEN, A.S.V. (1972). Homologues of benzilylcholine mustard. *J. Pharm. Pharmacol.*, **24**, 950–954.

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Induction of kinin B_1 receptor-dependent vasoconstriction following balloon catheter injury to the rabbit carotid artery

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1 Balloon catheter injury to the rabbit carotid artery damaged the endothelium and induced neointima formation over 7 days. The area of intima, expressed as a percentage of the media, was 16.2 ± 4.2% and 8.2 ± 0.1% in balloon catheter-injured and sham-operated arteries.

2 Seven days after arterial injury, carotid arteries were isolated and set up as ring preparations in organ baths for isometric tension measurements. Balloon catheter-injured arteries first contracted with noradrenaline (0.01–0.1 μM), contracted further in a concentration-dependent manner to bradykinin (BK; pD_2, 5.98 ± 0.22; E_{max}, 41.3 ± 5.2% of KCl) and to des-Arg^9-BK (pD_2, 7.12 ± 0.36; E_{max}, 46.0 ± 9.9% of KCl). In contrast, vessel segments with endothelium either intact or acutely removed were unresponsive to both BK receptor agonists.

3 The concentration-contraction curves for BK and for des-Arg^9-BK were shifted to the right by the B_1 receptor antagonist, [Leu^8]des-Arg^9-BK (3 μM), but not by the selective B_2 receptor antagonist, Hoe 140 (1 μM).

4 Thus, BK and its metabolite, des-Arg^9-BK act as vasoconstrictor agents following balloon catheter injury. These effects appear to be mediated by activation of B_1 receptors.

Keywords: Vascular injury; kinin B_1 receptor; des-Arg^9-BK; bradykinin; rabbit carotid artery

Introduction

As proposed by Regoli & Barabé in 1980, kinin receptors have been divided in two types, B_1 and B_2. Endothelial B_2 receptor-mediated vasodilatation is one of the most important cardiovascular effects of the nonapeptide, bradykinin (BK) (Regoli & Barabé, 1980; Bonner & Schunk, 1986; Pelc *et al.*, 1991). In contrast with B_2 receptors, vascular B_1 receptors are thought to be absent under normal conditions but can be induced *in vitro* by prolonged incubation (Deblois & Marceau, 1987; Pruneau & Bélichard, 1993) or *in vitro* and *in vivo* by treatment with endotoxin (Regoli *et al.*, 1981; Deblois & Marceau, 1987).

Balloon catheter angioplasty produces major mechanical injury to the arterial wall with ultimate death of endothelial cells and to some extent smooth muscle cells (Liu *et al.*, 1989; Schwartz *et al.*, 1992). The resultant inflammation which develops at the site of vascular damage is characterized by the release of cytokines, growth factors and the expression of nuclear oncogenes (Liu *et al.*, 1989; Schwartz *et al.*, 1992). These factors stimulate migration and proliferation of smooth muscle cells from the media to form a neointima (Liu *et al.*, 1989; Schwartz *et al.*, 1992).

We hypothesized that injury to a blood vessel such as that produced by angioplasty, induces the expression of B_1 receptors in the vascular wall. To test this possibility, we investigated the response to BK and to des-Arg^9-BK in balloon catheter-injured and sham-operated carotid arteries of the rabbit. In addition, the responses to various dilators (acetylcholine, ionophore A 23187, sodium nitroprusside) were obtained in the same vascular preparations to determine a possible involvement of the endothelium.

Methods

Balloon catheter injury

Male New-Zealand White rabbits weighing 2.5 to 3.5 kg each were anaesthetized with ketamine (35 mg kg^{-1}, i.m.) and xylazine (5 mg kg^{-1}, i.m.). An arterial embolectomy catheter (2F Fogarty, Baxter, CA, U.S.A.) was inserted into the right common carotid artery through a branch of the internal carotid artery. The balloon on the catheter was then inflated with 0.05 ml of distilled water and the inner surface of the artery rubbed three times from the aortic arch up to the external carotid artery branch. After withdrawal of the catheter, the internal carotid artery was ligated and the wound closed. Penicillin G (200,000 iu) was applied locally to the wound and the animals allowed to recover. Other rabbits that were sham-operated without the ballooning procedure were controls. Because the embolectomy catheter was re-used in consecutive rabbits and thus could have been contaminated with lipopolysaccharides (LPS), three rabbits were operated under sterile conditions and a newly purchased embolectomy catheter used only once for each animal.

Isolated carotid artery reactivity

At 7 days following balloon catheter injury, rabbits were killed by an overdose of i.v. sodium pentobarbitone. The right carotid artery was then carefully dissected out and transferred to a Krebs solution of the following composition (in mM): NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $NaHCO_3$ 25, $CaCl_2$ 2.5, ethylenediaminetetracetic acid (EDTA) 0.026, glucose 5.5, bubbled with 95% O_2 plus 5% CO_2. Carotid arteries of control animals were obtained and kept under the same conditions. Three rings of 3 mm in length were prepared from each carotid segment and were suspended on wire hooks (0.3 mm in diameter) in 20 ml jacketed organ baths maintained at 37°C. In some control carotid arteries, the vascular endothelium was rubbed off *in vitro* with a catheter which was introduced into the vessel lumen and gently moved back and forth several times. One hook was suspended from a Gould-Statham UC_2 or UTC_2 transducer, and the other was fixed to a plastic support leg. Changes in isometric tension were recorded continuously on two-channel recorders (Gould BS272 or Linseis Type 7025). Rings were left unstretched for 15 min and were then gradually stretched to a passive tension of 2 g. Preliminary experiments showed that a 2 g resting tension was at the

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optimal plateau of the length-tension relationship for potassium chloride (KCl, 75 mM).

After 1 h of equilibration, arterial rings were contracted with noradrenaline (NA, 0.01 to 0.1 μM) or angiotensin II (AII, 0.003 to 0.01 μM). At the plateau of contraction, the responses to cumulative concentrations of des-Arg^9-BK (0.001 to 3 μM) or of BK (0.001 to 3 μM) were obtained in the presence or absence of [Leu^8]des-Arg^9-BK (3 μM) or D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]-BK (Hoe 140, 1 μM). After washing, the maximum contractile responses were obtained with KCl (75 mM) and the bath washed. The vessels were then re-contracted to a stable level of active force with NA (0.01 to 0.1 μM) to determine relaxation responses to cumulative addition of acetylcholine (ACh, 0.001 to 1 μM), the calcium ionophore, A 23187 (0.001 to 1 μM) or sodium nitroprusside (SNP, 0.001 to 1 μM). Responses to des-Arg^9-BK and to BK in the absence and presence of antagonists were also obtained in vessels maintained under resting conditions for 1 h. Indomethacin (3 μM) was present throughout all experiments.

Morphology of the intraluminal surface

Two different methods were used to assess the integrity of the endothelium. First, *en face* preparations of vessel segments were prepared according to the method of Gabaldon (1987). In brief, perfusion fixation of the carotid artery was performed *in situ* at 100 mmHg via a catheter inserted into the abdominal aorta. Perfusion was as follows: (1) fixative for 10 min (1% paraformaldehyde, 2% glutaraldehyde, 6 mM NaCl in 0.1 M phosphate buffer, pH 7.4); (2) washing solution for 1 min (8.9% sucrose in 20 mM HEPES buffer, pH 7.4); (3) 0.05% $AgNO_3$ in washing solution for 1 min; (4) washing solution for 1 min; (5) fixative for 2 min. The carotid artery was then dissected out, mounted flat on a glass slide with the endothelium facing upward and exposed to light for 4 h.

The second method used to determine the presence of endothelium was the global *en face* technique of Kohler & Jawien (1992). The carotid arteries were fixed at 100–120 mmHg perfusion pressure by infusion of 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Evans blue dye in phosphate buffer saline (60 mg kg^{-1}) was infused intravenously 15 min before fixation to allow identification of endothelium-denuded (blue) regions as distinct from regions in which the endothelium had regenerated (white).

Morphometric measurements

One week following surgery, rabbits were anaesthetized and killed with an overdose of sodium pentobarbitone. A catheter was placed in the thoracic aorta and the vascular system was first rinsed with heparinized saline (100 iu ml^{-1}) for 2 min then fixed for 20 min with Bouin's fluid (Rhône Poulenc, France) under a perfusion pressure of 100 mmHg. The right carotid artery was removed, cleaned of connective tissue, placed in Bouin's fluid for 2 days for further fixation, and embedded in paraffin. The artery segments were divided in four equal parts and a cross-sectional ring (4 mm) was cut from each part and stained with haemotoxylin and eosin (Ridray *et al.*, 1991).

Computer-assisted morphometry was performed with a Samba Image Analysis System (Alcatel, France) with 256 levels of grey and a 512 × 512 pixel grid. For each carotid cross-sectional ring (4/carotid artery), eight to fifteen microscopic fields, in order to cover the entire vessel circumference, were analysed in a blind manner with a 75 × final magnification on the video screen. A specific programme was developed (C. Robert, Lab. Fournier, France) to trace automatically the boundary limits between adventitia, media and intima. The observer hand using the computer's mouse was needed to separate structures only when the field image did not allow the programme to identify the different arterial structures. Intima and media areas (mm^2 after internal calibration of the pixel size) and intima/media area ratios were automatically calculated.

Drugs

A 23187, acetylcholine chloride, angiotensin II acetate, bradykinin acetate, des-Arg^9-bradykinin acetate, [Leu^8]des-Arg^9-bradykinin acetate, indomethacin, noradrenaline hydrochloride and sodium nitroprusside were obtained from Sigma

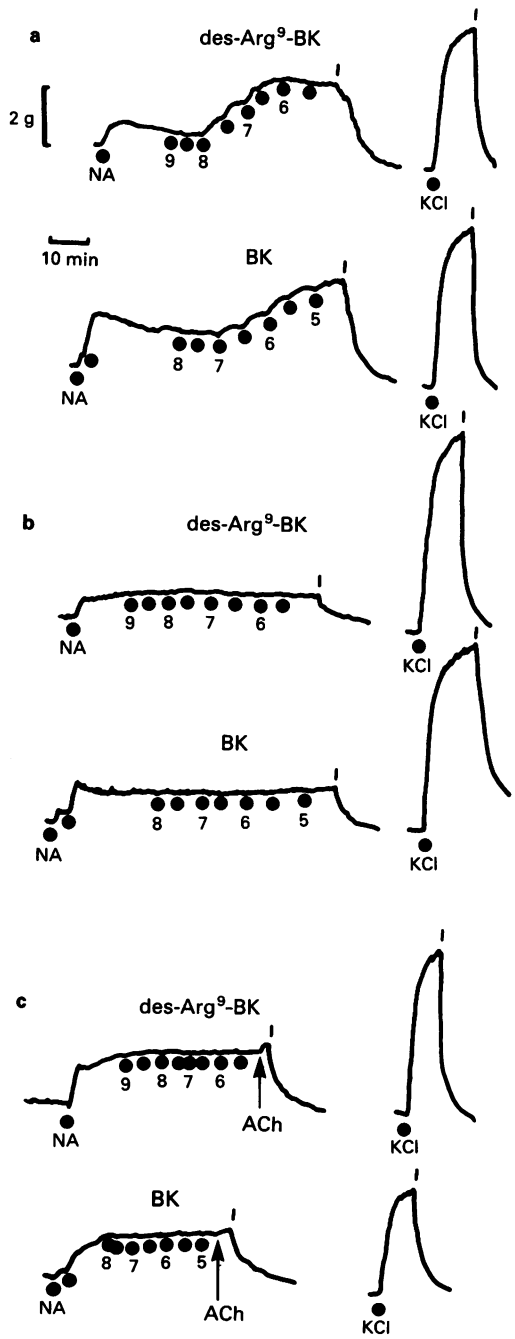


Figure 1 Representative recordings showing the effects of des-Arg^9-bradykinin (des-Arg^9-BK) and bradykinin (BK) on precontracted rings from balloon catheter-injured (a), control (b) and acutely denuded (c) carotid arteries. Precontraction was induced by noradrenaline (NA, 0.01 to 0.1 μM). Potassium chloride (KCl) was used at 75 mM. In acutely denuded arteries (c), 10 μM acetylcholine (ACh) produced a contraction (see arrow), confirming the absence of endothelium. Vertical black bars indicate washes (2 ×) with a fresh Krebs solution.

Chem. Co. (St Louis, MO, U.S.A.). D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]-bradykinin (Hoe 140) was a generous gift from Dr K. Wirth (Hoechst AG, Frankfurt, Germany). All drugs made up in distilled water or dimethylsulphoxide and kept on ice were protected from light.

Statistical analysis

Data are given as mean ± s.e.mean. Contraction responses are measured as a % of the maximum contraction obtained with KCl (75 mM). The negative logarithm of the concentration of agonist needed to reach 50% of its maximal response (pD_2) was calculated by using least square analysis (Tallarida & Murray, 1981). After testing for linearity and parallelism, pD_2 values and maximal responses were compared by Student's t test. The level of significance was $P<0.05$.

Results

Vascular reactivity

Stable precontractions of arteries were obtained with NA (0.01 to 0.1 μM) (Figure 1). The levels of precontraction before addition of des-Arg^9-BK were not significantly different between groups and represented 31 ± 7%, 35 ± 7% and 29 ± 8% of the response to KCl (75 mM) in vehicle-, [Leu^8]des-Arg^9-BK- and Hoe 140-treated arteries, respectively. Similarly, the contractile response to NA before addition of BK was not different and reached 36 ± 9%, 31 ± 9% and 26 ± 6% of KCl in vehicle-, [Leu^8]des-Arg^9-BK- and Hoe 140-treated arteries. Des-Arg^9-BK and BK caused further contractions only in the balloon-injured artery (Figures 1 and 2). This was not dependent on the precontracting agent (NA) since similar responses to des-Arg^9-BK and BK were obtained when the arteries were precontracted with AII (data not shown). Under similar conditions (NA precontraction), both agonists neither contracted nor relaxed acutely denuded and control arteries (Figure 1). The endothelium-dependent

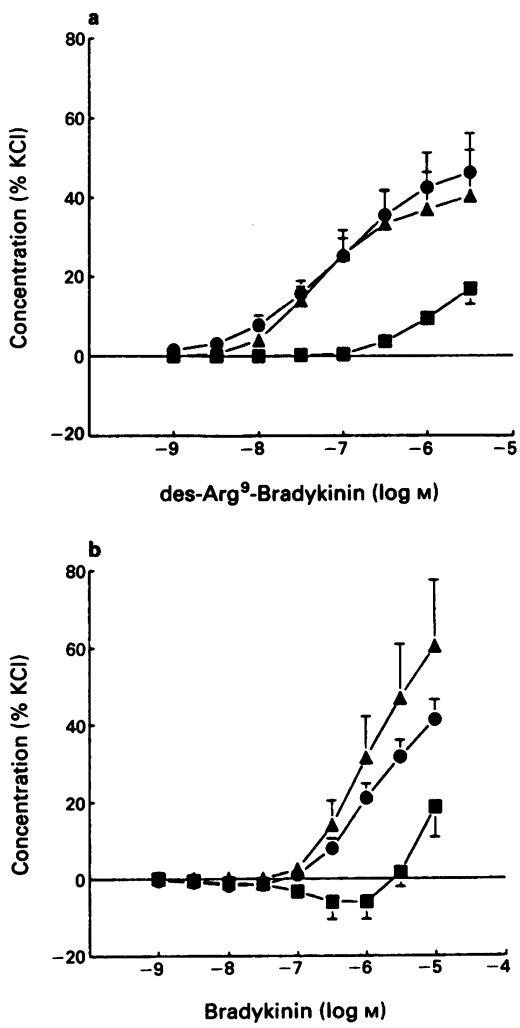


Figure 2 Concentration-contraction curves for des-Arg^9-bradykinin (a) and bradykinin (b) in balloon catheter-injured arteries precontracted with noradrenaline. Control responses (●) and responses in the presence of 3 μM [Leu^8]des-Arg^9-bradykinin (■) or 1 μM Hoe 140 (▲). Each point represents the mean ± s.e.mean of 6–9 animals. '0%' represents the level of contraction at the plateau of noradrenaline-induced response (see Results section for details).

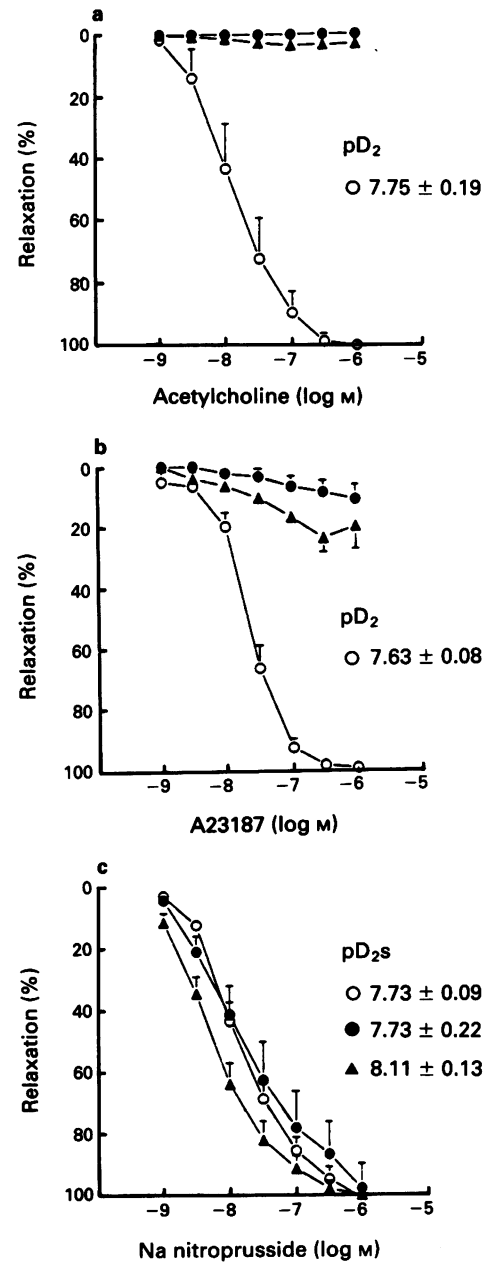


Figure 3 Cumulative concentration-relaxation curves for acetylcholine (a), A 23187 (b) and sodium nitroprusside (c) in balloon catheter-injured (▲), acutely denuded (●) and control (○) carotid arteries. Arterial rings were precontracted with noradrenaline (NA, 0.01 to 0.1 μM). Relaxation responses are expressed as percentages of contractions to NA. Each point represents the mean ± s.e.mean of 6 animals.

relaxing agent, ACh, was without effect in balloon-injured and acutely denuded arteries but relaxed maximally the control carotid artery (Figure 3). The calcium ionophore, A 23187, produced a small relaxation of balloon catheter-injured arteries (E_{max}, 19.7 ± 7.4%) and of acutely denuded arteries (E_{max}, 10.7 ± 4.8%) but fully relaxed control arteries (Figure 3). SNP relaxed maximally arterial rings from all three groups with similar pD_2 values (Figure 3). Figure 2 also shows the effects of [Leu^8]des-Arg^9-BK and Hoe 140 on concentration-contraction curves for des-Arg^9-BK and BK. In the absence of antagonist, the calculated pD_2 values for des-Arg^9-BK and BK were respectively 7.12 ± 0.36 and 5.98 ± 0.22 and the E_{max} values 46.0 ± 9.9% and 41.3 ± 5.2%. The B_1 receptor antagonist, [Leu^8]des-Arg^9-BK (3 μM) shifted to the right by 74 × and 14 ×, respectively, the concentration-contraction curves for des-Arg^9-BK and for BK (Figure 2). In contrast, Hoe 140 (1 μM) was without effect on the curves for des-Arg^9-BK (pD_2, 7.14 ± 0.48) and for BK (pD_2, 6.04 ± 0.38). Similar contractile responses to des-Arg^9-BK and to BK were obtained in arteries injured with a newly purchased balloon catheter (data not shown). The contraction to 75 mM KCl of balloon catheter-injured, acutely denuded and control arteries were 3.2 ± 0.3 g, 3.6 ± 0.3 g and 6.5 ± 0.2 g ($P<0.05$), respectively.

Balloon-catheter injured arteries under resting conditions contracted in response to des-Arg^9-BK and to BK with respective pD_2 values of 6.14 ± 0.12 and 5.73 ± 0.15 and E_{max} values of 37.2 ± 4.0% and 41.1 ± 5.4% of KCl ($n = 6$ in each group). Under these conditions, the responses to des-Arg^9-BK and to BK were unaffected by Hoe 140 (1 μM) but were shifted 7 and 10 × by [Leu^8]des-Arg^9-BK (3 μM).

Silver nitrate and Evan's blue staining

The luminal surface of sham-operated carotid arteries did not stain blue after administration of Evan's blue dye (Figure 4). In contrast, balloon catheter-injured arteries were uniformly stained blue indicating that, 7 days after surgery, most of the endothelium had not regenerated (Figure 4). However, patches of artery not stained blue were observed along the arterial surface indicating focal regeneration of endothelial cells around orifices of small artery branches (Figure 4). Endothelial cell regeneration also occurred at both ends of the injured arterial segment. Silver nitrate staining revealed intact endothelial junctions in control arteries whereas no staining was observed in injured vessels (Figure 4).

Morphometry

Morphological data are summarized in Table 1. Seven days after balloon catheter injury, significant increases in the cross-section area of both the intima and media were observed. The area of intima to media, expressed as a ratio, however was significantly increased 2.1 fold in injured arteries as compared with controls (sham-operated).

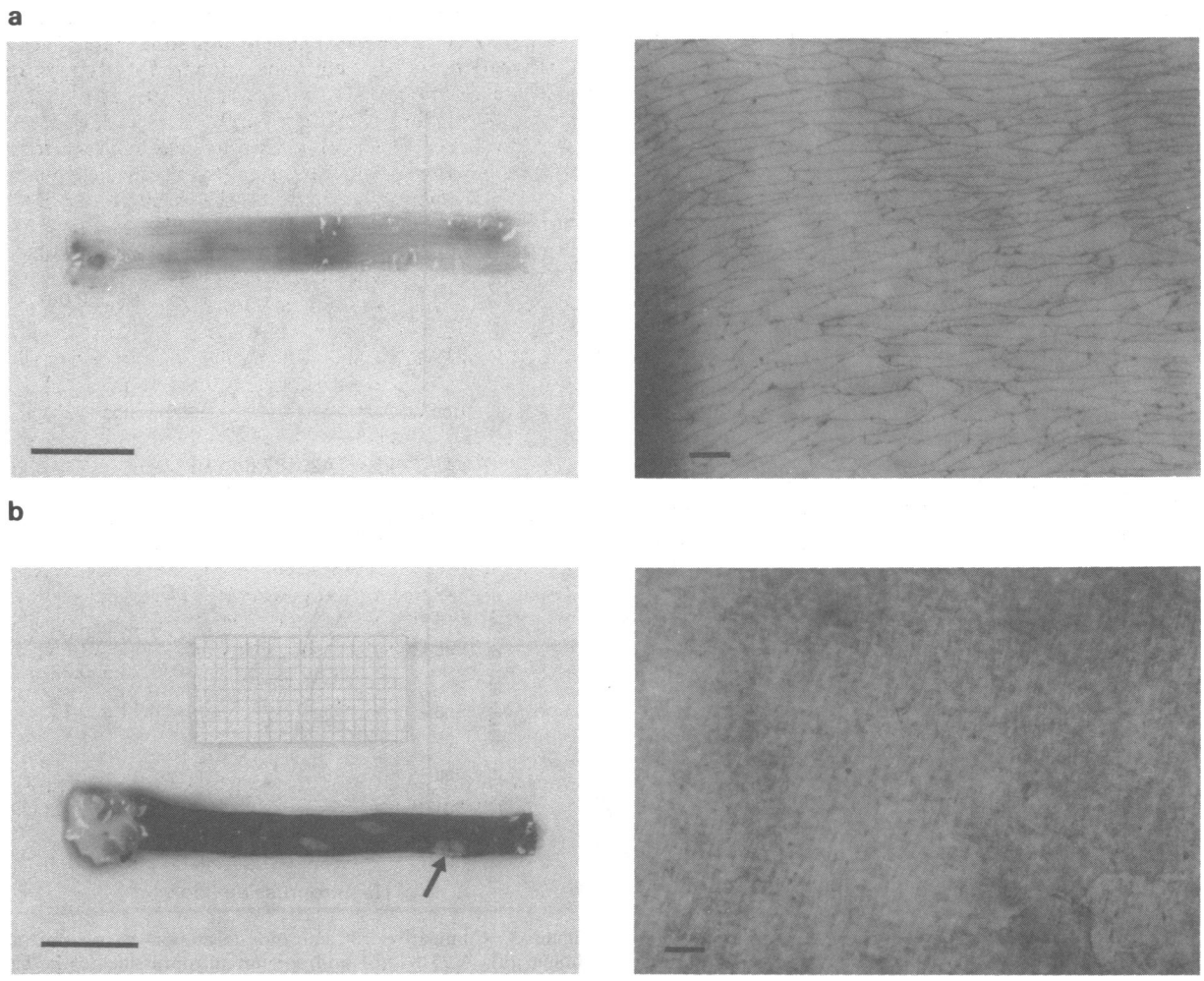


Figure 4 Intraluminal surface of a control (a) and a balloon catheter-injured (b) carotid artery stained with Evan's blue dye (left side) and silver nitrate (right side). See Methods for experimental details. Surfaces without endothelium which stained blue with Evan's blue dye appear black in the figure. The arrow indicates an area where the endothelium has regenerated. Note the absence of staining with silver nitrate in balloon catheter-injured arteries. Scale bar: 1 cm (left side) and 10 μm (right side).

Table 1 Morphological data obtained from vascular cross sections 7 days after angioplasty of the rabbit carotid artery

Group	*Intima area* (mm^2)	*Media area* (mm^2)	*Intima/media* (%)
Sham-operated (5)	0.030 ± 0.000	0.370 ± 0.007	8.2 ± 0.1
Balloon catheter-injured (9)	0.099 ± 0.018*	0.627 ± 0.032*	16.2 ± 4.2*

Results are given as mean ± s.e.mean. In parentheses are given the number of rabbits. See Methods section for details of histomorphometric techniques.
*Significantly different from sham-operated animals at $P<0.05$.

Discussion

Balloon catheter injury to the rabbit carotid artery produced a significant intimal thickening which was already apparent 7 days after surgery (see also Azuma *et al.*, 1992). At this time, the intima was predominantly devoid of endothelium although some endothelial regrowth was observed at both ends of the injured intraluminal surface and around the orifices of arterial branches. In these vessels that were chronically without endothelium, B_1 receptor-mediated contractions occurred, whereas, control vessels and vessels acutely without endothelium were unresponsive to B_1 receptor activation. Des-Arg^9-BK was about 10 times more potent than BK which is in agreement with the order of potency of both agonists towards the B_1 receptor subtype in other tissues (Regoli *et al.*, 1990). In addition, the contractions to des-Arg^9-BK and BK were inhibited by [Leu^8]des-Arg^9-BK but not by the specific B_2 antagonist, Hoe 140. Thus, the appearance of B_1 receptor-mediated contractions was related to the balloon catheter-induced injury. In balloon catheter-injured arteries maintained under resting conditions, contractions also developed in response to des-Arg^9-BK and to BK but with a reduced sensitivity. Although we have no adequate explanation for such a reduction in sensitivity, we suggest that it might be due to the absence of precontraction, a condition which has been shown in other pharmacological preparations to enhance the response to contractile agonists (see Cocks *et al.*, 1993). Similar to the observations made in precontracted arteries, the response to both kinin receptor agonists was probably the result of stimulation of B_1 receptors since it was antagonized by [Leu^8]des-Arg^9-BK but not by Hoe 140.

The absence of endothelium in the balloon catheter-injured artery was confirmed both by staining with Evan's blue dye and silver nitrate. Furthermore, absence of an endothelial layer was functionally confirmed by loss of endothelium-dependent relaxation responses to ACh. No staining to Evan's blue dye was observed around the orifices of small artery branches indicating the presence of regenerated endothelial cells which may have been responsible for the modest relaxation observed with BK and A 23187 but not with des-Arg^9-BK. However, no endothelium-dependent relaxation responses mediated by B_2 receptor activation were found in either intact (Pruneau & Bélichard, 1993) or sham-operated rabbit carotid arteries (this study). The reason why regenerated endothelial cells appear to respond to a B_2 receptor agonist is unknown.

Injury to the arterial wall, as that produced by angioplasty, is known to create a chronic inflammatory state (Berk *et al.*, 1991) which is reinforced by thrombus formation at the site of injury (Schwartz *et al.*, 1992). Expression of functional B_1 receptors has been demonstrated to occur in many inflammatory conditions (Marceau *et al.*, 1983). In most vascular experiments either *in vitro* or *in vivo*, B_1 receptor up-regulation was induced with LPS (Regoli *et al.*, 1981; Deblois & Marceau, 1987; Nwator & Whalley, 1989). Thus, our results could be explained by contamination of the balloon catheter with LPS. Although we did not measure LPS levels, this possibility was indirectly ruled out since des-Arg^9-BK induced similar contractions in arteries previously injured with newly purchased balloon catheters which were assumed to be LPS-free. In addition, responses to kinins were studied 90 min following the dissection of the arteries to avoid induction of B_1 receptors in response to prolonged incubation *in vitro* (Deblois & Marceau, 1987; Pruneau & Bélichard, 1993).

The physiological significance of B_1 receptor induction after intraluminal balloon injury is unknown. Des-Arg^9-BK is however likely to be produced *in situ* from BK. Circulating carboxypeptidase N, membrane anchored carboxypeptidase M or the lysosomal enzyme deamidase are able to remove terminal Arg from BK (Skidgel, 1992; Campbell *et al.*, 1993). In addition, BK is generated during inflammatory reactions by the action of plasma kallikrein (for review, see Bhoola *et al.*, 1992). Sufficient amounts of des-Arg^9-BK or BK may then be locally produced to activate B_1 receptors and to induce vasoconstriction after endothelium removal (Briner *et al.*, 1993). Interestingly, it has been reported that both BK and des-Arg^9-BK stimulated protein synthesis and cell division in human cultured fibroblasts (Goldstein & Wall, 1984) and that expression of a mutant *ras* protein in Rat 13 cells increased the expression of BK receptors and their sensitivity to ligand stimulation of mitogenesis (Roberts & Gullick, 1989). It raises the possibility that BK receptors may be involved in smooth muscle cell proliferation which occurs in the arterial wall following endothelium denudation.

In conclusion, we have provided evidence to support the view that induction of B_1 receptors occurs that mediate contraction at the site of vascular injury. Because BK is an autocrine hormone in many vascular tissues throughout the body, BK and its metabolite, des-Arg^9-BK, may become potent vasoconstrictors at the site of vascular injury following angioplasty.

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References

AZUMA, H., NIIMI, Y. & HAMASAKI, H. (1992). Prevention of intimal thickening after endothelial removal by a nonpeptide angiotensin II receptor antagonist, losartan. *Br. J. Pharmacol.*, **106**, 665–671.

BERK, B.C., GORDON, J.B. & ALEXANDER, J.W. (1991). Pharmacologic roles of heparin and glucocorticoids to prevent restenosis after coronary angioplasty. *J. Am. Coll. Cardiol.*, **17**, 111B–117B.

BHOOLA, K.D., FIGUEROA, C.D. & WORTHY, K. (1992). Bioregulation of kinins: kallikreins, kininogens, and kininases. *Pharmacol. Rev.*, **44**, 1–80.

BONNER, G. & SCHUNK, U. (1986). Hypotensive effect of bradykinin in normotensive and patients with renovascular hypertension. *Adv. Exp. Med. Biol.*, **198**, 321–327.

BRINER, V.A., TSAI, P. & SCHRIER, R.W. (1993). Bradykinin: potential for vascular constriction in the presence of endothelial injury. *Am. J. Physiol.*, **264**, F322–F327.

CAMPBELL, D.J., KLADIS, A. & DUNCAN, A.-M. (1993). Bradykinin peptides in kidney, blood, and other tissues of the rat. *Hypertension*, **21**, 155–165.

COCKS, T.M., KEMP, B.K., PRUNEAU, D. & ANGUS, J.A. (1993). Comparison of contractile response to 5-hydroxytryptamine and sumatriptan in human isolated coronary artery: synergy with the thromboxane A_2-receptor agonist, U46619. *Br. J. Pharmacol.*, **110**, 360–368.

DEBLOIS, D. & MARCEAU, F. (1987). The ability of des-Arg^9-bradykinin to relax rabbit isolated mesenteric arteries is acquired during in vitro incubation. *Eur. J. Pharmacol.*, **142**, 141–144.

GABALDON, M. (1987). Methodological approaches for the study of the aortic endothelium of the rat. *Atherosclerosis*, **65**, 139–149.

GOLDSTEIN, R.H. & WALL, M. (1984). Activation of protein formation and cell division by bradykinin and des-Arg^9-bradykinin. *J. Biol. Chem.*, **259**, 9263–9268.

KOHLER, T.R. & JAWIEN, A. (1992). Flow affects development of intimal hyperplasia after arterial injury in rats. *Arterioscler. Thromb.*, **12**, 963–971.

LIU, M.W., ROUBIN, G.S. & KING, S.B. (1989). Restenosis after coronary angioplasty. Potential biologic determinants and role of intimal hyperplasia. *Circulation*, **79**, 1374–1387.

MARCEAU, F., LUSSIER, A., REGOLI, D. & GIROUD, J.P. (1983). Pharmacology of kinins: their relevance to tissue injury and inflammation. *Gen. Pharmacol.*, **14**, 209–229.

NWATOR, I.A.A. & WHALLEY, E.T. (1989). Angiotensin converting enzyme inhibitors and expression of des-Arg^9-BK (kinin B_1) receptors in vivo. *Eur. J. Pharmacol.*, **160**, 125–132.

PELC, L.R., GROSS, G.J. & WARLTIER, D.C. (1991). Mechanism of coronary vasodilation produced by bradykinin. *Circulation*, **83**, 2048–2056.

PRUNEAU, D. & BÉLICHARD, P. (1993). Induction of bradykinin B_1 receptor-mediated relaxation in the isolated rabbit carotid artery. *Eur. J. Pharmacol.*, **239**, 63–67.

REGOLI, D. & BARABE, J. (1980). Pharmacology of bradykinin and related kinins. *Pharmacol. Rev.*, **32**, 1–46.

REGOLI, D., MARCEAU, F. & LAVIGNE, J. (1981). Induction of B_1-receptors for kinins in the rabbit by a bacterial lipopolysaccharide. *Eur. J. Pharmacol.*, **71**, 105–115.

REGOLI, D., RHALEB, N.-E., DRAPEAU, G. & DION, S. (1990). Kinin receptor subtypes. *J. Cardiovasc. Pharmacol.*, **15** (Suppl. 6), S30–S38.

RIDRAY, S., CAPRON, L., HEUDES, D., PICON, L. & KTORZA, A. (1991). Effects of fasting and refeeding on the proliferative response of rat aorta to injury. *Am. J. Physiol.*, **261**, H190–H196.

ROBERTS, R.A. & GULLICK, W.J. (1989). Bradykinin receptor number and sensitivity to ligand stimulation of mitogenesis is increased by expression of a mutant *ras* oncogene. *J. Cell Sci.*, **94**, 527–535.

SCHWARTZ, R.S., HOLMES, D. & TOPOL, E.J. (1992). The restenosis paradigm revisited: an alternative proposal for cellular mechanisms. *J. Am. Coll. Cardiol.*, **20**, 1284–1293.

SKIDGEL, R.A. (1992). Bradykinin-degrading enzymes: structure, function, distribution, and potential roles in cardiovascular pharmacology. *J. Cardiovasc. Pharmacol.*, **20** (Suppl. 9), S4–S9.

TALLARIDA, R.J. & MURRAY, R.B. (1981). Computational procedures. In *Manual of Pharmacologic Calculations with Computer Programs*. p. 3. ed. Tallarida, R.J. & Murray, R.B. New York: Springer Verlag.

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Reduced endothelium-dependent relaxation at enhanced NO release in hearts of hypercholesterolaemic rabbits

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1 Langendorff hearts, perfused at constant volume, were prepared from rabbits fed a cholesterol-enriched diet for 4 months. Coronary perfusion pressure and nitric oxide (NO) release (oxyhaemoglobin technique) into the coronary effluent were measured continuously. Prostacyclin (PGI_2) in the effluents was determined by radioimmunoassay (6-oxo-$PGF_{1\alpha}$).

2 Basal NO release was not different between control and hypercholesterolaemic rabbits. However, the coronary vasculature of hypercholesterolaemic rabbits showed a considerably (>50%) reduced endothelium-dependent relaxation in response to short-term (3 min) infusion of bradykinin (50 nM) and substance P (50 nM) ($P<0.05$, $n=8-9$). Under these conditions, NO release into the vessel lumen was increased, by 26%, in hypercholesterolaemic hearts ($P<0.05$, $n=8-9$). N^G-nitro-L-arginine (L-NOARG, 30 μM) significantly attenuated both bradykinin-induced NO formation and vessel relaxation in control hearts but only NO release in hypercholesterolaemia. L-Arginine (200 μM) restored the response to that before L-NOARG but did not improve the reduced endothelium-dependent relaxation in cholesterol-fed rabbits.

3 Superoxide dismutase (10 u ml^{-1}) significantly improved vessel relaxation without changing the hypercholesterolaemia-related coronary dysfunction. Vasodilatation in response to exogenous NO donors (linsidomine) was diminished in hypercholesterolaemia as compared to controls.

4 Basal PGI_2 release was unchanged in hypercholesterolaemic hearts. There was a tendency in these hearts for greater PGI_2 formation after stimulation by substance P and bradykinin ($P \geq 0.05$). The coronary relaxation to iloprost was unchanged.

5 The data demonstrate impaired endothelium-dependent relaxation of coronary arterial resistance vessels in hypercholesterolaemia. This diminished vascular response was not due to reduced NO generation but probably a reduced action of released NO, either by accelerated degradation and/or disturbed signal transduction pathways to vascular smooth muscle cells. There was no significant change in PGI_2 related pathways of vasomotor control in hypercholesterolaemia.

Keywords: EDRF; nitric oxide; hypercholesterolaemia; L-arginine; superoxide dismutase (SOD); prostacyclin (PGI_2)

Introduction

Local control of vessel tone is mainly exerted via generation and release of chemical mediators. Two different types of endothelium-derived vasodilators have received particular attention: nitric oxide (NO) and prostacyclin (PGI_2). Stimulation of endothelial function by hormones or mechanical stress results in increased generation of these compounds and subsequent vessel relaxation.

This physiological interaction between vascular endothelium and smooth muscle may be disturbed under conditions of endothelial dysfunction. For example, hypercholesterolaemia results in diminished endothelium-dependent relaxation. This observation, originally reported in man by Ludmer *et al.* (1986) suggested reduced formation of endothelium-derived relaxing factor (EDRF) and was confirmed in several later studies (Verbeuren *et al.*, 1986; Osborne *et al.*, 1989; Kappagoda *et al.*, 1990; Sellke *et al.*, 1990; Tagawa *et al.*, 1991). However, many of these studies were bioassay-type experiments where release of EDRF, i.e. NO, was measured in terms of vessel relaxation. Any reduced vessel relaxation could also have been caused by a more rapid degradation of the unstable mediator and/or reduced action on the vascular smooth muscle cells. Thus, the question whether reduced endothelium-dependent relaxation in hypercholesterolaemia is due to reduced mediator formation or reduced action remains unanswered.

A recent investigation by Minor *et al.* (1990), describing enhanced generation of nitrogen oxides in aortic tissue of hypercholesterolaemic rabbits gives a first hint that vascular NO formation might not always be reduced in hypercholesterolaemia.

These observations resemble earlier findings on prostacyclin when the original studies described reduced PGI_2 formation in vessels of hypercholesterolaemic rabbits (Gryglewski *et al.*, 1978) while later work demonstrated enhanced vascular PGI_2 production in both animal (Beetens *et al.*, 1986) and human studies (FitzGerald *et al.*, 1984). Thus, the question arises of whether the formation of both mediators is under control of common denominators, such as hypercholesterolaemia-related cytokine production that may up-regulate enzyme proteins at the message level (Pommerantz *et al.*, 1993).

The present study was designed to measure directly formation and action of NO and PGI_2 in the coronary microcirculation of the hypercholesterolaemic rabbit. Specifically, the following questions were studied: (i) Does hypercholesterolaemia impair endothelium-dependent NO and PGI_2 formation in the plaque-free coronary microcirculation as opposed to the conventional studies on plaque-containing aortic tissue, (ii) is such impairment correlated to changes in coronary vessel tone, i.e. coronary vascular resistance, and (iii) does any impairment of vessel relaxation involve an altered vessel reactivity towards exogenous mimetics, such as linsidomine (SIN-1) or iloprost.

Methods

Hypercholesterolaemia

New Zealand White rabbits (1800–2000 g initial weight; $n=8$) were fed for 12 weeks a standard laboratory diet,

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supplemented with 0.5% cholesterol and 3% olive oil. This was followed by 1% cholesterol and 3% olive oil for further 4 weeks. Age matched control animals ($n = 9$) were fed a cholesterol-free standard diet for 16 weeks. A blood lipid analysis was performed at the end of the feeding period in all animals. Some hearts were also investigated microscopically for plaque development in the coronary circulation. No overt atherosclerotic plaque formation was detected in the coronary circulation (not shown).

Heart preparation

At the end of the feeding period, the animals were killed by a blow to the head. The hearts were rapidly excised and perfused with Krebs-Henseleit-buffer (pH 7.4; 37°C) at a constant volume of 40 ml min^{-1} according to the Langendorff-technique. The buffer was supplemented with oxyhaemoglobin (3 μM) and equilibrated with 95% O_2 + 5% CO_2 by the use of a membrane oxygenator (Eschweiler, Kiel, Germany). The hearts were beating spontaneously.

Measurements

Actively developed left ventricular systolic pressure (LVP, balloon technique) and coronary perfusion pressure (CPP) were monitored continuously by Statham transducers and recorded on a multichannel recorder.

NO generation was measured by the oxyhaemoglobin technique as previously described (Schrör *et al.*, 1991; Woditsch & Schrör, 1992). Briefly, the coronary effluent passed a flow-through cuvette which was inserted in a dual-wavelength spectrophotometer (Aminco DW-2000, Colora, Lorch, Germany). NO release into the vessel lumen was calculated from the photometric signal by determination of the area under the curve (AUC) of the amount above basal levels induced by vasodilator infusion. The detection limit of the method was 0.3 nM.

Prostacyclin (PGI_2) was measured in aliquots of the coronary effluent by radioimmunoassay of the stable hydrolysis product 6-oxo-$PGF_{1\alpha}$. The detection limit was 10 pg ml^{-1}. PGI_2 formation was also calculated as total amount released from the area under the curve (AUC).

Vasodilator infusion

All vasodilators were administered as short-term (3 min) infusion directly into the coronary inflow tract by a perfusion pump (Braun Melsungen, Germany). The endothelium-dependent vasodilators, carbamoylcholine (0.1 μM), bradykinin (0.05 μM) and substance P (0.05 μM) were also used. These concentrations were previously found to be submaximal and equieffective with respect to release of NO and coronary vasodilatation (Woditsch & Schrör, 1992). As an endothelium-independent PGI_2-mimetic, iloprost (0.1 μM) was used, and as an NO-mimetic, linsidomine (SIN-1, 5 μM). SIN-1 has previously been shown in this model to release NO spontaneously in the perfusion buffer at vasoactive concentrations (Schrör *et al.*, 1991).

The NO synthase inhibitor N^G-nitro-L-arginine (L-NOARG, 30 μM), the NO precursor L-arginine (L-Arg, 200 μM) and superoxide dismutase (SOD, 10 u × ml^{-1}) were added to the perfusion buffer during the experiment (see Experimental protocol).

Substances and solutions

Bovine haemoglobin, N^G-nitro-L-arginine (L-NOARG), bradykinin, substance P, carbamoylcholine and superoxide dismutase (bovine) were purchased from Sigma (Deisenhofen, Germany). Iloprost (Schering AG, Berlin/Bergkamen, Germany) and linsidomine (SIN-1, Cassella-Riedel, Frankfurt/M., Germany) were kindly provided by the respective manufacturers. The other chemicals were obtained from Merck (Darmstadt, Germany).

L-NOARG was dissolved in hydrochloric acid (0.1 mM) and further diluted in Krebs-Henseleit-buffer. Fresh solutions of SIN-1 were prepared 15 min prior to use. Stock solutions of substance P were prepared in acetic acid (10 mM) and further diluted in Krebs-Henseleit-buffer. The other compounds were dissolved in Krebs-Henseleit-buffer.

Experimental protocol

Figure 1 demonstrates the experimental protocol. Experiments were started after a 30 min equilibration period, when stable conditions of CPP, heart rate and LVP were obtained (min 0). In contrast to the other vasodilators, the relaxations induced by carbamoylcholine and SIN-1 were inhibited by oxyhaemoglobin. These vasodilators were tested before the perfusion buffer was supplemented with oxyhaemoglobin, and NO formation by carbamoylcholine and SIN-1 could not be measured. Then (min 20), oxyhaemoglobin (3 μM) was added to the buffer and bradykinin, substance P, and iloprost were infused for 3 min each. At the 70th min of the experiment, L-NOARG was added to the perfusion buffer, followed by another infusion of bradykinin. The perfusion was then changed to Krebs-Henseleit-buffer containing L-arginine (min 115). After a short equilibration period, bradykinin was again infused for 3 min and the perfusion was continued until min 160. Additionally, in some experiments at the 60th min bradykinin was infused in the presence of SOD (10 u ml^{-1}).

Before, during and after each vasodilator infusion (0, 1, 2, 3, 5 and 10 min after start of infusion) aliquots of the effluent were taken for determination of 6-oxo-$PGF_{1\alpha}$. NO formation was measured continuously as described above.

Statistics

The data are mean ± s.e.mean of n determinations. Statistical analysis was performed by the two-tailed t test. P levels of <0.05 were considered significant.

Results

Plasma-cholesterol

Total plasma cholesterol levels were enhanced after cholesterol feeding, from 0.7 ± 0.1 mM (27 ± 4 mg dl^{-1}) in the control group to 28.5 ± 2.0 mM (1102 ± 77 mg dl^{-1}) ($P<0.01$).

Cardiac and coronary function

Left ventricular systolic pressure (LVP) and heart rate were not significantly different between cholesterol-fed and control animals at any time of the experiment. For example, LVP was 63 ± 4 mmHg in the controls and 69 ± 5 mmHg in cholesterol-fed animals ($P>0.05$, $n = 8-9$). The respective data for heart rate were 185 ± 5 beats min^{-1} vs. 180 ± 7 beats min^{-1} ($P>0.05$, $n = 8-9$). The initial (time 0) coronary perfusion pressure (CPP) was also not different, amounting to 46 ± 3 mmHg in controls and 43 ± 3 mmHg in the cholesterol-fed group ($P>0.05$, $n = 8-9$).

Figure 1 demonstrates alterations of the CPP during the acute experiment. Inhibition of luminally released NO by oxyhaemoglobin slightly enhanced basal levels of CPP. Inhibition of NO synthase by L-NOARG resulted in a significant increase of CPP. This increase was not reversed by subsequent addition of L-Arg. Apparently identical changes were obtained in hearts of hypercholesterolaemic rabbits and control animals. Because of this significant increase in CPP by L-NOARG, all relaxations induced by short-term infusion of vasodilators are expressed as % decrease of CPP from the

actual baseline to allow direct comparisons of the different treatments.

Vasodilator infusion

Figure 2 exemplifies the effect of vasodilator infusion by two original tracings, of a control and a hypercholesterolaemic heart, respectively. All vasodilators caused a significant decrease in CPP that returned rapidly to baseline level after the infusion was stopped. This vasodilatation in response to endothelium-dependent stimuli, i.e. carbamoylcholine, bradykinin, and substance P was markedly (by $>50\%$) reduced in hearts of hypercholesterolaemic rabbits. There was also a significantly reduced relaxation in response to SIN-1 (5 μM) in hypercholesterolaemia ($P<0.05$, $n=8-9$), while the vasodilatation induced by iloprost remained unchanged. The data of all experiments are summarized in Figure 3.

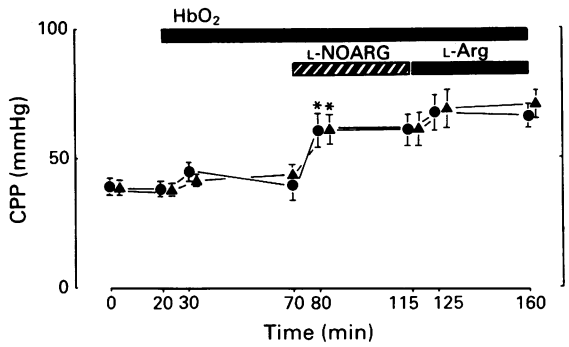


Figure 1 Changes in the coronary perfusion pressure (CPP) (mmHg) in Langendorff hearts of control rabbits (●) and hearts of cholesterol-fed animals (▲) during the standard experimental protocol. Initial buffer perfusion for 20 min is followed by perfusion with oxyhaemoglobin (3 μM). Additional perfusion with N^G-nitro-L-arginine (L-NOARG, 30 μM) and L-arginine (L-Arg, 200 μM) is started at time 70 and 115 min, respectively. The data are mean ± s.e.mean of $n=8-9$ experiments. There were no significant differences ($P>0.05$) between control and cholesterol treatment at the same time points. *$P<0.05$ by comparing 80 min (L-NOARG) with the time before (70 min).

Generation of nitric oxide (NO)

Basal release of NO into the coronary perfusate was not different between the two groups of hearts: 1.57 ± 0.21 nM min^{-1} in controls and 1.40 ± 0.23 nM min^{-1} in hearts of cholesterol-fed animals ($P>0.05$, $n=8-9$).

Infusion of bradykinin and substance P into the coronary inflow tract of control hearts stimulated NO release by 2.62 ± 0.17 and 2.22 ± 0.21 nM, respectively. In hearts of hypercholesterolaemic rabbits, this stimulated NO release was significantly higher, amounting to 3.30 ± 0.18 nM with bradykinin and 2.80 ± 0.16 nM with substance P. This corresponded to a 26% increase of NO release in hypercholesterolaemic hearts in both cases ($P<0.05$, $n=8-9$) (Figure 4).

Generation of PGI_2

Basal release of 6-oxo-$PGF_{1\alpha}$ was not different between the two groups of hearts. For example, at 20 min, basal release of 6-oxo-$PGF_{1\alpha}$ was 20 ± 5 pM min^{-1} in hearts of control rabbits and 21 ± 4 pM min^{-1} in hearts of cholesterol-fed animals ($P>0.05$, $n=8-9$).

Both bradykinin and substance P stimulated 6-oxo-$PGF_{1\alpha}$ release into the coronary effluent of control hearts by 0.44 ± 0.09 and 1.24 ± 0.20 nM, respectively. In hearts of hypercholesterolaemic rabbits, this release was slightly enhanced, amounting to 0.99 ± 0.33 nM for bradykinin and 1.47 ± 0.24 nM for substance P ($P\geqslant 0.05$, $n=8-9$). These data are summarized in Figure 4. This stimulated 6-oxo-$PGF_{1\alpha}$ generation was not significantly altered by subsequent treatment with L-NOARG or L-Arg (Table 1).

Effects of N^G-nitro-L-arginine (L-NOARG)

Inhibition of endogenous NO formation by L-NOARG was associated with a considerable increase in basal CPP, by

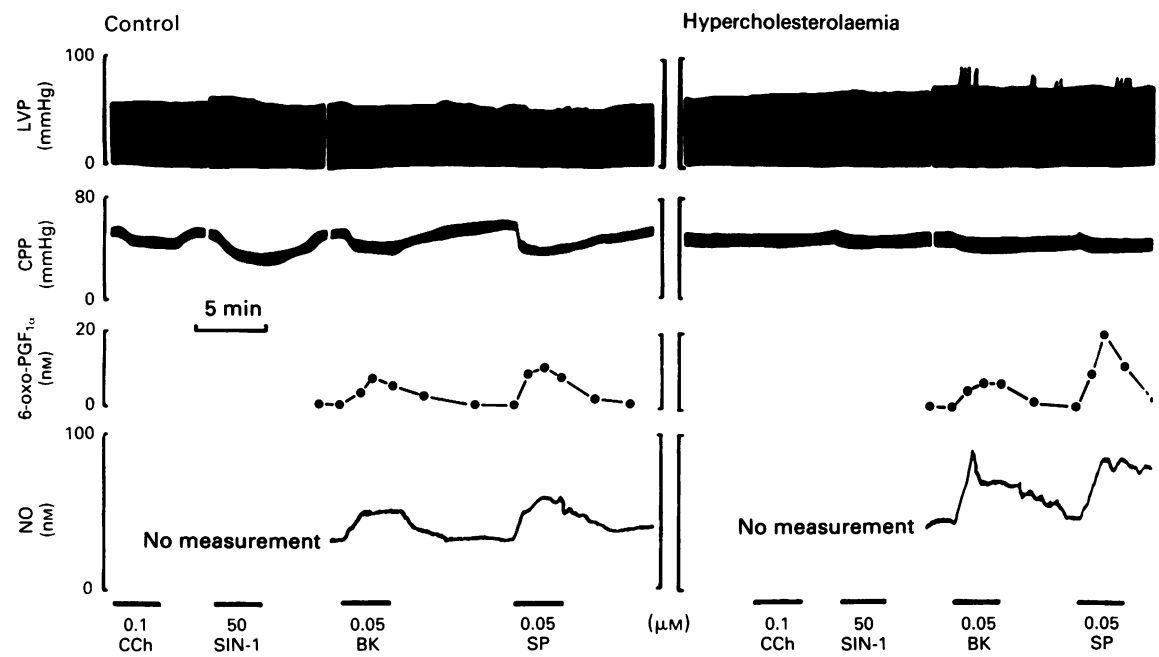


Figure 2 Original tracing of left ventricular systolic pressure (LVP), coronary perfusion pressure (CPP) and release of nitric oxide (NO) and prostacyclin (6-oxo-$PGF_{1\alpha}$) into the coronary effluent of a normocholesterolaemic control rabbit and a hypercholesterolaemic rabbit. The black lines below mark the infusion of carbamoylcholine (CCh, 0.1 μM), linsidomine (SIN-1, 50 μM), bradykinin (BK, 50 nM) or substance P (SP, 50 nM) into the coronary inflow tract.

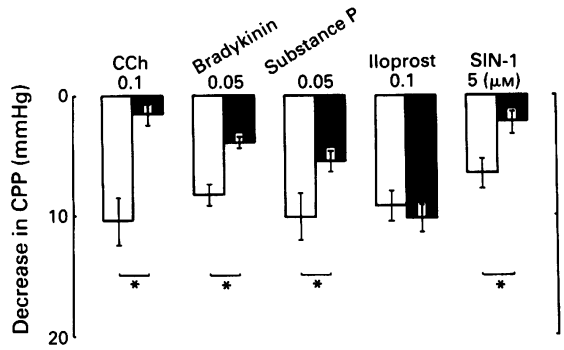


Figure 3 Decrease in coronary perfusion pressure (CPP) (mmHg) by carbamoylcholine (CCh, 0.1 μM), bradykinin (50 nM), substance P (50 nM), iloprost (0.1 μM) and linsidomine (SIN-1, 5 μM) in Langendorff hearts of control (□) and hypercholesterolaemic rabbits (■). The data are mean ± s.e.mean of $n = 8-9$ experiments. *$P < 0.05$ (cholesterol vs. control).

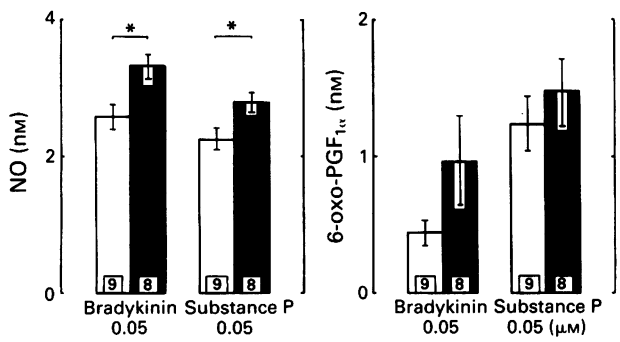


Figure 4 Release of NO (nM) and 6-oxo-$PGF_{1\alpha}$ (nM) by bradykinin (50 nM) and substance P (50 nM) into the coronary effluent of Langendorff hearts of control (□) and hypercholesterolaemic rabbits (■). The data are mean ± s.e.mean of the number of experiments indicated in the columns. *$P < 0.05$ (cholesterol vs. control).

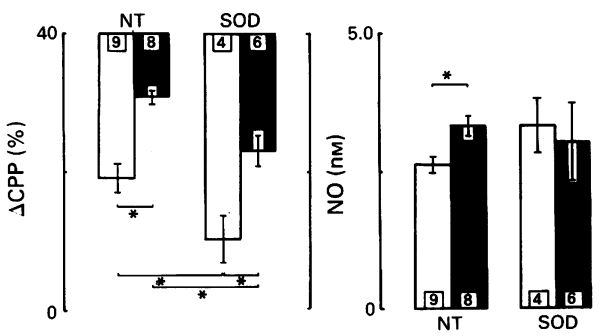


Figure 5 Bradykinin-induced decrease in coronary perfusion pressure (CPP) (%) and release of NO (nM) in isolated hearts of control (□) and hypercholesterolaemic rabbits (■). Hearts were untreated (NT) or treated with superoxide dismutase (SOD, 10 u ml^{-1}). The data are mean ± s.e.mean of the number of experiments indicated in the columns. *$P < 0.05$ as indicated.

21 ± 6 mmHg in hearts of controls and by 18 ± 3 mmHg in hearts of hypercholesterolaemic rabbits. These differences were not significant ($P > 0.05$, $n = 8-9$).

L-NOARG at the same concentration markedly reduced bradykinin-induced NO formation in both groups to 0.7 ± 0.1 and 0.5 ± 0.1 nM, respectively. This was equivalent to an inhibition of stimulated NO release by 75–80% of control ($P < 0.05$). L-NOARG also reduced bradykinin-induced vessel relaxation in control hearts. In these preparations, bradykinin diminished the CPP by 21.0 ± 2.0% in the absence but only 13.0 ± 2.4% in the presence of L-NOARG ($P < 0.05$). In contrast, no inhibition of vessel relaxation by L-NOARG was obtained in hypercholesterolaemic hearts: the bradykinin-induced decrease in CPP was 9.3 ± 1.0% in the absence and 11.6 ± 1.5% in the presence of L-NOARG ($P > 0.05$, $n = 9$).

Table 1 Effect of N^G-nitro-L-arginine (L-NOARG, 30 μM), L-arginine (L-Arg, 200 μM) and superoxide dismutase (SOD, 10 u ml^{-1}) on bradykinin (50 nM)-stimulated 6-oxo-$PGF_{1\alpha}$ release (nM) in Langendorff hearts of control (Con) and cholesterol-fed rabbits (Chol)

Group	*No treatment*	*+ L-NOARG*	*+ L-Arg*	*+ SOD*
Con	0.44 ± 0.09	0.35 ± 0.08	0.30 ± 0.05	0.42 ± 0.11
Chol	0.99 ± 0.33	0.41 ± 0.11	0.36 ± 0.07	0.51 ± 0.15

The data are mean ± s.e.mean of $n = 4-9$ experiments; no significant differences between cholesterol and control groups ($P \geq 0.05$).

Effects of L-arginine (L-Arg)

Inhibition of bradykinin-induced NO formation by L-NOARG was partially restored by subsequent infusion of L-Arg (200 μM) (cf. Figure 1). NO release was 1.65 ± 0.13 nM in controls and 1.98 ± 0.21 nM in hearts of hypercholesterolaemic animals. In controls, this was associated with a complete normalization of bradykinin-induced vasodilatation. No such effect was seen with L-Arg in the cholesterol group. There was still a significant reduction in endothelium-dependent relaxation ($P < 0.05$, $n = 8-9$). L-Arg did not alter the basal CPP (Figure 1).

Effects of superoxide dismutase (SOD)

In separate experiments, SOD (10 u ml^{-1}) was infused and, after a period of equilibration, bradykinin was administered as described above. No direct action of SOD on basal CPP, LVP or heart rate was observed at this concentration. In the presence of SOD, bradykinin-induced vasodilatation was significantly enhanced in both groups of hearts by comparable amounts. Thus, there was no specific effect of SOD on the CPP of normo- or hypercholesterolaemic hearts, respectively. Bradykinin-induced NO release and 6-oxo-$PGF_{1\alpha}$ formation were also not significantly altered by SOD treatment ($P > 0.05$, $n = 4-9$) (Table 1, Figure 5).

Discussion

This study demonstrates impaired endothelium-dependent relaxation in the coronary microcirculation of the rabbit at early stages of atherosclerosis, i.e. when overt plaque formation has not yet occurred. This confirms earlier data in coronary arteries and microvessels of cholesterol-fed rabbits (Osborne *et al.*, 1989), pigs (Cohen *et al.*, 1988) and monkeys (Sellke *et al.*, 1990) as well as several *in vivo* and *in vitro* studies on atherosclerotic human coronary arteries (Ludmer *et al.*, 1986; Bossaller *et al.*, 1987; Förstermann *et al.*, 1988). There was a reduced coronary vasodilatation against a variety of different endothelium-dependent relaxants, such as muscarinic agonists, bradykinin and substance P, suggesting that mechanisms distal to the receptor level are involved. Two possibilities exist: reduced receptor-dependent generation of vasodilator mediator(s) or reduced action of the mediator(s).

Reduced generation of endothelium-dependent vasodilators, such as NO or PGI_2, appears to be an attractive hypothesis

to explain reduced endothelium-dependent relaxation. However, in the present study, direct measurements of NO and PGI_2 clearly indicate that their formation is not reduced but rather enhanced in the coronary microcirculation. Beetens *et al.* (1986) described a biphasic change of vascular PGI_2 formation in cholesterol-fed rabbits. There was an enhanced 6-oxo-$PGF_{1\alpha}$ production by aortic segments in early stages of cholesterol feeding which was followed by a severely depressed formation at later times. Voss *et al.* (1983) reported that the amount of PGI_2 generation depends on the type of vascular tissue studied. Thus, it appears to be clear from the data from the present study that any reduced endothelium-dependent relaxation cannot be explained by reduced PGI_2 production. There is also no evidence for reduced sensitivity of the coronary vessels to PGI_2 as seen from the unchanged responses to iloprost.

Our results also demonstrate that there was unchanged basal NO release but enhanced stimulated NO release into the vessel lumen of hypercholesterolaemic hearts. This finding agrees with a previous report by Minor and colleagues (1990) who, using a chemiluminescence technique, found enhanced release of nitrogen oxides, i.e. mainly nitrite, from aortae of hypercholesterolaemic rabbits. The oxyhaemoglobin technique used in the present study is more selective for NO, and does not detect NO metabolites (Woditsch & Schrör, 1992). In addition, the NO signal was markedly suppressed by L-NOARG, confirming both the specificity of the assay and the endogenous NO biosynthesis from L-Arg.

There are several possible sources for enhanced NO formation in hypercholesterolaemia. First, hypercholesterolaemia *per se* may result in changes of the abundance of vascular NO synthases. Distinct isoforms may exist (Moncada, 1992) and may be regulated in different ways (Lamas *et al.*, 1992). Recently, induction of a non-endothelial NO synthase has been postulated from bioassay experiments in the hypercholesterolaemic rabbit aorta (Verbeuren *et al.*, 1993). Cell culture studies suggest an upregulation of cytokine-induced NO synthase in cholesterol-enriched vascular smooth muscle cells (Pommerantz *et al.*, 1993). These data as well as those from Kanno and colleagues (1993) would probably explain our findings of increased stimulated NO release in terms of cytokine-induced expression of new NO synthases in hypercholesterolaemia.

The question still remains why enhanced expression of the enzyme and enhanced NO liberation still results in impaired endothelium-dependent relaxation. The theoretical possibility of exhaustion of substrate supply can be excluded as in this study and in two earlier *in vitro* trials (Mügge & Harrison, 1991; Kerr *et al.*, 1992) no improvement of the impaired endothelium-dependent vessel relaxation was observed by addition of L-Arg.

Diminished endothelium-dependent relaxation may result from a more rapid inactivation of released NO. There is evidence for enhanced endothelium-derived superoxide anion production in hypercholesterolaemia (Ohara *et al.*, 1993) and improved recovery of EDRF-dependent relaxation by superoxide dismutase (SOD) in a previous (Mügge *et al.*, 1991) and in the present study, SOD also significantly enhanced the bradykinin-induced relaxation in both hypercholesterolaemic and control hearts but did not normalize the reduced endothelium-dependent relaxation of hypercholesterolaemic hearts. This suggests that inactivation of NO by superoxide radicals may not fully explain impaired endothelium-dependent relaxation in this model. Other mechanisms, such as inactivation of NO by low-density lipoproteins (Jacobs *et al.*, 1990) may be involved.

Finally, the possibility exists that NO is less active on vascular smooth muscle because of disturbed signal transduction within the vascular smooth muscle cell. If this were true, then relaxation by exogenous NO donors should also be disturbed. In fact, relaxations subsequent to SIN-1 were significantly reduced in coronary arteries from hypercholesterolaemic animals. SIN-1 has previously been shown *in vitro* to release NO spontaneously and almost exclusively into the vessel lumen (Schrör *et al.*, 1991). This effect was not changed by SOD. Thus, decreased relaxation by SIN-1 may rather suggest a diminished sensitivity of the cholesterol-loaded vascular smooth muscle cell to cyclic GMP-related relaxation. This hypothesis is in agreement with studies by others (Verbeuren *et al.*, 1990; Tagawa *et al.*, 1991) and suggests a reduced action of NO within the vessel lumen on vessel tone in hypercholesterolaemia.

In summary, this study demonstrates an enhanced vascular NO forming capacity despite of impaired endothelium-dependent relaxation in the coronary microcirculation of hypercholesterolaemic rabbits. The decreased vasodilator activity of NO might be due to a more rapid local inactivation by superoxide radicals or oxidized LDL in the subendothelial space. Additionally, cholesterol-loaded, cytokine-exposed vascular smooth muscle cells may become less sensitive to the relaxing action of NO. There is no altered formation or reduced action of prostacyclin.

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References

BEETENS, J.R., COENE, M.-C., VERHEYEN, A., ZONNEKEYN, L. & HERMAN, A.G. (1986). Biphasic response of intimal prostacyclin production during the development of atherosclerosis. *Prostaglandins*, **32**, 319–334.

BOSSALLER, C., HABIB, G.B., YAMAMOTO, H., WILLIAMS, C., WELLS, S. & HENRY, P.D. (1987). Impaired muscarinic endothelium-dependent relaxation and cyclic guanosine 5'-monophosphate formation in atherosclerotic human coronary artery and rabbit aorta. *J. Clin. Invest.*, **79**, 170–174.

COHEN, R.A., ZITNAY, K.M., HAUDENSCHILD, C.C. & CUNNINGHAM, L.D. (1988). Loss of selective endothelial cell vasoactive functions caused by hypercholesterolaemia in pig coronary arteries. *Circ. Res.*, **63**, 903–910.

FITZGERALD, G.A., SMITH, B., PEDERSEN, A.K. & BRASH, A.R. (1984). Increased prostacyclin biosynthesis in patients with severe atherosclerosis and platelet activation. *New Engl. J. Med.*, **310**, 1065–1068.

FÖRSTERMANN, U., MÜGGE, A., ALHEID, U., HAVERICH, A. & FRÖLICH, J.C. (1988). Selective attenuation of endothelium-mediated vasodilation in atherosclerotic human coronary arteries. *Circ. Res.*, **62**, 185–190.

GRYGLEWSKI, R.J., DEMBINSKA-KIEC, A., ZMUDA, A. & GRYGLEWSKA, T. (1978). Prostacyclin and thromboxane biosynthesis capacities of heart, arteries and platelets at various stages of experimental atherosclerosis in rabbits. *Atherosclerosis*, **31**, 385–394.

JACOBS, M., PLANE, F. & BRUCKDORFER, K.R. (1990). Native and oxidized low-density lipoproteins have different inhibitory effects on endothelium-derived relaxing factor in the rabbit aorta. *Br. J. Pharmacol.*, **100**, 21–26.

KANNO, K., HIRATA, Y., IMAI, T. & MARUMO, F. (1993). Induction of nitric oxide synthase gene by interleukin in vascular smooth muscle cells. *Hypertension*, **22**, 34–39.

KAPPAGODA, C.T., THOMSON, A.B.R. & SENARATNED, M.P.J. (1990). A model for demonstration of reversal of impairment of endothelium-dependent relaxation in the cholesterol-fed rabbit. *Can. J. Physiol. Pharmacol.*, **68**, 845–850.

KERR, P.M., BRUCKDORFER, K.R. & JACOBS, M. (1992). Inhibition of EDRF responses in isolated aorta by atherosclerosis or lysophosphatidylcholine are not reversed by L-arginine. *J. Vasc. Res.*, **2**, 149–150.

LAMAS, S., MARSDEN, P.A., LI, G.K., TEMPST, P. & MICHEL, T. (1992). Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 6348–6352.

LUDMER, P.L., SELWYN, A.P., SHOOK, T.L., WAYNE, R.R., MUDGE, G.H., ALEXANDER, R.W. & GANZ, P. (1986). Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *New Engl. J. Med.*, **315**, 1046–1051.

MINOR, R.L., MYERS, P.M., GUERRA, R., BATES, J.N. & HARRISON, D.G. (1990). Diet-induced atherosclerosis increases the release of nitrogen oxides from rabbit aortas. *J. Clin. Invest.*, **86**, 2109–2116.

MONCADA, S. (1992). The L-arginine:nitric oxide pathway. *Acta Physiol. Scand.*, **145**, 201–227.

MÜGGE, A., ELWELL, J.H., PETERSON, T.E., HOFMEYER, T.G., HEISTAD, D.D. & HARRISON, D.G. (1991). Chronic treatment with superoxide dismutase partially restores endothelium-dependent vascular relaxation in cholesterol-fed rabbits. *Circ. Res.*, **69**, 1293–1300.

MÜGGE, A. & HARRISON, D.G. (1991). L-arginine does not restore endothelial dysfunction in atherosclerotic rabbit aorta in vitro. *Blood Vessels*, **28**, 354–357.

OHARA, Y., PETERSON, T.E. & HARRISON, D.G. (1993). Hypercholesterolemia increases endothelial superoxide anion production. *J. Clin. Invest.*, **91**, 2546–2551.

OSBORNE, J.A., SIEGMAN, M.J., SEDAR, W.A., MOERS, S.U. & LEFER, A.M. (1989). Lack of endothelium-dependent relaxation in coronary arteries of cholesterol-fed rabbits. *Am. J. Physiol.*, **256**, C591–C597.

POMMERANTZ, K.B., HAJJAR, D.P., LEVI, L. & GROSS, S.S. (1993). Cholesterol enrichment of arterial smooth muscle cells upregulates cytokine-induced nitric oxide synthesis. *Biochem. Biophys. Res. Commun.*, **191**, 103–109.

SCHRÖR, K., FÖRSTER, S. & WODITSCH, I. (1991). On-line measurement of nitric oxide release from organic nitrates in the intact coronary circulation. *Naunyn-Schmied. Arch. Pharmacol.*, **344**, 240–246.

SCHRÖR, K., LATTA, G., SEIDEL, H., SMITH, E.F.III & PALMÉR, M. (1986). Thromboxane and prostacyclin generation in hearts, platelets and aorta of cholesterol fed rabbits in relation to platelet function. *Naunyn-Schmied. Arch. Pharmacol.*, **332** (Suppl.), R35.

SELLKE, F.W., ARMSTRONG, M.L. & HARRISON, D.G. (1990). Endothelium-dependent vascular relaxation is abnormal in the coronary microcirculation of atherosclerosis primates. *Circulation*, **81**, 1586–1593.

TAGAWA, H., TOMOIKE, H. & NAKAMURA, M. (1991). Putative mechanisms of the impairment of endothelium-dependent relaxtion of the aorta with atheromatous plaque in heritable hyperlipidemic rabbits. *Circ. Res.*, **68**, 330–337.

VERBEUREN, T.J., BONHOMME, E., LUBIE, M. & SIMONET, S. (1993). Evidence for induction of nonendothelial NO synthase in aortas of cholesterol-fed rabbits. *J. Cardiovasc. Pharmacol.*, **21**, 841–845.

VERBEUREN, T.J., JORDAENS, F.H., VAN HOVE, C.E., VAN HOYDONCK, A.-E. & HERMAN, A.G. (1990). Release and vascular activity of endothelium-derived relaxing factor in atherosclerotic rabbit aorta. *Eur. J. Pharmacol.*, **191**, 173–184.

VERBEUREN, T.J., JORDAENS, F.H., ZONNEKEYN, L.L., VAN HOVE, C.E., COENE, M.C. & HERMAN, A.G. (1986). Effect of hypercholesterolemia on vascular reactivity in the rabbit. *Circ. Res.*, **58**, 552–564.

VOSS, R., DON, J.A. & TEN HOOR, F. (1983). Prostacyclin formation by the rabbit aorta: relation to atherosclerosis. *Prostaglandin Leukot. Med.*, **11**, 451–456.

WODITSCH, I. & SCHRÖR, K. (1992). Prostacyclin rather than nitric oxide is a tissue protective factor in myocardial ischemia. *Am. J. Physiol.*, **263**, H1390–H1396.

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Stimulation of phospholipase C in cultured microvascular endothelial cells from human frontal lobe by histamine, endothelin and purinoceptor agonists

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1 Cultures of endothelial cells derived from the microvasculature of human frontal lobe have been investigated for phospholipase C (PLC) responses to histamine, endothelins and purinoceptor agonists.

2 Using cells prelabelled with [^{3}H]-inositol and measuring total [^{3}H]-inositol (poly)phosphates, histamine acting at H_1 receptors stimulated a substantial response with an EC_{50} of about 10 μM.

3 Endothelin-1 also gave a clear stimulation of phosphoinositide-specific phospholipase C. Both concentration-response curves and binding curves showed effective responses and binding in the rank order of endothelin-1>sarafotoxin S6b>endothelin-3, suggesting an ET_A receptor.

4 Assay of total [^{3}H]-inositol (poly)phosphates showed no response to the purinoceptor agonists, 2-methylthioadenosine 5'-trisphosphate (2MeSATP), adenosine 5'-O-(3-thiotrisphosphate) (ATPγS) or β,γ-methylene ATP. Both ATP and UTP gave a small PLC response.

5 Similarly, when formation of [^{32}P]-phosphatidic acid from cells prelabelled with ^{32}Pi was used as an index of both PLC and phospholipase D, a small response to ATP and UTP was seen but there was no response to the other purinoceptor agonists tested.

6 Study by mass assay of stimulation by ATP of inositol (1,4,5) trisphosphate accumulation revealed a transient response in the first few seconds, a decline to basal, followed by a small sustained response.

7 These results show that human brain endothelial cells in culture are responsive to histamine and endothelins in a manner which may regulate brain capillary permeability. Purines exert a lesser influence.

Keywords: Endothelial cells; blood brain barrier; histamine; endothelin; purinoceptors; phospholipase C

Introduction

An understanding and ability to manipulate the endothelial cells which form the blood brain barrier is of considerable practical and clinical importance. Morbidity and mortality following such events as ischaemia, infection, subarachnoid haemorrhage and physical insult are affected by oedema following increased permeability of the cerebral vascular endothelial cell layer. There is widespread acceptance of the notion that the special characteristics of this endothelial layer are sustained by the influence of local astrocytes (Janzer & Raff, 1987), although it has been suggested that the evidence for this relates to protein movement and not the stricter criterion of high electrical resistance. Pro-inflammatory mediators are likely to be involved in the pathological processes by direct action on the endothelial cells (causing increased permeability at the cell-cell junction) and contributing in other ways to the generation and maintenance of the inflammatory state. The nature of the local mediators which regulate cerebral vascular permeability is unclear. Various studies have shown receptors and responses to neurotransmitters and local mediators on cerebral microvessel preparations (Peroutka *et al.*, 1980), cerebral microvessels *in situ* (Butt & Jones, 1992) and cultured brain microvessel endothelial cells (e.g. Chamoux *et al.*, 1991; Durieu-Trautmann *et al.*, 1991; Joo, 1992; Xu *et al.*, 1992). In particular there is evidence that histamine (Peroutka *et al.*, 1980; Revest *et al.*, 1991; Butt & Jones, 1992), endothelins (Vigne *et al.*, 1990; 1991) and ATP/ADP acting on purinoceptors (Revest *et al.*, 1991; Frelin *et al.*, 1993) are all involved in the regulation of brain microvessel endothelium. However there is little information on the activation of phospholipase C (PLC) in these cells: endothelin and ATP/ADP stimulate PLC in bovine brain capillary endothelial cells (Vigne *et al.*, 1990; Frelin *et al.*, 1993). The PLC responses are likely to be particularly important since it has been suggested that intracellular Ca^{2+} is the principal regulator of transendothelial permeability (Revest & Abbott, 1991).

Given the clinical significance of this work it is important to know what happens in cells of human origin. In the present studies we have used cultured cells which originate from human brain microvascular endothelial cells. While this cell culture cannot be considered a model of the human blood brain barrier, the study of its regulation by cell surface receptors will bring us closer to understanding the control of permeability in the human brain. Here we describe the results of studies on the regulation of these cells by purinoceptor agonists, histamine and endothelin.

Methods

Cell cultures

Human brain microvessel endothelial cells (HBEC) were immunopurified according to the method of Clarke & West (1991) from fresh adult human brain tissue obtained from Professor J. Ironside (Department of Neuropathology, Leeds University). Right frontal lobe from a single patient was obtained on surgery (with local ethical committee approval) and processed immediately. It was cut into small pieces with scissors and homogenized in phosphate buffered saline with a teflon homogenizer (10 strokes, 0.025 mm clearance, 400

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r.p.m.). The homogenate was suspended in phosphate buffered saline containing 1 mM glucose and filtered through 150 μm nylon mesh. The filtrate was passed through a 25 μm filter and the small capillaries and vessels were washed from the nylon mesh with 10% foetal calf serum (FCS) in medium MCDB 131 containing 100 μg ml^{-1} endothelial cell growth factor. The suspension was plated onto 35 mm petri dishes and incubated at 37°C in 5% CO_2. The primary HBEC cultures were then purified by immunoadsorption using an endothelium specific antibody (Clarke & West, 1991). The endothelial nature of the cells in culture was confirmed by immunoreactivity to Factor VIII and *Ulex europus* (markers for endothelial cells, including those from brain: Gerritsen, 1988; Lathey *et al.*, 1990). The cells exhibited stable gross morphology and responsiveness to agonists throughout this study and were used to passage 10.

Bovine thoracic aorta endothelial cells (BAEC) were prepared essentially as described by Booyse *et al.* (1975). Briefly, an undamaged aorta was clamped at one end, arteries ligated and washed gently inside with balanced salt solution (BSS, mM: NaCl 125, KCl 5.4, $NaHCO_3$ 16.2, HEPES 30, NaH_2PO_4 1, $MgSO_4$ 0.8 mM, $CaCl_2$ 1.8, glucose 5.5; buffered to pH 7.4 with NaOH and gassed with 95%:5% CO_2). The aorta was then filled with collagenase solution (Sigma type 1, 0.5 mg ml^{-1} in BSS) and incubated for 20 min at 37°C. After gentle washing with BSS the aorta was half filled with complete medium (D-valine minimal essential medium, 5% FCS, 15% newborn calf serum, with 27 mg ml^{-1} glutamine, 25 units ml^{-1} penicillin, 25 μg ml^{-1} streptomycin, 0.5 mg ml^{-1} gentamycin and 25 μg ml^{-1} fungizone) and vigorously shaken. The resultant suspension was triturated and seeded into 80 cm^2 flasks. When confluent the cells were passaged into 6 or 24 well multidishes. Cells were used up to passage 3.

Measurement of total [^{3}H]-inositol phosphate

Confluent BAEC and near confluent HBEC cultures on 24 well plates were incubated with 0.5 μCi per well of *myo*-[^{3}H]-inositol in 0.5 ml of medium 199 supplemented with glutamine, fungizone, penicillin and streptomycin at concentrations indicated above for 48 h at 37°C. At the end of this loading period the monolayer cultures were washed twice with 0.5 ml of BSS with 10 mM lithium chloride. Solutions (agonist in BSS with lithium) were added to the cells after removal of the final wash and the cells incubated at 37°C for 30 min. At the end of this period 0.5 ml of ice cold methanol was added, followed by chloroform extraction and purification of total inositol (poly)phosphates (InsPx) on Dowex-1 (Cl^-).

Measurement of separated [^{3}H]-inositol (poly)phosphates

Cells on 6-well dishes were labelled as above but with 5 μCi per well in 1.5 ml of medium. Incubation at 37°C with 30 μM ATP for 0–300 s was stopped and inositol phosphates extracted by the addition of trichloroacetic acid to a final concentration of 1 M. Cells were scrape harvested, followed by ether extraction and separation of inositol phosphates on an AG1 × 8 (200 × 400 mesh) anion exchange column, prepared and eluted as described by Allsup & Boarder, 1990.

Measurement of mass Ins(1,4,5)P_3

The mass of inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) in acid extracts of cells in multiwells was measured by a protein binding procedure described in Challiss *et al.* (1991). These experiments were performed in the absence of lithium.

[^{125}I]-endothelin-1 (ET-1) binding

[^{125}I]-ET-1 binding to intact HBECs was investigated as described by Wilkes & Boarder (1991).

Measurement of [^{32}P]-phosphatidic acid

Accumulation of [^{32}P]-phosphatidic acid in cells prelabelled with ^{32}Pi for 24 h was as described in Purkiss *et al.* (1992).

Materials

[^{32}P]-H_3PO_4 was from ICN Flow Labs Ltd, Irvine, CA, U.S.A. [^{3}H]-*myo*-inositol and [^{3}H]-Ins(1,4,5)P_3 were from Du Pont, NEN, Stevenage.

[^{125}I]-ET-1 (2000 Ci mmol^{-1}) was from Amersham, Bucks. 2-Methylthioadenosine 5'-trisphosphate (2MeSATP) was from Research Biochemicals Inc, Natick, MA, U.S.A. Other chemicals were from Sigma (Dorset) or Fisons (Loughborough). All tissue culture reagents were from GIBCO, Paisley, Scotland.

Results

Histamine

When HBECs loaded with [^{3}H]-inositol were stimulated with histamine, there was a concentration-related increase in the formation of total [^{3}H]-inositol (poly)phosphates ([^{3}H]-InsP_x). Figure 1a shows the concentration-response curve for histamine with respect to [^{3}H]-InsP_x; the response at 300 μM was three fold basal and the response had a typical EC_{50} of 1.3×10^{-5} M (p[A_{50}] = 4.89 ± 0.04; mean ± s.e.mean from 3 separate experiments). Figure 1b shows the time course of stimulation with 100 μM histamine, indicating a rapid increase in the first 25 min followed by a plateau formed from 30 min. Further studies with mepyramine showed a concentration-related inhibition of the response to 30 μM histamine by this H_1-receptor antagonist, with an IC_{50} of 0.17 μM (mean of 2 experiments) and complete inhibition by 10 μM mepyramine. In the presence of nominally Ca^{2+}-free medium, the response to 30 μM histamine was 80% and with the addition of 0.5 mM EGTA the response was 23%, of that seen in 1.8 mM Ca^{2+} (mean values from 2 experiments). For example: in 1.8 mM Ca^{2+}, control 182 ± 24, 30 μM histamine 311 ± 19; in 0.5 mM EGTA, control 102 ± 6, 30 μM, histamine 138 ± 7; data are d.p.m., mean ± s.e.mean, $n = 3$. This indicates that the histamine stimulation of PLC was partly dependent on the entry of extracellular Ca^{2+}.

Endothelins

Figure 2 shows the concentration-response curve for both ET-1 and ET-3 with respect to [^{3}H]-InsP_x. There was a concentration-related response to ET-1, with a maximum at 100 nM of 5.29 ± 0.06 fold over basal (mean ± s.e.mean from 3 separate experiments). The EC_{50} was 7.2×10^{-10} M (p[A_{50}] = 9.14 ± 0.07; mean ± s.e.mean, $n = 3$)). However, there was characteristically no response to ET-3, except at the highest concentration used (100 nM) when a very small response was seen. Such a pattern has been reported in other systems (e.g. Wilkes & Boarder, 1991) and is consistent with the presence of an ET_A receptor. The time course of the response to 10 nM ET-1 was essentially similar to that for histamine described above, with a rapid rise in the first few minutes and a plateau from 20 min (data not shown). Again there was a dependency on extracellular Ca^{2+}. In this case 50 μM EGTA was added to nominally Ca^{2+}-free medium. The response to 10 nM ET-1 fell to 25% of that in 1.8 mM Ca^{2+} (mean of 2 experiments). For example: in 1.8 mM Ca^{2+}, control 95.9 ± 4.3, 10 nM ET-1 608 ± 73; in low Ca^{2+}, control 99.3 ± 9.2, 10 nM ET-1 233 ± 19; data are d.p.m., mean ± s.e.mean, $n = 3$.

To investigate further the endothelin receptors on HBECs we used a radioligand binding procedure looking at [^{125}I]-ET-1 binding to whole cells cultured close to confluency on 24 well plates. Competitive inhibition curves with ET-1, ET-3

and STX S6b are shown in Figure 3. The rank order of displacement of [^{125}I]-ET-1 was ET-1>STX S6b>ET-3. ET-1, but not ET-3, was effective at binding with high affinity for the binding site labelled by this radioligand. Analysis of isotope dilution data generated from 4 separate ET-1 curves gave an apparent K_D of $4.37 \pm 0.74 \times 10^{-10}$ M, and B_{max} of $2.31 \pm 0.88 \times 10^{-14}$ mol per well (mean ± s.e.mean, $n = 4$; typical protein per well was 70 μg).

P_2-purinoceptor agonists

In preliminary studies we were unable to demonstrate a substantial [^{3}H]-InsP$_x$ response to the P_2-purinoceptor agonist ATP in HBECs. We therefore undertook parallel experiments on cultured bovine aortic endothelial cells (BAECs) to provide a comparison with endothelial cells from a major vessel which we and others have previously shown to respond to ATP and related compounds with a clear PLC response (e.g. Wilkinson *et al.*, 1993; Motte *et al.*, 1993). Neither cell type responded to the P_{2X} agonist, β,γ-methylene ATP. We have shown that 2MeSATP is a selective P_{2Y}-purinoceptor agonist and UTP a selective nucleotide receptor agonist in BAECs (Wilkinson *et al.*, 1993). Both these agonists produced a clear response in BAECs: 1.70 ± 0.19 and 2.01 ± 0.57 fold over basal respectively for 30 μM 2MeSATP and UTP (mean ± s.e.mean pooled from 4 separate experiments). This is consistent with the presence of both categories of receptor on this cell type (Wilkinson *et al.*, 1993). Adenosine 5'-O-(thiotrisphosphate) (ATPγS), an ectonucleotidase resistant agonist at both P_{2Y}- and P_{2U}-receptors, produced a larger response on BAECs. However with HBECs there was little response to any of these agonists. Expressed as mean ± s.e.mean of fold over basal, pooled over 4 separate experiments each in triplicate, the effects of 30 min incubation with each agonist at 30 μM were: ATPγS 1.04 ± 0.09; 2MeSATP 1.18 ± 0.08; β,α-methylene ATP 1.14 ± 0.16; ATP 1.34 ± 0.09; UTP 1.28 ± 0.13. Only ATP and UTP generated a significant response ($P < 0.05$, Student's t test) when individual experiments were analysed.

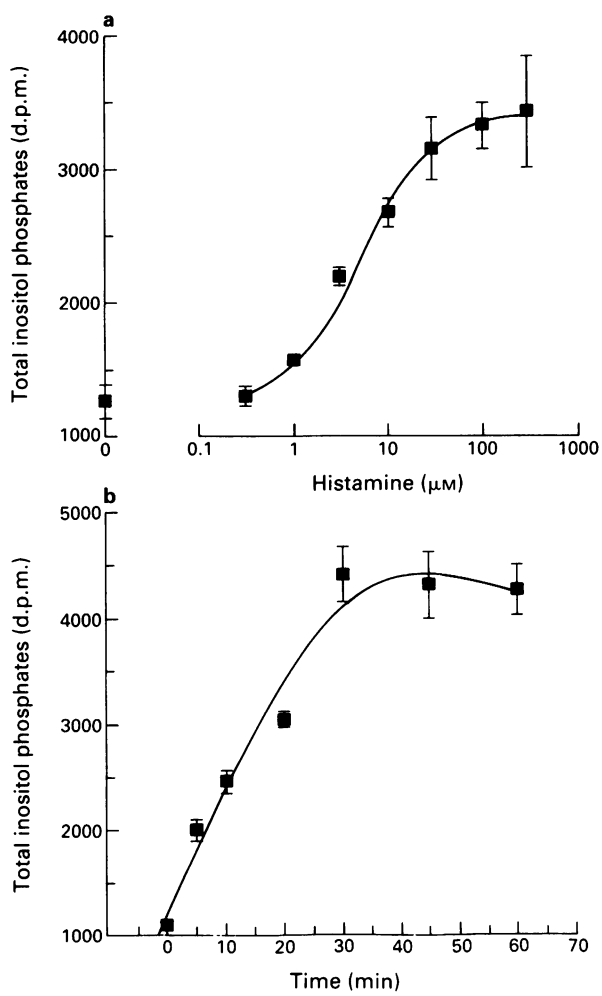


Figure 1 Stimulation of accumulation of total [^{3}H]-InsP$_x$ in HBECs by histamine. The response recorded is d.p.m. per well from 24 well plates. (a) Concentration-response curve with a 20 min incubation; (b) time course of the response stimulated with 100 μM histamine. Data are mean ± s.e.mean, $n = 4$, of an experiment representative of 3.

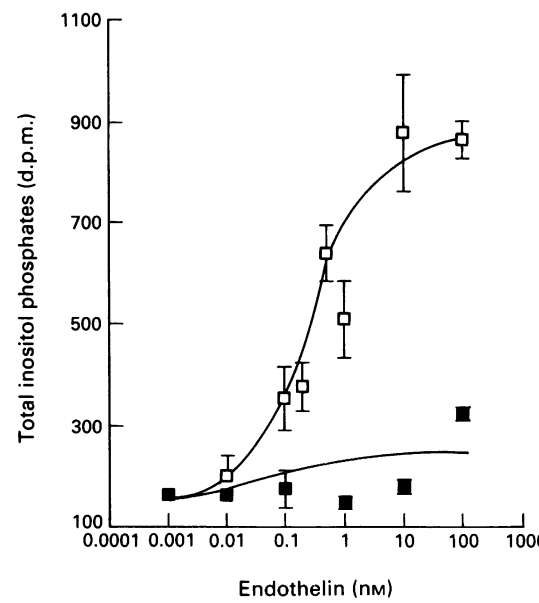


Figure 2 Stimulation of accumulation of total [^{3}H]-InsP$_x$ in HBECs by endothelins. Data are from a 20 min incubation with endothelin-1 (ET-1, □) and ET-3 (■), mean ± s.e.mean ($n = 4$), representative of three experiments.

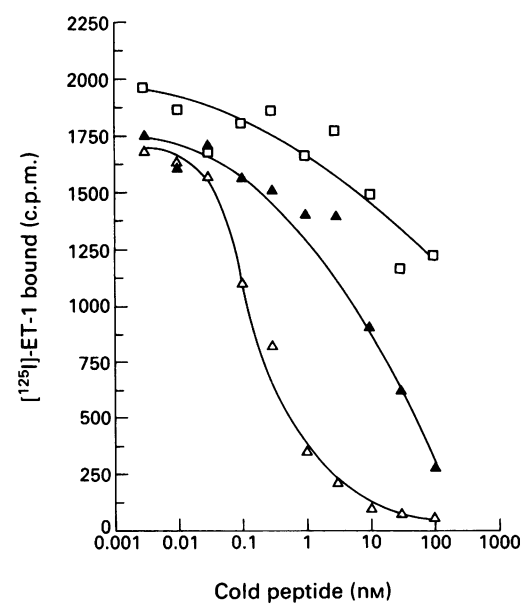


Figure 3 Competitive inhibition of binding of 2.5×10^{-11} M [^{125}I]-endothelin-1 ([^{125}I]-ET-1) to HBECs in 24 well plates: (△) ET-1; (▲) sarafotoxin S6b; (□) ET-3. Each point represents the mean of duplicates from a single experiment representative of 3.

The use of the total [^{3}H]-inositol (poly)phosphate assay in the presence of lithium is used as an index of inositol phospholipid PLC activity. A much broader phospholipase assay is the measurement of [^{32}P]-phosphatidic acid accumulation in cells loaded with ^{32}P-labelled Pi. This procedure, previously characterized in BAECs (Purkiss *et al.*, 1992; Purkiss & Boarder, 1992), provides an index of PLC/diacylglycerol kinase and PLD activities of all phospholipid classes, and would therefore reveal receptor linked phospholipase activities directed at non-inositol phospholipids as well as inositol phospholipids. In this assay (Figure 4) the BAECs showed a strong response to 2MeSATP, ATPγS, ATP and UTP consistent with our earlier reports. However the responses of HBECS were not substantial. 2MeSATP and ATPγS gave no response. ATP and UTP however gave small stimulations (1.36 ± 0.11 fold and 1.35 ± 0.02 fold over basal respectively; mean ± s.e.mean from three separate experiments each in quadruplicate) which were significant in each of the three experiments underaken (e.g. Figure 4).

To look for transient inositol phospholipid PLC responses to purinoceptor agonists we labelled cells with [^{3}H]-inositol and separated the major inositol phosphates on small ion exchange columns after brief stimulations with 30 μM ATP. Data in Figure 5 from a representative experiment show that a clear and rapid rise in each of $InsP_1$, $InsP_2$, $InsP_3$ and $InsP_4$ could be seen when BAECs were stimulated with 30 μM ATP. However, when HBECs were stimulated (Figure 6) there was only a very small apparent response (compare scales in Figures 5 and 6), the interpretation of which was confused by a rising baseline and variation between experiments.

Because of these difficulties we attempted to pursue the possibility of transient responses further, using a mass assay procedure for Ins(1,4,5)P_3. (The [^{3}H]-$InsP_3$ fractions of Figures 5c and 6c contained both Ins(1,4,5)P_3, the Ca^{2+} mobilising primary produce of PLC action on phosphatidylinositol 4,5-bisphosphate, and its metabolic product Ins(1,3,4)P_3.) The results of a typical experiment are shown in Figure 7. There is a clear biphasic response to ATP in HBECs which was seen in each of 3 experiments undertaken. The first rapid transient phase was above unstimulated con-

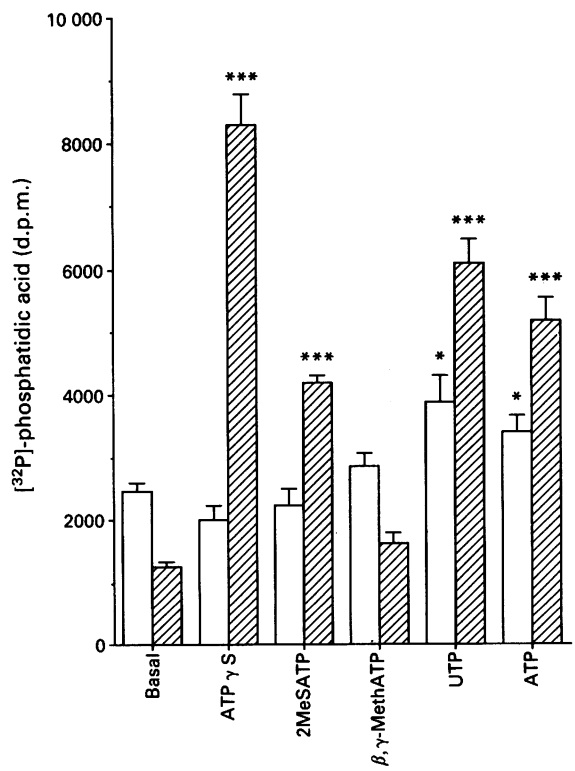


Figure 4 Stimulation of accumulation of [^{32}P]-phosphatidic acid in HBECs (open columns) and BAECs (hatched columns) in response to ATP and analogues and UTP. Nucleotides were at 30 μM in each case and the stimulation was for 3 min. Data are mean ± s.e.mean ($n = 4$) from a single experiment representative of 3. Significance of difference from the unstimulated control was by Student's t test: ***$P < 0.001$; *$P < 0.05$.

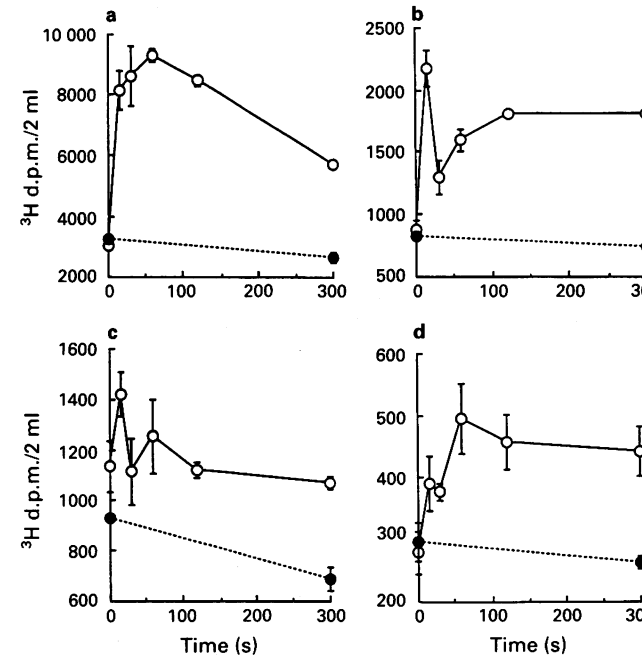


Figure 5 Accumulation of separated [^{3}H]-inositol phosphates in BAECs in response to incubation for the times shown with 30 μM ATP (○) and controls (●). (a) $InsP_1$; (b) $InsP_2$; (c) $InsP_3$; (d) $InsP_4$. Data are from one experiment of three carried out in triplicate (mean ± s.e.mean).

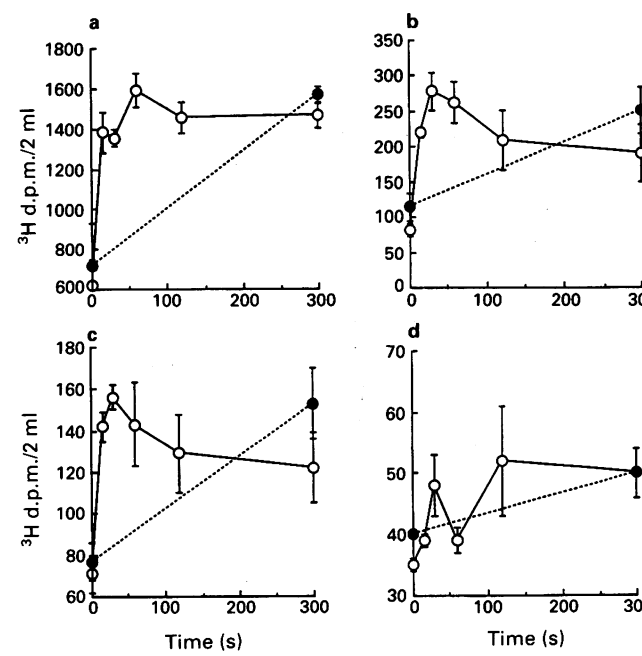


Figure 6 Accumulation of separated [^{3}H]-inositol phosphates in HBECs in response to incubation for the times shown with 30 μM ATP (○) and controls (●). (a) $InsP_1$; (b) $InsP_2$; (c) $InsP_3$; (d) $InsP_4$. Data are from one experiment of three carried out in triplicate (mean ± s.e.mean).

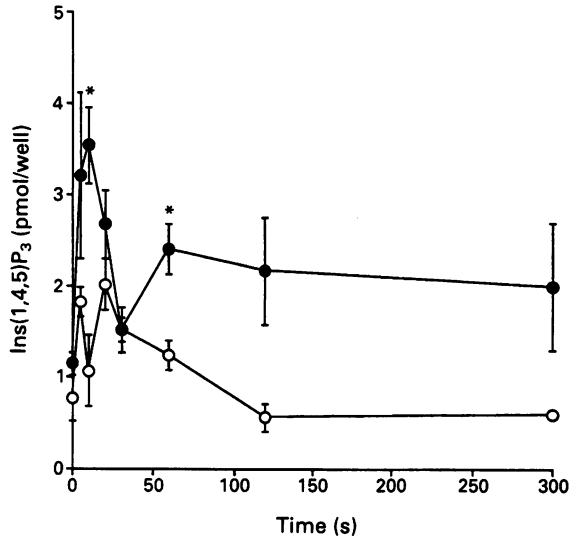


Figure 7 Mass assay of Ins(1,4,5)P_3 in HBEC cultures stimulated with 30 μM ATP (●) or unstimulated controls (○) for the times indicated. Data are mean of three determinations (± s.e.mean) from a single experiment representative of three. Asterisk indicates significantly different from unstimulated controls ($P<0.05$, Student's t test).

trol at 10–20 s, declining to control levels, followed by a small sustained elevation above control from 60–300 s. While this pattern was consistent across experiments, the absolute levels of Ins(1,4,5)P_3, the exact position of the first peak, and the rate of rise of the second sustained phase each varied, making pooling of data across experiments difficult. However, when expressed as fold over basal the first peak gave a response which was 2.35 ± 0.5 (mean ± s.e.mean) across the three separate experiments.

Discussion

The information available on the control of permeability of the brain microvessel endothelium is inadequate to contribute to a satisfactory model of the blood brain barrier. There have been no previous reports of studies on regulation of cultured endothelial cells of the human microvasculature by cell surface receptors to histamine, endothelin and P_2-purinoceptor agonists. In the present work we have made no attempt to evaluate the maintenance of blood brain barrier characteristics; neither have we used any special culture conditions which may sustain these characteristics. The cells we have used are of human brain microvascular endothelial origin, and have immunological and lipoprotein uptake characteristics of endothelial cells and as such are of a similar status as for example, the passaged bovine brain endothelial cultures used in recent studies on the influence of endothelin and purinoceptor agonists (Vigne *et al.*, 1990; 1991; Frelin *et al.*, 1993).

Evidence has been presented which suggests that maintenance of endothelium with the high electrical resistance characteristic of brain is dependent on the involvement of second messengers, namely adenosine 3':5'-cyclic monophosphate (cyclic AMP) and cyclic GMP, as well as glial influences (Rubin *et al.*, 1991). They report that elevated levels of cyclic AMP increase the tightness of the barrier. This is apparently in conflict with evidence for the involvement of H_2 receptors in the histamine-mediated increase in brain endothelial permeability (Butt & Jones, 1992), since H_2 receptors are positively linked to adenylate cyclase. Histamine acting at H_1 receptors is widely acknowledged to increase vascular permeability at non-brain vasculature, in a manner dependent on extracellular Ca^{2+} (Haraldsson *et al.*, 1986; Liddell *et al.*, 1981). In this paper we demonstrate activation of the PLC-inositol phospholipid system in brain endothelial cells by histamine acting on H_1 receptors. The response, expressed as absolute counts or fold over basal, was large compared with other systems we have examined. This suggests that histamine may play a particularly significant role in the regulation of Ca^{2+} and consequently of endothelial permeability. Our results are notably different from the report of no PLC response to histamine in murine brain endothelial cells (Xu *et al.*, 1992).

The results presented here with endothelins show that human brain endothelial cells respond to stimulation with ET-1, but not ET-3, by activation of (poly)phosphoinositide breakdown. This selectivity suggests the presence of an ET_A receptor linked to PLC: the ET_A receptor has a high affinity for ET-1 and low for ET-3, while the ET_B receptor is non-selective. This was confirmed by the binding studies presented here, with the binding of [^{125}I]-ET-1 being competitively inhibited with an order of potency: ET-1>sarafotoxin S6b> ET-3. These results suggest that ET-1 may be a potent mediator of increased permeability in human brain, in agreement with previous data on bovine brain (Vigne *et al.*, 1990). The predominant form of endothelin in brain is thought to be ET-3 from astrocytes (Ehrenreich *et al.*, 1991). An ET_B receptor sensitive to ET-3, but not PLC-linked, has been described in bovine brain endothelial cells (Vigne *et al.*, 1991). The origin of ET-1 acting on the ET_A receptors described here is unclear, although ET-1 mRNA has been described in extracts of rat cerebellar astroglial cells (MacCumber *et al.*, 1990) and has recently been reported to be secreted by an immortalised rat brain microvessel endothelial cell line (Durieu-Trautmann *et al.*, 1993).

With respect to purinoceptors, the present data do not provide clear evidence for P_2 purinoceptors in these HBEC cultures. While in some cases small stimulations of phospholipase linked responses could be seen, even in the instances when they reach significance the response was too marginal to permit characterization. This is despite using a variety of procedures to measure responses. We included stimulations with the P_{2Y}-selective agonist 2MeSATP, the P_{2U} (nucleotide) selective UTP, and the non-selective agonists ATP and ATPγS (see Wilkinson *et al.*, 1993). The only prior study with PLC data to which this can be related is Frelin *et al.* (1993) on bovine cerebral microvasculature endothelial cells, in which only the total [^{3}H]-inositol phosphate response was measured. They report that ADP gave no response below 0.1 mM; the response to ATP is only shown at 0.1 mM. Their results are therefore not at variance with those described in the present study. However, there are reports of purines regulating brain microvasculature endothelial cells (e.g. Hardebo *et al.*, 1987) including increased intracellular Ca^{2+} in response to ATP and ADP (Revest *et al.*, 1991; Frelin *et al.*, 1993).

The experimental results described here were with cultures derived from endothelial cells of the human cerebral microvasculature. As with other recent studies on non-human brain endothelial cells, the characteristics of these cells may differ from those present *in vivo*. However, the observations provide the first insights into the regulation of human brain endothelial cells by phospholipase linked receptors, showing a major influence by H_1 and ET_A receptors. By contrast P_2-purinoceptors linked to phospholipases have little effect. Subsequent studies should contribute to understanding of the role of local mediators in the generation of cerebral oedema and perhaps lead to rational therapy with specific antagonists.

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References

ALLSUP, D.J. & BOARDER, M.R. (1990). Comparison of P_2 purinergic receptors of aortic endothelial cells with those of adrenal medulla: evidence for heterogeneity of receptor subtype of inositol phosphate response. *Mol. Pharmacol.*, **38**, 84–91.

BOOYSE, F.M., SEDLAK, B.J. & RAFELSON, M.E. (1975). Culture of arterial endothelial cells: characterisation and growth of bovine aortic endothelial cells. *Thromb. Diath. Haemorrh.*, **34**, 825–839.

BUTT, A.M. & JONES, H.C. (1992). Effect of histamine and antagonists on electrical resistance across the blood brain barrier in rat brain-surface microvessels. *Brain Res.*, **569**, 100–115.

CHALLISS, R.A., JONES, J.A., OWEN, P.J. & BOARDER, M.R. (1991). Changes in inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate mass accumulations in cultured adrenal chromaffin cells in response to bradykinin and histamine. *J. Neurochem.*, **56**, 1083–1086.

CHAMOUX, M., DEHOUCK, M.P., FRUCHART, J.C., SPIK, G., MONTREUIL, J. & CECCHELLI, R. (1991). Characterisation of angiogenin receptors on bovine brain capillary endothelial cells. *Biochem. Biophys. Res. Commun.*, **176**, 833–839.

CLARKE, M.S.F. & WEST, D.C. (1991). The identification of, proliferation and tumour induced protein in human endothelial cells: a possible target for tumour therapy. *Electrophoresis*, **12**, 500–508.

DURIEU-TRAUTMANN, O., FEDERICI, C., CREMINON, C., FOIGNANT-CHAVEROT, N., ROUX, F., CLAIRE, M., STROSBERG, A.D. & COURAUD, P.O. (1993). Nitric oxide and endothelin secretion by brain microvessel endothelial cells: regulation by cyclic nucleotides. *J. Cell. Physiol.*, **155**, 104–111.

DURIEU-TRAUTMANN, O., FOIGNANT, N., STROSBERG, A.D. & COURAUD, P.O. (1991). Coexpression of β_1 and β_2 adrenergic receptors on bovine brain capillary endothelial cells in culture. *J. Neurochem.*, **56**, 775–781.

EHRENREICH, H., KEHRL, J.H., ANDERSON, R.W., RIECKMANN, P., VITKOVIC, L., COLIGAN, J.E. & FAUCI, A.S. (1991). A vasoactive peptide, endothelin 3, is produced by and binds to primary astrocytes. *Brain Res.*, **538**, 54–58.

FRELIN, C., BREITTMAYER, J.P. & VIGNE, P. (1993). ADP induces inositol phosphate-independent intracellular Ca^{2+} mobilisation in brain capillary endothelial cells. *J. Biol. Chem.*, **268**, 8787–8792.

GERRITSEN, M.E. (1988). Microvascular endothelial cells: isolation, identification and cultivation. *Adv. Cell Culture*, **6**, 35–67.

HARALDSSON, B., ZACKRISSON, U. & RIPPE, B. (1986). Calcium dependence of histamine induced increases in capillary permeability. *Am. J. Physiol.*, **258**, H1366–1374.

HARDEBO, J.H., KAHRSTROM, J. & OWMAN, C. (1987). P_1- and P_2-purine receptors in brain circulation. *Eur. J. Pharmacol.*, **144**, 343–352.

JANZER, R.C. & RAFF, M.C. (1987). Astrocytes induce blood-brain barrier properties in endothelial cells. *Nature*, **325**, 253–257.

JOO, F. (1992). The cerebral microvessels in culture, an update. *J. Neurochem.*, **58**, 1–17.

LATHEY, J.L., WILEY, C.A., VERITY, A. & NELSON, J.A. (1990). Cultured human brain capillary endothelial cells are permissive for infection by human cytomegalovirus. *Virology*, **176**, 266–273.

LIDDELL, R.H.A., SCOTT, A.R.W. & SIMPSON, J.G. (1981). Histamine induced changes in the endothelium of post capillary venules: effects of chelating agents and cytochalasin B. *Bibl. Anat.*, **20**, 109–112.

MACCUMBER, M.W., ROSS, C.A. & SNYDER, S.H. (1980). Endothelins in brain: receptors, mitogenesis and biosynthesis in glial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 2359–2363.

MOTTE, S., PIROTTON, S. & BOEYNAEMS, J.M. (1993). Heterogeneity of ATP receptors in aortic endothelial cells. Involvement of P_{2Y} and P_{2U} receptors in inositol phosphate response. *Circ. Res.*, **72**, 504–510.

PEROUTKA, S.J., MOSKOWITZ, M.A., REINHARD, J.F. & SNYDER, S.H. (1980). Neurotransmitter receptor binding in bovine cerebral microvessels. *Science*, **208**, 610–612.

PURKISS, J.R. & BOARDER, M.R. (1992). Stimulation of phosphatidate synthesis in endothelial cells in response to P_2-receptor activation. *Biochem. J.*, **287**, 31–36.

PURKISS, J., OWEN, P.J., JONES, J.A. & BOARDER, M.R. (1992). Stimulation of phosphatidic acid synthesis in bovine aortic endothelial cells in response to activation of P_2-purinergic receptors. *Biochem. Pharmacol.*, **43**, 1235–1242.

REVEST, P.A. & ABBOTT, N.J. (1991). Control of brain endothelial permeability. *Cerebrovasc. Brain Metab. Rev.*, **3**, 39–72.

REVEST, P.A., ABBOTT, N.J. & GILLESPIE, J.I. (1991). Receptor mediated changes in intracellular $[Ca^{2+}]$ in cultured rat brain capillary endothelial cells. *Brain Res.*, **549**, 159–161.

RUBIN, L.L., HALL, D.E., PORTER, S., BARBU, K., CANNON, C., HORNER, H.C., JANATPOUR, M., LIAW, C.W., MANNING, J., MORALES, J., TANNER, L.I., TOMASELLI, K.J. & BARD, F. (1991). A cell culture model of the blood brain barrier. *J. Cell. Biol.*, **115**, 1725–1735.

VIGNE, P., LADOUX, A. & FRELIN, C. (1991). Endothelins activate Na^+/H^+ exchange in brain capillary endothelial cells via a high affinity endothelin-3 receptor that is not coupled to phospholipase C. *J. Biol. Chem.*, **266**, 5925–5928.

VIGNE, P., MARSAULT, R., BREITTMAYER, J.P. & FRELIN, C. (1990). Endothelin stimulates phosphatidylinositol hydrolysis and DNA synthesis in brain capillary endothelial cells. *Biochem. J.*, **266**, 415–420.

WILKES, L.C. & BOARDER, M.R. (1991). Characterisation of endothelin receptors on a human neuroblastoma cell line: evidence for the ET_A subtype. *Br. J. Pharmacol.*, **104**, 750–754.

WILKINSON, G.F., PURKISS, J.R. & BOARDER, M.R. (1993). The regulation of aortic endothelial cells by purines and pyrimidines involves co-existing P_{2Y}-purinoceptors and nucleotide receptors linked to phospholipase C. *Br. J. Pharmacol.*, **108**, 689–693.

XU, J., QU, Z.-X., MOORE, S.A., HSU, C.Y. & HOGAN, E.L. (1992). Receptor linked hydrolysis of phosphoinositides and production of prostacyclin in cerebral endothelial cells. *J. Neurochem.*, **58**, 1930–1935.

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Relaxation of guinea-pig trachea by cyclic AMP phosphodiesterase inhibitors and their enhancement by sodium nitroprusside

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1 The effects of agents that elevate either cyclic AMP (the phosphodiesterase (PDE) III inhibitor siguazodan, salbutamol) or cyclic GMP (sodium nitroprusside (SNP)) on the relaxant activity of the PDE IV inhibitor, rolipram, were investigated in carbachol (0.1 μM) precontracted guinea-pig tracheal sheets.

2 Rolipram, siguazodan and SNP caused concentration-related reductions in tone of tissues precontracted with 0.1 μM carbachol (EC_{50} values 12.5; 2.73 and 0.35 μM respectively). Whilst the concentration-response relationship for the PDE III inhibitor, siguazodan, was monophasic that of the PDE IV inhibitor, rolipram, was biphasic.

3 The relaxant activity of rolipram was markedly enhanced in the presence of 10 μM siguazodan ($EC_{50} < 0.01$ μM), 0.1 μM salbutamol (EC_{50} 0.03 μM) and 0.3 μM SNP (EC_{50} 0.03 μM). In contrast, the relaxant activity of siguazodan was unaffected by SNP and only modestly enhanced by rolipram (10 μM) and salbutamol (0.1 μM).

4 The relaxant activity of SNP was enhanced by the PDE V inhibitor SK&F 96231 (30 μM: EC_{50} 0.06 μM) and rolipram (30 μM, EC_{50} 0.08 μM) but was unaffected by 30 μM siguazodan.

5 At concentrations up to 10 μM, neither siguazodan nor rolipram elevated tracheal cyclic AMP levels. However, the combination of 10 μM rolipram and siguazodan caused a two fold increase in the cyclic AMP content (from 2.19 to 4.36 pmol cyclic AMP mg^{-1} protein). SNP (0.1–10 μM) failed to produce a significant increase in tracheal cyclic AMP levels. At 0.1 μM the effect of SNP on tracheal cyclic AMP levels was significantly ($P < 0.05$) increased in the presence of rolipram but not siguadozan.

6 The results indicate that the relaxant effects of rolipram are markedly enhanced by agents that inhibit PDE III activity or elevate cyclic GMP. They support the hypothesis that SNP potentiates the effects of rolipram via the inhibitory action of cyclic GMP on hydrolysis of cyclic AMP by PDE III. The findings also suggest that whilst PDE III may be more significant in regulating basal smooth muscle tone in the absence of any exogenous stimulus to cyclic AMP accumulation, PDE IV activity may be more tightly coupled to the pool of adenylyl cyclase stimulated by β_2-adrenoceptor agonists.

Keywords: Sodium nitroprusside; rolipram; siguazodan; salbutamol; cyclic AMP; cyclic GMP; guinea-pig trachea; relaxation

Introduction

Relaxation of airways smooth muscle, elicited by a number of agents, is well correlated with increases in intracellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) concentrations and it is believed that these two phenomena are causally related (Torphy & Undem, 1991). Thus β-adrenoceptor agonists induce smooth muscle relaxation via activation of adenylyl cyclase and the consequent stimulation of cyclic AMP formation (Murray, 1990) whilst smooth muscle relaxation caused by sodium nitroprusside (SNP) is believed to be mediated by the increase in intracellular cyclic GMP levels following stimulation of soluble guanylyl cyclase (Waldman & Murad, 1987). Intracellular concentrations of cyclic AMP and cyclic GMP are also determined by their rate of hydrolysis, catalyzed by a related group of phosphodiesterase (PDE) isoenzyme families, PDE I–PDE V (Beavo & Reifsnyder, 1990). In tracheal smooth muscle, members of all five families have been identified (Torphy & Cieslinski, 1990; Shahid *et al.*, 1991; de Boer *et al.*, 1992; Cortijo *et al.*, 1993). Of these, PDE's I, II, and III can utilize both cyclic AMP and cyclic GMP as substrate, PDE IV is a cyclic AMP-specific PDE and PDE V a cyclic GMP-specific PDE (Beavo, 1988; Beavo & Reifsnyder, 1990).

Selective inhibitors of specific PDE isoenzymes (namely PDE III, PDE IV, PDE III/IV, PDE V and PDE I/V) have all been shown to relax airways smooth muscle, with variable potencies according to species (Harris *et al.*, 1989; Hall *et al.*, 1990; Murray *et al.*, 1991; Torphy *et al.*, 1991; Shahid *et al.*, 1991; de Boer *et al.*, 1992). In guinea-pig and human airways smooth muscle the concentration-response curves for the relaxant activity of selective PDE III and PDE IV inhibitors however are biphasic (Harris *et al.*, 1989; de Boer *et al.*, 1992; Cortijo *et al.*, 1993) suggesting that at higher concentrations their relaxant activity may not be solely due to inhibition of a single specific isoenzyme. Thus, in these tissues their effectiveness in increasing cyclic AMP levels and causing smooth muscle relaxation is likely to be related to their inhibition of total PDE activity. Implicit in this assumption therefore is the potential additive or synergistic interaction between selective PDE isoenzyme inhibitors. In guinea-pig trachea, Harris *et al.* (1989) have shown a marked synergism between the relaxant activity of PDE III and PDE IV inhibitors, with their combination having a monophasic concentration-response relationship. Similarly, in human bronchus the concentration-response relationship for mixed PDE III/IV inhibitors is monophasic (de Boer *et al.*, 1992; Cortijo *et al.*, 1993).

There is little information, however, relating to the potential functional interaction between cyclic AMP PDE inhibitors and agents that elevate cyclic GMP, other than that the relaxant activity of sodium nitroprusside is not enhanced by PDE III or IV inhibitors (Torphy & Undem, 1991). In contrast, in rat aorta Komas *et al.* (1991) have

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reported that the relaxant effects of PDE IV inhibitors are markedly enhanced by cyclic GMP elevating agents. The aim of the present study therefore was to examine the hypothesis that agents that elevate cyclic GMP may enhance the relaxant activity of cyclic AMP PDE inhibitors in airway smooth muscle.

Methods

Male Dunkin-Hartley guinea-pigs weighing from 350–450 g were killed by cervical dislocation. The trachea was rapidly excised, placed in oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit solution (composition in mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2SO_4 1.2, D-glucose 5.5, Na pyruvate 2, $CaCl_2$ 2.5) at room temperature and trimmed free of adherent fat and connective tissue.

Each trachea was cut into rings containing 3–4 adjacent cartilage plates, the rings were carefully opened opposite the trachealis to avoid damaging the epithelium and suspended in 25 ml organ baths containing Krebs solution at 37°C, under an initial load of 2.5 g. Following a 60 min equilibration period, during which time the tissues were washed at 15 min intervals, tissues were primed for contractile activity by addition of 1 μM carbachol (CCh). This procedure was repeated 3 times. Tissues were then equilibrated for a further 30 min to allow resting tone to return to 2.5 g, which was optimal for contraction as determined by the length tension relationship. In those tissues where spontaneous tone was above or below 2.5 g at the end of the equilibration period the load was mechanically stabilized at 2.5 g before addition of agonists. Sustained contractions were elicited by addition of 0.1 μM CCh.

When the increase in tension had stabilized the relaxant activity of the PDE IV selective inhibitor, rolipram (Livi *et al.*, 1990), the PDE III selective inhibitor, siguazodan (SK&F 94836; Reeves *et al.*, 1987; Torphy *et al.*, 1988) and sodium nitroprusside (SNP) were investigated by their cumulative addition to the organ bath. In interaction studies, tissues were preincubated with: rolipram, siguazodan, the PDE V inhibitor SK&F 96231 (Murray *et al.*, 1991); salbutamol or SNP for 20 min prior to contraction of the tissues with carbachol and subsequent assessment of the relaxant activity of the other spasmolytics. In these experiments, following the initial addition of spasmolytic, there was a reduction in spontaneous tone which plateaued within 20 min; resting tone was mechanically returned to 2.5 g prior to contraction of the tissue with carbachol.

Changes in tension were measured with a dynamometer UF1 isometric transducer coupled to a Lectromed MT8P chart recorder. Relaxations were expressed as a percentage of the maximum relaxation induced by 10^{-4} M SNP. EC_{50}'s were calculated as the concentrations producing 50% of the maximum relaxation to 10^{-4} M SNP.

Measurement of cyclic nucleotide levels

Guinea-pig tracheal tubes, 6–7 cartilage bands deep, were incubated, in triplicate, free-floating in oxygenated Krebs-Henseleit solution at 37°C. Following a 60 min equilibration period, during which time the Krebs-Henseleit solution was replaced at 15 min intervals, the tissues were incubated for a further 20 min in 0.1 μM carbachol with or without SNP, siguazodan or rolipram, alone or in combination. At the end of the incubation the tissues were rapidly removed, blotted dry, and frozen by immersion in liquid nitrogen. Tracheal tubes treated in this way were then stored at − 70°C. Tissues were finely chopped prior to homogenization (Polytron PTA7K1 probe 5 × 10 s burst, setting 8) in 1 ml cold 0.3 M perchloric acid. Following centrifugation, 500 μl of the supernatant was removed. Cyclic nucleotides were extracted by addition of 650 μl of a 50/50 (v/v) mixture of tri-n-octylamine and freon. Following centrifugation the upper layer (of 3) was removed and cyclic AMP levels measured by radioimmunoassay.

Protein estimations were determined on the pellet by the Bio-Rad protein assay based on the method described by Bradford (1976).

Materials

Rolipram (4-[(3-cyclopentyloxy-4-methoxy-phenyl)-2-pyrrolidinone]), siguazodan (SK&F 94836; 2-cyano-1-methyl-3-[4-(4-methyl-6-oxo-1,4,5,6-tetrahydropyridazin-3-yl)phenyl]guanidine and SK&F 96231 (1,7-dihydro-2-(2-propoxyphenyl)-6H purin-6-one) were synthesized by Dr W.J. Coates of the Department of Medicinal Chemistry, SmithKline Beecham, The Frythe, Welwyn, U.K. Cyclic AMP radioimmunassay kits were from Amersham International (Amersham, Bucks). The protein assay kit was from Bio-Rad (Hemel Hempstead). Sodium nitroprusside and carbachol were obtained from Sigma (Poole, Dorset); all other chemicals were obtained from BDH (Poole, Dorset).

Results

Effect of selective PDE inhibitors on relaxation of guinea-pig trachea

Rolipram, siguazodan, salbutamol and SNP caused concentration-related reductions in tone of guinea-pig tracheal sheets pre-contracted with 0.1 μM carbachol (EC_{50}'s 12.5 ± 4.19, 2.73 ± 1.0, 0.07 ± 0.029 and 0.35 ± 0.05 μM respectively). Whilst the concentration-response relationship for the PDE III inhibitor was monophasic that of the PDE IV inhibitor, rolipram, was biphasic, the initial component occurring over the concentration-range 0.01–10 μM and the second over the range 10–100 μM (Figure 1). The EC_{50} for the first component of the rolipram-induced relaxation was 0.08 ± 0.045 μM, the maximum response being 46.8 ± 7.7% of that to 10^{-4} M SNP.

Effects of PDE III or PDE IV inhibitors on relaxation of guinea-pig trachea in combination with agents which elevate cyclic AMP or cyclic GMP levels

The effects of pretreatment of tracheal sheets with agents that elevate either cyclic AMP (siguazodan, salbutamol) or cyclic GMP (SNP) on the relaxant activity of rolipram were investigated in tissues precontracted with 0.1 μM carbachol. Since the concentrations of each agent used reduced spontaneous tone (siguazodan 0.54 ± 0.055 g, salbutamol 1.37 ± 0.2 g, SNP 0.9 ± 0.23 g) the resting tone of the tissues was mechanically readjusted to 2.5 g prior to contraction. This was done in order to minimize the effects of variations in initial resting tone on the subsequent contractile response to carbachol and secondary relaxant responses to the PDE inhibitors. We had previously established that none of the agents used had any effect on the spasmogenic activity of carbachol (0.1 μM) but that the magnitude of the contractile response was dependent on the resting tension (data not shown).

The relaxant activity of rolipram was markedly enhanced in the presence of siguazodan (10 μM), salbutamol (0.1 μM) or SNP (0.3 μM); EC_{50}'s control 12.5 ± 4.2 μM; + siguazodan, <0.01 μM; + salbutamol, 0.03 ± 0.002 μM; + SNP, 0.034 ± 0.003 μM (Figure 2). Pretreatment of guinea-pig tracheal sheets with rolipram (10 μM) or salbutamol (0.1 μM) caused approximately a ten fold enhancement of the spasmolytic activity of siguazodan (EC_{50}'s 0.37 ± 0.02 and 0.31 ± 0.04 μM). In contrast to its marked effects on responses to rolipram, pretreatment with SNP (0.3 μM) was without effect on the relaxant activity of siguazodan (Figure 3). There was no significant difference in the relaxant activity of 0.3 μM SNP in the combination studies with rolipram (0.93 ± 0.24 g)

or siguazodan (1.2 ± 0.14 g) and the subsequent contractile response to carbachol was the same in both cases (0.6 ± 0.12 and 0.54 ± 0.15 g respectively).

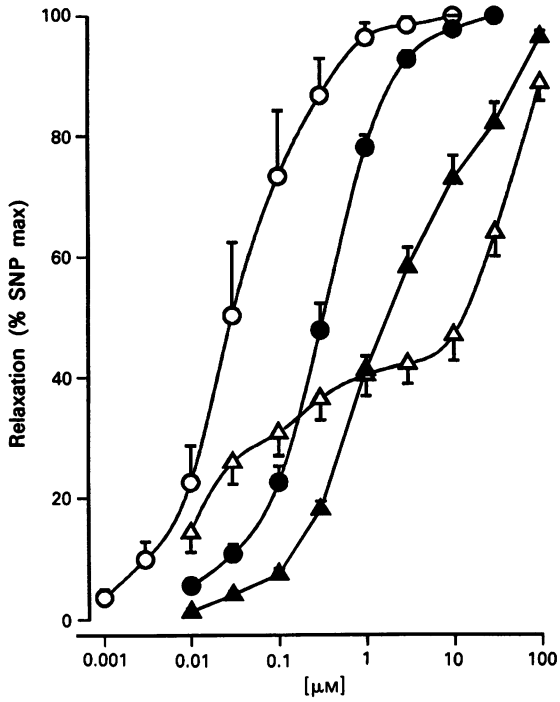


Figure 1 Comparison of the relaxant activity of salbutamol (○), sodium nitroprusside (●), siguazodan (▲) and rolipram (△) in guinea-pig tracheal sheets precontracted with 0.1 μM carbachol. Each point is the mean ± s.e.mean of at least 6 observations.

Effect of PDE inhibitors on SNP-induced relaxation of guinea-pig tracheal sheets

SNP alone was a potent relaxant of tracheal sheets precontracted with 0.1 μM carbachol (EC_{50} 0.35 ± 0.05 μM). The SNP concentration-response curves were shifted to the left following pre-incubation of tissues with the PDE V inhibitor, SK&F 96231 (30 μM; Figure 4; EC_{50} 0.06 ± 0.01 μM). Responses to SNP were not significantly affected by 30 μM siguazodan but were potentiated by rolipram (30 μM; EC_{50} 0.08 ± 0.01; Figure 4).

Effect of SNP and cyclic nucleotide PDE inhibitors on tracheal cyclic AMP levels

The effects of inhibition of PDE III and IV on tracheal cyclic AMP accumulation are summarized in Table 1. At concentrations up to 10 μM, neither siguazodan nor rolipram had any effect on cyclic AMP accumulation in carbachol (0.1 μM) pretreated tracheal rings. In contrast, at the same concentrations, siguazodan and rolipram caused 70–80% and 40–50% relaxations of carbachol precontracted tracheal sheets respectively (Figure 1). The combination of rolipram and siguazodan (both 10 μM), however did result in a two fold increase in cyclic AMP accumulation under the same conditions.

The effect of SNP is shown in Table 2. SNP alone (0.1–10 μM) failed to produce a significant change in tracheal cyclic AMP levels. Tracheal cyclic AMP levels however were significantly increased by 0.1 μM SNP, in the presence of rolipram but not siguazodan (both 30 μM).

Discussion

It is well established that SNP is a potent bronchorelaxant which stimulates soluble guanylyl cyclase and thus intracel-

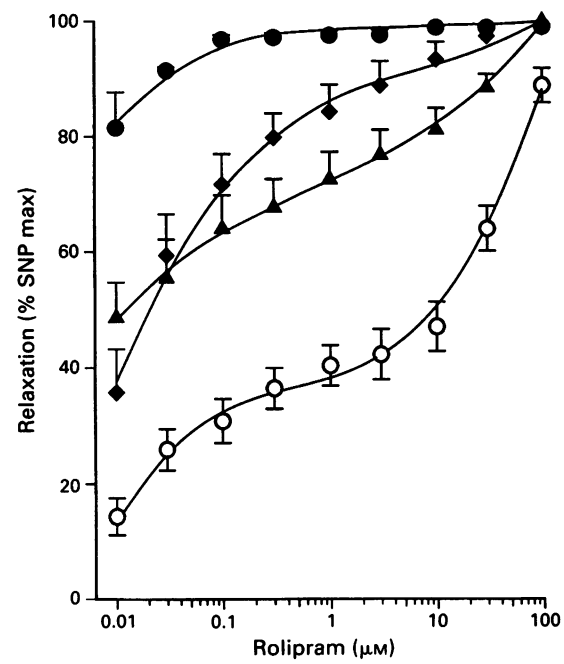


Figure 2 Cumulative concentration-response curves to rolipram in guinea-pig tracheal sheets precontracted with carbachol. Tissues were preincubated with 10 μM siguazodan (●, $n = 3$), 0.1 μM salbutamol (◆, $n = 6$) or 0.3 μM sodium nitroprusside (SNP, ▲, $n = 3$) for 20 min prior to contraction with 0.1 μM carbachol. Rolipram concentration-response studies were carried out at the plateau of the carbachol response; (○) are the responses to rolipram alone ($n = 6$). Data are expressed as a % of the relaxation induced by 10^{-4} M SNP added at the completion of the rolipram concentration-response curve. Each point is the mean ± s.e.mean.

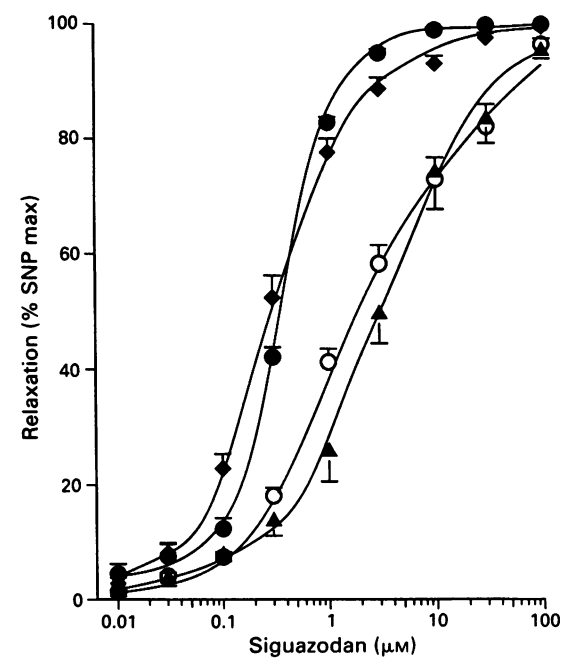


Figure 3 Cumulative concentration-response curves to siguazodan in guinea-pig tracheal sheets precontracted with carbachol. Tissues were preincubated with 10 μM rolipram (●, $n = 6$), 0.1 μM salbutamol (◆, $n = 6$) or 0.3 μM sodium nitroprusside (SNP, ▲, $n = 6$) for 20 min prior to contraction with 0.1 μM carbachol. Siguazodan concentration-response studies were carried out at the plateau of the carbachol response; (○) are the responses to siguazodan alone ($n = 12$). Data are expressed as a % of the relaxation induced by 10^{-4} M SNP added at the completion of the siguazodan concentration-response curve. Each point is the mean ± s.e.mean.

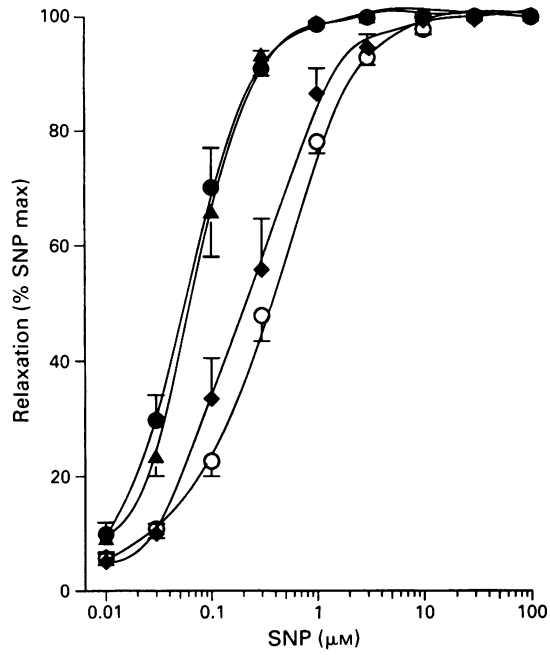


Figure 4 Cumulative concentration-response curves to sodium nitroprusside (SNP) in guinea-pig tracheal sheets precontracted with carbachol. Tissues were preincubated with 30 μM SK&F 96231 (▲, $n = 6$), 30 μM siguazodan (◆, $n = 6$) or 30 μM rolipram (●, $n = 6$) for 20 min prior to contraction with 0.1 μM carbachol. SNP concentration-response studies were carried out at the plateau of the carbachol response; (○) are the responses to SNP alone ($n = 6$). Data are expressed as a % of the relaxation induced by 10^{-4} M SNP. Each point is the mean ± s.e.mean.

Table 1 Effect of rolipram and siguazodan on cyclic AMP accumulation in guinea-pig trachea

	0.1 μM	1 μM	10 μM	+ *Siguazodan both* 10 μM
Rolipram	2.41 ± 0.59	3.02 ± 0.68	1.46 ± 0.33	4.36 ± 0.62*
Siguazodan	3.38 ± 0.62	2.36 ± 0.61	2.31 ± 1.05	

Tracheal rings were incubated for 20 min in Krebs-Henseleit solution at 37°C containing 0.1 μM carbachol with or without the phosphodiesterase (PDE) inhibitors. Tissues were frozen by immersion in liquid nitrogen and stored at −70°C prior to extraction and radioimmunoassay of cyclic AMP. Cyclic AMP levels are expressed in pmol mg^{-1} protein and values are the mean ± s.e. mean of 3 tissues, the cyclic AMP level in control tissues was 2.19 ± 0.46 pmol cyclic AMP mg^{-1} protein. Significantly different from control value, $*P < 0.05$, Mann Whitney U test.

Table 2 Effect of sodium nitroprusside (SNP) on cyclic AMP accumulation in guinea-pig trachea

	SNP 0.1 μM	1 μM	10 μM
	1.9 ± 0.01	2.29 ± 0.6	2.8 ± 1.06
+ Rolipram 30 μM	2.89 ± 0.25*		
+ Siguazodan 30 μM	1.84 ± 0.2		

Tracheal rings were incubated for 20 min in Krebs-Henseleit solution at 37°C containing 0.1 μM carbachol with or without SNP alone or in combination with the phosphodiesterase (PDE) inhibitors. Tissues were frozen by immersion in liquid nitrogen and stored at −70°C prior to extraction and radioimmunoassay of cyclic AMP. Cyclic AMP levels are expressed in pmol mg^{-1} protein, values are the mean ± s.e.mean of 3 tissues. Significantly different from 0.1 μM SNP alone, $*P < 0.05$, Mann Whitney U test.

lular cyclic GMP accumulation (Waldman & Murad, 1987). Since cyclic AMP PDE activity may also be modulated by cyclic GMP (Beavo, 1988) we were interested to see if SNP might also modulate the functional responses evoked by isoenzyme selective PDE inhibitors.

In the present study, SNP potently relaxed carbachol contracted guinea-pig trachea. In addition, the relaxant activity of SNP was enhanced in the presence of the PDE V inhibitor, SK&F 96231, supporting the involvement of cyclic GMP accumulation in this effect. In previous studies we have also shown that the converse is true, in that the relaxant activity of the PDE V inhibitor was potentiated by SNP (unpublished data, not shown). In the present study we have also found SNP to potentiate markedly the spasmolytic activity of the PDE IV inhibitor, rolipram. There is evidence to suggest that smooth muscle relaxation induced by agents that elevate intracellular levels of cyclic AMP and cyclic GMP may involve different mechanisms (Murray, 1990). Whilst the synergy between rolipram and SNP may therefore reflect the combined effects of cyclic AMP and cyclic GMP on the contractile process, the lack of effect of SNP on responses to the PDE III inhibitor, siguazodan, suggests that other mechanisms are involved. Indeed the specific enhancement of the relaxant activity of rolipram by SNP was paralleled by the synergism seen between rolipram and the PDE III inhibitor, siguazodan. The commonality of the effects of SNP and siguazodan, on the relaxant activity of rolipram, might therefore be accounted for by an inhibitory effect of SNP on cyclic AMP hydrolysis. In this regard Torphy *et al.* (1985) have reported that high concentrations of SNP (300 μM) significantly elevate cyclic AMP levels in canine tracheal smooth muscle and Maurice & Haslam (1990) have shown that at concentrations which increase cyclic GMP levels, SNP also enhances isoprenaline-induced increases in cyclic AMP content of rat aorta.

In agreement with the results of Harris *et al.* (1989) we have found that the concentration-relaxation curve for rolipram alone was shallow and biphasic. Biphasic concentration-response curves for the spasmolytic activity of PDE IV inhibitors have also been observed in human bronchus (de Boer *et al.*, 1992; Cortijo *et al.*, 1993). Such observations suggest that in these tissues inhibition of PDE IV activity alone only results in partial relaxation and that the full spasmolytic activity of rolipram is in part due to inhibition of a sufficient proportion of other PDE isoenzymes to cause a more complete inhibition of total cyclic AMP hydrolysis. The synergy between rolipram and siguazodan may therefore be explained by augmented suppression of cyclic AMP hydrolysis by combined PDE III and PDE IV inhibition, as has been suggested in cardiac muscle (Nicholson *et al.*, 1991). In this regard we have found that the potentiation of the relaxant activity of rolipram by siguazodan was associated with increased intracellular cyclic AMP levels compared to that seen with either agent alone. Since PDE III activity is also inhibited by low concentrations of cyclic GMP (K_i 0.1 μM; Beavo, 1988) then the specific enhancement of the relaxant activity of the PDE IV inhibitor, by SNP, may similarly be explained by inhibition of PDE III activity as a consequence of the accumulation of intracellular cyclic GMP stimulated by SNP.

Similarly, whilst the spasmolytic activity of SNP was unaffected by siguazodan, it was enhanced by rolipram. This was somewhat surprising since the PDE III also hydrolyses cyclic GMP with high affinity (Beavo, 1988). Some potentiation of the spasmolytic activity of SNP by siguazodan due to inhibition of cyclic GMP hydrolysis may therefore have been expected. Our results however are in agreement with those of

Torphy *et al.* (1988) who reported that siguazodan (30 μM) is without effect on SNP-induced relaxation or cyclic GMP accumulation in canine airway and suggests that other PDE isoenzymes (i.e I and V) are the major regulators of cyclic GMP hydrolysis.

Rolipram is a highly selective inhibitor of PDE IV and it is unlikely, therefore, that the enhancement of the relaxant activity of SNP by rolipram is due to non-specific inhibition of PDE V activity. Furthermore, the similarity between the enhancement of SNP responses by rolipram and the potentiation of siguazodan-induced relaxation by the latter suggests that a component of the SNP response may be attributed to cyclic AMP accumulation, secondary to PDE III inhibition. Whilst Torphy *et al.* (1985) have reported that high concentrations of sodium nitroprusside (300 μM) significantly elevate cyclic AMP levels in canine tracheal smooth muscle the effects of SNP on tracheal cyclic AMP levels in the present experiments are equivocal. Thus although SNP alone failed to cause a significant elevation in tracheal cyclic AMP levels, the cyclic AMP levels in tissues treated with 0.1 μM SNP were enhanced in the presence of 30 μM rolipram but not 30 μM siguazodan, paralleling the results of the mechanical studies. It is noteworthy however, that in tissues incubated in parallel with those used in functional studies, neither siguazodan nor rolipram had any significant effect on total tracheal cyclic AMP levels at concentrations that produced up to an 80% reduction in tone. In both canine trachealis (Torphy *et al.*, 1991) and bovine tracheal smooth muscle (Hall *et al.*, 1989) PDE III and PDE IV inhibitors, at concentrations up to 100 μM, have also been reported to be devoid of effects on basal cyclic AMP levels. Furthermore β-adrenoceptor agonist induced relaxation of bovine tracheal smooth muscle is not invariably associated with an increase in cyclic AMP content (Lohmann *et al.*, 1977; Lau & Lum, 1983) and in canine trachealis isoprenaline only causes a two fold increase in cyclic AMP levels (Torphy *et al.*, 1988). Thus, although the effects of SNP on cyclic AMP accumulation were modest they were similar to those of rolipram and siguazodan. Taken together therefore the functional and biochemical data are consistent with the proposal that a proportion of the relaxant activity of SNP in guinea-pig trachea is a consequence of an increase in cyclic AMP levels and activation of the cyclic AMP-dependent protein kinase, although cross activation of the cyclic GMP-dependent protein kinase (Jiang *et al.*, 1991) might also be involved.

The activity of PDE IV inhibitors as smooth muscle relaxants is species-dependent, with the relative efficacy of PDE IV inhibitors compared to PDE III inhibitors being greater in bovine airway than in man or guinea-pig (Harris *et al.*, 1989; Shahid *et al.*, 1991; de Boer *et al.*, 1992). The monophasic concentration-response relationship of the PDE III inhibitor, siguazodan, in our present experiments and the relatively smaller enhancement of its relaxant activity by rolipram further confirms that in the guinea-pig trachea, PDE III plays a more significant role in regulating basal smooth muscle tone than does PDE IV. In the presence of salbutamol, however, the potency of rolipram was more markedly enhanced than that of siguazodan. It is unlikely that this difference can be attributed to the initial reduction in resting tone caused by salbutamol since this was similar in both cases and in any case was negated by the return of resting tension to 2.5 g prior to contraction with carbachol. In addition there was no significant effect of salbutamol on the subsequent response to carbachol. Furthermore, that the marked synergy between salbutamol and rolipram is a specific phenomenon is confirmed by the much smaller increase in potency of siguazodan seen in the presence of the same concentration of salbutamol. The data suggest therefore that whilst the PDE III may be more significant in regulating airway tone in the absence of any exogenous stimulus to cyclic AMP accumulation, PDE IV activity may be more tightly coupled to the pool of adenylyl cyclase stimulated by β_2-adrenoceptor agonists.

The identification of PDE IV activity in inflammatory cells (Wright *et al.*, 1990; Souness *et al.*, 1991; Turner *et al.*, 1993) and bronchial smooth muscle (de Boer *et al.*, 1992; Cortijo *et al.*, 1993) has resulted in a growing interest in inhibitors of this isoenzyme as potential anti-asthma agents. The results of the present study show that the spasmolytic activity of the PDE IV inhibitor, rolipram, in guinea-pig trachea is potently enhanced by agents that elevate both cyclic AMP (salbutamol) and cyclic GMP (SNP). The synergistic interaction between rolipram and siguazodan or SNP and the lack of effect of SNP on siguazodan-induced relaxation further suggest that SNP exerts its potentiating effects through cyclic GMP inhibition of cyclic AMP hydrolysis by PDE III. Taken together these data and previous reports (de Boer *et al.*, 1992; Cortijo *et al.*, 1993) indicate that inhibitors of both PDE III and PDE IV or alternatively agents that inhibit PDE IV and elevate cyclic GMP, will provide a more effective mechanism for relaxation of airway smooth muscle than PDE IV inhibitors alone.

References

BEAVO, J.A. (1988). Multiple isozymes of cyclic nucleotide phosphodiesterase. In *Advances in Second Messenger and Phosphoprotein Research*, Vol 22. ed. Greengard, P. & Robinson, G.A. New York: Raven Press.

BEAVO, J.A. & REIFSNYDER, D.H. (1990). Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. *Trends Pharmacol. Sci.*, **11**, 150–155.

DE BOER, J., PHILPOTT, A.J., VAN AMSTERDAM, R.G.M., SHAHID, M., ZAAGSMA, J. & NICHOLSON, C.D. (1992). Human bronchial cyclic nucleotide phosphodiesterase isoenzymes: biochemical and pharmacological analysis using selective inhibitors. *Br. J. Pharmacol.*, **106**, 1028–1034.

BRADFORD, M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein using the principal of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

CORTIJO, J., BELETA, B.J., LLENAS, I.C.J., MORCILLO, E. & GRISTWOOD, R.W. (1993). Investigation into the role of phosphodiesterase IV in bronchorelaxation, including studies in human bronchus. *Br. J. Pharmacol.*, **108**, 562–568.

HALL, I.P., DONALDSON, J. & HILL, S.J. (1989). Inhibition of histamine-stimulated inositol phospholipid hydrolysis by agents which increase cyclic AMP levels in bovine tracheal smooth muscle. *Br. J. Pharmacol.*, **97**, 603–613.

HALL, I.P., DONALDSON, J. & HILL, S.J. (1990). Modulation of carbachol-induced inositol phosphate formation in bovine tracheal smooth muscle by cyclic AMP phosphodiesterase inhibitors. *Biochem. Pharmacol.*, **39**, 1357–1363.

HARRIS, A.L., CONNELL, M.J., FERGUSON, E.W., WALLACE, A.M., GORDON, R.J., PAGANI, E.D. & SILVER, P.J. (1989). Role of low Km cyclic AMP phosphodiesterase inhibition in tracheal relaxation and bronchodilatation in the guinea-pig. *J. Pharmacol. Exp. Ther.*, **251**, 199–206.

JIANG, H., COLBRAN, J.L., FRANCIS, S.H. & CORBIN, J.D. (1992). Direct evidence for cross-activation of cGMP-dependent protein kinase by cAMP in pig coronary arteries. *J. Biol. Chem.*, **267**, 1015–1019.

KOMAS, N., LUGNIER, C. & STOCLET, J.-C. (1991). Endothelium-dependent and independent relaxation of the rat aorta by cyclic nucleotide phosphodiesterase inhibitors. *Br. J. Pharmacol.*, **104**, 495–503.

LAU, Y.-S. & LUM, B.K.B. (1983). Role of cyclic AMP in adrenergically-induced tracheal smooth muscle relaxation. *Arch. Int. Pharmacodyn. Ther.*, **261**, 26–50.

LIVI, G.P., KMETZ, P., MCHALE, M.M., CIESLINSKI, L.B., SATHE, G.M., TAYLOR, D.P., DAVIS, R.L., TORPHY, T.J. & BALCAREK, J.M. (1990). Cloning and expression of cDNA for a human low-Km, rolipram-sensitive cyclic AMP phosphodiesterase. *Mol. Cell. Biol.*, **10**, 2678–2686.

LOHMANN, S., MIECH, R.P. & BUTLER, R.F. (1977). Effects of isoprenaline theophylline and carbachol on cyclic nucleotide levels and relaxation of bovine tracheal smooth muscle. *Biochim. Biophys. Acta*, **499**, 238–250.

MAURICE, D.H. & HASLAM, R.J. (1990). Nitroprusside enhances isoprenaline-induced increases in cAMP in rat aortic smooth muscle. *Eur. J. Pharmacol.*, **191**, 471–475.

MURRAY, K.J. (1990). Cyclic AMP and mechanisms of vasodilatation. *Pharmacol. Ther.*, **47**, 329–345.

MURRAY, K.J., EDEN, R.J., ENGLAND, P.J., DOLAN, J., GRIMSDITCH, D.C., STUTCHBURY, C.A., PATEL, B., REEVES, M.L., WORBY, A., TORPHY, T.J., WOOD, L.M., WARRINGTON, B.H. & COATES, W.J. (1991). Potential use of selective phosphodiesterase inhibition in the treatment of asthma. In *Agents and Actions Supplements*, Vol. 34. *New Drugs for Asthma Therapy*. ed. Anderson, G.P., Chapman, I.D. & Morley, J. pp. 27–46. Basel: Birkhanser Verlag.

NICHOLSON, C.D., CHALLIS, R.A.J. & SHAHID, M. (1991). Differential modulation of tissue function and therapeutic potential of selective inhibitors of cyclic nucleotide phosphodiesterase isoenzymes. *Trends Pharmacol. Sci.*, **12**, 19–27.

REEVES, M.L., GRISTWOOD, R.W., LEIGH, B.K. & ENGLAND, P.J. (1987). SK&F 94836, a potent ionotropic agent, is a selective inhibitor of type III phosphodiesterase activity from several tissues. *Br. J. Pharmacol.*, **92**, 773A.

SHAHID, M., VAN AMSTERDAM, R.G.M., DE BOER, J., TEN BERGE, R.E., NICHOLSON, C.D. & ZAAGSMA, J. (1991). The presence of five cyclic nucleotide phosphodiesterase isoenzyme activities in bovine tracheal smooth muscle and the functional effects of selective inhibitors. *Br. J. Pharmacol.*, **104**, 471–477.

SOUNESS, J.E., CARTER, C.M., DIOCEE, B.K., HASSAL, G.A., WOOD, L.J. & TURNER, N.C. (1991). Characterization of guinea-pig eosinophil phosphodiesterase activity. Assessment of its involvement in regulating superoxide generation. *Biochem. Pharmacol.*, **42**, 937–945.

TORPHY, T.J., BURMAN, M., HUANG, L.B.F. & TUCKER, S.S. (1988). Inhibition of the low km cyclic AMP phosphodiesterase in intact canine trachealis by SK&F 94836: mechanical and biochemical responses. *J. Pharmacol. Exp. Ther.*, **246**, 843–850.

TORPHY, T.J. & CIESLINSKI, L.B. (1990). Characterization and selective inhibition of cyclic nucleotide phosphodiesterase isoenzymes in canine tracheal smooth muscle. *Mol. Pharmacol.*, **37**, 206–214.

TORPHY, T.J. & UNDEM, B.J. (1991). Phosphodiesterase inhibitors: new opportunities for the treatment of asthma. *Thorax*, **46**, 512–523.

TORPHY, T.J., ZHENG, C., PETERSON, S.M., FISCUS, R.R., RINARD, G.A. & MAYER, S.E. (1985). Inhibitory effect of methacholine on drug-induced relaxation, cyclic AMP accumulation, and cyclic AMP-dependent protein kinase activation in canine tracheal smooth muscle. *J. Pharmacol. Exp. Ther.*, **233**, 409–417.

TORPHY, T.J., ZHOU, H.-L., BURMAN, M. & HUANG, L.B.F. (1991). Role of cyclic nucleotide phosphodiesterase isoenzymes in intact canine trachealis. *Mol. Pharmacol.*, **39**, 376–384.

TURNER, N.C., WOOD, L.J., BURNS, F.M., GUEREMY, T. & SOUNESS, J.E. (1993). The effect of cyclic AMP and cyclic GMP phosphodiesterase inhibitors on the superoxide burst of guinea-pig peritoneal macrophages. *Br. J. Pharmacol.*, **108**, 876–883.

WALDMAN, S.A. & MURAD, F. (1987). Cyclic GMP synthesis and function. *Pharmacol. Rev.*, **39**, 163–196.

WRIGHT, C.C., KNIPERS, P.J., KOBLARZ-SINGER, D., DEVALL, C.J., KLINKENFUS, B.A. & WEISHAAR, R.E. (1990). Differential inhibition of human neutrophil functions. Role of cyclic AMP specific GMP-insensitive phosphodiesterase. *Biochem. Pharmacol.*, **40**, 699–707.

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Cyclic GMP-independent relaxation and hyperpolarization with acetylcholine in guinea-pig coronary artery

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1 The effects of acetylcholine (ACh) on membrane potential, relaxation and cyclic GMP levels were compared to the NO donor L-nitrosocysteine (Cys-NO) in segments of guinea-pig coronary artery.

2 ACh and Cys-NO produced concentration-dependent relaxations of muscles contracted with the H_1 receptor agonist, 2-(2-aminoethyl)pyridine (AEP, 0.35 mM). The relaxation to ACh was unchanged in the presence of N^G-monomethyl-L-arginine (L-NMMA; 350 μM) or indomethacin (3 μM).

3 Oxyhaemoglobin (HbO; 20 μM) alone or in combination with L-NMMA increased the EC_{50} for ACh-induced relaxation whereas relaxation with Cys-NO was almost completely abolished with HbO.

4 Scorpion venom (SV; 8.7 μg ml^{-1}) increased the EC_{50} for relaxation with ACh but not Cys-NO. Combined L-NMMA, HbO and SV produced nearly complete abolition of ACh-induced relaxations.

5 Basal cyclic GMP levels (i.e., 20 pmol mg^{-1} protein) were significantly increased following addition of either ACh (190 pmol mg^{-1} protein) or Cys-NO (240 pmol mg^{-1} protein). L-NMMA significantly reduced the rise of cyclic GMP with ACh but not Cys-NO. In contrast, SV did not significantly reduce the rise in cyclic GMP produced with ACh. In the combined presence of L-NMMA and HbO neither ACh nor Cys-NO produced a significant increase in cyclic GMP levels.

6 ACh gave rise to significantly greater membrane hyperpolarization than Cys-NO both in the presence and absence of AEP. Combined L-NMMA and HbO did not reduce the amplitude of hyperpolarization with ACh.

7 These data indicate that some but not all of the actions of ACh in the coronary artery can be mimicked by the NO donor, Cys-NO, suggesting that ACh releases NO as well as a second hyperpolarizing factor (i.e., EDHF). Release of NO results in a large increase in tissue cyclic GMP levels and minimal change in membrane potential whereas release of EDHF results in a large membrane hyperpolarization which is independent of changes in tissue cyclic GMP levels. Both of these pathways appear to contribute to relaxation throughout the entire ACh concentration-relaxation relationship.

Keywords: EDHF; EDRF; relaxation; membrane potential; cyclic GMP; guinea-pig coronary artery; blood vessel; endothelium

Introduction

Acetylcholine (ACh) produces relaxation in blood vessels via an endothelium-dependent mechanism (Furchgott & Zawadzki, 1980; for review see Furchgott & Vanhoutte, 1989). An important factor involved in this response is nitric oxide (NO) which is released from the endothelium following the binding of ACh to muscarinic receptors (Hynes *et al.*, 1986; Dauphin & Hamel, 1990). NO diffuses to the adjacent vascular smooth muscle cells where it stimulates soluble guanylyl cyclase activity leading to increased cyclic GMP levels (Ignarro, 1989; Moncada *et al.*, 1991). Both the relaxation and enhanced guanylyl cyclase activity produced by ACh can be mimicked by addition of authentic NO (Gruetter *et al.*, 1981; Ignarro *et al.*, 1987).

ACh has also been reported to hyperpolarize smooth muscle cells in a number of different blood vessels via an endothelium-dependent mechanism (e.g., Komori & Suzuki, 1987; Taylor & Weston, 1988; Chen *et al.*, 1988; Keef & Bowen, 1989). However, in several blood vessels the ACh-induced hyperpolarization is not mimicked by NO (Bény & Brunet, 1988; Komori *et al.*, 1988; Brayden, 1990; Brayden *et al.*, 1991; Garland & McPherson, 1992) nor can hyperpolarization be blocked by manoeuvres which antagonize the NO pathway such as oxyhaemoglobin (Komori *et al.*, 1988; Chen *et al.*, 1988; Huang *et al.*, 1988; Nishiye *et al.*, 1989) arginine analogues (Nagao & Vanhoutte, 1991; Chen *et al.*, 1991; Garland & McPherson, 1992), and methylene blue (Komori *et al.*, 1988; Chen *et al.*, 1988; Brayden, 1990; Garland & McPherson, 1992). These observations have led to the proposal that a second factor, distinct from NO, is also released from the endothelium in response to ACh stimulation. This factor has been termed 'endothelium derived hyperpolarizing factor' or EDHF (Bény & Brunet, 1988; Chen *et al.*, 1988; Feletou & Vanhoutte, 1988; Taylor & Weston, 1988). Evidence has been presented to suggest that the hyperpolarization is due to the opening of K^+ channels in the smooth muscle membrane (Kitamura & Kuriyama, 1979; Brayden, 1990; Chen & Suzuki, 1990; Chen *et al.*, 1991; Eckman *et al.*, 1992; Garland & McPherson, 1992).

There is still considerable controversy regarding the contribution of EDHF to endothelium-dependent relaxation. In some blood vessels ACh-induced relaxation is almost completely abolished in the presence of antagonists of the NO pathway such as arginine analogues, oxyhaemoglobin and methylene blue (Martin *et al.*, 1985; Chen *et al.*, 1988; Huang *et al.*, 1988; Flavahan *et al.*, 1989; Rees *et al.*, 1990). In other studies the physiological relevance of EDHF is unclear since hyperpolarization has been reported to be partially or completely blocked without reducing the relaxation response to ACh (Komori & Suzuki, 1987; Feletou & Vanhoutte, 1988; Chen *et al.*, 1988; Komori & Vanhoutte, 1990; Nagao & Vanhoutte, 1991). Finally, in some studies it has been suggested that endothelium-dependent relaxation depends upon both NO and EDHF since partial inhibition of relaxation was obtained when either the NO or EDHF pathway was blocked (Brayden, 1990; Nagao & Vanhoutte, 1991; Suzuki *et al.*, 1992).

ACh produces significant endothelium-dependent hyper-

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polarization of the guinea-pig coronary artery (Kitamura & Kuriyama, 1979; Keef & Bowen, 1989; Chen et al., 1991; Eckman et al., 1992) and we have previously suggested that EDHF plays an important role in eliciting relaxation in this tissue (Keef & Bowen, 1989). To distinguish further the NO and the EDHF pathway in this blood vessel the effects of ACh on membrane potential, contractile tension and cyclic GMP levels were compared with the NO donor L-nitrosocysteine (Cys-NO).

Methods

Contractile experiments

Male albino guinea-pigs (350–550 g) were killed by CO_2 inhalation followed by exsanguination. The heart was immediately removed and placed in cold (10°C) oxygenated Krebs solution. A 1–1.5 cm segment of the left circumflex coronary artery was dissected out and cleaned of all adhering fat and myocardial tissue. Ring segments (3 mm long) were mounted onto two triangular tungsten wires (89 μm diameter) and hung vertically in an isolated organ chamber. The bottom triangle was mounted on a stable hook while the top triangle was attached to a Gould strain guage. The bath was maintained at 37°C in Krebs solution of the following composition (mM): NaCl 118.5, KCl 4.7, $MgCl_2$ 1.2, $NaHCO_3$ 23.8, KH_2PO_4 1.2, dextrose 11, $CaCl_2$ 2.5 and aerated with 95% O_2/5% CO_2.

A resting force of 0.3 g was applied to the guinea-pig coronary artery. In preliminary experiments this was found to stretch vessels to near the optimal length for tension development (i.e. L_o). Vessels were initially equilibrated for 2 h with alternating 4 min exposures every 15 min to the histamine H_1-receptor agonist, 2-(2-aminoethyl)pyridine (AEP 0.1–1 mM, Durant et al., 1975) and 100 mM KCl.

The experiments in which the effects of L-NMMA, HbO or scorpion venom were tested on contractile responses to ACh were performed in a sequential manner where each vessel segment served as its own control. On the other hand, experiments in which the effect of N^G-monomethyl-L-arginine (L-NMMA), oxyhaemoglobin (HbO) and scorpion venom were tested on responses to Cys-NO were undertaken in a parallel fashion where adjacent vessel segments from the same animal were used. This was done since it was often difficult to reverse completely the inhibition produced with high concentrations of Cys-NO. One artery segment therefore served as a control and the other was exposed to L-NMMA (350 μM) 20–30 min prior to contracting with AEP and addition of Cys-NO. To insure reproducibility further, each matched control and experimental tissue was exposed to the same stock solution of Cys-NO.

Cyclic GMP measurements

Cyclic GMP measurements were made on tissues under three different conditions including basal levels, during contraction with AEP and during relaxation with either ACh or Cys-NO (see inset to Figure 4). Artery segments were flash-frozen in liquid nitrogen while still attached to the stainless steel triangles. Cyclic GMP was assayed with commercially available reagents (Caymen Chemical Company, Ann Arbor, MI, U.S.A.). Samples were prepared for assay by homogenization in 6% TCA with glass Duall tissue grinders followed by extraction with water-saturated diethyl ether. Aqueous phases were then lyophilized to dryness and resuspended in 1.0 M potassium phosphate buffer (pH 7.4) before addition to duplicate microtiter plate wells. Cyclic GMP levels in samples and standards were detected following acetylation and competition between cyclic GMP and the acetylcholinesterase-linked cyclic GMP tracer for specific antiserum binding sites. The antiserum complex, linked to acetylcholinesterase, was used to cleave Ellman's reagent (5,5'-dithio-*bis*-(2-nitrobenzoic acid), and absorbance was measured at 412 nm. Cyclic GMP content of samples was determined from a standard curve constructed from determination of known amounts of cyclic GMP added to the plate. Levels of cyclic GMP are expressed as pmol cyclic GMP mg^{-1} protein (determined by method of Bradford, 1976). Duplicate variation in the cyclic GMP assay was less than 3%.

Intracellular measurements

For experiments measuring membrane potential, a 0.5–0.7 cm segment of left circumflex coronary artery was employed. Vessels rings were mounted onto two parallel wires and stretched to approximately 1.3–1.5 fold the resting diameter. All intracellular measurements were made through the adventitial surface with microelectrodes filled with 3 M KCl and having resistances between 70–100 MΩ. Intracellular measurements were made in the absence and presence of AEP-induced contractions. Impalements were judged on the basis of a rapid drop in potential upon entering the cell, a low noise level and minimal change in the electrode resistance and zero potential before and after impalement. Signals were viewed on a digital oscilloscope (Hitachi) and stored on tape with a Vetter PCM Recording Adapter attached to a video cassette recorder.

Statistics

Statistical significance was determined by the two-tailed paired or unpaired t test. The changes were considered significant at $P<0.05$. Data are expressed as mean ± s.e.mean, n values indicate the number of animals studied. The concentration of drug producing half maximal relaxation (i.e., EC_{50}) was calculated for each experiment by normalizing to the maximum amount of relaxation attained on that day. The software program InPlot (Graphpad software, Carlsbad, CA, U.S.A.) was used to calculate these EC_{50} values. This programme utilizes nonlinear regression according to the method of Marquardt (1963). However, the data in Figures 1–3 are plotted as a percentage of complete relaxation of the tissue so that the relative efficacy of the various drugs can be compared.

Drugs used

Acetylcholine HCl (ACh; Sigma, St. Louis, MO, U.S.A.), scorpion venom (*Leiurus quinquestriatus habraeus*, S.V; Sigma, St. Louis, MO, U.S.A.), 2-(2-aminoethyl)pyridine (AEP, Aldrich, Milwaukee, WI, U.S.A.), an indomethacin (Sigma), N^G-monomethyl-L-arginine, HOAc salt (L-NMMA, Calbiochem). L-Nitrosocysteine (Cys-NO) was made fresh daily according to Field et al. (1978). Haemolysate was made according to the method of Bowman & Gillespie (1982) and was stored at 2°C for up to 3 days. Since the active component of haemolysate is oxyhaemoglobin we have referred to haemolysate as oxyhaemoglobin (HbO) throughout the text. Three volume % of haemolysate solution was added to the bath to attain a final concentration of approximately 20 μM HbO.

Results

Contractile experiments

Comparison of the effect of N^G-monomethyl-L-arginine on acetylcholine- and L-nitrosocysteine-induced relaxations The concentration-relaxation relationships for ACh (0.0035–3.5 μM) and Cys-NO (0.0035–3.5 μM) were obtained by cumulative addition of drugs to segments of coronary artery contracted with AEP (100 μM). Both drugs were capable of producing full relaxation of the tissue and their potencies were very similar to one another (EC_{50} values -7.36 ± 0.12, $n = 10$ and -7.62 ± 0.16, $n = 8$ for ACh and

Cys-NO respectively, Figure 1). The ability of ACh and Cys-NO to relax vessels segments was also tested in the presence of the NO synthase (NOS) inhibitor, L-NMMA (350 μM, 25 min). This arginine analogue was used since it does not antagonize muscarinic receptors (Buxton *et al.*, 1993). The EC_{50} values for ACh and Cys-NO obtained in the presence of L-NMMA (i.e., -7.06 ± 0.13, $n = 10$ and -7.61 ± 0.11, $n = 9$ respectively) were not significantly different from their respective control values (Figure 1). The AEP-induced contraction was 137.5 ± 18.2% of control amplitude in the presence of L-NMMA suggesting that there is basal release of NO in this tissue.

Comparison of the effect of oxyhaemoglobin on acetylcholine- and L-nitrosocysteine-induced relaxations The concentration-relaxation relationships for ACh and Cys-NO were also measured following exposure to the NO scavenger, HbO (20 μM, 30 min). In the presence of HbO there was a small but significant shift to the right of the ACh relationship (-7.53 ± 0.06, $n = 18$ control versus -7.12 ± 0.08, $n = 18$ with HbO, Figure 2a). In contrast, relaxation with Cys-NO ($EC_{50} = -6.47 \pm 0.13$, $n = 12$) was almost completely abolished in the presence of HbO (Figure 2b) indicating that HbO effectively binds to the NO released from the NO donor Cys-NO. The AEP induced contraction was 158 ± 19% ($n = 12$) of control amplitude in the presence of HbO again suggesting that there is basal release of NO in this tissue.

The inability of either L-NMMA or HbO to diminsh substantially responses to ACh might be due to incomplete blockade of the NO pathway. To explore this possibility further, additional experiments were undertaken using combined L-NMMA (350 μM) and HbO (20 μM). The concentration relaxation relationship for ACh in the presence of L-NMMA and HbO was significantly to the right of the control relationship (-7.51 ± 0.13 control versus -7.04 ± 0.14, $n = 11$ with HbO and L-NMMA) but was not different from the responses observed with HbO alone (i.e., Figure 2a). These data suggest that ACh releases significant quantitities of NO since relaxations with ACh are reduced following blockade of the NO pathway. However, since relaxation is not abolished it also suggests that another factor, presumably EDHF, contributes to relaxations produced with each concentration of ACh.

Effect of scorpion venom on acetylcholine- and L-nitrosocysteine-induced relaxations We have previously reported that the crude scorpion venom (SV) from *Leiurus quinquestriatus hebraeus* (Eckman *et al.*, 1992) reduces the ACh-induced hyperpolarization in the guinea-pig coronary artery by 94% This venom contains a number of different substances known to block K^+ channels (Strong, 1990). In the present study we have investigated the effects of this venom on the concentration-relaxation relationships for ACh and Cys-NO. In the presence of SV (8.7 μg ml^{-1}, 30 min) the relaxation relationship for ACh was significantly shifted to the right (EC_{50}

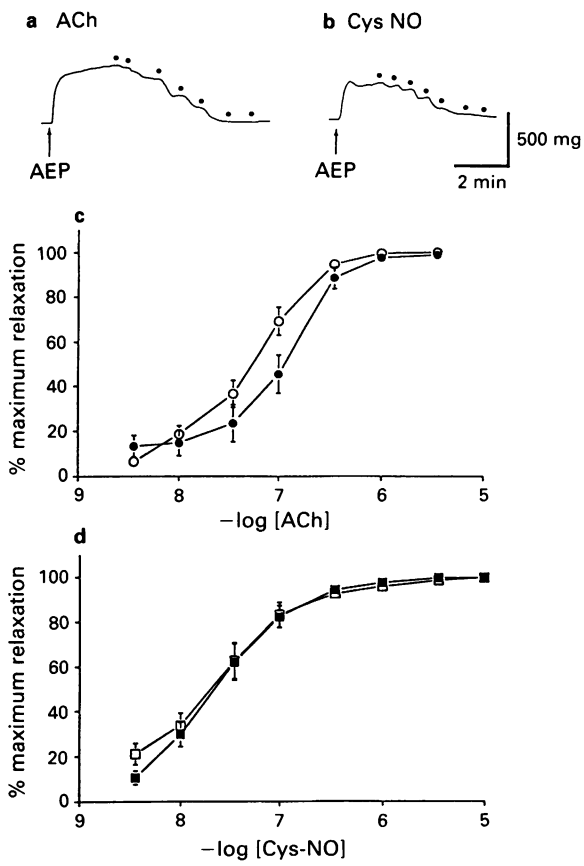


Figure 1 Comparison of the effects of N^G-monomethyl-L-arginine (L-NMMA, 350 μM) on the concentration-relaxation relationships for acetylcholine (ACh) and L-nitrosocysteine (Cys-NO). (a) and (b) Examples of the relaxation response to cumulative addition of either ACh (a) or Cys-NO (b) in two different blood vessels contracted with 2-(2-aminoethyl)pyridine (AEP). (c) Concentration-relaxation relationship for ACh in the absence (○) or presence (●) of L-NMMA. The EC_{50} values for ACh-induced relaxation were not significantly different (-7.36 ± 0.12, control versus -7.06 ± 0.13, $n = 10$, L-NMMA). (d) Concentration-relaxation relationship for Cys-NO in the absence (□) or presence (■) of L-NMMA. The EC_{50} values for Cys-NO induced relaxation (-7.62 ± 0.16, $n = 8$, control versus -7.61 ± 0.11, $n = 9$, L-NMMA) were not significantly different. Shown are mean values ± s.e.

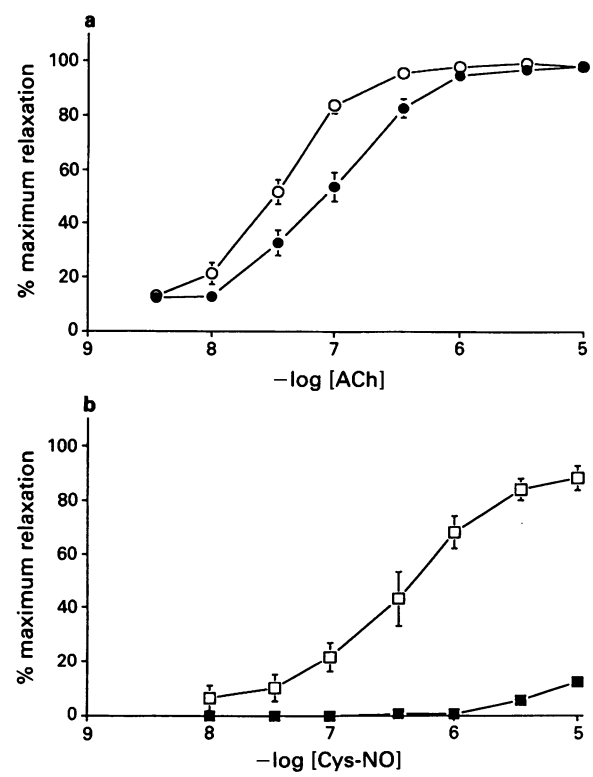


Figure 2 Comparison of the effects of oxyhaemoglobin (HbO, 20 μM) on the concentration-relaxation relationships for acetylcholine (ACh) and L-nitrosocysteine (Cys-NO). (a) Concentration-relaxation relationship for ACh in the absence (○) or presence (●) of HbO. The EC_{50} values for ACh-induced relaxation were significantly shifted to the right in the presence of HbO. (b) Concentration-relaxation relationship for Cys-NO in the absence (□) or presence (■) of HbO. The Cys-NO response was almost completely eliminated in the presence of HbO. Shown are mean values ± s.e.

-7.14 ± 0.11 control, $n = 9$, versus -6.39 ± 0.22 with SV, $n = 5$, Figure 3a) whereas responses with Cys-NO were unchanged (EC_{50} -7.49 ± 0.15 control versus -7.35 ± 0.06 with SV, $n = 4$, Figure 3b). Since SV significantly reduced responses to ACh it suggests that the EDHF pathway also contributes significantly to the ACh-induced relaxation.

Effect of combined N^G-monomethyl-L-arginine, oxyhaemoglobin and scorpion venom on acetylcholine-induced relaxation

Our results indicate that the ACh-induced relaxation persists in the presence of blockade of either the NO or the EDHF pathway. Experiments were therefore undertaken to determine the extent to which relaxation is reduced following combined blockade of both pathways. For these experiments tissues were bathed in L-NMMA (350 μM), HbO (20 μM) and SV (8.7 μg ml^{-1}) for 30 min before the concentration-relaxation relationship for ACh was measured. In the presence of all three blockers, relaxation with ACh was almost completely abolished ($n = 4$, Figure 3a). In contrast, both the control relaxation with ACh (-7.24 ± 0.08, $n = 14$) and the relaxation obtained in the presence of HbO and L-NMMA (-6.67 ± 0.15, $n = 5$) were unchanged in the presence of indomethacin (3 μM; -7.22 ± 0.25, $n = 4$ and -6.63 ± 0.11, $n = 5$, respectively).

Measurements of cyclic GMP levels

Relaxation of blood vessels by NO is known to involve stimulation of smooth muscle guanylyl cyclase activity leading to a rise in tissue cyclic GMP levels (Rapoport & Murad, 1983; Ignarro, 1989). Our results suggest that both NO and EDHF significantly contribute to relaxations induced with ACh. To assess directly the relationship of cyclic GMP to relaxations elicited with ACh and Cys-NO, experiments were undertaken in which cyclic GMP levels were measured in artery segments under the same conditions employed for contractile experiments (see methods).

Cyclic GMP levels in the tissue were measured at rest, during peak contraction with AEP and during relaxation with a concentration of ACh (0.35–1 μM) or Cys-NO (1–3.5 μM) which produced at least 90% maximum relaxation (see inset, Figure 4). Significant basal levels of cyclic GMP were detected in the absence of agonist (21 ± 5 pmol mg^{-1} protein, $n = 4$). During contraction with AEP, cyclic GMP levels were not significantly increased above the control level (33 ± 4 pmol mg^{-1} protein, $n = 4$). Upon application of ACh, cyclic GMP levels increased 5.7 fold ($n = 5$) above the level measured in the presence of AEP alone. Following application of Cys-NO, cyclic GMP levels were 7.5 fold greater ($n = 5$, Figure 4). Cyclic GMP levels in the presence of ACh and Cys-NO were not significantly different from one another.

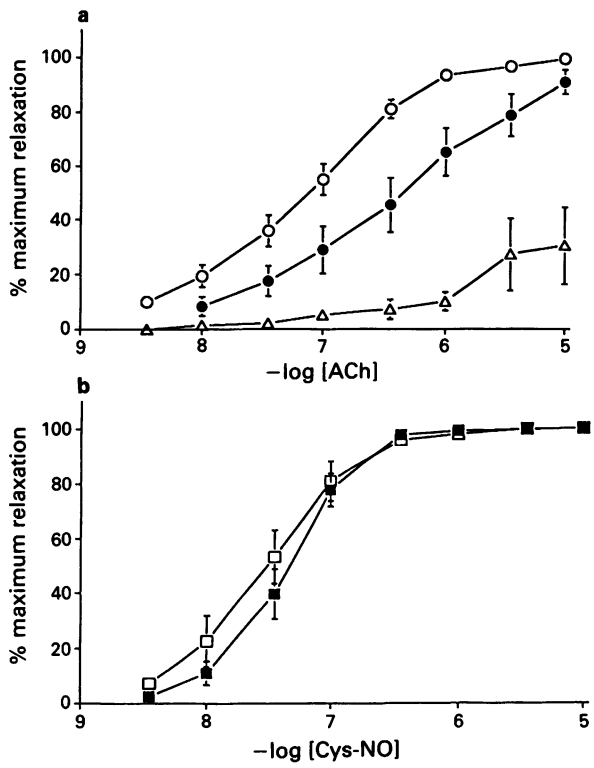


Figure 3 Comparison of the concentration-relaxation relationship for acetylcholine (ACh) and L-nitrosocysteine (Cys-NO) under various conditions. (a) ACh concentration-relaxation relationship in the absence (○) of blockers or following exposure to scorpion venom (SV, 8.7 μg ml^{-1}; ●) or combined SV, N^G-monomethyl-L-arginine (L-NMMA, 350 μM) and oxyhaemoglobin (HbO, 20 μM, △). The ACh response in the combined presence of SV, L-NMMA and HbO was nearly abolished. (b) Concentration-relaxation relationship for Cys-NO in the absence (□, $n = 4$) or presence (■) of SV. SV had no significant effect on this relationship. Shown are mean values ± s.e.

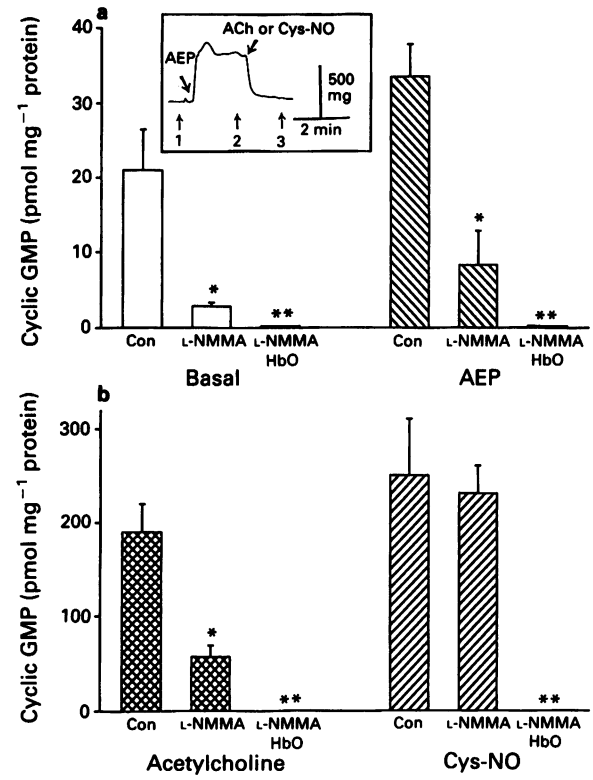


Figure 4 Cyclic GMP accumulation in guinea-pig coronary artery. Inset: Diagram of the conditions under which tissue cyclic GMP measurements are made. Shown is a representative contractile response to 2-(2-aminoethyl)pyridine (AEP, 100 μM) followed by relaxation with acetylcholine (ACh, 0.35 μM). Arrow (1) Basal GMP. These measurements were made in the absence of agonist on tissues equilibrated as described for other contractile experiments. Arrows (2) cyclic GMP during contraction. These measurements were made during peak contraction at an average time of 1.9 ± 0.1 min ($n = 15$) after addition of AEP. Arrow (3) cyclic GMP during relaxation. These measurements were made 1.3 ± 0.1 min ($n = 31$) after addition of either ACh or L-nitrosocysteine (Cys-NO) in the continued presence of AEP. All cyclic GMP measurements were made on endothelium intact vessel segments. (a) and (b) Cyclic GMP levels obtained either in the absence of blockers of the NO pathway (Con), in the presence of N^G-monomethyl-L-arginine (L-NMMA, 350 μM) or in the presence of combined L-NMMA and oxyhaemoglobin (HbO, 20 μM). L-NMMA significantly decreased cyclic GMP levels in basal, AEP and ACh tissues ($P < 0.05$) (a). In the presence of combined L-NMMA and HbO, cyclic GMP levels never exceeded 0.2 pmol mg^{-1} protein (i.e. less than 1% of basal control values) in all four experimental conditions. Asterisks indicate values which are significantly different from the control value of each group. Shown are mean values ± s.e.

The rise in cyclic GMP levels observed with ACh is probably due to release of NO. To provide direct evidence for this conclusion, additional experiments were undertaken in which NO synthase activity was inhibited with L-NMMA (350 μM, 30 min). In the presence of L-NMMA both the basal levels of cyclic GMP ($n = 4$) as well as the levels measured during contraction with AEP ($n = 5$) were significantly reduced. In addition, the ACh-induced increase in cyclic GMP was reduced 66% ($n = 5$) by L-NMMA. In contrast, there was no significant difference in the levels of cyclic GMP reached with Cys-NO in the presence ($n = 5$) or absence of L-NMMA (Figure 4). There was also no significant difference in the rise in cyclic GMP produced with ACh in the presence or absence of SV (8.7 μg ml^{-1}, $n = 4$, data not shown).

Although L-NMMA significantly reduced the rise in cyclic GMP levels elicited with ACh it did not abolish this action. To determine whether the L-NMMA resistant rise in cyclic GMP was due to incomplete blockade of NO synthase or to another mechanism, additional experiments were undertaken with combined L-NMMA (350 μM) and HbO (20 μM). Basal cyclic GMP levels in the presence of combined L-NMMA and HbO (30 min) were reduced to less than 1% of control ($n = 6$) and there was no significant increase in cyclic GMP levels upon application of either AEP ($n = 6$), ACh ($n = 5$) or Cys-NO ($n = 6$) (Figure 4). Since relaxation was still observed with ACh in the presence of combined L-NMMA and HbO our data suggest that a portion of the ACh-induced relaxation is independent of either NO or changes in cyclic GMP levels in the tissue.

Intracellular experiments

Comparison of the effects of acetylcholine and L-nitrosocysteine on membrane potential The results obtained from contractile experiments and measurements of cyclic GMP levels suggest that the actions of ACh are not identical to Cys-NO. To distinguish further between these two agents their effects on membrane potential were measured. The mean resting membrane potential of guinea-pig coronary artery cells was − 56 mV. Application of ACh (0.35 μM) hyperpolarized cells by 20 mV. In contrast, Cys-NO (3.5 μM) gave rise to a mean hyperpolarization of only 3.5 mV. Examples of these changes in membrane potential are shown in Figure 5 and results are summarized in Table 1.

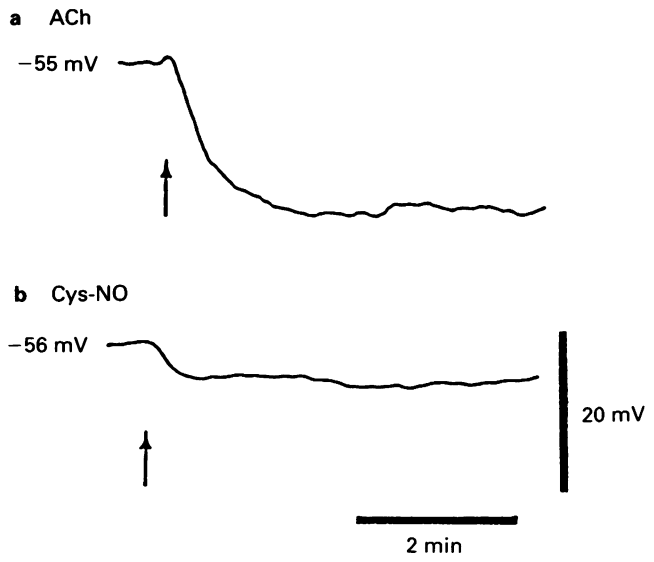


Figure 5 Representative examples of the effect of acetylcholine (ACh, 0.35 μM) and L-nitrosocysteine (Cys-NO, 3.5 μM) on resting membrane potential in two different cells in a segment of guinea-pig coronary artery. Each drug was added at the arrow followed by its respective hyperpolarization (downward deflection).

The effects of ACh and Cys-NO on membrane potential were also measured in tissues exposed to the contractile agonist AEP. AEP (100 μM) depolarized cells from a mean of − 56 mV to − 42 mV. Once a plateau of depolarization was reached either ACh or Cys-NO was included in the superfusate. ACh reversed the depolarization produced by AEP and hyperpolarized the tissue below the control level so that the total change in membrane potential with ACh in the presence of AEP (i.e., 33 mV) was greater than the change in membrane potential in the absence of AEP (i.e., 20 mV). In contrast, Cys-NO increased the polarization of the membrane by only 5 mV (see Table 1). This change in membrane potential was not significantly different from the change observed in the absence of AEP. In spite of the large difference in the effects of ACh and Cys-NO on membrane potential both of these agents relaxed tissues by at least 90%.

Effect of N^G-monomethyl-L-arginine and oxyhaemoglobin on acetylcholine-induced hyperpolarization In the presence of combined HbO and L-NMMA, ACh failed to produce a significant increase in cyclic GMP levels suggesting that this combination of drugs produces complete blockade of the NO pathway (Figure 4). However, under the same conditions there was only a small shift in the concentration-relaxation relationship for ACh. The relaxation which remains in the absence of an increase in cyclic GMP levels in the tissue is likely to be due to release of EDHF from the endothelium. To provide direct evidence for this hypothesis intracellular responses to ACh were measured in the presence of either L-NMMA (350 μM) or L-NMMA plus HbO (20 μM). A control response to ACh (0.35 μM) was obtained followed by a 15 min re-equilibration period in normal Krebs solution. The tissue was then superfused with L-NMMA or L-NMMA plus HbO. The resting membrane potential following 30 min exposure to either L-NMMA alone or L-NMMA plus HbO was not significantly different from control nor was there a significant reduction in the amplitude of hyperpolarization with ACh (Table 2). These data suggest that the ACh-

Table 1 Effect of acetylcholine (ACh) and L-nitrosocysteine (Cys-NO) on membrane potential in the presence and absence of 2-(2-aminoethyl)pyridine (AEP)

	E_m (mV)	n
Control	− 56 ± 1	45
	ΔE_m (mV)	n
ACh (0.35 μM)	20 ± 1	10
Cys-NO (3.5 μM)	3.5 ± 1	3
	E_m (mV)	n
AEP (100 μM)	− 42 ± 4	7
	ΔE_m (mV)	n
AEP + ACh (0.35 μM)	33 ± 6	4
AEP + Cys-NO (3.5 μM)	5 ± 3	3

E_m, membrane potential
ΔE_m (mV), change in E_m with the drugs indicated.

Table 2 Effect of acetylcholine (ACh) on membrane potential following blockade of the NO pathway

	E_m (mV)	*ACh* (0.35 μM) *hyperpolarization*	n
Control	− 56 ± 2	16 ± 1	8
L-NMMA (350 μM)	− 52 ± 2	16 ± 1	5
LNMMA + HbO (20 μM)	− 53 ± 3	17 ± 3	3

induced hyperpolarization is generated, in large part, by an NO-independent pathway.

Discussion

ACh elicits endothelium-dependent relaxation and hyperpolarization in the guinea-pig coronary artery (Kitamura & Kuriyama, 1979; Keef & Bowen, 1989; Chen *et al.*, 1991). The present study indicates that ACh also significantly increases cyclic GMP levels in this blood vessel. Our results suggest that the effects of ACh are due to the release of two distinctly different factors from the endothelium, namely, NO and EDHF. Although each factor is capable of producing complete relaxation of the tissue, the pathway by which they produce relaxation appears to differ significantly. Specifically, the NO pathway leads to an increase in tissue cyclic GMP levels and is relatively independent of changes in membrane potential whereas the EDHF pathway leads to membrane hyperpolarization and is independent of changes in cyclic GMP levels in the tissue.

The coronary artery exhibited significant basal levels of cyclic GMP. These levels were reduced to 12% of control with the arginine analogue by L-NMMA and to less than 1% of control with combined L-NMMA and HbO. The results suggest that basal cyclic GMP levels are almost exclusively due to spontaneous release of NO. The H_1 receptor agonist, AEP, was capable of eliciting contraction in the presence of these basal levels of cyclic GMP and interestingly, although H_1 receptors are also reported to be present on endothelial cells in some blood vessels (Chen & Suzuki, 1989; Malinowska & Schlicker, 1993) we did not observe a significant change in cyclic GMP levels when AEP was added to the tissue. Basal cyclic GMP levels appear to modulate contractions however, since significantly larger contractions were obtained in the presence of either L-NMMA or HbO.

To investigate the contribution of NO to the relaxation induced with ACh, experiments were undertaken with blockers of the NO pathway. L-NMMA did not shift the ACh concentration-relaxation relationship whereas some shift to the right was observed with combined HbO and L-NMMA. The results with L-NMMA alone are of particular interest since in some blood vessels this concentration of L-NMMA has been reported to produce almost complete abolition of the ACh-induced relaxation (e.g., rat aorta, Rees *et al.*, 1990). One possible explanation for the difference is that relaxation with ACh in the rat aorta is primarily due to NO whereas in the guinea-pig coronary artery EDHF also contributes significantly to relaxation. In fact, the limited effect which combined L-NMMA and HbO have on the ACh-induced relaxation relationship in guinea-pig coronary artery might lead one to speculate that only small quantities of NO are released upon exposure of the guinea-pig coronary artery to ACh. This is apparently not the case however since concentrations of ACh and Cys-NO which produced equivalent relaxation also produced equivalent increases in cyclic GMP levels and in both cases the rise in cyclic GMP was abolished with combined L-NMMA and HbO. The results therefore suggest that significant quantities of NO are released upon addition of ACh to the coronary artery. The fact that relaxation is only minimally reduced when the NO pathway is blocked must therefore be attributed to the actions of a second factor. The importance of both NO and EDHF to relaxation is further underscored by that fact that when either the NO or EDHF pathway is separately blocked only a small shift in the ACh concentration-relaxation relationship is observed whereas when both pathways are blocked almost complete abolition of relaxation is obtained.

The ACh-induced hyperpolarization was not significantly reduced in the presence of combined L-NMMA and HbO, suggesting that this response is largely dependent upon release of EDHF. A similar conclusion has been reached by others using various blockers of the NO pathway (Komori *et al.*, 1988; Chen *et al.*, 1988; Huang *et al.*, 1988; Nishiye *et al.*, 1989; Brayden, 1990; Nagao & Vanhoutte, 1991; Chen *et al.*, 1991; Garland & McPherson, 1992). Since combined L-NMMA and HbO entirely abolished the rise in cyclic GMP elicited with ACh our results also provide direct evidence that the EDHF induced hyperpolarization is independent of cyclic GMP. Interestingly, we observed that Cys-NO was also capable of producing a small but significant increase in membrane polarization. Nitric oxide induced hyperpolarization has been reported in some blood vessels (Garland & McPherson, 1992; Tare *et al.*, 1991; Kitamura *et al.*, 1993) but is absent in others (Bény & Brunet, 1988; Brayden, 1990; Brayden *et al.*, 1991; Komori *et al.*, 1988). Since the amplitude of the ACh-induced hyperpolarization before and after addition of combined L-NMMA and HbO in the guinea-pig coronary artery was not significantly different it suggests either that the NO which is released upon addition of ACh does not reach a concentration that is sufficient to affect membrane potential or alternatively that the actions of NO and EDHF on membrane potential combined in a manner which is less than additive so that removing the NO pathway does not lead to a detectable reduction in the ACh-induced hyperpolarization. Our experiments cannot distinguish between these two possibilities.

The hyperpolarization which occurs with ACh is likely to be due to the opening of K^+ channels in the smooth muscle membrane as previously suggested (Kitamura & Kuriyama, 1979; Brayden, 1990; Chen *et al.*, 1991; Eckman *et al.*, 1992; Garland & McPherson, 1992). In the rabbit basilar artery the ACh-induced hyperpolarization appears to involve the opening of ATP-dependent K^+ channels (Brayden *et al.*, 1991). However, in several other blood vessels including the guinea-pig coronary artery this does not appear to be the case since membrane hyperpolarization in these tissues is not blocked with glibenclamide, an antagonist of ATP-dependent K^+ channels (e.g., McPherson & Angus, 1991; Eckman *et al.*, 1992; for review see Edwards & Weston, 1993). Regardless of the K^+ channel involved, the relaxation induced by EDHF is likely to involve hyperpolarization-induced closing of voltage-dependent Ca^{2+} channels (Weir & Weston, 1986; Meisheri *et al.*, 1988; Standen *et al.*, 1989; Brayden *et al.*, 1991).

The most effective blocker of ACh-induced hyperpolarization in the guinea-pig coronary artery which we have observed to date is crude scorpion venom (SV). In a previous study we reported that this venom reduced the hyperpolarization elicited with ACh by 94% (Eckman *et al.*, 1992). Scorpion venom contains a number of different K^+ channel blockers (Strong, 1990) and we hypothesize that the effects of SV on hyperpolarization are due to the actions of one or more of these blockers on smooth muscle K^+ channels. Another possibility which cannot be excluded given the present results is that SV specifically antagonizes the synthesis or release of EDHF. However, in spite of this limitation, several lines of evidence suggest that the effects of SV are relatively specific for the EDHF pathway: (1) SV did not reduce the relaxant responses to Cys-NO indicating that the smooth muscle cells are still capable of relaxing and specifically of relaxing in response to NO; (2) SV reduced but did not abolish relaxations elicited with ACh indicating that some factor is still released from the endothelium in the presence of SV; (3) the relaxation which remains in the presence of SV is almost completely abolished when blockers of the NO pathway are also included suggesting that the relaxation which occurs in the presence of SV is due to NO. Point (3) is further supported by the observation that: (4) SV does not block the increase in cyclic GMP produced by ACh.

The relationship of NO and EDHF to the physiological control of coronary blood flow is still uncertain. Blood flow to the heart depends in large part upon local factors which serve to match the flow of blood to the work of the heart (Parratt, 1982). The precise mechanism of this matching is unknown but the endothelium may play an important role (Bigaud & Vatner, 1991). Our results suggest that the cor-

onary endothelium releases two distinctly different factors that are each capable of producing significant relaxation of the adjacent smooth muscle. Since the mechanism by which these two factors produce relaxation differ, their contribution to relaxation *in vivo* may also differ depending upon the circumstances which have led to contraction. If contraction involved the opening of voltage-dependent Ca^{2+} channels, then EDHF would be predicted to be an effective vasodilator. In contrast, under circumstances in which contraction involved a minimal change in Ca^{2+} entry (e.g., contraction induced with phorbol ester) NO would be predicted to be the more effective vasodilator. Since the chemical nature of 'EDHF' is not yet known, it is also possible that the distance over which NO and EDHF exert an effect could differ from one another or that their synthesis and release could be differentially regulated *in vivo*. Thus, although both NO and EDHF relax the coronary artery, the differences seen between these two factors may increase the effectiveness of regulating coronary blood flow under diverse metabolic conditions.

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References

BÉNY, J.-L. & BRUNET, P.C. (1988). Neither nitric oxide nor nitroglycerin accounts for all the characteristics of endothelially mediated vasodilation of pig coronary arteries. *Blood Vessels*, **25**, 308–311.

BIGAUD, M. & VATNER, S.F. (1991). Endothelium and blood flow mediated vasomotion in the conscious dog. *Basic Res. Cardiol.*, **86** (Suppl. II), 59–68.

BOWMAN, A. & GILLESPIE, J.S. (1982). Block of some non-adrenergic inhibitory responses of smooth muscle by a substance from hemolyzed erythrocytes. *J. Physiol.*, **328**, 11–25.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal. Biochem.*, **72**, 248–254.

BRAYDEN, J.E. (1990). Membrane hyperpolarization is a mechanism of endothelium-dependent cerebral vasodilation. *Am. J. Physiol.*, **259**, H668–H673.

BRAYDEN, J.E., QUAYLE, J.M., STANDEN, N.B. & NELSON, M.T. (1991). Role of potassium channels in the vascular response to endogenous and pharmacological vasodilators. *Blood Vessels*, **28**, 147–153.

BUXTON, I.L.O., CHEEK, D.J., ECKMAN, D., WESTFALL, D.P., SANDERS, K.M. & KEEF, K.D. (1993). N^G-nitro L-arginine methyl ester and other alkyl esters of arginine are muscarinic receptor antagonists. *Circ. Res.*, **72**, 387–395.

CHEN, G. & SUZUKI, H. (1989). Some electrical properties of the endothelium-dependent hyperpolarization recorded from rat arterial smooth muscle cells. *J. Physiol.*, **410**, 91–106.

CHEN, G. & SUZUKI, H. (1990). Endothelium-derived hyperpolarizing factor (EDHF): an endogenous potassium-channel activator. *News Physiol. Sci.*, **5**, 212–215.

CHEN, G., SUZUKI, H. & WESTON, A.H. (1988). Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. *Br. J. Pharmacol.*, **95**, 57–64.

CHEN, G., YAMAMOTO, Y., MIWA, K. & SUZUKI, H. (1991). Hyperpolarization of arterial smooth muscle induced by endothelial humoral substances. *Am. J. Physiol.*, **260**, H1888–H1892.

DAUPHIN, F. & HAMEL, E. (1990). Muscarinic receptor subtype mediating vasodilation in feline middle cerebral artery exhibits M_3 pharmacology. *Eur. J. Pharmacol.*, **178**, 203–213.

DURANT, G.J., GANELLIN, C.R. & PARSONS, M.E. (1975). Chemical differentiation of histamine H_1 and H_2 receptor agonists. *J. Med. Chem.*, **18**, 905–909.

ECKMAN, D.M., FRANKOVICH, J.D. & KEEF, K.D. (1992). Comparison of the action of acetylcholine and BRL 38227 in the guinea-pig coronary artery. *Br. J. Pharmacol.*, **106**, 9–16.

EDWARDS, G. & WESTON, A.H. (1993). The pharmacology of ATP-sensitive potassium channels. *Annu. Rev. Pharmacol. Toxicol.*, **33**, 597–637.

FELETOU, M. & VANHOUTTE, P.M. (1988). Endothelium-dependent hyperpolarization of canine coronary smooth muscle. *Br. J. Pharmacol.*, **93**, 515–524.

FIELD, L., DILTS, R.V., RAVICHANDRAN, R., LENHERT, P.G. & CARNAHAN, G.E. (1978). An unusually stable thionitrite from N-Acetyl-D,L-penicillamine; X-Ray crystal and molecular structure of 2-(acetylamino)-2-carboxy-1,1-dimethyl thionitrite. *J.C.S. Chem. Commun.*, **6**, 249–250.

FLAVAHAN, N.A., SHIMOKAWA, H. & VANHOUTTE, P.M. (1989). Pertussis toxin inhibits endothelium-dependent relaxations to certain agonists in porcine coronary arteries. *J. Physiol.*, **408**, 549–560.

FURCHGOTT, R.F. & VANHOUTTE, P.M. (1989). Endothelium-derived relaxing and contracting factors. *FASEB J.*, **3**, 2007–2018.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle of acetylcholine. *Nature*, **288**, 373–376.

GARLAND, C.J. & MCPHERSON, G.A. (1992). Evidence that nitric oxide does not mediate the hyperpolarization and relaxation to acetylcholine in the rat small mesenteric artery. *Br. J. Pharmacol.*, **105**, 429–443.

GRUETTER, C.A., GRUETTER, D.Y., LYON, J.E., KADOWTIZ, P.J. & IGNARRO, L.J. (1981). Relationship between cyclic GMP formation and relaxation of coronary arterial smooth muscle by glyceryl trinitrate, nitroprusside, nitrite, and nitric oxide: Effects of methylene blue and methemoglobin. *J. Pharmacol. Exp. Ther.*, **219**, 181–186.

HUANG, A.H., BUSSE, R. & BASSENGE, E. (1988). Endothelium-dependent hyperpolarization of smooth muscle cells in rabbit femoral arteries is not mediated by EDRF (nitric oxide). *Naunyn-Schmied. Arch. Pharmacol.*, **338**, 438–442.

HYNES, M.R., BANNER, W.J., YAMAMURA, H.I. & DUCKLES, P.S. (1986). Characterization of muscarinic receptors of the rabbit ear artery smooth muscle and endothelium. *J. Pharmacol. Exp. Ther.*, **238**, 100–105.

IGNARRO, L.J. (1989). Endothelium-derived nitric oxide: actions and properties. *FASEB J.*, **3**, 31–36.

IGNARRO, L.J., BYRNS, R.E. & WOOD, K.S. (1987). Endothelium-dependent modulation of cGMP levels and intrinsic smooth muscle tone in isolated bovine intrapulmonary artery and vein. *Circ. Res.*, **60**, 82–90.

KEEF, K.D. & BOWEN, S.M. (1989). Effect of ACh on electrical and mechanical activity in guinea pig coronary arteries. *Am. J. Physiol.*, **257**, H1096–H1103.

KITAMURA, K. & KURIYAMA, H. (1979). Effect of acetylcholine on the smooth muscle cell of isolated main coronary artery of the guinea pig. *J. Physiol.*, **293**, 119–133.

KITAMURA, K., LIAN, Q., CARL, A. & KURIYAMA, H. (1993). S-nitrosocysteine, but not sodium nitroprusside, produces apamin-sensitive hyperpolarization in rat gastric fundus. *Br. J. Pharmacol.*, **109**, 415–423.

KOMORI, K., LORENZ, R.R. & VANHOUTTE, P.M. (1988). Nitric oxide, ACh and electrical and mechanical properties of canine arterial smooth muscle. *Am. J. Physiol.*, **255**, H207–H212.

KOMORI, K. & SUZUKI, H. (1987). Heterogenous distribution of muscarinic receptors in rabbit saphenous artery. *Br. J. Pharmacol.*, **92**, 657–664.

KOMORI, K. & VANHOUTTE, P.M. (1990). Endothelium-derived hyperpolarizing factor. *Blood Vessels*, **27**, 238–245.

MALINOWSKA, B. & SCHLICKER, E. (1993). Identification of endothelial H_1, vascular H_2, and cardiac presynaptic H_3 receptors in the pithed rat. *Naunyn-Schmied. Arch. Pharmacol.*, **347**, 55–60.

MARTIN, W., VILLANI, G.M., JOTHIANDAN, D. & FURCHGOTT, R.F. (1985). Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. *J. Pharmacol. Exp. Ther.*, **232**, 708–716.

MARQUART, D.W. (1963). An algorithm for least-squares estimation of nonlinear parameters. *J. Soc. Indust. Appl. Math.*, **11**, 431–441.

MCPHERSON, G.A. & ANGUS, J.A. (1991). Evidence that acetylcholine-mediated hyperpolarization of the rat small mesenteric artery does not involve the K^+ channel opened by cromakalim. *Br. J. Pharmacol.*, **103**, 1184–1190.

MEISHERI, K.D., CIPKUS, L.A. & TAYLOR, C.J. (1988). Mechanism of action of minoxidil sulfate-induced vasodilation: a role for increased K^+ permeability. *J. Pharmacol. Exp. Ther.*, **245**, 751–760.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.

NAGAO, T. & VANHOUTTE, P.M. (1991). Hyperpolarization contributes to endothelium-dependent relaxations to acetylcholine in femoral veins of rats. *Am. J. Physiol.*, **261**, H1034–H1037.

NISHIYE, E., NAKAO, K., ITOH, T. & KURIYAMA, H. (1989). Factors inducing endothelium-dependent relaxation in the guinea-pig basilar artery as estimated from the actions of haemoglobin. *Br. J. Pharmacol.*, **96**, 645–655.

PARRATT, J.R. (1982). Myocardial metabolic activity as a determinant of coronary-vessel tone. In *The Coronary Artery*, ed. Kalsner pp. 309–339. New York: Oxford University Press.

RAPOPORT, R.M. & MURAD, F. (1983). Agonist-induced endothelium-dependent relaxation in rat thoracic aorta may be mediated through cGMP. *Circ. Res.*, **52**, 352–357.

REES, D.D., PALMER, R.M.J., SCHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **101**, 746–752.

STANDEN, N.B., QUAYL, J.M., DAVIES, N.W., BRAYDEN, J.E., HUANG, Y. & NELSON, M.T. (1989). Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science*, **245**, 177–180.

STRONG, P.N. (1990). Potassium channel toxins. *Pharmacol. Ther.*, **46**, 137–162.

SUZUKI, H., CHEN, G., YAMAMOTO, Y. & MIWA, K. (1992). Nitroarginine-sensitive and -insensitive components of the endothelium-dependent relaxation in the guinea-pig carotid artery. *Jpn. J. Physiol.*, **42**, 335–347.

TARE, M., PARKINGTON, H.C., COLEMAN, H.A., NEILD, T.O. & DUSTING, G.J. (1991). Nitric oxide hyperpolarizes arterial smooth muscle. *J. Cardiovasc. Pharmacol.*, **17** (Suppl. 3), S108–S112.

TAYLOR, S.G. & WESTON, A.H. (1988). Endothelium-derived hyperpolarizing factor: a new endogenous inhibitor from the vascular endothelium. *Trends Pharmacol. Sci.*, **9**, 272–274.

WEIR, S.W. & WESTON, A.H. (1986). The effects of BRL 34915 and nicorandil on electrical and mechanical activity and on ^{86}Rb efflux in rat blood vessels. *Br. J. Pharmacol.*, **88**, 121–128.

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Depression of high-threshold calcium currents by activation of human D_2 (short) dopamine receptors expressed in differentiated NG108-15 cells

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1 This study examined the regulation of calcium currents in differentiated NG108-15 cells that had been stably transfected with cDNA encoding the short isoform of the human D_2 dopamine receptor. Whole cell calcium currents were recorded by nystatin-perforated patch clamp recording.

2 Transient low-threshold calcium currents elicited by depolarizations from −100 mV to −20 mV were reversibly depressed by $NiCl_2$ (84 ± 8% at 30 μM; $n = 3$) and by ω-agatoxin IVA (15 ± 5%; 100 nM, $n = 7$). These currents were unaffected by hD_2 receptor activation.

3 High-threshold calcium currents elicited by depolarizations from −80 mV to 0 mV were partly blocked by ω-conotoxin GVIA (67 ± 6% at 100 nM, $n = 4$) and by the subsequent addition of the dihydropyridine, nisoldipine (94 ± 3% at 1 μM). Consistent with the presence of at least two distinct types of high-threshold calcium channels, nisoldipine alone (38 ± 15% at 1 μM, $n = 6$) did not preclude the inhibition caused by ω-conotoxin GVIA (69 ± 13% at 100 nM, $n = 4$). The residual current was completely blocked by 100 μM $CdCl_2$ (98.8 ± 0.4%, $n = 7$).

4 In hD_2-transfected cells, but not untransfected cells, high-threshold currents were depressed by quinpirole (30 ± 4% at 100 nM; $n = 15$) with a pEC_{50} of 8.61 ± 0.22 ($n = 5$), as well as by (−)-noradrenaline (28 ± 5% at 1 μM, $n = 9$). Responses to both agonists were selectively antagonized by S-(−)sulpiride (100 nM) but not by the α-adrenoceptor antagonist, phentolamine (10 μM). The depression caused by (−)-noradrenaline was positively correlated with that of quinpirole for each cell ($r^2 = 0.91$, slope = 0.99).

5 hD_2-receptor-mediated inhibition of high-threshold calcium currents was abolished by pretreatment of cells with ω-conotoxin GVIA (100 nM; $n = 4$). However, a component of the high-threshold current was reversibly depressed by ω-conotoxin GVIA (67% to 45% depression after 10 min wash). This current was also depressed by hD_2 receptor activation (59 ± 9% depression in 100 nM quinpirole, $n = 3$), and was completely blocked by nisoldipine (95 ± 2% at 1 μM).

6 These data demonstrate that activation of hD_2(short) dopamine receptors can regulate both ω-conotoxin GVIA, and dihydropyridine-sensitive high-threshold calcium currents in neuroblastoma cells. Morever, the ability of human D_2 dopamine receptors to regulate more than one type of calcium current supports the notion that these receptors have a diverse functional role in the central nervous system.

Keywords: D_2 dopamine receptor; quinpirole; nisoldipine; NG108-15 cells; sulpiride; ω-agatoxin IVA; ω-conotoxin GVIA; calcium currents

Introduction

Several studies have demonstrated that G-protein linked receptors can regulate the function of calcium currents in neuronal cell lines and that these include endogenous D_2-like dopamine receptors (Stack & Suprenant 1991; Keja *et al.*, 1992; Lledo *et al.*, 1992; Nussinovitch & Kleinhaus, 1992). Neurotransmitter regulation of calcium channel function is known to involve several G-protein types, which include the α subunits of $G_{o1\&2}$ and G_i, as well as in some cases their β and γ subunit counterparts (reviewed in Hille 1992; Kleuss *et al.*, 1993). The specificity, and promiscuity of G-proteins for different calcium channel subtypes is only just starting to be elucidated and it appears that in some neurones, such as those of sympathetic ganglia (Bernheim *et al.*, 1991; Beech *et al.*, 1992; Mathie *et al.*, 1992), subpopulations of calcium currents can be regulated by different second messenger systems.

Calcium channels have been conventionally classifed into L-, N-, P- and T-types according to their pharmacological and biophysical characteristics (reviewed in McCleskey *et al.*, 1986; Tsien *et al.*, 1988; Spedding & Paoletti, 1992). Recent molecular cloning techniques have revealed the existence of a much greater diversity of structurally distinct calcium channel types. At least five discrete families of the pore forming alpha subunit have been identified, which contain at least 13 different types (Snutch & Reiner, 1992). Of these some, but not all, have typical pharmacological profiles that include dihydropyridine-, or ω-conotoxin GVIA-sensitivity. The differences in regional distributions of these calcium channel types, as well as their divergent biophysical properties suggest that they subserve different cellular roles. Thus it is important to understand not only how neurotransmitter receptors can activate second messengers systems, but also how these systems influence the function of pharmacologically distinct calcium channel types.

At least five structural types of dopamine receptor have been identified (reviewed in Schwartz *et al.*, 1992; Sibley & Monsma, 1992), and expression studies have demonstrated that they can couple to different second messenger systems. D_1 and D_5 receptors couple to the activation of adenylate cyclase (Zhou *et al.*, 1990; Dearry *et al.*, 1990; Sunahara *et al.*, 1991), whereas activation of D_2, D_3 and D_4 receptors can

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inhibit adenylate cyclase activity (Freedman *et al.*, 1994; and unpublished observations). We have recently shown that activation of human D_3 dopamine receptors can regulate calcium currents when expressed in differentiated NG108-15 cells, but not in undifferentiated NG108-15 cells or GH_4C_1 cells (Seabrook *et al.*, 1992; 1994). Differentiated NG108-15 cells are known to have at least three types of calcium current (Fishman & Spector, 1981; Bodewei *et al.*, 1985; Hering *et al.*, 1985; Docherty, 1988; Kasai & Neher,1992), as well as an increased diversity of G-proteins (McFadzean *et al.*, 1989; Milligan, 1989; Mullaney & Milligan, 1990). To understand further the mechanism(s) by which human dopamine receptors may regulate calcium channels, this study investigated whether heterologously expressed human D_2 (short) receptors can also couple to calcium currents in this cell line and, if so, to identify the type of calcium currents involved.

Methods

Cell culture and differentiation procedures

NG108-15 cells were grown in tissue culture in a medium that contained Dulbecco's Modified Eagles Medium (GIBCO), 10% foetal calf serum, 2 mM L-glutamine (Geneticin), and were plated on poly-L-lysine coated coverslips. Cells were differentiated for 3 to 5 days using prostaglandin E_1 (10 μM) and isobutylmethylxanthine (50 μM) as previously described (Docherty, 1988). Human D_2 (short) receptor cDNA was subcloned into the mammalian expression vector pcDNAneo (Invitrogen) as described previously by McAllister *et al.* (1993). Stably transfected cell lines were obtained by transfecting this cDNA into NG108-15 cells by a standard calcium phosphate method (Cullen, 1987). Transfected cells were selected for their resistance to the antibiotic G418 and assayed for their ability to bind [^{125}I]-iodosulpiride. The identity of the receptor mRNA expressed in the cell line used for functional assays was confirmed using PCR analysis (e.g. McAllister *et al.*, 1993) and radioligand binding (e.g. Freedman *et al.*, 1994).

Nystatin perforated patch clamp recording

Nystatin perforated patch clamp recordings were made using borosilicate glass microelectrodes (Clark Electromedical Instruments), as previously described (Horn & Marty, 1988; Caulfield & Brown, 1991), using a pipette solution that contained (in mM); CH_3O_3SCs 100, CsCl 25, $MgCl_2$, HEPES 40, adjusted to pH 7.3 with CsOH. Cells were perfused at a rate of 1 to 2 ml min^{-1} with an extracellular solution that contained (in mM); NaCl 155, glucose 10, HEPES 10, KCl 5, $CaCl_2$ 5, and tetrodotoxin 0.5 μM, pH 7.4, at 22°C. Drugs were applied via the superfusion system. Cells were voltage-clamped at either − 80 mV or − 100 mV, and calcium currents were elicited by 100 ms depolarizing pulses to − 20 mV or 0 mV at a frequency of 0.033 Hz. Calcium currents were amplified with an Axopatch 200 patch clamp amplifier (Axon Instruments), and captured on line via a CED1401 interface (Cambridge Electronic Design) which was connected to a Compaq 386 microcomputer. Steady-state leak currents were subtracted with the amplifier, and voltage-step leak currents by the CED voltage-clamp analysis software. Data are expressed as the mean ± s.e.mean. Where indicated, the mean was determined from cells which responded with > 5% depression in the peak calcium current amplitude, cited along with the proportion of cells responding in each sample. Unless stated all percentage measurements are relative to the peak calcium current in control conditions.

Drugs

Drugs were applied in the superfusion medium in known concentrations, reaching the recording chamber after a delay of 20–30 s. The following drugs were used: (−)-noradrenaline (Arterenol), synthetic ω-conotoxin GVIA (Sigma), ω-agatoxin-IVA (Peptide Institute, Inc., Japan), (−)-quinpirole hydrochloride, S-(−)-sulpiride (Research Biochemicals, Inc., U.S.A.), and phentolamine mesylate (Ciba).

Results

Characterization of transfected cells

Transfected cells were assayed for their resistance to the antibiotic G418 (see Methods) and then assayed for their ability to bind [^{125}I]-iodosulpiride. Of ten clones examined all exhibited specific [^{125}I]-iodosulpiride binding, whereas, no specific binding was observed in the untransfected and differentiated NG108-15 cell line that was used for transfection studies. The clonal cell line selected for functional assays, clone Y, exhibited agonist binding (1 nM [^{3}H]-N-0437) that was selectively reduced by 70% following incubation with guanosine-5′-*O*-3-thiotri-phosphate (10 μM), but not by adenosine-5′-*O*-3-thiotri-phosphate (10 μM). This was considered to be indicative of the functional association of G-protein(s) with the hD_2 receptor (e.g. McAllister *et al.*, 1993), and was correlated with the ability of quinpirole to inhibit forskolin-stimulated adenylate cyclase activity in these cells. Experiments were carried out to confirm that the hD_2-NG108-15 cells were expressing the transfected hD_2 receptor mRNA, and not that of an endogenous rat-like D_3 receptor, by probing Southern blots with ^{32}P radiolabelled probes specific to D_2 and D_3 receptors (data not shown). In the cell line chosen for transfection studies, no effects of dopamine, quinpirole, or noradrenaline were observed on high-threshold calcium currents in differentiated cells (Seabrook *et al.*, 1994), consistent with absence of endogenous catecholamine receptors that might regulate these currents.

Depression by calcium channel antagonists

At least three types of calcium current were identified in hD_2 transfected NG108-15 cells. These included transient low-threshold calcium currents elicited by depolarizations from − 100 or − 80 mV to − 20 mV, and non-inactivating high-threshold currents elicited by depolarizations from − 80 or − 60 mV to 0 mV. Low-threshold currents were depressed by application of $NiCl_2$ (84 ± 8% at 30 μM; $n = 3$), and reversibly by ω-agatoxin IVA (15 ± 5% at 100 nM; $n = 7$). Block of these transient currents with $NiCl_2$ precluded the inhibition of ω-agatoxin IVA (Figure 1). In contrast, high-threshold calcium currents were unaffected by application of ω-agatoxin IVA (100 nM) but were partly blocked by ω-conotoxin GVIA (67 ± 6% at 100 nM, $n = 4$). The subsequent addition of the dihydropyridine, nisoldipine (1 μM) decreased the peak calcium current by 94 ± 3% compared to control values (see Figure 4). In a separate series of experiments, nisoldipine alone (38 ± 15% at 1 μM, $n = 6$) did not prevent the inhibition by subsequent addition of ω-conotoxin GVIA (69 ± 13% at 100 nM, $n = 4$). This was consistent with the presence of at least two pharmacologically distinct types of high-threshold calcium channels. The residual high-threshold calcium currents recorded in the presence of nisoldipine and ω-conotoxin GVIA was completely blocked by 100 μM $CdCl_2$ (98.8 ± 0.4%, $n = 7$).

hD_2 receptor mediated depression of high-threshold calcium currents

In differentiated hD_2-NG108-15 cells, the dopamine receptor agonist, quinpirole (0.01 nM to 1 μM), depressed high-thres-

hold calcium currents in two distinct ways. Low concentrations of quinpirole (<1 nM) caused a transient depression that desensitized upon sustained agonist application in some cells, whereas higher concentrations (>1 nM) caused the sustained inhibition of whole cell currents. The half-maximally effective concentration of quinpirole which caused sustained calcium current inhibition was 3 nM ($pEC_{50} = 8.61 \pm 0.22$, $n = 5$; Figure 2). Only part of the peak calcium current in any one cell was affected by hD_2 receptor activation. The maximal depression of calcium currents by quinpirole (100 nM) was $30 \pm 4\%$ ($n = 15$). These effects of quinpirole were completely, and reversibly antagonized by S-(−)-sulpiride (100 nM; Figure 3).

In contrast to the effects of quinpirole on high-threshold currents, low-threshold transient currents elicited by depolarizations from a membrane potential of −100 mV to −20 mV were unaffected by hD_2 receptor activation (Figure 2b). Similarly, morphologically undifferentiated hD_2-NG108-15 cells that had not been treated with IBMX or PGE_1 (see Methods) predominantly expressed low-threshold transient calcium currents ('T-type'), and calcium currents in these cells were likewise unaffected by hD_2 receptor activation (data not shown).

In differentiated hD_2-NG108-15 cells, high-threshold calcium currents were also depressed by (−)-noradrenaline ($28 \pm 5\%$ at 1 μM; $n = 9$), however, this effect was mediated by the direct activation of the transfected D_2 dopamine receptors. The magnitude of the depression achieved with (−)-noradrenaline (1 μM) was positively correlated with the maximum percentage depression seen with quinpirole in each cell ($r^2 = 0.91$, slope = 0.99, $n = 7$). Furthermore, the effect of (−)-noradrenaline, like that of quinpirole, was selectively antagonized by S-(−)-sulpiride and not by phentolamine (Figure 3).

The inhibition of high-threshold calcium currents by quinpirole (100 nM) was abolished by pretreatment of cells with 100 nM ω-conotoxin GVIA ($n = 4$; Figure 4). However, a component of the high-threshold current was reversibly depressed by ω-conotoxin (67% to 45% depression after 10 min wash). This current was also depressed following hD_2 receptor activation ($59 \pm 9\%$ depression in 100 nM quinpirole, $n = 3$), and was completely blocked by nisoldipine ($95 \pm 2\%$ at 1 μM).

Discussion

Calcium currents in NG108-15 cells can be modulated by the activation of a variety of G-protein linked neurotransmitter

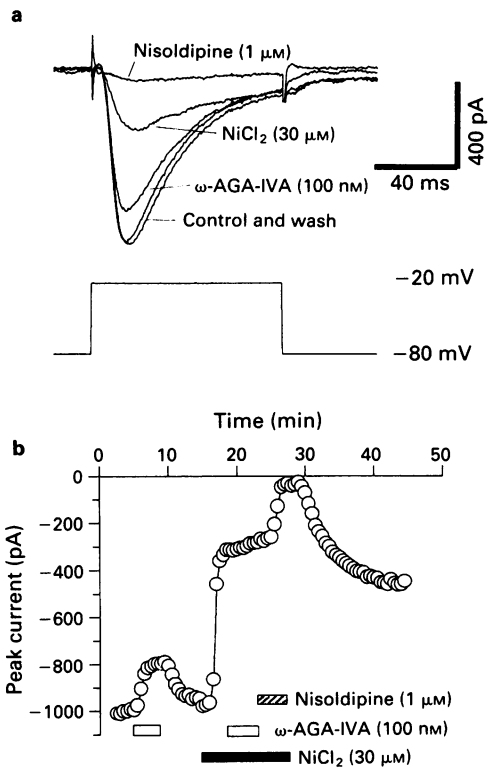


Figure 1 Depression of transient low-threshold calcium currents by calcium channel antagonists. (a) Superimposed calcium currents elicited by 100 ms depolarizations from a membrane potential of −80 mV to −20 mV. ω-Agatoxin IVA (ω-AGA-IVA) (100 nM) caused a reversible depression of these transient calcium currents, which were also blocked by $NiCl_2$ (30 μM). In this cell a residual current that was insensitive to $NiCl_2$ but blocked by nisoldipine was also observed. This residual dihydropyridine-sensitive current was insensitive to ω-agatoxin IVA. In contrast to the effects of ω-agatoxin IVA on transient currents which were fully reversible (Control & wash), the depression caused by nisoldipine was only partly reversible even after 30 min. (b) Time-course of the inhibition of low-threshold calcium currents by calcium channel antagonists in the same cell as in (a). ω-Agatoxin IVA (open bar), $NiCl_2$ (solid bar), and nisoldipine (hatched bar) were applied for the duration of the horizontal lines. Note that after block of low-threshold currents by $NiCl_2$ no further depression was seen with ω-agatoxin IVA.

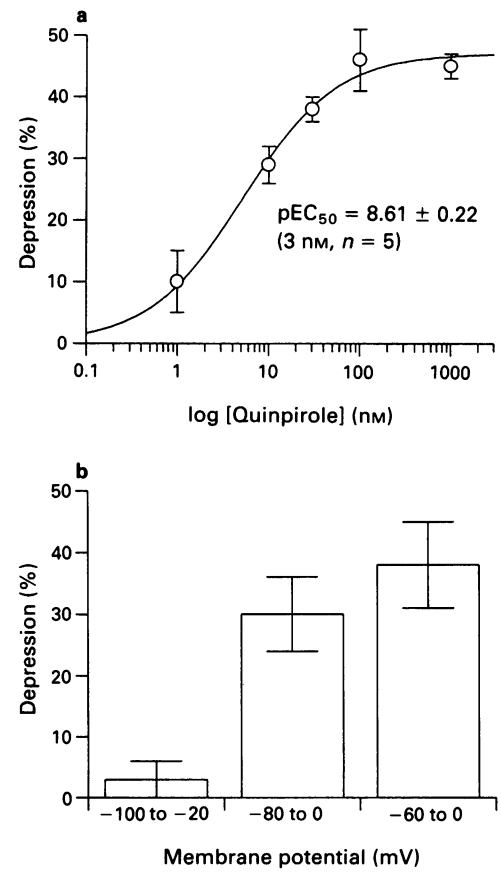


Figure 2 Depression of calcium currents by quinpirole in differentiated hD_2-NG108-15 cells. (a) Concentration-effect curve for the inhibition of the peak calcium current by quinpirole. Each data point represents the mean ± s.e.mean from five cells in which full concentration-effect data were determined. Calcium currents were elicited by 100 ms depolarizations from −80 mV to 0 mV. The log of the half-maximally effective concentration (pEC_{50}) was determined separately for each cell, and this was used to determine the geometric mean and standard error. (b) Comparison of the effects of quinpirole (100 nM) upon calcium currents elicited by depolarizations from different holding and step potentials ($n = 5$ cells). Low-threshold currents, elicited by depolarizations from −100 to −20 mV, were unaffected by hD_2 receptor activation.

receptors. These include T-type currents which are depressed by angiotensin AT_2 receptor activation (Buisson *et al.*, 1992), and ω-conotoxin GVIA-sensitive high-threshold calcium currents which are depressed by activation of endogenous α_{2B}-adrenoceptors (Docherty & McFadzean, 1989), somatostatin receptors (Tsunoo *et al.*, 1986), δ-opiate receptors (Tsunoo *et al.*, 1986; McFadzean & Docherty, 1989; Kasai, 1992), muscarinic receptors (Caulfield & Brown, 1991), and by heterologous expression of human D_3 dopamine receptors (Seabrook *et al.*, 1994). In the present study, activation of heterologously expressed human D_2(short) receptors also depressed calcium currents in NG108-15 cells. Furthermore these results demonstrate that hD_2 receptors can regulate both ω-conotoxin GVIA-sensitive and dihydropyridine-sensitive calcium currents.

The inhibition of calcium currents in NG108-15 cells following hD_2 receptor activation was restricted to high-threshold calcium currents (Figure 1). The EC_{50} of quinpirole that produced sustained calcium current inhibition (3 nM) was potent relative to its binding affinity for hD_2 receptor in CHO cells (K_i = 600 nM; Freedman *et al.*, 1994). This may in part reflect a high receptor reserve in the transfected cells, in that only fractional receptor occupancy may be required to achieve the maximal functional response. As has previously been observed with human D_3 receptors, only a fraction of the high-threshold calcium current was depressed following

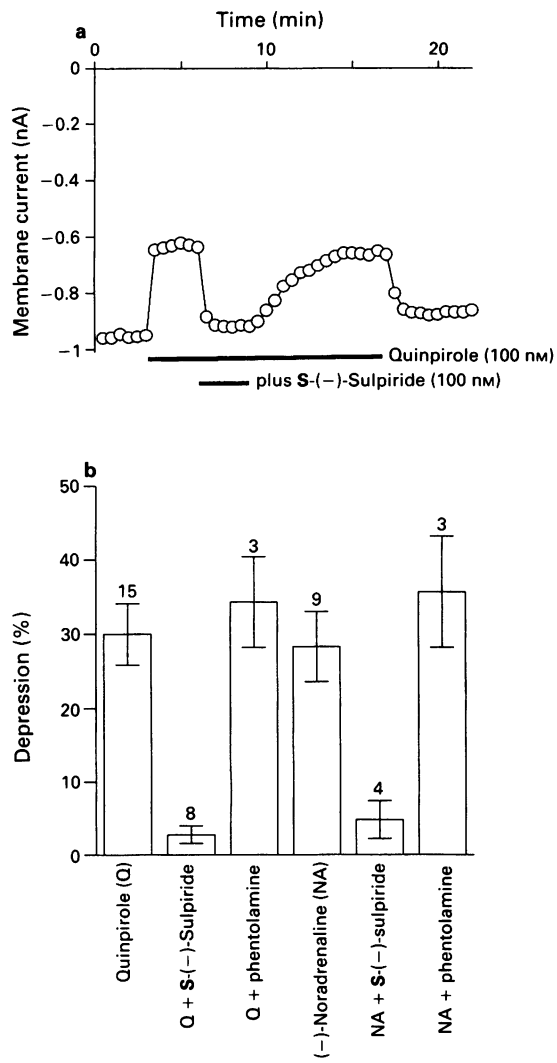


Figure 3 The depression of high-threshold calcium currents by quinpirole was reversible antagonized by S-(−)-sulpiride (100 nM). (a) Time course of the antagonism of hD_2 receptors by S-(−)-sulpiride in an individual cell. Drugs were applied for the duration of the horizontal bars. (b) (−)-Noradrenaline (1 μM) caused a comparable depression to that seen with quinpirole (100 nM). Responses to both agonists were blocked by S-(−)-sulpiride (100 nM) but not by the α-adrenoceptor antagonist, phentolamine (10 μM), consistent with both agonists directly activating the transfected hD_2 receptors (see text). Figures above columns represent the number of cells in each sample.

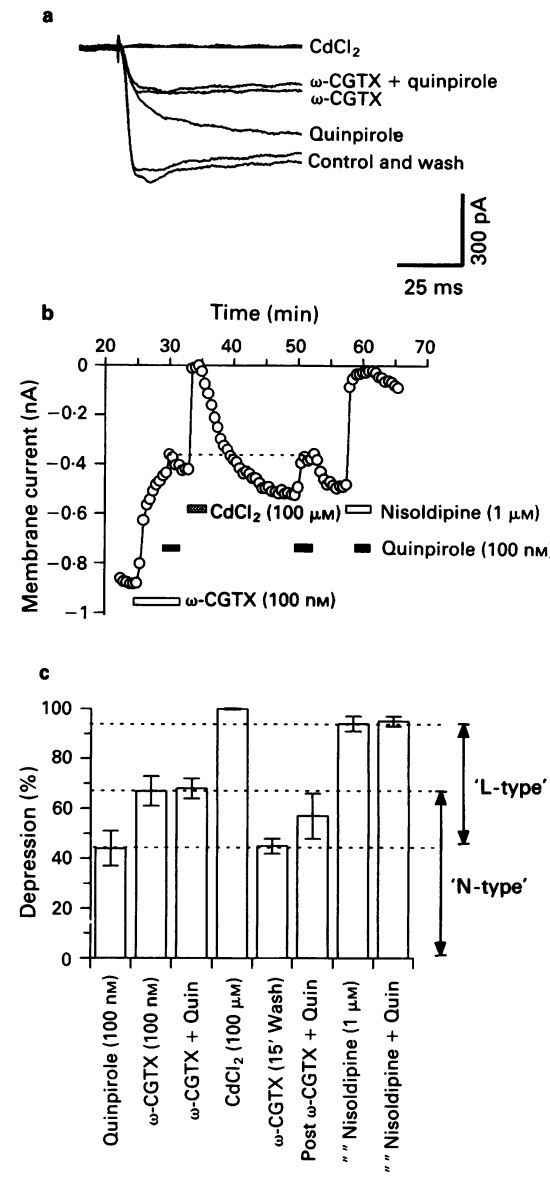


Figure 4 Identification of the component of the calcium current modulated by hD_2 receptor activation. (a) Superimposed calcium currents recorded from the same cell, during and after perfusion with quinpirole (100 nM), with ω-conotoxin GVIA (100 nM), with ω-conotoxin GVIA (100 nM) plus quinpirole (100 nM), and after perfusion with $CdCl_2$ (100 μM). Note that ω-conotoxin GVIA (ω-CGTX) occludes the inhibition by hD_2 receptor activation. (b) Time course of the depression of the peak calcium current in an individual cell using the same protocol as described in (a). ω-Conotoxin GVIA was allowed to wash off for 15 min after which time the cell was again treated with quinpirole. The component of the calcium current that was reversibly blocked by ω-conotoxin GVIA was completely antagonized by nisoldipine, consistent with the current flowing through dihydropyridine-sensitive calcium channels. (c) Pooled data from 4 cells summarising the results from the experimental protocol described in (b). The pharmacology of the high-threshold calcium currents did not fit into a discrete N- or L-type definition, and hD_2 receptor activation could modulate both ω-conotoxin GVIA-, and dihydropyridine-sensitive calcium currents.

D_2 receptor activation. It is unlikely that this fraction relates to an effect on just one type of calcium current since both ω-conotoxin GVIA and dihydropyridine-sensitive currents were affected (Figure 4). The fractional inhibition of high-threshold calcium currents by G-proteins has also been observed for endogenous D_2-like dopamine receptors in rat pituitary cells (e.g. Stack & Suprenant, 1991), as well as with other receptor types in neuroblastoma cells (McFadzean & Docherty, 1989; Caulfield & Brown, 1991; Kasai, 1992). The mechanism underlying the transient depression of high-threshold calcium currents in some cells caused by low concentrations of quinpirole (< 1 nM) is not clear.

The effects of quinpirole in hD_2-transfected NG108-15 cells were completely antagonized by the D_2 receptor antagonist S-(−)-sulpiride (100 nM). At this concentration S-(−)-sulpiride is selective for hD_2 and hD_3 receptors over hD_4 receptors (K_i for hD_4 receptor = 250 nM; Freedman *et al.*, unpublished observations). The absence of D_3 receptor mRNA in the differentiated cell line used for transfection studies lend further support to the conclusion that the depression of calcium currents by quinpirole was mediated by a selective action on the transfected hD_2 receptors.

Some differentiated NG108-15 cell lines have α_{2B}-adrenoceptors that can depress high-threshold calcium currents when activated by dopamine at high micromolar concentrations (Docherty & McFadzean, 1989). Similarly in untransfected NG108-15 cells dopamine can reduce forskolin-stimulated cyclic AMP levels presumably by a non-selective action on a non-D_2-like catecholamine receptors (Freedman *et al.*, 1994). It is unlikely that the depression of calcium currents by quinpirole seen in the present study was caused by a non-selective action on such receptors because (a) no effects of dopamine ($n = 10$) or quinpirole ($n = 4$) were seen on calcium currents in the differentiated cell line used for transfection studies, (b) the effects of the selective dopamine receptor agonist quinpirole were reversibly antagonized by the D_2-like antagonist S-(−)-sulpiride (Figure 3), and (c) responses to quinpirole were not antagonized by the α adrenoceptor antagonist, phentolamine (10 μM; Figure 3) which has a pA_2 equivalent to 170 nM against α_{2B}-receptors (Docherty & McFadzean, 1989). High-threshold calcium currents were depressed by (−)-noradrenaline (28% at 1 μM); however this effect was mediated by the direct activation of the transfected receptors because these responses were blocked by S-(−)-sulpiride and not phentolamine. The conclusion that noradrenaline inhibits calcium currents by activating the transfected hD_2 receptor is further supported by our observation that (−)-noradrenaline weakly displaces [^{125}I]-iodosulpiride binding to hD_2 receptors expressed in CHO cells (Freedman *et al.*, unpublished observations).

Identity of the hD_2-sensitive calcium current

Several peptide neurotoxins including ω-conotoxin GVIA and ω-agatoxin IVA, as well as dihydropyridines are commonly used to identify components of whole cell calcium currents that are believed to be carried by different calcium channel types. Indeed, several categories of calcium currents including L, N, P and T types have been identified by use of these agents (reviewed in McCleskey *et al.*, 1986; Tsien *et al.*, 1988; Spedding & Paoletti, 1992). A few studies have demonstrated that in some cell lines the pharmacological profile of these calcium channel antagonists may overlap, presumably because there is a much greater diversity of calcium channel types with intermediate pharmacological sensitivities than was at first appreciated (e.g. Snutch & Reiner, 1992). Indeed in the present study, the putative P-type selective calcium channel antagonist ω-agatoxin IVA (Mintz *et al.*, 1992) caused a reversible 15% depression (at 100 nM) of T-type calcium currents in hD_2-NG108-15 cells. ω-Agatoxin IVA also depressed calcium currents sensitive to both ω-conotoxin GVIA and dihydropyridines in GH_4C_1 cells (Seabrook *et al.*, unpublished observations), as well as ω-conotoxin GVIA-sensitive calcium currents in rat cerebellar neurones (Sutton *et al.*, 1993). These effects of ω-agatoxin IVA demonstrate that assignment of physiological processes to a specific calcium channel subtype using ω-agatoxin IVA should be carried out with caution.

The ability of G-protein-linked receptors to modulate high-threshold calcium currents in differentiated NG108-15 cells has been reported to be restricted to the ω-conotoxin GVIA-sensitive portion of the current (Caulfield *et al.*, 1992). Consistent with this view, the effects of quinpirole in hD_2-transfected cells were abolished by 100 nM ω-conotoxin GVIA. However, coupling was not restricted to dihydropyridine-insensitive calcium currents. Although, micromolar concentrations of ω-conotoxin GVIA has been shown to block reversibly both low-threshold and dihydropyridine-sensitive calcium currents in a number of cell types (McCleskey *et al.*, 1987; Kasai *et al.*, 1987), the inhibition of calcium currents in NG108-15 cells by ω-conotoxin GVIA has been reported to be effectively irreversible (Kasai & Neher, 1992; Caulfield *et al.*, 1992). In the present study, the depression of the high-threshold current by 100 nM ω-conotoxin GVIA did partly recover after washing for 5–10 min (ca. 20%). This fraction of the high-threshold calcium current was also depressed following hD_2 receptor activation and completely blocked by the dihydropyridine, nisoldipine (1 μM; Figure 4). Therefore, G-protein linked receptors can couple to transduction systems that regulate more than one component of the high-threshold current in these cells.

The proportion of high-threshold calcium current that was inhibited by the combined application of ω-conotoxin GVIA and nisoldipine was greater in NG108-15 cells that had been transfected with hD_2 receptor cDNA (this study), than in those with hD_3 receptor cDNA (Seabrook *et al.*, 1994). This heterogeneity may partly reflect inherent differences between the single cell subclones, differential effects of the transfection procedure, or tonic effects of the transfected receptors on cell function.

In conclusion, this study demonstrates that human D_2 (short) receptors, like hD_3 receptors, can couple to the depression of ω-conotoxin GVIA-sensitive, and dihydropyridine-sensitive calcium currents in differentiated NG108-15 cells. Electrophysiological studies have shown that in some central neurones, ω-conotoxin GVIA-sensitive calcium channels are utilised during synaptic transmission (e.g.Horne & Kemp, 1991). Consequently, G-proteins activated by D_2 dopamine receptors, in addition to their effects on potassium channels and adenylate cyclase activity, may have a direct influence on calcium channels involved in transmitter release processes. The ability of human D_2 dopamine receptors to couple to effector mechanisms that differentially regulate more than one type of calcium current, supports the notion that these receptors have a diverse functional role in the central nervous system.

References

BEECH, D.J., BERNHEIM, L. & HILLE, B. (1992). Pertussis toxin and voltage-dependence distinguish multiple pathways modulating calcium channels of rat sympathetic neurons. *Neuron*, **8**, 97–106.

BERNHEIM, L., BEECH, D.J. & HILLE, B. (1991). A diffusible second messenger mediates one of the pathways coupling receptors to calcium currents in rat sympathetic neurons. *Neuron*, **6**, 859–867.

BODEWEI, R., HERING, S., SCHUBERT, B. & WOLLENBERGER, A. (1985). Sodium and calcium currents in neuroblastoma X glioma hybrid cells before and after morphological differentiation by dibutyryl cyclic AMP. *Gen. Physiol. Biophys.*, **4**, 113–127.

BUISSON, B., BOTTARI, S.P., DE GASPARO, M., GALLO-PAYET, N. & PAYET, M.D. (1992). The angiotensin AT_2 receptor modulates T-type calcium current in non-differentiated NG108-15 cells. *FEBS Lett.*, **309**, 161–164.

CAULFIELD, M.P. & BROWN, D.A. (1991). Pharmacology of the putative M_4 muscarinic receptor mediating Ca-current inhibition in neuroblastomaxglioma hybrid (NG108-15) cells. *Br. J. Pharmacol.*, **104**, 39–44.

CAULFIELD, M.P., ROBBINS, J. & BROWN, D.A. (1992). Neurotransmitters inhibit the omega-conotoxin-sensitive component of Ca current in neuroblastoma × glioma hybrid (NG108-15) cells, not the nifedipine-sensitive component. *Pflügers Arch.*, **420**, 486–492.

CULLEN, B.R. (1987). Use of eukaryotic expression technology in the functional analysis of cloned genes. *Methods Enzymol.*, **152**, 684–704.

DEARRY, A., GINGRICH, J.A., FALARDEAU, P., FREMEAU, R.T., BATES, M.D. & CARON, M.G. (1990). Molecular cloning and expression of the gene for a human D1 dopamine receptor. *Nature*, **347**, 72–76.

DOCHERTY, R.J. (1988). Gadolinium blocks a component of calcium current in rodent neuroblastoma × glioma hybrid (NG108-15) cells. *J. Physiol.*, **398**, 33–47.

DOCHERTY, R.J. & MCFADZEAN, I. (1989). Noradrenaline-induced inhibition of voltage-sensitive calcium currents in NG108-15 hybrid cells. *Eur. J. Neurosci.*, **1**, 132–140.

FISHMAN, M.C. & SPECTOR, I. (1981). Potassium current suppression by quinidine reveals additional calcium currents in neuroblastoma cells. *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 5245–5249.

FREEDMAN, S.B., PATEL, S., MARWOOD, R., EMMS, F., SEABROOK, G.R., KNOWLES, M.R. & MCALLISTER, G.K (1994). Expression and pharmacological characterisation of the human D_3 receptor. *J. Pharmacol. Exp. Ther.*, **268**, 417–426.

HERING, S., BODEWEI, R., SCHUBERT, B., ROHDE, K. & WOLLENBERGER, A. (1985). A kinetic analysis of the inward current in 108CC15 neuroblastoma × glioma hybrid cells. *Gen. Physiol. Biophys.*, **4**, 129–141.

HILLE, R. (1992). G-protein coupled mechanisms and nervous signalling. *Neuron*, **9**, 187–195.

HORN, R. & MARTY, A.J. (1988). Muscarinic activation of ionic currents measured by a new whole-cell recording method. *Gen. Physiol.*, **92**, 145–159.

HORNE, A.L. & KEMP, J.A. (1991). The effect of ω-conotoxin GVIA on synaptic transmission within the nucleus accumbens and hippocampus of the rat *in vitro*. *Br. J. Pharmacol.*, **103**, 1733–1739.

KASAI, H. (1992). Voltage- and time-dependent inhibition of neuronal calcium channels by a GTP-binding protein in a mammalian cell line. *J. Physiol.*, **448**, 189–209.

KASAI, H., AOSAKI, T. & FUKUDA, J. (1987). Presynaptic Ca-antagonist ω-conotoxin irreversibly blocks N-type Ca-channels in chick sensory neurons. *Neurosci. Res.*, **4**, 228–235.

KASAI, H. & NEHER, E. (1992). Dihydropyridine-sensitive and ω-conotoxin-sensitive calcium channels in a mammalian neuroblastoma-glioma cell line. *J. Physiol.*, **448**, 161–188.

KEJA, J.A., STOOF, J.C. & KITS, K.S. (1992). Dopamine D_2 receptor stimulation differentially affects voltage-activated calcium channels in rat pituitary melanotropic cells. *J. Physiol.*, **450**, 409–435.

KLEUSS, C., SCHERUBL, H., HESCHELER, J., SCHULTZ, G. & WITTIG, B. (1993). Selectivity in signal transduction determined by γ subunits of heterotrimeric G proteins. *Science*, **259**, 832–834.

LLEDO, P.M., HOMBURGER, V., BOCKAERT, J. & VINCENT, J.-D. (1992). Differential G protein-mediated coupling of D_2 dopamine receptors to K^+ and Ca^{2+} currents in rat anterior pituitary cells. *Neuron*, **8**, 445–463.

MATHIE, A., BERNHEIM, L. & HILLE, B. (1992). Inhibition of N- and L-type calcium channels by muscarinic receptor activation in rat sympathetic neurons. *Neuron*, **8**, 907–914.

MCALLISTER, G., KNOWLES, M.R., PATEL, S., MARWOOD, R., EMMS, F., SEABROOK, G.R., GRAZIANO, M., BORKOWSKI, D., HEY, P.J. & FREEDMAN, S.B. (1993). Characterisation of a chimeric hD_3/hD_2 dopamine receptor expressed in CHO cells. *FEBS Lett.*, **324**, 81–86.

MCCLESKEY, E.W., FOX, A.P., FELDMAN, D.H., CRUZ, L.J., OLIVERA, B.M., TSIEN, R.W. & YOSHIKAMI, D. (1987). ω-Conotoxin: Direct and persistent blockade of specific types of calcium channels in neurons but not muscle. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 4327–4331.

MCCLESKEY, E.W., FOX, A.P., FELDMAN, D. & TSIEN, R.W. (1986). Different types of calcium channels. *J. Exp. Biol.*, **124**, 177–190.

MCFADZEAN, I. & DOCHERTY, R.J. (1989). Noradrenaline- and enkephalin-induced inhibition of voltage-sensitive calcium currents in NG108-15 hybrid cells: transduction mechanisms. *Eur. J. Neurosci.*, **1**, 141–147.

MCFADZEAN, I., MULLANEY, I., BROWN, D.A. & MILLIGAN, G. (1989). Antibodies to the GTP binding protein, G_o, antagonize noradrenaline-induced calcium current inhibition in NG108-15 hybrid cells. *Neuron*, **3**, 177–182.

MILLIGAN, G. (1989). Tissue distribution and subcellular location of guanine nucleotide binding proteins:- implications for cellular signalling. *Cell. Signalling*, **1**, 411–419.

MINTZ, I.M., ADAMS, M.E. & BEAN, B.P.(1992). P-type calcium channels in rat central and peripheral neurons. *Neuron*, **9**, 85–95.

MULLANEY, I. & MILLIGAN, G. (1990). Identification of two distinct isoforms of the guanine nucleotide binding protein Go in neuroblastoma × glioma hybrid cells: independent regulation during cyclic AMP-induced differentiation. *J. Neurochem.*, **55**, 1890–1898.

NUSSINOVITCH, I. & KLEINHAUS, A.L. (1992). Dopamine inhibits voltage-activated calcium channel currents in rat pars intermedia pituitary cells. *Brain Res.*, **574**, 49–55.

SCHWARTZ, J.-C., GIROS, B., MARTRES, M.-P. & SOKOLOFF, P. (1992). The dopamine receptor family: molecular biology and pharmacology. *Seminars in the Neurosci.*, **4**, 99–108.

SEABROOK, G.R., PATEL, S., MARWOOD, R., EMMS, F., KNOWLES, M.R., FREEDMAN, S.B. & MCALLISTER, G. (1992). Stable expressio of human D_3 dopamine receptors in GH_4C_1 pituitary cells. *FEBS Lett.*, **312**, 123–126.

SEABROOK, G.R., PATEL, S., MARWOOD, R., EMMS, F., KNOWLES, M.R., FREEDMAN, S.B. & MCALLISTER, G. (1994). Human D_3 dopamine receptors couple to the depression of calcium currents in a neuroblastoma- × -glioma cell line. *Br. J. Pharmacol.*, **111**, 391–393.

SIBLEY, D.R. & MONSMA, F.J. (1992). Molecular biology of dopamine receptors. *Trends Pharmacol. Sci.*, **13**, 61–69.

SNUTCH, T.P. & REINER, P.B. (1992). Ca^{2+} channels: diversity of form and function. *Curr. Opinion Neurobiol.*, **2**, 247–253.

SPEDDING, M. & PAOLETTI, R. (1992). Classification of calcium channels and sites of actions of drugs modifying channel function. *Pharmacol. Reviews*, **44**, 363–376.

STACK, J. & SUPRENANT, A. (1991). Dopamine actions on calcium currents, potassium currents and hormone release in rat melanotrophs. *J. Physiol.*, **439**, 37–58.

SUNAHARA, R.K., GUAN, H.-C., O'DOWD, B.F., SEEMAN, P., LAURIER, L.G., NG, G., GEORGE, S.R., TORCHIA, J., VAN TOL, H.H.M. & NIZNIK, H.B. (1991). Cloning of the gene for a human dopamine D_5 receptor with a higher affinity for dopamine than D1. *Nature*, **350**, 614–619.

SUTTON, K.G., PEARSON, H., SCOTT, R.H. & DOLPHIN, A.C. (1993). Inhibition of Ca^{2+} channel currents in cultured rat cerebellar granule neurones by the Funnel Web spider toxin ω-Aga IVA. *J. Physiol.*, **467**, 271P.

TSIEN, R.W., LIPSCOMBE, D., MADISON, D.V., BLEY, K.R. & FOX, A.R. (1988). Multiple types of neuronal calcium channels and their selective modulation. *Trends Neurosci.*, **11**, 431–438.

TSUNOO, A., YOSHI, M. & NARAHASHI, T. (1986). Block of calcium channels by enkephalin and somatostatin in neuroblastoma-glioma hybrid NG108-15 cells. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 9832–9836.

ZHOU, Q.-Y., GRANDY, D.K., THAMBI, L., KUSHNER, J.A., VAN TOL, H.H.M., CONE, R., PRIBNOW, D., SALON, J., BUNZOW, J.R. & CIVELLI, O. (1990). Cloning and expression of human and rat D1 dopamine receptors. *Nature*, **347**, 76–80.

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Effect of subthreshold ouabain on the tone of guinea-pig aortic strips following repeated noradrenaline stimulation

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1 The effect of ouabain at a concentration (0.8 μM) that does not induce contractile response in guinea-pig aortic strips has been studied on endothelium-denuded strips repeatedly stimulated with 1 μM noradrenaline or 60 mM K^+ applied for 5 min every 30 min.

2 The resting tone (i.e. the tone between one noradrenaline stimulation and the following) of the aortic strips exposed to ouabain increased progressively, whereas the control strips (no ouabain) completely relaxed on washout of the agonist. In the aortic strips stimulated by 60 mM K^+, the resting tone did not increase.

3 The calcium antagonist, verapamil, did not affect the increase in tone, that was nevertheless strictly dependent on external calcium, since the contracted strips completely relaxed on calcium removal and promptly contracted again on calcium readdition. This finding indicates a mechanism independent of voltage-gated calcium channels.

4 Caffeine-induced contractions, taken as a measure of sarcoplasmic reticulum calcium content, were amplified by the presence of ouabain in aortic strips either stimulated by noradrenaline or unstimulated, with a larger increase in the former.

5 These results suggest that the repeated stimulation of guinea-pig aortic strips by noradrenaline in the presence of ouabain, by raising both intracellular Na^+ and Ca^{2+}, decreases the ouabain threshold concentration required for contraction, thus increasing the responsiveness of vascular smooth muscle to the glycoside.

Keywords: Vascular smooth muscle; hypertension; ouabain; Na^+ pump; Na^+/Ca^{2+} exchange

Introduction

It has been proposed that in some forms of hypertension, circulating endogenous inhibitors of the plasmalemma Na^+ pump (Na^+-K^+ ATPase) could be responsible for the increased vascular smooth muscle tone (Blaustein, 1977; de Wardener & MacGregor, 1980; Hamlyn *et al.*, 1982). The recent identification (Hamlyn *et al.*, 1991) of an endogenous ouabain-like compound (OLC) identical to the digitalis glycoside, ouabain, in the blood of various mammals, including man and the observation that plasma levels of digitalis-like compounds increase in different forms of hypertension (Gruber *et al.*, 1980; 1982; Masugi *et al.*, 1986; Deray *et al.*, 1987; Moreth *et al.*, 1987) support the hypothesis that the inhibition of the Na^+ pump in vascular smooth muscle cells by endogenous factors could be involved in the pathophysiology of hypertension (Blaustein *et al.*, 1990; Bova *et al.*, 1990).

Cardiac glycosides induce a myogenic tonic contraction in isolated vascular smooth muscle preparations from animals and man (Moreland *et al.*, 1986; Bova *et al.*, 1988; Woolfson *et al.*, 1990) and long-term administration of ouabain produces hypertension in rats (Yuan *et al.*, 1993). However, since the OLC concentration (138 pmol l^{-1}) found in human plasma (Hamlyn *et al.*, 1991) is presumably not sufficient to induce an increase in vascular tone, mechanisms other than a direct contractile effect on vascular smooth muscle have been implicated, such as the inhibition of endothelium-induced vascular relaxation (Boulanger *et al.*, 1989; Woolfson & Poston, 1991) or the enhancement of endothelin secretion (Yamada *et al.*, 1990; Goligorsky *et al.*, 1991). Furthermore, OLC, at a concentration that does not stimulate a contractile response, increases the responsiveness to vasoconstrictor stimuli of isolated vascular smooth muscle preparations, possibly as a consequence of an increased calcium content of the sarcoplasmic reticulum (Bova *et al.*, 1991). In addition, an increase in the responsiveness to vasoconstrictor stimuli has been demonstrated in man after short-term treatment with the cardiac glycoside, digoxin (Guthrie, 1984). There is evidence therefore that the inhibition of the Na^+ pump, even though not sufficient to induce contraction of vascular smooth muscle, amplifies the response to vasoconstrictor stimuli.

In this context, we have hypothesized that vasoconstrictor stimuli can induce hyperresponsiveness of vascular smooth muscle to ouabain. In other words, the stimulation of vascular smooth muscle by vasoconstrictor stimuli could decrease the threshold for contraction to ouabain. To test this hypothesis, we have studied the effect of transient and repeated noradrenaline stimulation on guinea-pig aortic strips exposed to ouabain concentrations subthreshold for contraction. A preliminary account of these results was given to the American College of Cardiology (Bova *et al.*, 1992).

Methods

Aortae were excised from guinea-pigs (350–450 g) killed by a sharp blow to the neck. The thoracic part of the vessels was carefully cleaned of connective tissue, cut into helicoidal strips (10 × 1.5 mm) and the endothelium gently removed. The strips were then suspended vertically in 16 ml water-jacketed organ baths filled with physiological salt solution (PSS) aerated with 95% O_2 and 5% CO_2 and maintained at 35°C (pH 7.36–7.40). The composition of the solution was as follows (mM): NaCl 118, KCl 4.75, $CaCl_2 \cdot 2H_2O$ 2.7, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11. One end of each strip was anchored to the bottom of the bath and the other connected to a force transducer (Battaglia-Rangoni TRB/200/2) coupled to a pen recorder (Battaglia-Rangoni KV 220) for monitoring isometric force generation. The tissues

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were preloaded with approximately 1.8 g and allowed to equilibrate for at least 120 min before starting the experiment.

Ca^{2+}-free solution was made by omitting $CaCl_2$ from, and adding 1 mM EGTA to the PSS. The calcium antagonist, verapamil (10 μM), was present in the PSS throughout the experiments performed with noradrenaline. Preliminary experiments showed that this concentration of verapamil inhibited the contraction induced by 1 μM noradrenaline by about 10%. The 60 mM K^+ solution was made by adding the appropriate amount of KCl to the PSS, without substituting K^+ for Na^+, since lowering the Na^+ gradient affects Na^+/Ca^{2+} exchange activity. It has been reported that high K^+ (up to 60 mM) does not have a hyperosmotic effect on vascular smooth muscle contractility (Ozaki *et al.*, 1978).

Experimental protocol

The aortic strips were repeatedly stimulated by 1 μM noradrenaline or 60 mM K^+ applied for 5 min every 30 min. After the first reproducible response to noradrenaline or high K^+ was obtained, the stimulation was continued in one set of strips in the absence, and in another set in the presence of 0.8 μM ouabain (subthreshold ouabain). The subthreshold ouabain concentration was previously determined in unstimulated strips, where it was found that ouabain up to 1 μM did not elicit a contractile response within a period of exposure of 240 min (Figure 1a). In all the experiments, a concentration of 0.8 μM ouabain was used. The subthreshold ouabain concentration was occasionally checked in unstimulated strips. The subthreshold concentration of K^+ (0.5 mM) was determined as detailed in Results and aortic strips were repeatedly stimulated by 1 μM noradrenaline applied for 5 min every 30 min in the presence of 0.5 mM K^+.

Statistical analysis

Data are expressed as means ± s.e.mean. Tests of significance were performed using Student's t test; a $P<0.05$ was considered significant.

Drugs

The following drugs were used: noradrenaline bitartrate, ouabain, caffeine and amiloride (Sigma Chemical Co., St Louis, MO, U.S.A.); verapamil (Knoll, Ludwigshafen, Germany). All drugs, except caffeine, were made up as concentrated solutions in twice distilled water. Caffeine was dissolved in the PSS to obtain the desired concentration (10 mM).

Results

Effect of subthreshold ouabain

As shown in Figure 1a, in guinea-pig aortic strips the exposure for 240 min to ouabain up to 1 μM did not affect the resting tone. The aortic strips responded with reproducible contractions to repeated stimulation by 1 μM noradrenaline, applied for 5 min every 30 min, and full relaxation on washout (Figure 1b). When stimulation by noradrenaline was carried out in the presence of a subthreshold concentration of ouabain (0.8 μM), relaxation was progressively impaired upon washout of the agonist and an increase of the vascular tone occurred, the magnitude of which increased with successive stimuli (Figure 1c and Table 1). The increase in tone was well maintained in the presence of ouabain, completely reversed when ouabain was removed (Figure 1c), and was not prevented by the Ca^{2+}-entry blocker verapamil (10 μM) (Figure 2), indicating that voltage-operated Ca^{2+} channels were not involved. Furthermore, the maintenance of the increase in tone was strictly dependent on extracellular Ca^{2+}, since the removal of Ca^{2+} from the bath induced a rapid relaxation of the aortic strips, that responded with a verapamil-insensitive contraction upon reintroduction of external Ca^{2+} (Figure 3a). The contraction elicited by the reintroduction of Ca^{2+} was prevented by 0.5 mM amiloride (Figure 3b), indicating the involvement of Na^+/Ca^{2+} exchange (Bova *et al.*, 1988).

In isolated vascular smooth muscle preparations, caffeine-induced Ca^{2+} release from sarcoplasmic reticulum (SR) elicits a phasic contraction that appears to be a measure of the SR calcium content (Ashida & Blaustein, 1987; van Breemen & Saida, 1989). In some experiments, contractions were induced by 10 mM caffeine before and after exposure to ouabain. As shown in Figure 4 and Table 2, in the presence of ouabain, caffeine-induced contractions were amplified in both unstimulated and noradrenaline-stimulated aortic strips, with a larger increase in the latter. The strips previously stimulated by noradrenaline in the presence of ouabain and then relaxed by Ca^{2+}-free PSS did not respond to caffeine, indicating a low SR Ca^{2+} content.

When aortic strips exposed to subthreshold ouabain were stimulated by 60 mM K^+, instead of by noradrenaline, no increase in tone was observed.

Effect of low K^+-PSS

In isolated vascular smooth muscle preparations, a low K^+-medium induces slowly developing, well maintained, contractile responses that have been ascribed to Ca^{2+} entry through Na^+/Ca^{2+} exchange activated by the decrease of the Na^+ gradient consequent on Na^+ pump inhibition (Karaki *et al.*, 1978; Ozaki *et al.*, 1978; Bova *et al.*, 1988). The degree of inhibition is dependent on the extent of K^+ lowering, since it has been shown that the activity of the Na^+ pump is directly related to the extracellular K^+ concentration (Lindenmayer & Schwartz, 1975; Moromizato *et al.*, 1992).

In our experiments, the exposure of aortic strips to external K^+ concentrations lower than 0.5 mM induced a contractile response, while no response was observed at 0.5 mM external K^+ or above (Figure 5a), so this concentration of K^+ has been considered as subthreshold for the contractile response. In the aortic strips incubated in 0.5 mM K^+-PSS, repeated stimulation by 1 μM noradrenaline for 5 min every 30 min elicited a progressive increase in the tone between each stimulus (Figure 5b and Table 1). This effect was not affected by 10 μM verapamil and was strictly dependent on extracellular Ca^{2+}, since the contracted strips relaxed on Ca^{2+} removal and contracted again on Ca^{2+} readmission (Figure 5b and Table 1). This Ca^{2+}-induced contraction was prevented by 0.5 mM amiloride (Figure 5c). The response to caffeine (10 mM) was increased both in the unstimulated and noradrenaline-stimulated aortic strips, with a greater increase in the latter (Figure 6 and Table 2). Therefore, the effects of subthreshold concentrations of ouabain and potassium on the tone of the aortic strips after noradrenaline stimulation were identical.

Discussion

The results of this study show that ouabain, at a concentration subthreshold for contraction, elicits an increase in tone between successive stimuli in guinea-pig aortic strips repeatedly stimulated by noradrenaline. This effect, the magnitude of which increases with the number of stimuli, is unaffected by the calcium antagonist, verapamil, indicating that voltage-operated calcium channels are not involved. Similar results are obtained when a low concentration of potassium is used instead of ouabain. In our experiments, the enhancement of agonist-induced contraction caused by ouabain (Ashida & Blaustein, 1987; Bova *et al.*, 1991) is not observed, presumably because a concentration of noradrenaline very close to that giving maximal contraction has been chosen to show better the effect of ouabain.

Taking into account that ouabain and low potassium elicit myogenic, slow developing contractile responses in guinea-pig aortic strips and human subcutaneous resistance vessels (Ozaki *et al.*, 1978; Moreland *et al.*, 1986; Bova *et al.*, 1988; Woolfson *et al.*, 1990), our results indicate that noradrenaline stimulation apparently triggers the contractile effect of either ouabain or low potassium. The contractile response of vascular smooth muscle to ouabain or low potassium has been ascribed to Ca^{2+} entry through the Na^+/Ca^{2+} exchange system as a consequence of Na^+ pump inhibition (Ozaki *et al.*, 1978; Bova *et al.*, 1988; Woolfson *et al.*, 1990). Na^+/Ca^{2+} exchange was first recognized as one of the two mechanisms, together with the ATP-dependent Ca^{2+} pump, that extrude calcium from the cytosol through the plasmalemma of vascular smooth muscle cells (Schatzmann, 1985). More recent evidence indicates that the exchanger is a bidirectional transport system operating in the Ca^{2+} entry mode when the Na^+ gradient across the plasma membrane decreases (Ashida & Blaustein, 1987; Nabel *et al.*, 1988; Battle *et al.*, 1991).

In our experiments, the exposure of aortic strips to subthreshold ouabain increases the contractile response to caffeine in either unstimulated or noradrenaline-stimulated strips,

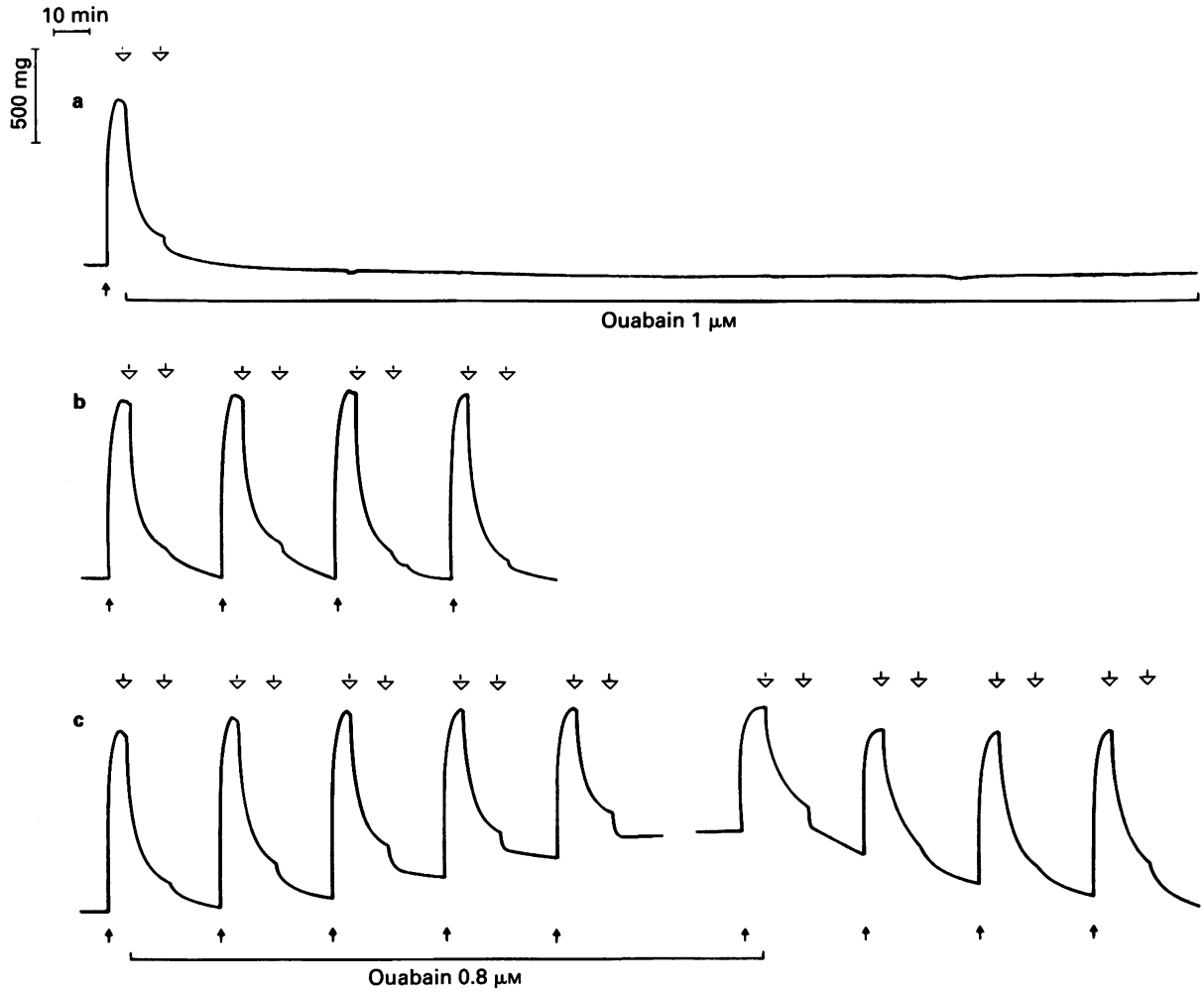


Figure 1 Traces of the contractile responses to 1 μM noradrenaline of three separate guinea-pig aortic strips. (a) Trace of the first reproducible response to noradrenaline and resting tone of the strip exposed to 1 μM ouabain for over 240 min. (b) Trace of the response of one strip to successive noradrenaline stimulations in the absence of ouabain. (c) Trace of the responses of one aortic strip to noradrenaline in the presence of 0.8 μM ouabain (left) and after removal of ouabain (right).

Table 1 Resting tone between successive contractions induced by 1 μM noradrenaline in the presence of ouabain or low K^+ in guinea-pig aortic strips

	Resting tone (mg)			
Number of contractions	I	II	III	IV
Ouabain (0.8 μM)	0	103 ± 13	213 ± 20	300 ± 32
K^+(0.5 mM)	137 ± 41	258 ± 26	323 ± 19	398 ± 24

Resting tone is the tone maintained by the aortic strips before each noradrenaline addition. Noradrenaline-induced contractions are numbered after the beginning of the exposure to 0.8 μM ouabain or 0.5 mM K^+. Data are mean values ± s.e.mean of at least nine experiments performed as shown in Figures 1 and 5b.

with a higher increase in the latter. This indicates that the glycoside increases the Ca^{2+} concentration in SR of vascular smooth muscle cells and that the stimulation by noradrenaline amplifies this effect. Taking into account that ouabain promotes Ca^{2+} entry via the Na^+/Ca^{2+} exchanger (Ozaki *et al.*, 1978; Bova *et al.*, 1988; Woolfson *et al.*, 1990), the lack of effect of the concentration of ouabain used here on the resting tone of the unstimulated strips may be explained by a partial inhibition of the Na^+ pump that does not increase the intracellular Na^+ concentration to a sufficient extent to transport enough Ca^{2+} for contraction. However, Ca^{2+} transported into the cells, although not sufficient to elicit a contraction, enhances the size of the SR Ca^{2+} store, as shown also by Ashida & Blaustein (1987). The increase in tone in noradrenaline-stimulated strips may be accounted for by the further enhancement of the SR Ca^{2+} concentration (as shown by higher caffeine-induced contraction in stimulated versus unstimulated strips), that could be explained by noradrenaline-induced Ca^{2+} influx through receptor-operated calcium channels in cells where Na^+/Ca^{2+} exchange is functionally excluded as a Ca^{2+} extrusion mechanism and operates as a Ca^{2+} influx mechanism.

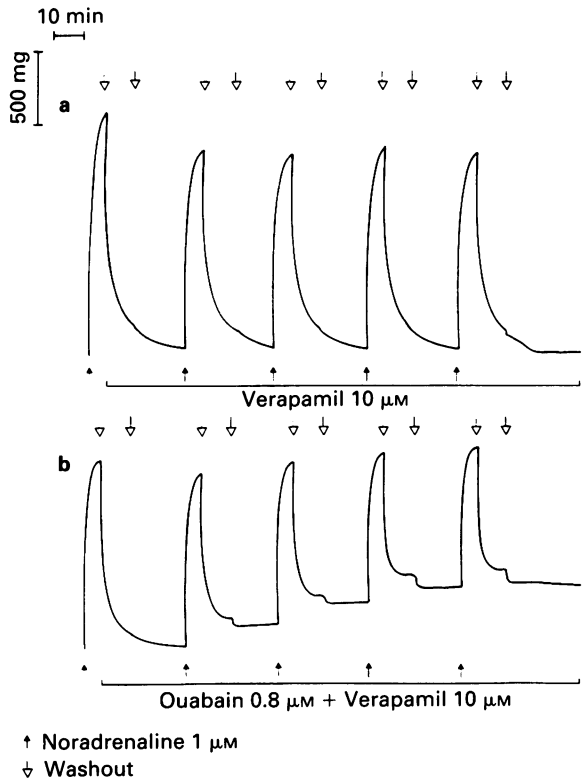


Figure 2 Traces of the contractile responses to 1 μM noradrenaline of two separate guinea-pig aortic strips exposed to 10 μM verapamil and repeatedly stimulated in the absence (a) and presence (b) of 0.8 μM ouabain.

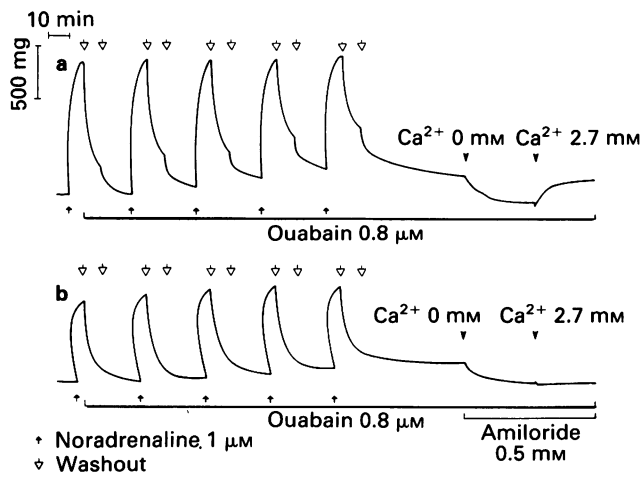


Figure 3 Effect of Ca^{2+} removal and readmission in the absence (a) and presence (b) of 0.5 mM amiloride in two separate guinea-pig aortic strips previously stimulated by 1 μM noradrenaline in the presence of ouabain.

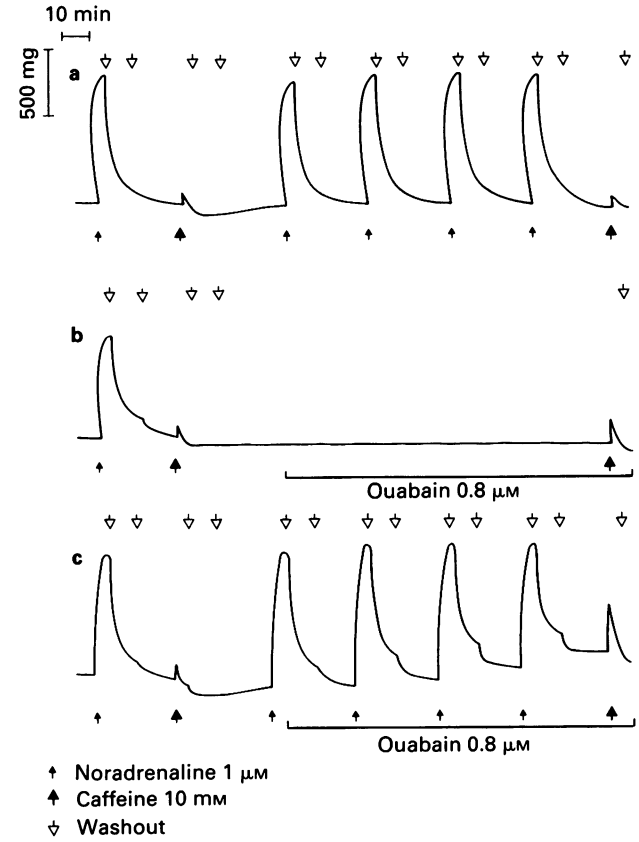


Figure 4 Traces of the contractile responses of three separate aortic strips to 10 mM caffeine applied both before and after repeated stimulation by 1 μM noradrenaline (a), exposure to 0.8 μM ouabain (b), and repeated stimulation by 1 μM noradrenaline in the presence of ouabain (c).

Table 2 Caffeine-induced contractions in guinea-pig aortic strips

	Peak developed tension (mg)	
	Unstimulated	*Stimulated*
Control	92 ± 18	92 ± 18
Ouabain (0.8 μM)	211 ± 30*	362 ± 55**
K^+(0.5 mM)	240 ± 30*	380 ± 20**

Caffeine (10 mM) was applied before and after the exposure of either unstimulated or noradrenaline-stimulated aortic strips to ouabain or low K^+. The values of peak developed tension for the stimulated strips refer to caffeine-induced contractions measured from the maintained tension to the peak contraction. The unstimulated strips were exposed to ouabain or low K^+-PSS for the same period of time as the stimulated strips (about 150 min). Data are mean values ± s.e.mean of at least five experiments performed as shown in Figures 4 and 6. *$P<0.05$ vs control in unstimulated strips (Student's t test for paired data); **$P<0.05$ stimulated vs unstimulated strips (Student's t test for unpaired data).

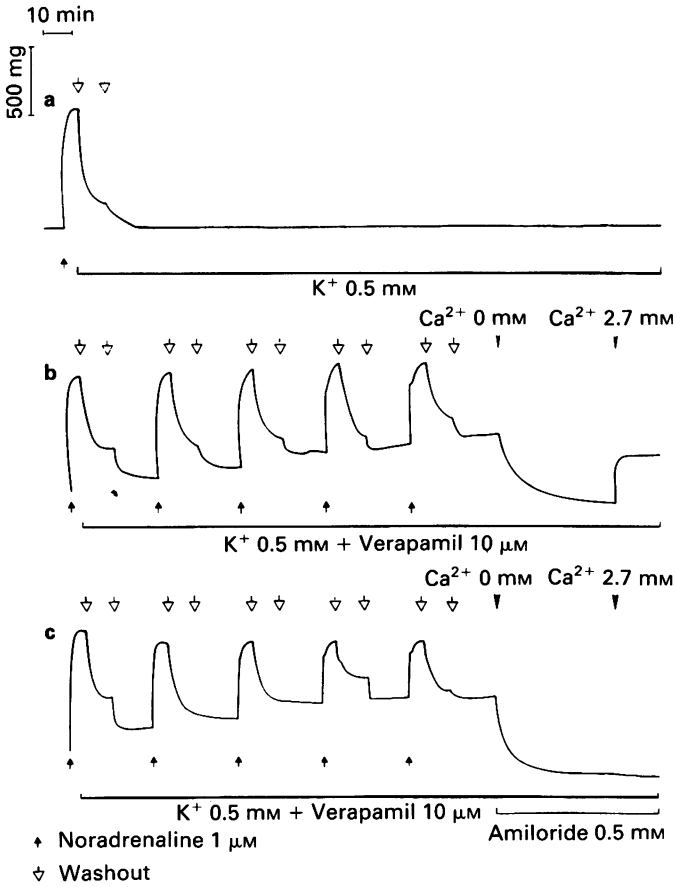


Figure 5 Traces of the contractile responses to 1 μM noradrenaline of three separate aortic strips. (a) Trace of the first reproducible response to noradrenaline and resting tone of the strip exposed to low K^+-PSS for over 240 min. (b) Trace of the responses of strip repeatedly stimulated by 1 μM noradrenaline in low K^+-PSS and in the presence of 10 μM verapamil, followed by Ca^{2+} removal and readmission. (c) Effect of 0.5 mM amiloride on Ca^{2+}-induced contraction.

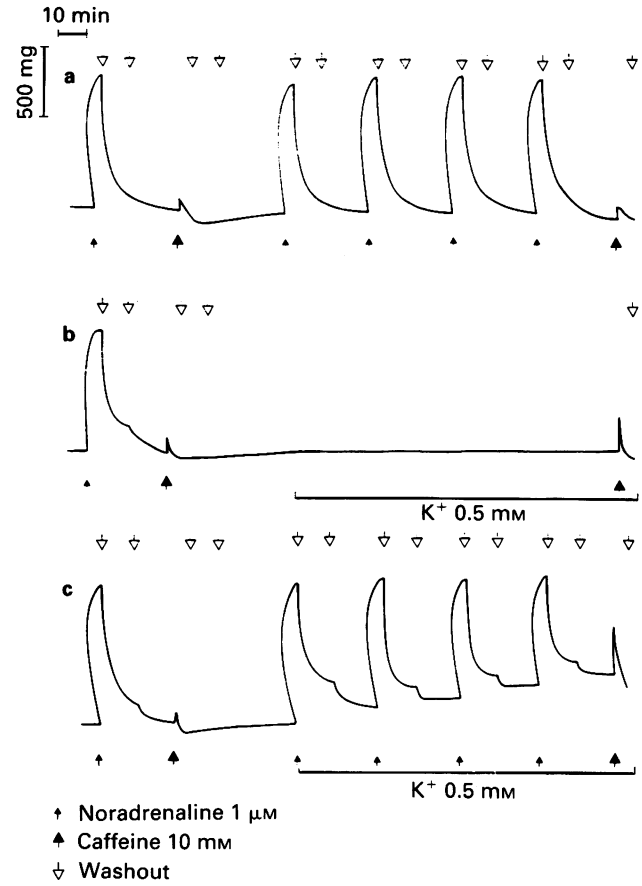


Figure 6 Traces of the contractile responses of three separate aortic strips to 10 mM caffeine applied both before and after repeated stimulation by 1 μM noradrenaline (a), exposure to low K^+-PSS (b), and repeated stimulation by 1 μM noradrenaline in the presence of low K^+-PSS (c).

However, the stimulation of ouabain-exposed strips by noradrenaline should have produced some other modification in addition to the increase in stored Ca^{2+}. In fact, strips previously stimulated by noradrenaline and then relaxed by Ca^{2+}-free PSS respond with a verapamil-insensitive, amiloride-inhibited, tonic contraction when Ca^{2+} is readmitted. This in spite of the low SR Ca^{2+} content, as shown by the lack of response to caffeine. Since it has been reported that in vascular smooth muscle cells several agonists induce a dose-dependent increase in Na^+ influx following the increase in intracellular Ca^{2+} (Smith & Brock, 1983; Aaronson & Jones, 1988; Vallega *et al.*, 1988; Okada *et al.*, 1991), it can be assumed that Na^+ entry at each noradrenaline stimulation results in a progressive intracellular Na^+ accumulation because the Na^+ pump is inhibited by ouabain. The enhancement of intracellular Na^+ concentration increases the driving force for Ca^{2+} influx through Na^+/Ca^{2+} exchange. The inhibition by amiloride of the contraction induced by Ca^{2+} in Ca^{2+}-free PSS-relaxed aortic strips supports this explanation, since it has been shown that amiloride prevents contractile responses of guinea-pig aortic strips induced by Ca^{2+} entry through Na^+/Ca^{2+} exchange (Bova *et al.*, 1988). Therefore, it can be proposed that repeated noradrenaline stimulation decreases the threshold of ouabain for contraction of aortic strips not only by contributing to Ca^{2+} entry (via receptor-operated calcium channels) but also by inducing influx of Na^+. A further mechanism may be postulated: since intracellular Na^+ activates the turnover rate of the Na^+ pump (Eisner, 1986), the increase in intracellular Na^+ induced by noradrenaline could result in an increased affinity of ouabain for the pump, thus explaining the response to the subthreshold concentration of the glycoside. However, the observation that the rise in tone is induced also by subthreshold K^+-PSS indicates that an increased binding of ouabain is not required to account for our results.

The increase in intracellular Na^+ induced by noradrenaline could also explain why repeated stimulation by high K^+ of aortic strips in the presence of subthreshold ouabain does not induce an increase in tone, since Na^+ influx associated with high K^+-mediated contraction is smaller than that induced by noradrenaline stimulation (Aaronson & Jones, 1988). Furthermore, the mutually antagonistic actions of potassium and digitalis glycosides on the Na^+ pump (Schwartz *et al.*, 1975) could contribute to the ineffectiveness of high K^+ stimulation.

In conclusion, the repeated stimulation by noradrenaline of guinea-pig vascular smooth muscle *in vitro* lowers the threshold concentration of ouabain required for contraction. If vasoactive stimuli have similar effects in man *in vivo*, OLC could contribute to enhance vascular tone in hypertension not only by amplifying vasoconstrictor-evoked contractions (Bova *et al.*, 1991), but also by a direct contractile action. A physiological role and pathophysiological implications of the presence of OLC in human plasma have indeed been suggested (Hamlyn *et al.*, 1991). Even though the concentration of ouabain used in this study is much higher than the plasma level of OLC found in man, it should be taken into account that, at least for the positive inotropic effect of digitalis, the *in vitro* sensitivity of the guinea-pig to ouabain is roughly ten

fold lower than that in man (Brown *et al.*, 1986). Furthermore, the ratio between active concentrations of ouabain on human myocardium *in vitro* and therapeutic plasma levels of cardiac glycosides *in vivo* is about 50.

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References

AARONSON, P.I. & JONES, A.W. (1988). Ca dependence of Na influx during treatment of rabbit aorta with NE and high K solutions. *Am. J. Physiol.*, **254**, C75–C83.

ASHIDA, T. & BLAUSTEIN, M.P. (1987). Regulation of cell calcium and contractility in mammalian arterial smooth muscle. The role of sodium-calcium exchange. *J. Physiol.*, **392**, 617–635.

BATTLE, D.C., GODINICH, M., LAPOINTE, M.S., MUNOZ, E., CARONE, F. & MEHERING, N. (1991). Extracellular Na^+ dependency of free cytosolic Ca^{2+} regulation in aortic vascular smooth muscle cells. *Am. J. Physiol.*, **261**, C845–C856.

BLAUSTEIN, M.P. (1977). Sodium ions, calcium ions, blood pressure regulation and hypertension: a reassessment and a hypothesis. *Am. J. Physiol.*, **232**, C165–C173.

BLAUSTEIN, M.P., BOVA, S., YUAN, X.-J. & GOLDMAN, W.F. (1990). The role of sodium/calcium exchange in the regulation of vascular contractility. In *Calcium Transport and Intracellular Calcium Homeostasis*. ed. Datsu, T. & Bronner, F. pp. 123–132. Berlin: Springer-Verlag.

BOULANGER, C., HENDRICKSON, H., LORENZ, R.R. & VANHOUTTE, P.M. (1989). Release of different relaxing factors by cultured porcine endothelial cells. *Circ. Res.*, **64**, 1070–1078.

BOVA, S., BLAUSTEIN, M.P., HARRIS, D.W., LUDENS, J.H., DUCHARME, D.W. & HAMLYN, J.M. (1991). Effects of an endogenous ouabainlike compound on heart and aorta. *Hypertension*, **17**, 944–950.

BOVA, S., CARGNELLI, G. & LUCIANI, S. (1988). Na/Ca exchange and tension development in vascular smooth muscle: effect of amiloride. *Br. J. Pharmacol.*, **93**, 601–608.

BOVA, S., CARGNELLI, G., ROSSI, G.P. & PESSINA, A.C. (1992). Subthreshold ouabain concentrations affect resting tone of aortic strips contracted by noradrenaline (Ne) and histamine (H) (abstract). *J. Am. Coll. Cardiol.*, **19**, 75A.

BOVA, S., GOLDMAN, W.F., YUAN, X.-J. & BLAUSTEIN, M.P. (1990). Influence of the Na^+ gradient on Ca^{++} transients and contraction in vascular smooth muscle. *Am. J. Physiol.*, **259**, H409–H423.

BROWN, L., LORENZ, B. & ERDMAN, E. (1986). Additive and non-additive positive inotropic effects in human and guinea-pig myocardium. In *Cardiac Glycosides 1785–1985*. ed. Erdman, E., Greef, K. & Skou, J.C. pp. 195–205. New York: Springer-Verlag.

DE WARDENER, H.E. & MACGREGOR, G.A. (1980). Dahl's hypothesis that a saluretic substance may be responsible for a sustained rise in arterial pressure: its possible role in essential hypertension. *Kidney Int.*, **18**, 1–9.

DERAY, G., RIEU, M., DEVYNCK, M.A., PERNOLLET, M.G., CHANSON, P., LUTON, J.P. & MEYER, P. (1987). Evidence for an endogenous digitalis-like factor in the plasma of patients with acromegaly. *New Engl. J. Med.*, **316**, 575–580.

EISNER, D.A. (1986). The Na-K pump in cardiac muscle. In *The Heart and Cardiovascular System*. ed. Fozzard, H.A., Haber, E., Jennings, R.B., Katz, A.M. & Morgan, H.E. pp. 489–507. New York: Raven Press.

GOLIGORSKY, M.S., IIJIMA, K., MORGAN, M., YANAGISAWA, M., MASAKI, T., LIN, L., NASJILETTI, A., KASKEL, F., FRAZER, M. & BADR, K.F. (1991). Role of endothelium in the development of Dahl hypertension. *J. Cardiovasc. Pharmacol.*, **17** (suppl 7), S84–S91.

GRUBER, K.A., RUDEL, L.L. & BULLOCK, B.C. (1982). Increased circulating levels of an endogenous digoxin-like factor in hypertensive monkeys. *Hypertension*, **4**, 348–354.

GRUBER, K.A., WHITAKER, J.M. & BUCKALEW, V.H. (1980). Endogenous digitalis-like substance in plasma volume-expanded dogs. *Nature*, **287**, 743–745.

GUTHRIE, T.P.Jr. (1984). Effects of digoxin on responsiveness to the pressor actions of angiotensin and noradrenaline. *J. Clin. Endocrinol. Metab.*, **58**, 76–80.

HAMLYN, J.M., BLAUSTEIN, M.P., BOVA, S., DUCHARME, D.W., HARRIS, D.W., MANDEL, F., MATHEWS, W.R. & LUDENS, J.H. (1991). Identification and characterization of a ouabain-like compound from human plasma. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 6259–6263.

HAMLYN, J.M., RINGEL, R., SCHAEFFER, J., LEVINSON, P.D., HAMILTON, B.P., KOWARSKI, A.A. & BLAUSTEIN, M.P. (1982). A circulating inhibitor of (Na-K)-ATPase associated with essential hypertension. *Nature*, **300**, 650–652.

KARAKI, H., OZAKI, H. & URAKAWA, N. (1978). Effects of ouabain and potassium-free solution on the contraction of isolated blood vessels. *Eur. J. Pharmacol.*, **48**, 439–443.

LINDENMAYER, G.E. & SCHWARTZ, A. (1975). A kinetic characterization of calcium on (Na^+/K^+)-ATPase and its potential role as a link between extracellular and intracellular events: hypothesis for digitalis-induced inotropism. *J. Mol. Cell. Cardiol.*, **7**, 591–612.

MASUGI, F., OGIHARA, T., HASEGAWA, T., TOMII, A., NAGANO, M., HIGASHIMORI, K., KUMAHARA, K. & TERANO, Y. (1986). Circulating factor with ouabain-like immunoreactivity in patients with primary aldosteronism. *Biochem. Biophys. Res. Commun.*, **135**, 41–45.

MORELAND, R.S., TERRY, C.M. & WEBB, R.C. (1986). Contractile responses to ouabain and K^+-free solution in aorta from hypertensive rats. *Am. J. Physiol.*, **250**, H612–H619.

MORETH, K., RENNER, D. & SCHONER, W. (1987). A quantitative receptor assay for 'digitalis-like' compounds in serum: demonstration of raised concentrations in essential hypertension and correlation with arterial blood pressure. *Klin. Wochenschr.*, **65**, 179–184.

MOROMIZATO, H., MIYAGI, H., NAKAYAMA, M., HIGUCHI, M. & SAKANASHI, M. (1992). Effects of ouabain on contractile response to noradrenaline in isolated rat aorta. *Gen. Pharmacol.*, **23**, 279–283.

NABEL, E.G., BERK, B.C., BROCK, T.A. & SMITH, T.W. (1988). Na^+–Ca^{2+}-exchange in cultured vascular smooth muscle cells. *Circ. Res.*, **62**, 486–493.

OKADA, K., ISHIKAWA, S. & SAITO, T. (1991). Mechanisms of vasopressin-induced increase in intracellular Na^+ in vascular smooth muscle cells. *Am. J. Physiol.*, **261**, F1007–F1012.

OZAKI, H., KARAKI, H. & URAKAWA, N. (1978). Possible role of Na-Ca exchange mechanism in the contractions induced in guinea-pig aorta by potassium free solution and ouabain. *Arch. Pharmacol.*, **304**, 203–209.

SCHATZMANN, H.J. (1985). Calcium extrusion across the plasma membrane by the calcium-pump and the Ca^{2+}-Na^+ exchange system. In *Calcium and Cell Physiology*. ed. Marmé, D. pp. 18–52. Berlin: Springer-Verlag.

SCHWARTZ, A., LINDENMAYER, G.E. & ALLEN, J.C. (1975). The sodium-potassium adenosine triphosphatase: pharmacological, physiological and biochemical aspects. *Pharmacol. Rev.*, **27**, 4–134.

SMITH, J.B. & BROCK, T.A. (1983). Analysis of angiotensin-stimulated sodium transport in cultured smooth muscle cells from rat aorta. *J. Cell Physiol.*, **114**, 284–290.

VALLEGA, G.A., CANESSA, M.L., BERCK, B.C., BROCK, T.A. & ALEXANDER, R.W. (1988). Vascular smooth muscle Na^+–H^+ exchanger kinetics and its activation by angiotensin II. *Am. J. Physiol.*, **254**, C751–C758.

VAN BREEMAN, C. & SAIDA, K. (1989). Cellular mechanisms regulating $[Ca^{2+}]_i$ smooth muscle. *Annu. Rev. Physiol.*, **51**, 315–329.

WOOLFSON, R.G., HILTON, P.J. & POSTON, L. (1990). Effects of ouabain and low sodium on contractility of human subcutaneous resistance arteries. *Hypertension*, **15**, 583–590.

WOOLFSON, R.G. & POSTON, L. (1991). Effect of ouabain on endothelium-dependent relaxation of human resistance arteries. *Hypertension*, **17**, 619–625.

YAMADA, H., GOTO, A., HUI, C. & SUGIMOTO, T. (1990). Endogenous digitalis-like factor as a stimulator of endothelin secretion from endothelial cells. *Biochem. Biophys. Res. Commun.*, **172**, 178–183.

YUAN, C.M., MANUNTA, P., HAMLYN, J.M., CHEN, S., BOHEN, E., YEUN, J., HADDY, F.J. & PAMNANI, M.B. (1993). Long-term ouabain administration produces hypertension in rats. *Hypertension*, **22**, 178–187.

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Behavioural and neurochemical adaptations to nicotine in rats: influence of NMDA antagonists

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1 The repeated co-administration of the non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist dizocilpine (0.1 and 0.3 mg kg^{-1}, i.p.) with nicotine (0.4 mg kg^{-1}, s.c.) attenuated the development of tolerance to the locomotor depressant effect of the nicotine in rats.

2 The repeated co-administration of the competitive NMDA antagonist D-CPPene (SDZ EAA 494; 3-(2-carboxypiperazin-4-yl)-1-propenyl-1-phosphonic acid, 2 and 8 mg kg^{-1}, i.p.) also attenuated tolerance to the locomotor depressant effect of nicotine.

3 Dizocilpine (0.3 mg kg^{-1}, i.p.) pretreatment attenuated sensitization to the locomotor stimulant effect of nicotine (0.4 mg kg^{-1}, s.c.) and prevented sensitization of nicotine-induced dopamine release in the nucleus accumbens. However, pretreatment with dizocilpine alone caused a modest enhancement of the behavioural response to a subsequent acute dose of nicotine.

4 D-CPPene (2.0 mg kg^{-1}, i.p.) pretreatment prevented sensitization to the nicotine-induced dopamine release in the nucleus accumbens. There was no enhanced locomotor response that could be attributed to nicotine pretreatment when D-CPPene was co-administered with nicotine. However, pretreatment with D-CPPene alone enhanced the locomotor response to an acute dose of nicotine.

5 The results suggest the involvement of NMDA receptors in adaptations of the behavioural and neurochemical effects of nicotine that occur as a result of repeated administration of the drug.

Keywords: Nicotine; locomotor activity; extracellular dopamine; NMDA receptor antagonists; dizocilpine; D-CPPene; MK-801; SDZ EAA 494

Introduction

In view of the addictive properties of tobacco use, there has been considerable interest in the way that the central nervous system adapts to chronic exposure to nicotine. The effects of nicotine on locomotor activity in rodents are thought to be centrally mediated and have been extensively studied with regard to changes with repeated exposure to the drug. The acute administration of nicotine at a dose of 0.4 mg kg^{-1} (s.c.) to experimentally naive rats can depress the high levels of locomotor activity seen upon exposure to a novel environment; tolerance develops readily to this effect and it is characterized by a 2–3 fold shift to the right in the dose-response curve (Morrison & Stephenson, 1972; Stolerman *et al.*, 1974). On the other hand, when the basal level of activity of rats is low because they have been habituated to the test apparatus, the acute administration of the same dose of nicotine can increase the locomotor activity (Imperato *et al.*, 1986; Benwell & Balfour, 1992a). Under these conditions, there is sensitization (reverse tolerance) to the effect of nicotine following repeated, intermittent exposure to the drug; this sensitization is characterized by an upward shift (elevation) of the dose-response curve (Ksir *et al.*, 1987).

The mechanisms underlying chronic tolerance and sensitization are not known, although both effects are robust and reproducible. However, in common with other psychostimulant drugs of abuse such as cocaine and the amphetamines (Wise & Bozarth, 1987), nicotine is thought to produce locomotor stimulation through an action on the mesolimbic dopamine system (Clarke *et al.*, 1988; Reavill & Stolerman, 1990; Balfour *et al.*, 1991), a neural pathway which arises in the ventral tegmental area (VTA) and terminates in target structures such as the nucleus accumbens (Fallon & Moore, 1978). This neural pathway is rich in nicotinic receptors located both pre- and post-synaptically (Clarke & Pert, 1985), although current data suggest that both the increase in mesolimbic dopamine secretion and locomotor stimulation, evoked by the systemic administration of nicotine at behaviourally relevant doses, are principally mediated by stimulation of the somatodendritic nicotinic receptors in the VTA (Reavill & Stolerman, 1990; Benwell *et al.*, 1993). Recently, Benwell & Balfour (1992a) have reported sensitization of the effect on mesolimbic dopamine following repeated exposure to nicotine and this is accompanied by sensitization of the nicotine-induced locomotor stimulant effect.

The mechanisms of the adaptive responses to nicotine have not yet been fully elucidated. Chronic exposure to nicotine increases the number of nicotinic receptors as assessed in binding assays (Marks *et al.*, 1985; Schwartz & Kellar, 1985) and this phenomenon has been proposed as a basis for both sensitization (where it is assumed that the increased number reflects fully functional receptors) and chronic tolerance, where the up-regulation is cited as evidence for receptor desensitization. It has also been proposed that the sensitivity of nicotinic receptors may be modulated by corticosteroids such that the release of steroids as a function of chronic exposure to nicotine reduces the sensitivity of the receptors and thus contributes to the observed tolerance (Pauly *et al.*, 1992). In addition, some reports suggest that conditioning may play a role in the development of tolerance to nicotine (Caggiula *et al.*, 1991) although evidence on this point has been inconsistent (Hendry & Rosecrans, 1982).

There have been recent reports that pretreatment with dizocilpine (MK-801), a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist, weakens or prevents the development of adaptations to diverse drugs including CNS stimulants such as amphetamine, methamphetamine and cocaine (Karler *et al.*, 1989; 1990; Kuribara *et al.*, 1992; Wolf & Khansa, 1991; Schenk *et al.*, 1993), opioids such as morphine (Trujillo & Akil, 1990; Ben-Eliyahu *et al.*, 1992) and alcohol (Morriset *et al.*, 1990). Studies also suggest that NMDA

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receptors may play a role in the adaptive responses to nicotine since, when rats are exposed chronically to dizocilpine, as well as to nicotine, the usual sensitization reaction to the locomotor stimulant effect of nicotine fails to develop (Shoaib & Stolerman, 1992b). These data have been interpreted as evidence that activation of NMDA receptors may be a necessary step in the development of sensitization to nicotine. Preliminary studies have also suggested that dizocilpine inhibited adaptation to nicotine under conditions where tolerance to the behavioural depressant effects of nicotine developed (Shoaib & Stolerman, 1992c).

The present experiments were undertaken to test more extensively the effects of NMDA receptor antagonists on tolerance and sensitization to nicotine. The experiments have been carried out with lower doses of dizocilpine than those used in previous work (Shoaib & Stolerman, 1992b; 1992c) and with the competitive NMDA antagonist D-CPPene (SDZ EAA 494; 3-(2-carboxy-piperazin-4-yl)-1-propenyl-1-phosphonic acid) described by Aebischer *et al.* (1989). In addition, in view of the importance of dopamine in mediating the effects of nicotine on stimulating locomotor activity, some of the studies were carried out with determinations of the extracellular concentration of dopamine, using *in vivo* microdialysis, in parallel with behavioural measurements. Akbar *et al.* (1993) have given a preliminary account of some of the experiments.

Methods

Animals

For experiments on tolerance to nicotine, male Lister hooded rats weighing 180–250 g were purchased from Harlan Olac Ltd. (Bicester, Oxfordshire). For experiments on sensitization to nicotine, male Sprague-Dawley rats were bred in the Biomedical Services Unit, Dundee University Medical School and weighed 250–350 g at the start of the experiments.

Locomotor activity

Tolerance to the locomotor depressant effects of nicotine was assessed in photocell activity cages described previously (Clarke & Kumar, 1983; Reavill & Stolerman, 1990), each cage measuring 30 × 30 × 30 cm. For behavioural assessments of sensitization to nicotine in conjunction with microdialysis, the rats were placed in photocell boxes described by Vale & Balfour (1989), each of these cages measuring 40 × 40 × 25 cm.

Drug pretreatments

The four experiments consisted of two studies of tolerance to nicotine using behavioural assessments only and two studies of sensitization to nicotine with both behavioural and neurochemical determinations. One study of each type was carried out with dizocilpine and one with D-CPPene. In each study rats were injected intraperitoneally (i.p.) with saline or either one of the NMDA antagonists, followed 30 min later by subcutaneous (s.c.) injections of saline or nicotine (0.4 mg kg^{-1}). These procedures were carried out once per day for 7 days and are called 'pretreatments'. After the period of pretreatment, tests of the responses to single injections of saline or nicotine (0.4 mg kg^{-1}, s.c.) were carried out. During this stage of the studies, no further doses of the NMDA antagonists were given.

In the two studies of tolerance, each rat was tested in photocell cages for 30 min beginning immediately after injection of the saline or nicotine (0.4 mg kg^{-1}, s.c.). The first test took place 24 h after the last pretreatment and the drug and saline were tested in a counterbalanced order within each group. One clear day without injections or tests was interposed between the tests. For the two studies of sensitization, both of which involved microdialysis, the probes were implanted 3 h after the seventh of the daily pretreatments with nicotine, dizocilpine or D-CPPene. Recordings of locomotor activity and collection of dialysates began 18–24 h later and the responses to saline and nicotine (0.4 mg kg^{-1}, s.c.) were tested during 220 min sessions in photocell cages.

Implantation of microdialysis probes

The dialysis probe used and its implantation were similar to that described by Benwell & Balfour (1992a). Rats were anaesthetized with a mixture of Hypnovel (10 mg midazolam/2 ml water), Hypnorm (10 mg fluanisone + 0.315 mg fentanyl citrate/ml water) and water, in a ratio of 1:1:2 and injected in a volume of 1 ml kg^{-1} (i.p.). Dialysis loops were implanted in the nucleus accumbens using co-ordinates of 1.7 mm rostral and 1.5 mm lateral to bregma and 7.5 mm from the surface of the brain, according to Paxinos & Watson (1986). At the end of the experiments, the position of the probes was determined histologically from sections prepared post mortem from tissue fixed in formalin.

Microdialysis

Eighteen to twenty-four hours after implantation of the dialysis probes, the rats were placed in the photocell boxes and the dialysis probes connected via liquid swivels to syringe pumps containing Ringer solution (mM: NaCl 147, $CaCl_2$ 1.25, KCl 4.0) which was perfused at a rate of 1.7 µl min^{-1}. Samples were collected at intervals of 20 min for 80 min to establish a baseline of dopamine release. Then, all animals were injected with saline (1 ml kg^{-1}, s.c.) to act as an injection control, followed 60 min later by an injection of nicotine (0.4 mg kg^{-1}, s.c.).

The concentrations of dopamine, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the brain dialysates were assayed by injecting the samples directly into a high performance liquid chromatographic (h.p.l.c.) system using electrochemical detection as described by Benwell & Balfour (1992a). Nicotine, dizocilpine and D-CPPene did not produce any peaks on the h.p.l.c. traces. In each experiment, the mean concentrations of dopamine and its metabolites for the three samples collected before any test injections were calculated and defined as 100%. The concentrations of these substances in each fraction collected were then expressed as percentages of the mean baseline values.

Drugs

(−)-Nicotine hydrogen tartrate was purchased from British Drug Houses and Sigma (Poole, Dorset). Merck, Sharp and Dohme and Sandoz generously donated dizocilpine (MK-801) and D-CPPene (SDZ EAA 494) respectively. EDTA and octane sulphonic acid (SOS) were purchased from Fisons plc., h.p.l.c. grade methanol was supplied by Rathburn Chemical Ltd. All other laboratory and h.p.l.c grade reagents were obtained from British Drug Houses. Drug doses are expressed in terms of their free base concentrations. Nicotine was administered subcutaneously in a volume of 1 ml kg^{-1} and dizocilpine and D-CPPene were administered intraperitoneally in volumes of 2 ml kg^{-1}. Corresponding volumes of saline (0.9%) served as controls.

Statistical analysis

Statistical analysis of behavioural and biochemical results was performed by two and three factor analysis of variance for repeated measures. Individual comparisons were made, where appropriate, by means of Duncan's test and Dunnett's *t* tests.

Results

Experiment 1: Effect of dizocilpine on nicotine tolerance

The results from the tests after administration of saline are presented first and are taken as baseline data. Table 1 (left section) shows the baseline amounts of activity for different groups of animals that had been pretreated for 7 days with either saline, nicotine (0.4 mg kg^{-1}, s.c.) or the same dose of nicotine plus dizocilpine (0.03, 0.1 or 0.3 mg kg^{-1}). Each group consisted of 8 rats. Two-factor analysis of variance failed to show any significant overall differences between the groups for activity over the whole 30-min test ($F(4,35) = 1.20$). There was an overall decline in activity in all the groups as a function of time ($F(5,175) = 49.4$, $P < 0.001$) and this decline was similar in all cases (interaction $F(20,175) < 1$).

In rats that had no previous exposure to nicotine or dizocilpine, nicotine (0.4 mg kg^{-1}, s.c.) markedly reduced locomotor activity. Figure 1b (inset) shows the time course of this effect, which was strongest early in the 30 min test and had dissipated by the end. The remainder of the results for this experiment are presented as differences from saline control data (i.e., activity after saline) because the baseline data did not differ significantly (see above).

Figure 1a shows the differences between groups in the effects of nicotine on the total amount of locomotor activity in the test sessions. These effects of nicotine were substantially altered by the previous administration of nicotine and dizocilpine ($F(4,35) = 8.97$, $P < 0.001$). The strong depressant effect of nicotine in rats not previously exposed to the drug, is clearly apparent, as is the lack of depressant effect in rats previously exposed to nicotine. These latter rats were taken as the control group for statistical purposes and the data from every other group were compared with them. Thus, the effect of pretreatment with nicotine was highly significant ($F(1,35) = 27.4$, $P < 0.001$) and is taken as evidence of tolerance. The results for rats pretreated with both nicotine and a small dose of dizocilpine (0.03 mg kg^{-1}) did not differ from those for rats pretreated with nicotine only. However, rats pretreated with nicotine plus either 0.1 or 0.3 mg kg^{-1} of dizocilpine were more sensitive to the depressant effects of nicotine than rats pretreated with nicotine only ($F(1,35) = 5.45$ and 7.39, $P < 0.05$ in each case).

Time courses of locomotor activity are shown in Figure 1b. The short-lasting depressant effect of nicotine in drug-naive rats was abolished by pretreatment with nicotine and partly restored when dizocilpine (0.3 mg kg^{-1}, i.p.) was administered during the pretreatment phase. Time course data for other doses of dizocilpine have been omitted for clarity.

Experiment 2: Effect of D-CPPene on nicotine tolerance

Table 1 shows the baseline amounts of activity for six different groups of animals that had been pretreated for 7 days with either saline, nicotine (0.4 mg kg^{-1}, s.c.), the same dose of nicotine plus D-CPPene (1, 2 or 8 mg kg^{-1}) or D-

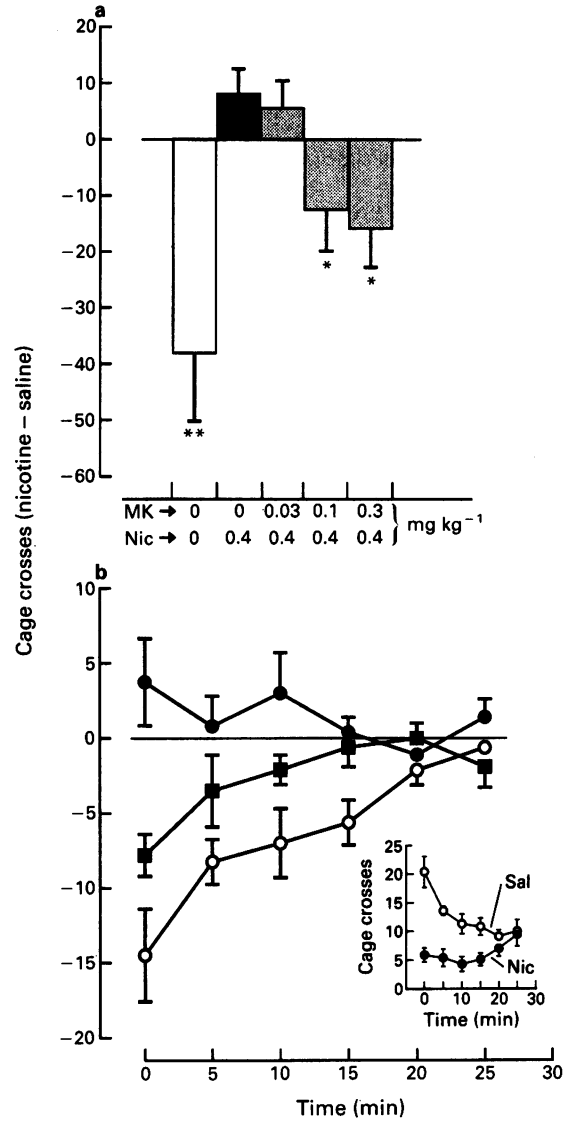


Figure 1 Pretreatment for 7 days with dizocilpine (MK) and nicotine (Nic) influences effects of nicotine (0.4 mg kg^{-1}) administered immediately before locomotor activity test sessions. (a) Effects of nicotine on total activity in 30 min session as differences from saline. Open column shows results for drug-naive rats; solid column shows nicotine pretreatment; stippled columns show pretreatment with nicotine plus dizocilpine in doses shown (statistical significance of comparisons with rats pretreated with nicotine only: $*P < 0.05$; $**P < 0.01$). (b) Time-course for effects of nicotine as differences from saline (○, drug-naive rats; ●, nicotine (0.4 mg kg^{-1}) pretreatment; ■, nicotine plus dizocilpine (0.3 mg kg^{-1}) pretreatment). Inset shows absolute numbers of cage crosses to illustrate the decline in activity within the session in drug-naive rats (○), and the time-course of the depressant effect of nicotine in the same rats (●). All results are shown as means ± s.e.mean; overlapping s.e.means are omitted for clarity ($n = 8$).

Table 1 Baseline levels of locomotor activity of rats in photocell cages

Experiment 1: Dizocilpine (MK-801)		*Experiment 2: D-CPPene*	
Group	*Mean ± s.e.mean*	*Group*	*Mean ± s.e.mean*
Sal + Sal	75.1 ± 7.8	Sal + Sal	68.4 ± 4.4
Sal + Nic	69.1 ± 9.8	Sal + Nic	84.3 ± 6.6
MK-801 (0.03 mg kg^{-1}) + Nic	86.8 ± 4.2	CPPene (1 mg kg^{-1}) + Nic	81.9 ± 5.5
MK-801 (0.1 mg kg^{-1}) + Nic	69.5 ± 7.0	CPPene (2 mg kg^{-1}) + Nic	76.8 ± 5.2
MK-801 (0.3 mg kg^{-1}) + Nic	68.6 ± 4.7	CPPene (8 mg kg^{-1}) + Nic	69.8 ± 4.9
		CPPene (8 mg kg^{-1}) + Sal	82.3 ± 6.2

The rats were pretreated for 7 days with saline (1 ml kg^{-1}), nicotine (0.4 mg kg^{-1}), dizocilpine and D-CPPene as indicated above, prior to the collection of the data shown. Results are mean ± s.e.mean of numbers of cage crosses in 30 min beginning immediately after injection of saline ($n = 8$).

CPPene (8 mg kg^{-1}) only. Each group consisted of eight rats. Two-factor analysis of variance failed to show any significant overall differences between the groups for activity over the whole 30-min test ($F(5,42) = 1.51$). There was an overall decline in activity as a function of time ($F(5,210) = 42.9$, $P < 0.001$) and this decline was similar in all groups (interaction $F(25,210) < 1$).

In rats which had no previous exposure to either nicotine or D-CPPene, nicotine (0.4 mg kg^{-1}, s.c.) markedly reduced locomotor activity. Figure 2a shows the very different effects of nicotine on the total amount of locomotor activity in the different groups of rats ($F(5,42) = 6.98$, $P < 0.001$). The strong depressant effect of nicotine, in rats not previously exposed to the drug, is clearly apparent. Pretreatment with D-CPPene alone, in a dose of 8 mg kg^{-1}, did not affect the acute depressant response to nicotine. The depressant effect was abolished in rats pretreated with nicotine alone and replaced by a trend towards stimulation. For statistical purposes, the data for every other group were compared with those for the rats pretreated with nicotine alone. Thus, the effect of pretreatment with nicotine alone, as compared with saline pretreatment, was highly significant ($F(1,42) = 18.1$, $P < 0.001$) and is taken as evidence for tolerance. The results for rats pretreated with both nicotine and a small dose of D-CPPene (1.0 mg kg^{-1}) did not differ from those for rats pretreated with nicotine only. However, in rats pretreated with nicotine plus either 2.0 or 8.0 mg kg^{-1} of D-CPPene, there was less activity after the administration of nicotine than in rats pretreated with nicotine only ($F(1,42) = 8.70$ and 8.49, $P < 0.01$ in each case).

Time courses of locomotor activity are shown in Figure 2b. The short lasting depressant effect of nicotine, in drug-naive rats, was abolished by pretreatment with nicotine and partly restored when D-CPPene (8 mg kg^{-1}, i.p.) was administered during the pretreatment phase. Time course data for other doses of D-CPPene have been omitted for clarity.

Experiment 3: Effect of dizocilpine on nicotine sensitization

Initially, locomotor activity did not differ appreciably between the groups that had been pretreated for 7 days with either saline, nicotine (0.4 mg kg^{-1}, s.c.), the same dose of nicotine plus dizocilpine (0.3 mg kg^{-1}, i.p.), or dizocilpine (0.3 mg kg^{-1}, i.p.) alone ($n = 4-6$). Locomotor activity declined to a low level prior to the injection of saline or nicotine (0.4 mg kg^{-1}, s.c.) and injections of saline had no detectable effects (Figure 3a).

In rats pretreated with saline only, the acute administration of nicotine during the activity test sessions increased locomotor activity ($F(8,24) = 8.9$, $P < 0.01$). This effect was enhanced in rats pretreated with nicotine ($F(8,136) = 4.77$, $P < 0.001$), and the enhanced response was attenuated when dizocilpine as well as nicotine was administered during the pretreatment phase ($F(8,136) = 3.07$, $P < 0.01$). However, di-

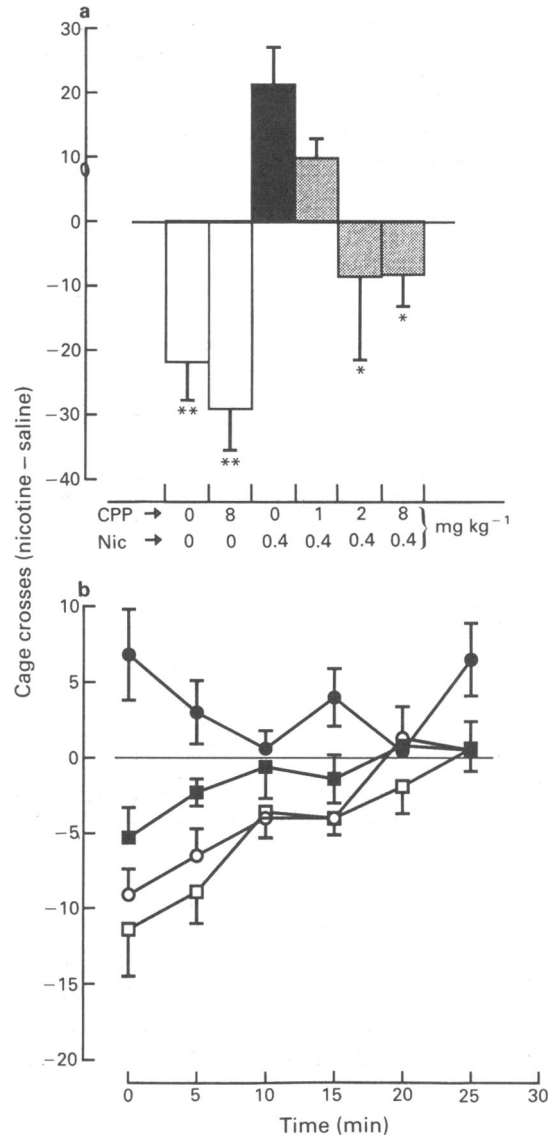


Figure 2 Pretreatment for 7 days with D-CPPene and nicotine (Nic) influences effects of nicotine (0.4 mg kg^{-1}) administered immediately before locomotor activity test sessions. (a) Effects of nicotine on total activity in 30 min session. Open columns show results for drug-naive rats and for rats pretreated with D-CPPene only; solid column shows nicotine pretreatment; stippled columns show pretreatment with nicotine plus D-CPPene in doses shown (statistical significance of comparisons with rats pretreated with nicotine only: $*P < 0.01$; $**P < 0.001$). (b) Time-course for effects of nicotine (0.4 mg kg^{-1}, s.c.) on activity (○, drug-naive rats; ● nicotine pretreatment; □ D-CPPene (8 mg kg^{-1}) pretreatment; ■ nicotine plus D-CPPene pretreatment). All results are shown as mean differences from saline ± s.e.mean; overlapping s.e.means are omitted for clarity ($n = 8$).

Table 2 Basal extracellular concentrations of dopamine (DA), dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in nucleus accumbens of rats

Pretreatment	n	*DA* (pmol 20 μl^{-1})	*DOPAC* (pmol 20 μl^{-1})	*HVA* (pmol 20 μl^{-1})
Sal + Sal	4	0.053 ± 0.006	19.12 ± 4.76	6.08 ± 1.60
Sal + Nic	6	0.098 ± 0.025	13.40 ± 1.51	5.68 ± 0.83
MK-801 + Sal	5	0.058 ± 0.015	14.65 ± 3.29	7.73 ± 2.27
MK-801 + Nic	6	0.064 ± 0.009	15.13 ± 1.94	6.30 ± 0.57
CPPene + Sal	5	0.093 ± 0.019	17.25 ± 4.06	6.94 ± 0.98
CPPene + Nic	4	0.097 ± 0.021	11.85 ± 1.78	4.78 ± 0.63

The rats were pretreated for 7 days with saline (1 ml kg^{-1}), nicotine (0.4 mg kg^{-1}, s.c.), dizocilpine (MK-801, 0.1 mg kg^{-1}, i.p.) and D-CPPene (2 mg kg^{-1}, i.p.) as indicated above, prior to collection of the data. The results are means ± s.e.mean of the concentrations of dopamine and its metabolites in dialysate samples collected in the first 60 min of test sessions, uncorrected for recovery through the membrane (10%, 14% and 13% for dopamine, DOPAC and HVA respectively).

zocilpine did not completely abolish the enhanced response to nicotine and pretreatment with dizocilpine only (i.e. without nicotine) was sufficient to enhance the response ($F(8,56) = 3.11$, $P < 0.01$).

Table 2 shows the basal concentrations of dopamine and its metabolites for the different groups of rats. There was a tendency for the extracellular dopamine to be higher in rats pretreated with nicotine. However, there was no significant differences between the groups associated with any of the pretreatments involving nicotine, dizocilpine or D-CPPene.

Following pretreatment with nicotine, an injection of nicotine increased the extracellular concentration of dopamine in

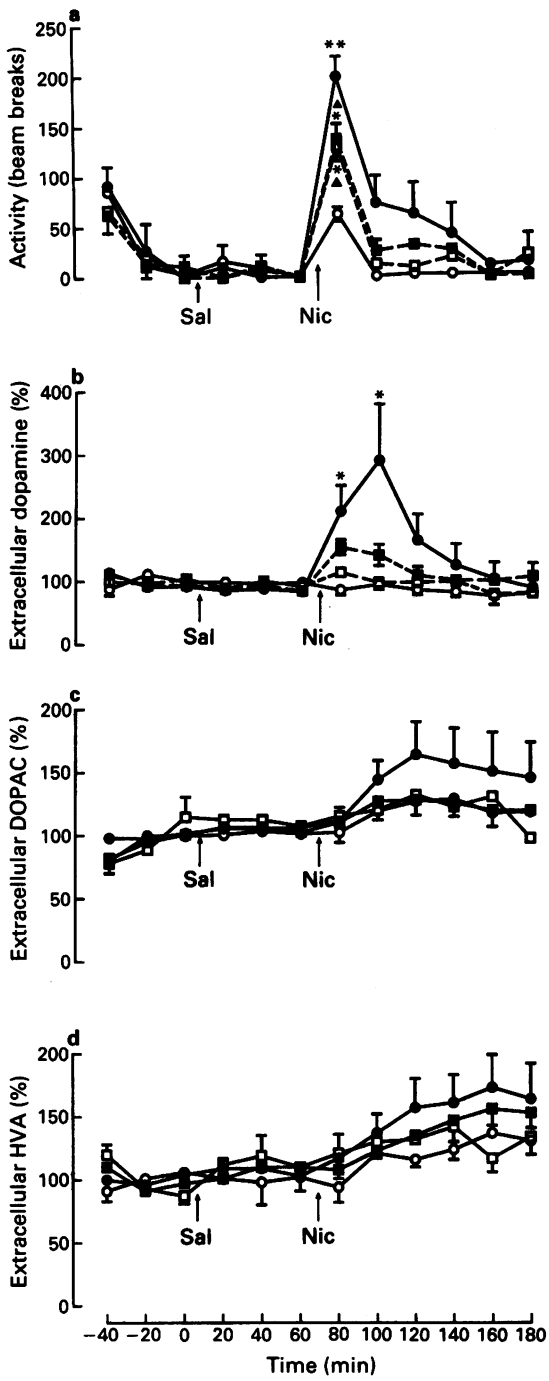


Figure 3 Pretreatment for 7 days with dizocilpine (MK-801, 0.3 mg kg^{-1}, i.p.) and nicotine (Nic, 0.4 mg kg^{-1}, s.c.) influenced effects of nicotine when locomotor activity was recorded simultaneously with collection of dialysate for successive 20 min periods (pretreatments: ○, saline; ●, nicotine; □, dizocilpine; ■, nicotine plus dizocilpine). Saline (Sal) and nicotine (0.4 mg kg^{-1}, s.c.) were injected at times shown. (a) Locomotor activity as numbers of light-beam breaks (means ± s.e.mean). For dopamine (b), dihydroxyphenylacetic acid (DOPAC, c) and homovanillic acid (HVA, d) results are shown as percentages of the means ± s.e.mean for the three samples collected prior to the injection of saline. Statistical significance: comparisons with rats pretreated with saline: $^{*}P < 0.05$; $^{**}P < 0.01$; comparisons with rats pretreated with nicotine: $^{\blacktriangle}P < 0.05$.

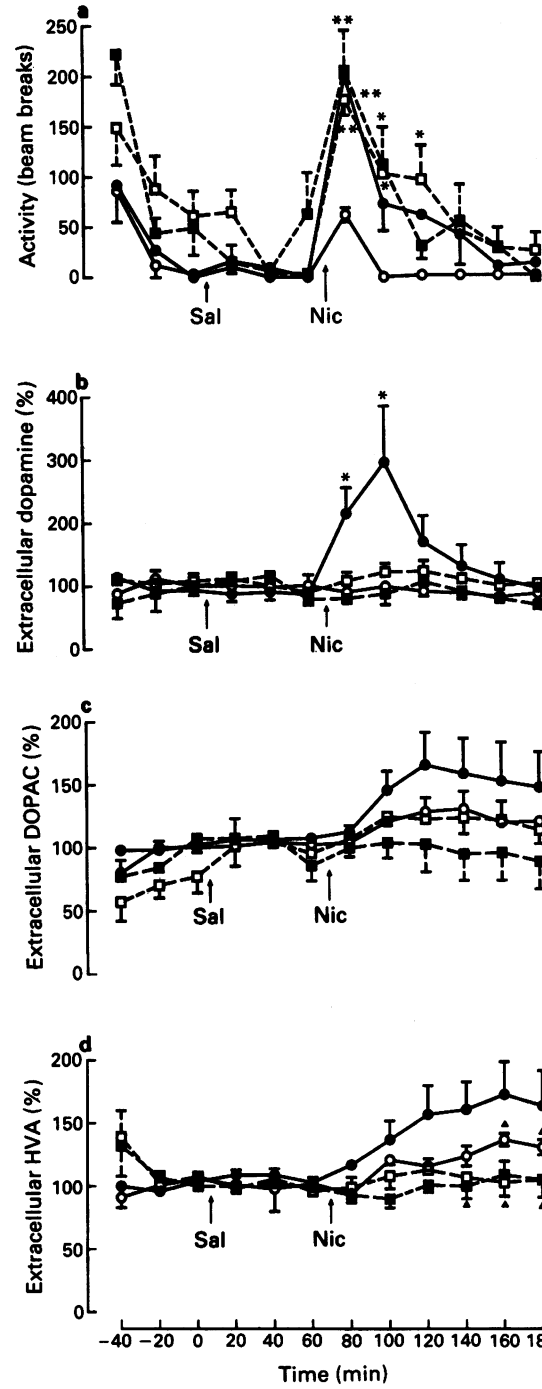


Figure 4 Pretreatment for 7 days with D-CPPene (2 mg kg^{-1}, i.p.) and nicotine (Nic, 0.4 mg kg^{-1}, s.c.) influenced the effects of nicotine when locomotor activity was recorded simultaneously with collection of dialysate for successive 20 min periods (pretreatments: ○, saline; ●, nicotine; □, D-CPPene; ■, nicotine plus D-CPPene). Saline and nicotine (0.4 mg kg^{-1}, s.c.) were injected at times shown. (a) Locomotor activity as numbers of light-beam breaks (means ± s.e.mean). For dopamine (b), dihydroxyphenylacetic acid (DOPAC, c) and homovanillic acid (HVA, d) results are shown as percentages of the means ± s.e.mean for the three samples collected prior to the injection of saline. Statistical significance: comparisons with rats pretreated with saline: $^{*}P < 0.05$; $^{**}P < 0.01$; comparisons with rats pretreated with nicotine: $^{\blacktriangle}P < 0.05$.

the nucleus accumbens ($F(8,136) = 5.04$, $P < 0.01$). Figure 3b shows that dizocilpine co-administered with the nicotine during the pretreatment phase prevented the development of the enhanced response ($F(8,136) = 2.11$, $P < 0.05$). Pretreatment with dizocilpine alone was without effect on the response to an acute dose of nicotine. The administration of nicotine on the test day increased the extracellular concentrations of DOPAC ($F(8,136) = 7.21$, $P < 0.001$) and HVA ($F(8,136) = 13.2$, $P < 0.001$) in the nucleus accumbens of rats in all groups. The trend for the concentrations of DOPAC and HVA to be greater in the rats pretreated with nicotine than in those pretreated with saline was not significant. Pretreatment with dizocilpine had no effect on the concentrations of either DOPAC or HVA (Figure 3c and d).

Experiment 4: Effect of D-CPPene on nicotine sensitization

The spontaneous locomotor activity of the rats pretreated with D-CPPene was greater than in the saline and nicotine pretreated rats ($F(3,19) = 3.74$, $P < 0.05$) when they were first placed in the activity boxes ($n = 4-6$). However, the activity of all groups declined to a low level prior to injections of saline or nicotine and the injection of saline had no detectable effects in any group (Figure 4a). The enhanced response seen with pretreatment with nicotine ($F(8,128) = 2.72$, $P < 0.01$) was not influenced by concurrent pretreatment with D-CPPene and, in fact, the increase in activity observed was similar in magnitude to that seen with nicotine pretreatment alone. An enhanced response to nicotine was also obtained in rats which had received D-CPPene only prior to the test day ($F(8,64) = 2.43$, $P < 0.05$).

Figure 4b shows the findings on the extracellular concentration of dopamine in the nucleus accumbens. The co-administration of D-CPPene with nicotine, during the pretreatment phase, completely abolished the development of the enhanced response to nicotine ($F(8,128) = 4.19$, $P < 0.001$). Pretreatment with D-CPPene alone was without effect on this parameter. The administration of nicotine on the test day increased the extracellular concentrations of DOPAC ($F(8,128) = 3.79$, $P < 0.001$) in the nucleus accumbens. There were no significant changes in DOPAC in this brain region which could be attributed to pretreatment with D-CPPene (Figure 4c). However, nicotine administered on the test day also increased ($F(8,126) = 6.13$, $P < 0.001$) the extracellular concentration of HVA in the nucleus accumbens and this effect was prevented ($F(8,128) = 3.58$, $P < 0.01$) by pretreatment with D-CPPene, as shown in Figure 4d.

Discussion

Earlier studies suggested that dizocilpine could attenuate both tolerance to the depressant effect of nicotine on locomotor activity and sensitization to nicotine-induced locomotor stimulation (Shoaib & Stolerman, 1992b,c). The present experiments extend these observations to behavioural studies with the competitive NMDA receptor antagonist D-CPPene and to experiments on dopamine release with both dizocilpine and D-CPPene. Experiment 1 confirmed the effect of dizocilpine on tolerance to nicotine and provided evidence for the efficacy of a relatively small dose of dizocilpine (0.1 mg kg^{-1}). Shoaib & Stolerman (1992c) showed that under similar conditions, pretreatment with dizocilpine (0.3 mg kg^{-1}) did not sensitize rats to the depressant effect of a subsequent acute exposure to nicotine.

Experiment 2 showed that a competitive antagonist, D-CPPene, could also attenuate tolerance to nicotine. This substance had not been used previously in experiments of this type and therefore, the effects were examined of pretreatment with D-CPPene on the response to an acute dose of nicotine. D-CPPene did not sensitize to the acute locomotor depressant effect of nicotine and the apparent attenuation of tolerance cannot be explained by a directly opposing effect of D-CPPene. However, although two doses of both dizocilpine and D-CPPene attenuated tolerance in the present experiments, neither dose of either drug appeared to prevent tolerance completely. Both the present studies and those of Shoaib & Stolerman (1992c) employed 7 day periods of pretreatment with the drugs and a fixed 30 min interval between the daily administrations of the NMDA receptor antagonists and nicotine. The effects of the antagonists on tolerance produced by different durations of pretreatment and when administered at different times in relation to nicotine are not known. The mechanism of the decreases in locomotor activity may involve the mesolimbic dopamine system. Bilateral infusions of nicotine into the accumbens can decrease locomotor activity in experimentally naive rats but are without effect in animals made tolerant by subcutaneous administration of nicotine (Welzil *et al.*, 1990). In contrast, tolerance does not develop readily to the locomotor depressant effect obtained when nicotine is infused into the fourth ventricle of rats and this effect, therefore, seems to be different from the locomotor depression seen after subcutaneous administration of nicotine (Shoaib & Stolerman, 1992a).

As in previous studies using a 5 day pretreatment schedule (Benwell & Balfour, 1992a), pretreatment with 7 daily injections of nicotine in experiments 3 and 4 was associated with sensitization of both the mesolimbic dopamine and locomotor stimulant responses to nicotine. The different acute and chronic effects of nicotine on locomotor activity in these studies, as compared with experiments 1 and 2, may be attributed primarily to the prior habituation of the animals to the test apparatus, although some contributions from strain and apparatus differences are also possible. There were no changes in the baseline levels of dopamine or its metabolites (DOPAC and HVA) in the nucleus accumbens which could be attributed to 7 days pretreatment with either dizocilpine or D-CPPene. In addition, neither of these drugs influenced the dopamine response when the animals were acutely challenged with nicotine. However, the sensitization of the dopamine response to nicotine was prevented by both dizocilpine and D-CPPene when they were administered concurrently with the nicotine pretreatment. Pretreatment with D-CPPene also influenced HVA levels, but there was no other significant effects on metabolites of dopamine.

The locomotor responses obtained in the dialysis studies were rather more complex. Pretreatment with dizocilpine attenuated the sensitization of the locomotor stimulant response to nicotine that occurred following repeated exposure to nicotine alone. However, dizocilpine pretreatment alone sensitized the locomotor stimulant effect of an acute exposure to nicotine. Interestingly, it has been reported that dizocilpine inhibited methamphetamine-induced changes in the striatum while at the same time sensitizing the behavioural response (Weihmuller *et al.*, 1991), effects which show similarity to those reported here.

Despite the finding that D-CPPene completely abolished the nicotine-induced sensitization of the mesolimbic dopaminergic system to nicotine, the locomotor activity of these rats was not significantly different from that in the rats pretreated with nicotine alone. In rats that had been pretreated with D-CPPene only, the locomotor response to an acute injection of nicotine, was of the same magnitude as that in the sensitized rats. In view of the stimulatory influence of D-CPPene pretreatment on the locomotor activity of these animals, the interpretaton of the effect of this pretreatment on the nicotine-induced enhanced locomotion is difficult. However, the effects of nicotine plus D-CPPene pretreatment on the locomotor stimulation were not additive. This is unlikely to be due to a ceiling effect since D-amphetamine can induce much greater increases in locomotor activity than this dose of nicotine (Benwell & Balfour, 1992a). Therefore, the data are consistent with the possibility that the D-CPPene pretreatment inhibited the dopamine-

related enhanced locomotor stimulation and that the locomotor stimulation seen is, in this instance, solely related to the D-CPPene pretreatment. Stewart & Druhan (1993) recently put forward a similar argument when seeking to interpret the effects of dizocilpine on amphetamine-induced behavioural sensitization and conditioning.

These results suggest that the nicotine-induced sensitization of the mesolimbic dopamine response is mediated, to some extent at least, by activation of NMDA receptors. However, the locomotor results suggest that the influence of NMDA receptors may be relatively complex. Under the conditions of the present experiment, where nicotine is administered at a time when the basal activity of the animals is very low, even the acute administration of nicotine is associated with stimulation of locomotor activity. There is some evidence to suggest that this component of nicotine-induced locomotion is independent of the mesolimbic dopaminergic system and may be mediated by another neural pathway through a different subpopulation of neuronal nicotinic receptors (Benwell & Balfour, 1992a,b). In order to explain the effects of the NMDA receptor antagonists, it could be proposed that NMDA receptors are involved in mediating the effects of nicotine via both mechanisms, but in opposing fashions. For instance, the stimulation of the NMDA receptors associated with the mesolimbic dopaminergic system may increase nicotine-induced locomotion. Conversely, it is possible that stimulation of NMDA receptors weakens the action of nicotine via the mechanism independent of mesolimbic dopamine.

There are recent reports that dizocilpine, which blocks the ion channels of NMDA receptors (Wong *et al.*, 1986; Heuttner & Bean, 1988), is also capable of blocking the ion channels of nicotinic receptors (Ramoa *et al.*, 1990; Amador & Dani, 1991). Administration of nicotinic channel-blocker, mecamylamine, repeatedly together with nicotine can prevent the development of tolerance that would otherwise occur to the behavioural depressant effect of nicotine (Mariathasan & Stolerman, 1993). Therefore, dizocilpine could have influenced tolerance and sensitization by blocking nicotinic receptors in a manner similar to mecamylamine. However, several lines of argument suggest that a blockade of nicotine receptors is unlikely to explain the present results.

Firstly, dizocilpine is more potent at the NMDA than the nicotinic receptor (Amador & Dani, 1991) and it is not clear whether the concentrations required to block nicotinic receptors *in vitro* are attained by the doses employed in the present experiments. Secondly, the discriminative stimulus effects of mecamylamine are different from those of dizocilpine and D-CPPene, suggesting differences between the dominant mechanisms of action of these drugs in behavioural studies (Garcha & Stolerman, 1993). Thirdly, it seems unlikely that D-CPPene, a competitive NMDA receptor antagonist, acts on nicotinic receptors in the way reported for channel-blockers such as mecamylamine or dizocilpine. Finally, as cited above, dizocilpine attenuates adaptive changes to other psychostimulants, opioids and alcohol, substances that do not interact potently with nicotinic receptors (Karler *et al.*, 1989; 1990; Wolf & Khanza, 1991; Kuribara *et al.*, 1992). It seems reasonable to conclude that an antagonist effect at NMDA receptors is more likely than nicotinic blockade to be the mechanism by which dizocilpine and D-CPPene influenced responses to nicotine.

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References

AEBISCHER, B., FREY, P., HAERTER, H.-P., HERRLING, P.L., MULLER, W., OLVERMAN, H.J. & WATKINS, J.C. (1989). Synthesis and NMDA antagonist properties of the enantiomers of (3-phosphonopropyl)-piperazine-2-carboxylic acid (CPP) and of the unsaturated analogue (E)-4-(3-phosphonoprop-2-enyl)-piperazine-2-carboxylic acid (CPP-ene). *Helv. Chem. Acta.*, **72**, 1043–1051.

AKBAR, M.T., BENWELL, M.E.M., SHOAIB, M., BALFOUR, D.J.K. & STOLERMAN, I.P. (1993). Influence of NMDA antagonists on behavioural and neurochemical adaptation to repeated nicotine administration. *Br. J. Pharmacol.*, **108**, 264P.

AMADOR, M. & DANI, J.A. (1991). MK-801 inhibition of nicotinic acetylcholine receptor channels. *Synapse*, **7**, 207–215.

BALFOUR, D.J.K., BENWELL, M.E.M. & VALE, A.L. (1991). Studies on the role of mesolimbic dopamine in behavioural responses to chronic nicotine. In *Effect of Nicotine on Biological Systems*, ed. Adlkofer, F. & Thurau, K. pp. 407–416. Berlin: Birkhauser.

BEN-ELIYAHU, S., MAREK, P., VACCARINO, A.L., MOGIL, J.S., STERNBERG, W.F. & LIEBESKIND, J.C. (1992). The NMDA receptor antagonist MK801 prevents long-lasting non-associative morphine tolerance in the rat. *Brain Res.*, **575**, 304–308.

BENWELL, M.E.M. & BALFOUR, D.J.K. (1992a). The effects of acute and repeated nicotine treatment on nucleus accumbens dopamine and locomotor activity. *Br. J. Pharmacol.*, **105**, 849–856.

BENWELL, M.E.M. & BALFOUR, D.J.K. (1992b). The effects of nicotine infusions on mesolimbic dopamine responses to injected nicotine. *J. Psychopharmacology*, Abstract book, A9.

BENWELL, M.E.M., BALFOUR, D.J.K. & LUCCHI, H.M. (1993). Influence of tetrodotoxin and calcium on changes in extracellular dopamine levels evoked by systemic nicotine. *Psychopharmacology*, (in press).

CAGGIULA, A., EPSTEIN, L.H., ANTELMAN, S.M., SAYLOR, S.S., PERKINS, K.A., KNOPF, S. & STILLER, R. (1991). Conditioned tolerance to the anorectic and corticosterone-elevating effects of nicotine. *Pharmacol. Biochem. Behav.*, **40**, 53–59.

CLARKE, P.B.S. & KUMAR, R. (1983). The effects of nicotine on locomotor activity in non-tolerant and tolerant rats. *Br. J. Pharmacol.*, **78**, 329–337.

CLARKE, P.B.S. & PERT, A. (1985). Autoradiographic evidence for nicotine receptors on nigrostriatal and mesolimbic dopaminergic neurons. *Brain Res.*, **348**, 355–358.

CLARKE, P.B.S., FU, D.S., JAKUBOVIC, A. & FIBIGER, H.C. (1988). Evidence that mesolimbic dopaminergic activation underlies the locomotor stimulant action of nicotine in rats. *J. Pharmacol. Exp. Ther.*, **246**, 701–708.

FALLON, J.H. & MOORE, R.Y. (1978). Catecholamine innervation of the basal forebrain IV. Topography of the dopamine projection to the basal forebrain and neostriatum. *J. Comp. Neurol.*, **180**, 545–580.

GARCHA, H.S. & STOLERMAN, I.P. (1993). Discriminative stimulus effects of the nicotine antagonist mecamylamine in rats. *J. Psychopharmacol.*, **7**, 43–51.

HENDRY, J.S. & ROSECRANS, J.A. (1982). The development of pharmacological tolerance to the effect of nicotine on schedule-controlled responding in mice. *Psychopharmacology*, **77**, 339–343.

HEUTTNER, J.E. & BEAN, B.P. (1988). Block of *N*-methyl-D-aspartate-activated current by the anticonvulsant MK-801: selective binding to open channels. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 1307–1311.

IMPERATO, A., MULAS, A. & DI CHIARA, G. (1986). Nicotine preferentially stimulates dopamine release in the limbic system of freely moving rats. *Eur. J. Pharmacol.*, **132**, 337–338.

KARLER, R., CALDER, L.D., CHAUDHRY, I.A. & TURKANIS, S.A. (1989). Blockade of 'reverse tolerance' to cocaine and amphetamine by MK-801. *Life Sci.*, **45**, 599–606.

KARLER, R., CHAUDHRY, I.A., CALDER, L.D. & TURKANIS, S.A. (1990). Amphetamine behavioural sensitization and the excitatory amino acids. *Brain Res.*, **537**, 76–82.

KSIR, C., HAKAN, R.L. & KELLAR, K.J. (1987). Chronic nicotine and locomotor activity: influences of exposure dose and test dose. *Psychopharmacology*, **92**, 25–29.

KURIBARA, H., ASAMI, T., IDA, I., IIJIMA, Y. & TADOKORA, S. (1992). Effects of repeated MK-801 on ambulation in mice and in sensitization following methamphetamine. *Psychopharmacology*, **108**, 271–275.

MARIATHASAN, E.A. & STOLERMAN, I.P. (1993). Discrimination of agonist-antagonist mixtures: experiments with nicotine plus mecamylamine. *Behav. Pharmacol.*, **4**, 555–561.

MARKS, M.J., STITZEL, J.A. & COLLINS, A.C. (1985). Time course study of the effects of chronic nicotine infusion on drug response and brain receptors. *J. Pharmacol. Exp. Ther.*, **235**, 619–628.

MORRISETT, R.A., REZVANI, A.H., OVERSTREET, D., JANOWSKY, D.S., WILSON, W.A. & SWARTZWELDER, H.S. (1990). MK-801 potently inhibits alcohol withdrawal seizures in rats. *Eur. J. Pharmacol.*, **176**, 103–105.

MORRISON, C.F. & STEPHENSON, J.A. (1972). The occurrence of tolerance to a central depressant effect of nicotine. *Br. J. Pharmacol.*, **46**, 151–156.

PAULY, J.R., GRUN, E.U. & COLLINS, A.C. (1992). Tolerance to nicotine following chronic treatment by injections: a potential role for corticosterone. *Psychopharmacology*, **108**, 33–39.

PAXINOS, G. & WATSON, C. (1986). *The Rat Brain in Stereotaxic Co-ordinates*. 2nd Edition. New York: Academic Press.

RAMOA, A.S., ALKONDON, M., ARACAVA, Y., IRONS, J., LUNT, G.G., DESHPANDE, S.S., WONNACOTT, S., ARONSTAM, R.S. & ALBUQUERQUE, E.X. (1990). The anticonvulsant MK-801 interacts with the peripheral and central nicotinic acetylcholine receptor ion channels. *J. Pharmacol. Exp. Ther.*, **254**, 71–82.

REAVILL, C. & STOLERMAN, I.P. (1990). Locomotor activity in rats after administration of nicotinic agonists intracerebrally. *Br. J. Pharmacol.*, **99**, 273–278.

SCHENK, S., VALADEZ, A., MCNAMARA, C., HOUSE, D.T., HIGLEY, D., BANKSON, M.G., GIBBS, S. & HORGER, B.A. (1993). Development and expression of sensitization to cocaine's reinforcing properties: role of NMDA receptors. *Psychopharmacology*, **111**, 332–338.

SCHWARTZ, R.D. & KELLAR, K.J. (1985). In vivo regulation of [^{3}H]acetylcholine recognition sites in brain by nicotinic cholinergic drugs. *J. Neurochem.*, **45**, 427–433.

SHOAIB, M. & STOLERMAN, I.P. (1992a). Locomotor activity after nicotine infusions into the fourth ventricle of rats. *Br. J. Pharmacol.*, **105**, 167P.

SHOAIB, M. & STOLERMAN, I.P. (1992b). MK801 attenuates behavioural adaptation to chronic nicotine administration in rats. *Br. J. Pharmacol.*, **105**, 514–515.

SHOAIB, M. & STOLERMAN, I.P. (1992c). MK801 attenuates tolerance to nicotine in rats. *J. Psychopharmacol.*, Abstract Book, A9.

STEWART, J. & DRUHAN, J.P. (1993). Development of both conditioning and sensitization of the behavioural activating effects of amphetamine is blocked by the non-competitive NMDA receptor antagonist, MK-801. *Psychopharmacology*, **110**, 125–132.

STOLERMAN, I.P., BUNKER, P. & JARVIK, M.E. (1974). Nicotine tolerance in rats: role of dose and dose interval. *Psychopharmacologia*, **34**, 317–324.

TRUJILLO, K.A. & AKIL, H. (1991). Inhibition of morphine tolerance and dependence by the NMDA receptor antagonist MK-801. *Science*, **251**, 85–87.

VALE, A.L. & BALFOUR, D.J.K. (1989). Aversive environmental stimuli as a factor in the psychostimulant response to nicotine. *Pharmacol. Biochem. Behav.*, **32**, 857–860.

WEIHMULLER, F.B., O'DELL, S.J., COLE, B.N. & MARSHALL, J.F. (1991). MK-801 attenuates the dopamine-releasing but not the behavioural effects of methamphetamine: an in vivo microdialysis study. *Brain Res.*, **549**, 230–235.

WELZIL, H., BATTIG, K. & BERZ, S. (1990). Acute effects of nicotine injection into the nucleus accumbens on locomotor activity in nicotine-naive and nicotine-tolerant rats. *Pharmacol. Biochem. Behav.*, **37**, 743–746.

WISE, R.A. & BOZARTH, M.A. (1987). A psychomotor stimulant theory of addiction. *Psychol. Rev.*, **91**, 469–492.

WOLF, M.E. & KHANSA, M.R. (1991). Repeated administration of MK-801 produces sensitization to its own locomotor stimulant effects but blocks sensitization to amphetamine. *Brain Res.*, **562**, 164–168.

WONG, E.H.F., KEMP, J.A., PRIESTLEY, T., KNIGHT, A.R., WOODRUFF, G.N. & IVERSEN, L.L. (1986). The anticonvulsant MK-801 is a potent *N*-methyl-D-aspartate antagonist. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 7104–7108.

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Possible role of cyclic AMP phosphodiesterases in the actions of ibudilast on eosinophil thromboxane generation and airways smooth muscle tone

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1 The possible role of cyclic AMP phosphodiesterase (PDE) in the inhibitory actions of ibudilast on tracheal smooth muscle contractility and eosinophil thromboxane generation was investigated.

2 Ibudilast was a non-selective inhibitor of partially purified cyclic nucleotide PDE isoenzymes from pig aorta and bovine tracheal smooth muscle, exhibiting only moderate potency against bovine tracheal PDE IV ($IC_{50} = 12 \pm 4\,\mu M$, $n = 3$). Similar or slightly lower potencies were displayed against PDEs I, II, III and V. In contrast, rolipram exhibited selectivity for PDE IV ($3 \pm 0.5\,\mu M$, $n = 3$).

3 Ibudilast ($IC_{50} = 0.87 \pm 0.37\,\mu M$, $n = 3$), like rolipram ($IC_{50} = 0.20 \pm 0.04\,\mu M$, $n = 3$), was a more potent inhibitor of membrane-bound PDE IV from guinea-pig eosinophils than of partially purified PDE IV from bovine tracheal smooth muscle. The potency of ibudilast increased when the eosinophil enzyme was solubilised with deoxycholate and NaCl ($IC_{50} = 0.11 \pm 0.05\,\mu M$, $n = 3$) or exposed to vanadate/glutathione complex (V/GSH) ($IC_{50} = 0.11 \pm 0.02\,\mu M$, $n = 3$). The potency of rolipram was also increased by solubilization ($IC_{50} = 0.012 \pm 0.003$, $n = 3$) or V/GSH ($IC_{50} = 0.012 \pm 0.003$, $n = 3$).

4 In intact eosinophils, ibudilast ($0.032\,\mu M - 20\,\mu M$) potentiated isoprenaline-induced cyclic AMP accumulation in a concentration-dependent manner, being approximately 20 fold less potent than rolipram. Little or no effect on basal cyclic AMP levels was observed with either compound. The cyclic AMP-dependent protein kinase activity ratio was significantly increased following incubation of eosinophils with either ibudilast ($20\,\mu M$) or rolipram ($20\,\mu M$) in the absence or presence of isoprenaline.

5 Leukotriene B_4 (300 nM)-induced thromboxane generation from guinea-pig eosinophils was inhibited by ibudilast ($IC_{50} = 11.3 \pm 3.7\,\mu M$, $n = 5$) and rolipram ($IC_{50} = 0.280 \pm 0.067\,\mu M$, $n = 5$) in a concentration-dependent manner.

6 Ibudilast ($10\,nM - 1\,\mu M$), whilst generally less potent than rolipram ($1\,nM - 1\,\mu M$), produced concentration-dependent relaxation of spasmogen (methacholine, histamine, LTD_4)-induced tone in the guinea-pig isolated tracheal strip. Ibudilast was less potent in reversing the methacholine ($IC_{50} = 1.95 \pm 0.40\,\mu M$, $n = 6$)-induced contraction than those of histamine ($IC_{50} = 0.18 \pm 0.70\,\mu M$, $n = 6$) or leukotriene D_4 (LTD_4, $IC_{50} = 0.12 \pm 0.05\,\mu M$, $n = 6$). Rolipram also exhibited a similar pattern of activity, although the difference in potency against methacholine ($IC_{50} = 0.1 \pm 0.01\,\mu M$, $n = 6$) compared with the other two spasmogens, histamine ($IC_{50} = 0.034 \pm 0.017\,\mu M$, $n = 7$) and LTD_4 ($IC_{50} = 0.026 \pm 0.008\,\mu M$, $n = 7$), was not as great.

7 These results demonstrate that ibudilast, like rolipram, has several biological actions on the eosinophil and airways smooth muscle which may be attributed to inhibition of cyclic AMP PDE. These actions may account, at least in part, for the recently reported anti-asthma effects of ibudilast.

Keywords: Cyclic AMP-specific phosphodiesterase; ibudilast; rolipram; eosinophil thromboxane B_2 generation; tracheal smooth muscle relaxation

Introduction

Ibudilast is an anti-asthma drug (Kawasaki *et al.*, 1992) the mechanism of action of which has not been clearly defined. The studies of Ohashi *et al.* (1986a) suggested that low concentrations (less than $1.3\,\mu M$) of ibudilast inhibited leukotriene D_4 (LTD_4)-induced contraction of guinea-pig lung strips in an apparently competitive manner; however, the deviation from competitive inhibition observed at higher concentrations of ibudilast coupled with its inability to displace [^{3}H]-LTD_4 from guinea-pig lung membranes (Etoh *et al.*, 1990) suggest that ibudilast is not a specific LTD_4 receptor antagonist. This conclusion is reinforced by the finding that ibudilast can inhibit histamine, platelet activating factor as well as LTD_4-induced inositol phosphate formation in guinea-pig lung slices (Etoh *et al.*, 1990).

Inhibition of adenosine 3':5'-cyclic monophosphate (cyclic AMP) phosphodiesterase (PDE) has been implicated in the potentiation by ibudilast of the platelet anti-aggregatory activity of prostacyclin (Ohashi *et al.*, 1986b). This is likely to result from an effect on guanosine 3':5'-cyclic monophosphate (cyclic GMP)-inhibited PDE (PDE III) which is the predominant cyclic AMP hydrolysing isoenzyme in these cells (Simpson *et al.*, 1988). If PDE inhibition contributes to the anti-asthma profile of ibudilast, it is of interest that some of its *in vitro* and *in vivo* actions, including bronchodilatation (Mue *et al.*, 1986), airways hypersensitivity (Hoshino *et al.*, 1989), mediator release from leukocytes (Tamura *et al.*, 1986) and inhibition of antigen-induced microvascular permeability (Eda *et al.*, 1987) are shared with compounds which act on cyclic AMP-specific PDE (PDE IV) (see reviews by Giembycz, 1992; Raeburn *et al.*, 1993).

Since smooth muscle relaxant and anti-inflammatory properties are useful in treating asthma, we have investigated the possible role of cyclic AMP PDE inhibition in the actions of ibudilast on tracheal smooth muscle contractility and eosinophil function *in vitro*. We have compared the effects of

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ibudilast with those of rolipram, a selective inhibitor of PDE IV (Reeves *et al.*, 1987). Preliminary results of these studies have been presented to the British Pharmacological Society (Souness *et al.*, 1993; Tomkinson *et al.*, 1993).

Methods

Preparation of guinea-pig eosinophils

Male Dunkin Hartley guinea-pigs (250–400 g) were injected (i.p.) with 0.5 ml of donor horse serum twice weekly. At least 5 days after the second injection, the guinea-pigs were killed by CO_2 asphyxiation. A ventral incision was made into the peritoneum and 30 ml of Hanks buffered salt solution (HBSS) without Ca^{2+} (Gibco, U.K. Ltd., Uxbridge) poured into the abdominal cavity. The abdomen was gently massaged for approximately 1 min and the peritoneal exudate was aspirated and centrifuged at 250 *g* for 10 min at 4°C. The supernatant was discarded and the pellet washed once (10 ml HBSS) and resuspended in HBSS. One ml aliquots of the cell suspension were layered onto a discontinuous (18.5% and 22.5%, w/v) metrizamide gradient prepared in conical tubes by dissolving metrizamide in Tyrode buffer (composition, mM: NaCl 137, KCl 2.7, $NaHCO_3$ 11.9, Na_2HPO_4 0.35, glucose 5.5, pH 7.3) containing 0.1% gelatin. The gradients were centrifuged (250 *g*, 20 min, 20°C) and the eosinophil-rich pellet resuspended in 10 ml HBSS. Total cell counts were determined using a Coulter counter and differential cell counts were obtained from cytospin slides fixed in methanol and stained with Wright-Giemsa. Cell viability, determined by trypan blue exclusion, was greater than 99% and eosinophil purity greater than 97%.

Preparation of eosinophil subcellular fractions

Cells ($100-200 \times 10^6$), suspended in HBSS, were centrifuged (250 *g*, 10 min, 4°C), the supernatant removed and the resulting cell pellet resuspended in 5 ml homogenization buffer (composition, mM: Tris/HCl 20, pH 7.5, $MgCl_2$ 2, dithiothrietol 1, EDTA 5, sucrose 0.25, *p*-tosyl-L-lysine-chloromethyl ketone 20, leupeptin 10 $\mu g\ ml^{-1}$, aprotinin 2000 u ml^{-1}). Cells were homogenized on ice with a Dounce homogenizer (10 strokes). The homogenate was centrifuged at 105,000 *g* for 60 min, the supernatant was collected and the pellet resuspended in an equal volume of homogenization buffer.

Solubilization of membrane-associated cyclic AMP PDE from eosinophils

The membrane-bound cyclic AMP PDE was solubilized by homogenizing freshly prepared membranes with a Dounce homogenizer (10 strokes) in 4 ml of homogenization buffer containing deoxycholate (0.5%) and NaCl (100 mM). The homogenate was centrifuged at 100,000 *g* for 30 min and the supernatant containing the solubilized activity removed.

Partial purification of smooth muscle PDE isoenzymes

Ca^{2+}/calmodulin-dependent PDE (PDE I), cyclic GMP-inhibited PDE (PDE III) and cyclic GMP-specific PDE (PDE V) were isolated from pig aortic smooth muscle. Isoenzymes were partially purified by DEAE-trisacryl anion exchange chromatography (IBF, Villeneuve La Garenne, France) from the 100,000 *g* supernatant fraction of aortic smooth muscle strips which were prepared and processed as previously described (Souness & Scott, 1993). Cyclic GMP-stimulated PDE (PDE II) and cyclic AMP-specific PDE (PDE IV) were partially purified by the same homogenization and chromatography procedure except that the source of the isoenzymes was fresh bovine cervical trachealis isolated according to Giembycz & Barnes (1991).

Measurement of PDE activity

PDE activity was determined by the two-step radioisotopic method of Thompson *et al.* (1979). The reaction mixture contained (mM): Tris/HCl 20 (pH 8.0), $MgCl_2$ 10, 2-mercaptoethanol 4, EGTA 0.2 and bovine serum albumin, 0.05 mg ml^{-1}. Unless otherwise stated, the concentration of substrate was 1 μM.

The IC_{50} values (concentration which produced 50% inhibition of substrate hydrolysis) for the compounds were determined from concentration-response curves in which concentrations ranged from 3 nM to 1 mM. At least three concentration-response curves were generated for each agent.

Categorization of PDE isozymes

The nomenclature for the different cyclic nucleotide PDEs adopted in this paper is based on that of Beavo & Reifsnyder (1990).

Preparation of Na_3VO_4/GSH complex (V/GSH)

The V/GSH complex was prepared according to Souness *et al.* (1985). Briefly, reduced glutathione (GSH-224 mM) and Na_3VO_4 (112 mM) were mixed together to form a green complex; 10 μl of this soluton were added to the 400 μl assay mix, immediately prior to addition of enzyme. The final concentrations of GSH and Na_3VO_4 were 2.8 mM and 1.4 mM, respectively.

Measurement of eosinophil thromboxane generation

Eosinophils were resuspended at a concentration of 1×10^7 cells ml^{-1} in ice-cold HEPES-buffered physiological salt solution (PSS) of the following composition (mM): HEPES 10, (pH 7.4), NaCl 124, KCl 4, NaH_2PO_4 0.64, K_2HPO_4 0.66, $NaHCO_3$ 5.2, $CaCl_2$ 1.6, $MgCl_2$ 1, glucose 5.6. Assays were performed using a modification of the method described previously by Giembycz *et al.* (1990). Briefly, incubations were conducted and were performed in triplicate at 37°C in a shaking water bath ($\sim$120 strokes min^{-1}) in a total volume of 125 μl and were initiated by the addition of 25 μl ($1-2 \times 10^6$ cells) of eosinophil suspension to 87.5 μl pre-warmed (5 min) HEPES-buffered PSS. The cell suspensions were incubated for 5 min after which 12.5 μl of rolipram (1 nM to 100 μM), ibudilast (1 nM to 100 μM), or their respective vehicles were added. After a further 5 min incubation, cells were challenged with LTB_4 (300 nM; $\sim EC_{90}$ for thromboxane release). Reactions were terminated 5 min later by the addition of 600 μl of ice-cold HEPES-buffered PSS (without Ca^{2+} and Mg^{2+}) supplemented with the cyclo-oxygenase inhibitor, flurbiprofen (8 μM). After vortex mixing, the eosinophils were pelleted rapidly by centrifugation in a microfuge and the resultant supernatant used as the source of elaborated prostanoids. The concentration of thromboxane A_2 (TXA_2) measured as its non-enzymatic degradation product, thromboxane B_2 (TXB_2) was quantified by RIA as described previously (Giembycz *et al.*, 1990) and which had a detection limit and sensitivity (IC_{50}) of 5 fmol and 100 fmol, respectively. In all cases the conversion of TXA_2 to TXB_2 was assumed to follow a 1:1 stoichiometry.

Measurement of eosinophil cyclic AMP accumulation

For measurement of cyclic AMP, freshly prepared eosinophils (1×10^6 cells ml^{-1}) were preincubated in HBSS containing Ca^{2+} and Mg^{2+}. To test the effects of PDE inhibitors, compounds (0.032 μM–100 μM) were routinely added to cell suspensions for 10 min, after which incubations were continued for a further 2 min, either in the absence or presence of isoprenaline (10 μM). Incubations were terminated with 50 μl of 100% trichloroacetic acid.

The trichloroacetic acid extract was briefly sonicated (10 s),

centrifuged (3000 *g*) for 15 min and the supernatant removed to a clean tube. Trichloroacetic acid was removed with 3 washes of water-saturated ether (5 vol). The last traces of ether were removed by gassing with nitrogen, and sodium acetate (pH 6.2) was added to a final concentration of 50 mM. Samples were acetylated and cyclic AMP quantified by radioimmunoassay (RIA, NEN Chemicals GmbH).

Measurement of cyclc AMP-dependent protein kinase activity

Eosinophils were suspended in HBSS solution at a concentration of 1×10^7 cells ml^{-1} and incubated with test substances for 10 min at 37°C. Incubations were terminated by centrifuging cells at 3000 *g* for 20 s. The incubation buffer was removed and 0.5 ml of extraction buffer (KH_2PO_4 50 mM [pH 6.8], containing EDTA 10 mM, dithiothreitol 0.2 mM, NaCl 125 mM, 3-isobutyl-1-methylxanthine, 0.5 mM, Triton X-100 0.2%) were added, after which the mixture was vortexed for 5 s.

The cyclic AMP-dependent protein kinase (PKA) activity of eosinophil Triton X-100 extracts was determined with an assay kit (GIBCO) in which incorporation of ^{33}P from [γ-^{33}P] ATP into the synthetic specific substrate, kemptide, was measured. Briefly, 10 μl of the eosinophil extract were added to an assay mixture (total volume 40 μl) containing kemptide 50 μM, [γ-^{33}P]-ATP 0.1 mM (20 μ Ci ml^{-1}), $MgCl_2$ 10 mM, bovine serum albumin 0.25 mg ml^{-1}, Tris/HCl 50 mM (pH 7.5) in the presence and absence of cyclic AMP (10 μM) and/or synthetic cyclic AMP-dependent protein kinase inhibitor (GIBCO) (1 μM). Incubations were allowed to proceed for 15 min before the reaction was stopped by pipetting 20 μl of the assay mixture onto phosphocellulose ion-exchange paper discs, which were then washed twice, extensively in phosphoric acid (1%, v/v) and then twice with water. Individual discs were placed in scintillation vials and ^{33}P measured by liquid scintillation spectrometry after addition of scintillation cocktail (lumagel, Lumac-LSC B.V., Olen, Belgium). The extent of PKA activation was expressed as the activity ratio, which is that ratio of specific activity in the absence of added cyclic AMP to that in the presence of sufficient cyclic AMP to activate the enzyme fully (− cyclic AMP/+ cyclic AMP activity ratio). Data are presented as means ± s.e.mean and analysed by one-way analysis of variance (ANOVA). Values were considered to be statistically significant when P was less than 0.05.

Preparations of guinea-pig trachealis and experimental protocol

Guinea-pigs (male, Dunkin-Hartley, 350–450 *g*), were killed by stunning and exsanguination. Tracheae were removed and placed in Krebs-Ringer Bicarbonate (KRB) solution and fat and connective tissue dissected away. Transverse tracheal strips were prepared and suspended in KRB under an applied load such that they were at their optimal length (L_O), as determined in preliminary experiments using a standard maximum contraction to methacholine (100 μM) (adjusting resting tension to achieve maximal active tension). The KRB was maintained at 37°C and gassed with 5% CO_2 in O_2, pH was 7.4. The composition of the KRB was as follows (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 10.1. Changes in force of contraction were measured with an isometric force displacement-transducer (Grass FT03) connected to a pen recorder (Lectromed, Multitrace 8).

Initial experiments were performed to establish suitable spasmogen concentrations for use in relaxant studies. Tissues were allowed to equilibrate for 60 min in KRB containing indomethacin (5.6 μM) and were washed at 15 min intervals. Cumulative (1/2 log incremental) concentration-response curves were constructed to either methacholine (10 nM–100 μM), histamine (100 nM–100 μM) or LTD_4 (0.1 nM–100 nM). In a preliminary series of experiments, the concentration of spasmogen producing 30% of maximum contraction (EC_{30}) was determined by computerised linear regression analysis. The mean EC_{30} values were histamine, 0.49 μM ± 0.04 μM ($n = 6$); methacholine, 0.09 μM ± 0.01 μM, ($n = 10$); LTD_4, 0.41 nM ± 0.07 nM ($n = 6$). These concentrations of spasmogens were chosen to reduce complications of functional antagonism seen at higher concentrations of the agonists. To determine the airways smooth muscle relaxing properties of ibudilast and rolipram, tissues were allowed to equilibrate as described above. Tissues were then contracted with spasmogen (EC_{30}) and when the response had plateaued, the relaxants or vehicle control (dimethylsulphoxide, DMSO final concentration $<0.5\%$) were added (1/2 log increments) at 8 min intervals. For relaxant studies in tissues under basal tone, tracheal strips were allowed to equilibrate for 30 min, during which they were washed at 10 min intervals with fresh KRB solution. Isoprenaline (2 μM) was then added to the tissue baths to determine zero tone. The isoprenaline was subsequently washed from the tissues and when the tone was fully recovered, relaxant studies with ibudilast and rolipram were performed.

Relaxant responses are expressed as % relaxation of spasmogen-induced contraction or % relaxation of basal tone where appropriate. Final results are expressed as mean ± s.e. mean from n animals. IC_{50} values were taken as the concentration of relaxant producing 50% relaxation of either spasmogen-induced contraction. EC_{50} values were calculated for drug effects on basal tone.

Materials

[5, 6, 8, 9, 11, 12, 14, 15 (n)-^3H]-thromboxane B_2, cyclic [2,8-^3H]-AMP (41 Ci $mmol^{-1}$) and cyclic [8-^3H]-GMP (13.8 Ci $mmol^{-1}$) were purchased from Amersham International (Amersham, Bucks.). Rolipram [4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrolidone] was synthesized by the Department of Discovery Chemistry, Rhône-Poulenc Rorer Ltd, (Dagenham, Essex). Ibudilast (3-isobutyryl-2-isopropylpyrazolo[1,5-a]pyridine,KC-404) was a gift from Kyorin Pharmaceutical Co. Ltd., (Chiyoda-ku, Tokyo, Japan). The cyclic AMP radioimmunoassay kit and [γ-^{33}P]-ATP (3000 Ci $nmol^{-1}$) were purchased from NEN Chemicals GmbH. Donor horse serum was purchased from Flow Laboratories Ltd. (Irvine, Scotland). Other cell culture reagents and the PKA assay kit were from Gibco BRL (Paisley, Scotland). All other chemicals were obtained from Sigma Chemical Co., BDH Chemicals (both of Poole, Dorset) and Prolabo (Eccles, Manchester). Male Dunkin Hartley guinea-pigs were purchased from David Hall (Burton-upon-Trent, Staffs.).

Results

Inhibition of partially purified smooth muscle PDE isoenzymes

Ibudilast non-selectively inhibited all PDE isoenzymes isolated from pig aorta and bovine tracheal smooth muscle with moderate potency being shown against PDE IV and PDE V (Table 1). Slightly lower activity (3–5 fold) was observed against PDE I, PDE II and PDE III. In contrast, rolipram was a highly selective inhibitor (> 30 fold) of PDE IV (Table 1).

Inhibition of eosinophil PDE IV

Previous studies have demonstrated a tightly membrane-bound PDE IV associated with guinea-pig peritoneal eosinophils (Souness *et al.*, 1991). Both ibudilast ($IC_{50} = 0.87 \pm 0.37$ μM, $n = 3$) and rolipram ($IC_{50} = 0.20 \pm 0.04$ μM, $n = 5$) were more potent inhibitors of this activity than of the partially purified PDE IV from bovine trachea (Figures 1 & 2). As previously demonstrated (Souness *et al.*, 1992), the potency

Table 1 Inhibition of partially purified smooth muscle phosphodiesterase isoenzymes by ibudilast and rolipram

IC_{50} (μM) PDE isoenzymes	Ibudilast	Rolipram
Ia (pig aorta)	53 ± 20	> 100
II (bovine trachea)	35 ± 2	> 100
III (pig aorta)	48 ± 13	> 100
IV (bovine trachea)	12 ± 4	3 ± 0.5
V (pig aorta)	10 ± 4	> 100

IC_{50} values were determined as described in the Methods section. The Ca^{2+} PDE (I) was assayed in the presence of Ca^{2+} (2 mM) + calmodulin (2.5 u). Cyclic GMP-stimulated PDE (II) was assayed in the presence of 10 μM cyclic GMP. The results represent the means ± s.e.mean of three separate experiments.

of rolipram (IC_{50} = 0.012 ± 0.003 μM, n = 10) was increased 10 fold when the particulate PDE IV was solubilized with deoxycholate and NaCl (Figure 1). An increased potency of ibudilast (IC_{50} = 0.11 ± 0.05 μM, n = 3) was also observed under these conditions (Figure 1). Exposure of membrane-bound PDE IV to V/GSH, which activates PDE III (Souness *et al.*, 1985) and PDE IV (Souness *et al.*, 1992) through an uncertain mechanism likely to involve an active complex of a vanadyl ion and two or more electron donors (Thompson *et al.*, 1991), similarly increased the potencies of both ibudilast (IC_{50} = 0.11 ± 0.02 μM, n = 3) and rolipram (IC_{50} = 0.02 ± 0.01 μM, n = 3) (Figure 2). The 8 fold increase in ibudilast potency observed when the PDE IV was solubilized or exposed to V/GSH was slightly less than that seen with rolipram (10–16 fold).

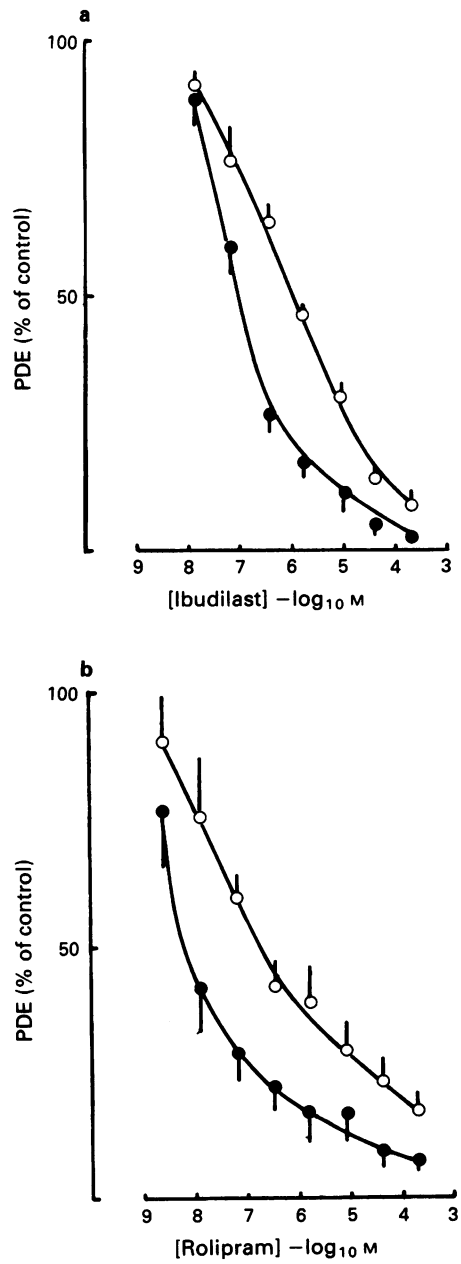


Figure 1 The effect of solubilization of eosinophil phosphodiesterase (PDE) IV on the inhibitory potencies of ibudilast and rolipram. Membrane-bound (○) or solubilized (●) PDE IV was assayed in the presence of the indicated concentrations of ibudilast (a) or rolipram (b). The results represent the means ± s.e.mean of 3–5 experiments.

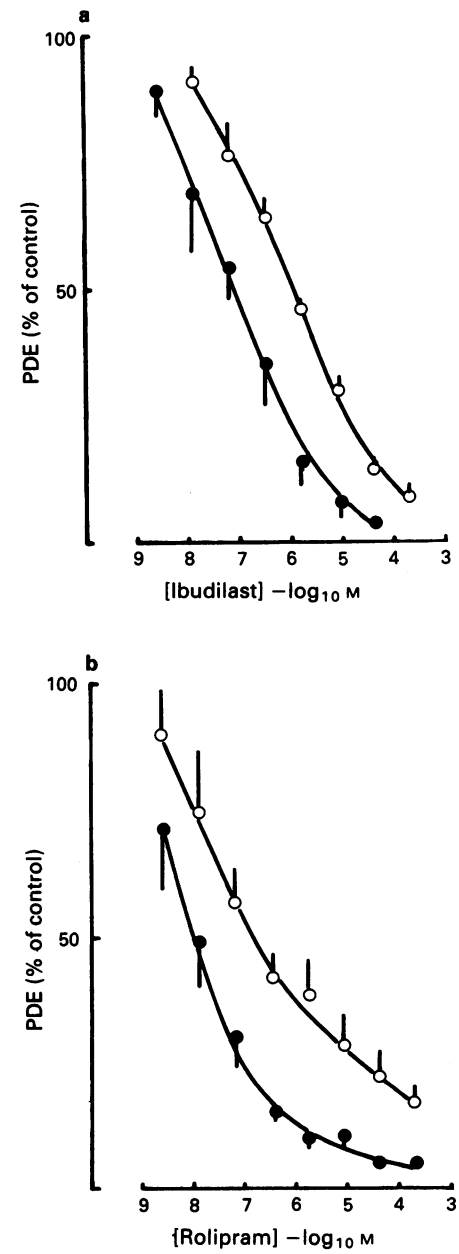


Figure 2 The effect of V/GSH on the inhibitory potencies of ibudilast and rolipram against eosinophil membrane-bound phosphodiesterase (PDE) IV. Membrane-bound PDE IV was assayed without (○) or with (●) vanadate/glutathione complex (V/GSH) in the presence of the indicated concentrations of ibudilast (a) or rolipram (b). The results represent the means ± s.e.mean of 4–5 experiments.

Stimulation of eosinophil cyclic AMP accumulation in intact eosinophils

In intact eosinophils, ibudilast and rolipram (0.032 μM–20 μM) potentiated isoprenaline (10 μM)-induced cyclic AMP accumulation in a concentration-dependent manner (Figure 3). Ibudilast was approximately 10–20 fold less potent than rolipram. Little or no effect of either ibudilast or rolipram on cyclic AMP accumulation was observed in the absence of isoprenaline.

Activation of eosinophil cyclic AMP-dependent protein kinase

Incubation of eosinophils with ibudilast (20 μM) or rolipram (20 μM) induced significant increases in the PKA activity ratio (Table 2). Isoprenaline (10 μM) alone, significantly ($P<$ 0.01) increased the PKA activity ratio and this effect was significantly enhanced by both ibudilast and rolipram (Table 2).

Inhibition of LTB_4-induced thromboxane generation from eosinophils

As shown in Figure 4, ibudilast and rolipram inhibited LTB_4 (300 nM, EC_{90})-induced TXB_2 generation from guinea-pig eosinophils. Ibudilast ($IC_{50} = 11.3 \pm 3.7$ μM, $n = 5$) was approximately 40 fold less potent than rolipram ($IC_{50} = 0.28 \pm 0.07$ μM, $n = 5$).

Relaxation of airways smooth muscle

Figure 5 shows the relaxant actions of rolipram and ibudilast on guinea-pig tracheae precontracted with methacholine (a), histamine (b) or LTD_4 (c). Ibudilast (10 nM–1 μM) and rolipram (1 nM–1 μM) exhibited concentration-dependent spasmolytic activity against all three agonists. Ibudilast was less potent in reversing the contraction elicited by methacholine ($IC_{50} = 1.95 \pm 0.40$ μM, $n = 6$) compared to its actions on histamine ($IC_{50} = 0.18 \pm 0.70$ μM, $n = 6$) or LTD_4 ($IC_{50} = 0.12 \pm 0.05$ μM, $n = 6$)-induced tone. Rolipram exhibited an IC_{50} value of 0.1 ± 0.01 μM, ($n = 6$) on methacholine-induced contraction while being approximately 3 fold more potent when the tissue was contracted with histamine ($IC_{50} = 0.034 \pm 0.17$ μM, $n = 7$) or LTD_4 ($IC_{50} = 0.026 \pm 0.008$ μM, $n = 7$). Both compounds relaxed tissues under basal tone with rolipram ($EC_{50} = 0.011 \pm 0.003$ μM, $n = 5$) being 25 fold more potent than ibudilast ($EC_{50} = 0.27 \pm 0.04$ μM, $n = 4$).

Discussion

The anti-asthma potential of PDE inhibitors is attracting much interest since they exhibit bronchodilator and anti-inflammatory actions (Torphy & Undem, 1991; Giembycz, 1992; Raeburn *et al.*, 1993). The results presented here show ibudilast to be a moderately potent but non-selective inhibitor of partially purified PDEs from smooth muscle. Interestingly, like rolipram, ibudilast exhibited approximately 10 fold greater potency against membrane-bound PDE IV from eosinophils compared to its activity against the partially purified, bovine tracheal smooth muscle enzyme. The potencies of both compounds were increased a further 10 fold when eosinophil PDE IV was solubilized or exposed to V/GSH. Thus, under the aforementioned conditions, ibudilast is a relatively potent PDE IV inhibitor, exhibiting IC_{50} values

Table 2 Stimulation of cyclic AMP-dependent protein kinase in intact eosinophils by ibudilast and rolipram

Addition	*Cyclic AMP-dependent protein kinase activity ratio*	
	– Isoprenaline	*+ Isoprenaline*
None	0.28 ± 0.04	0.49 ± 0.04
Ibudilast	0.43 ± 0.05*	0.92 ± 0.08†
Rolipram	0.55 ± 0.14†	0.87 ± 0.09†

Eosinophils were incubated with or without isoprenaline (10 μM) and/or PDE inhibitors (20 μM) for 10 min. Incubations were terminated and protein kinase activity measured as described in the Methods section. The specific activity of cyclic AMP-dependent protein kinase (+ cyclic AMP) in control cells was 157 ± 31 pmol min^{-1} per 10^6 cells. The results represent means ± s.e.mean ($n = 3$). *$P<0.05$; †$P<$ 0.01 for significant difference from control.

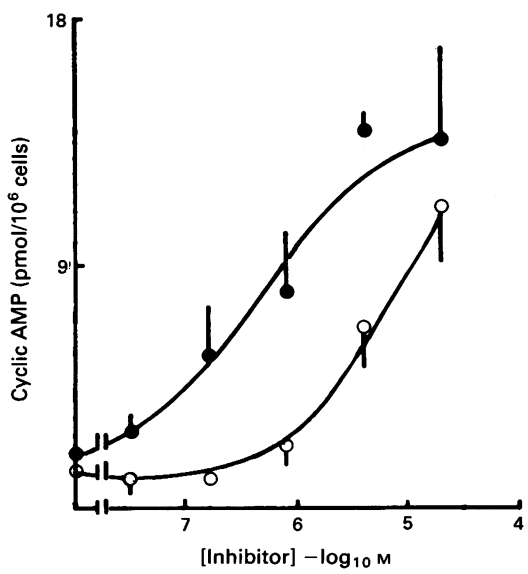


Figure 3 Effects of ibudilast and rolipram on cyclic AMP accumulation in intact eosinophils. Cells were preincubated with ibudilast (○) or rolipram (●) for 10 min before exposure to isoprenaline (10 μM) or its vehicle (saline) for a further 2 min. The results represent means ± s.e.mean of at least 3–4 separate experiments.

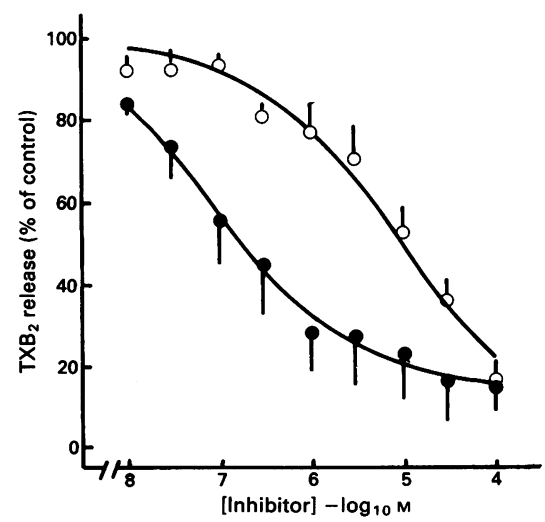


Figure 4 Effects of ibudilast and rolipram on leukotriene B_4 (LTB_4) induced thromboxane B_2 (TXB_2) generation from eosinophils. Cell incubations were conducted with LTB_4 (300 nM) in the presence of the indicated concentrations of ibudilast (○) or rolipram (●). TXB_2 was quantified by RIA as described in the Methods section. The results represent means ± s.e.mean of five experiments.

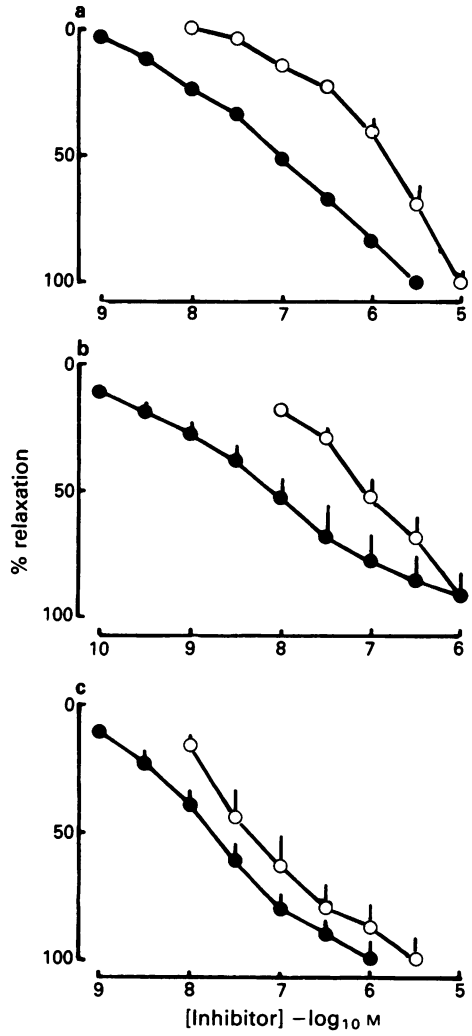


Figure 5 Relaxant effects of ibudilast and rolipram on guinea-pig tracheal smooth muscle contracted with various spasmogens. The relaxant effects of ibudilast (○) and rolipram (●) were tested as described in the Methods section on tissue precontracted (EC_{30}) with methacholine (a), histamine (b) and leukotriene D_4 (c). The results represent means ± s.e.mean of 6–7 experiments.

which are at least 100 fold lower than those against other PDE isoenzymes.

The increased potencies of some, but not all, PDE inhibitors against the eosinophil PDE IV caused by solubilization or V/GSH led us to propose the existence of a biologically important high-affinity site on the enzyme at which rolipram-like compounds can interact (Souness *et al.*, 1992). This site was envisaged as concealed in freshly prepared membranes and exposed by solubilization or V/GSH treatment. Furthermore, we highlighted similarities in the potency order of compounds against the solubilized enzyme and the high affinity rolipram binding site in brain membranes (Souness & Scott, 1993). The increased potency of ibudilast, exhibited when eosinophil PDE IV is solubilized or treated with V/GSH, suggests it interacts with the enzyme in a manner similar to rolipram. Indeed, ibudilast displaces (±)-[^{3}H]-rolipram from brain membranes, exhibiting a potency approximately 10 fold less than (±)-rolipram (unpublished observations).

The ability of ibudilast to interact with PDE IV in intact cells is demonstrated by its enhancement of isoprenaline-induced cyclic AMP accumulation, a property it shares with rolipram. Elevation of cyclic AMP in eosinophils is associated with the dampening of various cell functions (Souness *et al.*, 1991). Both compounds inhibit LTB_4-induced thromboxane generation from guinea-pig eosinophils in the absence of a stimulus of adenylate cyclase. Under these conditions a minimal increase in cyclic AMP was observed, suggesting a dissociation between the biological responses to ibudilast and rolipram and changes in second messenger levels; however, as shown previously (Souness *et al.*, 1991), rolipram alone can activate eosinophil cyclic AMP-dependent protein kinase and this property is also exhibited by ibudilast. This, and the dissimilar stimulatory interactions of isoprenaline and PDE inhibitors on cyclic AMP accumulation (synergistic) and PKA activity ratio (additive) may indicate compartmentalization of a functionally important pool of the second messenger as has been suggested in other tissues (Zhou *et al.*, 1992). The potency difference between rolipram and ibudilast on the untreated or treated membrane-bound eosinophil PDE IV was approximately 10 fold; however, rolipram was approximately 20–40 fold more potent than ibudilast in elevating cyclic AMP or inhibiting thromboxane release in intact cells. This may reflect differences in the abilities of the two compounds to accumulate in cells or indicate that the native enzyme exists in a conformation state slightly different from that of the membrane-bound (− or + V/GSH) or solubilized PDE IV.

As well as dampening the functions of a variety of inflammatory cells, PDE IV inhibitors, as well as PDE III inhibitors, functionally antagonize the bronchoconstrictor actions of a variety of agonists (Raeburn *et al.*, 1993). The data presented here show that ibudilast and rolipram inhibit the constrictor actions of methacholine, histamine and LTD_4. Furthermore, both compounds are less potent in reversing contraction induced by methacholine compared to the latter two agonists. If PDE inhibition is causally related to their relaxant actions, the potencies of both ibudilast and rolipram are surprising in view of their relatively weak activities against partially purified bronchial smooth muscle PDE IV. However, it has been argued (Souness & Scott, 1993) that the rigorous homogenization and purification procedures employed in enzyme isolation may alter the PDE IV in such a way as to reduce the potencies of certain inhibitors. Whether a higher-affinity site, which may be altered or destroyed during enzyme preparation exists on smooth muscle PDE IV is uncertain. It is apposite to point out that a close correlation exists between the bronchorelaxant potencies of several PDE inhibitors and their abilities to displace [^{3}H]-(±)-rolipram from brain membranes (Harris *et al.*, 1989). Whether inhibition of PDE III, which plays a role in regulating airways smooth muscle tone (Torphy & Undem, 1991) contributes to the bronchorelaxant action of ibudilast is uncertain.

In comparison with rolipram and some of the other recently described selective inhibitors of PDE IV, ibudilast is relatively weak in terms of its actions on the isolated enzyme or intact cells/tissues. In spite of this, our results add to the growing list of biological effects *in vitro* and *in vivo* which ibudilast shares with this class of compound. The inhibitory effect of ibudilast on inositol phosphate formation in guinea-pig lung (Etoh *et al.*, 1990) may be due to cyclic AMP elevation as a result of PDE inhibition – rolipram inhibits histamine-stimulated inositol phospholipid hydrolysis in bovine tracheal smooth muscle (Hall *et al.*, 1989). The inhibitory effect of ibudilast on eicosanoid mediator release (Tamura *et al.*, 1986) from leukocytes is a property shared with PDE IV inhibitors (Raeburn *et al.*, 1993). *In vivo* actions of ibudilast are also consistent with an action involving PDE IV: ibudilast (Mue *et al.*, 1986) and rolipram (Harris *et al.*, 1989) inhibit bronchoconstriction induced by a number of agonists. Inhibition of bronchial hyperresponsiveness in guinea-pig models in response to a variety of stimuli is observed with ibudilast and rolipram (Hoshino *et al.*, 1989; Raeburn & Lewis, 1991). Both compounds inhibit anaphylactic bronchospasm (Hoshino *et al.*, 1989; Underwood *et al.*, 1992) and, microvascular leakage (Eda *et al.*, 1987; Ortiz *et al.*, 1992). Ibudilast (Nagai *et al.*, 1983) and other PDE IV inhibitors (Davies & Evans,

1973) also reduce passive cutaneous anaphylaxis. Surprisingly, ibudilast had no effect on PAF-induced accumulation of eosinophils in the airways of guinea-pigs, whereas another PDE inhibitor, benzafentrine (AH-21-132) reduced cell numbers (Sanjar *et al.*, 1989). Rolipram has been reported to reduce airways eosinophil accumulation following antigen challenge in guinea-pigs (Hand *et al.*, 1990).

In conclusion, ibudilast, a non-selective PDE inhibitor, shares several biological actions on eosinophils and airways smooth muscle with rolipram which can be attributed to cyclic AMP PDE inhibition. It is likely that this action accounts, at least in part, for the anti-asthma effects of ibudilast.

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References

BEAVO, J.A. & REIFSNYDER, D.H. (1990). Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. *Trends Pharmacol. Sci.*, **11**, 150–155.

DAVIES, G.E. & EVANS, D.P. (1973). Studies with two new phosphodiesterase inhibitors (ICI 58,301 and ICI 63,197) on anaphylaxis in guinea-pigs, mice and rats. *Int. Arch. Allergy Appl. Immunol.*, **45**, 467–478.

EDA, K., OHASHI, M. & NISHINO, K. (1987). Effect of KC 404 on antigen-induced increase in vascular permeability in guinea-pig airway smooth muscles. *Jpn. J. Pharmacol.*, **43** (Suppl.) 178P.

ETOH, S., OHASHI, M., BABA, A. & IWATA, H. (1990). Inhibition by ibudilast of leukotriene D_4-induced formation of inositol phosphates in guinea-pig lung. *Br. J. Pharmacol.*, **100**, 564–568.

GIEMBYCZ, M.A. (1992). Could isoenzyme-selective phosphodiesterase inhibitors render bronchodilator therapy redundant in the treatment of bronchial asthma? *Biochem. Pharmacol.*, **43**, 2041–2051.

GIEMBYCZ, M.A. & BARNES, P.J. (1991). Selective inhibition of a high affinity Type IV cyclic AMP phosphodiesterase in bovine trachealis by AH-21-132: Relevance to the spasmolytic and anti-spasmogenic actions of AH-21-132 in the intact tissue. *Biochem. Pharmacol.*, **42**, 663–677.

GIEMBYCZ, M.A., KROEGEL, C. & BARNES, P.J. (1990). Platelet activating factor stimulates cyclo-oxygenase activity in guinea-pig eosinophils: concerted biosynthesis of thromboxane A_2 and E-series prostaglandin. *J. Immunol.*, **144**, 3489–3497.

HALL, I.P., DONALDSON, J. & HILL, S.J. (1989). Inhibition of histamine – stimulated inositol phospholipid hydrolysis by agents which increase cyclic AMP levels in bovine tracheal smooth muscle. *Br. J. Pharmacol.*, **97**, 603–613.

HAND, J.M., GADALETA, P.E., NOVAK, L., FERRACONE, J. & OSBORN, R.R. (1990). Effect of rolipram (Ro), salbutamol (SA), chlorpheniramine (CPA) and SK&F 106203 on bronchoalveolar lavage (BAL) cells following antigen challenge in guinea-pigs. *Pharmacologist*, **32**, 172.

HARRIS, A.L., CONNELL, M.J., FERGUSON, E.W., WALLACE, A.M., GORDON, R.J., PAGANI, E.D. & SILVER, P.J. (1989). Role of low Km cyclic AMP phosphodiesterase inhibition in tracheal relaxation and bronchodilation in the guinea-pig. *J. Pharmacol. Exp. Ther.*, **251**, 199–206.

HOSHINO, H., KOBAYASHI, H., KONNO, S., OKAZAWA, A., OKADA, Y., SUGANUMA, T., MARUYANA, S., ADACHI, M. & TAKAHASHI, T. (1989). Allergen-induced late bronchoconstriction and airway hyperresponsiveness in guinea-pigs. *Arerugi*, **38**, 314–325.

KAWASAKI, A., HOSHINO, K., OSAKI, R., MIZUSHIMA, Y. & YANO, S. (1992). Effect of ibudilast: A novel antiasthmatic agent, on airway hypersensitivity in bronchial asthma. *J. Asthma*, **29**, 245–252.

MUE, S., ISHIHARA, T., HIDA, W., SEKIZAWA, K., TAMURA, G., YAMAUCHI, K., OHTSU, H. & TAKISHIMA, T. (1986). Bronchodilating effect of KC-404, a novel anti-asthmatic agent, and its derivatives in monkey. *Arch. Int. Pharmacodyn. Ther.*, **283**, 153–162.

NAGAI, H., IWAMOTO, T., NISHIYORI, T., TAKIZAWA, T., TSUCHIYA, H. & KODA, A. (1983). Pharmacological studies on newly synthesized anti-allergic agents, 2-methyl-3-piperidino-beta-propionaphtone hydrochloride (KZ-III) and 3-isobutyryl-2-isopropyl-pyrazolo-[1, 5-a] pyridine (KC-404). *Jpn. J. Pharmacol.*, **33**, 1215–1223.

OHASHI, M., KANAI, R., NISHINO, K., SATO, T. & TAKAYANAGI, I. (1986a). Antagonistic effect of KC-404, a new anti-asthmatic agent, on leukotriene D_4-induced contractile responses in isolated guinea-pig smooth muscles. *Prostaglandins*, **32**, 875–888.

OHASHI, M., OHKUBO, H., KITO, J. & NISHINO, K. (1986b). A new vasodilator 3-isobutyryl-2-isopropyl-pyrazolol [1,5-a] pyridine (KC-404) has a dual mechanism of action on platelet aggregation. *Arch. Int. Pharmacodyn. Ther.*, **283**, 321–334.

ORTIZ, J.L., CORTIJO, J., VALLES, J.M., BOU, J. & MORCILLO, E.J. (1992). Rolipram inhibits PAF-induced airway microvascular leakage in guinea-pig: a comparison with milrinone and theophylline. *Fundam. Clin. Pharmacol.*, **6**, 247–249.

RAEBURN, D. & LEWIS, S. (1991). Rolipram inhibits PAF-induced bronchial hyperreactivity (BHR) in the guinea-pig in vivo: a comparison with theophylline and salbutamol. *Eur. Resp. J.*, **4**, (suppl. 14), 307S.

RAEBURN, D., SOUNESS, J.E., TOMKINSON, A. & KARLSSON, J.-A. (1993). Isozyme-selective cyclic nucleotide phosphodiesterase inhibitors: biochemistry, pharmacology and therapeutic potential in asthma. *Prog. Drug Res.*, **40**, 9–32.

REEVES, M.L., LEIGH, B.K. & ENGLAND, P.J. (1987). The identification of a new cyclic nucleotide phosphodiesterase in human and guinea-pig cardiac ventricle. *Biochem. J.*, **241**, 535–541.

SANJAR, S., AOKI, S., BOUBEKEUR, K., BURROWS, L., COLDITZ, I., CHAPMAN, I. & MORLEY, J. (1989). Inhibition of PAF-induced eosinophil accumulation in pulmonary airways of guinea-pigs by anti-asthma drugs. *Jpn. J. Pharmacol.*, **51**, 167–172.

SIMPSON, A.W.M., REEVES, M.L. & RINK, T.J. (1988). Effects of SK&F 94120, an inhibitor of cyclic nucleotide phosphodiesterase type III, on human platelets. *Biochem. Pharmacol.*, **37**, 2315–2320.

SOUNESS, J.E., CARTER, C.M., DIOCEE, B.K., HASSALL, G.A., WOOD, L.J. & TURNER, N.C. (1991). Characterisation of guinea-pig eosinophil phosphodiesterase activity: Assessment of its involvement in regulating superoxide generation. *Biochem. Pharmacol.*, **42**, 937–945.

SOUNESS, J.E., MASLEN, C. & SCOTT, L.C. (1992). Effects of solubilisation and vanadate/glutathione complex on inhibitor potencies against eosinophil cyclic AMP-specific phosphodiesterase. *FEBS Lett.*, **302**, 181–184.

SOUNESS, J.E. & SCOTT, L.C. (1993). Stereospecificity of rolipram actions on eosinophil cyclic AMP-specific phosphodiesterase. *Biochem. J.*, **291**, 389–395.

SOUNESS, J.E., SCOTT, L.C., TOMKINSON, A., GIEMBYCZ, M.A. & RAEBURN, D. (1993). Inhibition of cyclic nucleotide phosphodiesterase by ibudilast: Comparison with rolipram. *Br. J. Pharmacol.*, **108**, 71P.

SOUNESS, J.E., THOMPSON, W.J. & STRADA, S.J. (1985). Adipocyte cyclic nucleotide phosphodiesterase activation by vanadate. *J. Cyclic Nucleotide Protein Phosphorylation Res.*, **10**, 383–396.

TAMURA, H., OHTSU, H., FUJIMOTO, Y., AIZAWA, T., MUE, H. & TAKIJIMA, M. (1986). Inhibition of KC-404 against leukotriene C_4 and B_4 production from human peripheral white blood cells by stimulation of Ca-ionophore A23187. *Clin. Rep.*, **20**, 181–185.

THOMPSON, W.J., TAN, B.H. & STRADA, S.J. (1991). Activation of rabbit liver high-affinity cAMP (Type IV) phosphodiesterase by a vanadyl-glutathione complex. *J. Biol. Chem.*, **266**, 17011–17019.

THOMPSON, W.J., TERASAKI, W., EPSTEIN, P.M. & STRADA, S.J. (1979). Assay of cyclic nucleotide phosphodiesterase and resolution of multiple molecular forms of the enzyme. *Adv. Cyclic Nucleotide Res.*, **10**, 69–92.

TOMKINSON, A., SHARMA, A., GIEMBYCZ, M.A., SOUNESS, J.E. & RAEBURN, D. (1993). Effects of ibudilast on airways smooth muscle contraction in vitro and in vivo: Comparison with rolipram. *Br. J. Pharmacol.*, **108**, 73P.

TORPHY, T.J. & UNDEM, B.J. (1991). Phosphodiesterase inhibitor: New opportunities for the treatment of asthma. *Thorax*, **46**, 512–523.

UNDERWOOD, D.C., MATTHEWS, J.K., OSBORN, R.R., NOVAK, L.B. & TORPHY, T.J. (1992). The significance of β-adrenergic activity on the inhibitory effects of rolipram against early and late phase response to antigen in the guinea-pig. *Am. Rev. Resp. Dis.*, **145**, A392.

ZHOU, H.-L., NEWSHOLME, S.J. & TORPHY, T.J. (1992). Agonist-related differences in the relationship between cAMP content and protein kinase activity in canine trachealis. *J. Pharmacol. Exp. Ther.*, **261**, 1260–1267.

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Effects of ethanol and other aliphatic alcohols on NO-mediated relaxations in rat anococcygeus muscles and gastric fundus strips

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1 In anococcygeus muscles, ethanol (20–500 mM) slightly increased the tone and inhibited relaxations elicited by nitrergic nerve stimulation (0.5–5 Hz) in a concentration-dependent manner.

2 Other aliphatic alcohols decreased the tone but had inhibitory effects similar to ethanol on stimulation-induced relaxations, the EC_{50} (mM) values being: methanol 280, ethanol 80, propan-1-ol 20, propan-2-ol 55, propan 1,2-diol 135, butan-1-ol 120, butan-2-ol 15 and pentan-1-ol 3.

3 Relaxations induced by sodium nitroprusside (SNP, 10 nM) were inhibited by ethanol (20–500 mM) in a concentration-dependent manner and by propan-2-ol (100 mM). Relaxations induced by NO (1 μM) were inhibited by high concentrations of ethanol (200–300 mM) and by propan-2-ol (100 mM).

4 In gastric fundus strips, ethanol (60–200 mM) did not affect the resting tone but inhibited NO-mediated relaxations elicited by low frequency (1 Hz) field stimulation and reduced the initial relaxation by high frequency field stimulation (10 Hz) and by SNP (50 nM). The relaxant action of isoprenaline (10 nM) was not reduced although it was slightly slower in onset. Other aliphatic alcohols tested decreased the tone and inhibited relaxations elicited by field stimulation.

5 Acetaldehyde (1–10 mM) inhibited relaxations elicited by field stimulation and SNP in both the rat anococcygeus muscles and gastric fundus strips. The tone of gastric fundus strips was decreased by acetaldehyde but it was transiently increased in anococcygeus muscles.

6 The dehydrogenase inhibitors, 4-methylpyrazole (100 μM) and disulfiram (100 μM) did not significantly affect the inhibition by ethanol of nitrergic nerve stimulation-induced relaxations in anococcygeus muscles, which suggests that ethanol acts directly.

7 The inhibition of NO-mediated relaxations by alcohols is attributed to sequestration of NO to form nitroso-alcohols.

Keywords: Acetaldehyde; alcohols (aliphatic); anococcygeus muscle (rat); ethanol; gastric fundus strips (rat); nitric oxide; nitrergic transmission; sodium nitroprusside

Introduction

It has been previously reported that relaxations elicited by stimulation of non-adrenergic, non-cholinergic (NANC) nerves in the rat and rabbit anococcygeus and the bovine retractor penis muscles are inhibited by ethanol (Gillespie & McKnight, 1978; Gillespie *et al.*, 1982). However, the mechanism of this inhibitory effect of ethanol has not been elucidated. More recently, it has been shown that nitrergic transmission is responsible for the NANC relaxations in the above-mentioned tissues as well as in a number of other tissues, such as the rat gastric fundus, the relaxations being mediated by nitric oxide (NO) or a NO-like substance synthesized from L-arginine (for reviews, see Rand, 1992a,b; Sanders & Ward, 1992; Sneddon & Graham, 1992). Substances that inhibit NO production, such as N^G-nitro-L-arginine methyl ester (L-NAME), or reduce the availability of NO, such as haemoglobin, or inhibit guanosine 3':5'-cyclic monophosphate (cyclic GMP) generation by guanylate cyclase, such as methylene blue, inhibit nitrergic transmission (for reviews, see Rand, 1992a,b). Hence it is possible that ethanol may interfere with NO-mediated mechanisms in such a way that nitrergic transmission is inhibited. In a preliminary study, we confirmed the findings of Gillespie and his colleagues in the rat anococcygeus muscle (Li & Rand, 1991). Additionally, we showed that nitrergic relaxations of the rat gastric fundus strips as well as relaxations to the NO donor, sodium nitroprusside (SNP) in both anococcygeus muscles and gastric fundus strips were inhibited by ethanol, suggesting a common mechanism may be involved. Furthermore, acetaldehyde was found to have a similar but more potent effect than ethanol in inhibiting nitrergic relaxations in the rat anococcygeus muscles (Li & Rand, 1991). Therefore, we have now further tested the possibility that effects of ethanol may be mediated by acetaldehyde by using inhibitors of alcohol and aldehyde dehydrogenase.

One possible explanation for the inhibition by ethanol of NO-mediated responses is that the alcoholic hydroxyl group is nitrosylated by NO originating either from nitrergic nerves or NO-releasing compounds such as SNP, resulting in the formation of ethyl nitrite (CH_3-CH_2-O-N = O), and this sequestering of NO reduces its availability for activating guanylate cyclase. If this is the case, other aliphatic alcohols would be expected to have similar activity. In this study we investigated the effects of eight aliphatic alcohols, differing in the numbers of carbon atoms and the position of the hydroxyl group, on the relaxations elicited by nitrergic stimulation in the rat anococcygeus muscle and in some cases compared their effects on relaxations elicited by exogenously applied nitric oxide (NO) and sodium nitroprusside (SNP). Some of these findings have been communicated to a meeting of the Australian Physiological and Pharmacological Society (Rand *et al.*, 1992).

Methods

Male Sprague-Dawley rats (250–400 g) were killed by decapitation and the anococcygeus muscles and stomach were removed.

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Anococcygeus muscles were set up as described previously (Gillespie 1972; Li & Rand, 1989). The tone was raised and nitrergic relaxations in response to field stimulation were revealed by guanethidine (10–30 μM) or the α_2-adrenoceptor agonist, UK14304 (1 μM): there were no apparent differences between the findings with either agent.

A longitudinal strip of the gastric fundus was prepared and set up for recording field stimulation or drug-induced relaxations as described by Li & Rand (1990).

The composition of the physiological salt solution (PSS) used for both tissues was as follows (mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 0.45, KH_2PO_4 1.03, $CaCl_2$ 2.5, D-(+)-glucose 11.1, disodium edetate 0.067 and ascorbic acid 0.14.

The effects of alcohols were studied after initial control responses to nerve stimulation or agonists had been obtained and the alcohol was added to the organ bath in cumulatively increasing concentrations at intervals of 3–5 min. Only one alcohol was used on each preparation. In the experiments with dehydrogenase inhibitors in the anococcygeus muscle, the inhibitor was added 30 min before the tone was raised. Parallel experiments, without addition of alcohols, were elicited in the contralateral muscle from the same donor rat to serve as a time control.

The drugs used were acetaldehyde (BDH), guanethidine sulphate (Ciba), 5-hydroxytryptamine creatinine sulphate (serotonin, Sigma), isoprenaline hydrochloride (Winthrop, Australia), L-phenylephrine hydrochloride (Sigma), 4-methylpyrazole hydrochloride (Sigma), disulfiram (Sigma), nitric oxide (compressed gas, CIG, Melbourne), N^G-nitro-L-arginine methyl ester (L-NAME, Wellcome Research Laboratory, UK), sodium nitroprusside (SNP, Sigma) and UK14304 (5-bromo-6-[2-imidazoline-2-Y1-amino]-quinoxaline; Pfizer). The following alcohols were purchased from BDH: methanol, ethanol, propan-1-ol, propan-2-ol, propan-1,2-diol, butan-1-ol, butan-2-ol and pentan-1-ol. Saturated aqueous solutions of NO were prepared from NO gas as previously described (Rajanayagam *et al.*, 1993).

Differences between means were compared by Student's *t* test, and values of $P<0.05$ were taken to indicate statistical significance.

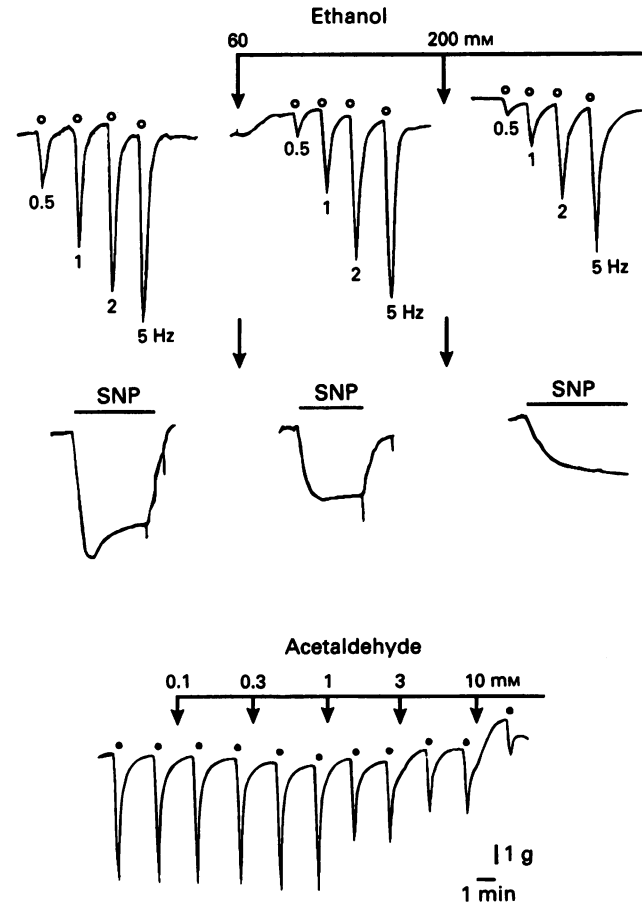


Figure 1 Records of effects of ethanol (upper tracings) and acetaldehyde (lower tracing) on relaxations of anococcygeus muscles elicited by field stimulation (0.5–5 Hz for 10 s, at (○) in the upper tracings and 5 Hz for 10 s at (●) in the lower tracings). Middle tracings show the effect of ethanol on relaxations of an anococcygeus muscle elicited by sodium nitroprusside (SNP, 10 nM). In these records, the tone of the muscles was raised by guanethidine. Similar results were obtained on each type of response in at least 5 separate experiments.

Results

Effects of ethanol and other alcohols on field stimulation-induced relaxations and resting tension of rat anococcygeus muscles

Ethanol (20–500 mM) produced a concentration-dependent inhibition of relaxations elicited by field stimulation at various frequencies (0.5–5 Hz) of partially contracted anococcygeus muscle, as shown for 60 and 200 mM ethanol in Figure 1. The reductions were more marked at the lower than at the higher frequencies of stimulation. The resting tone of the anococcygeus muscle was slightly but regularly increased (Figure 1); however, the increase in tone in the prolonged presence of a high concentration of ethanol was not sustained.

The effects of the five primary aliphatic alcohols from methanol to pentan-1-ol, two secondary alcohols (propan-2-ol and butan-2-ol) and the di-alcohol propan-1,2-diol were tested. All of these alcohols produced concentration-dependent reductions of nitrergic nerve stimulation-induced (1 Hz, 10 s) relaxations as shown in Figure 2a,b. The mean concentrations that reduced these relaxations by 50% (EC_{50} values) are shown in Table 1. All of the alcohols except ethanol decreased tone, and this created difficulties for the precise assessment of the ED_{50} values; hence, only approximate means are given. The relationship between the reduction in tone and the inhibition of nitrergic relaxation by the alcohols is shown in Figure 3.

Effects of ethanol and propan-2-ol on SNP- and NO-induced relaxations of anococcygeus muscles

Relaxations elicited by SNP (10 nM) were inhibited by ethanol (60–200 mM) in a concentration-dependent manner (Figures 1 and 4). Relaxations elicited by NO (1 μM) were not significantly inhibited by 100 mM ethanol but there was significant inhibition with 200–300 mM ethanol (Figure 4). Propan-2-ol (100 mM) significantly inhibited relaxations induced by both SNP (10 nM) and NO (1 μM) (results not shown).

Table 1 The EC_{50} (mM) of the alcohols tested in inhibiting nitrergic relaxations of the anococcygeus

Alcohol	*EC_{50}* (mM)	*Alcohol*	*EC_{50}* (mM)
Methanol	280	Pentan-1-ol	3
Ethanol	80	Butan-2-ol	15
Propan-1-ol	20	Propan-1-ol	20
Propan-2-ol	55	Propan-2-ol	55
Propan-1,2-diol	135	Ethanol	80
Butan-1-ol	120	Butan-1-ol	120
Butan-2-ol	15	Propan-1,2-diol	135
Pentan-1-ol	3	Methanol	280

The first two columns are in order of size and complexity of the alcohols; the second two columns are in order of potency.

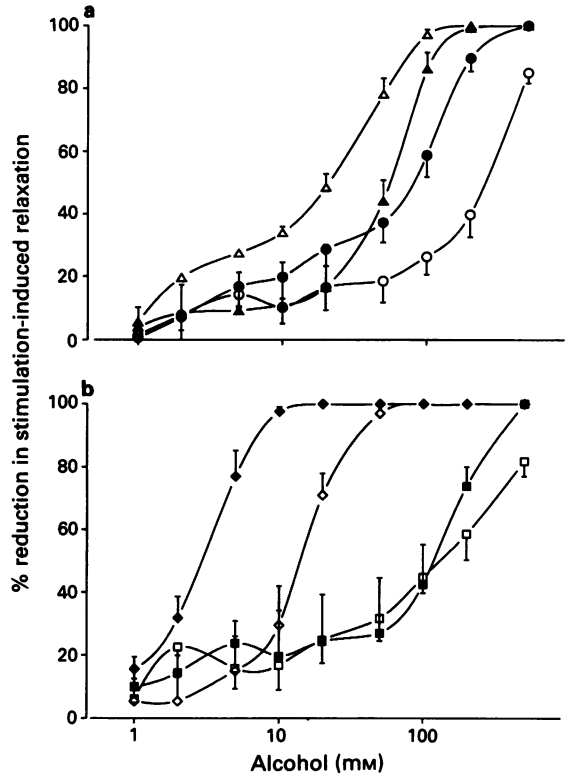


Figure 2 Effects of aliphatic alcohols on field stimulation-induced (1 Hz, 10 s) relaxations of anococcygeus muscles, expressed as percentage reduction of the relaxations (a and b). (a) (○) methanol; (●) ethanol; (▲) propan-1-ol; (△) propan-2-ol. (b) (□) Propan-1,2-diol; (■) butan-1-ol; (◇) butan-2-ol; (◆) pentan-1-ol. Symbols represent means ($n = 3-7$) with standard errors of means, which in some cases are less than the size of the symbol indicating the mean.

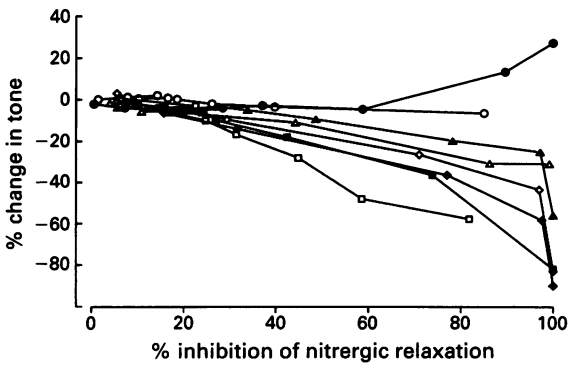


Figure 3 The relationship between the mean changes in tone and mean inhibitions of nitrergic relaxations by aliphatic alcohols: (○) methanol; (●) ethanol; (▲) propan-1-ol; (△) propan-2-ol; (□) propan-1,2-diol; (■) butan-1-ol; (◇) butan-2-ol; (◆) pentan-1-ol.

Effects of ethanol and other alcohols on NO-mediated relaxations in gastric fundus strips

In gastric fundus strips, as in anococcygeus muscles, relaxations elicited by field stimulation were reduced in a concentration-dependent manner by ethanol (60–200 mM), which did not greatly affect the resting tone (Figure 5); the effect of ethanol was considerably greater on the relaxation elicited by 1 Hz for 60 s than that elicited by 5 Hz for 10 s. Ethanol also inhibited the initial rapid phase of the relaxation elicited by high frequency stimulation (10 Hz for 60 s), as shown in Figure 5 with 200 mM of ethanol. The relaxant action of SNP was almost abolished by 200 mM ethanol, as was the

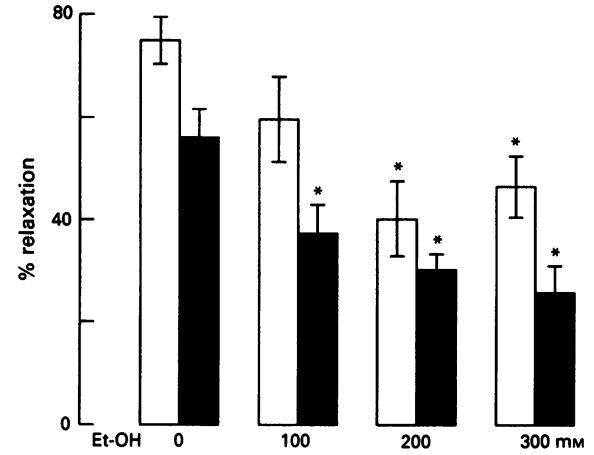


Figure 4 Effects of ethanol on relaxations elicited by NO (open columns) and sodium nitroprusside (SNP) (solid columns) in rat anococcygeus muscles, expressed as percentage of the tone of the muscles raised by guanethidine. Columns represent means ($n = 3-7$) with standard errors of means. *Indicates significantly different ($P < 0.05$) from control (no ethanol).

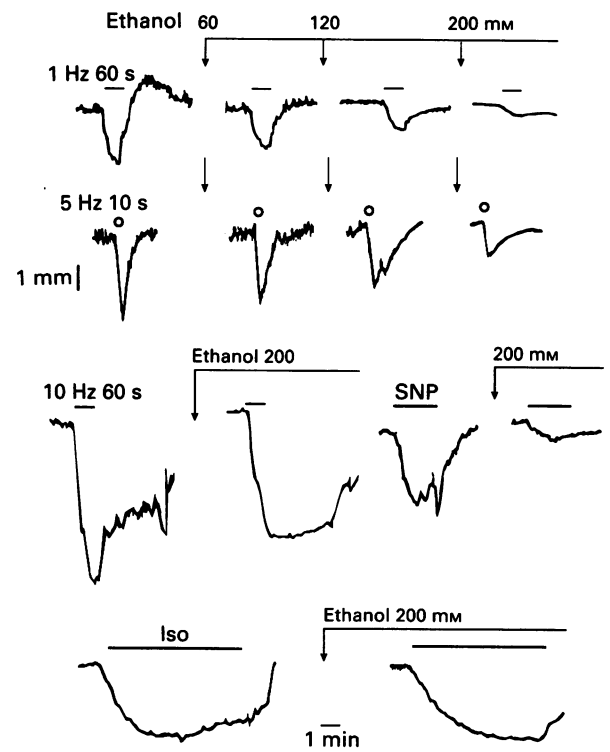


Figure 5 Records of effects of ethanol on relaxations of rat gastric fundus strips, in which the tone was raised by 5-hydroxytryptamine (5-HT, 10 μM), elicited by field stimulation at 1 Hz for 60 s (indicated by horizontal lines), 5 Hz for 10 s (indicated by ○) and 10 Hz for 60 s (indicated by horizontal lines), sodium nitroprusside (SNP, 50 nM) and isoprenaline (Iso, 10 nM). Periods of exposure to drugs are indicated by horizontal lines. Similar results were obtained on each type of response in at least 4 separate experiments.

stimulation (1 Hz)-induced relaxation, but the relaxant action of isoprenaline was not reduced although it was slightly slower in onset (Figures 5 and 6).

The other alcohols tested on gastric fundus strips (propan-2-ol and butan-1-ol) produced concentration-dependent inhibitions of relaxations to field stimulation but also greatly reduced the tone of the muscle (results not shown).

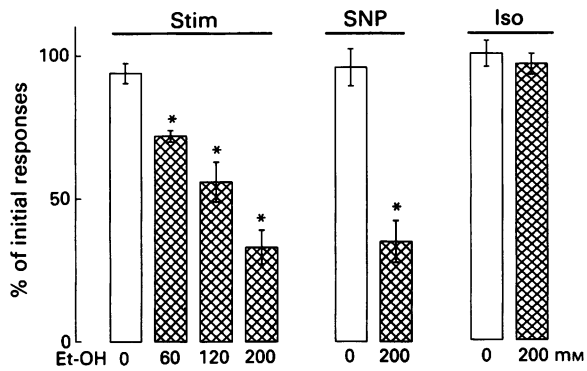


Figure 6 Effects of ethanol on relaxation elicited by field stimulation (Stim, 5 Hz, 10 s), sodium nitroprusside (SNP, 50 nM) and isoprenaline (Iso, 10 nM) in rat gastric fundus, expressed as percentage of the initial relaxations. Columns represent means ($n = 4-7$) with standard errors of means. *Indicates significantly different ($P<0.05$) from control (no ethanol).

Effects of acetaldehyde on NO-mediated relaxations in anococcygeus muscles and gastric fundus strips

In anococcygeus muscles, acetaldehyde (1–10 mM) had concentration-dependent effects in increasing the tone and inhibiting nitrergic nerve stimulation-induced relaxations as shown in Figure 1. Thus, acetaldehyde was about 100 times more potent than ethanol in both actions.

In the gastric fundus strip, acetaldehyde (1–10 mM) also reduced nitrergic nerve stimulation-induced relaxations and was again more potent than ethanol, but reduced the resting tone (results not shown).

Relaxations of anococcygeus muscles and gastric fundus strips elicited by SNP (10 nM) were inhibited by 3–10 mM acetaldehyde to about the same extent as by 200 mM ethanol (results not shown).

Effects of 4-methylpyrazole and disulfiram on the inhibition of nitrergic relaxation by ethanol in the anococcygeus muscle

Neither 4-methylpyrazole (100 μM) nor disulfiram (100 μM) had any significant effect on the inhibition by ethanol (20–200 mM) of relaxations elicited by nitrergic nerve stimulation or SNP (10 nM) (results not shown).

Discussion

The present study confirmed and extended the previous observations that ethanol inhibited NANC-mediated relaxations in the rat anococcygeus muscle and related tissues (Gillespie & McKnight, 1978; Gillespie *et al.*, 1982; Li & Rand, 1991). In addition we have shown that acetaldehyde and other aliphatic alcohols have effects similar to ethanol in both the rat anococcygeus muscle and the gastric fundus.

Ethanol slightly increased the tone of anococcygeus muscle, as previously reported by Gillespie *et al.* (1982), who also found that ethanol potentiated the contractile responses to noradrenergic nerve stimulation. Both these effects are produced by other agents that inhibit nitrergic nerve-stimulation-induced relaxations, such as NO synthase inhibitors, haemoglobin and methylene blue (Rand, 1992a), and are attributable to removal of a NO-mediated relaxant influence. However, ethanol also reduced the contractile action of noradrenaline (Gillespie *et al.*, 1982).

In gastric fundus strips, ethanol selectively inhibited NO-mediated relaxations elicited by field stimulation at a low frequency (1 Hz) and reduced the initial rapid relaxation at high frequency (10 Hz), and it has been established that this relaxation is mediated by a nitrergic mechanism (Li & Rand, 1990). The inhibition of SNP- but not isoprenaline-induced relaxations by ethanol is in accord with the concept of a selective inhibition by ethanol of a NO-mediated process.

It has been reported that ethanol inhibited the NO-mediated, endothelium-dependent relaxant actions of acetylcholine and ATP but not the NO-independent relaxant action of papaverine in the rat mesenteric artery (Criscione *et al.*, 1989). We have also found that both acetylcholine- and SNP-induced relaxations of rat aortic rings were inhibited by ethanol and propan-2-ol (Li & Rand, unpublished observations). However, our finding that ethanol inhibited the relaxant action of SNP in the rat aorta differs from that reported by Hatake *et al.* (1989), who found that ethanol inhibited only acetylcholine-but not SNP-induced relaxations. The reason for this discrepancy is not clear.

Acetaldehyde had effects similar to ethanol in the rat anococcygeus and gastric fundus. The possibility of production of acetaldehyde from ethanol in isolated extrahepatic tissues has been discussed by Altura *et al.* (1978). Therefore, we tested the possibility that the inhibitory effect of ethanol was mediated by acetaldehyde by using high concentrations of 4-methylpyrazole and disulfiram which are selective inhibitors of alcohol and aldehyde dehydrogenases, respectively. However, since neither 4-methylpyrazole nor disulfiram significantly affect the actions of ethanol in the rat anococcygeus muscle, it seems unlikely that the effects of ethanol were mediated by acetaldehyde. Thus, acetaldehyde may, in its own right, inhibit the nitrergic responses by either the same or a different mechanism from ethanol. More experiments will be necessary before any conclusion can be drawn.

Gillespie *et al.* (1982) suggested that the inhibition by ethanol of NANC-mediated relaxations in anococcygeus muscles and the bovine retractor penis were most probably due to an alteration in the level of intracellular calcium in the smooth muscle cells. At that time, the mechanism involved in mediating the NANC relaxations had not been elucidated, but more recently it has been shown that the NANC relaxant transmitter in these tissues is NO, or a NO-like substance (for reviews, see: Rand, 1992a,b; Sanders & Ward, 1992; Sneddon & Graham, 1992). In this context, an alternative explanation for the effect of ethanol is that it may inhibit one or more stages in the nitrergic transmitter mechanism.

Since the relaxations of anococcygeus muscle to exogenously applied NO and the NO-donor SNP were also inhibited, it is unlikely that the effect of ethanol was due to inhibition of transmitter release from nitrergic nerves. The findings could be explained either by inhibition of the guanylate cyclase/cyclic GMP mechanism mediating the relaxation of the smooth muscle, or by an interaction of ethanol with the released NANC transmitter, NO or a NO-related compound, resulting in its inactivation. However, SNP-induced relaxations of the bovine retractor penis muscle were not affected by ethanol (Gillespie *et al.*, 1982), although it reduced the response to the inhibitory factor (IF) isolated from the bovine retractor penis muscle (Gillespie *et al.*, 1982), which has recently been identified as a NO-donating nitrosothiol (Kerr *et al.*, 1992).

One of the possible interactions of ethanol with NO is formation of the nitroso compound ethyl nitrite (CH_3-CH_2-O-N = O). If this is the case, other alcohols may be expected to have a similar effect. The finding that all the other aliphatic alcohols tested inhibited nitrergic relaxations is in accord with this concept. According to Taylor & Baker (1945), the reaction of NO with alcohols to form nitroso-alcohols (NO + R-OH → R-O-N = O) proceeds readily. Certain nitroso-alcohols (e.g. butyl and amyl nitrite, or nitrosobutanol and nitrosopentanol, respectively, as they may also be termed) are well known as nitrovasodilators, which donate NO to guanylate cyclase and thereby relax smooth muscle. We suggest that the reaction of NO with an alcohol is reversible, and NO is sequestered when the alcohol is in excess, but NO is released when the nitroso-alcohol is in

excess. The differences in potency of the alcohols may reflect the equilibrium position of their reactions with NO under the prevailing conditions. In general, an increase in chain length of an alcohol resulted in an increase in its ability to inhibit nitrergic relaxations; however, as other factors are also involved (such as the reduction in tone), the potency for each alcohol is only approximate. Evidence for an interaction between NO and ethanol (as we propose), or a competition between NO and ethanol for a binding site, was provided by Nelson (1987), who observed that the pH-dependent changes in the electron paramagnetic resonance spectrum of the NO complex with the non-haem iron containing enzyme soybean lipoxygenase were abolished by 100 mM ethanol (Nelson, 1987).

The amount of endogenously formed NO in the exhaled air of anaesthetized rabbits was decreased in a dose-dependent manner by infusions of 3, 10 and 30 mmol kg^{-1} ethanol (Persson & Gustafsson, 1992). The ED_{50} for inhibition of exhaled NO was 23 mmol kg^{-1} and, given that the volume of distribution of ethanol in rabbits is about 0.65 l kg^{-1}, the ethanol concentration in body water would be about 35 mM ethanol, which is not too divergent from our estimate of an EC_{50} of about 80 mM for inhibition of nitrergic relaxations of anococcygeus muscles. Persson & Gustafsson (1992) attributed the effect of ethanol to inhibition of NO synthesis; however, the finding is compatible with our suggestion that the NO is sequestered by formation of ethyl nitrite.

It was noted that there were some differences in the sensitivity to inhibition of NO-mediated relaxations by ethanol in different tissues. For example, ethanol inhibited nitrergic relaxations in the rat anococcygeus muscle and gastric fundus strip at a much lower concentration than that needed to inhibit endothelium-dependent relaxations in the aorta (unpublished observation). It is not clear whether the difference is due to a difference in the fate of the NO after it is released from its source (nerve terminals or endothelial cells) or to the possible different nature of nerve-derived and endothelium-derived NO (Rand & Li, 1993). Furthermore, ethanol was found to be less effective against NO-induced relaxation than that induced by SNP or nitrergic nerve stimulation. Again, it is not clear whether this reflects the nature of the equilibrium of the proposed reaction between ethanol and NO or whether ethanol has an additional effect on NO synthesis. In addition, effects other than interaction with NO may also be involved in the actions produced by alcohols, especially at the relatively high concentrations used, since the tone of the muscle was decreased by all the alcohols except ethanol. It is possible that this reduction in tone is due to disordering of the cell membrane by the alcohols, causing a reduction of the intracellular calcium concentration in the smooth muscle cells, as suggested by Gillespie *et al.* (1982).

The demonstration that ethanol as well as other alcohols impair nitrergic transmission may be of some significance in understanding the possible mechanism of functional changes in a variety of physiological systems that are observed in alcohol abuse. For example, in the central nervous system, the activation of neuronal guanylate cyclase by excitatory amino acid agonists, such as glutamate and NMDA, is mediated by NO (Bredt & Snyder, 1989). Ethanol reduces the levels of cyclic GMP in the brain, particularly in the cerebellum (for references, see Hoffman *et al.*, 1989a), and the production of cyclic GMP stimulated by agonists in the cerebellar granule cells (Hoffman *et al.*, 1989a,b) and other cell lines (Stenstrom *et al.*, 1986) was inhibited by ethanol. Ethanol also reduced the neurotoxicity of glutamate and NMDA in cultures of rat cerebral cortical cells (Lustig *et al.*, 1992), and this effect is thought to be mediated by NO. However, as was pointed by Hoffman *et al.* (1989b) and Stenstrom *et al.* (1986), the inhibition of agonist-stimulated production of cyclic GMP by ethanol may not be due to inhibition of guanylate cyclase. Ethanol inhibited SNP-stimulated cyclic GMP production in cultured neuroblastoma cells (Stenstrom *et al.*, 1986); this effect could be due to inhibition of guanylate cyclase, or to sequestration of the NO released from SNP.

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References

ALTURA, B.M., CARELLA, A. & ALTURA, B.T. (1978). Acetaldehyde on vascular smooth muscle: possible role in vasodilator action of ethanol. *Eur. J. Pharmacol.*, **52**, 73–83.

BREDT, D.S. & SNYDER, S.H. (1989). Nitric oxide mediates glutamate-linked enhancement of cyclic GMP levels in the cerebellum. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 9030–9033.

CRISCIONE, L., POWELL, J.R., BURDET, R., ENGESSER, S., SCHLAGER, F. & SCHOEPFER, A. (1989). Alcohol suppresses endothelium-dependent relaxation in rat mesenteric vascular beds. *Hypertension*, **13**, 964–967.

GILLESPIE, J.S. (1972). The rat anococcygeus muscle and its response to nerve stimulation and to some drugs. *Br. J. Pharmacol.*, **45**, 404–416.

GILLESPIE, J.S. & MCKNIGHT, A.T. (1978). The actions of some vasoactive polypeptides and their antagonists on rat anococcygeus and bovine retractor penis muscles. *Br. J. Pharmacol.*, **62**, 267–274.

GILLESPIE, J.S., HUNTER, J.C. & MCKNIGHT, A.T. (1982). The effect of ethanol on inhibitory and motor responses in the rat and rabbit anococcygeus and the bovine retractor penis muscles. *Br. J. Pharmacol.*, **75**, 189–198.

HATAKE, K., WAKABAYASHI, I., KAKISHITA, E., TANIGUCHI, T., OUCHI, H., SAKAKI, N. & HISHIDA, S. (1989). Inhibitory effect of ethanol endothelium-dependent vascular responsiveness. *Eur. J. Pharmacol.*, **168**, 277–283.

HOFFMAN, P.L., MOSES, F. & TABAKOFF, B. (1989a). Selective inhibition by ethanol of glutamate-stimulated cyclic GMP production in primary cultures of cerebellar granule cells. *Neuropharmacol.*, **28**, 1239–1242.

HOFFMAN, P.L., RABE, C.S., MOSES, F. & TABAKOFF, B. (1989b). *N*-Methyl-D-aspartate receptors and ethanol: inhibition of calcium flux and cyclic GMP production. *J. Neurochem.*, **52**, 1937–1940.

KERR, S.W., BUCHANAN, L.V., BUNTING, S. & MATHEWS, W.R. (1992). Evidence that S-nitrosothiols are responsible for the smooth muscle relaxing activity of the bovine retractor penis inhibitory factor. *J. Pharmacol. Exp. Ther.*, **263**, 285–292.

LI, C.G. & RAND, M.J. (1989). Evidence for a role of nitric oxide in the NANC-mediated relaxations in rat anococcygeus muscle. *Clin. Exp. Pharmacol. Physiol.*, **16**, 933–938.

LI, C.G. & RAND, M.J. (1990). Nitric oxide and vasoactive intestinal polypeptide mediate non-adrenergic non-cholinergic inhibitory transmission to smooth muscle of the rat gastric fundus. *Eur. J. Pharmacol.*, **191**, 303–309.

LI, C.G. & RAND, M.J. (1991). Effects of ethanol and acetaldehyde on nitrergic relaxations of rat anococcygeus muscle, gastric fundus and aorta. *Proc. Austral. Physiol. Pharmacol. Soc.*, **22**, 103P.

LUSTIG, H.S., CHAN, J. & GREENBERG, D.A. (1992). Ethanol inhibits excitotoxicity in cerebral cortical cultures. *Neurosci. Lett.*, **15**, 259–261.

NELSON, M.J. (1987). The nitric oxide complex of ferrous soybean lipoxygenase-1: substrate pH, and ethanol effects on the active-site iron. *J. Biol. Chem.*, **262**, 12137–12142.

PERSSON, M. & GUSTAFSSON, L.E. (1992). Ethanol can inhibit nitric oxide production. *Eur. J. Pharmacol.*, **224**, 99–100.

RAJANAYAGAM, M.A.S., LI, C.G. & RAND, M.J. (1993). Differential effects of hydroxocobalamin on NO-mediated relaxations in rat aorta and anococcygeus muscle. *Br. J. Pharmacol.*, **108**, 3–5.

RAND, M.J. (1992a). Nitrergic transmission: nitric oxide as a mediator of non-adrenergic, non-cholinergic neuroeffector transmission. *Clin. Exp. Pharmacol. Physiol.*, **19**, 147–169.

RAND, M.J. (1992b). New perspectives in neurotransmission: nitric oxide, the mediator of nitrergic transmission. *Proc. Austral. Physiol. Soc.*, **23**, 1–16.

RAND, M.J., BEESLEY, A. & LI, C.G. (1992). Effects of some aliphatic alcohols on NO-mediated relaxations in the rat anococcygeus muscle and aorta. *Proc. Austral. Physiol. Pharmacol. Soc.*, **23**, 221P.

RAND, M.J. & LI, C.G. (1993). Differential effects of hydroxocobalamin on relaxations induced by nitrosothiols in rat aorta and anococcygeus muscle. *Eur. J. Pharmacol.*, **241**, 249–254.

SANDERS, K.M. & WARD, S.M. (1992). Nitric oxide as a mediator of nonadrenergic noncholinergic neurotransmission. *Am. J. Physiol.*, **262**, G379–G392.

SNEDDON, P. & GRAHAM, A. (1992). Role of nitric oxide in the autonomic innervation of smooth muscle. *J. Auton. Pharmacol.*, **12**, 445–456.

STENSTROM, S., ENLOE, L., PFENNING, M. & RICHELSON, E. (1986). Acute effects of ethanol and other short-chain alcohols on the guanylate cyclase system of murine neuroblastoma cells (Clon N1E-115). *J. Pharmacol. Exp. Ther.*, **236**, 458–463.

TAYLOR, T.W.J. & BAKER, W. (1945). *Sidgwick's Organic Chemistry of Nitrogen*. Oxford: Oxford University Press.

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Inhibition of excitatory non-adrenergic non-cholinergic bronchoconstriction in guinea-pig airways *in vitro* by activation of an atypical 5-HT receptor

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1 The effect of 5-hydroxytryptamine (5-HT) was studied on excitatory neurally mediated non-adrenergic non-cholinergic (NANC) contractions evoked by electrical field stimulation (EFS) in guinea-pig isolated bronchi.

2 5-HT (0.1–100 μM) produced a concentration-dependent inhibition of the excitatory NANC response with 50.9 ± 5.0% ($n = 5$, $P < 0.01$) inhibition at 100 μM. This inhibition was not significantly affected by the 5-HT_2 antagonist, ketanserin (1 μM) when inhibitions (± ketanserin) at each concentration of 5-HT were compared by unpaired t tests; however, this concentration appeared to produce a leftward shift (≈10 fold) of the 5-HT concentration-inhibition curve. Ketanserin (1 μM) was effective in blocking bronchoconstriction evoked by activation of 5-HT_{2A} receptors on airway smooth muscle. In the presence of ketanserin (1 μM) 5-HT (100 μM) evoked an inhibition of 57.4 ± 5.9% ($n = 5$, $P < 0.01$) with an EC_{50} of 0.57 μM.

3 Inhibition evoked by 5-HT (0.1–100 μM) was unaffected by the α-adrenoceptor antagonist phentolamine (1 μM), the β_2-adrenoceptor antagonist, ICI 118551 (0.1 μM), the 5-$HT_{1A/B}$ antagonist, cyanopindolol (1 μM) or the 5-$HT_{3/4}$ antagonist, ICS 205-930 (1 μM).

4 Methiothepin (0.1 μM) produced an insurmountable inhibition of the effect of 5-HT (0.1–100 μM), reducing the maximum inhibition produced by 5-HT (100 μM) to 30.2 ± 5.0% ($n = 5$, $P < 0.001$) and suggesting a non-competitive antagonism. Methiothepin inhibited the effect of 5-HT (10 μM) in a concentration-dependent manner with an IC_{50} of 81 nM.

5 Selective 5-HT receptor agonists were also tested on excitatory NANC responses. 5-Carboxamidotryptamine (5-CT, 0.1–100 μM) was the most potent, producing a concentration-dependent inhibition with an EC_{50} of 0.13 μM. Calculation of approximate IC_{25} values (concentration of the agonist required to give a 25% inhibition of the excitatory NANC response) gave a rank order of potency 5-CT ≥ 5-HT >> 8-hydroxy-dipropylaminotetralin (8-OH-DPAT) > α-methyl-5-hydroxytryptamine (α-Me-5HT). Sumatriptan, 5-methoxytryptamine (5-MeOT) and 2-methyl-5-hydroxytryptamine (2-Me-5HT) were essentially inactive with $IC_{25} > 100$ μM.

6 5-HT (10 μM) did not significantly affect contractile responses to exogenously applied substance P (1 nM–10 μM).

7 The effect of 5-HT was unchanged after incubation with the nitric oxide (NO) synthase inhibitor L-N^G-nitroarginine methyl ester (L-NAME, 100 μM). However, pretreatment with charybdotoxin (ChTX, 0.1–30 nM), a blocker of the large conductance Ca^{2+}-activated K^+channel (K^+_{Ca}), produced a concentration-dependent inhibition of the effect of 5-HT (10 μM).

8 5-HT evokes a concentration-dependent inhibition of e-NANC bronchoconstriction in guinea-pig isolated bronchi but does not affect cumulative concentration-dependent contractile responses to substance P, suggesting that inhibition is via a prejunctional receptor. Effects of selective antagonists and agonists suggest that an atypical 5-HT receptor mediates this inhibition. The inhibitory effect of 5-HT does not involve the production of NO, but may involve the opening a ChTX-sensitive K^+_{Ca} channel. These data suggest that an atypical 5-HT receptor inhibits the release of neuropeptides from sensory C fibres and may act as other inhibitory neuromodulators via the opening of a common K^+channel.

Keywords: Guinea-pig airways; bronchial smooth muscle; non-adrenergic non-cholinergic nerves; bronchoconstriction; 5-hydroxytryptamine; prejunctional inhibition; Ca^{2+}-activated K^+ channels; charybdotoxin; substance P

Introduction

Antidromic stimulation of capsaicin-sensitive primary afferent nerves (C-fibres) in the airways evokes release of neuropeptides which can cause a wide range of effects including vasodilatation, plasma extravasation, smooth musle contraction and a variety of effects on inflammatory cells (Barnes *et al.*, 1991). In guinea-pig airways, *in vivo*, antidromic stimulation of C-fibres by vagal stimulation results in bronchoconstriction due to the release of neuropeptides, including substance P and neurokinin-A (Lundberg & Saria, 1987). *In vitro*, electrical field stimulation (EFS) leads to a biphasic contractile response comprising a rapid transient cholinergic contraction and a long-lasting non-adrenergic non-cholinergic (NANC) contraction (Grundstrom *et al.*, 1981) due to the release of tachykinins from C fibres as the response is abolished by capsaicin pretreatment which irreversibly depletes neuropeptides (Martling *et al.*, 1984), as well as tachykinin receptor antagonists (Maggi *et al.*, 1991).

Several agonists inhibit excitatory NANC bronchoconstriction both *in vivo* and *in vitro* by a mechanism which involves prejunctional receptors on C-fibres. Inhibitory receptors include μ-opioid receptors, β_2/α_2-adrenoceptors, NPY receptors, histamine-H_3 receptors, galanin receptors, $GABA_B$ receptors, and vasoactive intestinal peptide (VIP) receptors (Barnes *et*

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al., 1991). Prejunctional inhibition of excitatory NANC responses by activation of a number of receptors (α_2-adrenoceptors, NPY receptors and μ-opioid receptors) appears to be mediated by a common mechanism involving the activation of a large conductance Ca^{2+}-activated K^+ channel (K^+_{Ca}) as charybdotoxin (ChTX) a K^+_{Ca} blocker (Gimenez-Gallego et al., 1988) attenuates prejunctional inhibition of excitatory NANC responses in guinea-pig bronchi in vitro (Stretton et al., 1992).

5-Hydroxytryptamine (5-HT, serotonin) has long been known to depolarize sensory nerves (Douglas & Ritchie, 1957), including the sensory fibres of the rabbit nodose ganglion (NG) (Wallis et al., 1982) and rat dorsal root ganglion (DRG) (Todorovic & Anderson, 1990). Autoradiographic mapping studies have demonstrated both 5-HT_3 (Hamon et al., 1989) and 5-HT_1 (Daval et al., 1987; Huang & Peroutka, 1987) receptors to be present on C-fibres in the rat spinal cord. Electrophysiological studies of sensory nerves have shown that activation of 5-HT_3/5-HT_1-like receptors in the rabbit NG (Christian et al., 1989) and 5-HT_2/5-HT_3 receptors in the rat DRG (Todorovic & Anderson, 1990) caused depolarization. Functionally, 5-HT has been shown both to stimulate and inhibit C-fibres to modulate efferent release of neuropeptides. In guinea-pig isolated atria (Bernoussi & Rioux, 1989) 5-HT_3 receptors stimulate efferent neuropeptide release (Tramontana et al., 1993); however, in the rat and guinea-pig dura mater 5-HT receptor activation inhibits plasma extravasation indicating an inhibition of extracranial perivascular sensory fibres via 5-HT_{1B} and 5-HT_{1D} receptors, respectively (Buzzi & Moskowitz, 1990; Matsubara et al., 1991).

5-HT has a wide range of effects on airway smooth muscle which appear to be species-dependent. In guinea-pig airways in vitro, 5-HT acts directly on smooth muscle causing contraction at low doses and relaxation at high doses both via activation of 5-HT_{2A} receptors (Baumgartner et al., 1990) and 5-HT_3 receptor activation causes facilitation of parasympathetic post-ganglionic cholinergic neurotransmission (Rizzo et al., 1993). In vivo 5-HT causes bronchoconstriction via 5-HT_{2A}, 5-HT_1-like and 5-HT_3 receptors. 5-HT_{2A} bronchoconstriction is partly mediated by activation of parasympathetic cholinergic nerves (Macquin-Mavier et al., 1991) and furthermore 5-HT_1-like/5-HT_3 bronchconstriction is due to release of neuropeptides from C-fibres; however, it has not been shown whether these receptors are central or peripheral (Buckner et al., 1991).

In this study we have investigated the effects of 5-HT on excitatory NANC contractile responses evoked by EFS in isolated guinea-pig bronchi, characterized the 5-HT receptor responsible for any effect by using selective receptor agonists and antagonists, and investigated whether this receptor is pre- or post-junctional by comparison of the effects of 5-HT on NANC responses with those on concentration-contraction relationships to exogenously applied substance P. Finally we have studied two possible mechanisms for the effect of 5-HT, firstly that it may be acting to release nitric oxide (NO) as in the canine intestine where 5-HT has shown to evoke relaxations which are mediated by NO (Bogers et al., 1991), or that it may be acting via a ChTX-sensitive K^+_{Ca} channel in the same manner as other prejunctional receptors which inhibit the release of neuropeptides from airway C-fibres.

Methods

Tissue preparation

Male outbred Dunkin–Hartley guinea-pigs (Harlan-Olac, UK) with body weights 300–550 g were killed by cervical dislocation and the lungs with trachea and bronchi attached were rapidly removed. Two main and four hilar bronchi were carefully dissected, ring preparations were then prepared and silk loops attached so that tissues could be connected to steel hooks at both the bottom of the 10 ml organ baths and on the force transducer. The organ baths contained Krebs-Henseleit (KH) solution of the following composition (mM): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 25.5 and glucose 5.6. KH solution was maintained at 37°C and gassed with 95% O_2/5% CO_2, giving a pH of 7.4. Eight tissues, four from each of two animals were studied each day. Indomethacin (10 μM) was present throughout to prevent modulation of neural responses by endogenously synthesized prostaglandins. Propranolol was not present as it is an antagonist at 5-HT_1 receptors (Glennon, 1987). Tissues were allowed to equilibrate for 1 h with frequent washing, and the resting tension was adjusted to 500 mg, which was found to be optimal for measuring changes in tension. Isometric changes in tension were measured by Grass FT.03 force-displacement transducers (Grass Instruments, Quincy, Massachusetts, U.S.A.) and responses were recorded on a polygraph (Grass model 7D).

Measurement of NANC bronchoconstriction

NANC-induced bronchoconstriction was measured in the presence of 1 μM atropine in order to block cholinergic responses. EFS was applied via two platinum wire field electrodes inserted in parallel (10 mm apart) inside the organ baths with the tissue samples placed between them. A Grass S88 stimulator and a unity grain inverting amplifier provided biphasic square wave pulses with a supramaximal voltage of 40 V at source and 0.5 ms pulse width. EFS was delivered at 8 Hz as this frequency was shown to elicit measurable, reproducible NANC responses which were $\approx$50% of the maximal NANC response and was found to be optimal for detection of modulatory effects (Stretton et al., 1992). Preparations were stimulated for 20 s once every 30 min or when the tension returned to baseline levels. Contractile responses evoked by EFS were shown to be completely abolished by tetrodotoxin (TTX; 3 μM) confirming their neural origin. Responses could also be abolished by pretreatment of the guinea-pig with capsaicin (Martling et al., 1984) confirming that it was due to the release of neuropeptides from C-fibres. At the end of each experiment tissues were washed and a maximum contractile response was obtained to histamine (1 mM).

Effects of 5-HT agonists and antagonists

Agonists were added after two stable responses to EFS had been obtained and at least 10 min before two test responses to EFS, in a non-cumulative manner with only one concentration of agonist added per tissue. The effect of 5-HT (0.1–100 μM) alone on NANC responses was examined; however, 5-HT causes contraction of bronchial smooth muscle directly via 5-HT_{2A} receptors (Baumgartner et al., 1990) and so 5-HT-evoked tension was allowed to return to baseline before NANC responses were tested. In all subsequent experiments ketanserin (1 μM) was added 20 min prior to NANC stimulation to abolish the contractile effect of 5-HT.

The effects of 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), 5-carboxamidotrytamine (5-CT), α-methyl-5-hydroxytryptamine (α-Me-5HT), sumatriptan, 2-methyl-5-hydroxytryptamine (2-Me-5HT) and 5-methoxytryptamine (5-MeOT) (all 0.1–100 μM) were also studied. In each case ketanserin (1 μM) was added prior to the first NANC stimulation and drugs were added after two control EFS and at least 10 min before two test EFS, in a non-cumulative manner with only one concentration of agonist added per tissue.

The effect of the selective α and β_2-adrenoceptor antagonists, phentolamine (1 μM) and ICI 118551 (0.1 μM), the 5-HT receptor antagonists methiothepin (0.1 nM–10 μM), cyanopindolol (1 μM) and ICS 205–930 (1 μM) was also studied both on NANC responses alone and on the inhibi-

tion of these responses produced by 5-HT (0.1–100 μM). For experiments looking at their effects on NANC responses, antagonists were added after two control excitations of NANC and at least 10 min before two test responses. Subsequently antagonists were added at least 10 min before the first NANC stimulation. In a separate series of experiments the effects of the K^+_{Ca} blocker ChTX (0.1–30 nM) or the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 0.1 mM) were also studied in the same way as the antagonists above; however, both ChTX and L-NAME were incubated with the tissue for at least 20 min before NANC stimulations.

In each case only one concentration of agonist was given per preparation, as only 5–6 stable NANC responses could be obtained. As a consequence it was not possible to provide a control preparation from each animal, as only 4 preparations were obtained per animal and each agonist had to be given at 4 concentrations, hence a continuous control group was used for studies comparing the effects of 5-HT in the absence or presence of antagonists.

Contractile-response studies

To confirm that the modulatory effects produced by 5-HT in this study are not by postjunctional mechanisms, the effect of 5-HT (10 μM) on cumulative concentration-contraction relationships to exogenously applied substance P (0.1 nM–10 μM) was also studied. Paired tissues were used and 5-HT (10 μM) was added to alternate tissues 30 min prior to construction of cumulative substance P concentration-contraction relationships.

Drugs

Drugs were obtained from the following sources: indomethacin, 5-HT hydrochloride, histamine dihydrochloride, atropine sulphate, tetrodotoxin, 5-MeOT hydrochloride, substance P acetate, L-NAME, capsaicin, phentolamine hydrochloride (Sigma); ketanserin tartrate, 8-OH-DPAT hydrobromide, 5-CT maleate, α-Me-5-HT maleate, 2-Me-5-HT maleate (RBI); ICI 118551 [erythro-DL-1-(7-methyl indian-4-yloxy)-3-(isopropyl amino butan-2-ol) hydrochloride] (ICI); sumatriptan was a generous gift from Glaxo, ICS 205-930 [(3α-tropanyl)-1H-indole-3-carboxylic acid ester] and cyanopindolol from Sandoz and methiothepin maleate from Roche. All drugs were made up daily and dissolved in distilled water except methiothepin (100% methanol); 5-HT, 2-Me-5-HT and α-Me-5-HT (1 mg ml^{-1} ascorbic acid); indomethacin (made up at 1 mg ml^{-1} in alkaline phosphate buffer [pH 7.8] of composition [mM]: KH_2PO_4 20, Na_2HPO_4 120). Drug additions did not exceed 1% of the bath volume. All concentrations refer to final bath volumes.

Statistical analysis

Data are expressed as mean ± standard error (s.e.). Contractile responses were expressed as a percentage of a maximum contraction to histamine (10^{-3} M). In most experiments using EFS where each tissue was its own control, results before and after single concentration drug addition were compared by a Students two-tailed t test for paired data. In experiments where maximum responses to agonists (or antagonists) were obtained (ie. 5-HT in the presence of ketanserin, 5-CT, and methiothepin) EC_{50} (or IC_{50}s) were calculated by iterative curve fitting using Graphpad Inplot (Graphpad Inc., San Diego, CA., U.S.A.). However, these values were obtained only for the mean curve data as only one concentration of agonist was tested per tissue and hence it was not possible to calculate s.e. or statistically compare the EC_{50} (or IC_{50}) values obtained. For experiments involving cumulative concentration-dependent contractions to substance P (± 5-HT) approximate EC_{50} values were calculated for each tissue, as a maximum response to substance P had to be estimated. Significant differences were determined by comparisons of EC_{50} (± 5-HT) by Student's two-tailed t test for unpaired data. For most experiments involving concentration-response curves to 5-HT ± antagonists, maximum responses were not obtained and hence EC_{50} values could not be calculated even for the mean concentration-response curves. Here the inhibition produced by 5-HT in the presence of the antagonist was compared to that produced by the same concentration of 5-HT alone using Student's two-tailed t test for unpaired data. P values <0.05 were considered significant. $*P<0.05$, $**P<0.01$, $***P<0.001$, NS represents not significant.

Results

The effect of 5-HT on excitatory NANC responses

5-HT (0.1–100 μM) produced a concentration-dependent inhibition of NANC neural contractile responses (Figure 1) with 100 μM 5-HT producing 50.9 ± 5.0% inhibition ($n = 5$, $P<0.01$) (Figure 2a), however it was not clear if this inhibition was maximal so an EC_{50} value was not calculated. At these concentrations, 5-HT evokes a direct contractile effect on airway smooth muscle via 5-HT_{2A} receptors which was allowed to subside before test NANC contractile responses could be measured. The addition of ketanserin (1 μM) prevented this direct action of 5-HT (Figure 1) without affecting NANC responses *per se* (1 μM: 8.3 ± 3.5% inhibition, $n = 6$, NS). Ketanserin (1 μM) had no significant effect on the inhibition caused by 5-HT (0.1–100 μM) when each data point in the presence of ketanserin was compared to its control by Student's t test for unpaired data (5-HT (1 μM): 20.8 ± 2.5% inhibition, $n = 4$; + ketanserin: 35.2 ± 6.7% inhibition, $n = 10$, NS). Addition of ketanserin (1 μM) appeared to produce a leftward shift (≈10 fold) of the 5-HT concentration-response curve however, as 5-HT alone did not reach a maximum response and as 5-HT was added in a non-cumulative manner, comparison of EC_{50} values was not possible and hence the statistical significance of this leftward shift could not be assessed. In the presence of ketanserin (1 μM) the 5-HT concentration-response relationship appears to reach a plateau with 100 μM 5-HT producing 57.4 ± 5.9% inhibition ($n = 5$, $P<0.01$), allowing iterative curve fitting and calculation of an EC_{50} of 0.57 μM. In all subsequent experiments ketanserin (1 μM) was present throughout.

The effect of adrenoceptor antagonists on 5-HT induced inhibition of excitatory NANC responses

Neither the non-selective α-adrenoceptor antagonist, phentolamine (1 μM) nor the selective β_2-adrenoceptor antagonist, ICI 118551 (0.1 μM) had any significant effect on either EFS evoked NANC contractile responses (ICI 118551:2.3 ± 4.0% inhibition, $n = 5$, NS; phentolamine:6.9 ± 5.2% enhancement, $n = 4$, NS), or on the concentration-dependent inhibition of the NANC contractile response evoked by 5-HT (0.1–100 μM). 5-HT (1 μM) produced an inhibition of 35.2 ± 6.7% ($n = 10$, $P<0.001$), 25.9 ± 3.7% ($n = 5$, NS compared to 5-HT alone) in the presence of phentolamine (1 μM) and 24.2 ± 3.8% ($n = 4$, NS compared to 5-HT alone) in the presence of ICI 118551 (0.1 μM).

Effect of selective 5-HT receptor antagonists on 5-HT-induced inhibition of NANC responses

Pre-incubation with ICS 205-930 at a concentration that has been demonstrated to block both 5-HT_3 and 5-HT_4 receptors (1 μM) did not affect either excitatory NANC responses alone (ICS 205-930, 1 μM: 3.4 ± 4.5% inhibition, $n = 5$, NS) or the concentration-dependent inhibition produced by 5-HT (0.1–100 μM) (5-HT [1 μM]: control: 35.2 ± 6.7% inhibition, $n = 10$; + ICS 205-930: 20.9 ± 3.5% inhibition, $n = 5$, NS compared to control) (Figure 2a).

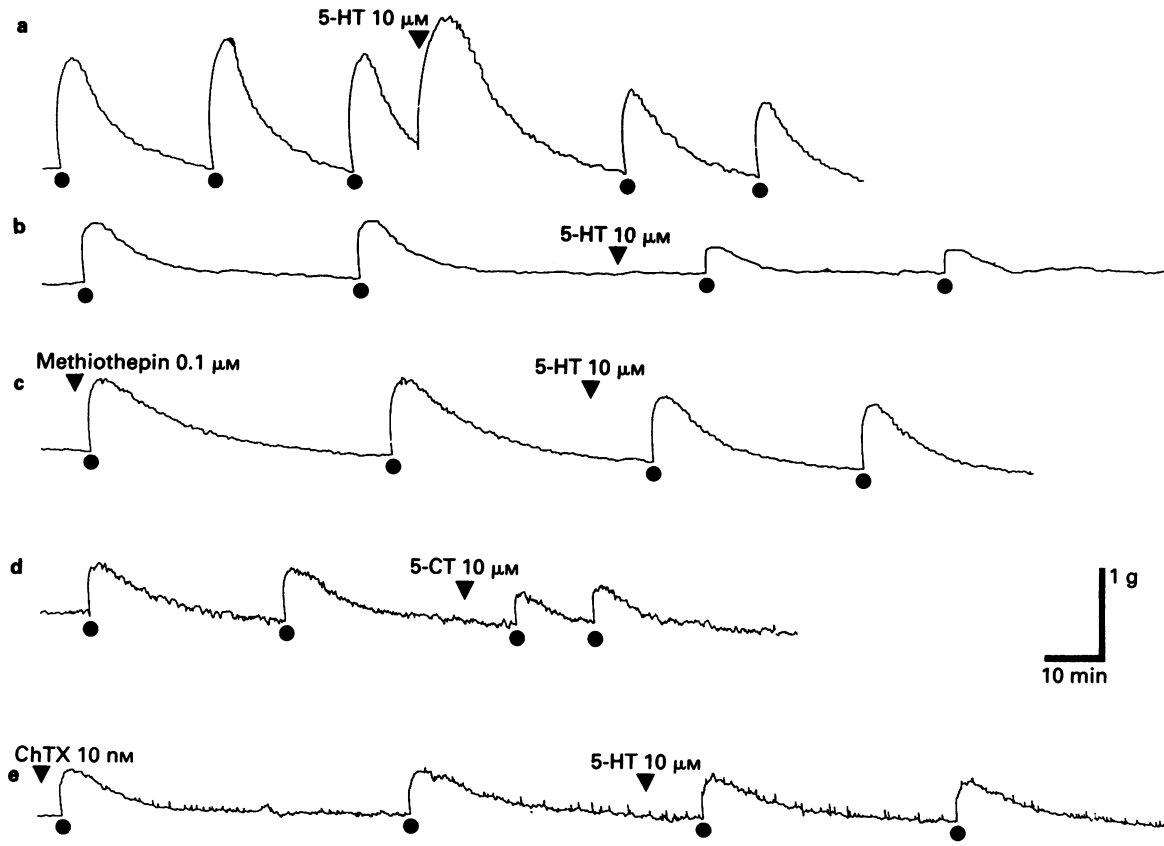


Figure 1 Trace showing the effects of 5-HT and selective agonists/antagonists on NANC bronchoconstriction elicited by electrical field stimulation (40 V, 0.5 ms, 8 Hz for 20 s; represented by ●) in guinea-pig bronchi. Atropine (1 μM) was present throughout and ketanserin (1 μM) was present in all traces except (a). (a) Inhibition by 5-HT in the absence of ketanserin. (b) Inhibition by 5-HT (10 μM). (c) Antagonism of 5-HT inhibition by methiothepin (0.1 μM). (d) Inhibition by 5-carboxamidotryptamine (5-CT, 10 μM). (e) Antagonism of 5-HT inhibition by charybdotoxin (ChTX, 10 nM).

The non-selective 5-$HT_{1/2}$ antagonist, methiothepin (0.1 μM) did not affect NANC contractile responses alone (methiothepin, 0.1 μM; 0.7 ± 0.9% inhibition, $n = 4$, NS) however it significantly attenuated the effects of 5-HT (Figure 1) at all concentrations studied (5-HT [1 μM] control: 35.2 ± 6.7% inhibition, $n = 10$; + methiothepin [0.1 μM]: 17.2 ± 3.7% inhibition, $n = 5$, $P < 0.05$ compared to control) (Figure 2b). Methiothepin elicited a concentration-dependent inhibition of the effect of 5-HT (10 μM) (Figure 3) with an IC_{50} of 81 nM.

Cyanopindolol (1 μM) the 5-$HT_{1A/1B}$ antagonist did not significantly affect the 5-HT induced inhibition of the NANC response (5-HT [1 μM] control: 35.2 ± 6.7% inhibition, $n = 10$; + cyanopindolol: 31.2 ± 7.3% inhibition, $n = 4$, NS compared to control) (Figure 2b).

Effect of selective 5-HT receptor agonists on excitatory NANC responses

5-CT (0.1–100 μM) was found to be the most potent agonist (Figure 1) and inhibited NANC contractile responses in a concentration-dependent manner (Figure 4a) with an EC_{50} of 0.13 μM. 5-CT (100 μM) produced an inhibition of 51.1 ± 4.7% ($n = 5$, $P < 0.001$). 8-OH-DPAT (0.1–100 μM), the selective 5-HT_{1A} receptor agonist, also produced a concentration-dependent inhibition as did the selective 5-$HT_{2A/B/C}$ receptor agonist α-Me-5-HT (0.1–100 μM) (Figure 4a). The 5-HT_{1D} receptor agonist, sumatriptan (Figure 4a), 5-HT_3 receptor agonist 2-Me-5-HT and the putative 5-HT_4 receptor agonist, 5-MeOT (all 0.1–100 μM) (Figure 4b) were essentially inactive. Calculation of approximate IC_{25} values (the concentration of the agonist required to produce a 25% inhibition of the NANC contractile response [μM]) gave a rank order of potency 5-CT [0.13] ⩾ 5-HT [0.32] >> 8-OH-DPAT [14] > α-Me-5-HT [80]. Sumatriptan, 5-MeOT and 2-Me-5-HT were essentially inactive with $IC_{25} > 100$ μM.

Effect of 5-HT on cumulative concentration-contraction relationships to exogenously applied substance P

To determine whether the inhibitory effect of 5-HT was due to activation of pre- or post-junctional receptors we studied the effect of 5-HT (10 μM) on cumulative concentration-response relationships to exogenously applied substance P (0.1 nM–10 μM) and compared these to control responses in different tissues from the same animal (Figure 5). Pretreatment with 5-HT (10 μM) did not significantly affect either the response to 10 μM substance P (control: 843 ± 44 mg, $n = 4$; + 5-HT: 878 ± 144 mg, $n = 4$, NS compared to control) or the approximate EC_{50} (control: 3.0 ± 0.9 μM, $n = 4$; + 5-HT: 4.0 ± 0.3 μM, $n = 4$, NS compared to control). EFS (8 Hz) produced a contraction 42.8 ± 1.1% ($n = 65$) of the maximum contraction to histamine ($n = 65$) which was approximately equal in magnitude to 3 μM substance P (42.4 ± 0.9% of histamine maximum, $n = 4$), but even at this concentration there was clearly no significant effect of 5-HT (10 μM) (Figure 5).

Effect of ChTX on 5-HT induced inhibition of excitatory NANC responses

Pretreatment of tissues with ChTX (0.1–30 nM; Figure 1) had no significant effect on NANC contractile responses alone (ChTX, 30 nM: 0.5 ± 2.3% inhibition, $n = 5$, NS) but

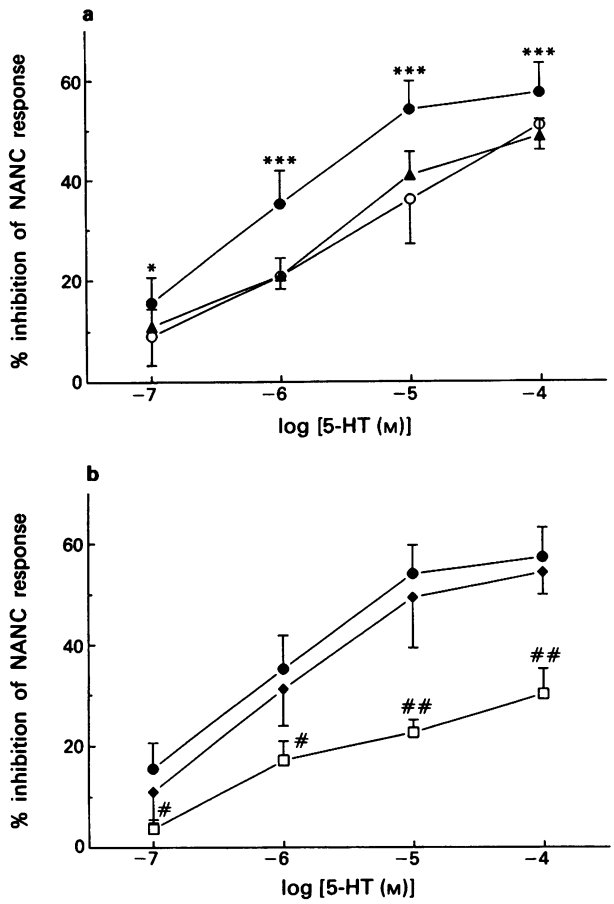


Figure 2 The effect of 5-HT on NANC bronchoconstriction elicited by electrical field stimulation (40 V, 0.5 ms, 8 Hz for 20 s) in guinea-pig isolated bronchi. Effects are displayed as a mean percentage inhibition of the mean initial response preceding drug administration. Atropine (1 μM) was present throughout. (a and b) 5-HT + ketanserin (1 μM, ●). (a) 5-HT alone (○), 5-HT + ketanserin (1 μM) + ICS 205-930 (1 μM, ▲). (b) 5-HT + ketanserin (1 μM) + cyanopindolol (1 μM, ◆); 5-HT + ketanserin (1 μM) + methiothepin (0.1 μM, □). Points represent mean ± s.e.mean of at least $n = 5$ observations. Significance values are shown for the inhibition caused by 5-HT in the presence of ketanserin (●): $*P<0.05$, $**P<0.01$, $***P<0.001$ compared to control NANC responses preceding 5-HT administration. Significance values are also shown for the blockade of this inhibition by methiothepin (□): $\#P<0.05$, $\#\#P<0.01$, $\#\#\#P<0.001$ compared to inhibition preceding methiothepin administration.

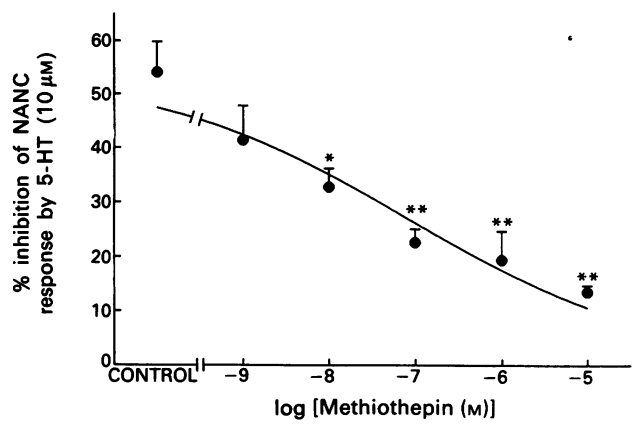


Figure 3 The effect of methiothepin on 5-HT (10 μM)-induced inhibition of the NANC response elicited by electrical field stimulation (40 V, 0.5 ms, 8 Hz for 20 s) in guinea-pig isolated bronchi. Atropine and ketanserin (both 1 μM) were present throughout. Effects are displayed as a mean percentage inhibition of the mean initial response preceding drug administration. Points represent mean ± s.e.mean of at least $n = 5$ observations. $*P<0.05$, $**P<0.01$, $***P<0.001$ compared to the inhibition preceding methiothepin administration.

evoked a concentration-dependent inhibition of the effect of 5-HT (10 μM) (Figure 6). ChTX, at concentrations ≥ 1 nM, produced significant inhibition of the effect of 5-HT (10 μM) with almost complete inhibition at 30 nM (5-HT: 46.4 ± 5.0% inhibition, $n = 4$, $P<0.01$; 5-HT + ChTX 30 nM: 1.7 ± 2.3% inhibition, $n = 5$, $P<0.001$ compared to 5-HT alone).

Effect of NO synthase inhibitors on 5-HT-induced inhibition of excitatory NANC responses

Incubation of tissues with L-NAME (100 μM) did not significantly affect either NANC responses alone (L-NAME, 100 μM: 3.0 ± 4.5% enhancement, $n = 5$, NS) or inhibition of NANC contractile responses evoked by 5-HT (10 μM) (Figure 6). 5-HT (10 μM) inhibited NANC responses by 46.4 ± 5.0% ($n = 5$, $P<0.01$) in the absence and 42.0 ± 5.8% ($n = 5$, NS compared to control) in the presence of L-NAME (100 μM).

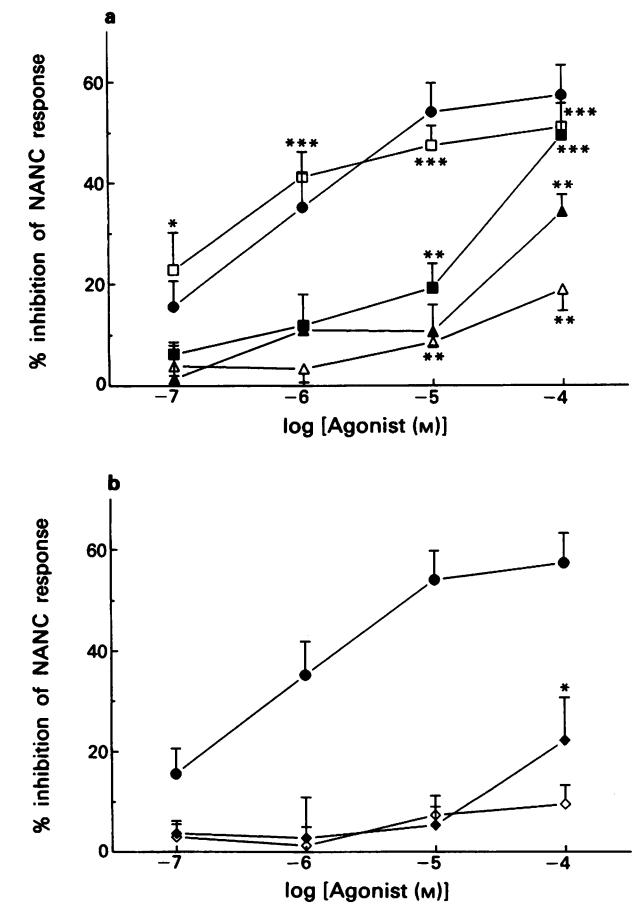


Figure 4 The effects of selective 5-HT receptor agonists on NANC bronchoconstriction elicited by electrical field stimulation (40 V, 0.5 ms, 8 Hz for 20 s) in guinea-pig isolated bronchi. Effects are displayed as a mean percentage inhibition of the mean initial response preceding drug administration. Atropine and ketanserin (both 1 μM) were present throughout. (a and b) 5-HT (●); (a) 5-CT (□); 8-OH-DPAT (■); α-Me-5HT (▲) and sumatriptan (△). (b) 2-Me-5-HT (◇); and 5-MeOT (◆). Points represent mean ± s.e.mean of at least $n = 5$ observations. $*P<0.05$, $**P<0.01$, $***P<0.001$ compared to control values preceding drug administration.

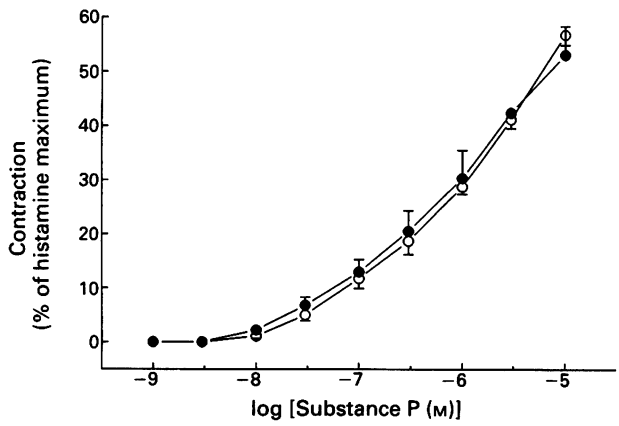


Figure 5 The effect of 5-HT (10 μM) on cumulative concentration-contraction relationships to exogenously applied substance P. Effects are displayed as a percentage of the maximum contraction to histamine (1 mM), added at the end of each experiment. Curves are shown for substance P in the absence (●) or presence (○) of 5-HT (10 μM). Points represent the mean ± s.e.mean of $n = 4$ observations.

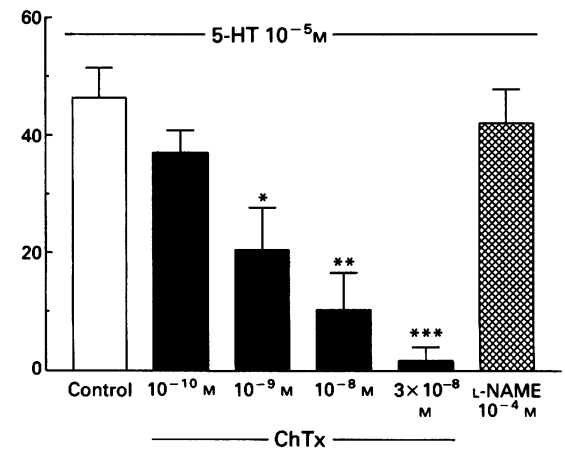


Figure 6 The effects of charybdotoxin (ChTX) (filled columns) and N^G-nitro-L-arginine methyl ester (L-NAME) (cross-hatched column) on 5-HT (10 μM, open column)-induced inhibition of the NANC bronchoconstriction evoked by electrical field stimulation (40 V, 0.5 ms, 8 Hz for 20 s) in guinea-pig isolated bronchi. Atropine and ketanserin (both 1 μM) were present throughout. Effects are displayed as a mean percentage inhibition of the mean initial response preceding drug administration. Columns represent mean ± s.e.mean of at least $n = 5$ observations. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to inhibition preceding ChTX administration.

Discussion

We have demonstrated that exogenously applied 5-HT inhibits excitatory NANC neural bronchoconstriction elicited by EFS in guinea-pig bronchi *in vitro*. This inhibition is not due to 5-HT behaving as a simple biogenic amine at either α- or β_2-adrenoceptors as neither phentolamine, at a concentration selective for α-adrenoceptors (1 μM), nor ICI 118551, at a concentration selective for β_2-adrenoceptors (0.1 μM) affected the 5-HT concentration-response relationship.

The 5-$HT_{3/4}$ selective antagonist, ICS 205-930 (Richardson *et al.*, 1985; Bockaert *et al.*, 1992) was without effect on the 5-HT concentration-response relationship suggesting these receptors play no part in the effect of 5-HT. The 5-HT_2 receptor antagonist, ketanserin (Richardson & Hoyer, 1990) had no significant effect on the inhibition produced by 5-HT when each data point in the presence of ketanserin was compared to its control by Student's *t* test for unpaired data, but the 5-HT concentration-response curve did appear to be shifted in a leftward direction (≈10 fold). However, as 5-HT alone did not reach a maximum response, and 5-HT was added in a non-cumulative manner, comparison of the 5-HT (± ketanserin) concentration-response curve EC_{50} values was not possible, hence the statistical significance of this leftward shift could not be assessed. It is possible that ketanserin may enhance the effect of 5-HT on NANC responses; however, experimental limitations of NANC responses (i.e. only 5 stable responses per tissue), makes confirmation of this shift impossible. In contrast, methiothepin which we have used as a non-selective 5-$HT_{1/2}$ receptor antagonist, (Richardson & Hoyer, 1990), although it has been found to have equal or higher affinity at α_1, H_1 and dopamine receptors (Leysen *et al.*, 1981), produced an apparently non-surmountable attenuation of the 5-HT concentration-response relationship. Methiothepin also evoked a concentration-dependent attenuation of the inhibition produced by 5-HT (10 μM) with an IC_{50} of 81 nM. However, methiothepin reduced the maximum response to 5-HT suggesting a non-competitive blockade of the effect of 5-HT by methiothepin which is inconsistent with 5-HT acting at a 5-HT_1 receptor (Richardson & Hoyer, 1990). Further characterization of the 5-HT receptor responsible for inhibition of NANC bronchoconstriction was possible with agonists, however, this was difficult due to the poor selectivity of most agonists. The 5-HT_3 receptor agonist 2-Me-5-HT (Richardson *et al.*, 1985) and 5-MeOT, the ligand we have used as a 5-HT_4 receptor agonist (Bockaert *et al.*, 1992) although it does show high affinity at 5-$HT_{1A/D}$ and 5-HT_{2C} receptors (Glennon, 1987), were essentially inactive, providing further evidence that 5-$HT_{3/4}$ receptors are not involved. 5-CT shows some selectivity for 5-$HT_{1A/B/D}$ receptors and was the most potent agonist tested. This coupled with the relative ineffectiveness of 8-OH-DPAT, a highly selective 5-HT_{1A} receptor agonist, and of the 5-$HT_{2A/B/C}$ receptor agonist, α-Me-5-HT (Richardson & Hoyer, 1990) would suggst that the receptor responsible is of the 5-$HT_{1B/D}$ subtype. However the inactivity of the 5-HT_{1D} agonist, sumatriptan and of the 5-$HT_{1A/B}$ receptor antagonist, cyanopindolol, the low affinity of 5-HT/5-CT compared to previously published results at 5-HT_1 receptors (Richardson & Hoyer, 1990) and the non-competitive nature of the blockade by methiothepin, suggests that it may be of an atypical nature. Recently genes encoding 5-HT_{1E} and 5-HT_{1F} receptor subtypes have been cloned (McAllister *et al.*, 1992; Adham *et al.*, 1993) and it is possible that the receptor responsible for the inhibition of NANC responses could correspond to one of these novel 5-HT receptors. However definitive characterization awaits further functional correlates on these recently described receptors and development of selective receptor ligands.

The inhibitory effect of this atypical 5-HT receptor is likely to be due to inhibition of neuropeptide release from C fibre nerve terminals rather than postjunctional receptors on the bronchial smooth muscle leading to functional antagonism of the contractile effects of neuropeptides, since 5-HT had no effect on exogenously applied substance P concentration-contraction relationships. To confirm the prejunctional nature it will be necessary in future experiments to measure neuropeptide release directly possibly via radioimmunoassay for calcitonin gene-related peptide (CGRP) (Lou *et al.*, 1991) or neurokinins (e.g. substance P) (Ray *et al.*, 1991). This evidence would suggest that the atypical 5-HT receptor responsible could be part of a group of prejunctional receptors, including α_2-adrenoceptors, μ-opioid receptors and NPY receptors which have previously been shown to have similar inhibitory effects in guinea-pig bronchi (see Barnes, 1992).

Our results are consistent with other studies which have demonstrated the presence of 5-HT receptors on sensory nerves. Recent studies have demonstrated that 5-HT_{1B} and 5-HT_{1D} receptors are involved in inhibition of neurogenic

plasma extravasation in the dura mater of the rat and guinea-pig, respectively (Buzzi & Moskowitz, 1990; Matsubara *et al.*, 1991).

We investigated two possible mechanisms for the inhibitory effect of 5-HT. 5-HT evokes the release of NO, leading to relaxation of the canine terminal ileum and ileocolonic junction (Bogers *et al.*, 1991). We investigated the possibility that 5-HT was acting in a similar manner in guinea-pig bronchi, evoking the release of NO which could then act either at nerve terminals or directly at the level of the bronchial smooth muscle to inhibit NANC contractile responses. Since 5-HT did not affect exogenous substance P concentration-contraction relationships and the NO synthase inhibitor L-NAME had no effect on 5-HT-induced inhibition of NANC responses, it is unlikely that NO mediates the effect of 5-HT either through inhibition of neuropeptide release or postjunctional functional antagonism.

Stimulation of α_2-adrenoceptors, μ-opioid receptors and NPY receptors have all been shown to evoke inhibition via activation of a common ChTX-sensitive K^+_{Ca} channel (Stretton *et al.*, 1992). We have demonstrated that ChTX pretreatment can completely abolish the effect of 5-HT on NANC responses, suggesting that 5-HT is acting to open a K^+_{Ca} channel and providing further evidence that the 5-HT receptor responsible for this inhibition is acting via a prejunctional mechanism involving a common K^+ channel. Although ChTX was originally considered to be specific for K^+_{Ca}, its selectivity has been questioned as it has been suggested that it blocks Ca^{2+}-insensitive voltage-dependent K^+ channels (K^+_V) in rat brain and DRG cells (Schweitz *et al.*, 1989). However, comparison of the effects of iberiotoxin (IbTX), a highly selective blocker of K^+_{Ca} channels and noxioustoxin (NxTX), a selective blocker of K^+_V channels, on prejunctional inhibition of NANC responses by μ-opioids in guinea-pig bronchi has shown that the ChTX-sensitive channel which mediates this inhibition is sensitive to IbTX but not NxTX, confirming the involvement of a K^+_{Ca} channel (Miura *et al.*, 1993). Therefore it is likely that the ChTX-sensitive channel which is opened, either directly or indirectly, by 5-HT_1-like receptor activation is indeed a K^+_{Ca} channel.

It has been confirmed that 5-HT acts as a neurotransmitter in the gastrointestinal tract as its release has been demonstrated from nerves in response to electrical stimulation (Gershon & Tamir, 1981). It is not certain whether 5-HT acts as a neurotransmitter in the airways. Although our experiments were not designed to determine whether there is a role for endogenous 5-HT in guinea-pig bronchi, it is unlikely that there is any basal release of 5-HT as the 5-HT_2 antagonist, ketanserin, which would block the action of 5-HT acting directly at 5-HT_2 receptors on bronchial smooth muscle, has no effect on resting tone. A 5-hydroxytryptaminergic neural innervation allowing EFS-evoked release of 5-HT also appears unlikely, as selective 5-HT receptor antagonists alone had no effect on EFS-induced excitatory NANC bronchoconstriction with the stimulation parameters we have tested. However this does not rule out the *in vivo* presence of endogenous 5-HT either from neuroepithelial cells, as in the rabbit (Lauweryns *et al.*, 1973) or released from platelets activated during tissue damage. This released 5-HT may then serve to offset neurogenic inflammation resulting from sensory nerve activation.

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References

ADHAM, N., KAO, H.-T., SCHECHTER, L.E., BARD, J., OLSEN, M., URQUHART, D., DURKIN, M., HARTIG, P.R., WEINSHANK, R.L. & BRANCHEK, T.A. (1993). Cloning of another human serotonin receptor (5-HT_{1F}): a fifth 5-HT_1 receptor subtype coupled to the inhibition of adenylate cyclase. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 408–412.

BARNES, P.J. (1992). Modulation of neurotransmission in the airways. *Physiol. Rev.*, **72**, 699–729.

BARNES, P.J., BARANIUK, J.M. & BELVISI, M.G. (1991). State of the art review. Neuropeptides in the respiratory tract. *Am. Rev. Respir. Dis.*, **144**, 1187–1198; 1391–1399.

BAUMGARTNER, R.A., WILLS-KARP, M., KAUFMAN, M.J., MUNAKATA, M. & HIRSHMAN, C. (1990). Serotonin induces constriction and relaxation of the guinea pig airway. *J. Pharmacol. Exp. Ther.*, **255**, 165–173.

BERNOUSSI, A. & RIOUX, F. (1989). Effects of capsaicin desensitization on the stimulatory effect of kinins, prostaglandins, biogenic amines and various drugs in guinea-pig isolated atria. *Br. J. Pharmacol.*, **96**, 563–572.

BOCKAERT, J., FOZARD, J.R., DUMUIS, A. & CLARKE, D.E. (1992). The 5-HT_4 receptor: a place in the sun. *Trends Pharmacol. Sci.*, **13**, 141–145.

BOGERS, J.J., PELCKMANS, P.A., BOECKXSTAENS, G.E., DE MAN, J.G., HERMAN, A.G. & VAN MAERCKE, Y.M. (1991). The role of nitric oxide in serotonin-induced relaxations in the canine terminal ileum and the ileocolonic junction. *Naunyn-Schmied. Arch. Pharmacol.*, **344**, 716–719.

BUCKNER, C.K., DEA, D., LIBERATI, N. & KRELL, R.D. (1991). A pharmacologic examination of receptors mediating serotonin-induced bronchoconstriction in the anaesthetised guinea-pig. *J. Pharmacol. Exp. Ther.*, **257**, 26–34.

BUZZI, M.G. & MOSKOWITZ, M.A. (1990). The antimigraine drug, sumatriptan (GR 43175), selectively blocks neurogenic plasma extravasation from blood vessels in dura mater. *Br. J. Pharmacol.*, **99**, 202–206.

CHRISTIAN, E.P., TAYLOR, G.E. & WEINREICH, D. (1989). Serotonin increases excitability of rabbit C-fiber neurons by two distinct mechanisms. *J. Appl. Physiol.*, **67**, 584–591.

DAVAL, G., VERGE, D., BASBAUM, A., BOURGOIN, S. & HAMON, M. (1987). Autoradiographic evidence of serotonin 1 binding sites on primary afferent fibres in the dorsal horn of the rat spinal cord. *Neurosci. Letts.*, **83**, 71–76.

DOUGLAS, W.W. & RITCHIE, J.M. (1957). On excitation of nomedullated afferent fibres in the aortic nerves by pharmacological agents. *J. Physiol.*, **138**, 31–43.

GERSHON, M.D. & TAMIR, H. (1981). Release of endogenous 5-hydroxytryptamine from resting and stimulated enteric neurons. *Neuroscience*, **6**, 2277–2286.

GIMENEZ-GALLEGO, G., NAVIA, M.A., REUBEN, J.P., KATZ, G.M., KACZOROWSKI, G.J. & GARCIA, M.L. (1988). Purification, sequence and structure of charybdotoxin, a potent selective inhibitor of calcium-activated potassium channels. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 3239–3333.

GLENNON, R.A. (1987). Central serotonin receptors as targets for drug research. *J. Med. Chem.*, **30**, 1–12.

GRUNDSTROM, N., ANDERSSON, R.G.G. & WIKBERG, J.E.S. (1981). Pharmacological characterization of the autonomous nervous innervation of the guinea pig tracheobronchial smooth muscle. *Acta Pharmacol. Toxicol.*, **49**, 150–157.

HAMON, M., GALLISSOT, M.C., MENARD, F., GOZLAN, H., BOURGOIN, S. & VERGE, D. (1989). 5-HT_3 receptor binding sites are on capsaicin-sensitive fibres in the rat spinal cord. *Eur. J. Pharmacol.*, **164**, 315–322.

HUANG, J.C. & PEROUTKA, S.J. (1987). Identification of 5-hydroxytryptamine$_1$ binding site subtypes in rat spinal cord. *Brain Res.*, **436**, 173–176.

LAUWERYNS, J.M., COLEAERE, M. & THEUNYNCK, P. (1973). Serotonin producing neuroepithelial bodies in rabbit respiratory mucosa. *Science*, **180**, 410–413.

LEYSEN, J.E., AWOUTERS, F., KENNIS, L., LADURON, P.M., VANDENBERK, J. & JANSSEN, P.A. (1981). Receptor binding profile of R41 468, a novel antagonist at 5-HT_2 receptors. *Life Sci.*, **28**, 1015–1022.

LOU, Y.-P., KARLSSON, J.-A., FRANCO-CERECEDA, A. & LUNDBERG, J.M. (1991). Selectivity of ruthenium red in inhibiting bronchoconstriction and CGRP release induced by afferent C-fibre activation in guinea-pig lung. *Acta Physiol Scand.*, **142**, 191–199.

LUNDBERG, J.M. & SARIA, A. (1987). Polypeptide containing neurons in airway smooth muscle. *Am. Rev. Physiol.*, **49**, 557–572.

MACQUIN-MAVIER, I., JARREAU, P., ISTIN, N. & HARF, A. (1991). 5-hydroxytryptamine-induced bronchoconstriction in the guinea-pig: effect of 5-HT_2 receptor activation on acetylcholine release. *Br. J. Pharmacol.*, **102**, 1003–1007.

MAGGI, C.A., PATACCHINI, R., ROVERO, P. & SANTICOLI, P. (1991). Tachykinin receptors and non-cholinergic bronchoconstriction in the guinea-pig isolated bronchi. *Am. Rev. Respir. Dis.*, **144**, 363–367.

MARTLING, C.R., SARIA, A., ANDERSSON, P. & LUNDBERG, J.M. (1984). Capsaicin pretreatment inhibits vagal cholinergic and non-cholinergic control of pulmonary mechanics in the guinea pig. *Naunyn-Schmied. Arch. Pharmacol.*, **325**, 343–348.

MATSUBARA, T., MOSKOWITZ, M.A. & BYUN, B. (1991). CP-93,129, a potent and selective 5-HT_{1B} receptor agonist blocks neurogenic plasma extravasation within rat but not guinea-pig dura mater. *Br. J. Pharmacol.*, **104**, 3–4.

MCALLISTER, G., CHARLESWORTH, A., SNOWDIN, C., BEER, M.S., NOBLE, A.J., MIDDLEMISS, D.N., IVERSEN, L.L. & WHITING, P. (1992). Molecular cloning of a serotonin receptor from human brain (5HT1E): a fifth 5-HT1-like subtype. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 5517–5521.

MIURA, M., BELVISI, M.G., WARD, J.K. & BARNES, P.J. (1993). Role of Ca^{2+}-activated K^+ channels blockers on opioid-induced prejunctional modulation of airway sensory nerves. *Am. Rev. Respir. Dis.*, **147**, A815.

RAY, N.J., JONES, A.J. & KEEN, P. (1991). $GABA_B$ modulation of the release of substance P from capsaicin-sensitive neurones in the rat trachea *in vitro*. *Br. J. Pharmacol.*, **102**, 801–804.

RICHARDSON, B.P., ENGEL, G., DONATSCH, P. & STRADLER, P.A. (1985). Identification of serotonin M-receptor subtypes and their specific blockade by a new class of drugs. *Nature*, **316**, 126–131.

RICHARDSON, B.P. & HOYER, D. (1990). Selective agonists and antagonists at 5-hydroxytryptamine receptor subtypes. In *Serotonin: From Cell Biology to Pharmacology and Therapeutics* ed. Paoletti, R., Vanhoutte, P.M., Brunell, N. & Maggi, F.M. pp. 265–376. Dordrecht: Kluwer Academic Publisher.

RIZZO, C.A., KREUTNER, W. & CHAPMAN, R.W. (1993). 5-HT_3 receptors augment neuronal, cholinergic contractions in guinea pig trachea. *Eur. J. Pharmacol.*, **234**, 109–112.

SCHWEITZ, H., STANSFIELD, C.E., BIDARD, J.-N., FAGNI, L., MEAS, P. & LAZDUNSKI, M. (1989). Charybdotoxin blocks dendrotoxin-sensitive voltage-activated K^+ channels. *FEBS Lett.*, **250**, 519–522.

STRETTON, D., MIURA, M., BELVISI, M.G. & BARNES, P.J. (1992). Calcium-activated potassium channels mediate prejunctional inhibition of peripheral sensory fibres. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 1325–1329.

TRAMONTANA, M., GIULIANI, S., DEL BIANCO, E., LECCI, A., MAGGI, C.A., EVANGELISTA, S. & GEPPETTI, P. (1993). Effects of capsaicin and 5-HT_3 antagonists on 5-hydroxytryptamine-evoked release of calcitonin gene-related peptide in the guinea-pig heart. *Br. J. Pharmacol.*, **108**, 431–435.

TODOROVIC, S.M. & ANDERSON, E.G. (1990). Pharmacological characterization of 5-hydroxytryptamine$_2$ and 5-hydroxytryptamine$_3$ receptors in rat dorsal root ganglion cells. *J. Pharmacol. Exp. Ther.*, **254**, 109–115.

WALLIS, D.I., STANSFIELD, C.E. & NASH, H.L. (1982). Depolarizing responses recorded from nodose ganglion cells of the rabbit evoked by 5-hydroxytryptamine and other substances. *Neuropharmacology*, **21**, 31–40.

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Effect of the putative cognitive enhancer, linopirdine (DuP 996), on quantal parameters of acetylcholine release at the frog neuromuscular junction

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1 The subcellular mechanism and site of action of linopirdine or DuP 996 (3,3-*bis*(4-pyridinylmethyl)-1-phenylindolin-2-one) was investigated at the frog neuromuscular junction, using miniature endplate potential (m.e.p.p.) counts and a new method for obtaining unbiased estimates of n (number of functional release sites), p (probability of release), and $var_s p$ (spatial variance in p).

2 DuP 996 produced an increase in m (no. of quanta released), which was due to an increase in n and p. The increase in m was concentration-dependent over a range of 0.1–100 μM and completely reversible with 15 min of wash. There was a saturation in the increase in p, but not in the increase in m and n, for [DuP 996] > 10 μM. By contrast, there was no major change in $var_s p$.

3 Block of presynaptic Na^+- and Ca^{2+}-channels with 3 μM tetrodotoxin and 1.8 mM Co^{2+} prevented the m.e.p.p. frequency increase to DuP 996, and this effect was completely reversed by washing.

4 Application of the neuronal Ca^{2+}-channel blocker, ω-conotoxin GVIA (1 μM) brought about a rapid and profound decrease in the m.e.p.p. frequency increase produced by DuP 996. The effect of the toxin was not reversed by prolonged washing.

5 Block of voltage-gated K^+-channels with 100 μM 4-aminopyridine (4-AP) resulted in only a small (28%) increase in m. The combination of 4-AP (100 μM) and DuP 996 (10 μM) produced an increase in m (189%) which was much greater than the sum of the responses to each agent alone. This increase in m was due solely to an increase in n, as p and $var_s p$ were unchanged.

6 For [DuP 996] up to 100 μM, there was no apparent change in the mean size, amplitude distribution, or time course of m.e.p.ps, signifying that it had no anticholinesterase activity.

7 It is concluded that DuP 996 increases the release of quantal transmitter but not the postsynaptic response to the quanta. This appears to involve an effect at the nerve terminal membrane, most likely an increase in Ca^{2+}-conductance, and not an action to block K^+-conductance or to release Ca^{2+} from intraterminal organelles.

Keywords: Acetylcholine; 4-aminopyridine; Ca^{2+} influx; ω-conotoxin GVIA; DuP 996; intracellular organelles; linopirdine; miniature endplate potentials; neurosecretion; quantal release parameters

Introduction

One of the widely held hypotheses on the pathophysiology underlying Alzheimer's disease (AD) is that of a cholinergic deficit in the nucleus basalis of Meynert (Whitehouse *et al.*, 1982; Katzman, 1986). Pharmacological approaches to providing a palliative treatment for AD have thus been aimed at enhancing transmission at the cholinergic synapses remaining in this region (Reinikainen *et al.*, 1990; Kumar & Calache, 1991). Agents affecting the cholinergic system which have been examined with varying success include arecoline (Christie, 1982), bethanechol (Penn *et al.*, 1988; Read *et al.*, 1990), physostigmine (Muramoto *et al.*, 1984; Harrell *et al.*, 1990), and tetrahydroaminoacridine (THA) (Summers *et al.*, 1986; Molloy *et al.*, 1991). Agents which have received more serious consideration are Synapton, a controlled release physostigmine (Forest Labs), Cognex, a formulation of THA (Warner-Lambert), Mentane, a THA analogue (Hoechst-Roussel), and HP 290, an analogue of physostigmine (Hoechst-Roussel) (reviewed by Sarter, 1991).

Linopirdine or DuP 996 (3,3-*bis*(4-pyridinylmethyl)-1-phenylindolin-2-one, Du Pont Merck) is a compound currently undergoing Phase III testing for possible use in the treatment of AD. Preclinical studies have shown that DuP 996 enhances the K^+-evoked release of acetylcholine (ACh), dopamine and 5-hydroxytryptamine (5-HT) in brain slices, without affecting the resting release of these transmitters (Nickolson *et al.*, 1990). A similar increase in ACh release has been seen in synaptosomes (Vickroy, 1993). The ability to release ACh is highly correlated with the affinities of DuP 996 and thirty of its analogues for specific receptors (Tam *et al.*, 1991) located in areas of the brain associated with cognition (De Souza *et al.*, 1992). DuP 996 also enhances the performance of rats in behavioural tests and increases the amount of their alpha and beta wave activity recorded with EEGs (Cook *et al.*, 1990).

Recent studies with rodent skeletal muscle have shown that DuP 996 increases the direct and indirect twitch, the miniature endplate potential (m.e.p.p.) frequency, and the quantal content of the endplate potential (e.p.p.) in Mg^{2+}-depressed preparations (Tsai *et al.*, 1992). Despite this report, there remain several questions regarding the actions of DuP 996. First, it is possible that the release of ACh measured neurochemically consists of both quantal and non-quantal ACh. A significant increase in *quantal* ACh release should therefore be documented in order to relate the effect of DuP 996 on ACh output with that on cognitive function. Second, the increase in ACh output with DuP 996 may be due to either (a) an enhancement of the normal processes involved in neurosecretion or (b) an inhibition of, or damage to, critical cellular components, which could lead to leakage of ACh (e.g. DuP 996 could inhibit quantal packaging, in which case the increased output might be due to overflow of non-

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quantal ACh). Identification of the underlying mechanism is thus important to assess the ultimate effects of an agent earmarked for long-term patient use.

We have examined the effect of DuP 996 using a new method for obtaining the quantal release parameters, m (number of quanta released), n (number of functional release sites), p (probability of release), and $var_s p$ (spatial variance in p) (Provan & Miyamoto, 1993a). This approach circumvents the problem of nonuniformity and nonstationarity in the quantal estimates (Brown *et al.*, 1976), as well as the problem of inaccuracies in the nerve-evoked synaptic potential (Zucker, 1977). The aims of this study were (a) to examine whether DuP 996 produces a significant increase in quantal ACh release, as monitored by the release parameters, and (b) to elucidate the cellular or subcellular mechanism by which DuP 996 enhances neurosecretion. A preliminary account of these results was presented at the 1992 Meeting of the American Society for Pharmacology and Experimental Therapeutics in Orlando, FL (Provan & Miyamoto, 1992).

Methods

Experimental procedures

Conventional intracellular microelectrodes (3 M KCl, 8–15 MΩ) were used to record m.e.p.ps from isolated cutaneous pectoris muscles of frogs (*Rana pipiens*), after decapitating and double pithing the animals. Preparations were mounted in a 1 ml Perspex chamber which was continuously perfused with Ringer solution by means of a dual chambered pump. Junctions were selected on the basis of focal recording (m.e.p.p. rise time <2 ms), large m.e.p.p. size (>0.4 mV), moderately high m.e.p.p. frequency (>10 Hz), good signal-to-noise ratio, few giant-m.e.p.ps and sub-m.e.p.ps, and high and stable muscle resting potential. Continuous single junction recordings were held throughout each series of solution changes, and only one trial was carried out on each preparation. The problem of maintaining impalements for these periods was minimized by use of an air-suspension table and a hydraulic microdrive which allowed fine repositioning of the electrode. Tracings of resting potential, bath temperature, and experimental notations with time were made on a dual channel chart recorder. Studies were conducted at the ambient temperature (range 22–25°C), which varied less than 2°C during any single experiment.

Instrumentation

Bioelectric signals were fed into a high impedance preamplifier and viewed on an oscilloscope. Signal-to-noise ratio was increased by a band-pass filter set at 0–1 kHz. Signals were boosted by the rear-output amplifier of the oscilloscope to allow interfacing with an R.C. Electronics analogue-to-digital data acquisition unit (Goleta, CA). The unit was coupled to a Zenith Z-386SX computer with 2 MB of RAM and 40 MB hard drive, and data were analysed directly on-line or stored on FM magnetic tape for off-line analysis.

Experimental solutions and drugs

$[K^+]$ in the Ringer solution was elevated to 10 mM (equimolar substitution of KCl for NaCl) to raise the basal m.e.p.p. frequency and reduce the possibility of a Poisson-distributed release of transmitter (Provan & Miyamoto, 1993a). The control solution thus contained (in mM): NaCl 102.5, KCl 10.0, $CaCl_2$ 1.8, Tris 2.0 (adjusted to pH 7.2), and glucose 5.6. Muscles were equilibrated for at least 30 min before use, to allow re-establishment of the Donnan equilibrium and ensure that results were obtained during the long-term, steady-state effect of K^+ (Cooke & Quastel, 1973). Tetrodotoxin (TTX)-Co^{2+} solution was made by adding 3 μM TTX to the control solution and replacing the Ca^{2+} with 1.8 mM Co^{2+} to maintain a constant [divalent cation] and avoid changes in membrane surface charge (Hille, 1984). DuP 996 was dissolved in ethanol, with the final [ethanol] in the Ringer solution being 40 μM. Although this was considered too low to affect transmitter release (Deitrich *et al.*, 1989), an equivalent amount of the vehicle was added to all solutions as a precaution. DuP 996 was obtained from The Du Pont Merck Pharmaceutical Co. (Wilmington, DE, U.S.A.), and TTX, $CoCl_2$ hexahydrate, 4-AP, and ω-conotoxin GVIA were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Data collection, analysis and statistics

Specific details of the overall procedure are described elsewhere (Provan & Miyamoto, 1993a). Briefly, the number of quanta released by one nerve impulse (m) was replaced by the number of m.e.p.ps occurring in a 50 ms time interval (bin), and 500 sequential bins were used for each estimate. Data were divided into subgroups of 100 before analysis to minimize nonstationarity, and results which were nonstationary according to statistical tests were discarded. Unbiased estimates of m, n, p, and $var_s p$ were then computed by use of equations previously reported (Miyamoto, 1986).

M.e.p.p. amplitudes were obtained from digitized data and adjusted for baseline fluctuations with a double vertical cursor tool. Results were corrected for shifts in membrane potential (Katz & Thesleff, 1957), after recalculating the reversal potential for ionic changes in the solution (Takeuchi, 1963). The number required for each estimate was determined by collecting 500 m.e.p.ps and designating the mean amplitude as the 'control'. These were then divided into subgroups of 50, and the means of the cumulative subgroups plotted as percentages of control. A sample size of two subgroups (100 m.e.p.ps) was found to be sufficient, since accuracy was not improved with the use of more subgroups.

Results were obtained from 5–6 different preparations for each experimental procedure. In several instances, data were converted to percentage of control and then averaged, to illustrate better the percentage increase or decrease. In these cases, the values equal to 100% are given in the Figure legends. One-way analysis of variance (ANOVA) was used to test for differences between groups of data, with $P<0.05$ chosen as the level of significance. Results are expressed as means ± s.e.mean.

Results

Effect of DuP 996 at K^+-depolarized nerve terminals

The first step was to find a working concentration of DuP 996. Junctions were selected as described, and m.e.p.ps recorded every 5 min. DuP 996 was added to the bath starting at 0.1 μM, and recordings made after 15 min. The concentration was then increased in half-logarithmic increments (0.3, 1, 3 μM, etc.), and the response recorded after another 15 min. The time course of effect (onset, peak, and reversal) was found to be basically the same for each concentration tested, and the concentration finally used was 10 μM. An illustration of the response is presented in Figure 1, in which sample records of m.e.p.ps are shown for control (Figure 1a), onset of effect (Figures 1b–d), and wash (Figure 1e).

The time course and magnitude of the response was examined in more detail by recording m.e.p.ps for 25 min in control solution, 60 min in 10 μM DuP 996, and 25 min during wash. Data from six continuous single cell recordings were combined and plotted as m.e.p.p. frequency vs. time (Figure 2a). The results showed that, following a stable control period, addition of DuP 996 ($t=0$, first dotted line) produced an immediate increase in m.e.p.p. frequency. The frequency reached a plateau after 15 min and remained at that level (179% of control) for the duration of exposure to

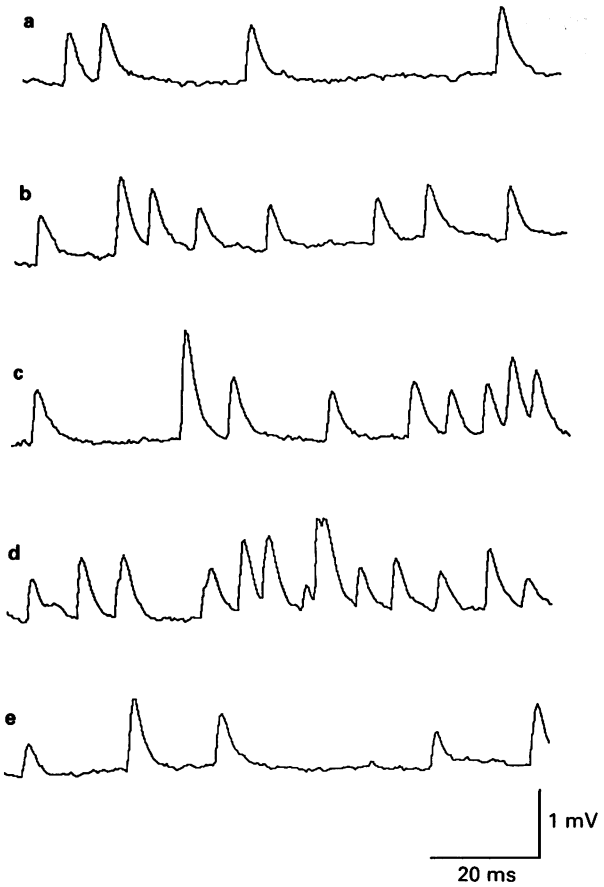


Figure 1 Effect of DuP 996 demonstrated by data from a representative experiment: m.e.p.ps were recorded continuously from a single junction, and sample traces (100 ms long for clarity) show m.e.p.ps: (a) in control 10 mM K^+-Ringer (45.2 quanta s^{-1}); (b) 5 min after introducing 10 μM DuP 996 (72.2 quanta s^{-1}); (c) 10 min with DuP 996 (73.8 quanta s^{-1}); (d) 15 min with DuP 996 (92.2 quanta s^{-1}); and (e) 15 min after washing out DuP 996 (40.6 quanta s^{-1}). Analogue signals were digitized with a 1 MHz R.C. Electronics data acquisition unit and printed with a Hewlett-Packard Laserjet III. The high signal-to-noise ratio is due to impalement of a small muscle fibre and low-pass filtering (1 kHz). Calibration bars: 1 mV (vertical) by 20 ms (horizontal).

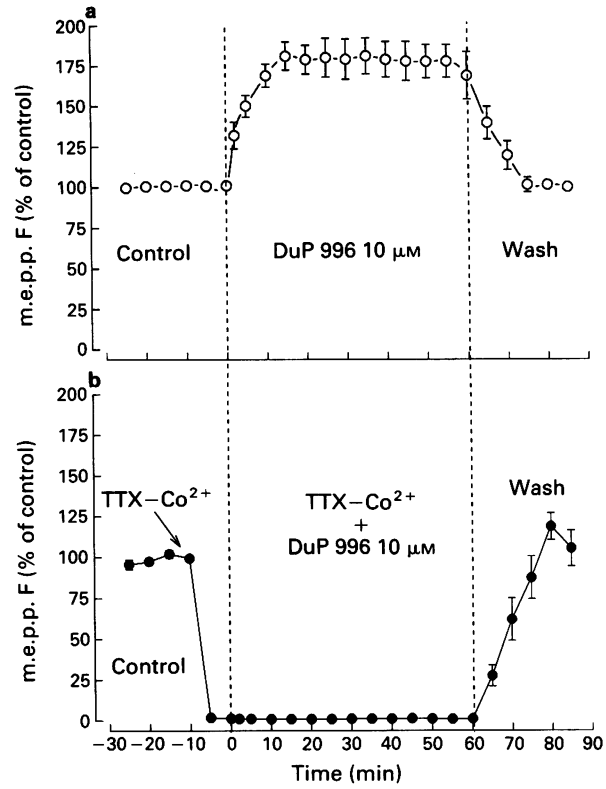


Figure 2 (a) Effect of DuP 996 on m.e.p.p. frequency (F): m.e.p.ps were continuously recorded from single junctions exposed to 10 mM K^+-Ringer (control), 10 μM DuP 996, and during wash. Data were normalised to percentage of the control values at time (t) zero. Addition of DuP 996 ($t = 0$) caused a rapid, sustained increase in m.e.p.p. F, and washing with 10 mM K^+-Ringer ($t = 60$ min) produced a prompt, complete reversal of effect (100% = 46.5 m.e.p.ps s^{-1}). (b) Effect of tetrodotoxin (TTX) and Co^{2+} on the m.e.p.p. F increase produced by DuP 996. Experiments were identical to those above except that TTX-Co^{2+} Ringer was added 10 min prior to DuP 996. In the continued presence of TTX and Co^{2+}, the m.e.p.p. F increase expected with DuP 996 was completely blocked. M.e.p.p. F returned quickly to control levels after washing with control Ringer (100% = 37.7 m.e.p.ps s^{-1}). Points in (a) and (b) represent means ± s.e.means of six experiments each.

DuP 996. Washing with control Ringer ($t = 60$ min, second dotted line) resulted in a decrease in m.e.p.p. frequency to control values within 15 min. The rapidity of recovery, as well as the similarity in time course of onset and washout, suggested that the effect of DuP 996 was readily reversible.

Effect of DuP 996 at K^+-depolarized nerve terminals in the presence of TTX and Co^{2+}

The increase in m.e.p.p. frequency with DuP 996 (Figure 2a) could have been due to (a) an action at the nerve membrane to enhance Na^+ and/or Ca^{2+} influx, or (b) an action at an intracellular site which by-passed the membrane. One way of distinguishing between these two possibilities was to block the Na^+- and Ca^{2+}-channels, then re-examine the response to DuP 996. Under these conditions, the absence of a m.e.p.p. frequency increase would imply that the effect of DuP 996 was mediated by Na^+ and/or Ca^{2+} influx, whereas a recurrence of the m.e.p.p. frequency increase would indicate that the site of action was further downstream.

The effect of Na^+- and Ca^{2+}-channel block (using 3 μM TTX and 1.8 mM Co^{2+}, respectively) on the response to DuP 996 was examined by recording m.e.p.ps for 15 min in control solution, 10 min in TTX-Co^{2+} solution, 60 min in TTX-Co^{2+} solution plus 10 μM DuP 996 and 25 min during wash. Data from six trials were combined to give Figure 2b.

As anticipated, there was a marked decrease in the control m.e.p.p. frequency on exposure to TTX-Co^{2+} solution ($t = -10$ min in Figure 2b). In the continued presence of TTX and Co^{2+}, addition of 10 μM DuP 996 ($t = 0$ min, first dotted line) failed to produce either an immediate or delayed increase in m.e.p.p. frequency (monitored for 60 min exposure to DuP 996). The inhibitory effect of TTX and Co^{2+} was completely reversed on return to control solution ($t = 60$ min, second dotted line), except for a slight overshoot and return to baseline at $t = 80$ min (this may have been a rebound effect due to the prolonged block or a delayed increase due to residual amounts of DuP 996).

Time course of effect of DuP 996 on unbiased quantal release parameters

The increase in m.e.p.p. frequency in K^+-depolarized preparations (Figure 2a) was, according to the method of Provan & Miyamoto (1993a), equivalent to an increase in m. This increase in m could in turn be due to an increase in n, p, or both n and p (since $m = np$). To dissociate the effect on m into its quantal components and examine the temporal relationships of the changes, data were recorded for 15 min in control solution, 60 min in 10 μM DuP 996 and 15 min of wash. Results from six experiments were combined and the effect on each parameter plotted as a function of time (Figure 3).

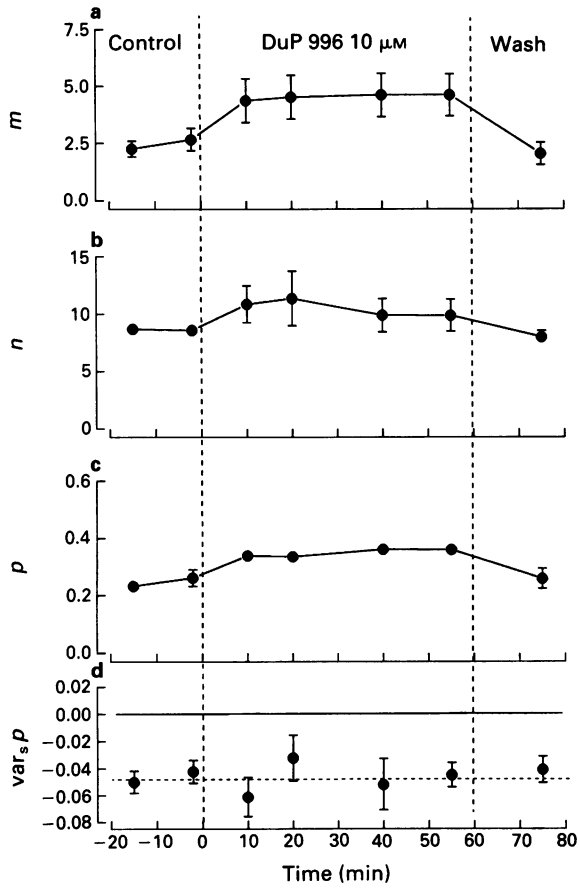


Figure 3 Effect of 10 μM DuP 996 on unbiased estimates of quantal release parameters as a function of time. Continuous single cell recording was maintained throughout for control ($t = -15$ min), DuP 996 ($t = 0$ min), and wash ($t = 60$ min). Plots show effect of DuP 996 on (a) number of quanta released, m; (b) number of functional release sites, n; (c) probability of release, p; and (d) spatial variance in p, var$_s p$. Note the increase in m, n and p but not var$_s p$. Values for m and n are in quanta, whereas p and var$_s p$ are dimensionless. Points represent means ± s.e.mean of six separate experiments.

As before (Figure 2a), the effect of DuP 996 on m occurred rapidly, with a peak increase after 10 min and a plateau thereafter (Figure 3a). There were also immediate increases in n and p (Figure 3b, c), which meant that the increase in m was due to increases in both parameters. By the end of 60 min exposure to DuP 996, there was a slight decrease in n (Figure 3b). This was offset by a small increase in p (Figure 3c), so the net effect was a sustained increase in m (Figure 3a) (the decrease in n may have been due to a reduced number of available quanta during the prolonged release of transmitter). On washing with control solution, the increases in n and p, like that for m, returned to control levels within 15 min (Figures 3a–c). By contrast, DuP 996 produced no significant changes in var$_s p$ (Figure 3d).

Concentration-dependent effects of DuP 996 on unbiased quantal release parameters

The effect of [DuP 996] on m, n, p and var$_s p$ was examined to provide information on (a) the threshold concentration which elicited a response, (b) the nature of the concentration-effect relationship, and (c) whether desensitization or inhibition occurred at the higher levels.

Junctions were sequentially exposed to half-logarithmic increments of [DuP 996] from 0.1 to 100 μM, and m.e.p.ps recorded after 15 min exposure to each concentration (as the response took 10–15 min to reach plateau). The combined results from six experiments showed concentration-dependent increases in m, n and p (Figures 4a–c), with the threshold effect occurring at 0.3 μM (Figure 4a). The effect on p appeared to peak at [DuP 996] > 10 μM (Figure 4c), such that the increase in m at this level was due primarily to an increase in n (Figure 4b). The 50% effective concentration (EC_{50}) for p (using 4-parameter logistic curve-fitting) was 1.1 μM (equivalent EC_{50} could not be determined accurately for m and n since these curves did not saturate). Lastly, increasing [DuP 996] produced no significant effects on var$_s p$ (Figure 4d), except for a modest increase at the highest concentration examined (100 μM).

Effect of ω-conotoxin GVIA (ω-CgTX) on the increase in quantal release produced by DuP 996

The effect of ω-CgTX, an irreversible N-type Ca^{2+}-channel blocker (Sher & Clementi, 1991), was tested to elucidate further the mechanism of action of DuP 996. Recordings were carried out for 20 min in control solution, 30 min in DuP 996 (10 μM), 7 min in DuP 996 plus ω-CgTX (1 μM), and 23 min of wash (Figure 5). As before, DuP 996 produced a rapid increase in m.e.p.p. frequency (Figure 5, first dotted line) which was maintained at 165% of control. Subsequent

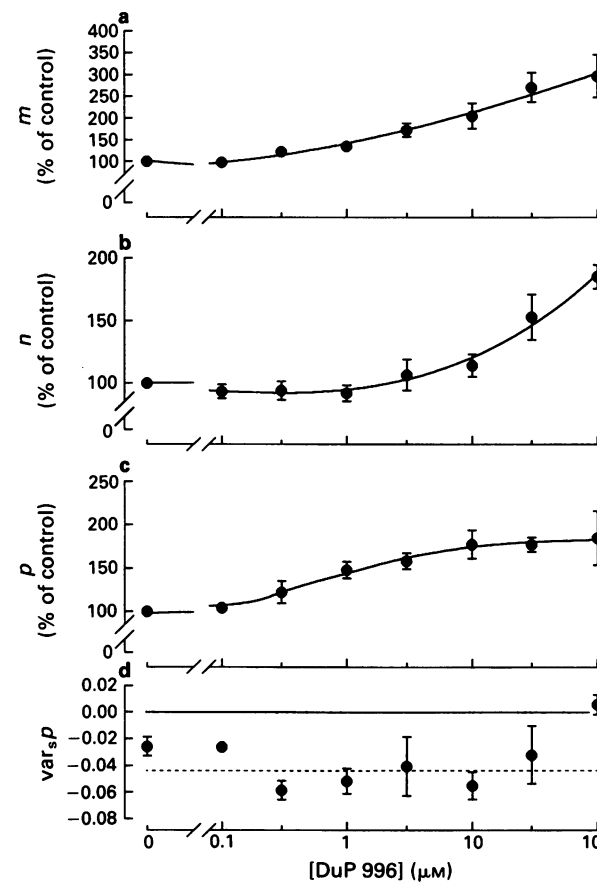


Figure 4 Concentration-effect curves of [DuP 996] vs. unbiased estimates of quantal release parameters, (a) number of quanta released, m, (b) number of functional release sites, n, (c) probability of release, p, and (d) spatial variance in p, var$_s p$. Data were continuously recorded from six junctions exposed first to control 10 mM K^+-Ringer, then to increasing [DuP 996]. Results (except those for var$_s p$) are normalised to percentages of the values obtained in the absence of DuP 996 (points represent means ± s.e.mean). Results show a marked concentration-dependent increase for each of the parameters except var$_s p$, with no indication of inhibition (reversal in effect) at the highest concentrations. Note the logarithmic scale for the abscissae. 100% = 1.09 quanta for m, 6.70 quanta for n, and 0.165 for p.

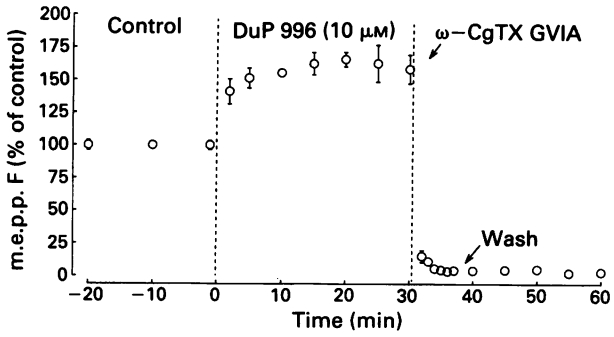


Figure 5 Effect of ω-conotoxin-GVIA (ω-CgTX) on the m.e.p.p. frequency (F) increase produced by DuP 996: m.e.p.ps were continuously recorded from junctions exposed to 10 mM K^+-Ringer control solution, 10 μM DuP 996, DuP 996 plus 1 μM ω-CgTX, and during wash. Experiments were identical to those in Figure 2a, except for addition of ω-CgTX during the exposure to DuP 996. An aliquot of stock ω-CgTX solution was added to the chamber (to give a final concentration of 1 μM) and perfusion stopped for 7 min (at $t = 31$ min). Data were normalised to percentages of the values at time zero. The inhibition produced by the toxin was not reversed by washing with either control solution (three trials) or control solution with DuP 996 (three trials), so these data were simply combined. Points represent means ± s.e.mean for six experiments (100% = 40.2 m.e.p.ps s^{-1}).

addition of 1 μM ω-CgTX (second dotted line) produced a rapid and dramatic decline in m.e.p.p. frequency, which continued to near zero and which was not reversed with >20 min of wash. These results indicated that the effect of DuP 996 was mediated primarily by voltage-gated N-type Ca^{2+}-channels in the presynaptic membrane.

Effect of DuP 996 in the presence of 4-aminopyridine (4-AP)

Influx of Ca^{2+} through voltage-gated Ca^{2+}-channels could be the result of either an increase in Ca^{2+}-conductance or a decrease in K^+-conductance (which would indirectly increase Ca^{2+}-conductance by depolarizing the membrane (Harvey, 1993)). One way of differentiating between these two mechanisms was to examine the effect of DuP 996 after voltage-gated K^+-channels were completely blocked, e.g., with supramaximal concentrations of 4-AP (Lundh, 1978). An increase in transmitter release under these circumstances would then indicate that the effect of DuP 996 could not have been due to block of voltage-gated K^+-channels.

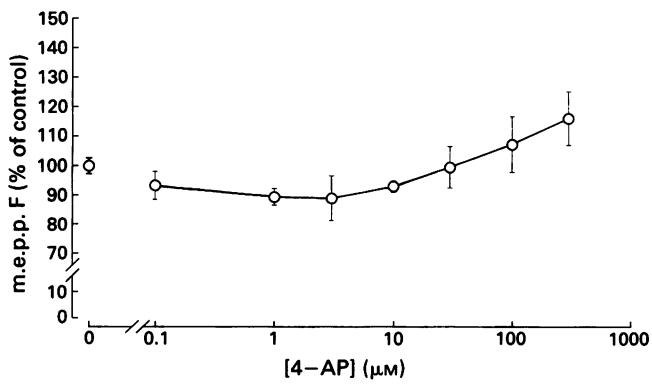


Figure 6 Concentration-effect curve of 4-aminopyridine (4-AP) vs. m.e.p.p. frequency (F). Data were continuously recorded from junctions exposed to 10 mM K^+-Ringer solution (control), then to increasing [4-AP]. Results are normalized to percentages of values obtained in the absence of 4-AP. Note the logarithmic scale for the abscissae. Points represent means ± s.e.mean for five trials (100% = 30.5 m.e.p.ps s^{-1}).

Preliminary experiments were carried out to re-examine the concentration-dependent effects of 4-AP on m.e.p.p. frequency. Junctions were exposed to increasing [4-AP] in half-logarithmic increments up to 300 μM, and m.e.p.ps recorded after 20 min perfusion at each concentration. The results (Figure 6) revealed a biphasic effect, with m.e.p.p. frequency decreasing slightly for [4-AP] <30 μM (cf. Molgó *et al.*, 1977) and increasing slightly for concentrations >30 μM. A concentration of 100 μM 4-AP was chosen since it produced a measurable effect on m.e.p.p. frequency (Bowman *et al.*, 1977 and Figure 6) and on synaptic delay (Molgó *et al.*, 1977).

To examine the effect of voltage-gated K^+-channel block on the response to DuP 996, recordings were carried out for 20 min in control solution, 50 min in 100 μM 4-AP, 30 min in 100 μM 4-AP plus 10 μM DuP 996, and 25 min during wash (Figure 7). As before (Figure 6), 4-AP produced only a modest increase in m (to 128% of control). However, in the continued presence of 4-AP, addition of DuP 996 produced a marked increase in m to 289% of control. This was 117% greater than the average increase of 72% produced by DuP 996 alone (Figures 2a, 5). The increase in m was due to an increase in n (Figure 7b), as both p and $var_s p$ were unchanged (Figures 7c, d). The effect was reversed on washing, with a return to control levels in 15–20 min. The marked response in the presence of 4-AP indicated that

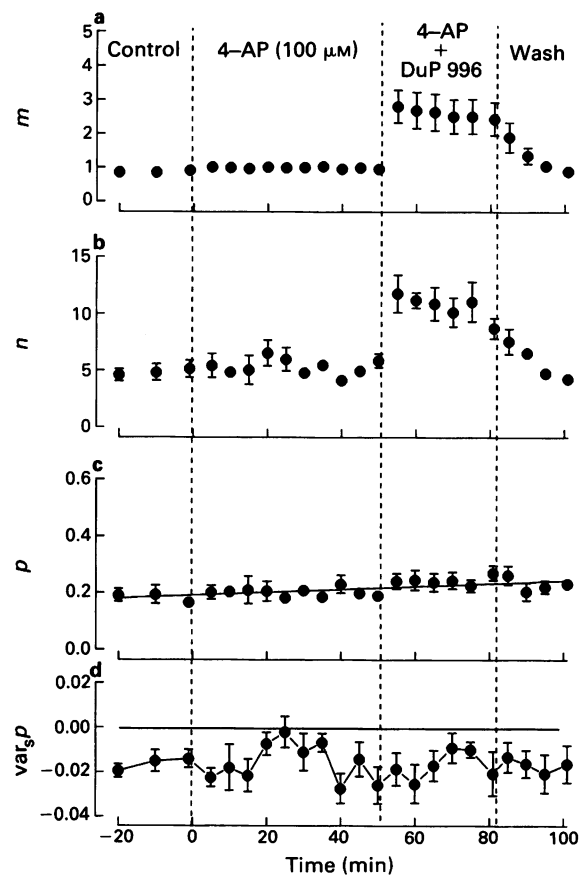


Figure 7 Effect of 4-aminopyridine (4-AP) on the response to DuP 996 with regard to (a) number of quanta released, m, (b) number of functional release sites, n, (c) probability of release, p, and (d) spatial variance in p, $var_s p$. M.e.p.ps were continuously recorded from single junctions exposed to 10 mM K^+-Ringer, 100 μM 4-AP, 4-AP plus 10 μM DuP 996, and wash. Addition of 4-AP ($t = 0$) produced only a modest increase (28%) in m. The response to DuP 996 ($t = 50$ min) was not attenuated but enhanced (by 189%) by the presence of 4-AP, and washing with control Ringer ($t = 81$ min) produced a complete reversal of effect. Note that the increase in m was due solely to an increase in n, as p and $var_s p$ were unaffected. Points represent means ± s.e.mean of six experiments.

DuP 996 was not working by decreasing voltage-gated K^+-conductance.

Effect of DuP 996 on postsynaptic sensitivity

The effect of DuP 996 on m.e.p.p. amplitude was examined to test for alterations in postsynaptic sensitivity; e.g., an increase in m.e.p.p. size might indicate a drug action to (a) inhibit cholinesterase (ChE), (b) increase receptor sensitivity to ACh, or (c) potentiate ion fluxes resulting from postsynaptic receptor activation.

Digitized data from the concentration-effect experiments (Figure 4) were retrieved from storage. Amplitudes of 100 m.e.p.ps were measured and corrected for differences in muscle resting potential, for the control period and for each sequential increase in [DuP 996] from 0.1 to 100 μM (recalling that results were recorded only during the plateau phase occurring 15 min after changing solutions). The results showed that DuP 996 produced no significant changes in m.e.p.p. amplitude at any of the concentrations tested ($P > 0.05$, ANOVA).

Discussion

Unbiased estimates of quantal release parameters

The present study uses a new procedure which avoids the problem of nonuniformity and nonstationarity (Brown *et al.*, 1976) to obtain unbiased estimates of n and p (Provan & Miyamoto, 1993a). Important features of the method are the use of direct quantal counts (m.e.p.ps) and elevated K^+ to evoke transmitter release. Direct counting of quanta eliminates the need to measure e.p.p. amplitudes, which may give inaccurate estimates of m (Zucker, 1977) and which may reflect, at least in theory, both quantal and non-quantal release (Zemková *et al.*, 1990). Use of elevated K^+ mimics the conditions used in measuring total ACh release, thereby allowing a more direct comparison between results obtained for quantal and total ACh release.

Comparison of results with neurochemical studies

The present findings are in basic agreement with those of neurochemical studies which monitored K^+-stimulated ACh release in brain slices (Nickolson *et al.*, 1990) and synaptosomes (Vickroy, 1993). For example, DuP 996 was found to produce a sizeable increase in quantal ACh release (Figures 1, 2a, 3–5), which was consistent with the increase in total (quantal plus non-quantal) ACh release found by Nickolson *et al.* (1990). Second, the threshold concentration for the effect on m was 0.3 μM (Figure 4a), which compared favourably with the 0.1 to 1 μM range found for brain slices (Table 1 of Nickolson *et al.*, 1990) and synaptosomes (Figures 2–4 of Vickroy, 1993). Third, the increase in m (to 300% of control with 100 μM, Figure 4a) was comparable to the average increase in ACh release for cortex, hippocampus, and caudate nucleus (to 335% of control with 100 μM; Table 1 of Nickolson *et al.*, 1990) and for hippocampal synaptosomes (to 260% of control with 30 μM; Figures 3, 4 of Vickroy, 1993). Finally, the effect of DuP 996 on m, n and p was readily reversible (Figures 2a, 3a, 7), which was consistent with the notion of a saturable, reversible binding to a specific receptor site (Tam *et al.*, 1991). While a direct comparison obviously cannot be made, the similarity of results between quantal and total ACh release does suggest that quantal release may comprise a substantial fraction of the total ACh release evoked by DuP 996.

DuP 996 acts at the presynaptic membrane

Several lines of evidence suggest that DuP 996 promotes ACh release by an action at the nerve terminal membrane. First, the effect of the drug is readily reversible (Figures 2a, 3a, 7), and the time course of onset is similar to that for washout. Both these findings are compatible with an extracellular rather than an intracellular site of action. Second, the effect of DuP 996 is reversibly inhibited by TTX and Co^{2+} (Figure 2b), which suggests that the action must occur at, or prior to, influx of Ca^{2+} at the membrane. Third, the effect of DuP 996 is rapidly and irreversibly blocked by ω-CgTX (Figure 5). This indicates the site of action may be at, or near, the voltage-gated Ca^{2+}-channel. Fourth, DuP 996 does not produce an increase in $var_s p$ (Figures 3d, 4d, 7d). According to recent studies (Provan & Miyamoto, 1993a), this indicates that DuP 996 does not work by releasing Ca^{2+} from intracellular organelles (see below). Taken together, the results are consistent with a drug action at an extracellular site on the presynaptic membrane.

Alterations in spatial variance in p

The use of $var_s p$ to distinguish between a drug action at intracellular organelles vs. one at the membrane is based on the premise that release of Ca^{2+} from intracellular organelles produces a non-uniform increase in $[Ca^{2+}]_i$ at transmitter release sites, which results in an increase in $var_s p$. By contrast, influx of Ca^{2+} through Ca^{2+}-channels adjacent to transmitter release sites is presumed to produce a uniform increase in $[Ca^{2+}]_i$, which would produce no increase in $var_s p$ (Figure 8 of Provan & Miyamoto, 1993a). Accordingly, the absence of effect on $var_s p$ (Figures 3d, 4d, 7d) would be consistent with the idea that DuP 996 does not promote Ca^{2+}-release from internal organelles. An increase in $var_s p$ did occur at very high levels (100 μM), which suggests that DuP 996 may eventually enter the terminal and release Ca^{2+} from intracellular organelles.

Effects of DuP 996 on quantal release parameters resemble effects associated with Ca^{2+} influx at the terminal

The concentration-dependent effects of DuP 996 on quantal transmitter release include an increase in m, n and p (Figures 4a, b, c) a saturation in p at the higher concentrations, and no change in $var_s p$ (Figure 4d). This pattern of response is the same as that found with elevating $[K^+]_o$ or $[Ca^{2+}]_o$ (Provan & Miyamoto, 1993a) (the link being that both manoeuvres enhance influx of Ca^{2+} at the terminal). The similarity in the response to DuP 996 further suggests that DuP 996 acts, like elevated K^+ or Ca^{2+}, to promote Ca^{2+} influx at the presynaptic membrane.

Possible mechanism of action of DuP 996

DuP 996 acts in a selective manner, since it enhances the release of ACh, dopamine and 5-HT but not noradrenaline (NA) or γ-aminobutyric acid (GABA). However, it is not associated with activation of nicotinic or muscarinic receptors (Nickolson *et al.*, 1990), and it does not involve histamine, benzodiazepines, opioids, or a variety of peptides (Tam *et al.*, 1991).

DuP 996 does not act by inhibiting voltage-gated K^+-conductance, because (a) block with 4-AP produces only a small increase in m.e.p.p. frequency (Figures 6, 7a), and (b) the effect of DuP 996 is not prevented by prior block with 4-AP (Figure 7a). These findings coincide with the report that DuP 996 does not inhibit voltage-gated K^+-channels (Nickolson *et al.*, 1990). A block of calcium-activated K^+-channels by DuP 996 is unlikely, because block of these channels with charybdotoxin produces no increase in m.e.p.p. frequency (Robitaille & Charlton, 1992). Finally, block of

K^+-channels would have been manifested by a prolongation of the nerve action potential, which is not observed with DuP 996 (Tsai *et al.*, 1992).

This means that DuP 996 works by depolarizing the presynaptic membrane or by increasing Ca^{2+}-channel conductance. While we cannot at present differentiate between these two possibilities, we tend to favour the latter explanation. The results with ω-CgTX (Figure 5) suggest that DuP 996 promotes the influx of Ca^{2+} through N-type Ca^{2+}-channels. If this is so, it may be that the action is not specific for a particular type of Ca^{2+}-channel, since ACh release at mammalian junctions, which is not mediated by N-type-Ca^{2+}-channels (Wray & Porter, 1993), is also enhanced by DuP 996 (Tsai *et al.*, 1992).

The increase in m which results from 4-AP plus DuP 996 (Figure 7a) is of interest for two reasons: first, the increase is due solely to an increase in n (Figure 7), which is what is found with tetraethylammonium (Volle & Branisteanu, 1976) and guanidine derivatives (Volle & Branisteanu, 1976; Farley *et al.*, 1979) using nerve-evoked e.p.ps and biased estimates of n and p. Second, assuming that DuP 996 increases Ca^{2+}-channel conductance, the finding of a supra-additive response with 4-AP would be compatible with the idea of overlapping effects of Ca^{2+} and K^+ channels at the active zone (cf. Robitaille *et al.*, 1993).

DuP 996 does not alter postsynaptic sensitivity

DuP 996 produced no significant change in m.e.p.p. amplitude, and no visible change in m.e.p.p. rise time or decay (e.g., Figure 1). Accordingly, DuP 996 must have produced little or no ChE inhibition, which is in agreement with the neurochemical findings (Nickolson *et al.*, 1990). This lack of effect differs from the increases in m.e.p.p. amplitude found with 3 μM THA (22%), physostigmine (46.5%) and neostigmine (79.2%) (Provan & Miyamoto, 1991), all of which are known antiChEs. This again implies that the facilitatory action of DuP 996 on cholinergic transmission must be due to pre- and not postsynaptic effects.

Potential advantages of DuP 996 as a palliative treatment for AD

One way of enhancing cholinergic transmission in AD is to inhibit ChE. However, chronic administration of antiChEs may produce receptor desensitization, so an alternative is to increase ACh release at the remaining nerve terminals, using agents such as DuP 996. The finding of an increase in *quantal* ACh release with DuP 996 affirms that the increase in ACh output is not due to interference with quantal packaging and overflow of non-quantal ACh, as may occur with physostigmine (Alderdice, 1979; Provan & Miyamoto, 1991).

An agent used in the treatment of AD should work by accelerating existing processes and not by inhibiting cellular components. Agents known to inhibit mitochondrial function, e.g., La^{3+}, Hg^{2+}, methyl Hg^+, and flufenamic acid, have been found to increase p and $var_s p$ (Provan & Miyamoto, 1993a,b). Accordingly, the finding that DuP 996 does not alter $var_s p$ is important, since it implies that facilitation of ACh release is probably not due to mitochondrial inhibition. The absence of such adverse effects would be advantageous for DuP 996 or any other agent relegated to long-term clinical use.

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References

ALDERDICE, M.T. (1979). Physostigmine, but not neostigmine, inhibits acetylcholine release. *Brain Res.*, **178**, 596–599.

BOWMAN, W.C., HARVEY, A.L. & MARSHALL, I.G. (1976). Facilitatory actions of aminopyridines on neuromuscular transmission. *J. Pharm. Pharmacol.*, **28**, 79P.

BROWN, T.H., PERKEL, D.H. & FELDMAN, M.W. (1976). Evoked neurotransmitter release: statistical effects of nonuniformity and nonstationarity. *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 2913–2917.

CHRISTIE, J.E. (1982). Physostigmine and arecoline infusions in Alzheimer's disease. In *Alzheimer's Disease: A Report of Progress in Research*, Vol. 19, ed. Corkin, S., Davis, K.L., Growdon, J.H., Usdin, E. & Wurtman, R.J. pp. 413–419. New York: Raven Press.

COOK, L., NICKOLSON, V.J., STEINFELS, G.F., ROHRBACH, K.W. & DENOBLE, V.J. (1990). Cognition enhancement by the acetylcholine releaser DuP 996. *Drug Dev. Res.*, **19**, 301–314.

COOKE, J.D. & QUASTEL, D.M.J. (1973). The specific effect of potassium on transmitter release by motor nerve terminals and its inhibition by calcium. *J. Physiol.*, **228**, 435–458.

DE SOUZA, E.B., RULE, B.L. & TAM, S.W. (1992). [^{3}H] linopirdine (DuP 996) labels a novel binding site in rat brain involved in the enhancement of stimulus-induced neurotransmitter release: autoradiographic localization studies. *Brain Res.*, **582**, 335–341.

DEITRICH, R.A., DUNWIDDIE, T.V., HARRIS, R.A. & ERWIN, V.G. (1989). Mechanism of action of ethanol: initial central nervous system actions. *Pharmacol. Rev.*, **41**, 489–537.

FARLEY, J.M., GLAVINOVIĆ, M.I., WATANABE, S. & NARAHASHI, T. (1979). Stimulation of transmitter release by guanidine derivatives. *Neurosci.*, **4**, 1511–1519.

HARRELL, L.E., CALLAWAY, R., MORERE, D. & FALGOUT, J. (1990). The effect of long-term physostigmine administration in Alzheimer's disease. *Neurology*, **40**, 1350–1354.

HARVEY, A.L. (1993). Neuropharmacology of potassium ion channels. *Med. Res. Rev.*, **13**, 81–104.

HILLE, B. (1984). *Ionic Channels in Excitable Membranes*. Sunderland, MA: Sinauer.

KATZ, B. & THESLEFF, S. (1957). On the factors which determine the amplitude of the 'miniature end-plate potential'. *J. Physiol.*, **137**, 267–278.

KATZMAN, R. (1986). Alzheimer's disease. *N. Engl. J. Med.*, **314**, 964–973.

KUMAR, V. & CALACHE, M. (1991). Treatment of Alzheimer's disease with cholinergic drugs. *Int. J. Clin. Pharmacol. Ther. Toxicol.*, **29**, 23–37.

LUNDH, H. (1978). Effects of 4-aminopyridine on neuromuscular transmission. *Brain Res.*, **153**, 307–318.

MIYAMOTO, M.D. (1986). Probability of quantal transmitter release from nerve terminals: theoretical considerations in the determination of spatial variation. *J. Theor. Biol.*, **123**, 289–304.

MOLGÓ, J., LEMEIGNAN, M. & LECHAT, P. (1977). Effects of 4-aminopyridine at the frog neuromuscular junction. *J. Pharmacol. Exp. Ther.*, **203**, 653–663.

MOLLOY, D.W., GUYATT, G.H., WILSON, D.B., DUKE, R., REES, L. & SINGER, J. (1991). Effect of tetrahydroaminoacridine on cognition, function and behaviour in Alzheimer's disease. *Can. Med. Assoc. J.*, **144**, 29–34.

MURAMOTO, O., SUGISHITA, M. & ANDO, K. (1984). Cholinergic system and constructional praxis: a further study of physostigmine in Alzheimer's disease. *J. Neurol. Neurosurg. Psychiatry*, **47**, 485–491.

NICKOLSON, V.J., TAM, S.W., MYERS, M.J. & COOK, L. (1990). DuP 996 (3,3-bis(4-pyrindinylmethyl)-1-phenylindolin-2-one) enhances the stimulus-induced release of acetylcholine from rat brain in vitro and in vivo. *Drug Dev. Res.*, **19**, 285–300.

PENN, R.D., MARTIN, E.M., WILSON, R.S., FOX, J.H. & SAVOY, S.M. (1988). Intraventricular bethanechol infusion for Alzheimer's disease: results of double-blind and escalating-dose trials. *Neurology*, **38**, 219–222.

PROVAN, S.D. & MIYAMOTO, M.D. (1991). Tetrahydroaminoacridine and physostigmine have opposing effects on probability of transmitter release at the frog neuromuscular junction. *Neurosci. Lett.*, **123**, 127–130.

PROVAN, S.D. & MIYAMOTO, M.D. (1992). Effect of the putative cognitive enhancer, linopirdine (DuP 996), on the quantal release of acetylcholine. *Pharmacologist*, **34**, 188.

PROVAN, S.D. & MIYAMOTO, M.D. (1993a). Unbiased estimates of quantal release parameters and spatial variation in the probability of neurosecretion. *Am. J. Physiol.*, **264**, 1051–1060.

PROVAN, S.D. & MIYAMOTO, M.D. (1993b). An increase in spatial variance in the probability of neurosecretion is associated with drug-induced inhibition of mitochondria. *Soc. Neurosci. Abstr.*, **19**, 1748.

READ, S.L., FRAZEE, J., SHAPIRA, J., SMITH, C., CUMMINGS, J.L. & TOMIYASU, U. (1990). Intracerebroventricular bethanechol for Alzheimer's disease: variable dose-related responses. *Arch. Neurol.*, **47**, 1025–1030.

REINIKAINEN, K.J., SOININEN, H. & RIEKKINEN, P.J. (1990). Neurotransmitter changes in Alzheimer's disease: implications to diagnostics and therapy. *J. Neurosci. Res.*, **27**, 576–586.

ROBITAILLE, R. & CHARLTON, M.P. (1992). Presynaptic calcium signals and transmitter release are modulated by calcium-activated potassium channels. *J. Neurosci.*, **12**, 297–305.

ROBITAILLE, R., GARCIA, M.L., KACZOROWSKI, G.J. & CHARLTON, M.P. (1993). Functional colocalization of calcium and calcium-gated potassium channels in control of transmitter release. *Neuron*, **11**, 645–655.

SARTER, M. (1991). Taking stock of cognition enhancers. *Trends Pharmacol. Sci.*, **12**, 456–461.

SHER, E. & CLEMENTI, F. (1991). ω-Conotoxin-sensitive voltage-operated calcium channels in vertebrate cells. *Neurosci.*, **42**, 301–307.

SUMMERS, W.K., MAJOVSKI, L.V., MARSH, G.M., TACHIKI, K. & KLING, A. (1986). Oral tetrahydroaminoacridine in long-term treatment of senile dementia, Alzheimer type. *N. Engl. J. Med.*, **315**, 1241–1245.

TAKEUCHI, N. (1963). Some properties of conductance changes at the endplate membrane during the action of acetylcholine. *J. Physiol.*, **167**, 128–140.

TAM, S.W., ROMINGER, D. & NICKOLSON, V.J. (1991). Novel receptor site involved in enhancement of stimulus-induced acetylcholine, dopamine, and serotonin release. *Mol. Pharmacol.*, **40**, 16–21.

TSAI, M.-C., SU, J.-L., CHEN, M.-L., FAN, S.-Z. & CHENG, C.-Y. (1992). The effect of 3,3-dipyridylmethyl-1-phenyl-2-indolinone on the neuromuscular transmission in the rodent skeletal muscles. *Neuropharmacol.*, **31**, 89–94.

VICKROY, T.W. (1993). Presynaptic cholinergic actions by the putative cognitive enhancing agent DuP 996. *J. Pharmacol. Exp. Ther.*, **264**, 910–917.

VOLLE, R.L. & BRANISTEANU, D.D. (1976). Statistical parameters of transmitter release at frog neuromuscular junctions treated with guanidine or tetraethylammonium. *J. Pharmacol. Exp. Ther.*, **197**, 653–661.

WHITEHOUSE, P.J., PRICE, D.L., STRUBLE, R.G., CLARK, A.W., COYLE, J.T. & DELONG, M.R. (1982). Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. *Science*, **215**, 1237–1239.

WRAY, D. & PORTER, V. (1993). Calcium channel types at the neuromuscular junction. *Ann. N.Y. Acad. Sci.*, **681**, 356–367.

ZEMKOVÁ, H., VYSKOČIL, F. & EDWARDS, C. (1990). The effects of nerve terminal activity on non-quantal release of acetylcholine at the mouse neuromuscular junction. *J. Physiol.*, **423**, 631–640.

ZUCKER, R.S. (1977). Synaptic plasticity at crayfish neuromuscular junctions. In *Identified Neurons and Behavior of Arthropods*. ed. Hoyle, G. pp. 49–65. New York: Plenum.

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Effect of PAF on rat lung vascular permeability: role of platelets and polymorphonuclear leucocytes

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1 The objectives of the present experiments were to assess the contribution of polymorphonuclear leucocytes (PMNLs), platelets and their products such as thromboxane A_2 (TxA_2), histamine and 5-hydroxytryptamine to platelet activating factor (PAF)-mediated protein extravasation in rat lungs.

2 Intravenous injection of PAF (1.0 and 5.0 $\mu g\,kg^{-1}$) increased dose-dependently (up to 7.5 fold) the vascular permeability of the trachea, upper and lower bronchi to Evans blue dye (EB), a marker of albumin extravasation. The permeability of the pulmonary parenchyma was not affected significantly by PAF.

3 Thrombocytopenia induced by administration of the IgG fraction of goat anti-rat platelet serum (APS; 15 mg $100\,g^{-1}$, i.p., 16–18 h) reduced by 55, 58 and 40% the effects of the lower dose of PAF (1.0 $\mu g\,kg^{-1}$) and by 31, 23 and 15% the effects of the higher dose of PAF (5.0 $\mu g\,kg^{-1}$) on the permeability of the trachea, upper and lower bronchi respectively to albumin.

4 PMNL depletion induced by administration of rabbit anti-rat polymorphonuclear serum (ANS; 2 mg kg^{-1}, i.v., 24 h) did not reduce significantly the effects of the lower dose of PAF (1.0 $\mu g\,kg^{-1}$) on the airways, however the effects of the higher dose of PAF (5.0 $\mu g\,kg^{-1}$) on the permeability of the trachea, upper and lower bronchi to albumin were reduced by 43, 25 and 23% respectively.

5 The injection of both the anti-platelet and the anti-PMNL sera reduced by 61, 62 and 96% the effects of the lower dose of PAF (1.0 $\mu g\,kg^{-1}$) and by 44, 39 and 47% the effects of the higher dose of PAF (5.0 $\mu g\,kg^{-1}$) on the permeability of the trachea, upper and lower bronchi respectively.

6 The combined injection of the TxA_2-mimetic (U-44069; 5.0 $\mu g\,kg^{-1}$) and PAF (1.0 and 5.0 $\mu g\,kg^{-1}$) in thrombocytopenic rats overcame the vascular permeability decrease induced by APS treatment.

7 Pretreatment of the animals with a combination of antagonists to histamine (mepyramine; 3.0 mg kg^{-1}) and 5-hydroxytryptamine (methysergide; 2.5 mg kg^{-1}) did not cause a significant inhibition of the effect of PAF (1.0 and 5.0 $\mu g\,kg^{-1}$) on EB extravasation in the airways.

8 These data show that the effect of intravenous PAF on rat vascular permeability is partly modulated by polymorphonuclear leucocyte and platelet activation. Our results suggest that following its release, TxA_2 could increase postcapillary hydrostatic pressure by inducing a venoconstriction and potentiate the extravasation elicited by PAF. These results do not suggest a major role for histamine and/or 5-hydroxytryptamine on PAF-induced albumin extravasation.

Keywords: Protein extravasation; airways; thromboxane A_2; Evans blue dye; thrombocytopenia; neutropenia; rat lung; platelets

Introduction

Majno & Palade (1961) provided the first evidence that endothelial cells play a major role in the control of vascular permeability and correlated the effects of inflammatory mediators on vascular permeability with the formation of junctional gaps between the endothelial cells of the postcapillary venules. Many attempts have been made to determine the role of various inflammatory mediators and haemodynamic parameters on oedema formation. Modulation of vascular tone by arterial vasodilators and/or increase in hydrostatic postcapillary pressure by non-inflammatory mediators can increase fluid leakage and produce mild oedema, but fail to induce protein extravasation. In contrast, inflammatory mediators can induce marked oedema by causing postcapillary protein and fluid extravasation; this phenomenon could be potentiated by venoconstriction and/or arterial vasodilatation which both increase postcapillary hydrostatic pressure and vascular protein exudation (Grega *et al.*, 1972).

Platelet activating factor (PAF) is an inflammatory mediator which exhibits a wide spectrum of pharmacological activities including increase of airway resistance and decrease of lung compliance (Halonen *et al.*, 1980; 1981), release of mediators from various cell types such as platelets (Henson, 1970) and neutrophils (Goetzl *et al.*, 1980) and increased systemic vascular permeability (Vargaftig & Ferreira, 1981; Humphrey *et al.*, 1982). Results of intravital and light microscopy showed that the action of PAF on vascular permeability is mediated by an early alteration of vascular integrity of the postcapillary venules by formation of gaps between endothelial cells (Bjork & Smedegard, 1983; Humphrey *et al.*, 1984). These changes of endothelial cell integrity have also been demonstrated to occur in response to other inflammatory mediators such as histamine (Majno & Palade, 1961), bradykinin (Svensjo *et al.*, 1979) and peptidoleukotrienes (Dahlen *et al.*, 1981). Heffner *et al.* (1983) showed that the perfusion of PAF together with platelets in isolated perfused lung *in vitro* caused an acute pulmonary hypertension and oedema which was attributed to TxA_2 released from platelets. Hamasaki *et al.* (1984) observed the same phenomenon in lungs perfused with a platelet-free solution and suggested that vascular permeability was not related to TxA_2 synthesis. Bolin *et al.* (1987) obtained similar increases of arterial pulmonary pressure following PAF treatment and

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showed that platelets potentiated this phenomenon. However the authors did not detect the presence of oedema. Sakai *et al.* (1989) observed that PAF perfusion increased pulmonary arterial and microvascular pressure as well as oedema and protein extravasation in rat isolated lung. These findings have been confirmed recently by Seale *et al.* (1991). Intravenous injection of PAF induced systemic hypotension *in vivo* (Caillard *et al.*, 1982), pulmonary hypertension (Heffner *et al.*, 1983), and increased protein extravasation in airways perfused by the systemic circulation, whereas oedema and protein permeability were not affected by PAF in lung parenchyma perfused by the pulmonary circulation (O'Donnell & Barnett, 1987; Sirois *et al.*, 1988; 1990; Chen *et al.*, 1990). Inarrea *et al.* (1984) reported that rat platelets did not bear PAF receptors and Pirotzky *et al.* (1984) and Humphrey *et al.* (1984) showed that intradermal injection of PAF in rat induced a plasma exudation which was independent of platelet and neutrophil contribution. However, Martins *et al.* (1988) and Klee *et al.* (1991) showed that intravenous PAF injection induces platelet aggregation in rats which might suggest that PAF can activate platelets in this species.

The aims of the following experiments were to study the effects of intravenous PAF injection on systemic and pulmonary vascular permeability of selected rat tissues and to elucidate the possible involvement of platelets and polymorphonuclear leucocytes in these activities.

Methods

Unanaesthetized male Wistar rats (225–275 *g*) were used in these experiments. Protein extravasation, a marker of vascular permeability was evaluated by measuring the Evans blue dye (EB) contents of tissues, as described previously (Sirois *et al.*, 1988). In brief, EB (25 mg ml^{-1} in 0.9% NaCl; 20 mg kg^{-1}) was injected together with PAF (1.0 or 5.0 $\mu g\ kg^{-1}$) in the caudal vein of the animals. In another series of experiments, the animals received a pretreatment with the IgG fraction of goat anti-rat platelet serum (APS; 15 mg 100 g^{-1} body weight, i.p.) 16–18 h before the PAF treatment, to produce a platelet depletion as described by Johnson *et al.* (1988). In another series of experiments, the animals received pretreatment with a rabbit anti-rat polymorphonuclear serum (ANS; 2 mg kg^{-1}, i.v.) 24 h before the PAF treatment, to produce a polymorphonuclear cell (PMNLs) depletion as described by Kurtel *et al.* (1991). In another series of experiments, the animals received pretreatment with ANS (2 mg kg^{-1}, i.v., 24 h) plus APS (15 mg 100 g^{-1} body weight, i.p., 16–18 h) before the PAF treatment, to produce PMNL and platelet depletion. In other experiments, the thromboxane A_2-mimetic U-44069 (5.0 $\mu g\ kg^{-1}$) was given in combination with PAF. For the last series of experiments, a solution of mepyramine (3.0 mg kg^{-1}) and methysergide (2.5 mg kg^{-1}) was injected in the tail vein 5 min before PAF injection.

The rats were killed 5 min after EB injection, the thorax was cut open and the lungs were perfused with 15 ml of Krebs solution (10 ml min^{-1}) via a cannula inserted in the pulmonary artery to remove the excess of intravascular pulmonary dye. The trachea, upper and lower bronchi and the pulmonary parenchyma were dissected, weighed and a portion of each organ was put in formamide (4 ml g^{-1} wet weight tissue at 20°C for 24 h) while another part was dried at 60°C for 24 h. The upper bronchi corresponded to the parts of the airways that begin at the trachea up to its entry into the lung parenchyma. The lower airways corresponded to major airways surrounded by lung parenchyma which can be easily dissected without magnification. To evaluate PAF-induced lung oedema, the lung parenchyma of PAF-treated rats was weighed and compared to that of the control group.

Platelet and leucocyte counts

An aliquot of blood (0.2 ml) was withdrawn from the tail vein in a syringe containing 0.1 ml of potassium oxalate (0.43% w/v). Part of the cell suspension (0.1 ml) was mixed with 1.9 ml of ammonium oxalate (1% w/v) and left at room temperature for 15 min; platelets were counted in a Neubauer chamber with a phase contrast microscope. Another part (0.1 ml) of the collected blood was mixed with 0.4 ml of ammonium oxalate (1% w/v) and left for 15 min at room temperature; the leucocytes were counted in a Neubauer chamber. Differential cell counts were performed on Wright-Giemsa stained blood smears.

Evans blue assay

The concentration of EB dye extracted in formamide was determined by spectrophotometry at a wavelength of 620 nm using a Titertek Multiskan (Flow Lab.) and 96 well plates. The results were plotted on a standard curve of EB dye (0.5–25 $\mu g\ ml^{-1}$). The Evans blue content of each sample was expressed as EB $\mu g\ g^{-1}$ dry weight of tissue. The expression of our results as a function of dry weight of tissue avoided underevaluation of changes due to oedema formation.

Chemicals used

The following drugs were used: Evans blue (Sigma Chem., St Louis, U.S.A.), PAF (1-O-hexadecyl-2-O-acetyl-sn-glycero-3-phosphorylcholine, Bachem, Switzerland), U-44069 (15(S)-hydroxy-9α,11α-(epoxymethano)-prosta-5-*cis*,13-trans-dienoic acid) gift from Dr J. Pike, Upjohn, Kalamazoo, U.S.A., mepyramine maleate (Rhône Poulenc Rorer, Montreal, Canada), methysergide hydrogen maleate (Sandoz, Switzerland). Anti-rat platelet serum (35 mg ml^{-1}) was raised in a goat and the IgG fraction purified using caprylic acid as described previously (Johnson *et al.*, 1988). Rabbit anti-rat polymorphonuclear serum was purchased from Accurate Chemicals & Scientific Corp, Westbury, U.S.A.

Statistical analysis

Results are expressed as mean ± s.e.mean and significance has been determined by analysis of variance. P values <0.05 were considered as significant.

Table 1 Effects of PAF on rat lung oedema

Lung parenchyma	*Control* EB (20 mg kg^{-1}) ($n = 5$)	*PAF* (1.0 $\mu g\ kg^{-1}$) ($n = 5$)	*PAF* (5.0 $\mu g\ kg^{-1}$) ($n = 5$)
Wet weight (*g*)	1.105 ± 0.020	1.148 ± 0.054	1.158 ± 0.068
Dry weight (*g*)	0.219 ± 0.032	0.236 ± 0.039	0.238 ± 0.052
Wet-to-dry weight ratio	5.04	4.87	4.85

The rats (250–275 g) received either a bolus intravenous injection of PAF (1.0 and 5.0 $\mu g\ kg^{-1}$) together with Evans blue dye (EB, 20 mg kg^{-1}), or EB alone, and were killed 5 min after the injection. Values are mean ± s.e.mean of 5 animals.

Results

Effects of PAF on protein extravasation in rat tissues

In the first series of experiments, EB dye was injected together with PAF (1.0 and 5.0 $\mu g\ kg^{-1}$) in the caudal vein of conscious rats that were killed 5 min after the injection. A group of rats received EB only and were used as controls. The following organs were taken out: trachea, upper and lower bronchi and pulmonary parenchyma. The injection of a low dose of PAF (1.0 $\mu g\ kg^{-1}$) increased the content of EB in the trachea, upper and lower bronchi by 72%, 114% and 125% respectively (Figures 1 and 2). The higher concentration of PAF (5.0 $\mu g\ kg^{-1}$) was more potent and the EB extravasation increased by 229%, 774% and 551% in the trachea, upper and lower bronchi respectively (Figures 1 and 2). In the pulmonary parenchyma neither EB permeability nor fluid balance in PAF-treated rats were affected significantly (Figure 1 and Table 1).

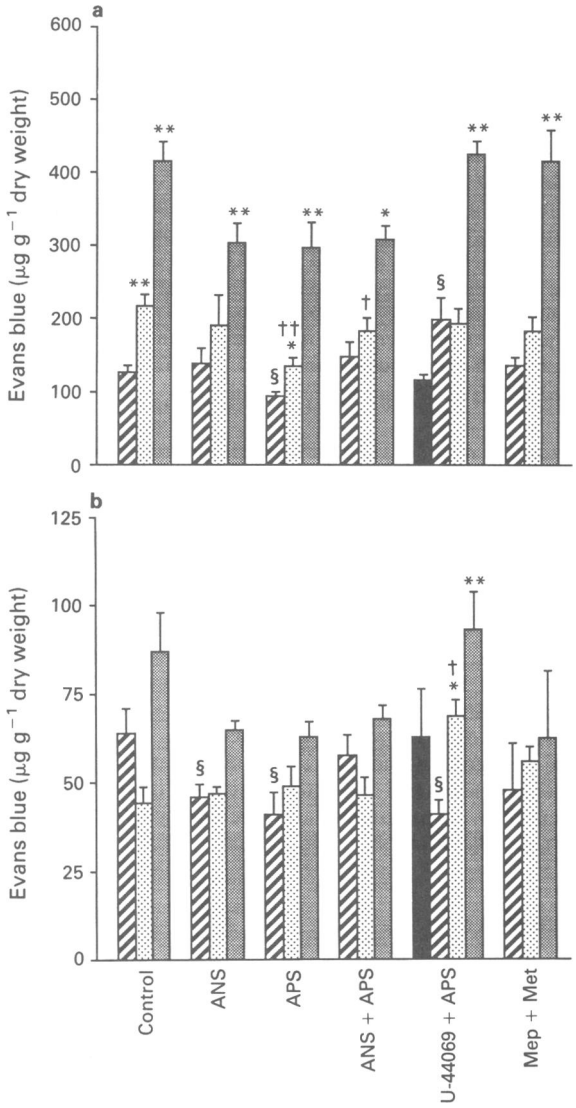


Figure 1 Effects of selected treatments on protein extravasation elicited by PAF treatment in rat trachea (a) and pulmonary parenchyma (b). Control animals received an intravenous injection of Evans blue dye only (20 $mg\ kg^{-1}$; hatched columns) or an injection of Evans blue dye plus PAF (1.0 $\mu g\ kg^{-1}$; stippled columns and 5.0 $\mu g\ kg^{-1}$; cross-hatched columns) and the rats were killed 5 min after the injection. In the other groups, the rats were pretreated with: a rabbit anti-rat polymorphonuclear leucocyte serum (ANS, 2 mg kg^{-1}, i.p. 24 h); an IgG fraction of goat anti-rat platelet serum (APS, 15 mg 100 g^{-1}, i.p. 16–18 h); ANS (2 $mg\ kg^{-1}$, i.p. 24 h) plus APS (15 mg 100 g^{-1}; i.p. 16–18 h); a mixture of mepyramine plus methysergide (Mep, 3 $mg\ kg^{-1}$, Met, 2.5 $mg\ kg^{-1}$, i.v. 5 min) before the injection of EB alone or combined with PAF. In another group, compound U-44069 (5.0 $\mu g\ kg^{-1}$) was injected with EB in naive rats (solid columns) or in APS pretreated rats with EB (hatched columns) and with PAF (1.0 $\mu g\ kg^{-1}$; stippled columns or 5.0 $\mu g\ kg^{-1}$; cross-hatched columns). Values are means ± s.e.mean of 5–8 experiments. $*P<0.05$ and $**P<0.01$ as compared with their respective Evans blue control, $\dagger P<0.05$ and $\dagger\dagger P<0.01$ as compared with control PAF (first group), $\S P<0.05$ as compared to control Evans blue (first group).

Polymorphonuclear leucocyte depletion induced with an anti-PMNL serum

The rabbit anti-rat polymorphonuclear leucocyte serum (2 $mg\ kg^{-1}$, i.v.) induced a severe neutropenia and eosinophilia 24 h after its administration in rats and the PMNL number decreased to values lower than 120 mm^{-3}. This effect represents a PMNL decrease of 87%. The PMNLs recovered after ANS-treatment represented less than 3% of the total leucocyte number. The number of mononuclear leucocytes increased by 23% (non-significant) whereas the ANS treatment did not affect the platelet number significantly (Table 2).

Effects of ANS on PAF-induced vascular permeability

In these experiments pretreatment with the ANS (2 $mg\ kg^{-1}$) was administered i.v., 24 h before the injection of EB alone or combined with PAF (1.0 and 5.0 $\mu g\ kg^{-1}$) and the animals were killed 5 min later as described above. Given in the absence of PAF, ANS decreased the control EB extravasation in the lower bronchi and lung parenchyma by 38 and 28% respectively (Figures 1 and 2). The ANS did not reduce significantly the protein extravasation elicited by the low dose of PAF (1.0 $\mu g\ kg^{-1}$) (Figures 1 and 2). At the higher dose of PAF (5.0 $\mu g\ kg^{-1}$), the ANS-treatment reduced by 43, 25 and 23% the protein extravasation in the trachea, upper and lower bronchi respectively (Figures 1 and 2).

Thrombocytopenia induction with anti-platelet serum

The IgG fraction of goat anti-rat platelet serum (15 mg 100 g^{-1}, i.p.) evoked a severe thrombocytopenia 12 to 24 h following its injection in rats and the platelet number decreased to values lower than 20,000 mm^{-3}. These experiments on EB vascular extravasation were carried out 16–18 h following the APS treatment. The APS induced a platelet depletion of 98% whereas it increased slightly but non-significantly by 17% and 19% the number of polymorphonuclear and mononuclear leucocytes respectively (Table 2).

Effects of APS on PAF-induced vascular permeability

In these experiments pretreatment with APS (15 mg 100 g^{-1}) was administered 16–18 h before the injection of PAF (1.0 and 5.0 $\mu g\ kg^{-1}$) and the animals were killed 5 min later as described previously. Given in the absence of PAF, APS decreased significantly the basal EB content in the trachea and lung parenchyma by 25 and 36% (Figure 1) as compared to the control values of EB dye. APS reduced by 55, 58 and 40% the protein extravasation induced by PAF (1.0 $\mu g\ kg^{-1}$) in the trachea, upper and lower bronchi respectively (Figures 1 and 2). At the high dose of PAF (5.0 $\mu g\ kg^{-1}$), the APS-treatment reduced significantly the protein extravasation in the trachea, upper and lower bronchi by 31, 23 and 15%, respectively (Figures 1 and 2).

Effects of combined treatment with ANS and APS on PAF-induced vascular permeability

In these experiments rats were pretreated with ANS (2 $mg\ kg^{-1}$, i.v., 24 h) and APS (15 mg 100 g^{-1} body weight, i.p., 16 h) before the injection of PAF. This combined treatment reduced the platelets counts under 25,000 mm^{-3} and the

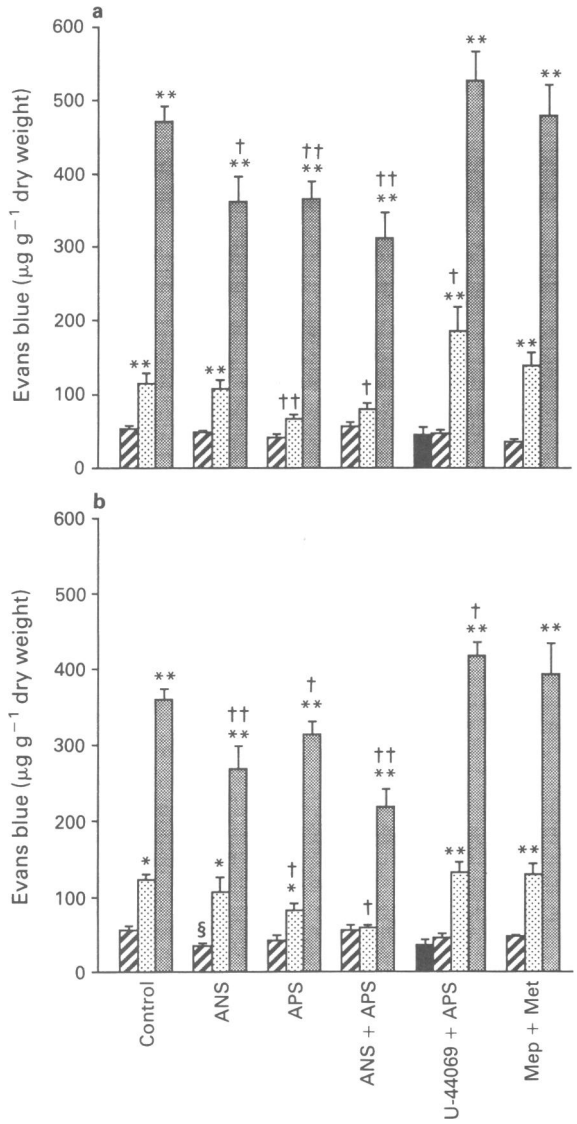


Figure 2 Effects of selected treatments on protein extravasation elicited by PAF treatment in rat upper bronchi (a) and lower bronchi (b). Other details as in Figure 1.

PMNL number to less than 150 mm^{-3} (Table 2). The neutropenia and thrombocytopenia reduced the protein extravasation mediated by PAF (1.0 $\mu g\,kg^{-1}$) in the trachea, the upper and lower bronchi by 61, 62 and 96% (Figures 1 and 2). At the higher dose of PAF (5.0 $\mu g\,kg^{-1}$), the ANS-APS-treatments significantly reduced the protein extravasation in the trachea, upper and lower bronchi by 44, 39 and 47% respectively (Figures 1 and 2). Given in the absence of PAF, ANS plus APS did not modify significantly the EB content of any tissues.

Modulation of the vascular permeability by compound U-44069

This series of experiments was performed to evaluate the role of TxA_2 in protein extravasation induced by PAF in the rat. PAF (1.0 and 5.0 $\mu g\,kg^{-1}$) was administered in combination with a TxA_2-mimetic (U-44069; 5.0 $\mu g\,kg^{-1}$) to thrombocytopenic animals that were killed 5 min later. The injection of U-44069 in naive rats did not modify the EB dye extravasation (Figures 1 and 2). In APS-treated rats, the injection of U-44069 increased the EB extravasation in the trachea by 56% and decreased it in the lung parenchyma by 35% (Figure 1). As described earlier, APS pretreatment reduced the EB extravasation mediated by the injection of PAF (1.0 and 5.0 $\mu g\,kg^{-1}$), but the combination of U-44069 with PAF (1.0 and 5.0 $\mu g\,kg^{-1}$) counteracted the decrease of EB extravasation induced by APS pretreatment in the trachea, upper and lower bronchi (Figures 1 and 2). Furthermore, in APS treated rats, the combination of U-44069 plus PAF (1.0 $\mu g\,kg^{-1}$) elicited a significant increase in protein extravasation by 55 and 125% in the lung parenchyma and the upper bronchi as compared to PAF (1.0 $\mu g\,kg^{-1}$) alone. The same phenomenon was observed in the upper and lower bronchi where the combination of U-44069 and PAF (5.0 $\mu g\,kg^{-1}$) produced an increase of the protein extravasation by 15% (non-significant) and 22% (significant) as compared to PAF (5.0 $\mu g\,kg^{-1}$) (Figure 2).

Histamine and 5-hydroxytryptamine contribution to PAF-induced protein extravasation

As the contribution of TxA_2 release by the platelets on PAF-induced protein extravasation was considered, it became imperative to evaluate the modulator role of histamine and 5-hydroxytryptamine which can both be released by platelets. In the last series of experiments, mepyramine (3.0 $mg\,kg^{-1}$) and methysergide (2.5 $mg\,kg^{-1}$) were given 5 min before the injection of PAF (1.0 and 5.0 $\mu g\,kg^{-1}$) in naive rats to study the modulatory role of histamine and 5-hydroxytryptamine on PAF-induced protein extravasation. Pretreatment with these antagonists did not affect significantly PAF (1.0 and 5.0 $\mu g\,kg^{-1}$)-induced EB extravasation (Figures 1 and 2). The injection of mepyramine and methysergide failed to alter control EB values.

Discussion

The present results show that the intravenous injection of PAF evoked a dose-dependent increase of protein extravasation in rat airways. The PAF effects were attenuated by platelet depletion and reappeared following treatment with U-44069. Polymorphonuclear leucocyte depletion also contributed to reduce the PAF effects on protein extravasation. Histamine and 5-hydroxytryptamine receptor antagonists did not modify the response of these target tissues to PAF.

These results confirmed earlier observations indicating that PAF increases protein extravasation in the airways (Evans *et*

Table 2 Effects of anti-platelet and anti-neutrophil sera on rat blood cell counts

Cell type	*Control*	*APS treatment*	*ANS treatment*	*ANS + APS treatment*
Platelets (cells × 10^3 mm^{-3})	857 ± 141	16.8 ± 3***	780 ± 117	24.3 ± 5***
Polymorphonuclear leucocytes (cells mm^{-3})	915 ± 51	1076 ± 147	119 ± 37**	147 ± 21**
Mononuclear leucocytes (cells mm^{-3})	5042 ± 502	6000 ± 478	6201 ± 484	5954 ± 391

IgG fraction of goat anti-rat platelet serum (APS, 15 mg 100 g^{-1}, i.p. 16–18 h); rabbit anti-rat polymorphonuclear leucocytes serum (ANS, 2 $mg\,kg^{-1}$, i.v. 24 h); ANS (2 $mg\,kg^{-1}$, i.v. 24 h) plus APS (15 mg 100 g^{-1}, i.p. 16–18 h). Data shown are means ± s.e.mean of 5–8 experiments. **$P<0.01$ and ***$P<0.001$ as compared to the control groups (analysis of variance).

al., 1987; O'Donnell & Barnett, 1987; Sirois *et al.*, 1990). The permeability of pulmonary parenchyma to albumin and fluid was not affected by PAF treatment. This is also in agreement with the results of O'Donnell & Barnett (1987) and Chen *et al.* (1990) who showed that PAF increased vascular permeability in guinea-pig airways (from the trachea to the small bronchi), but did not affect fluid and protein leakage in guinea-pig and rat pulmonary parenchyma. Pietra & Magno (1978) observed similar effects with histamine.

Induction of profound thrombocytopenia with the IgG fraction of goat anti-rat platelet serum (APS) decreased the protein extravasation induced by PAF (1.0 and 5.0 $\mu g\,kg^{-1}$). The APS treatment was more efficient against the lower dose of PAF (1.0 $\mu g\,kg^{-1}$) with a marked inhibition in the respiratory tract. At the higher dose of PAF (5.0 $\mu g\,kg^{-1}$), the effect of APS treatment was less marked. The APS *per se* induced a mild decrease of EB dye extravasation in the airways which could be related to the inhibition of endogenous release of inflammatory mediators such as PAF, histamine and/or 5-hydroxytryptamine from platelets. However, this modest decrease of protein extravasation is not sufficient to explain the high inhibitory efficacy (up to 90%) of APS on the vascular permeability of rat airways that followed PAF injection. These results suggest that the effects of PAF on rat systemic vasculature are mediated by PAF-induced platelet recruitment.

Our results also showed that compound U-44069, a TxA_2-mimetic, counteracted the inhibitory effect of platelet depletion on PAF-induced increase of vascular permeability. Considering the platelets as a major source of TxA_2, these results strongly support the contribution of TxA_2 as a critical mediator of PAF vascular actions. These results are in agreement with previous reports, which demonstrated the contribution of TxA_2 on PAF-induced protein extravasation in rat airways by using a cyclo-oxygenase inhibitor (indomethacin), a thromboxane synthase inhibitor (OKY-046) and a thromboxane antagonist (L-655,240) (Sirois *et al.*, 1990). They are also in agreement with the results of Heffner *et al.* (1983) who showed that PAF induced the release of TxA_2 which in turn raised microvascular hydrostatic pressure. In view of the fact that the contractile activity of the TxA_2-mimetic (U-46619) was shown to be more pronounced in the venous vasculature than in the arteriolar vessels (Warner, 1990), it is most likely that TxA_2, by its predominant venocontractile activity, increases hydrostatic postcapillary pressure and contributes to potentiate the protein extravasation through postcapillary endothelial cell gaps induced by PAF. This dual physiological effect might explain the reduction of protein extravasation induced by intravenous PAF injection in thrombocytopenic rats. Evans *et al.* (1987) also showed a slight inhibition of PAF-induced increase of vascular permeability by platelet depletion with an anti-platelet serum in guinea-pigs. Their results were statistically significant in the nasal mucosa and peripheral airways but the authors concluded that PAF effects were independent of platelet activation in their model. The discrepancy between their conclusions and ours which definitely showed the platelet involvement in PAF-induced EB extravasation might be explained by the different animal species studied.

The induction of a neutropenia did not decrease significantly the protein extravasation mediated by the low dose of PAF (1.0 $\mu g\,kg^{-1}$). However, ANS treatment decreased significantly the EB extravasation mediated by the high dose of PAF (5.0 $\mu g\,kg^{-1}$) in the trachea, upper and lower bronchi. This suggests that the postcapillary microvascular alterations induced by the low dose of PAF are not mediated by PMNLs as it is with the larger dose of PAF. Our results are in agreement with those of Bjork & Smedegard (1983) who showed in hamster cheek pouch that superfusion of PAF at a final concentration of 5 and 20 nM induced a massive macromolecular permeability, but only the high concentration of PAF (20 nM) induced a marked adhesion of leucocytes in postcapillary and larger venules. Their results suggest that PAF acts as a PMNL chemoattractant only at high concentrations in this model. Furthermore, they observed that the macromolecular permeability changes mediated by a 10 min superfusion with the high dose of PAF (20 nM) and not with the low dose of PAF (5 nM) was attenuated 5 min after the end of the superfusion in the neutropenic hamster.

Harlan *et al.* (1981) reported that PMNLs contribute to the genesis of endothelial injury in a variety of inflammatory conditions. The activation of PMNLs by inflammatory mediators such as PAF occurs very rapidly, and induces the expression of the glycoprotein complex CD11/CD18 on the surface of polymorphonuclear cells; the cells adhere to the endothelium within 2 min (Tonnesen, 1989). Furthermore, by their ability to secrete other inflammatory mediators such as PAF and leukotrienes as well as deleterious compounds such as oxygen free radicals, proteases and cationic molecules, it is likely that neutrophil activation contributes to protein extravasation induced by inflammatory mediators. Kubes *et al.* (1991) have shown that an intravenous PAF infusion induced the adherence of neutrophils to postcapillary venules in the cat. In addition, pretreatment with monoclonal antibodies directed against the common β-subunit of the adhesive glycoprotein complex CD11/CD18 inhibited the vascular protein leakage.

The induction of both a neutropenia and a thrombocytopenia decreased the protein extravasation induced by PAF (1.0 and 5.0 $\mu g\,kg^{-1}$). At the low dose of PAF, ANS-APS-treatment produced an inhibition equivalent to the effects of APS treatment alone in the upper and lower bronchi. As the ANS-pretreatment alone did not prevent the effect of the low dose of PAF, our results suggest that the effect of the low dose of PAF (1.0 $\mu g\,kg^{-1}$) was partly mediated by platelet activation but was independent of PMNLs. Since rat platelets do not bear PAF receptors, it might suggest that another cell type is necessary for the platelet activation. At the high dose of PAF (5.0 $\mu g\,kg^{-1}$), the induction of thrombocytopenia and neutropenia decreased the EB extravasation in the trachea, upper and lower bronchi. In the trachea, the inhibitory activity of a ANS-APS treatment was equivalent to the inhibition observed in APS or ANS treated animals, whereas in upper and lower bronchi the ANS-APS pretreatment produced a more potent inhibitory action than the APS or ANS treatment given separately. These results suggest that the platelet and PMNL activation contributes to the EB extravasation mediated by the large dose of PAF. It is possible that the PMNLs contributed to the platelet activation by stimulating TxA_2 release. However, as the PMNL depletion produced a smaller effect on PAF-induced increase of vascular permeability than the depletion of both neutrophils and platelets, the results suggest that another cell type could also be involved in platelet activation.

Pretreatment with mepyramine and methysergide did not prevent the protein extravasation induced by PAF (1.0 and 5.0 $\mu g\,kg^{-1}$) on airways. These results suggest that histamine and 5-hydroxytryptamine do not contribute to the protein extravasation elicited by PAF. Humphrey *et al.* (1982) and Pirotzky *et al.* (1984) reported similar findings following an intradermal PAF injection in guinea-pig and rat respectively. O'Donnell & Barnett (1987) also reported that the effect of intravenous PAF injection on airway vascular macromolecule leakage was not inhibited by antagonists of histamine or 5-hydroxytryptamine in guinea-pigs.

In conclusion, the intravenous injection of PAF increased systemic protein extravasation of bronchial airways without affecting the permeability of the lung parenchyma. Platelet depletion inhibited partly the PAF effects, an inhibition that was counteracted when PAF was injected with a TxA_2-mimetic. At the high dose of PAF, PMNL depletion partly inhibited the PAF effects. These results support a role for platelets, PMNL and TxA_2 release in the expression of intravenous PAF effects on vascular permeability.

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References

BJORK, J. & SMEDEGARD, G. (1983). Acute microvascular effects of PAF-acether, as studied by intravital microscopy. *Eur. J. Pharmacol.*, **96**, 87–94.

BOLIN, R.W., MARTIN, T.R. & ALBERT, R.K. (1987). Lung endothelial and epithelial permeability after platelet-activating factor. *J. Appl. Physiol.*, **63**, 1770–1775.

CAILLARD, K.G., MONDOT, S., ZUNDEL, J.L. & JULOU, L. (1982). Hypotensive activity of PAF-acether in rats. *Agents Actions*, **12**, 725–730.

CHEN, C.-R., VOELKEL, N.F. & CHANG, S.-W. (1990). PAF potentiates protamine-induced lung oedema: role of pulmonary venoconstriction. *J. Appl. Physiol.*, **68**, 1059–1068.

DAHLEN, S.E., BJORK, J., HEDQVIST, P., ARFORS, K.E., HAMMARSTROM, S., LINDGREN, J.A. & SAMUELSSON, B. (1981). Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: in vivo effects with relevance to the acute inflammatory response. *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 3887–3891.

EVANS, T.W., CHUNG, K.F., ROGERS, D.F. & BARNES, P.J. (1987). Effect of platelet-activating factor on airway vascular permeability: possible mechanisms. *J. Appl. Physiol.*, **63**, 479–484.

GOETZL, E.J., DERAIN, C.K., TAUBER, A.I. & VALONE, F.H. (1980). Novel effects of 1-0-hexadecyl-1-acyl-sn glycero 3-phosphorylcholine mediators on human leukocyte function. Delineation of the specific roles of the aryl substituents. *Biochem. Biophys. Res. Commun.*, **94**, 881–888.

GREGA, G.J., KLINE, R.L., DOBBINS, D.E. & HADDY, F.J. (1972). Mechanisms of oedema formation by histamine administered locally into canine forelimbs. *Am. J. Physiol.*, **223**, 1165–1171.

HALONEN, M., PALMER, J.D., LOHMAN, I.C., MCMANUS, L.M. & PINCKARD, R.N. (1980). Respiratory and circulatory alterations induced by acetyl glyceryl ether phosphorylcholine (AGEPC), a mediator of IgE anaphylaxis in the rabbit. *Am. Rev. Respir. Dis.*, **122**, 915–924.

HALONEN, M., PALMER, J.D., LOHMAN, I.C., MCMANUS, L.M. & PINCKARD, R.N. (1981). Differential effects of platelet depletion on the cardiovascular and pulmonary alterations of IgE anaphylaxis and AGEPC infusion in the rabbit. *Am. Rev. Respir. Dis.*, **124**, 416–421.

HAMASAKI, Y., MOJARAD, M., SAGA, T., TAI, H.-H. & SAID, S.I. (1984). Platelet-activating factor raises airway and vascular pressures and induces oedema in lungs perfused with platelet-free solution. *Am. Rev. Respir. Dis.*, **129**, 742–746.

HARLAN, J.M., KILLEN, P.D., HARKER, L.A., STRIKER, G.E. & WRIGHT, D.G. (1981). Neutrophil-mediated endothelial injury in vitro. *J. Clin. Invest.*, **68**, 1394–1403.

HEFFNER, J.E., SHOEMAKER, S.A., CANHAM, E.M., PATEL, M., MCMURTRY, I.F., MORRIS, H.G. & REPINE, J.E. (1983). Acetyl glyceryl ether phosphorylcholine-stimulated human platelets cause pulmonary hypertension and oedema in isolated rabbit lungs. *J. Clin. Invest.*, **71**, 351–357.

HENSON, P.M. (1970). Release of vasoactive amines from rabbit platelets induced by sensitized mononuclear leukocytes and antigen. *J. Exp. Med.*, **131**, 287–306.

HUMPHREY, D.M., MCMANUS, L.M., HANAHAN, D.J. & PINCKARD, R.N. (1984). Morphologic basis of increased vascular permeability induced by acetyl glyceryl ether phosphorylcholine. *Lab. Invest.*, **50**, 16–25.

HUMPHREY, D.M., MCMANUS, L.M., SATOUCHI, K., HANAHAN, D.J. & PINCKARD, R.N. (1982). Vasoactive properties of acetyl glyceryl ether phosphorylcholine and analogues. *Lab. Invest.*, **46**, 422–427.

INARREA, P., GOMEZ-AMBRONERO, J., NIETO, M. & SANCHEZ-CRESPO, M. (1984). Characteristics of the binding of platelet-activating factor to platelets of different animal species. *Eur. J. Pharmacol.*, **105**, 309–315.

JOHNSON, R.J., ALPERS, C.E., PRITZL, P., SCHULZE, M., BAKER, P., PRUCHNO, C. & COUSER, W.G. (1988). Platelets mediate neutrophil-dependent immune complex nephritis in the rat. *J. Clin. Invest.*, **82**, 1225–1235.

KLEE, A., SCHMID-SCHONBEIN, G.W. & SEIFFGE, D. (1991). Effects of platelet activating factor on rat platelets in vivo. *Eur. J. Pharmacol.*, **209**, 223–230.

KUBES, P., GRISHAM, M.B., BARROWMAN, J.A., GAGINELLA, T. & GRANGER, D.N. (1991). Leukocyte-induced vascular protein leakage in cat mesentery. *Am. J. Physiol.*, **261**, H1872–H1879.

KURTEL, H., FUJIMOTO, K., ZIMMERMAN, B.J., GRANGER, D.N. & TSO, P. (1991). Ischemia-reperfusion-induced mucosal dysfunction: role of neutrophils. *Am. J. Physiol.*, **261**, G490–G496.

MAJNO, G. & PALADE, G.E. (1961). Studies on inflammation. I. Effect of histamine and serotonin on vascular permeability: an electron microscopic study. *J. Biophys. Biochem. Cytol.*, **11**, 571–605.

MARTINS, M.A., MARTINS, P.M.R.E.S., CASTRO FARIA NETO, H.C., BOZZA, P.T., DIAS, P.M.F.L., CORDEIRO, R.S.B. & VARGAFTIG, B.B. (1988). Intravenous injections of PAF-acether induce platelet aggregation. *Eur. J. Pharmacol.*, **149**, 89–96.

O'DONNELL, S.R. & BARNETT, J.K. (1987). Microvascular leakage to platelet activating factor in guinea-pig trachea and bronchi. *Eur. J. Pharmacol.*, **138**, 385–396.

PIETRA, G.G. & MAGNO, M. (1978). Pharmacological factors influencing permeability of the bronchial microcirculation. *Fed. Proc.*, **37**, 2466–2470.

PIROTZKY, E., PAGE, C.P., ROUBIN, R., PFISTER, A., PAUL, W., BONNET, J. & BENVENISTE, J. (1984). PAF-acether induced plasma exudation in rat skin is independent of platelets and neutrophils. *Microcirc. Endoth. Lymph.*, **1**, 107–122.

SAKAI, A., CHANG, S.-W. & VOELKEL, N.F. (1989). Importance of vasoconstriction in lipid mediator-induced pulmonary oedema. *J. Appl. Physiol.*, **66**, 2667–2674.

SEALE, J.P., NOURSHARGH, S., HELLEWELL, P.G. & WILLIAMS, T.J. (1991). Mechanism of action of platelet activating factor in the pulmonary circulation: an investigation using a novel isotopic system in rabbit isolated lung. *Br. J. Pharmacol.*, **104**, 251–257.

SIROIS, M.G., JANCAR, S., BRAQUET, P., PLANTE, G.E. & SIROIS, P. (1988). PAF increases vascular permeability in selected tissues: effect of BN-52021 and L-655,240. *Prostaglandins*, **36**, 631–644.

SIROIS, M.G., PLANTE, G.E., BRAQUET, P. & SIROIS, P. (1990). Role of eicosanoids in PAF-induced increases of the vascular permeability in rat airways. *Br. J. Pharmacol.*, **101**, 896–900.

SVENSJO, E., ARFORS, K.E., RAYMOND, R.M. & GREGA, G.J. (1979). Morphological and physiological correlation of bradykinin-induced macromolecular efflux. *Am. J. Physiol.*, **236**, H600–H606.

TONNESEN, M.G. (1989). Neutrophil-endothelial cell interactions: mechanism of neutrophil adherence to vascular endothelium. *J. Invest. Dermatol.*, **93**, 53S–58S.

VARGAFTIG, B.B. & FERREIRA, S.H. (1981). Blockade on the inflammatory effects of PAF by cyclo-oxygenase inhibitors. *Br. J. Med. Res.*, **14**, 187–189.

WARNER, T.D. (1990). Simultaneous perfusion of rat isolated superior mesenteric arterial and venous beds: comparison of their vasocontrictor and vasodilator responses to agonists. *Br. J. Pharmacol.*, **99**, 427–433.

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Regional haemodynamic effects of dopamine and its prodrugs L-dopa and gludopa in the rat and in the glycerol-treated rat as a model for acute renal failure

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1 In this study the renal selectivity of dopamine and its prodrugs L-dopa and gludopa, with respect to their effects on regional blood flow, vascular resistance and central haemodynamics was investigated in normal rats and in rats with glycerol-induced acute renal failure (ARF).

2 In normal, anaesthetized rats, dopamine as well as its prodrugs caused a dose-dependent reduction of vascular resistance in the kidney (RR), mesentery (MR) and hindquarters (HQR) (dose range: dopamine: 0.1–5 μmol kg^{-1} h^{-1}; L-dopa and gludopa: 1–200 μmol kg^{-1} h^{-1}). Blood pressure and heart rate were affected at the highest dose only.

3 Administration of glycerol induced a preferential renal vasoconstriction; renal blood flow (−60%) and vascular resistance (+190%) were significantly more affected than MR (+40%) and HQR (+60%). This was only ameliorated by a low rate (10 μmol kg^{-1} h^{-1}) infusion of gludopa: the glycerol-induced reduction of renal flow and increase in RR were significantly attenuated. A high dose of gludopa (100 μmol kg^{-1} h^{-1}) or any dose of L-dopa or dopamine did not induce this beneficial effect. The glycerol-induced increase in MR and HQR was not attenuated by any of the treatments used.

4 The results indicate that gludopa is not renally selective at a pharmacodynamic level in normal, anaesthetized rats. Contrary to this, a low dose of gludopa does cause a renal selective vasodilatation and reduction of RR in rats with glycerol-induced ARF. This difference could be explained by a difference in renal vascular tone between normal rats and glycerol-induced ARF rats. A high dose of gludopa does not cause these renal-selective effects: renal resistance and renal flow are at the same level as following glycerol and saline. This is probably due to the systemic effects of the released dopamine.

Keywords: Dopamine; gludopa; glycerol; L-dopa; haemodynamics; renal failure; prodrug

Introduction

Gludopa is a prodrug of dopamine (Wilk *et al.*, 1978). It is converted by γ-glutamyl transpeptidase to L-dopa and subsequently by aromatic aminoacid decarboxylase to dopamine. This metabolism occurs predominantly in the kidney (Barthelmebs *et al.*, 1990; Boateng *et al.*, 1990; Cummings *et al.*, 1990). The effects of gludopa on the kidney have been described by several investigators in normal rats (Wilk *et al.*, 1978; 1979), rats with decreased renal function (Casson *et al.*, 1982; 1983; Boateng *et al.*, 1990; Barthelmebs *et al.*, 1991), rabbit (Wang, Z.-Q. *et al.*, 1992) and man (Jeffrey *et al.*, 1988a; Casagrande *et al.*, 1989; MacDonald *et al.*, 1989). Based on these results it was concluded that gludopa is a kidney-specific dopamine precursor and has kidney-selective actions. This selectivity was defined by comparison of renal actions with effects on heart and blood pressure, but no comparison has been made with other vascular regions, except for hindlimb flow in the conscious rabbit (Wang, Z-Q. *et al.*, 1992): it was found that gludopa has renal effects at doses that do not change hindlimb flow.

Other γ-glutamyl derived renal selective prodrugs are for instance N-acetyl-γ-glutamyl sulphamethoxazole (Orlowski *et al.*, 1980; Drieman *et al.*, 1990a), the renal vasodilator CGP 22979 (Smits & Struijker Boudier, 1985) and the dopamine-β-hydroxylase inhibitor prodrug, SC-47792 (Koepke *et al.*, 1991). The selectivity of these prodrugs is determined in part by the presence of a transport system for the prodrug in the kidney and of intracellular converting enzymes (Drieman *et al.*, 1990a,b; Hwang & Elfarra, 1991). N-acetyl-γ-glutamyl prodrugs are handled this way by the kidney, unlike γ-glutamyl prodrugs, which are converted extracellularly and cause relatively high plasma concentrations of active drug, released through the action of the extracellular enzyme γ-glutamyl transpeptidase (Drieman *et al.*, 1990a; 1993).

As gludopa is not an acetylated prodrug, it is likely that the dopamine released from gludopa is also systemically available. This led us to investigate the selectivity of gludopa with respect to different vascular regions in the rat.

The second question investigated in this study is whether the protective effects of gludopa on the kidney in the glycerol-treated rat model of acute renal failure (ARF), as has been described by Casson *et al.* (1982, 1983), is due exclusively to local renal effects of gludopa or also to systemic effects. The decreased glomerular filtration rate in this model of ARF is associated with preglomerular vasoconstriction (Hsu *et al.*, 1979; Wolfert & Oken, 1989). Dopamine increases renal plasma flow and glomerular filtration rate through DA_1 receptor stimulation in efferent and afferent arteries (see for review e.g. Lee, 1982; Felder *et al.*, 1989). Furthermore, DA_1 receptor stimulation results in normalization of the glomerular filtration rate (GFR) in a state of hyperfiltration caused by glycine (Wang Y.W. *et al.*, 1992) or streptozotocin-induced diabetes (Barthelmebs *et al.*, 1991). Because we found gludopa to have a vasodilator action not only in the kidney, but also in the mesentery and hindlimb, we investigated whether gludopa has normalizing effects only on renal blood flow or also on other flows in the glycerol-treated rat model for acute renal failure.

Methods

Animals

Male wistar rats weighing 220–300 g (Winkelman, Borchen, Germany) were used throughout the study.

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Surgery

In all experiments, miniaturized Doppler flowprobes were implanted on the left renal artery, superior mesenteric artery and abdominal aorta of the rats under pentobarbitone anaesthesia (50 mg kg^{-1}, i.p.), as described by Smits & Struyker Boudier (1985). Doppler flow probes record changes in blood flow; therefore only relative and not absolute blood flows are recorded. The animals were provided with polyethylene catheters for infusion of pentobarbitone and drugs in both femoral veins, and in the abdominal aorta via the left femoral artery for blood pressure measurements. The body temperature of the rats was kept constant at 37°C.

Anaesthesia was maintained by a constant rate infusion of pentobarbitone (24 mg kg^{-1} h^{-1}). Following stabilization, mean arterial pressure (MAP), systolic pressure (SP), heart rate (HR) and the relative blood flows through the left renal artery (renal flow: RF), superior mesenteric artery (mesenteric flow: MF) and abdominal aorta (hindquarter flow: HQF) were recorded.

Experiments

Dose-response curves of dopamine (0.1–5 μmol kg^{-1} h^{-1}) and dopamine prodrugs (1–20 μmol kg^{-1} h^{-1}) on MAP, SP, HR (flow rate: 5 ml kg^{-1} h^{-1}) and the changes in regional blood flows were recorded during cumulative infusions of the appropriate drugs. When all recorded parameters were stable, a 20 min control period was followed by the infusions. Infusion of each dose lasted 20 min, as it was found that all responses on a dose of a dopamine prodrug were maximal at approximately 15 min. Vascular resistances of kidney (RR), mesentery (MR) and hindquarter (HQR) were derived from the MAP values and flow changes in each rat.

Acute renal failure (ARF) was induced by subcutaneous injection of 7 ml kg^{-1} of glycerol (50% v/v in water) to the anaesthetized rats. This dose was chosen because it causes considerable renal failure with a minimal of losses (Finckh, 1957; Carrol *et al.*, 1965; Casson *et al.*, 1983).

The effect of glycerol-induced ARF on haemodynamic parameters and regional blood flows was studied as follows. Rats, instrumented as described above, were infused with pentobarbitone (24 mg kg^{-1} h^{-1}; 2.5 ml kg^{-1} h^{-1}) and saline (2.5 ml kg^{-1} h^{-1}). A pilot experiment showed that the rate of total saline infusion was important: when the rate was reduced by 50% most of the animals did not survive for 4 h; a higher (200%) rate of infusion had a too large protective effect on the kidneys, as has been described for conscious rats (Casson *et al.*, 1982; Cushner *et al.*, 1986). For the same reason, the rats were not dehydrated 24 h prior to induction of ARF. After the rats were stable for about 30 min, glycerol was injected s.c. ($t = 0$) and all parameters were recorded for 4 h. Changes in blood flow and vascular resistance are expressed as percentage change at $t = 4$ h (average of the last 15 min of these 4 h) compared to control period at $t = -15$ min to $t = 0$, changes in the other haemodynamic parameters in absolute differences (beats per min (b.p.m.) and mmHg).

Effects of dopamine and dopamine prodrugs on ARF Experiments were conducted as described above, except that at $t = -30$ min (i.e. 30 min before glycerol injection, which is $t = 0$), a continuous infusion of dopamine (0.5 and 5 μmol kg^{-1} h^{-1}), L-dopa (10 and 100 μmol kg^{-1} h^{-1}) or gludopa (10 and 100 μmol kg^{-1} h^{-1}) in saline was started instead of the saline infusion; total infusion rate (5 ml kg^{-1} h^{-1}) was the same in all experiments. Changes in parameters measured at $t = 4$ h (average of the last 15 min of these 4 h) are compared to the drug infusion control period ($t = -15$ min to $t = 0$).

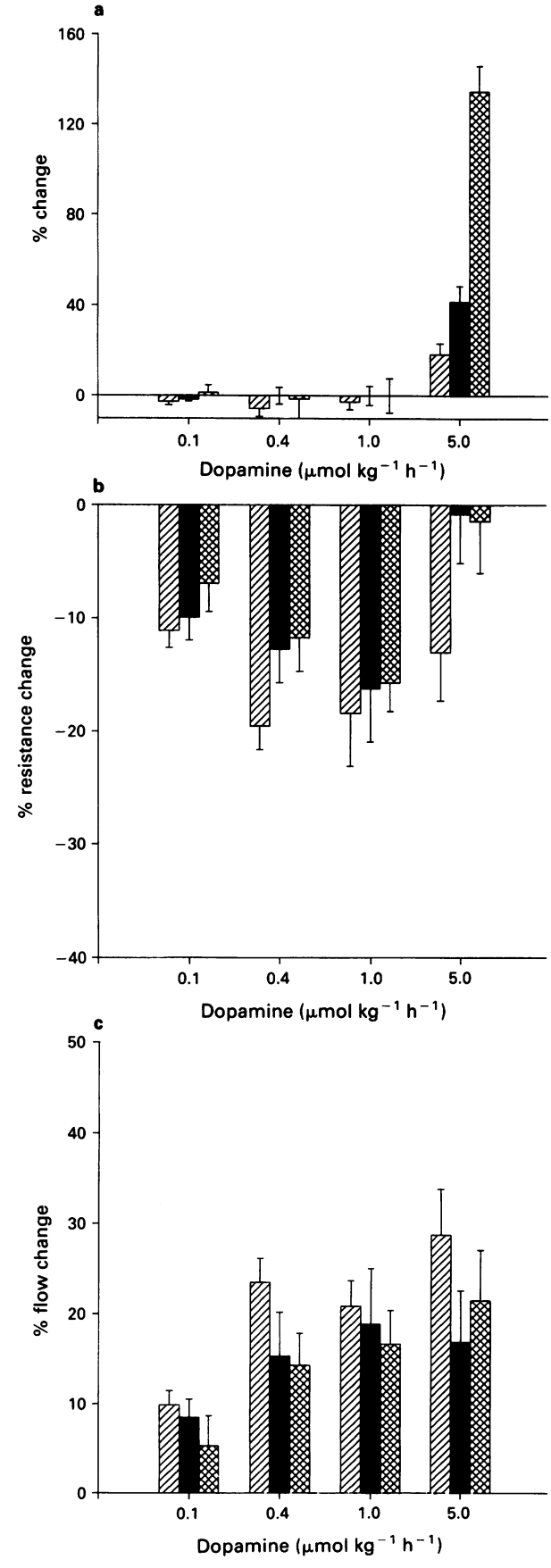


Figure 1 Dose-response curve of dopamine on haemodynamics (a), vascular resistance; (b) and regional blood flows (c). In (a), hatched columns, mean arterial pressure; solid columns, systolic pressure; cross-hatched columns, heart rate; In (b), hatched columns, renal resistance; solid columns, mesenteric resistance; cross-hatched columns, hindquarter resistance. In (c), hatched columns, renal blood flow (%); solid columns, mesenteric blood flow (%); cross-hatched columns, hindquarter blood flow (%).

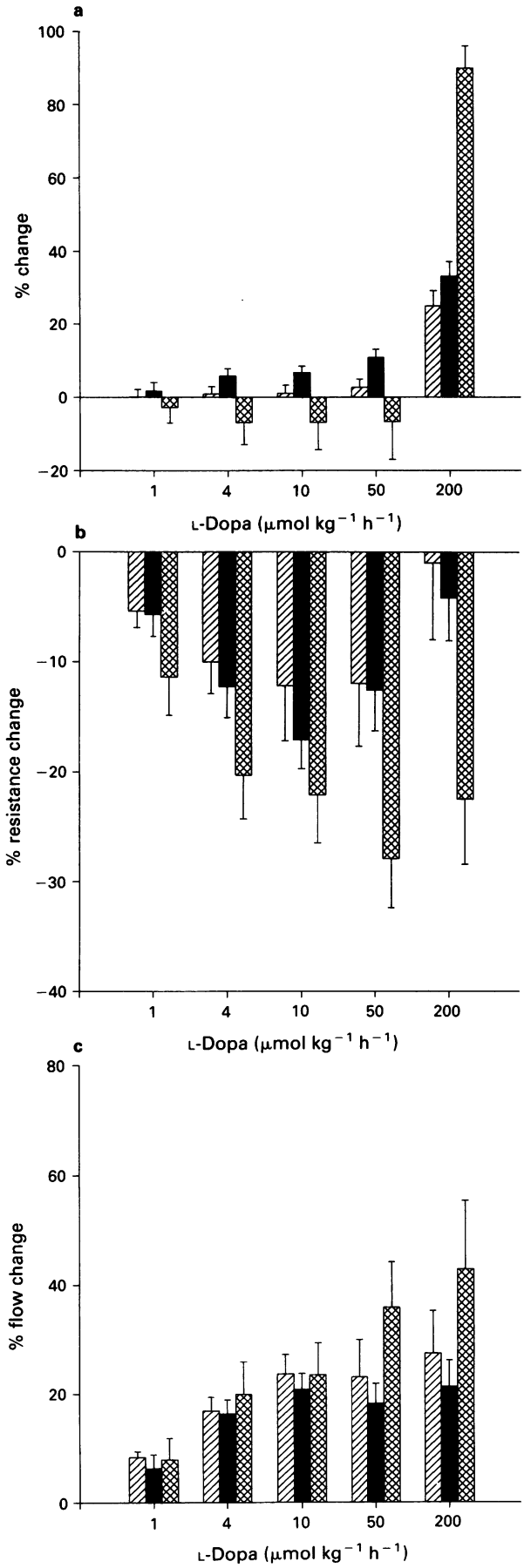


Figure 2 Dose-response curve of L-dopa on haemodynamics (a), vascular resistance (b) and regional blood flows (c). Columns as in Figure 1.

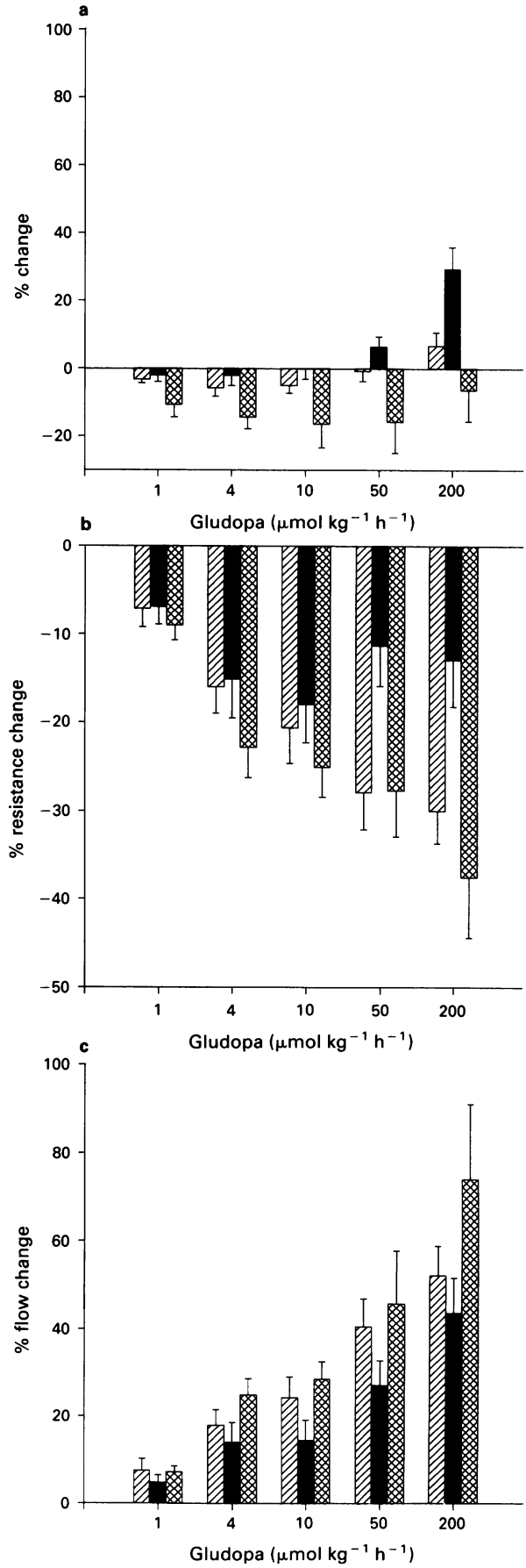
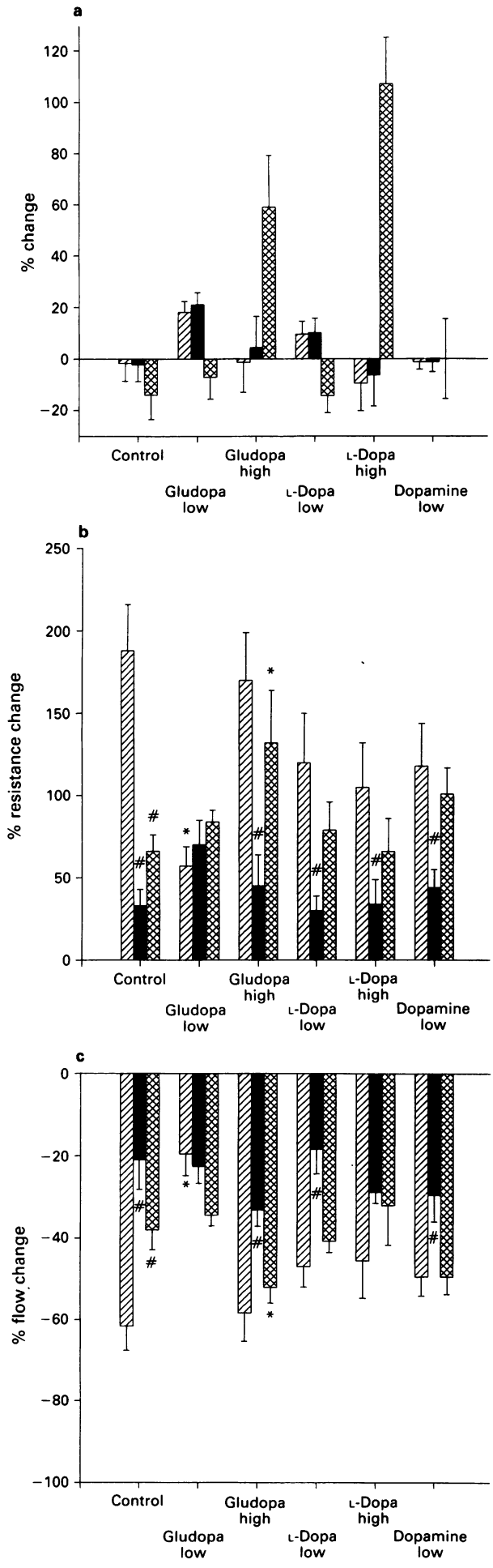


Figure 3 Dose-response curve of gludopa on haemodynamics (a), vascular resistance; (b) and regional blood flow (c). Columns as in Figure 1.



Drugs

Dopamine and glycerol were obtained from Sigma Chemical company (St Louis, U.S.A.), L-Dopa from Janssen Chimica (Beerse, Belgium); gludopa was a kind gift from Prof. M.R. Lee (University of Edinburgh, Scotland) and from Prof. C. Casagrande (Simes Cardiovascular Research Centre, Milan, Italy).

Statistics

Data are presented as mean ± s.e. Groups were compared by one-way analysis of variance, followed by Duncan's range test. Differences were considered to be statistically significant when $P<0.05$.

Results

Effects of dopamine, L-dopa and gludopa on regional blood flows and haemodynamic parameters

The results of these experiments are summarized in Figures 1–3. Baseline values (in the absence of drug) of MAP (mmHg) and HR (b.p.m.) were: dopamine, MAP: 108 ± 5, HR: 370 ± 15; L-dopa, MAP: 112 ± 4, HR: 378 ± 12; gludopa, MAP: 107 ± 5, HR: 385 ± 11. Dopamine decreased vascular resistance and increased blood flow in all regions studied (Figure 1b,c). At the highest dose the changes in MR and HQR, but not RR were diminished. This is probably due to stimulation of cardiac adrenoceptors by dopamine. No significant differences between changes in RR, MR and HQR were found at 0.1–1.0 μmol kg^{-1} h^{-1}, indicating that dopamine is not renal selective in the rat at low dose. L-Dopa and gludopa, both in a 10 fold higher dose, increased blood flows and decreased vascular resistance dose-dependently in all regions studied (Figures 2b,c and 3b,c), suggesting no renal selectivity for gludopa at the level of renal resistance under the circumstances studied. At a dose of 200 μmol kg^{-1} L-dopa had similar effects as dopamine at 5 μmol kg^{-1}.

The effects of dopamine and L-dopa on MAP, SP and HR were similar: no effects in low doses and a significant rise of all parameters at high doses (Figures 1a and 2a). At the high dose of 200 μmol kg^{-1} h^{-1} gludopa caused an increase in systolic pressure, but not in HR or MAP (Figure 3a).

These results indicate that dopamine and its prodrugs L-dopa and gludopa are not renal-selective at low doses in the rat, but that all resistances measured decrease at a dose which does not affect heart rate or blood pressure. Based on the results of these experiments, we chose to use doses of gludopa, L-dopa and dopamine in the ARF studies that decreased RR, MR and HQR without affecting blood pressure and heart rate, and higher doses which do affect these parameters.

Glycerol-induced ARF

The administration of glycerol had the following effects. Immediately following injection a large, transient reduction (typically about 30–40%) of all blood flows was observed which lasted for about 30 min. After this period, all flows

Figure 4 Effects of dopamine (0.5 μmol kg^{-1} h^{-1}), L-dopa (10 and 100 μmol kg^{-1} h^{-1}) and gludopa (10 and 100 μmol kg^{-1} h^{-1}) on haemodynamics (a), vascular resistance; (b) and regional blood flows (c) in glycerol-treated rats at $t=4$ h. In (a), hatched columns, mean arterial pressure; solid columns, systolic pressure; cross-hatched columns, heart rate. In (b), hatched columns, renal resistance; solid columns, mesenteric resistance; cross-hatched columns, hindquarters resistance. In (c), hatched columns, renal blood flow (%); solid columns, mesenteric blood flow (%), cross-hatched columns, hindquarters blood flow (%). #Significantly ($P<0.05$) different from kidney; *Significantly ($P<0.05$) different from saline control.

decreased slowly. At $t = 4$ h, MAP, SP and HR were not changed significantly (Figure 4a). RR showed a significantly larger increase than HQR and MR (Figure 4b). The latter two did not differ significantly.

Effects of dopamine and its prodrugs on glycerol-induced ARF

Baseline values of MAP (mmHg) and HR (b.p.m.) in these experiments (before start of drug infusion) were: saline, MAP: 110 ± 6, HR: 383 ± 15; dopamine, MAP: 106 ± 5, HR: 373 ± 11; L-dopa low dose, MAP: 110 ± 3, HR: 385 ± 10; L-dopa high dose, MAP: 111 ± 4, HR: 391 ± 10; gludopa low dose, MAP: 101 ± 4, HR: 384 ± 9; gludopa high dose, MAP: 110 ± 4, HR: 378 ± 12.

Dopamine at a low dose (0.5 μmol kg^{-1} h^{-1}) did not significantly affect the changes in vascular resistance or flow compared to saline control (Figure 4b and 4c), nor were MAP, SP or HR significantly different from control (Figure 4a). Unlike the saline control it was found that not only RF, but also HQF was significantly lower than MF. Most of the glycerol-treated animals did not survive the high dose (5 μmol kg^{-1} h^{-1} dopamine) for 4 h.

L-dopa at a low dose (10 μmol kg^{-1} h^{-1}) caused effects comparable to dopamine (0.5 μmol kg^{-1} h^{-1}). The high dose (100 μmol kg^{-1} h^{-1}) caused a large increase in HR (significantly different from saline control), but no change of MAP or SP. No amelioration of the glycerol-effects on RF, MF and HQF were found, compared to saline control.

Gludopa at a low dose (10 μmol kg^{-1} h^{-1}) prevented the sharp rise in renal resistance: RR was significantly less increased in this case than in the case of saline control or any of the other treatments and was now of the same magnitude as MR and HQR (no significant differences between ΔRR, ΔMR and ΔHQR). Gludopa at a high dose (100 μmol kg^{-1} h^{-1}) did not produce this amelioration of RR and RF, but had similar effects on regional blood flow and resistance to dopamine or L-dopa at a low dose. HQR was significantly increased. The effects of gludopa at a high dose on the haemodynamics were comparable to the haemodynamic effects of L-dopa at a high dose.

Discussion

The kidney is rich in dopamine receptors. D_1 and D_2 receptors are found in renal arterioles, proximal tubule and at juxtaglomerular cells (see for review Felder *et al.*, 1989), where they may cause vasodilatation, decrease sodium reabsorption and influence renin secretion (D_1: increase, D_2: decrease). Selective stimulation of D_1 receptors in the kidney may have beneficial effects in several renal disease states (Casson *et al.*, 1983; Barthelmebs *et al.*, 1991; Brooks *et al.*, 1991; Wang, Y.-W. *et al.*, 1992). Hence, a renal selective dopamine prodrug, without systemic side-effects, would be of great therapeutic value.

Gludopa has been described as renal selective in mice, rat and man (e.g. Wilk *et al.*, 1978; 1979; Lee, 1987; Jeffrey *et al.*, 1988a,b; MacDonald *et al.*, 1989; Cummings *et al.*, 1990). Dopamine concentrations following gludopa administration were found to be much higher in the kidney than in mouse heart (Wilk *et al.*, 1978) or rat liver (Cummings *et al.*, 1990). Furthermore, in rats it was found that renal plasma flow increased more following gludopa compared to dopamine and L-dopa at a dose at which no cardiac responses were observed (Wilk *et al.*, 1979). Our results show however, that in normal, anaesthetized rats, gludopa does not cause a renal selective increase in blood flow; mesenteric and hindquarter flow were increased to the same extent. This is in contrast with the findings in the conscious rabbit, where no increase in hindquarter flow was found after gludopa (Wang, Z.-Q. *et al.*, 1992). Species differences in receptor sensitivity or abundance, as well as differences between the state of consciousness may underlie this difference. Since blood pressure was not decreased, our results indicate that cardiac output must have been increased. This can be due to baroreflex activation, but a direct cardiac action of dopamine may also be involved.

The lack of renal selectivity of gludopa implies that the dopamine, released from gludopa is systemically available. Pharmacokinetic studies with gludopa in the rat support our pharmacodynamic findings. After a bolus injection of gludopa, considerable amounts of L-dopa and dopamine were found in plasma (Boateng *et al.*, 1990; Cummings *et al.*, 1990). Due to the short halflife time of dopamine, it is difficult to estimate the amount of dopamine that is available systemically *in vivo*. *In vitro* in rat isolated kidney it was found that the rate of dopamine release from gludopa to the perfusate was about 3 times higher than to the urine (Barthelmebs *et al.*, 1990). This high leakage from the kidney is proably due to the nature of the prodrug. Gludopa is the γ-glutamyl prodrug of L-dopa. We have found with the model prodrugs γ-glutamyl sulphamethoxazole and N-acetyl-γ-glutamyl sulphamethoxazole that the N-acetyl group is essential for high kidney-selectivity: the site of conversion is intracellular in the latter case, unlike that of the γ-glutamyl prodrug (Drieman *et al.*, 1990a). Sulphamethoxazole, released intracellularly, is retained by the kidney, whereas extracellularly released drug causes relatively high plasma concentrations. It would therefore be very interesting to study the N-acetyl derivative of gludopa with respect to its renal selectivity. Casson *et al.* (1983) did, however, report that gludopa has a protective effect on the glycerol-treated rat model of ARF. In the present study we investigated whether this protective effect is due to local or systemic dopamine effects. We chose to do this in the early phases of the induction of ARF, because it has been suggested that maintenance of renal blood flow during the first hours after glycerol administration protects the kidney from ARF (Cushner *et al.*, 1986).

Our results show that glycerol decreases renal blood flow significantly more than mesenteric flow or hindlimb flow (Figure 4c). This decrease was ameliorated only by a continuous infusion of gludopa at a low rate. Gludopa at a high rate reversed this beneficial effect, which indicates that the systemic effects of dopamine (on adrenoceptors) counteract the local beneficial effects of dopamine. The effects of the low dose gludopa on renal blood flow, in combination with the finding of Casson *et al.* (1983) that gludopa protects from ARF, confirm the suggestion of Cushner *et al.* (1986) that preservation of the renal blood flow may cause a reduction or prevention of ARF.

The dose of gludopa was not adjusted to the decreased kidney function, since the pharmacokinetics of gludopa are not changed in rats with glycerol-induced ARF (Boateng *et al.*, 1990). The renal effects of gludopa in glycerol-induced ARF in rats are different from those in normal rats. This could be caused by the difference in vascular diameter in these situations. When the diameter is near maximal, a dopamine-induced arteriolar vasodilatation will not result in a significant decrease of renal resistance, whereas when the renal vessels are contracted, a dilatation will have large effects on the resistance.

The pharmacokinetic studies combined with the pharmacodynamic studies lead to the conclusion that although gludopa may cause relatively high renal concentrations of dopamine, it does not seem to be kidney-selective under physiological circumstances. In pathological circumstances, on the other hand, gludopa has beneficial effects on the kidney (Casson *et al.*, 1983; Barthelmebs *et al.*, 1991). This does not seem to be due to systemic dopamine effects, as dopamine and L-dopa did not increase kidney function. Local stimulation of D_1 receptors in the kidney is supposed to be responsible for this effect: fenoldopam ameliorated kidney function in amphotericin B nephrotoxicity in dogs (Brooks *et al.*, 1991), and glycine-induced hyperfiltration (Wang, Y.-W. *et al.*, 1992) and hyperfiltration caused by streptozotocin-induced diabetes

(Barthelmebs *et al.*, 1991) in the rat. In the latter two models fenoldopam and gludopa decrease filtration fraction, thus counteracting the existing hyperfiltration. This decrease in filtration fraction is caused by preferential efferent arteriolar vasodilatation, causing an increase in renal blood flow, a decrease in renal vascular resistance and little effect on glomerular filtration rate (Wang, Y.-W. *et al.*, 1992). The restoration or potentiation of the tubuloglomerular feedback mechanism by dopamine probably plays a significant role in the reversal of hyperfiltration in these models (Premen, 1988; Schnermann *et al.*, 1990).

In consious rats, gludopa reverses the polyuria caused by glycerol, but has no effect on urine production in normal rats (Casson *et al.*, 1982; 1983). This is consistent with the mechanism described above and with our results: no renal selective blood flow increase in normal rats but a renal selective attenuation of the glycerol-induced fall in renal flow.

The dose of gludopa should not be too high in order to avoid systemic effects that counteract the beneficial renal effects. A more renal selective prodrug of dopamine would have a higher therapeutic index. N-acetyl gludopa is in our opinion an interesting candidate, considering the effects of gludopa itself and the beneficial effects of an N-acetyl group on the renal selectivity of prodrugs (Drieman *et al.*, 1990a; 1993). An alternative would be a renal selective D_1 agonist. Such a compound might become a useful therapeutic agent, for instance in protecting patients against the nephrotoxic side effects of cytostatics.

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References

BARTHELMEBS, M., CAILETTE, A., EHRHARDT, J.-D., VELLY, J. & IMBS, J.-L. (1990). Metabolism and vascular effects of gamma-L-glutamyl-L-dopa on the isolated rat kidney. *Kidney Int.*, **37**, 1414–1422.

BARTHELMEBS, M., VAILLY, B., GRIMA, M., VELLY, J., STEPHAN, D., FROEHLY, S. & IMBS, J.-L. (1991). Effects of dopamine prodrugs and fenoldopam on glomerular hyperfiltration in streptozotocin-induced diabetes in rats. *J. Cardiovasc. Pharmacol.*, **18**, 243–253.

BOATENG, Y.A., BARBER, H.E., MACDONALD, T.M., PETRIE, J.C., LEE, M.R. & WHITING, P.H. (1990). The pharmacokinetics of gamma-glutamyl-L-dopa in normal and anephric rats and rats with glycerol-induced acute renal failure. *Br. J. Pharmacol.*, **101**, 301–306.

BROOKS, D.P., MITCHELL, M.P., SHORT, B.G., RUFFOLO, R.R. & NICHOLS, A.J. (1991). Attenuation of amphotericin B nephrotoxicity in the dog by the fenoldopam prodrug, SK&F R-105058. *J. Pharmacol. Exp. Ther.*, **257**, 1243–1247.

CARROL, R., KOVACS, K. & TAPP, E. (1965). The pathogenesis of glycerol-induced renal tubular necrosis. *J. Path. Bact.*, **89**, 573–580.

CASAGRANDE, C., MERLO, L., FERRINI, R., MIRAGOLI, G. & SEMERARO, C. (1989). Cardiovascular and renal action of dopaminergic prodrugs. *J. Cardiovasc. Pharmacol.*, **14** (Suppl. 8), S40–S59.

CASSON, I.F., ANDERSON, C.K., COPE, G.F. & LEE, M.R. (1982). The effect of dietary sodium chloride and gamma-glutamyl dopa on tubular necrosis following glycerol administration in the rat. *Br. J. Exp. Path.*, **63**, 426–431.

CASSON, I.F., CLAYDEN, D.A., COPE, G.F. & LEE, M.R. (1983). The protective effect of gamma-glutamyl L-dopa on the glycerol treated rat model of acute renal failure. *Clin. Sci.*, **65**, 159–164.

CUMMINGS, J., MATHESON, L.M., MAURICE, L. & SMYTH, J.F. (1990). Pharmacokinetics, bioavailability, metabolism, tissue distribution and urinary excretion of gamma-glutamly-L-dopa in the rat. *J. Pharm. Pharmacol.*, **42**, 242–246.

CUSHNER, H.M., BARNES, J.L., STEIN, J.H. & REINECK, H.J. (1986). Role of volume depletion in the glycerol model of acute renal failure. *Am. J. Physiol.*, **250**, F315–F321.

DRIEMAN, J.C., THIJSSEN, H.H.W. & STRUYKER BOUDIER, H.A.J. (1990a). Renal selective N-acetyl-gamma-glutamyl prodrugs. II. Carrier-mediated transport and intracellular conversion as determinants in the renal selectivity of N-acetyl-gamma-glutamyl sulfamethoxazole. *J. Pharmacol. Exp. Ther.*, **252**, 1255–1260.

DRIEMAN, J.C., THIJSSEN, H.H.W., ZEEGERS, H.H.M., SMITS, J.F.M. & STRUYKER BOUDIER, H.A.J. (1990b). Renal selective N-acetyl-γ-glutamyl prodrugs: a study on the mechanism of activation of the renal vasodilator prodrug CGP 22979. *Br. J. Pharmacol.*, **99**, 15–20.

DRIEMAN, J.C., THIJSSEN, H.H.W. & STRUYKER-BOUDIER, H.A.J. (1993). Renal selective N-acetyl-L-γ-glutamyl prodrugs: studies on the selectivity of some model prodrugs. *Br. J. Pharmacol.*, **108**, 204–208.

FELDER, R.A., FELDER, C.C., EISNER, G.M. & JOSE, P.A. (1989). The dopamine receptor in adult and maturing kidney. *Am. J. Physiol.*, **257**, F315–F327.

FINCKH, E.S. (1957). Experimental acute tubular necrosis following subcutaneous injection of glycerol. *J. Path. Bact.*, **73**, 69–85.

HSU, C.H., KURTZ, T.W. & SANDS, C.E. (1979). Intrarenal vascular resistance in glycerol-induced acute renal failure in the rat. *Circ. Res.*, **45**, 583–587.

HWANG, I.Y. & ELFARRA, A.A. (1991). Kidney-selective prodrugs of 6-mercaptopurine. Biochemical basis of the kidney-selectivity of S-(6-purinyl)-L-cysteine and metabolism of new analogs in rats. *J. Pharmacol. Exp. Ther.*, **258**, 171–177.

JEFFREY, R.F., MACDONALD, T.M. & LEE, M.R. (1988a). A comparison of the renal actions of gamma-L-glutamyl-L-dopa and gamma-L-glutamyl-L-tyrosine in normal man. *Clin. Sci.*, **74**, 37–40.

JEFFREY, R.F., MACDONALD, T.M., MARWICK, K. & LEE, M.R. (1988b). The effect of carbidopa and indomethacin on the renal response to gamma-L-glutamyl-L-dopa in normal man. *Br. J. Clin. Pharmacol.*, **25**, 195–201.

KEOPKE, J.P., TYLER, L.D., SCHUH, J.R., REITZ, D.B., REINHARD, E.J. & BLAINE, E.H. (1991). Renal selective dopamine beta-hydroxylase inhibition is antihypertensive in spontaneously hypertensive rats. *Hypertension*, **18**, 413.

LEE, M.R. (1982). Dopamine and the kidney. *Clin. Sci.*, **62**, 439–448.

LEE, M.R. (1987). Dopamine, the kidney and essential hypertension. Studies with gludopa. *Clin. Exp. Theory Pract.*, **A9** (5&6), 977–986.

MACDONALD, T.M., JEFFREY, R.F. & LEE, M.R. (1989). The renal and haemodynamic effects of a 10 h infusion of glutamyl-l-dopa in normal man. *Br. J. Clin. Pharmacol.*, **27**, 811–822.

ORLOWSKI, M., MIZOGUSHI, N. & WILKS, S. (1980). N-acyl-gamma-glutamyl derivatives of sulphamethoxazole as models of kidney-selective prodrugs. *J. Pharmacol. Exp. Ther.*, **212**, 167–172.

PREMEN, A.J. (1988). Potential mechanisms mediating the renal hyperemia and hyperfiltration. *FASEB J.*, **2**, 131–137.

SCHNERMANN, J., TODD, K.M. & BRIGGS, J.P. (1990). Effects of dopamine on the tubuloglomerular feedback mechanism. *Am. J. Physiol.*, **258**, F790–F798.

SMITS, J.F.M. & STRUYKER-BOUDIER, H.A.J. (1985). Preferential renal vasodilator effects of CGP 22979A in conscious spontaneously hypertensive rats. *J. Pharmacol. Exp. Ther.*, **232**, 845–849.

WANG, Y.-W., GELLAI, M. & BROOKS, D.P. (1992). Dopamine DA1 receptor agonist, fenoldopam, reverses glycine-induced hyperfiltration in rats. *Am. J. Physiol.*, **262**, F1055–F1060.

WANG, Z.-Q., WAY, D., TRIGG, L. & MCGRATH, B.P. (1992). DA-1 receptors mediate renal effects of the dopamine prodrug, gludopa, in conscious rabbits. *Clin. Exp. Pharmacol. Physiol.*, **19**, 369–372.

WILK, S., MIZOGUSHI, H. & ORLOWSKI, M. (1978). Gamma-Glutamyl dopa: a kidney-specific dopamine precursor. *J. Pharmacol. Exp. Ther.*, **206**, 227–232.

WILK, S., MIZOGUSHI, H., KUPFER, S. & ORLOWSKI, M. (1979). Gamma-Glutamyl dopa and gamma-glutamyl dopamine as kidney specific pro-drugs. In *Catecholamines: Basic and Clinical Frontiers*. ed. Usdin, E., Kopin, I.J. & Barchas, J. New York, Pergamon Press.

WOLFERT, A.I. & OKEN, D.E. (1989). Glomerular hemodynamics in established glycerol-induced acute renal failure in the rat. *J. Clin. Invest.*, **84**, 1967–1973.

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Effect of duration of ischaemia on reduction of myocardial infarct size by inhibition of neutrophil accumulation using an anti-CD18 monoclonal antibody

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1 Neutrophil accumulation is a characteristic feature of the inflammatory response in myocardial tissue which has undergone a period of ischaemia. The aim of this study was to examine whether inhibition of myocardial neutrophil infiltration, using an antibody to the CD18 leukocyte adhesion molecule, was effective in reducing infarct size in anaesthetized rabbits.

2 Anaesthetized rabbits underwent coronary artery occlusion (CAO) for periods of 30 or 45 min followed by reperfusion for 3 h. Animals were treated intravenously 10 min prior to reperfusion with IB4, a monoclonal antibody to CD18 (1 mg kg^{-1}) or saline (1 ml kg^{-1}). In one group undergoing 45 min CAO, a control antibody, OKMI (1 mg kg^{-1}) was given.

3 Following either 30 or 45 min of CAO, administration of IB4 resulted in a $<75\%$ inhibition in neutrophil accumulation in the area at risk myocardium (AR) compared with control animals.

4 With the 30 min occlusion period, IB4 significantly reduced myocardial infarct size, $27.2 \pm 3.2\%$ vs $67.4 \pm 5.6\%$ in the saline control group ($n = 5$ $P < 0.01$). In contrast, IB4 did not reduce infarct size following a 45 min period of ischaemia.

5 In the same animals administration of IB4 significantly inhibited oedema formation in skin elicited by intradermal administration of the neutrophil chemoattractant f-Met-Leu-Phe, but had no effect on coronary microvascular plasma protein leakage in the AR.

6 Our results indicate that infiltrating neutrophils exacerbate tissue injury following a relatively short, 30 min period of myocardial ischaemia in the rabbit. However, protection with IB4 was no longer seen if the period of CAO was extended to 45 min. The results in this model suggest neutrophils are not a major determinant of tissue injury following more than a very short period of ischaemia.

Keywords: Myocardial infarction; neutrophils; CD11/CD18; IB4; oedema formation

Introduction

It is well established that neutrophil infiltration is a characteristic feature of myocardial infarcts (Mallory *et al.*, 1939). More recently it has been reported in a number of experimental studies that myocardial infarct size is reduced by interventions which deplete circulating neutrophils (Romson *et al.*, 1983; Mullane *et al.*, 1984). Protection has also been observed by use of other agents such as ibuprofen and prostacyclin, which suppress neutrophil infiltration into infarcted myocardium (Romson *et al.*, 1982; Simpson *et al.*, 1987). As a result of these studies it has been suggested that neutrophils may extend the area of tissue damage through injury of viable myocytes. However, in some studies no myocardial protection was observed using anti-neutrophil interventions such as ibuprofen or leukocyte filters (Reimer *et al.*, 1985; Chatelain *et al.*, 1987) and consequently there is still controversy over this hypothesis.

An essential prerequisite for neutrophil accumulation at sites of inflammation is an initial adherence of the leukocytes to the endothelium, which involves the expression of adhesion molecules on the surface of both cell types. In the case of leukocytes the CD11/CD18 β_2 integrins have been implicated in this process (Harlan *et al.*, 1985; Lo *et al.*, 1989). Monoclonal antibodies to the leukocyte CD18 antigen have been shown to inhibit neutrophil accumulation at sites of inflammation (Arfors *et al.*, 1987). We have previously shown that pretreatment of ^{111}In-neutrophils with a monoclonal antibody to the CD18 adhesion molecule, significantly reduces their accumulation in skin sites injected with chemoattractants (Nourshargh *et al.*, 1989) and in ischaemic/reperfused rabbit myocardium (Williams *et al.*, 1990). In this study we have examined the effect of systemic administration of IB4, a monoclonal antibody to CD18 (Wright *et al.*, 1983), on myocardial infarct size in anaesthetized rabbits using two different periods of ischaemia.

Methods

Coronary artery occlusion and reperfusion

New Zealand White rabbits, anaesthetized with intravenously administered sodium pentobarbitone (60 mg kg^{-1}) were used for this study. The trachea was cannulated to allow positive pressure ventilation with room air. Arterial blood pressure was monitored from a cannula in the carotid artery and a lead I electrocardiogram (ECG) from subcutaneous limb leads. The heart was exposed by a left thoracotomy at the 5th intercostal space. A silk ligature was positioned around a branch of the left main coronary artery and both ends passed through a short length of polythene tubing. Following a stabilisation period of 15 min, the coronary artery was occluded by pulling on the suture and clamping the ends against the tubing. After a period of either 30 or 45 min coronary artery occlusion (CAO), tension on the ligature was released to allow reperfusion of the myocardium for a further 3 h. At the end of this time, 500 units of heparin was administered intravenously as an anti-coagulant. The experiment was then terminated by anaesthetic overdose. The heart was rapidly removed and cannulated via the aorta to allow retrograde perfusion of the coronary circulation. Intravascular blood was washed out with saline. The coronary artery

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was reoccluded with the original ligature and the heart perfused with Monastral blue dye to stain the normal myocardium. Small samples of myocardium were taken from the normal zone (NZ) and area at risk (AR) and stored at − 20°C prior to assay for myeloperoxidase as an index of neutrophil accumulation. The rest of the heart was frozen prior to staining for infarct size (Thornton *et al.*, 1990).

Measurement of infarct size

The heart, while still frozen was sectioned transversely into 2 mm thick slices from apex to base. Each slice was weighed and traced onto transparency film, carefully demarcating normal (stained with Monastral blue) and ischaemic (unstained) regions. The heart sections were then incubated, in the dark in 1% triphenyltetrazolium chloride (TTC) in phosphate-buffered saline (PBS, pH 7.4) for 30 min at 37°C. The viable tissue stained dark red while the infarcted tissue appeared pale brown. This differential staining was enhanced by storing the heart in 1% formalin overnight. The slices were subsequently retraced to delineate the regions of viable and infarcted tissue. Computerised planimetry was used to determine the areas of risk and infarction on each slice. Measurement of AR and infarct areas were carried out blind.

Measurement of myeloperoxidase concentration

Measurement of myeloperoxidase (MPO) activity was carried out according to a previously described method (Rubin *et al.*, 1990). Myocardial samples were weighed and then homogenized in 0.02 M $NaPO_4$ buffer (pH 4.7) containing 0.1 M NaCl and 0.015 M Na_2EDTA. Homogenates were centrifuged at 20,000 *g* for 15 min at 4°C and the supernatant, containing the majority of the haemoglobin, discarded. The pellets were homogenized a second time in 0.05 M $NaPO_4$ with 0.5% hexadecyltrimethylammonium-bromide (HETAB) and freeze/thawed in liquid nitrogen three times. After a second centrifugation at 20,000 *g* for 15 min at 4°C, the supernatant containing the majority of the MPO was taken for assay. This was carried out using the following: 1.6 mM tetramethylbenzidine, 0.3% H_2O_2, 12% dimethyl formamide and 40% Dulbecco's PBS made up in 0.08 M $NaPO_4$, pH 5.4. The change in absorbance was measured at 690 nm. One unit of MPO was defined as the amount of enzyme reducing 1 μmol of H_2O_2 min^{-1}.

Measurement of plasma protein leakage in myocardial tissue and skin

Plasma protein leakage was measured as the local accumulation of intravenously administered ^{125}I-labelled albumin (5 μCi), given 10 min prior to coronary artery occlusion. Samples of myocardium were taken from the NZ and the AR, weighed and the radioactivity measured in a gamma counter. The effect of treatment with IB4 was also examined on plasma protein leakage in skin, following intradermal injection of f-Met-Leu-Phe (FMLP) and bradykinin in the same animals. These two inflammatory mediators cause plasma exudation through mechanisms which are neutrophil-dependent and neutrophil-independent respectively (Wedmore & Williams, 1981). The mediators were injected in a volume of 0.1 ml into the shaved flank of the rabbit, just after reperfusion. Treatments were given in replicates of six. FMLP was given at a dose of 5×10^{-11} mol per site and bradykinin at a dose of 1×10^{-10} mol per site. Control sites received saline. At the end of the experiment, a blood sample was taken for preparation of plasma. The skin was removed and the injection sites excised with a 17 mm punch. Plasma and skin samples were then counted in a gamma counter. Results are expressed in terms of volume of plasma exudate and are calculated by dividing the number of ^{125}I counts in tissue samples by the number in 1 μl of plasma.

Drug treatment

IB4 is a murine IgG_2 monoclonal antibody directed against an epitope on the common β-chain (CD18) of the leukocyte adhesion complex (Wright *et al.*, 1983) and has been shown to bind to rabbit neutrophils (Tuomanen *et al.*, 1989). The antibody was administered intravenously at a dose of 1 mg kg^{-1} since this dose has been shown to suppress neutrophil accumulation in rabbit skin elicited by zymosan-activated serum (Lundberg & Wright, 1990). In order to investigate if the deleterious effects of neutrophils were exerted during the reperfusion phase, treatment was administered 10 min before the end of the occlusion period. The effect of IB4 on infarct size was compared following CAO for 30 ($n = 5$) or 45 min ($n = 6$). Control animals received 1 ml kg^{-1} saline ($n = 5$, 30 min CAO or $n = 6$, 45 min CAO). One group of animals undergoing 45 min CAO was treated with a control antibody, OKM1 (1 mg kg^{-1}, i.v., $n = 7$). OKM1 is also a murine IgG_2 antibody but it binds to CD11b on the leukocyte surface without inhibiting binding of these cells to endothelial cells (Wallis *et al.*, 1986). Animals were assigned randomly to control or treatment groups.

Measurement of circulating neutrophil numbers

Arterial blood samples were taken into 0.01 M EDTA at the following time points: pre-occlusion, prior to antibody administration and at 60, 120 and 180 min of reperfusion. The total leukocyte count and the % of neutrophils present were determined with a haemocytometer and by differential staining respectively. From these values the number of circulating neutrophils was calculated.

Materials

Monastral blue dye, TTC, HETAB, tetramethylbenzidine, bradykinin and FMLP were obtained from Sigma Chemical Company, Dorset. ^{125}I-labelled human serum albumin was obtained from Amersham International, Buckinghamshire. Sodium pentobarbitone was obtained from RMB Animal Health Ltd, Essex. IB4 and OKM1 were provided by Dr E. MacIntyre, Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey, U.S.A.

Data analysis

Results are expressed as the mean ± s.e.mean of *n* observations. Statistical analysis was carried out by analysis of variance.

Results

Occlusion of the left main coronary artery was confirmed by changes in the colour of the distal portion of the artery and of the epicardial surface. Characteristic alterations in the ECG, which are indicative of myocardial ischaemia, were seen in all animals. Of the 29 animals that entered the study, two suffered irreversible ventricular fibrillation during CAO (one each in the saline and OKM1 groups) and one animal receiving OKM1 developed profound hypotension during reperfusion.

Myeloperoxidase measurements

In control animals receiving saline, CAO for either a 30 or 45 min period followed by 3 h of reperfusion, resulted in a marked increase in the concentration of myeloperoxidase activity in AR myocardium compared with the NZ (Figure 1). This increase in MPO activity in the AR was not affected by administration of the control antibody, OKM1. However, administration of IB4 resulted in a substantial and significant

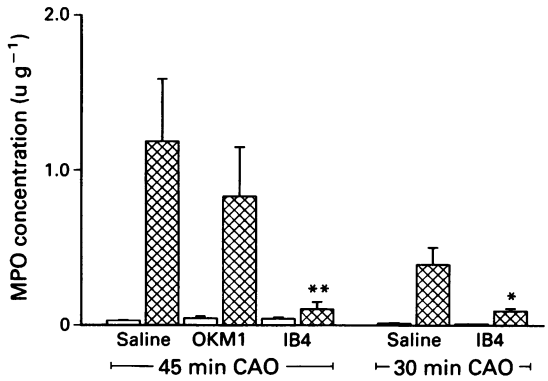


Figure 1 The concentration of myeloperoxidase (MPO) in normal zone (NZ, open columns) and area at risk (AR, cross-hatched columns) myocardium from animals undergoing 30 or 45 min coronary artery occlusion (CAO) and 3 h reperfusion. Animals were treated with saline (1 ml kg^{-1}, $n = 5$), OKM1 (1 mg kg^{-1}, $n = 5$) or IB4 (1 mg kg^{-1}, $n = 5$, 30 min CAO and $n = 6$, 30 min CAO). $^{*}P < 0.05$; $^{**}P < 0.01$ treated vs control.

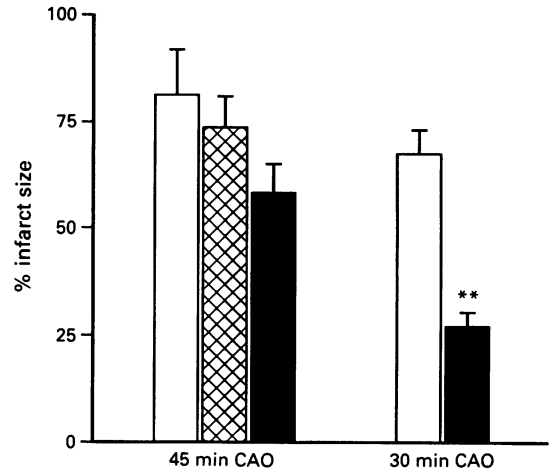


Figure 2 Infarct size as a % of the area at risk following 30 or 45 min coronary artery occlusion (CAO) and 3 h reperfusion in animals treated with saline (1 ml kg^{-1}, open columns, $n = 5$), OKM1 (1 mg kg^{-1}, cross-hatched columns, $n = 5$) or IB4 (1 mg kg^{-1}, filled columns, $n = 5$, 30 min CAO and $n = 6$, 45 min CAO). $^{**}P < 0.01$ IB4 vs control.

reduction in this response following both 30 and 45 min periods of CAO (Figure 1).

Myocardial infarct size

The AR was expressed as a % of the area of the left ventricle and infarct size was expressed as a % of the AR. Following the 30 min period of CAO, IB4 significantly reduced myocardial infarct size, 27.2 ± 3.2% compared with the saline control group, 67.4 ± 5.6% (Figure 2, $n = 5$, $P < 0.01$). There was no difference in the size of the AR for these two groups, 38.9 ± 3.4 and 32.0 ± 4.0% respectively. In contrast, infarct size resulting from a 45 min period of CAO, was not significantly reduced by the administration of IB4, 58.3 ± 6.7% ($n = 6$) compared with that of animals receiving either saline, 81.8 ± 8.7% ($n = 5$) or the control antibody, OKM1, 73.6 ± 7.3% ($n = 5$, Figure 2). The AR in the IB4 group, 59.9 ± 6.3% did not differ from that in the OKM1, 53.9 ± 7.9% and saline-treated groups, 46.8 ± 7.6%.

Plasma protein leakage in ischaemic/reperfused myocardium

In the control group, myocardial plasma volume increased by 254% from 54.6 ± 7.0 µl g^{-1} in the NZ to 192.7 ± 25.3 µl g^{-1} in the AR ($P < 0.01$, $n = 5$, Figure 3a) following a period of 30 min CAO and 3 h reperfusion. Administration of IB4 had no effect on this oedema formation (Figure 3), the plasma volume in the AR, 170.9 ± 35.3 µl g^{-1} being 307% greater than that in the NZ, 46.2 ± 9.6 µl g^{-1} ($P < 0.01$, $n = 5$). IB4 was similarly without effect on myocardial oedema formation following the longer 45 min period of CAO (results not shown).

Plasma protein leakage in response to intradermal administration of inflammatory mediators

Both FMLP and bradykinin elicited local oedema formation when injected into skin. The plasma volume of FMLP-injected sites was significantly reduced from 24.0 ± 1.2 µl in control animals compared with 14.8 ± 2.2 µl g^{-1} in rabbits treated with IB4 (Figure 3b, $n = 5$, $P < 0.01$). The values in the IB4-treated group were indistinguishable from those in saline-injected sites. In contrast, plasma protein leakage in response to the directly-acting mediator, bradykinin was unaffected by administration of IB4.

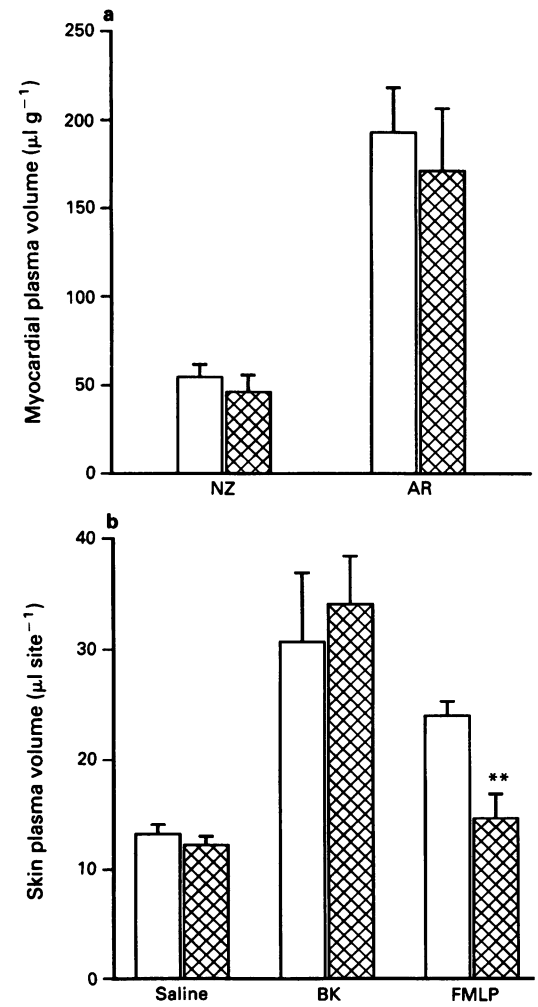


Figure 3 Plasma volume of (a) normal zone (NZ) and area at risk (AR) myocardium following 30 min coronary artery occlusion (CAO) and 3 h reperfusion and of (b) skin injected with saline, bradykinin (BK, 1×10^{-10} mol per site) or FMLP (5×10^{-11} mol per site) in animals receiving saline (open columns) or IB4 (1 mg kg^{-1}, cross-hatched columns, $n = 5$). $^{*}P < 0.01$ IB4 vs control.

Table 1 Number of circulating neutrophils in control and IB4-treated animals undergoing coronary artery occlusion (CAO) and reperfusion

		No. of neutrophils ml^{-1}		
Treatment	n	*Pretreatment*	*1 h reperfusion*	*3 h reperfusion*
Saline (30' CAO)	5	1.6 ± 0.5	1.8 ± 0.3	3.4 ± 0.7
IB4 (30' CAO)	5	1.0 ± 0.2	2.7 ± 0.8	3.9 ± 1.2
Saline (45' CAO)	5	1.8 ± 0.5	2.4 ± 0.6	2.4 ± 0.8
OKM1 (45' CAO)	5	1.3 ± 0.2	1.6 ± 0.6	2.4 ± 0.6
IB4 (45' CAO)	6	0.7 ± 0.2	1.9 ± 0.5	2.0 ± 0.6

Table 2 Systemic haemodynamic measurements in control and IB4-treated animals

		Mean arterial blood pressure (mmHg)			*Heart rate* (beats min^{-1})		
Treatment	n	*Pre-occlusion*	*30' CAO*	*3 h REP*	*Pre-occlusion*	*30' CAO*	*3 h REP*
Saline (30' CAO)	5	70 ± 7	73 ± 8	71 ± 6	230 ± 9	225 ± 14	180 ± 17*
IB4 (30' CAO)	5	74 ± 6	71 ± 4	68 ± 3	248 ± 7	231 ± 3	232 ± 15
Saline (45' CAO)	5	62 ± 3	65 ± 3	65 ± 3	212 ± 12	208 ± 8	203 ± 7
OKM1 (45' CAO)	5	68 ± 2	68 ± 4	64 ± 5	230 ± 13	224 ± 10	215 ± 14
IB4 (45' CAO)	6	72 ± 2	70 ± 2	71 ± 3	229 ± 9	222 ± 9	217 ± 7

Values are means ± s.e.mean.
*$P < 0.05$ 3 h REP vs Pre-occlusion.

Circulating neutrophil numbers

The number of circulating neutrophils in each group is shown in Table 1. Administration of IB4 did not result in a significant alteration in the number of circulating neutrophils. The control antibody, OKM1 was also without effect on this parameter. None of the experimental groups differed in the number of neutrophils present in blood samples at any of the measurement time points.

Blood pressure and heart rate measurements

Blood pressure and heart rate measurements for the control and treated groups are shown in Table 2. Neither of these parameters was altered during the course of the experiment in any of the treatment groups with the exception of heart rate in one of the control groups, which was significantly lower at 3 h of reperfusion.

Incidence of ventricular arrhythmias

Ventricular ectopic beats were observed in most rabbits during either CAO or reperfusion, although the incidence of the more severe arrhythmias was low in all groups. There was no significant difference in the incidence of arrhythmias in any of the treatment groups.

Discussion

In this study we have examined the effect of administration of an antibody to the leukocyte CD18 adhesion molecule on both neutrophil accumulation and infarct size following myocardial ischaemia and reperfusion. In addition, we have also examined whether the outcome of this intervention is dependent on the duration of the ischaemic period.

Measurement of MPO activity is well established as a method for the quantification of neutrophil accumulation in myocardial tissue (Bednar *et al.*, 1985; Mullane *et al.*, 1985). In saline-treated rabbits both the 30 and 45 min periods of CAO resulted in a substantial increase in the MPO concentration in the AR, indicating neutrophil accumulation. Whilst the control antibody, OKM1 did not affect the increase in MPO concentration, administration of IB4 resulted in a marked suppression of this response following both ischaemic periods. Administration of IB4 did not, however, affect the number of circulating neutrophils. The small residual increase in MPO activity in IB4-treated animals may reflect non-specific trapping of neutrophils.

Administration of IB4 towards the end of the 30 min period of CAO also resulted in a significant reduction in myocardial infarct size, suggesting that infiltrating neutrophils have a deleterious effect on reperfused tissue. When activated, neutrophils release proteolytic enzymes and free radicals (Weiss, 1989) and these toxic agents may well cause further damage to myocytes within the AR. However, extending the duration of ischaemia to 45 min resulted in a loss of the protective effect of IB4 on infarct size. The concentration of MPO measured in the AR of IB4-treated animals was, however, comparable for both occlusion periods. These results suggest that although infiltrating neutrophils contribute to myocyte injury following a relatively short ischaemic period of 30 min, the role of these leukocytes in reperfusion injury appears to be less important following a longer 45 min period of ischaemia. A possible reason for this is that the extent of direct ischaemic injury to myocytes following 45 min CAO in rabbit heart may be so great that further exacerbation by activated neutrophils is not detectable.

Previous studies examining the role of neutrophils in myocardial reperfusion injury have given mixed results. Whereas administration of anti-neutrophil serum has been reported to reduce myocardial infarct size in some experimental studies (Romson *et al.*, 1983; Jolly *et al.*, 1986; Simpson *et al.*, 1988a) it was ineffective in others (Jolly *et al.*, 1986; Chatelain *et al.*, 1987). Administration of monoclonal antibodies to leukocyte adhesion molecules has likewise been reported to reduce infarct size in some studies (Simpson *et al.*, 1988b; Ma *et al.*, 1991); however, Tanaka *et al.* (1993) recently reported that IB4 was ineffective in a canine model of acute myocardial infarction. The majority of these studies were carried out using dogs which have a relatively high, but variable, component of collateral blood flow in the myocardium. The absence of measurements of myocardial blood flow in many of these studies has been suggested as a possible reason for the contradictory results reported in the literature (Reimer *et al.*, 1989) since there could be differences in the level of collateral blood flow in control and treated groups. This was not considered to be a problem in our study since rabbits have been shown to have negligible coronary collateral blood flow (Maxwell *et al.*, 1987).

In agreement with our findings that IB4 was only effective with a short period of ischaemia, other studies in which

protection was reported with anti-neutrophil interventions were characterized by a shorter duration of coronary artery occlusion (Litt *et al.*, 1989; de Lorgeril *et al.*, 1989; Simpson *et al.*, 1990), compared with most of those reporting no effect (Reimer *et al.*, 1985; Chatelain *et al.*, 1987). Furthermore, Jolly *et al.* (1986) have previously reported that protection with anti-neutrophil antiserum in a canine model of acute myocardial infarction was lost when the duration of ischaemia was extended from 90 min to 4 h. Thus, there appears to be a difference between dogs and rabbits regarding the duration of ischaemia which can be tolerated. In species such as dogs in which there is a relatively high collateral blood flow, it is likely that the window of opportunity for use of anti-neutrophil interventions will be longer than in those with low collateral flow such as rabbits.

In most of the studies carried out using anti-neutrophil interventions, including that of Jolly *et al.* (1986), treatment was instituted prior to CAO. An important feature of our study is that administration of IB4 was made towards the end of the ischaemic period. Protection afforded by IB4 therefore appears to be exerted during the reperfusion period. Treatment just prior to reperfusion has also been shown to reduce myocardial infarct size in cats following administration of an anti-CD18 antibody (Ma *et al.*, 1991) and in dogs with anti-leukocyte filters (Litt *et al.*, 1989).

Administration of IB4 completely inhibited plasma protein leakage in response to intradermal injection of the neutrophil-dependent mediator FMLP whilst that elicited by bradykinin, a neutrophil-independent mediator, was unaffected. However, IB4 treatment did not reduce the oedema formation seen in the AR myocardium. This is in agreement with previous findings with this model, in which depletion of circulating neutrophils using either a polyclonal anti-neutrophil serum or a nitrogen mustard was found to be ineffective in reducing oedema formation in ischaemic/reperfused myocardium (Williams *et al.*, 1990). This oedema formation appears therefore to be due either to the release of neutrophil-independent mediators of increased microvascular permeability, or to direct injury to the vascular endothelium.

In conclusion, we have found that administration of IB4 prior to reperfusion significantly reduces myocardial infarct size following 30 min coronary artery occlusion in the anaesthetized rabbit. However, this protective action was lost when a longer 45 min occlusion period was used. This suggests that infiltrating neutrophils do exacerbate myocardial tissue injury following ischaemia and reperfusion but, in terms of potential therapy targeted at the neutrophil, the window of opportunity may be limited to relatively short periods of ischaemia.

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References

ARFORS, K.-E., LUNDBERG, C., LINDBOM, L., LUNDBERG, K., BEATTY, P.G. & HARLAN, J.M. (1987). A monoclonal antibody to the membrane glycoprotein complex CD18 inhibits polymorphonuclear leukocyte accumulation and plasma leakage in vivo. *Blood*, **69**, 338–340.

BEDNAR, M., SMITH, B., PINTO, A. & MULLANE, K.M. (1985). Nafazatrom-induced salvage of ischemic myocardium in anaesthetized dogs is mediated through inhibition of neutrophil function. *Circ. Res.*, **57**, 131–141.

CHATELAIN, P., LATOUR, J.-G., TRAN, D., LE LORGERIL, D., DUPRAS, G. & BOURASSA, M. (1987). Neutrophil accumulation in experimental myocardial infarcts: relation with extent of injury and effect of reperfusion. *Circulation*, **75**, 1083–1090.

DE LORGERIL, M., BASMADJIAN, A., LAVALLEE, M., CLEMENT, R., MILLETTE, D., ROUSSEAU, G. & LATOUR, J.-G. (1989). Influence of leukopenia on collateral flow, reperfusion flow, reflow ventricular fibrillation, and infarct size in dogs. *Am. Heart J.*, **117**, 523–532.

HARLAN, J.M., KILLEN, P.D., SENECAL, F., SCHWARTZ, B.R., YEE, E.K., TAYLOR, R.F., BEATTY, P.G., PRICE, T. & OCHS, H.D. (1985). The role of neutrophil membrane glycoprotein GP-150 in neutrophil adherence to endothelium in vitro. *Blood*, **66**, 167–178.

JOLLY, S.R., KANE, W.J., HOOK, B.G., ABRAMS, G.D., KUNKEL, S.L. & LUCCHESI, B.R. (1986). Reduction of myocardial infarct size by neutrophil depletion: effect of duration of occlusion. *Am. Heart J.*, **112**, 682–690.

LITT, M.R., JEREMY, R.W., WEISMAN, H.F., WINKELSTEIN, J.A. & BECKER, L.C. (1989). Neutrophil depletion limited to reperfusion reduces myocardial infarct size after 90 minutes of ischemia. Evidence for neutrophil-mediated reperfusion injury. *Circulation*, **80**, 1816–1827.

LO, S.K., DETMERS, P.A., LEVIN, S.M. & WRIGHT, S.D. (1989). Transient adhesion of neutrophils to endothelium. *J. Exp. Med.*, **169**, 1779–1793.

LUNDBERG, C. & WRIGHT, S.D. (1990). Relation of the CD11/CD18 family of leukocyte antigens to the transient neutropenia caused by chemoattractants. *Blood*, **76**, 1240–1245.

MA, X.-L., TSAO, P.S. & LEFER, A.M. (1991). Antibody to CD-18 exerts endothelial and cardiac protective effects in myocardial ischemia and reperfusion. *J. Clin. Invest.*, **88**, 1237–1243.

MALLORY, G.K., WHITE, P.D. & SALCEDO-SALGAR, J. (1939). The speed of healing of myocardial infarction. A study of the pathologic anatomy in seventy-two cases. *Am. Heart J.*, **18**, 647–671.

MAXWELL, M.P., HEARSE, D.J. & YELLON, D.M. (1987). Species variation in the coronary collateral circulation during regional myocardial ischaemia: a critical determinant of the rate of evolution and extent of myocardial infarction. *Cardiovasc. Res.*, **21**, 737–746.

MULLANE, K.M., KRAEMER, R. & SMITH, B. (1985). Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. *J. Pharmacol. Methods*, **14**, 157–167.

MULLANE, K.M., READ, N., SALMON, J.A. & MONCADA, S. (1984). Role of leukocytes in acute myocardial infarction in anesthetized dogs: relationship to myocardial salvage by anti-inflammatory drugs. *J. Pharmacol. Exp. Ther.*, **228**, 510–522.

NOURSHARGH, S., RAMPART, M., HELLEWELL, P.G., JOSE, P.J., HARLAN, J.M., EDWARDS, A.J. & WILLIAMS, T.J. (1989). Accumulation of ^{111}In-neutrophils in rabbit skin in allergic and non-allergic inflammatory reactions in vivo: inhibition by neutrophil pretreatment in vitro with a monoclonal antibody recognising the CD18 antigen. *J. Immunol.*, **142**, 3193–3198.

REIMER, K.A., JENNINGS, R.B., COBB, F.R., MURDOCK, R.H., GREENFIELD, J.C., BECKER, L.C., BULKLEY, B.R., HUTCHINS, G.M., SCHWARTZ, R.P., BAILEY, K.R. & PASSAMANI, E.R. (1985). Animal models for protecting ischemic myocardium: results of the NHLBI cooperative study. Comparison of unconscious and conscious dog models. *Circ. Res.*, **56**, 651–665.

REIMER, K.A., MURRY, C.E. & RICHARD, V.J. (1989). The role of neutrophils and free radicals in the ischemic-reperfused heart: why the confusion and controversy? *J. Mol. Cell Cardiol.*, **21**, 1225–1239.

ROMSON, J.L., HOOK, B.G., KUNKEL, S.L., ABRAMS, G.D., SCHORK, M.A. & LUCCHESI, B.R. (1983). Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. *Circulation*, **67**, 1016–1023.

ROMSON, J.L., HOOK, B.G., RIGOT, V.H., SCHORK, M.A., SWANSON, D.P. & LUCCHESI, B.R. (1982). The effect of ibuprofen on accumulation of indium-111-labelled platelets and leukocytes in experimental myocardial infarction. *Circulation*, **66**, 1002–1011.

RUBIN, B.B., SMITH, A., LIAUW, S., ISENMAN, D., ROMASCHIN, A.D. & WALKER, P.M. (1990). Complement activation and white cell sequestration in postischemic skeletal muscle. *Am. J. Physiol.*, **259**, H525–H531.

SIMPSON, P.J., FANTONE, J.C., MICKELSON, J.K., GALLAGHER, K.P. & LUCCHESI, B.R. (1988a). Identification of a time window for therapy to reduce experimental canine myocardial injury: suppression of neutrophil activation during 72 hours of reperfusion. *Circ. Res.*, **63**, 1070–1079.

SIMPSON, P.J., MITSOS, S.E., VENTURA, A., GALLAGHER, K.P., FANTONE, J.C., ABRAMS, G.D., SCHORK, M.A. & LUCCHESI, B.R. (1987). Prostacyclin protects ischemic reperfused myocardium in the dog by inhibition of neutrophil activation. *Am. Heart J.*, **113**, 129–137.

SIMPSON, P.J., TODD III, R.F., FANTONE, J.C., MICKELSON, J.K., GRIFFIN, J.D. & LUCCHESI, B.R. (1988b). Reduction of experimental canine myocardial reperfusion injury by a monoclonal antibody (Anti-Mol, Anti-CD11b) that inhibits leukocyte adhesion. *J. Clin. Invest.*, **81**, 624–629.

SIMPSON, P.J., TODD, R.F., MICKELSON, J.K., FANTONE, J.C., GALLAGHER, K.P., LEE, K.A., TAMURA, Y., CRONIN, M. & LUCCHESI, B.R. (1990). Sustained limitation of myocardial reperfusion injury by a monoclonal antibody that alters leukocyte function. *Circulation*, **81**, 226–237.

TANAKA, M., BROOKS, S.E., RICHARD, V.J., FITZHARRIS, G.P., STOLER, R.C., JENNINGS, R.B., ARFORS, K.-E. & REIMER, K.A. (1993). Effect of anti-CD18 antibody on myocardial neutrophil accumulation and infarct size after ischemia and reperfusion in dogs. *Circulation*, **87**, 526–535.

THORNTON, J., STRIPLIN, S., LIU, G.S., SWAFFORD, A., STANLEY, A.W.H., VAN WINKLE, D.M. & DOWNEY, J.M. (1990). Inhibition of protein synthesis does not block myocardial protection afforded by preconditioning. *Am. J. Physiol.*, **259**, H1822–H1825.

TUOMANEN, E.I., SAUKKONEN, K., SANDE, S., CIOFFE, C. & WRIGHT, S.D. (1989). Reduction of inflammation, tissue damage and mortality in bacterial meningitis in rabbits treated with monoclonal antibodies against adhesion-promoting receptors of leucocytes. *J. Exp. Med.*, **170**, 959–968.

WALLIS, W.J., HICKSTEIN, D.D., SCHWARTZ, B.R., JUNE, C.H., OCHS, H.D., BEATTY, P.G., KLEBANOFF, S.J. & HARLAN, J.M. (1986). Monoclonal antibody-defined functional epitopes on the adhesion-promoting glycoprotein complex (CDw18) of human neutrophils. *Blood*, **67**, 1007–1013.

WEDMORE, C.V. & WILLIAMS, T.J. (1981). Control of vascular permeability by polymorphonuclear leukocytes in inflammation. *Nature*, **289**, 646–650.

WEISS, S.J. (1989). Tissue destruction by neutrophils. *N. Engl. J. Med.*, **320**, 365–375.

WILLIAMS, F.M., COLLINS, P.D., TANNIERE-ZELLER, M. & WILLIAMS, T.J. (1990). The relationship between neutrophils and increased microvascular permeability in a model of myocardial ischaemia and reperfusion in the rabbit. *Br. J. Pharmacol.*, **100**, 729–734.

WRIGHT, S.D., RAO, P.E., VAN VOORHIS, W.C., CRAIGMYLE, L.S., IIDA, K., TALLE, M.A., WESTBERG, E.F., GOLDSTEIN, G. & SILVERSTEIN, S.C. (1983). Identification of the C3bi receptor on human monocytes and macrophages by using monoclonal antibodies. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 5699–5703.

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Functional effects and ligand binding of chimeric galanin-neuropeptide Y (NPY) peptides on NPY and galanin receptor types

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1 The effects and binding characteristics of a series of chimeric galanin-neuropeptide Y (NPY) peptides were examined in various preparations known to contain a predominant population of either Y_1 or Y_2 receptors for NPY or galanin receptors.

2 NPY suppressed the electrically stimulated twitches of the rat vas deferens (Y_2 receptors), while galanin enhanced the electrically stimulated twitches. The galanin-NPY peptides M 32 (galanin(1-13)-NPY(25-36)), M69A (galanin(1-13)-Lys-[εNH-Gly-NPY(4-1)]NPY(25-36)) and M88 (galanin(1-12)-Ala-NPY(25-36)) evoked a concentration-dependent suppression of the electrically stimulated twitches. These chimeric peptides were about equipotent with NPY, while NPY (13-36) was about five times less potent than NPY itself. Also a stochiometric combination of the N- and C-terminal fragments NPY (1-24)NH_2 and NPY (25-36) (each at 1 μM) was inactive in vas deferens. M120 (galanin (1-13)-NPY(14-36) (1 μM) did not affect the NPY-mediated suppression of the stimulated twitches.

3 NPY evoked a concentration-dependent contraction in the guinea-pig isolated caval vein (Y_1 receptors), while galanin ($\leqslant 1$ μM) was inactive. M32, M69A and M88 induced a slight contraction at very high concentrations only ($\geqslant 0.3$ M), while M120 was inactive at 1 μM. None of the four chimeric peptides affected the contraction evoked by NPY.

4 Since the number of NPY receptors in the rat vas deferens and guinea-pig caval vein were too low, the affinities of the galanin-NPY peptides for [^{3}H]-NPY binding sites were examined in membranes from rat brain areas known to contain predominant populations of Y_1 receptors (cerebral cortex) and Y_2 receptors (hippocampus), respectively. The chimeric peptides M32, M69A, M88, M120 and NPY (13-36) all had higher affinities for hippocampal binding sites than for cerebral cortical binding sites. These peptides were 90–440 times less potent than NPY at cerebral cortical binding sites and 15–125 times less potent than NPY at hippocampal binding sites. The most selective chimeric peptide was M32, which had a 20 fold higher affinity for hippocampal than for cerebral cortical binding sites.

5 At hypothalamic [^{125}I]-galanin binding sites M32, M88 and M69A were equipotent with galanin, while M120 was about 10 times less potent than galanin. M32, M88 and M69A, like galanin contracted the rat isolated jejunum.

6 The N-terminal portion (1–12) of galanin seems to permit a steric conformation of the attached NPY (25-36) part of the chimeric galanin-NPY peptides, which results in a facilitated Y_2 but not Y_1 receptor recognition and activation. None of the galanin-NPY peptides appeared to act as antagonists at either type of NPY receptor, probably due to their low affinity. Instead, they displayed a very high affinity for hypothalamic galanin receptors and probably act as galanin agonists in the rat jejunum.

Keywords: Neuropeptide Y; galanin; ligand binding; chimeric peptides; neuropeptide Y receptors; vas deferens; blood vessels; brain

Introduction

Neuropeptide Y (NPY) occurs in many central and peripheral neurones, including a prominent population of sympathetic neurones (e.g. Sundler *et al.*, 1986; De Quidt *et al.*, 1986) from which NPY is released in conjunction with noradrenaline. At the sympathetic neuroeffector junction NPY activates receptors referred to as Y_1 and Y_2 (for a review see Grundemar & Håkanson, 1993). The postjunctional receptor, which is mainly of the Y_1 type, causes vasoconstriction (cf. Grundemar *et al.*, 1992; 1993b). The Y_1 receptor is quite stringent in its demand on the N-terminal end of the NPY-molecule; the loss of Tyr[1] in (NPY 2-36) results in a marked loss of potency and shorter fragments are several orders of magnitude less potent than NPY itself (Grundemar *et al.*, 1992). Recently, the rat and human Y_1 receptor has been cloned and shown to belong to the family of G-protein coupled receptors (Eva *et al.*, 1990; Herzog *et al.*, 1992; Larhammar *et al.*, 1992). Pharmacological studies using cell cultures expressing cloned Y_1 receptors have confirmed the necessity for an intact N-terminal portion of NPY (Krause *et al.*, 1992; Larhammar *et al.*, 1992). The prejunctional NPY receptor, which is of the Y_2 type, is known to suppress the release of transmitters from sympathetic nerve endings (e.g. Wahlestedt *et al.*, 1986; Grundemar & Håkanson, 1990). In contrast to the Y_1 receptor, the Y_2 receptor recognizes a wide range of C-terminal NPY fragments (Grundemar & Håkanson, 1990). A common feature of both Y_1 and Y_2 receptors is the requirement for an amidated NPY C-terminus (Tyr^{36}-NH_2) (cf. Grundemar *et al.*, 1993b).

Several attempts have been made to synthesize Y_1 and Y_2 receptor-selective ligands. [Pro^{34}]NPY and variants thereof have been shown to interact with the Y_1 but not with the Y_2 receptor (Fuhlendorff *et al.*, 1990; Krstenansky *et al.*, 1990; Grundemar *et al.*, 1993a,b). Fairly selective Y_2 receptor agonists have been developed based on NPY analogues having the centre part of the molecule substituted by a spacer sequence (Beck *et al.*, 1989; Krstenansky *et al.*, 1989; 1990; Beck-Sickinger *et al.*, 1992; Grundemar *et al.*, 1993a). How-

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ever, none of these analogues seem to be more selective or potent than NPY (13-36), which is virtually inactive at Y_1 receptors and about 10 times less potent than NPY (1-36) at Y_2 receptors (e.g. Grundemar *et al.*, 1993b).

Some chimeric peptides have recently been shown to behave as potent antagonists at galanin receptors (Bartfai *et al.*, 1991; Langel *et al.*, 1992) and at vasoactive intestinal polypeptide (VIP) receptors (Gozes *et al.*, 1991), respectively. In these cases the recognition units of the peptides (such as galanin (1-12) or VIP (7-28) were joined to another peptide to yield high affinity peptide antagonists for galanin receptors and for VIP receptors, respectively. We have synthesized a series of galanin-NPY chimeric peptides, based on the information that at least the Y_2 type NPY receptors recognize a range of C-terminal NPY fragments (Abens *et al.*, 1989; Grundemar & Håkanson, 1990) and that galanin receptors require for recognition the N-terminal (1-12) fragment of galanin (Land *et al.*, 1991).

The chimeric peptide, M32 (galanin (1-13)-NPY (25-36)) is a high affinity galanin receptor ligand and results from a recent ^{1}H n.m.r. study (Arvidsson *et al.*, 1993) have suggested that this peptide may assume a hair pin loop similar to that of NPY (cf. Schwartz *et al.*, 1990). The structural similarity of the galanin-NPY analogue M32 to NPY suggested that it may act as a ligand at NPY receptors. We have examined possible interactions of M32 and related chimeric galanin-NPY peptides in various experimental set ups known to contain either of the two NPY receptor types, Y_1 and Y_2. In addition, we describe some preliminary results regarding the ability of these peptides to interact with galanin receptors.

Methods

Functional experiments

Male Sprague-Dawley rats (200–250 g) were killed and the prostatic segment of each vas deferens was cut out and placed in Krebs solution of the following composition (mM): NaCl 133, $NaHCO_3$ 16.3, KCl 4.7, $MgCl_2$ 1.0, NaH_2PO_4 1.4, $CaCl_2$ 2.5 and glucose 7.8. Segments (1.5 cm in length) were mounted vertically on Perspex holders in a 7 ml tissue bath maintained at 33°C. The solution was bubbled continuously with 95% O_2 and 5% CO_2, yielding a pH of 7.4. The mechanical activity was recorded isometrically with a Grass FT03 force displacement transducer and a Grass model 7 polygraph. The preparations were allowed to equilibrate for about 60 min with a tension of about 10 mN. Electrical field (submaximal) stimulation with square wave pulses (25 V, 1 ms and 0.15 Hz) was then applied by means of a pair of platinum electrodes connected to a Grass S4C stimulator. As soon as a series of pulses gave identical responses, increasing doses of a peptide were added in a cumulative manner. The preparation was stimulated repeatedly until the effect of each concentration of peptide attained a stable level. The inhibition of the electrically evoked twitches for each peptide concentration was expressed as a percentage of the response of a parallel control preparation not exposed to peptides. A new concentration of each peptide was added every 5 min.

Caval vein Guinea-pigs (Dunklin-Hartley, 350–540 g) were killed and the proximal part of the inferior caval vein close to the right auricle was removed. The vessel was divided into three circular segments (each 2 mm wide) which were transferred to a constant temperature bath (37°C), containing Krebs solution of the following composition (in mM): NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15.0, NaH_2PO_4 1.2 and D-glucose 6.0. The solution was bubbled continuously with 95% O_2 and 5% CO_2, yielding a pH of 7.4. The vascular segments were mounted on two L-shaped stainless steel wires (0.1 mm diameter), one of which was connected to a force-displacement transducer (Grass Instrument FT 03C) for continuous tension recording on a Grass polygraph (Högestätt *et al.*, 1983). The resting tension was gradually adjusted to 2 mN. The contractile capacity of the blood vessel was examined by adding an isotonic 124 mM K^+ Krebs solution (NaCl replaced by KCl); the resulting contraction which was maximal served as an internal control. After washing out the K^+-enriched solution, increasing doses of the different peptides were added in a cumulative manner. Each vascular segment was exposed to one peptide only. Contractile responses were expressed in relation to the initial K^+-evoked contraction. The IC_{50} values for each peptide (concentration eliciting 50% of the maximum response) were estimated by linear interpolation of the concentration-response curve in the 10–90% range.

Since bacitracin (1 mg ml^{-1}) did not affect the responses to the peptides, the peptidase inhibitor was not present in subsequent smooth muscle experiments.

Binding experiments

Male Sprague-Dawley rats (180–200 g) were decapitated and the hippocampus, cerebral cortex and hypothalamus were rapidly dissected and frozen at − 70°C. The tissue was homogenized on ice with a tight fitting teflon-glass homogenizer in 0.32 M sucrose buffered with 5 mM HEPES (pH 7.4). The homogenate was diluted and centrifuged at 1000 *g* for 10 min. The supernatant was centrifuged at 10,000 *g* for 45 min and the pellet was resuspended in 5 mM HEPES buffered binding buffer (HEPES-RBB) containing (in mM): NaCl 137, KCl 2.68, $MgCl_2$ 2.05, $CaCl_2$ 1.8, D-glucose 5.6, supplemented with bovine serum albumin 0.05% (w/v) and bacitracin 1 mg ml^{-1}, pH 7.4.

The experiments were performed in a final volume of 400 µl 5 mM HEPES-RBB containing 0.4–0.9 nM N[propionyl-^{3}H]-NPY or [^{125}I]-galanin (Amersham), membranes and increasing concentrations of unlabelled peptides (10 pM–10 µM). Samples were incubated for 45 min at 37°C in a shaking water bath. The incubation was terminated by the addition of 2 × 10 ml of ice cold 5 mM HEPES-RBB, followed by rapid filtration over Whatman GF/C glass-fibre filters precoated 3–4 h in 0.3% (w/v) polyethylenimine solution. Radioactivity retained on the filters was determined in a Packard scintillation spectrometer counter. The specific binding amounted to 55–65% of the total binding. All tubes and pipette tips used were silanized before the experiments with Sigmacote from Sigma. IC_{50} values were calculated by non-linear regression analysis (Kaleidagraph Macintosh) and corrected as described by Cheng & Prusoff (1973) to yield K_D values.

Peptide synthesis

The linear chimeric peptides were assembled in a stepwise manner in a solid support (MBHA resin to achieve the C-terminally amidated peptides) using t Boc chemistry on an Applied Biosystems Model 431A Peptide Synthesizer on a 0.1 mmol (small) scale. The synthesis, cleavage from resin and purification of the peptides have been described in detail before (Land *et al.*, 1991; Langel *et al.*, 1992). Purity of the individual peptides was checked by analytical h.p.l.c. on a Nucleosil 120-3 C_{18} reversed-phase h.p.l.c. columns (0.4 × 10 cm) and determined using Pharma Desorption Mass Spectrometer (PDMS) Model Bioion 20, Applied Biosystems, the calculated values were obtained in each case.

For synthesis of the branched peptide, M69A, solid phase peptide synthesis was carried out in the conventional way (see above) to produce [$N^{\alpha,1}$-tert-Boc-$N^{\varepsilon,14}$-Fmoc-[Lys^{14}]]-galanin(1-13)-Lys-NPY(25-36)-MBHA resin. The εNH-Fmoc protective group on Lys^{14} was removed by treating the peptide resin 20% piperidine (v/v) in N,N-dimethylformamide for 20 min. After that, the sequence GKSPY was coupled manually stepwise by adding 3 eq of HObt- and DCC-activated Fmoc-amino acids in N,N-dimethylformamide; the coupling was complete after 30 min at room temperature as

estimated by ninhydin test. After coupling of Fmoc-amino acids, the resin was deprotected, peptide cleaved from the resin, purified and characterized as described above.

The following was also used: porcine (p)NPY, (Richeliux Biotech, QC, Canada), (p) galanin (Peninsula Lab Europe Ltd., UK); (p) NPY 13-36 was synthesized by solid-phase synthesis and purified to at least 96% by h.p.l.c. (W. Krzeminsky, Ferring AB, Malmö, Sweden).

Results

Effects on the rat vas deferens

We confirmed that NPY suppressed, while galanin enhanced electrically stimulated twitches (Lundberg & Tatemoto, 1982; Ohhashi & Jacobowitz, 1985). The galanin-NPY peptides M32 (galanin(1-13)-NPY(25-36)),M69A (galanin (1-13)-Lys-[εNH-Gly-NPY(4-1)]NPY(25-36)) and M88 (galanin(1-12)-Ala-NPY(25-36)) evoked a concentration-dependent suppression of the electrically-stimulated twitches. For the primary structure of the chimeric peptides, see Table 1. The three chimeric peptides were about equipotent with NPY (Figure 1 and Table 2). In comparison, NPY (13-36) was about five times less potent than NPY itself (Figure 1 and Table 2). Also a stochiometric combination of the N- and C-terminal fragments NPY (1-24)NH_2 and NPY (25-36) (each at 1 μM) was inactive ($n = 4$) (data not shown). M120 (galanin (1-13)-NPY(14-36)) (1 μM) did not affect the NPY-evoked suppression of the stimulated twitches ($n = 4$) (data not shown).

Effects on the guinea-pig caval vein

As shown in Figure 2, NPY ($\leqslant 0.1$ μM) evoked a concentration-dependent contraction in the caval vein, while galanin ($\leqslant 1$ μM) was inactive. M32, M69A and M88 induced slight contractions at concentrations $\geqslant 0.3$ μM, while M120, was inactive at 1 μM (Figure 2). None of the four chimeric peptides (each peptide at 1 μM) affected the contraction evoked by NPY (data not shown).

Affinity for NPY binding sites in rat brain

The four chimeric galanin-NPY peptides were examined as ligands at [^{3}H]-NPY binding sites in areas known to contain predominant populations of Y_1 receptors (cerebral cortex) and Y_2 receptors (hippocampus). Table 3 and Figure 3 shows that while NPY bound with equally high affinity to cortical

Table 1 Alignment of amino acid sequences of chimeric galanin neuropeptide Y (NPY) peptides: NPY and galanin (porcine) are shown for comparison

Galanin

G-W-T-L-N-S-A-G-Y-L-L-G-P-H-A-I-D-N-H-R-S-F-H-D-K-Y-G-L-A amide

NPY

Y-P-S-K-P-D-N-P-G-E-D-A-P-A-E-D-L-A-R-Y-Y-S-A-L-R-H-Y-I-N-L-I-T-R-Q-R-Y amide

M32
Galanin(1-13)-NPY(25-36)

G-W-T-L-N-S-A-G-Y-L-L-G-P-R-H-Y-I-N-L-I-T-R-Q-R-Y amide

M88
Galanin(1-12)-Ala-NPY(25-36)

G-W-T-L-N-S-A-G-Y-L-L-G-A-R-H-Y-I-N-L-I-T-R-Q-R-Y amide

M69A
Galanin(1-13)-Lys-[εNH-Gly-NPY(4-1)]-NPY(25-36)

G-W-T-L-N-S-A-G-Y-L-L-G-P-K-(εNH-G-K-S-P-Y)-R-H-Y-I-N-L-I-T-R-Q-R-Y amide

M120
Galanin(1-13)-NPY(14-36)

G-W-T-L-N-S-A-G-Y-L-L-G-P-A-E-D-L-A-R-Y-Y-S-A-L-R-H-Y-I-N-L-I-T-R-Q-R-Y amide

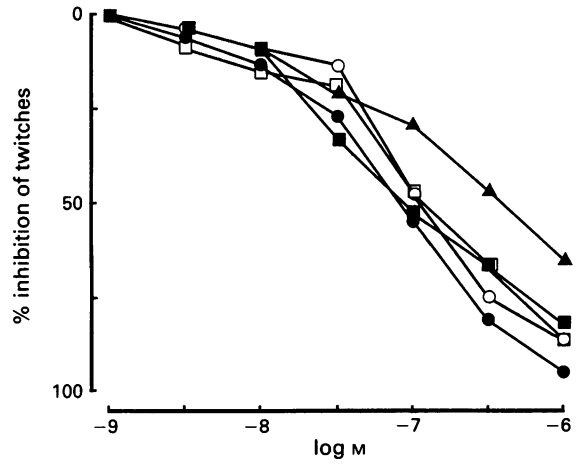


Figure 1 Concentration-response curves illustrating the suppression of electrically-evoked twitches of rat vas deferens in response to increasing concentrations of neuropeptides Y (NPY, ●), NPY 13-36 (▲), M32 (○), M69A (■) and M88 (□). For clarity only mean values are indicated. For each point s.e.mean was less than 10%. $n = 4-12$ for each peptide.

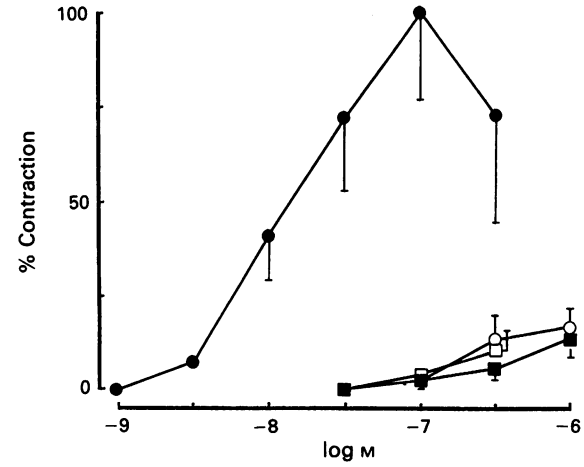


Figure 2 Contractile responses to neuropeptide Y (NPY, ●), M32 (○), M69A (■) and M88 (□) in the guinea-pig caval vein. Responses are expressed as a percentage of the potassium-induced contraction. $n = 4$ for each peptide.

Table 2 Ability of neuropeptide Y (NPY) and galanin-NPY chimeric peptides to suppress the electrically stimulated twitches of rat vas deferens

Peptide	IC_{50} (nM)	n
NPY	94 ± 14	12
NPY (13-36)	455 ± 173	4
M32	121 ± 28	6
M88	150 ± 47	6
M69A	140 ± 50	6
M120	0	4

Data shown are mean ± s.e.mean. 0, inactive at 1 μM. *n*, number of experiments. For primary amino acid sequences of the chimeric peptides see Table 1.

and hippocampal binding sites, the C-terminal NPY (13-36) and the four galanin-NPY peptides all had higher affinities for the hippocampal NPY binding sites than for cerebral cortical NPY binding sites. The most NPY receptor type selective chimeric analogue was M32, which had 20 fold higher affinity for Y_2 type binding sites in the hippocampus than for Y_1 type sites in the cerebral cortex. Table 3 shows the replacement of a single amino acid residue in M32; that of Pro^{13}, with Ala in M88 resulted in a marked loss of affinity for hippocampal NPY binding sites, but did not affect the already low affinity of the peptide for cortical NPY binding sites (Table 3). The 'tertraheder shaped' or branched galanin-NPY chimeric ligand M69A, which carries both NPY (1-4) and NPY (25-36) along with the galanin (1-12) sequence, bound to both cerebral cortical and hippocampal binding sites with a similar affinity in the low nanomolar range (Table 3). When the C-terminal portion of the chimeric peptide comprises NPY (13-36), i.e. peptide M120, the affinities for cerebral cortical and hippocampal NPY-binding sites, respectively, were found to be similar (Table 3).

Affinity for galanin binding sites and functional effects

As shown in Table 3 the chimeric peptides M32, M88 and M69A were at least equipotent with galanin at [^{125}I]-galanin binding sites in the rat hypothalamus, while M120 was about 10 times less potent than galanin. In a series of preliminary experiments M32, M69A and M88 (>0.1 μM) and galanin contracted the rat isolated jejunum (data not shown).

Discussion

The electrically stimulated rat vas deferens has been a much used model for the study of Y_2 receptors (e.g. Wahlestedt *et al.*, 1986; Danho *et al.*, 1988; Grundemar & Håkanson, 1990; Jörgensen *et al.*, 1990; Grundemar *et al.*, 1993a). In the present series of experiments we confirmed that vas deferens, contains both Y_2 and galanin receptors, which inhibit and facilitate electrically-evoked contractions in the rat vas deferens, respectively (Ohhashi & Jacobowitz, 1985; Grundemar & Håkanson, 1990). The Y_2 receptor is not stringent in its requirement for the N-terminal part of NPY, since NPY (13-36) is a fairly potent agonist. However, NPY (25-36) and shorter fragments are virtually inactive (e.g. Grundemar & Håkanson, 1990). In the present study we examined whether the presence of N-terminal fragments of NPY may facilitate Y_2 receptor activation: NPY (1-24)NH_2 and NPY (25-36) were administered in stochiometric amounts simultaneously. However, there was no response to the mixture of the two NPY fragments, suggesting that the N- and C-terminal parts must be covalently linked in order to activate Y_2 receptors in the rat vas deferens.

Galanin receptors appear to recognize the N-terminal part of the galanin molecule (Ekblad *et al.*, 1985; Fox *et al.*, 1988; Murakami *et al.*, 1989; Xu *et al.*, 1989; Kuwahara *et al.*, 1990). The rat vas deferens, thus, contains both inhibitory Y_2 receptors and enhancing galanin receptors. Using various chimeric galanin-NPY peptides with N-terminal galanin fragments (1-12) linked to C-terminal fragments of NPY, it was found that M32 (galanin(1-13)-NPY(25-36)), M69A (galanin (1-13)-Lys-[εNH-Gly-NPY(4-1)]NPY(25-36)) and M88 (galanin(1-12)-Ala-NPY(25-36)) potently and effectively suppressed the stimulated twitches in the rat vas deferens. In fact, the three chimeric galanin-NPY peptides were equipotent with NPY itself. However, these results contrast with the low ligand binding affinity of the chimeric peptides at NPY binding sites in the hippocampus, which is known to contain a predominant population of Y_2 receptors (Sheikh *et al.*, 1989; Dumont *et al.*, 1990; Aicher *et al.*, 1991). M32, M69A and M88 had a 30, 25 and 90 fold lower affinity, respectively, than NPY itself at Y_2 receptors in the hippocampus. These discrepancies of apparent high NPY agonist potency vs. low binding affinity in the two Y_2 receptor-containing preparations could possibly be explained by the fact that the chimeric galanin-NPY peptides may act as full Y_2 receptor agonists in the rat vas deferens, while their low affinity in hippocampus reflects the possible presence of additional

Table 3 Binding affinities to neuropeptide Y (NPY)-Y_1, NPY-Y_2 and galanin receptors

	Displacement of [^{3}H]-NPY				*Displacement of [^{125}I]-galanin*	
	Cerebral cortex (Y_1 receptors)		*Hippocampus* (Y_2 receptors)		*Hypothalamus*	
Peptide	K_D (nM)	n_H	K_D (nM)	n_H	K_D (nM)	n_H
NPY	0.32 ± 0.2	0.9 ± 0.2	0.2 ± 0.1	0.8 ± 0.1	>10,000*	
Galanin	>10,000*		-		0.8 ± 0.1	1.0 ± 0.1
NPY (13-36)	78 ± 31	0.7 ± 0.0	3.2 ± 0.8	0.8 ± 0.1	-	
M32	132 ± 35	0.6 ± 0.1	6.2 ± 5.5	0.6 ± 0.2	0.1 ± 0.1	0.5 ± 0.1
M88	115 ± 56	0.9 ± 0.1	19 ± 10	0.9 ± 0.1	1.1 ± 0.1	1.0 ± 0.2
M69A	26 ± 10	0.8 ± 0.2	4.9 ± 2.9	0.7 ± 0.1	0.1 ± 0.0	1.0 ± 0.2
M120	50 ± 22	0.8 ± 0.2	25 ± 16	0.9 ± 0.2	6.6 ± 1.8	1.0 ± 0.2

K_D values are determined as described in the methods. The values given are the mean ± s.e.mean from at least three independent experiments carried out using the concentration range 10^{-11}–10^{-5} M for the peptides. n_H, Hills coefficient.
*Data from Arvidsson *et al.*, 1993.

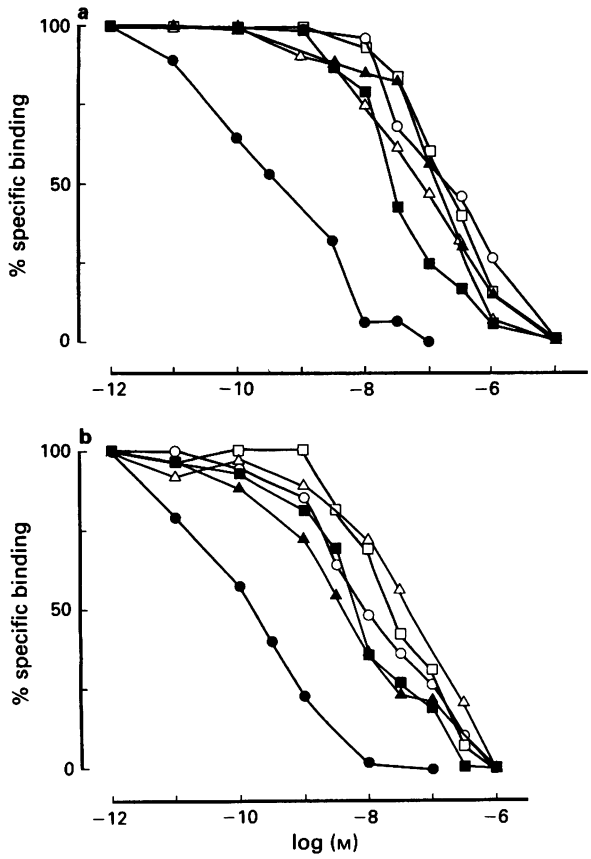


Figure 3 Displacement of [N-propionyl-^{3}H]-neuropeptide Y by NPY (●), NPY (13-36) (▲) and the chimeric galanin-NPY peptides M32 (○), M69A (■), M88 (□) and M120 (△) from Y_1 type binding sites in the rat cerebral cortex (a) and Y_2 type binding sites in the rat hippocampus (b). Each point represents the mean of data obtained from at least three experiments, each performed in triplicate and expressed as the percentage of specific binding. Specific d.p.m. mg^{-1} protein was 237 ± 553 in cerebral cortex and 962 ± 122 in hippocampus.

NPY receptor subtypes. The existence of different Y_2 receptor subtypes has recently been suggested, based on rank order of potency of centrally truncated NPY peptides in various Y_2 receptor containing tissues (Grundemar *et al.*, 1993a). In contrast, M120 (galanin (1-13)-NPY(14-36)) was essentially without any NPY agonist activity and did not affect responses to NPY, indicating also a lack of antagonistic properties at the Y_2 receptor. These results are in accordance with the low affinity obtained with M120 at Y_2 receptors in the hippocampus (125 fold lower than NPY itself).

NPY evoked a concentration-dependent contraction in the guinea-pig caval vein. This response is mainly mediated by Y_1 receptors (Grundemar *et al.*, 1992; 1993a). In agreement with findings obtained with other blood vessels, this study has shown that galanin was inactive in the caval vein (Ekblad *et al.*, 1985). M32, M69A and M88 evoked a slight contraction at very high concentrations only (0.3 μM), while M120 was inactive at ⩽ 1 μM. Neither chimeric peptide affected the response to NPY. Thus, the chimeric peptides do not appear to be Y_1 receptor antagonists. The binding of the galanin-NPY peptides in the rat cerebral cortex, which is known to contain Y_1 receptors (Dumont *et al.*, 1990; Aicher *et al.*, 1991) confirmed that the chimeric galanin-NPY peptides have very low affinities (90–440 fold lower than NPY) for Y_1 type binding sites. The results from the NPY binding and bioactivity studies both support the suggestion that, in order to activate the Y_1 receptor, the ligand must present the proximity of N- and C-terminal ends of NPY to the signal epiptope (Schwartz *et al.*, 1990; Grundemar *et al.*, 1993a,b).

The present study shows that the chimeric galanin-NPY peptides all had a very high affinity for galanin receptors in the hypothalamus. Several studies have suggested that various N-terminal galanin containing chimeric peptides act as antagonists at certain galanin receptors (Bartfai *et al.*, 1991; Langel *et al.*, 1992). Using a series of N- and C-terminal galanin fragments, it has recently been proposed that they recognize different galanin receptor subtypes in rat pancreas and jejunum, respectively (Rossowski *et al.*, 1993). In this study we have confirmed results from a very recent report that N-terminal galanin chimeric peptides, instead of being galanin antagonists, act as galanin agonists in the rat jejunum (Gu *et al.*, 1993).

In conclusion, the N-terminal portion (1-12) of galanin seems to permit a steric conformation of the attached NPY (25-36) part of the chimeric galanin-NPY peptides, which results in a facilitated Y_2 but not Y_1 receptor recognition and activation. None of the chimeric galanin-NPY peptides appeared to act as antagonists at either type Y_1 or Y_2 receptors, probably due to their low affinity. Instead, they display a very high affinity for hypothalamic galanin receptors and probably act as galanin agonists in the rat jejunum.

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References

ABENS, J., UNDEN, A., ANDELL, S., TUM, J.P. & BARTFAI, T. (1989). Fragments and analogs of NPY are ligands at NPY receptors in the rat cerebral cortex. In *Neuropeptide Y*. ed. Mutt, V., Fuxe, K., Hökfelt, T. & Lundberg, J.M. pp. 137–142. New York: Raven Press.

AICHER, S.A., SPRINGSTON, M., BERGER, S.B., REIS, D.J. & WAHLESTEDT, C. (1991). Receptor-selective analogs demonstrate NPY/PYY receptor heterogeneity in rat brain. *Neurosci. Lett.*, **130**, 32–36.

ARVIDSSON, K., LAND, T., LANGEL, Ü., BARTFAI, T. & EHRENBERG, A. (1993). Solution structure by 2D ^{1}H-NMR of a chimeric peptide recognized by galanin and neuropeptide Y receptors. *Biochemistry*, **32**, 7787–7798.

BARTFAI, T., BEDECS, K., LAND, T., LANGEL, Ü., BERTORELLI, R., GIROTTI, P., CONSOLO, S., XU, X., WIESENFELD-HALLIN, Z., NILSSON, S., PIERIBONE, V.A. & HÖKFELT, T. (1991). M-15: High-affinity chimeric peptide that blocks the neuronal actions of galanin in the hippocampus, locus coeruleus and spinal cord. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 10961–10965.

BECK, A., JUNG, G., GAIDA, W., KÖPPEN, H., LANG, R. & SCHNORRENBERG, G. (1989). Highly potent and small neuropeptide Y agonist obtained by linking NPY 1-4 via spacer to α-helical NPY 25-36. *FEBS Lett.*, **244**, 119–122.

BECK-SICKINGER, A.G., GRONZMAN, E., HOFFMAN, E., GAIDA, W., VAN MEIR, E.G., WAEBER, B. & JUNG, G. (1992). A novel cyclic analog of neuropeptide Y specific for the Y_2 receptor. *Eur. J. Biochem.*, **206**, 957–964.

CHENG, Y.C. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_{i}) and the concentration of inhibitor which causes 50% inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.*, **22**, 3099–3108.

DANHO, W., TRISCARI, J., VINCENT, G., NAKAJIMA, T., TAYLOR, J. & KAISER, E.T. (1988). Synthesis and biological evaluation of pNPY fragments. *Int. J. Pept. Protein Res.*, **32**, 496–505.

DEQUIDT, M.E. & EMSON, P.C. (1986). Distribution of neuropeptide Y-like immunoreactivity in the rat central nervous system. II. Immunohistochemical analysis. *Neuroscience*, **18**, 545–618.

DUMONT, Y., FOURNIER, A., ST-PIERRE, S., SCHWARTZ, T.W. & QUIRION, R. (1990). Differential distribution of neuropeptide Y1 and Y2 receptors in the rat brain. *Eur. J. Pharmacol.*, **191**, 501–503.

EKBLAD, E., HÅKANSON, R., SUNDLER, F. & WAHLESTEDT, C. (1985). Galanin: Neuromodulatory and direct contractile effects on smooth muscle preparations. *Br. J. Pharmacol.*, **86**, 241–246.

EVA, C., KEINÄNEN, K., MONYER, A., SEEBURG, P. & SPRENGEL, R. (1990). Molecular cloning of a novel co-protein-coupled receptor that may belong to the neuropeptide receptor family. *FEBS Lett.*, **271**, 81–84.

FOX, J.E.T., BROOKS, B., MCDONALD, T.J., BARNETT, W., KOSTOLANSKA, F., YANAIHARA, C., YANAIHARA, N. & RÖKEUS, A. (1988). Actions of galanin fragments on guinea-pig and canine intestinal motility. *Peptides*, **9**, 1183–1189.

FUHLENDORFF, J., GETHER, U., AAKERLUND, L., LANGELAND-JOHANSEN, J., THOGERSEN, H., MELBERG, S.G., BANG-OLSEN, U., THASTRUP, O. & SCHWARTZ, T.W. (1990). [Leu^{31},Pro^{34}] Neuropeptide Y – a specific Y1 receptor agonist. *Proc. Natl. Acad. Sci. U.S.A.*, **187**, 182–186.

GOZES, Y., BRENNEMAN, D.E., FRIDKIN, M., ASOFSKY, R. & GOZES, I. (1991). A VIP antagonist distinguishes VIP receptors on spinal cord cells and lymphocytes. *Brain Res.*, **540**, 319–321.

GRUNDEMAR, L. & HÅKANSON, R. (1990). Effects of various neuropeptide Y/peptide YY fragments on electrically-evoked contractions of the rat vas deferens. *Br. J. Pharmacol.*, **100**, 190–192.

GRUNDEMAR, L. & HÅKANSON, R. (1993). Multiple neuropeptide Y receptors are involved in cardiovascular regulation. Peripheral and central mechanisms. *Gen. Pharmacol.*, **24**, 785–796.

GRUNDEMAR, L., KRSTENANSKY, J.L. & HÅKANSON, R. (1993a). Activation of Y1 and Y2 receptors by substituted and truncated neuropeptide Y analogs: identification of signal epitopes. *Eur. J. Pharmacol.*, **232**, 271–278.

GRUNDEMAR, L., MÖRNER, S.E.J.M., HÖGESTÄTT, E.D., WAHLESTEDT, C. & HÅKANSON, R. (1992). Characterization of vascular neuropeptide Y receptors. *Br. J. Pharmacol.*, **105**, 45–50.

GRUNDEMAR, L., SHEIKH, S.P. & WAHLESTEDT, C. (1993b). Characterization of receptor types for neuropeptide Y and related peptides. In *The Biology of Neuropeptide Y and Related Peptides*. ed. Colmers, W.F. & Wahlestedt, C. pp. 197–239. Totowa, NJ, U.S.A: Humana Press.

GU, Z.F., ROSSOWSKI, W.J., COY, D.H., PRADHAN, T.K. & JENSEN, R.T. (1993). Chimeric galanin analogs that function as antagonists in the CNS are full agonists in gastrointestinal smooth muscle. *J. Pharmacol. Exp. Ther.*, **266**, 912–918.

HERZOG, H., HORT, Y.I., BALL, H.J., HAYES, G., SHINE, I. & SELBIE, L.D. (1992). Cloned human neuropeptide Y receptor couples to different second messenger systems. *Proc. Acad. Sci.*, **89**, 5794–5798.

HÖGESTÄTT, E.D., ANDERSSON, K.-E. & EDVINSSON, L. (1983). Mechanical properties of rat cerebral arteries studied by a sensitive device for recording of mechanical activity in isolated small blood vessels. *Acta Physiol. Scand.*, **117**, 49–61.

JÖRGENSEN, J.C.H., FUHLENDORFF, J. & SCHWARTZ, T.W. (1990). Structure-function studies on neuropeptide Y and pancreatic polypeptide – evidence for two PP-fold receptors in vas deferens. *Eur. J. Pharmacol.*, **186**, 105–114.

KRAUSE, J., EVA, C., SEEBURG, P.H. & SPRENGEL, R. (1992). Neuropeptide Y, subtype pharmacology or a recombinately expressed neuropeptide receptor. *Mol. Pharmacol.*, **41**, 817–821.

KRSTENANSKY, J.L., OWEN, T.J., BUCK, S.H., HAGAMAN, K.A. & LEAN, L.R. (1989). Centrally truncated and stabilized porcine neuropeptide Y analogs: Design, synthesis, and mouse brain receptor binding. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 4377–4381.

KRSTENANSKY, J.L., OWEN, T.J., PAYNE, M.H,. SHATZER, S.A. & BUCK, S.H. (1990). C-terminal modifications of neuropeptide Y and its analogs leading to selectivity of the mouse brain receptor over the porcine spleen receptor. *Neuropeptides*, **17**, 117–120.

KUWAHARA, A., OZAKI, T. & YANAIHARA, N. (1990). Structural requirements for galanin action in the guinea-pig ileum. *Regul. Pept.*, **29**, 23–29.

LAND, T., LANGEL, Ü., LÖW, M., BERTHOLD, M., UNDEN, A. & BARTFAI, T. (1991). Linear and cyclic N-terminal galanin fragments and analogs as ligands at the hypothalmic galanin receptor. *Int. J. Pept. Protein Res.*, **38**, 267–272.

LANGEL, Ü., LAND, T. & BARTFAI, T. (1992). Design of chimeric peptide ligands to galanin to receptors and substances receptors. *Int. J. Pept. Protein. Res.*, **39**, 516–522.

LARHAMMAR, D., BLOMQVIST, A.G., YEE, F., JAZIN, E., YOO, H. & WAHLESTEDT, C. (1992). Cloning and functional expression of a human neuropeptide Y/peptide YY receptor of the Y1 type. *J. Biol. Chem.*, **267**, 10935–10938.

LUNDBERG, J.M. & TATEMOTO, K. (1982). Pancreatic polypeptide family (APP, BPP, NPY and PYY) in relation to α-adrenoceptor-resistant sympathetic vasoconstrictor. *Acta Physiol. Scand.*, **116**, 393–402.

MURAKAMI, Y., KATO, Y., SHIMATSU, A., HOSHIYAMA, H., HATTORI, N., YANAIHARA, N. & IMURA, H. (1989). Possible mechanisms involved in growth hormone secretion induced by galanin in the rat. *Endocrinology*, **124**, 1224–1229.

OHHASHI, T. & JOCOBOWITZ, D.M. (1985). Galanin potentiates electrical stimulation and exogenous norepinephrine-induced contractions in the rat vas deferens. *Regul. Pept.*, **12**, 163–171.

ROSSOWSKI, W.J., ZUCHARIA, S., JIANG, N.-Y., MUNGAN, Z., MILLS, M., ERTAN, A. & COY, D.H. (1993). Galanin: structure-dependent effect on pancreatic analyse secretion and jejunum strip contraction. *Eur. J. Pharmacol.*, **240**, 259–267.

SCHWARTZ, T.W., FUHLENDORFF, J., KJEMS, L.L., KRISTENSEN, M.S., VERVELDE, M., O'HARE, M., KRSTENANSKY, J.L. & BJÖRNHOLM, B. (1990). Signal epitopes in the three-dimensional structure of neuropeptide Y. Interaction with Y1, Y2 and pancreatic polypeptide receptors. *Ann. N.Y. Acad. Sci.*, **611**, 35–47.

SHEIKH, S.P., HÅKANSON, R. & SCHWARTZ, T.W. (1989). Y1 and Y2 receptors for neuropeptide Y. *FEBS Lett.*, **245**, 209–214.

SUNDLER, F., HÅKANSON, R, EKBLAD, E., UDDMAN, R. & WAHLESTEDT, C. (1986). Neuropeptide Y in peripheral and enteric nervous systems. *Annu. Rev. Cytol.*, **102**, 234–269.

UNDEN, A., TATEMOTO, K., MUTT, V. & BARTFAI, T. (1984). Neuropeptide Y in the rat brain. *Eur. J. Biochem.*, **145**, 525–530.

WAHLESTEDT, C., YANAIHARA, H. & HÅKANSON, R. (1986). Evidence for different pre- and postjunctional receptors for neuropeptide Y and related peptides. *Regul. Pept.*, **13**, 307–318.

XU, X.J., WIESENFELD-HALLIN, Z., FISONE, G., BARTFAI, T. & HÖKFELT, T. (1989). The N-terminal 1-16 but not C-terminal 17-29 galanin fragment affects the flexor reflex in rats. *Eur. J. Pharmacol.*, **182**, 137–141.

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A nucleotide receptor that mobilizes Ca^{2+} in the mouse submandibular salivary cell line ST_{885}

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1 We have identified a Ca^{2+}-mobilizing receptor that responds to extracellular ATP (0.1–1000 μM) in a salivary epithelial cell line (ST_{885}). The activated receptor also stimulates Ca^{2+} entry from the extracellular fluid.

2 The receptor shows an agonist selectivity profile consistent with the behaviour of the nucleotide receptor class. In particular, the pyrimidine nucleotide, UTP, was equipotent with ATP.

3 Analysis of concentration-response data for the ATP and UTP-activated receptor using the Hill equation yielded EC_{50} values (concentrations of agonist inducing a half-maximal response) for ATP of 4.2 μM and for UTP of 4.6 μM.

4 ATP and UTP induced cross-desensitization and were not additive when administered simultaneously at maximal concentration. These findings are consistent with the hypothesis that both nucleotides act upon a common receptor.

5 The ATP analogue, 2-methylthio ATP, mobilized Ca^{2+} ions with higher potency ($EC_{50} = 0.23$ μM) but lower efficacy than either ATP or UTP. However, 2-methylthio ATP was not a partial agonist of the ATP/UTP receptor. At maximal concentration (30 μM), it failed to antagonize the effects of near maximal concentrations of ATP or UTP. We conclude that 2-methylthio ATP acts upon a distinct receptor.

Keywords: Extracellular ATP; extracellular UTP; nucleotide receptor; purinoceptor; mouse submandibular gland; cell culture; cytosolic Ca^{2+}

Introduction

Salivary acinar and ductal epithelial cells are responsible for the controlled secretion of fluid and electrolytes and of specific proteins and growth factors (Cook & Young, 1989). There are several well-recognised Ca^{2+}-mobilizing receptors that stimulate the secretion of fluid and electrolytes in salivary epithelia. These include muscarinic receptors, α-adrenoceptors and substance P receptors (Cook & Young, 1989). However, it has been recently recognised that there are also Ca^{2+}-mobilizing receptors for extracellular ATP in mouse and rat parotid acini (Gallacher, 1982; McMillian *et al.*, 1987; 1988; Soltoff *et al.*, 1990) and human submandibular duct cells (Yu & Turner, 1991). To establish whether there are receptors for extracellular ATP in other salivary epithelia we have examined the effect of extracellular ATP on cells of the mouse submandibular epithelial cell line (ST_{885}) which, by histological criteria, is of ductal origin. We find that extracellular ATP activates a Ca^{2+}-mobilizing receptor in these cells. Characterization of the receptor according to the potency of a series of ATP analogues including adenosine, AMP, ADP, α,β-methylene-ATP (α,β-MeATP), 2-methylthio-ATP and UTP has allowed us to identify it as a nucleotide receptor (O'Connor *et al.*, 1991). Nucleotide receptors respond to the purine nucleotide ATP and the pyrimidine nucleotide UTP with increases in $[Ca^{2+}]_i$ and the generation of inositol 1,4,5-trisphosphate and prostaglandins (O'Connor *et al.*, 1991).

Methods

Tissue culture techniques

ST_{885} cells (passage 19–33) were grown in culture Medium 199 (5 ml) with Earle's salts supplemented with foetal bovine serum (10%). The cells were incubated at 37°C in 95% air and 5% CO_2 and grown to confluence in culture flasks prior to plating onto coverslips. To dislodge the cells from the culture flasks, trypsin-EDTA was added to the flask following the removal of Medium 199. Trypsin activity was quenched 2 min later by the addition of fresh culture medium. The cell suspension was then centrifuged and the pellet resuspended in fresh Medium 199. A portion of the cell suspension was transferred onto six sterile coverslips (size 0), each in a covered Petri-dish. The cells were allowed to settle for 10 min before each Petri-dish was filled with fresh Medium 199. The remaining cell suspension was used to seed two fresh culture flasks. Cells were grown on coverslips for at least 48 h and the culture medium was changed 12–18 h prior to study.

Loading cells with fura-2 AM

ST_{885} cells were loaded with fura-2 AM (10 μM) in culture medium for 30 min at 37°C. Following the incubation, the cells were washed twice in physiological saline solution (37°C) containing (in mM): NaCl 145, KCl 5.0, H-HEPES 10, $MgCl_2$ 1.2, D-glucose 10 and $CaCl_2$ 1.0. The pH was adjusted to 7.40 at 37°C with NaOH. The fura-2 loaded cells were kept in the dark at room temperature (about 20–25°C) until required for fluorescence experiments. Fura-2 leakage and photobleaching as assessed by the time-dependent decline in the 380 nm and 340 nm fluorescence signals was typically less than 10% over the period of a 30 min perfusion experiment at 37°C. The ratio method of $[Ca^{2+}]_i$ determination is largely independent of fura-2 concentration (Grynkiewicz *et al.*, 1985).

Microfluorescence experiments

Fluorescence experiments were performed in a perfusion chamber (300 μl) on the stage of a Nikon Diaphot microscope adapted for microfluorescence studies. A coverslip was affixed to the base of the perfusion chamber with high vacuum grease. A small fragment of coverslip supporting

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ST_{885} cells was positioned on the base of the chamber and a second coverslip was fixed in place with vacuum grease to the top of the chamber. The cells were continuously superfused with control or modified physiological saline solutions (37°C) at 1.5 ml min^{-1} using a peristaltic pump. The superfused cells were illuminated with a 100 W xenon lamp housed in a Nikon light source. Alternating excitation wavelengths (340 nm and 380 nm) were obtained using a computer-controlled filter wheel (Lambda-10, Sutter Instrument Co. Novato, CA, U.S.A.). A neutral density filter (D = 1) was interposed between the light source and the excitation filter as required to reduce the intensity of illumination. Control of the filter wheel was achieved using the digital output facility of a MacPacq 10 analog to digital converter (BIOPAC Systems Inc. Goleta, CA. U.S.A.) which in turn was controlled by a Macintosh computer system using AcqKnowledge 2.0 software (BIOPAC Systems Inc. Goleta, CA. U.S.A.). Ultraviolet light (340 nm or 380 nm) was reflected onto the cell preparation by a dichroic mirror (cut-off 430 nm). Light emitted by the intracellular fluorescent probe, fura-2 (peak 510 nm) was passed through the dichroic mirror then reflected by a second dichroic mirror (cut-off 550 nm) and passed through an emission filter (peak 509 nm, half peak bandwidth 10.3 nm) to a photomultiplier tube (Oriel Corp., CT, U.S.A.). The resultant current signal generated by the photomultiplier was converted to a voltage signal by a preamplifier (Oriel Corp., CT, U.S.A.) then digitized by the MacPacq 10. Digital data were displayed and stored on computer using AcqKnowledge 2.0 software. With the fluorescence emission set at 510 nm, the fura-2 excitation ratio (340/380) was calculated for each time-point and used as a measure of the corresponding $[Ca^{2+}]_i$ (Grynkiewicz *et al.*, 1985).

In some experiments we used a simple calibration procedure to convert fluorescence ratio data to estimates of $[Ca^{2+}]_i$ using the equation (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}]_i = K_d \left(\frac{R-R_{min}}{R_{max}-R}\right)\left(\frac{S_{f2}}{S_{b2}}\right)$$

where K_d is the apparent dissociation constant for the Ca^{2+}/fura-2 complex (224 nM in cytosol-like physiological saline solutions at 37°C according to Grynkiewicz *et al.*, 1985), R_{min} is the minimum fluorescence ratio, R_{max} is the maximum fluorescence ratio and S_{f2} and S_{b2} are the fluorescence readings obtained at 380 nm in the presence of low and high $[Ca^{2+}]_i$ respectively. In our calibration experiments R_{min}, R_{max}, S_{f2} and S_{b2}, were determined at the end of each experiment. First a Ca^{2+}-free perfusion solution containing the Ca^{2+} ionophore, ionomycin (2 μM) and EGTA (5 mM) was used to obtain values for R_{min} and S_{f2}. R_{max} and S_{b2} were then determined using a perfusion solution that contained ionomycin (2 μM) and $CaCl_2$ (5 mM).

Concentration-response curves

Concentration-response data were obtained from separate batches of cells that had not been previously exposed to exogenous nucleotides. Concentration-response curves were derived from data obtained from cells exposed to different concentrations of agonist in the following way. First, the increase in the fluorescence ratio from its baseline level to the peak of the initial transient response was measured. The measured shifts in fluorescence ratio for each test concentration of an agonist were then expressed as a percentage of the shift in fluorescence ratio observed at an extracellular ATP concentration of 30 μM. The data were then fitted to the Hill equation:

$$y = \frac{y_{max}x^n}{c^n + x^n}$$

using non-linear regression analysis (MacCurveFit 1.0 for Macintosh). The symbols have the following meanings: x, the extracellular nucleotide concentration, y, the pharmacological response, y_{max}, the maximal response, c, the EC_{50} and n the Hill coefficient.

Materials

Medium 199 with Earle's salts was obtained from Flow Laboratories. FBS (heat inactivated) was obtained from Cytosystems (Aust.). Trypsin-EDTA was obtained from Difco Laboratories (Detroit, Michigan, U.S.A.). Adenosine, albumin (bovine fraction V), α,β-MeATP, ATP, DMSO, EGTA, H-HEPES and UTP were obtained from Sigma (St. Louis, MO, U.S.A.). Ionomycin (free acid) was from Calbiochem (La Jolla, CA, U.S.A.). ADP and AMP were from Boehringer (Germany). Fura-2 AM was from Molecular Probes Inc. (Eugene, Oregon, U.S.A.). 2-methylthio-ATP was obtained from Research Biochemicals Incorporated (Natick, MA, U.S.A.). All other chemicals were of analytical grade.

Results

The mean basal $[Ca^{2+}]_i$ in ST_{885} cells was 66.2 ± 7.2 nM ($n = 20$). The cells responded to extracellular ATP (0.1–1000 μM) with a transient increase in $[Ca^{2+}]_i$ of 2–3 min duration followed immediately by an extended phase in which the increase in $[Ca^{2+}]_i$ persisted at a much lower level (Figure 1). During the transient phase of the response to extracellular ATP (100 μM), $[Ca^{2+}]_i$ peaked at a value of 256 ± 33 nM ($n = 6$). The initial transient was also seen in Ca^{2+}-free media (Figure 2) implying that the source of Ca^{2+} ions involved in this response was predominantly intracellular. The extended phase of the response, however, was not seen in Ca^{2+}-free media, implying that the source of Ca^{2+} ions involved in this phase was extracellular. When ST_{885} cells were exposed to ATP (or UTP) on second and subsequent occasions the magnitude of the response was always

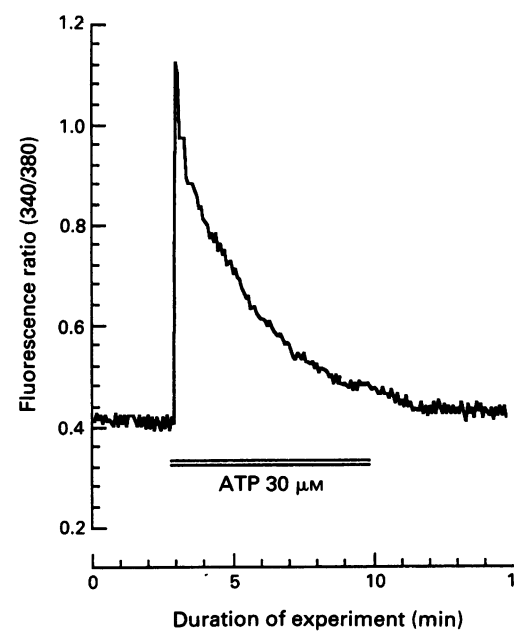


Figure 1 The effect of extracellular ATP (30 μM) on $[Ca^{2+}]_i$. ST_{885} cells growing on coverslips were loaded with fura-2 AM (10 μM, 37°C, 30 min) and then superfused with physiological saline solutions in a microfluorescence apparatus. The fluorescence ratio (340/380) was used as a measure of $[Ca^{2+}]_i$.

markedly reduced (Figure 3). This behaviour could not be altered by prolonging the recovery period between episodes of exposure by up to 30 min.

To characterize the receptor involved in the response, we examined the relative potencies of adenosine, AMP, ADP, ATP and of several different ATP analogues. ADP, ATP and UTP evoked similar maximal responses, that were all approximately 120% of the response evoked by ATP (30 μM),

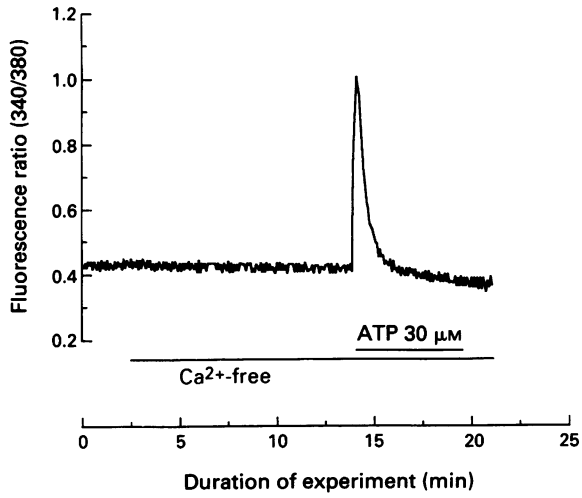


Figure 2 The effect of extracellular ATP (30 μM) on $[Ca^{2+}]_i$ in a Ca^{2+}-free medium. The initial transient response was unaffected by the removal of extracellular Ca^{2+}. The extended phase of the response was abolished.

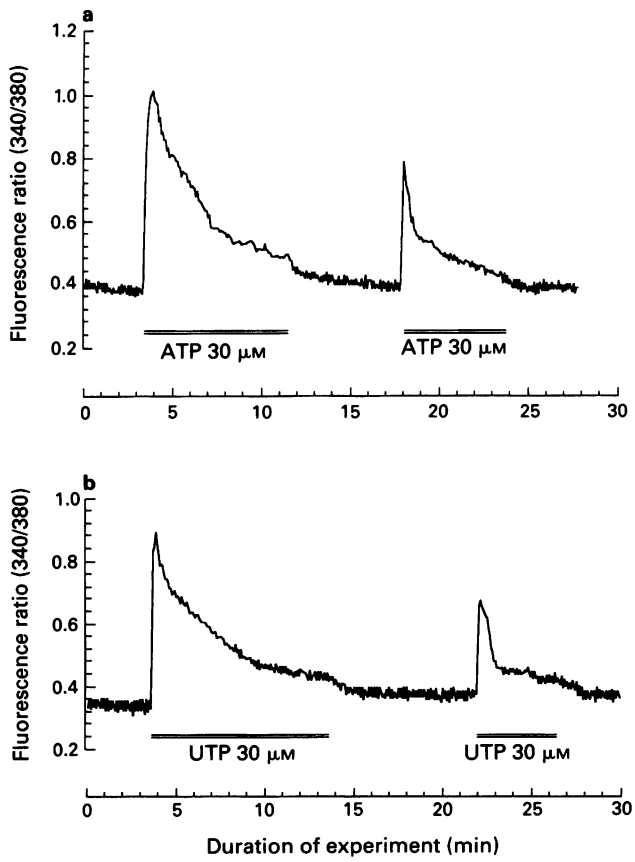


Figure 3 Desensitization of the Ca^{2+} responses to extracellular ATP (30 μM) (a) and UTP (30 μM) (b). The second stimulus resulted in much lower transient and extended responses in both cases.

but differed in their potencies. Adenosine and AMP were ineffective in concentrations up to 100 μM. α,β-MeATP induced a response that was about 10% of the maximal response induced by ATP or UTP at a concentration of 100 μM and was ineffective at lower concentrations. ADP (EC_{50} = 275 ± 48.7 μM) was less potent than ATP or UTP. We found the following rank order of potency: adenosine, AMP < α,β-MeATP < ADP < ATP = UTP. This order of potency is consistent with the behaviour of a novel class of extracellular ATP receptors described, because of their sensitivity to both the purine nucleotide ATP and the pyrimidine nucleotide UTP, as nucleotide receptors (O'Connor *et al.*, 1991). In an attempt to make a clear distinction between the nucleotide receptor subclass and the P_{2Y} subclass of purinoceptors we compared the concentration-response data for ATP, 2-methylthio ATP (which is a selective agonist at P_{2Y} receptors) and UTP using the peak of the initial transient as a measure of the nucleotide-induced response (Figure 4). We derived estimates of the EC_{50} values for ATP, UTP and 2-methylthio-ATP using non-linear regression analysis to fit the Hill equation to each data set. The calculated EC_{50} values were ATP (4.2 ± 2.4 μM), UTP (4.6 ± 2.2 μM) and 2-methylthioATP (0.23 ± 0.24 μM). The approximate equipotency of ATP and UTP is consistent with the behaviour of the nucleotide receptor subclass. However, 2-methylthio ATP behaved as a much higher potency agonist that induced a clearly lower maximal response (about 30% of the maximal responses observed with ATP and UTP; see Figure 4). We attempted to determine whether 2-methylthio ATP was a partial agonist at the ATP/UTP receptor by examining whether at a maximally effective concentration (30 μM) it could inhibit the responses to a near maximal concentration of ATP (30 μM) or UTP (30 μM). However, in neither case was 2-methylthio ATP effective in suppressing the response. The data are presented in Table 1. These data suggest that 2-methylthio ATP acts on a receptor distinct to the receptor/s that recognises ATP and UTP.

The major source of the rise in $[Ca^{2+}]_i$ induced by UTP was, as in the case of ATP, an intracellular store (not shown). We examined whether there was any difference in the

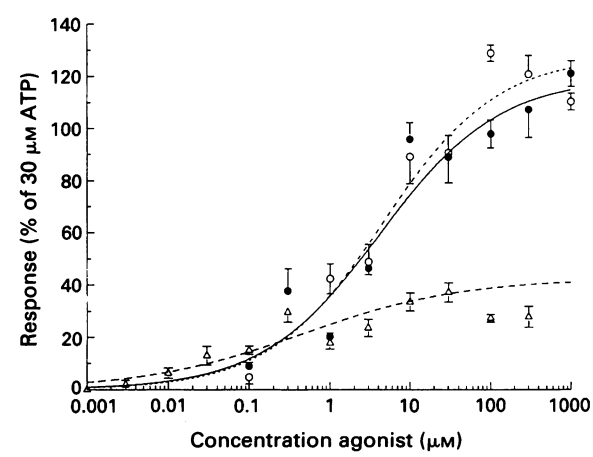


Figure 4 Concentration-response data for the effects of extracellular ATP (●), UTP (○) and 2-methylthio-ATP (Δ). Each data point was obtained from separate batches of cells that had not been exposed previously to exogenous nucleotides. First, the increase in fluorescence ratio from its baseline level to the peak of the initial transient response was measured. These shifts in fluorescence ratio were then expressed as a percentage of the shift in fluorescence ratio observed at an extracellular ATP concentration of 30 μM. In each experimental series ATP (30 μM) was tested on two or three occasions. The second response was routinely used as the reference. The data shown for ATP (30 μM) were derived by expressing the responses observed on the first or third occasion as a percentage of the second.

Table 1 Effect of 2-methylthio ATP (2-MeSATP) on ATP and UTP-induced responses

Agonist	2-MeSATP (30 μM)	ATP (30 μM)	ATP plus 2-MeSATP	UTP (30 μM)	UTP plus 2-MeSATP
Response	48.4 ± 6.3†§ (16)	95.5 ± 8.4* (15)	107.5 ± 9.6†* (8)	96.5 ± 10.2** (8)	77.6 ± 12.5§** (10)

The peak fluorescence responses ± s.e.mean, with the number of observations (n) in parentheses, obtained after exposure of ST_{885} cells to 2-methylthio ATP, ATP, UTP and combinations of these agonists are shown. The data were tested for statistically significant differences with Student's t test. †2-MeSATP v. ATP plus 2-MeSATP: $P<0.001$. *ATP v. ATP plus 2-MeSATP: $P>0.15$. §2-MeSATP v. UTP plus 2-MeSATP; $P<0.025$. **UTP v. UTP plus 2-MeSATP: $P>0.10$.

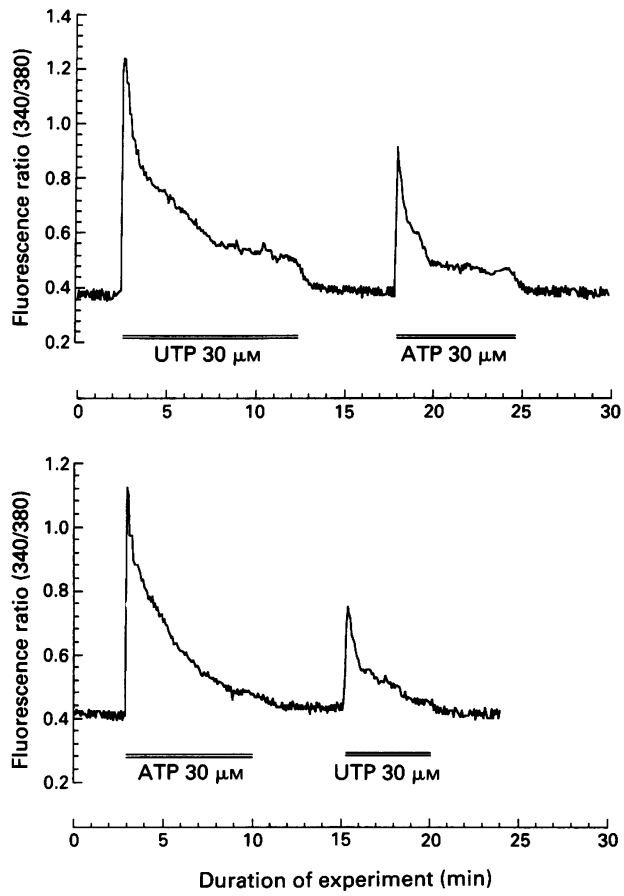


Figure 5 Desensitization and cross-desensitization of the nucleotide receptor by ATP and UTP. Regardless of the order in which the cells were exposed to ATP or UTP, these nucleotides (30 μM) desensitized the cells to subsequent stimuli.

desensitizing effects of UTP and ATP that might have indicated the operation of separate receptors. However, ATP and UTP induced similar desensitization responses regardless of the order in which they were applied. Desensitization appeared to affect both the initial transient and the subsequent extended phase (Figure 5). To explore further whether the ATP- and UTP-dependent effects might arise at a common receptor, we exposed ST_{885} cells to ATP and UTP simultaneously at maximal concentration. Consistent with the hypothesis that ATP and UTP share a common receptor there was no significant increase in the Ca^{2+} response above that observed in response to either ATP or UTP alone. When expressed as a percentage of the peak response to ATP (100 μM), UTP (100 μM) induced a peak response of 119 ± 17% (s.e.mean, $n = 6$) and ATP plus UTP (each 100 μM) induced a response of 113 ± 16% ($n = 6$); neither of these responses was significantly different from ATP (100 μM) alone (Student's t test).

Discussion

The responses that we have identified in ST_{885} cells to extracellular ATP, UTP and other nucleotides appear to have arisen from a nucleotide receptor (O'Connor *et al.*, 1991). Three of the four commonly recognised P_2 purinoceptor sub-classes (Gordon, 1986) have been excluded as follows: (i) P_{2T}; by the preference of the receptor for ATP over ADP, (ii) P_{2X}, by the preference of the receptor for ATP over α,β-MeATP, and (iii) P_{2Z}, by the effectiveness of ATP at concentrations less than 100 μM in the presence of saturating concentrations of Mg^{2+}. The failure of 2-methylthio ATP to induce maximal responses of similar magnitude to those induced by ATP is not consistent with the behaviour of P_{2Y} receptors. We considered the possibility that 2-methylthio ATP is a partial agonist on the nucleotide receptors present in ST_{885} cells. However, we have excluded this possibility in experiments in which a maximal concentration of 2-methylthio ATP failed to inhibit significantly the effects of either ATP or UTP. Partial agonists behave as antagonists of full agonists. The failure of 2-methylthio ATP to inhibit the effects of ATP and UTP suggests that 2-methylthio ATP acts on a separate population of receptors for extracellular ATP, presumably of the P_{2Y} subclass. Similar examples have been described in rat aortic endothelium (O'Connor *et al.*, 1991) and rat hepatocytes (Keppens & DeWulf, 1991).

Previously, mouse parotid acinar ATP receptors have been classified in the P_{2Y} subclass on the basis of co-existent ATP-dependent Ca^{2+} mobilization and Ca^{2+}-activated K^+ channels (Gordon, 1986). Rat parotid acinar cells also respond to extracellular ATP with elevations in $[Ca^{2+}]_i$ and K^+ efflux (Soltoff *et al.*, 1990). However, ATP^{4-} was found to be the preferred species and, in the presence of Mg^{2+} ions, no effects of ATP were seen at concentrations less than 100 μM (Soltoff *et al.*, 1990). Mouse lacrimal acinar cells also require high concentrations of ATP for activation (Sasaki & Gallacher, 1990). It is likely that the ATP receptors on rat and mouse parotid acini and on mouse lacrimal acinar cells are, in fact, P_{2Z} in type. Despite this, we found that neither the P_{2Z} nor P_{2Y} receptor subtypes alone could explain the effects we observed in ST_{885} cells. Furthermore, the finding that ATP and UTP are equipotent agonists for the Ca^{2+} responses seen in ST_{885} cells strongly favours the conclusion that nucleotide receptors are involved (O'Connor *et al.*, 1991). Nucleotide receptors that mobilize Ca^{2+} and activate K^+ efflux have recently been identified in a human submandibular cell line, HSG-PA (Yu & Turner, 1991). The EC_{50} values we calculated for the ATP (4.2 μM) and UTP (4.6 μM) responses are consistent with reported data for nucleotide receptors (O'Connor *et al.*, 1991).

In the absence of selective inhibitors it has not been possible to establish conclusively that ATP, a purine nucleotide, and UTP, a pyrimidine nucleotide, act upon a common receptor in any of the tissues in which ATP and UTP effects have been observed. However, the similarity of the agonist activity profiles across a diversity of tissues suggests that both ATP and UTP act upon a common receptor (O'Connor *et al.*, 1991; O'Connor, 1992). Furthermore, the case for a common receptor is strengthened, but not proved, by the finding that the divalent cation-free forms of the two

nucleotides i.e. ATP^{4-} and UTP^{4-} are preferentially recognised (Davidson *et al.*, 1990; Lustig *et al.*, 1992). Our finding that ATP and UTP induced mutual cross-desensitization of both the initial transient response and the lower extended phase of the response is also consistent with the hypothesis that both nucleotides act upon a common receptor. However, we have been unable to prove that this desensitization occurs at a receptor rather than at a post-receptor level because we have been unable to identify another Ca^{2+}-mobilizing receptor type. We have found, so far, that muscarinic receptors, α-adrenoceptors and substance P receptors are not present in ST_{885} cells. Therefore, we have pursued the question in another way by examining whether ATP and UTP were additive in their effects when administered simultaneously in maximal concentration (100 μM). This did not, however, result in responses that were significantly greater than the responses to either ATP or UTP alone. This finding is also consistent with the concept of a common receptor.

Although we have shown that the release of Ca^{2+} ions from an intracellular store, or stores, is responsible for the transient component of the Ca^{2+} response we have yet to identify the nature of the stores or second messengers involved. It is likely, based on the effects of extracellular nucleotides in other tissues, that inositol-1,4,5-trisphosphate (Ins-1,4,5P_3) is the second messenger responsible and that the activated nucleotide receptor is linked to the hydrolysis of phosphatidyl-4,5-bisphosphate (PI-4,5P_2) via a G-protein (Okajima *et al.*, 1987; Davidson *et al.*, 1990). The much smaller extended component of the Ca^{2+} response in ST_{885} cells relies on extracellular Ca^{2+}. The mechanism responsible for this effect is, at present, also uncharacterized. ATP-activated Ca^{2+} entry in parotid and lacrimal acinar cells that express P_{2Z} receptors appears to rely upon the direct activation of Ca^{2+}-permeable channels (McMillian *et al.*, 1988; Sasaki & Gallacher, 1990). However, nucleotide receptors have been linked to effects on inositol phosphate and prostaglandin metabolism that are likely to rely on G-protein activation (O'Connor *et al.*, 1991). Based on these effects, it is likely that a G-protein mediates the activating effect of nucleotide receptors on Ca^{2+} entry.

References

COOK, D.I. & YOUNG, J.A. (1989). Fluid and electrolyte secretion by salivary glands. In *Handbook of Physiology. the Gastrointestinal System. Salivary, Pancreatic, Gastric and Hepatobiliary Secretion*, Section 6, Volume III, ed. Forte, J.G. pp. 1–23. Bethesda: American Physiological Society.

DAVIDSON, J.S., WAKEFIELD, I.K., SOHNIUS, U., VAN DER MERWE, P.A. & MILLAR, R.P. (1990). A novel extracellular nucleotide receptor coupled to phosphoinositidase-C in pituitary cells. *Endocrinology*, **126**, 80–87.

GALLACHER, D.V. (1982). Are there purinergic receptors on parotid acinar cells? *Nature*, **296**, 83–86.

GORDON, J.L. (1986). Extracellular ATP: effects, sources and fate. *Biochem. J.*, **233**, 309–319.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3455.

KEPPENS, S. & DEWULF, H. (1991). Characterization of the biological effects of 2-methylthio-ATP on rat hepatocytes: clear-cut differences with ATP. *Br. J. Pharmacol.*, **104**, 301–304.

LUSTIG, K.D., SPORTIELLO, M.G., ERB, L. & WEISMAN, G.A. (1992). A nucleotide receptor in vascular endothelial cells is specifically activated by the fully ionized forms of ATP and UTP. *Biochem. J.*, **284**, 733–739.

MCMILLIAN, M.K., SOLTOFF, S.P., CANTLEY, L.C. & TALAMO, B.R. (1987). Extracellular ATP elevates intracellular free calcium in rat parotid acinar cells. *Biochem. Biophys. Res. Commun.*, **149**, 523–530.

MCMILLIAN, M.K., SOLTOFF, S.P., LECHLEITER, J.D., CANTLEY, L.C. & TALAMO, B.R. (1988). Extracellular ATP increases free cytosolic calcium in rat parotid acinar cells. Differences from phospholipase C-linked receptor agonists. *Biochem. J.*, **255**, 291–300.

O'CONNOR, S.E. (1992). Recent developments in the classification and functional significance of receptors for ATP and UTP, evidence for nucleotide receptors. *Life Sci.*, **50**, 1657–1664.

O'CONNOR, S.E., DAINTY, I.A. & LEFF, P. (1991). Further subclassification of ATP receptors based on agonist studies. *Trends Pharmacol. Sci.*, **12**, 137–141.

OKAJIMA, F., TOKUMITSU, Y., KONDO, Y. & UI, M. (1987). P2-Purinergic receptors are coupled to two signal transduction systems leading to inhibition of cAMP generation and to production of inositol trisphosphate in rat hepatocytes. *J. Biol. Chem.*, **262**, 13483–13490.

SASAKI, T. & GALLACHER, D.V. (1990). Extracellular ATP activates receptor-operated cation channels in mouse lacrimal acinar cells to promote calcium influx in the absence of phosphoinositide metabolism. *FEBS Lett.*, **264**, 130–134.

SOLTOFF, S.P., MCMILLIAN, M.K., CRAGOE, E.J., CANTLEY, L.C. & TALAMO, B.R. (1990). Effects of extracellular ATP on ion transport systems and $[Ca^{2+}]_i$ in rat parotid acinar cells: comparison with the muscarinic agonist carbachol. *J. Gen. Physiol.*, **95**, 319–346.

YU, H.X. & TURNER, J.T. (1991). Functional studies in the human submandibular duct cell line, HSG-PA, suggest a second salivary gland receptor subtype for nucleotides. *J. Pharmacol. Exp. Ther.*, **259**, 1344–1350.

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Modulation by central postsynaptic α_2-adrenoceptors of the jaw-opening reflex induced by orofacial stimulation in rats

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1 The modulation by α_2-adrenoceptors of the jaw-opening reflex (digastric electromyographic responses) elicited by orofacial electrical stimulation (OF-JOR) in pentobarbitone anaesthetized rats was investigated.

2 Increasing doses of clonidine (0.1–1000 $\mu g\,kg^{-1}$, i.v.) reduced, in a dose-dependent manner until abolition, the amplitude and duration of the OF-JOR and increased the latency to onset. The sum of amplitudes of the reflex was the most sensitive parameter to the inhibitory effects of clonidine ($ED_{50} = 13.9\,\mu g\,kg^{-1}$).

3 Pretreatment with the α_2-adrenoceptor antagonist, idazoxan (0.03–1 $mg\,kg^{-1}$, i.v.), caused a dose-dependent shift (1.5 to 37 fold) to the right of the dose-response curve for clonidine without significant change of maximum inhibitory effect, in a manner compatible with competitive antagonism ($ED_{50B} = 29.0\,\mu g\,kg^{-1}$). Pretreatment with yohimbine (0.3 $mg\,kg^{-1}$, i.v.) also antagonized the inhibitory effect of clonidine on the OF-JOR. In contrast, the α_2-adrenoceptor antagonist ARC-239 (0.3 $mg\,kg^{-1}$, i.v.) did not antagonize the effect of clonidine on the reflex.

4 In rats pretreated with reserpine (5 $mg\,kg^{-1}$, s.c., 18 h) the OF-JOR was not modified, but the potency of clonidine in inhibiting the reflex was potentiated (ED_{50} value decreased to 6.8 $\mu g\,kg^{-1}$) without a significant change of maximum inhibitory effect.

5 Increasing doses of amphetamine (0.1–3000 $\mu g\,kg^{-1}$, i.v.) caused a dose-related, but partial, inhibition of the OF-JOR ($ED_{50} = 135\,\mu g\,kg^{-1}$; $E_{max} = 67\%$). Pretreatment with idazoxan (0.1 $mg\,kg^{-1}$, i.v.) induced a nine fold shift to the right of the dose-response curve for amphetamine, while treatment with the depleting drug α-methyl-*p*-tyrosine (150 $mg\,kg^{-1}$ daily, i.p., for 14 days) abolished the inhibitory effect of this indirect adrenoceptor agonist on the OF-JOR.

6 Morphine (0.1–3000 $\mu g\,kg^{-1}$, i.v.) also reduced the OF-JOR in a dose-dependent manner (ED_{50} value about 325 $\mu g\,kg^{-1}$) but, in contrast to clonidine, it failed to inhibit the reflex fully ($E_{max} = 48\%$). As expected, pretreatment with the opioid antagonist naloxone (1 $mg\,kg^{-1}$, i.v.) abolished the inhibitory effect of morphine on the OF-JOR, while it did not alter that of clonidine.

7 Chronic, but not acute, pretreatment with idazoxan (3 $mg\,kg^{-1}$ daily, i.p. for 14 days) led to a marked potentiation of the inhibitory effect of clonidine on the OF-JOR (ED_{50} value decreased to 4.2 $\mu g\,kg^{-1}$), without a significant change of maximum inhibitory effect.

8 Together the results indicate that clonidine evokes a potent inhibition of the OF-JOR in rats through the activation of postsynaptic α_2-adrenoceptors. It is suggested that this functional response represents a simple and useful *in vivo* model for studying various regulatory mechanisms of central α_2-adrenoceptors.

Keywords: Central postsynaptic α_2-adrenoceptors; jaw-opening reflex; clonidine; idazoxan; reserpine; amphetamine; morphine

Introduction

Mechanical or electrical stimulation of perioral or intraoral structures evokes, in most experimental animals, a jaw-opening reflex (JOR) with activation of the jaw-opening muscles and simultaneous inhibition of the jaw-closing ones (for review see Luschei & Goldberg, 1981; Mason *et al.*, 1985). The JOR can be elicited by electrical stimulation of either low threshold mechanoreceptors, such as those mainly existing in the periodontal and oral mucosa, or of high threshold receptors, predominantly nociceptive, such as those in the tooth pulp cavity (Anderson & Mahan, 1971).

The JOR elicited by tooth pulp stimulation (TP-JOR) has been widely used as a model for studying the central electrophysiological and pharmacological mechanisms of pain and its control (for review, see Mylinski & Matthews, 1987). In animals the TP-JOR is suppressed by a variety of procedures which are known to reduce either clinical pain or experimental nociceptive reflexes. Thus, the TP-JOR is suppressed by stimulation of endogenous antinociceptive circuits such as the periaqueductal gray region and certain raphe nuclei (Oliveras *et al.*, 1974; Sessle & Hu, 1981; Dostrovsky *et al.*, 1982; Tanaka & Toda, 1982), as well as by opiate agonists (Chan & Fung, 1976; Ha *et al.*, 1978), peripheral electrical stimulation (Tal *et al.*, 1981; Toda, 1982a), acupuncture and electroacupuncture (Toda *et al.*, 1981; Toda, 1982b; Kawakita & Funakoshi, 1982). Recently, it has also been shown that noradrenergic systems, acting via α_2-adrenoceptors, play a fundamental role in the modulation of the TP-JOR. Systemic administration of clonidine increases the threshold of the digastric electromyogram evoked by tooth pulp stimulation in rodents (Curtis & Marwah, 1986a,b; 1987). Furthermore, administration of other α_2-adrenoceptor agonists such as medetomidine also produces a dose-dependent increase in the threshold of the TP-JOR in the cat (Pertovaara *et al.*, 1991).

When compared with the TP-JOR, which is considered a reflex expression of a noxious stimulus, less attention has been paid to the jaw-opening reflex elicited by orofacial

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stimulation (OF-JOR), which is believed to be elicited by non-noxious stimuli, although it appears to have physiological relevance (Lund & Rossignol, 1981; Sessle, 1981; Katakura & Chandler, 1991). The aim of the present study was to evaluate the involvement of α_2-adrenoceptors in the OF-JOR.

Methods

Animals, preparation, stimulation and recording

Male albino Sprague-Dawley rats (240–280 g) were used. They received a standard diet with water freely available and were housed at 21 ± 2°C with a 12 h light/dark cycle. The rats were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.), the trachea was intubated, the left digastric muscle located and the right femoral vein was cannulated for drug administration. Animals were placed in a supine position over a heating pad to maintain body temperature at 37 ± 1°C recorded by a rectal thermistor. A supplementary dose of pentobarbitone (15 mg kg^{-1}, i.v.) was routinely administered 40 min before the beginning of the experiment.

Orofacial bipolar stimulation was carried out with stainless steel clip electrodes (10 mm^2 surface) placed in the left labial commissure (affecting mucosa and skin), and in the left superior incisor (affecting gingival and labial adjacent mucosa). Electrical stimulation was delivered by trains of 10 rectangular monophasic pulses (0.1 ms duration each pulse) presented at 1 Hz (pulse generator NS6, Medelec Limited, Surrey, England) at supramaximal voltage (60 V) that was found to be 4 to 5 times the threshold value (12 ± 4 V) for the appearance of the reflex.

As an expression of the JOR the digastric electromyographic responses were recorded from the anterior belly of the left digastric muscle, using a concentric bipolar needle electrode (DMC 25, Medelec Limited). The evoked responses were amplified and filtered (bandwidth 8 Hz to 8 kHz) by a differential preamplifier (AA6 MK III, Medelec Limited) and displayed on a storage oscilloscope. For each recording of the JOR ten succesive digastric electromyographic responses were averaged (digital average DAV 62, Medelec Limited) and subsequently plotted. The JOR was induced and recorded every 4 min for 52 min.

In all experiments two initial recordings were obtained in the absence of drugs to assess the basal value of the OF-JOR. Immediately after the second recording, saline (1 ml kg^{-1}) was administered i.v. and a new basal value was obtained which did not differ from the previous ones. Then, electromyographic responses were measured after cumulative i.v. administration of increasing doses (× 3.3) of clonidine (0.1–1000 μg kg^{-1}), morphine (0.1–3000 μg kg^{-1}) or amphetamine (0.1–3000 μg kg^{-1}) at 4 min intervals until the complete abolition of the reflex (see Figure 1). Cumulative dose-response curves for these compounds were constructed by evaluating the sum of amplitudes (Σ amplitude; see Table

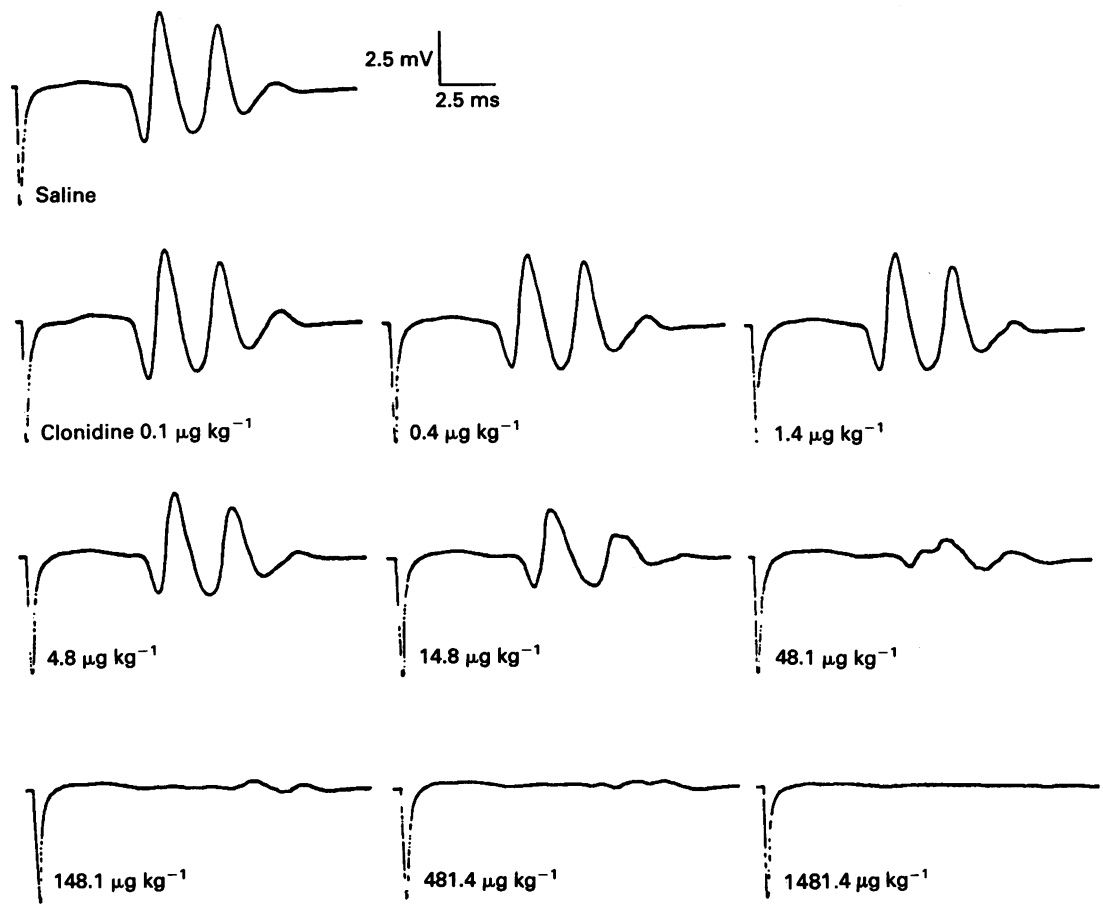


Figure 1 Inhibitory effects of cumulative and increasing doses (× 3.3) of clonidine (0.1–1000 μg kg^{-1}, i.v.) on electromyographic responses recorded from the anterior belly of the digastric muscle, as expression of the jaw-opening reflex, after orofacial electrical supramaximal stimulation (60 V). The recordings show a representative experiment performed in a naive rat. Each tracing is the digital average of the ten digastric electromyographic signals evoked by a train of 10 rectangular pulses (0.1 ms duration each pulse) presented at 1 Hz. Note that clonidine altered the configuration of the recordings and reduced, in a dose-dependent manner, the amplitudes and the duration of the reflex while it increased its latency to onset. See Table 1 for parameters of the dose-response curve.

Table 1 Effect of clonidine on the jaw-opening reflex induced by orofacial electrical stimulation (OF-JOR)

		Clonidine-induced inhibition of the OF-JOR			
Parameters	*Basal values*	ED_{50} ($\mu g\ kg^{-1}$)	E_{max} (% decrease or increase)	n	r^2
Σ Amplitude	11.2 ± 1.1 (mV)	13.9 ± 1.7	102.9 ± 2.9	0.97 ± 0.12	0.87
Maximum amplitude	7.0 ± 0.5 (mV)	21.7 ± 1.2	102.0 ± 4.7	0.96 ± 0.14	0.80
Duration	6.3 ± 0.3 (ms)	62.8 ± 10.0	109.1 ± 8.5	0.90 ± 0.14	0.78
Latency	5.6 ± 0.2 (ms)	14.3 ± 1.9	49.5 ± 5.5	0.86 ± 0.24	0.58

Cumulative dose-response curves for clonidine (0.1–1000 $\mu g\ kg^{-1}$; i.v.) were constructed estimating, for each dose of agonist, the reduction in the sum (Σ) of amplitudes, maximum amplitude and duration, as well as the increase in the latency of the OF-JOR. Parameter r^2 is the square of the correlation coefficient which was calculated after nonlinear regression (perfect fit, $r^2 = 1.00$) (for further details see Motulsky & Ransnas, 1987). The basal values represent the mean ± s.e.mean of 29 determinations. Dose-response parameters for clonidine were determined directly by simultaneous analysis of 29 experiments and are expressed as the best fit values ± s.e. calculated by nonlinear regression analysis.

1) of the JOR with respect to the previously obtained saline basal value. The sum of amplitudes was defined as the arithmetic addition of each peak-to-peak value of every wave that configured the reflex. Cumulative dose-response curves for clonidine, morphine and amphetamine were also determined after various pretreatments specified in the Results section.

Data analysis and statistics

The parameters of the OF-JOR (sum of amplitudes, maximum amplitude, duration and latency) are expressed as mean ± s.e.mean. One-way analysis of variance (ANOVA) followed by Scheffé's test or Student's two tailed t test was used for the statistical evaluations.

Cumulative dose-response curves were analysed by nonlinear analysis according to the equation proposed by Parker & Waud (1971):

$$E = \frac{E_{max} * A^n}{ED_{50}^n + A^n} \quad (1)$$

where A is the dose of agonist, ED_{50} the dose of agonist that produces 50% of the maximum effect (E_{max}) and n is the slope factor of the inhibitory function.

To describe the nature and quantitate the antagonism exerted by idazoxan, the inhibition of the OF-JOR induced by clonidine in the absence and presence of increasing doses of this antagonist (0.03–1 $mg\ kg^{-1}$) were fitted, by nonlinear analysis, to a modification of the equation proposed by Gaddum (1937):

$$\frac{E}{E_{max}} = \frac{A}{A + ED_{50A} \cdot (1 + B^n/ED_{50B})} \quad (2)$$

where A is the dose of agonist, ED_{50A} the dose of agonist that produces 50% of the maximum effect (E_{max}) in the absence of antagonist, B the dose of antagonist, and ED_{50B} the dose of antagonist that necessitates a doubling of the dose of agonist to reach the same level of effect (E). In this equation, n is a slope factor included to check the deviation of the antagonism from a competitive model and corresponds with the slope of the Schild regression (Arunlakshana & Schild, 1959). This nonlinear analysis technique is statistically optimal when compared with linearizing transformations as experimental errors are not systematically distorted (Motulsky & Ransnas, 1987). Moreover, this approach permits the contrast of different hypotheses and allows the user to obtain the statistical significance of differences in the parameters between different ligands or experimental circumstances (Ratkowsky, 1983). A similar approach to calculate antagonist affinity (K_B values) from functional studies has been published recently (Lazareno & Birdsall, 1993). Schild analysis (Arunlakshana & Schild, 1959) using the obtained ED_{50} values for clonidine in inhibiting the OF-JOR after the various doses of idazoxan was also carried out.

Dose-response parameters for the inhibition and antagonism of the OF-JOR are expressed as the best fit values ± standard error determined by the matrix inversion method, using the simple nonlinear least squares fitting programme GraFit (Leatherbarrow, 1990). r^2 values (square of the correlation coefficient) were calculated after nonlinear regression to assess further the goodness of fit as described elsewhere (Motulsky & Ransnas, 1987). Error values determined by nonlinear regression were not used in further formal statistical calculations. Comparisons of experimental data sets were performed by comparing the goodness of fit of a model with and without a set of constraints by means of an F-test. First, the sets of data were analysed separately (with no constraints). Next, the sets of data were pooled, analysed simultaneously, and constrained to share one or more common parameters. The analysis that permitted one or more of the parameters to be shared without a significant increase in the residual variance was taken as the best fit (Ratkowsky, 1983; De Lean *et al.*, 1978). In the present model, equality was tested during the simultaneous analysis by inspecting the consequences, in terms of increasing residual variance, of forcing the parameters to be equal. The statistical significance of the improvement was determined with an F-test at a level of significance of $P = 0.05$.

Drugs

The following drugs were used: clonidine HCl and 2-[2-[4-(*o*-methoxyphenyl) piperazine-1-yl]-ethyl]-4,4-dimethyl-1,3 (2H, 4H)-isoquinoline-dione HCl, ARC 239 (Boehringer Ingelheim, Spain); (±)-idazoxan HCl (Reckitt & Colman, U.K.); reserpine (Serpasol, Ciba-Geigy, Spain); (±)-amphetamine sulphate (S.A. Lasa Laboratories, Spain); α-methyl-*p*-tyrosine methylester HCl, corynanthine HCl and (±)-naloxone HCl (Sigma Chemical, U.S.A.); morphine HCl (Unión Químico-Farmacéutica, Spain). All drugs were dissolved in saline prepared with distilled and purified water (Milli Q).

Results

Characteristics of the OF-JOR and inhibitory effect of clonidine

Orofacial electrical stimulation by trains of 10 rectangular pulses of 60 V (supramaximal voltage) at 1 Hz elicited polyphasic electromyograms from the ipsilateral digastric muscle (see Figure 1). Under basal conditions, the elicited electromyograms showed a mean latency to onset of 5.6 ms, a mean duration of 6.3 ms, a maximum amplitude of 7.0 mV and a sum (Σ) of amplitudes of 11.2 mV (Table 1). These

basal parameters were not modified by the i.v. injection of saline (0.1 ml each dose, total volume: 0.9 ml). Furthermore, vanishing of the OF-JOR due to fatigue or adaptation was not likely because no change in the recordings were found along the entire period of the experiment (52 min).

Administration of increasing doses of the α_2-adrenoceptor agonist, clonidine (0.1–1000 $\mu g\,kg^{-1}$, i.v.) resulted in a progressive reduction of the OF-JOR, altering the configuration of the recordings and affecting simultaneously several parameters of the reflex (Figure 1). Thus, clonidine reduced, in a dose-dependent manner, the amplitudes (Σ and maximum amplitudes) and the duration of the reflex while it increased its latency to onset. These changes were further analysed using the untransformed experimental data for each parameter according to the generic hyperbolic function proposed by Parker & Waud (1971) (Table 1). This model well described the relationship between the doses of clonidine and the changes in sum of amplitudes, maximum amplitude, duration and latency, as revealed by the existence of significant correlations ($r^2 = 0.58–0.87$; $P<0.001$) and non systematic deviation from the model was found for any of these parameters. Furthermore, the slope factors (n) were not different from unity ($P>0.05$), and the maximum effects (E_{max}) obtained were, with the exception of that for latency, not different from 100 (Table 1).

The sum of amplitudes (Σ amplitude) of the OF-JOR was the most susceptible parameter to the inhibitory effects of clonidine ($ED_{50} = 13.9\,\mu g\,kg^{-1}$, i.v.), as well as the one most adequate to the description of the dose-response relationship ($r^2 = 0.87$) (Table 1 and Figure 2). Based on these results, the variations in the sum of amplitudes were used to construct the cumulative dose-response curves for clonidine, amphetamine and morphine when inhibiting the OF-JOR.

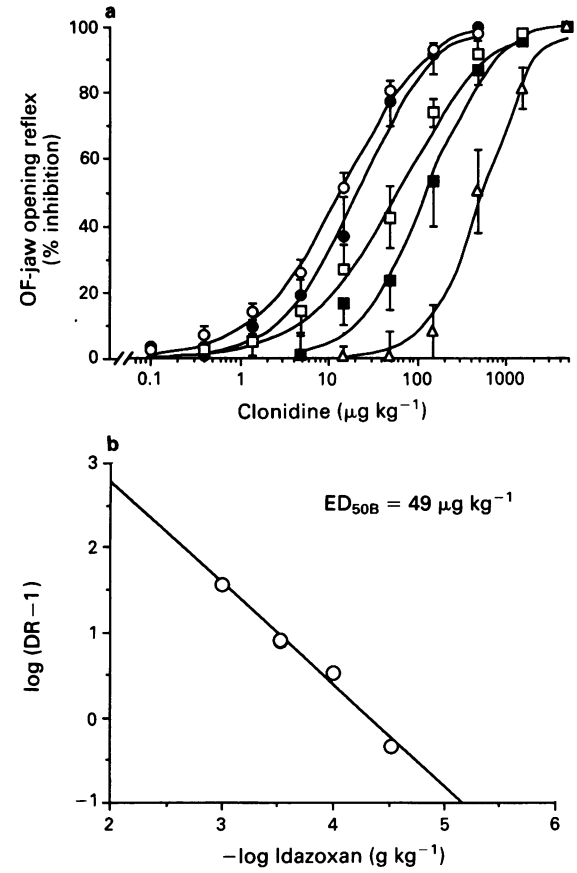


Figure 2 (a) Cumulative dose-response curves for clonidine-induced inhibition of the jaw-opening reflex (Σ of amplitudes) elicited by orofacial electrical stimulation (OF-JOR). For each dose the digital average of ten digastric electromyographic signals evoked by a train of 10 rectangular pulses (0.1 ms duration each pulse) presented at 1 Hz was obtained and then the Σ of amplitudes calculated. Eight min before determination of the dose-response curve, rats were treated either with saline (1 ml kg^{-1}, i.p., ○) or idazoxan (0.03, ●; 0.1, □; 0.3 ■; and 1 mg kg^{-1} i.p., △). Data shown are the mean ± s.e.mean (vertical bars) of 4 to 29 experiments per treatment. Curves represent the computer fitted hyperbolic functions described by equation 2. (b) Corresponding Schild plot constructed by plotting the log of the dose-ratio minus one (DR-1) against the negative log of the antagonist concentration. The data were best described by the expression $y = 5.19 - 1.20\ x$ ($r = -0.99$; $P<0.001$). The ED_{50} B value represents the dose of idazoxan (49 $\mu g\,kg^{-1}$) that necessitates a doubling of the dose of clonidine to reach the same level of response.

Effects of α_2-adrenoceptor antagonists on clonidine-induced inhibition of the OF-JOR

Pretreatment with the α_2-adrenoceptor antagonist, idazoxan (0.03–1 mg kg^{-1}, i.v., 8 min) caused a dose-dependent shift (1.5 to 37 fold) to the right of the dose-response curve for clonidine, without significant changes of the basal values, maximum effects (E_{max}) or slope factors (n), except for the highest dose of the antagonist (Figure 2). Thus, pretreatment with 1 mg kg^{-1} idazoxan increased the basal value for the sum of amplitudes of the OF-JOR (Σ amplitude increased by 15%; $P<0.05$) (data not shown) and the slope (n increasesd by 75%; $P<0.05$) of the dose-response curve for clonidine (Figure 2a). Multidimensional nonlinear fitting of the results obtained for the increasing doses of clonidine after different doses of idazoxan to a modification of the equation proposed by Gaddum (1937), suggested that, in the current functional model, idazoxan acted as a competitive antagonist with respect to clonidine and revealed a high potency for the antagonism. Thus, the slope factor of the function was no different from unity ($n = 1.22 \pm 0.11$) and the estimated dose of the antagonist idazoxan that would necessitate a doubling of the dose of clonidine to reach the same level of response (ED_{50B}) was $29 \pm 4.5\,\mu g\,kg^{-1}$. Similar results were obtained when using the Schild plot technique ($n = 1.20$; $ED_{50B} = 49\,\mu g\,kg^{-1}$) (Figure 2b).

Similarly, pretreatment with yohimbine (0.3 mg kg^{-1}, i.v., 8 min) also resulted in a marked decrease in the sensitivity of the OF-JOR to the inhibitory effect of clonidine (ED_{50} increased by 182%; $P<0.001$). In contrast to idazoxan and yohimbine, two nonselective $\alpha_{2A/B}$-adrenoceptor antagonists, the selective α_{2B}-antagonist ARC-239 (0.3 mg kg^{-1}, i.v., 8 min) did not antagonize the inhibitory effect of clonidine on the OF-JOR (data not shown).

Effects of reserpine and amphetamine on the OF-JOR

In rats pretreated with reserpine (5 mg kg^{-1}, s.c. for 18 h) the basal sum of amplitudes of the OF-JOR was not modified but the inhibitory effect of clonidine on the reflex was potentiated, probably reflecting the well-known phenomenon of reserpine-induced supersensitivity. Thus, reserpine significantly increased the potency of clonidine in inhibiting the reflex, without changing the slope of the curve or the estimated E_{max} value (saline, $ED_{50} = 13.9 \pm 1.7\,\mu g\,kg^{-1}$, i.v., $n = 29$; reserpine, $ED_{50} = 6.8 \pm 1.4\,\mu g\,kg^{-1}$, i.v., $n = 10$; $P<0.001$).

As with clonidine, the administration of increasing doses of amphetamine (0.1–3000 $\mu g\,kg^{-1}$, i.v.) resulted in a progressive reduction of the OF-JOR, that fitted well to the proposed dose-response model (equation 1) ($r^2 = 0.65$; $P<0.001$). Cumulative dose-response curves for amphetamine showed a shallow shape ($n = 0.5 \pm 0.2$; $P<0.05$) and revealed a relatively high potency for this indirectly acting amine ($ED_{50} = 135 \pm 28\,\mu g\,kg^{-1}$) in inhibiting the reflex with an estimated E_{max} of 67.1 ± 14.9% (Figure 3). The inhibitory effect of amphetamine on the JOR was highly sensitive to both the acute blockade of α_2-adrenoceptors and the prolonged inhibition of noradrenaline synthesis. Thus, pretreatment with idazoxan (0.1 mg kg^{-1}, i.v., 8 min) induced a nine

fold shift to the right of the dose-response curve for amphetamine, while treatment with the depleting drug α-methyl-*p*-tyrosine (150 mg kg^{-1} day^{-1}; i.p. for 14 days) abolished the inhibitory effect (Figure 3).

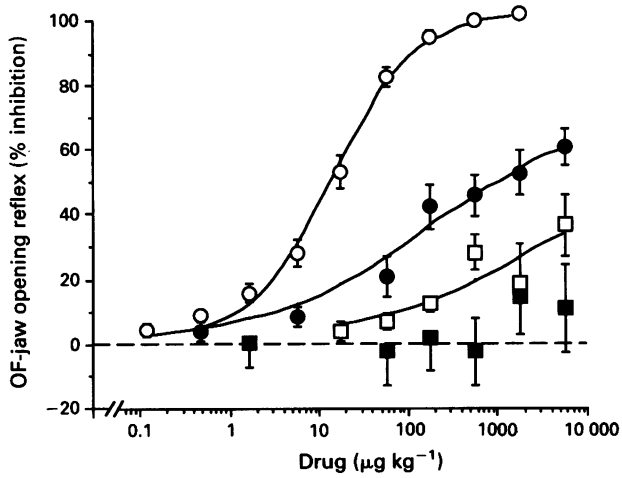


Figure 3 Cumulative dose-response curves for amphetamine-induced inhibition of the jaw-opening reflex (Σ of amplitudes) elicited by orofacial electrical stimulation (OF-JOR) in rats treated 8 min before either with saline (1 ml kg^{-1}, i.p., ●) or idazoxan (0.1 mg kg^{-1}, i.p., □), or treated for 14 days with the depleting drug α-methyl-*p*-tyrosine (150 mg kg^{-1} daily; i.p., ■). For each dose of amphetamine the digital average of ten digastric electromyographic signals evoked by a train of 10 rectangular pulses (0.1 ms duration each pulse) presented at 1 Hz was obtained and then the Σ of amplitudes calculated. The inhibitory dose-response curve for clonidine in saline pretreated rats is included for comparison (○). Data shown are the mean ± s.e.mean of 6 to 29 experiments per treatment. Solid lines represent the computer-assisted curve fitting of experimental data to the hyperbolic function described by equation 1.

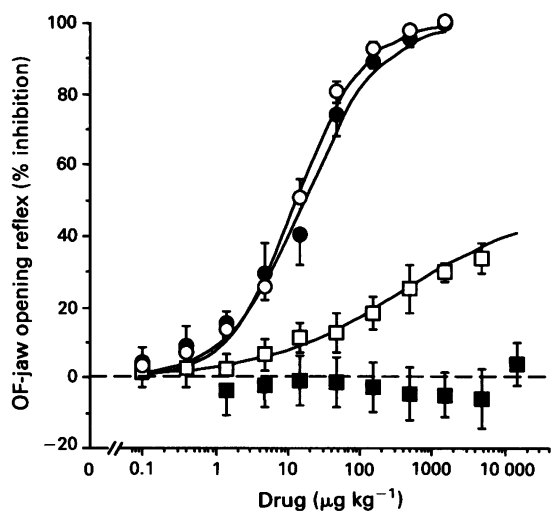


Figure 4 Cumulative dose-response curves for clonidine and morphine-induced inhibition of the jaw-opening reflex (Σ of amplitudes) elicited by orofacial electrical stimulation (OF-JOR). For each agonist dose the digital average of ten digastric electromyographic signals evoked by a train of 10 rectangular pulses (0.1 ms duration each pulse) presented at 1 Hz was obtained and then the Σ of amplitudes calculated. Eight min before determination of the dose-response curves, rats were treated either with saline (1 ml kg^{-1}, i.p., clonidine ○, morphine □) or naloxone (1 mg kg^{-1}, i.p., clonidine ●, morphine ■). Data shown are the mean ± s.e.mean of 5 to 29 experiments per treatment. Solid lines represent the computer-assisted curve fitting of experimental data to the hyperbolic function described by equation 1.

Effect of morphine on the OF-JOR

Administration of increasing doses of morphine (0.1–3000 μg kg^{-1}, i.v.) also reduced in a dose-dependent manner ($r^2 = 0.68$; $P<0.01$) the JOR evoked by orofacial stimulation but, in contrast to clonidine, it failed to inhibit the reflex fully (Figure 4). The estimated E_{max} for the inhibition of the sum of amplitudes of the OF-JOR according to equation 1 was only of 47.9 ± 19.9% with an ED_{50} value of about 325 μg kg^{-1} and a slope factor of 0.5 ± 0.2 (Figure 4).

As expected, pretreatment with the opioid antagonist, naloxone (1 mg kg^{-1}; i.v., 8 min) abolished the inhibitory effect of morphine on the OF-JOR but it did not alter the effect of clonidine (Figure 4).

Effects of acute and long-term treatments with idazoxan on clonidine-induced inhibition of the OF-JOR

Acute treatment with idazoxan (3 mg kg^{-1}; i.p. for 1 day) failed to modify significantly the dose-response relationship for clonidine-induced inhibition of the OF-JOR (Figure 5). In contrast, sustained blockade of α_2-adrenoceptors with idazoxan (3 mg kg^{-1} daily, i.p. for 14 days) led to a marked increase in clonidine sensitivity (ED_{50} decreased by 81 ± 37%; $P<0.05$), without affecting the estimated E_{max} for this agonist. The sensitization of the dose-response curve for clonidine was accompanied by a slight decrease in the slope of the inhibitory function (n decreased by 30 ± 10%; $P<0.01$). Neither the acute nor the long-term treatment with idazoxan altered the basal values of the sum of amplitudes of the OF-JOR (Figure 5).

Discussion

Averaged electromyographic responses recorded from the anterior belly of the left digastric muscle after supramaximal OF-JOR showed, when compared with those obtained after stimulation of the periodontal ligament in cats (Anderson & Mahan, 1971), a similar configuration with a mean latency to onset of the same order, consistent with a proposed disynaptic relay (Mason *et al.*, 1985). Also, the electromyographic

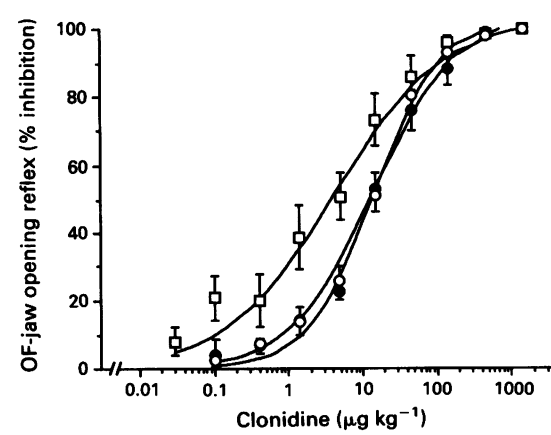


Figure 5 Cumulative dose-response curves for clonidine-induced inhibition of the jaw-opening reflex (Σ of amplitudes) elicited by orofacial electrical stimulation (OF-JOR) in rats acutely treated for 1 day with saline (1 ml kg^{-1}, i.p., ●) or idazoxan (3 mg kg^{-1}, i.p., ○), or chronically treated for 14 days with idazoxan (3 mg kg^{-1}, i.p., □). For each dose of clonidine the digital average of ten digastric electromyographic signals evoked by a train of 10 rectangular pulses (0.1 ms duration each pulse) presented at 1 Hz was obtained and then the Σ of amplitudes calculated. The experiments were carried out 12–24 h after the last injection. Data shown are the mean ± s.e.mean of 6 to 29 experiments per treatment. Curves represent the computer fitted hyperbolic functions described by equation 1.

responses after intrapulpal stimulation (TP-JOR) in the rat (Rajaona *et al.*, 1986), rabbit (Chan & Fung, 1976) and cat (Pertovaara *et al.*, 1991) showed similarities with the OF-JOR in terms of morphology, latency and duration of the reflex, suggesting, in agreement with Anderson & Mahan (1971), that the neural circuitries underlying the TP-JOR and OF-JOR may partially overlap (Curtis & Marwah, 1986a,b; Pertovaara *et al.*, 1991). However, no previous work has evaluated the effect of sustained and progressive activation of α_2-adrenoceptors on the recordings of the JOR after either orofacial or intrapulpal stimulation. The results of the present study demonstrate that clonidine induces a progressive reduction of electromyographic responses to supramaximal OF-JOR, until abolition, by altering the configuration of the recordings and by affecting in a dose-dependent manner several parameters of the reflex. Among the various parameters affected by clonidine, the reduction in the sum (Σ) of amplitudes was the most sensitive and showed the best agreement with the logistic function. The potency of clonidine obtained for the inhibition of the OF-JOR in the present study (Σ of amplitudes, ED_{50}: 14 $\mu g\,kg^{-1}$) reveals a relatively high sensitivity of the current model. Thus, the ED_{50} values reported for clonidine in causing CNS depression and in relaxing skeletal muscle in the rat were eight and 23 fold greater, respectively (Colpaert, 1986), when comapred with the results of the present study; while the ED_{50} value for clonidine in inducing mydriasis in rats was about two fold greater (Menargues *et al.*, 1990). The potency of clonidine in depressing the OF-JOR was similar to that reported for this agonist in lowering body temperature in rats (Colpaert, 1986).

As demonstrated in the present study, the OF-JOR could be completely abolished by α_2-adrenoceptor stimulation, but not by morphine administration acting through opioid receptors. The dose-response curve for morphine in inhibiting the OF-JOR fitted poorly to the dose-response model, showed a shallow shape and only reached half the level of inhibition of clonidine, suggesting that opioid control of the reflex is probably partial and indirectly exerted. Furthermore, the inhibitory effect of clonidine was not related to the opioid system since it was not reversed by naloxone. Therefore, the present results reinforce the possible physiological relevance of the OF-JOR as a propioceptive reflex involved in rhythmic chewing and protective oral mechanisms (Lund & Rossignol, 1981; Sessle, 1981) rather than a role in the response to pain.

The conclusion that an α_2-adrenoceptor mechanism is involved in the inhibition by clonidine of the OF-JOR is based on the findings that both idazoxan and yohimbine antagonized the inhibitory effect. Clonidine and idazoxan are prototypical α_2-adrenoceptor ligands (Starke *et al.*, 1974; Doxey *et al.*, 1983). Recently, however, it has been demonstrated that both drugs, and related imidazoline compounds, can also bind to noradrenaline-insensitive, non-adrenoceptor sites, the so-called imidazoline sites (Ernsberger *et al.*, 1987; Miralles *et al.*, 1993). In this context, functional responses involving clonidine and idazoxan could be attributable, at least in part, to the activation of these imidazoline sites (Pineda *et al.*, 1993). However, in the present model the antagonism induced by yohimbine confirmed the involvement of an α_2-adrenoceptor in the inhibition by clonidine of the OF-JOR, as yohimbine is a non-imidazoline compound with no affinity for the imidazoline sites (Miralles *et al.*, 1993).

A postsynaptic location for the α_2-adrenoceptor involved in the inhibition of OF-JOR was suggested by the results with the indirectly acting amine, amphetamine. Thus, inhibition of the reflex by amphetamine was highly sensitive to either the inhibition of noradrenaline synthesis with α-methyl-*p*-tyrosine or to the blockade of α_2-adrenoceptors with idazoxan. Moreover, the inhibitory effect of clonidine on the OF-JOR was potentiated by reserpine treatment, further suggesting the induction of postsynaptic receptor supersensitivity. Katakura & Chandler (1991) recently demonstrated that iontophoretic application of clonidine to individual digastric motoneurones during activation of the OF-JOR resulted in a suppression of synaptic transmission that was antagonized by yohimbine. This study and the present findings lead to suggest that activation of α_2-adrenoceptors located on trigeminal motoneurones may be involved in the control of the OF-JOR. This hypothesis is further strengthened by anatomical data which demonstrate a rich noradrenergic innervation of the trigeminal motor nucleus (Vornov & Sutin, 1983; Card *et al.*, 1986). Using similar experimental approaches, a postsynaptic location has also been proposed, inter alia, for central inhibitory α_2-adrenoceptors mediating mydriasis in the rat (Koss & San, 1976; Menargues *et al.*, 1990 and other references therein).

Finally, long-term treatment with the antagonist, idazoxan, led to an increase in the sensitivity of the α_2-adrenoceptor involved in the inhibition by clonidine of the OF-JOR, in good agreement with the observed sensitization of the dose-pupillary response curve for clonidine after chronic idazoxan in rats (Menargues *et al.*, 1990). This observation is also in line with recent findings which indicate that the density of brain α_2-adrenoceptors can be down- or up-regulated after sustained activation or blockade of the receptor (Giralt & García-Sevilla, 1989), probably as a consequence of modifications in the rate of receptor degradation (Barturen & García-Sevilla, 1992). The remarkable sensitivity of the OF-JOR to clonidine and its plasticity after chronic pharmacological treatment indicates that this functional response represents a simple and useful *in vivo* model to study various regulatory mechanisms of central postsynaptic α_2-adrenoceptors.

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References

ANDERSON, K.V. & MAHAN, P. (1971). Interaction of tooth pulp and periodontal ligament receptors in a jaw-depression reflex. *Exp. Neurol.*, **32**, 295–302.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BARTUREN, F. & GARCIA-SEVILLA, J.A. (1992). Long term treatment with desipramine increases the turnover of α_2-adrenoceptors in the rat brain. *Mol. Pharmacol.*, **42**, 846–855.

CARD, J.P., RILEY, J.N. & MOORE, R.Y. (1986). The motor trigeminal nucleus of the rat: analysis of neuronal structure and the synaptic organization of noradrenergic afferents. *J. Comp. Neurol.*, **250**, 469–484.

CHAN, S.H.H. & FUNG, S.J. (1976). The effects of morphine and nalorphine on the jaw-opening reflex in rabbit. *Exp. Neurol.*, **53**, 363–370.

COLPAERT, F.C. (1986). Maximal magnitude of effect and potency of putative α-adrenoceptor agonists in causing CNS depression, in relaxing muscle, and in lowering body temperature in rat. *Drug Dev. Res.*, **7**, 209–220.

CURTIS, A.L. & MARWAH, J. (1986a). Alpha$_2$ adrenoceptors modulate the nociceptive jaw-opening reflex in rats. *Exp. Neurol.*, **91**, 524–531.

CURTIS, A.L. & MARWAH, J. (1986b). Evidence for alpha adrenoceptor modulation of the nociceptive jaw-opening reflex in rats and rabbits. *J. Pharmacol. Exp. Ther.*, **238**, 576–579.

CURTIS, A.L. & MARWAH, J. (1987). Alpha adrenoceptor modulation of the jaw-opening reflex. *Neuropharmacology*, **26**, 649–655.

DE LEAN, A., MUNSON, P.J. & RODBARD, D. (1978). Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay and physiological dose-response curves. *Am. J. Physiol.*, **235**, E97–E92.

DOSTROVSKY, J.O., HU, J., SESSLE, B. & SUMINO, R. (1982). Stimulation sites in periaqueductal gray, nucleus raphe magnus and adjacent regions effective in suppressing oral-facial reflexes. *Brain Res.*, **252**, 287–297.

DOXEY, J.C., ROACH, A.G. & SMITH, C.F.C. (1983). Studies on RX 781094: a selective, potent and specific antagonist of α_2-adrenoceptors. *Br. J. Pharmacol.*, **78**, 489–505.

ERNSBERGER, P., MEELEY, M.P., MANN, J.J. & REIS, D.J. (1987). Clonidine binds to imidazole binding sites as well as α_2-adrenoceptors in the ventrolateral medulla. *Eur. J. Pharmacol.*, **134**, 1–13.

GADDUM, J.H. (1937). The quantitative effects of antagonistic drugs. *J. Physiol.*, **89**, 7–9P.

GIRALT, M.T. & GARCIA-SEVILLA, J.A. (1989). Acute and long-term regulation of brain α_2-adrenoceptors after manipulation of noradrenergic transmission in the rat. *Eur. J. Pharmacol.*, **164**, 455–466.

HA, H., WU, R.S., CONTRERAS, R.A. & TAN, E.C. (1978). Measurement of pain threshold by electrical stimulation of tooth pulp afferents in the monkey. *Exp. Neurol.*, **61**, 260–269.

KATAKURA, N. & CHANDLER, S.H. (1991). Iontophoretic analysis of the pharmacologic mechanisms responsible for initiation and modulation of trigeminal motoneuronal discharge evoked by intra-oral afferent stimulation. *Brain Res.*, **549**, 66–77.

KAWAKITA, K. & FUNAKOSHI, M. (1982). Suppression of the jaw-opening reflex by conditioning A-delta fiber stimulation and electroacupuncture in the rat. *Exp. Neurol.*, **78**, 461–465.

KOSS, M.C. & SAN, L.C. (1976). Analysis of clonidine-induced mydriasis. *Invest. Ophthalmol.*, **15**, 566–570.

LAZARENO, S. & BIRDSALL, N.J.M. (1993). Estimation of competitive antagonist affinity from functional inhibition curves using the Gaddum, Schild and Cheng-Prusoff equations. *Br. J. Pharmacol.*, **109**, 1110–1119.

LEATHERBARROW, M. (1990). *Grafit*, Version 2.0. Erithacus Software Ltd., Staines, U.K.

LUND, J.P. & ROSSIGNOL, S. (1981). Modulation of the amplitude of the digastric jaw opening reflex during the masticatory cicle. *Neuroscience*, **6**, 95–98.

LUSCHEI, E.S. & GOLDBERG, L.J. (1981). Neural mechanism of mandibular control: mastication and voluntary biting. In *Handbook of Physiology*, Section I, vol. 2, pp. 1237–1274. ed. Brookhart, J.M. & Mountcastle, V.B. New York, U.S.A.: Academic Press.

MASON, P., STRASSMAN, A. & MACIEWICZ, R. (1985). Is the jaw-opening reflex a valid model of pain? *Brain Res. Rev.*, **10**, 137–146.

MENARGUES, A., OBACH, R. & GARCIA-SEVILLA, J.A. (1990). Modulation by antidepressant drugs of CNS postsynaptic α_2-adrenoceptors mediating mydriasis in the rat. *Naunyn-Schmied. Arch. Pharmacol.*, **341**, 101–107.

MIRALLES, A., OLMOS, G., SASTRE, M., BARTUREN, F., MARTIN, I. & GARCIA-SEVILLA, J.A. (1993). Discrimination and pharmacological characterization of I_2-imidazoline sites with [^{3}H]-idazoxan and alpha-2 adrenoceptors with [^{3}H]-RX 821002 (2-methoxy idazoxan) in human and rat brains. *J. Pharmacol. Exp. Ther.*, **264**, 1187–1197.

MOTULSKY, H.J. & RANSNAS, L.A. (1987). Fitting curves to data using nonlinear regression: a practical and nonmathematical review. *FASEB J.*, **1**, 365–374.

MYLINSKI, N. & MATTHEWS, B. (1987). Intrapulpal nerve stimulation in the rat. *J. Neurosci. Methods*, **22**, 73–78.

OLIVERAS, J.L., WODA, A., GUILBAUD, G. & BESSON, J. (1974). Inhibition of the jaw opening reflex by electrical stimulation of the periaqueductal gray matter in the awake unrestrained cat. *Brain Res.*, **72**, 328–331.

PARKER, R.B. & WAUD, D.R. (1971). Pharmcological estimation of drug-receptor dissociation constants. Statistical evaluation. I. Agonists. *J. Pharmacol. Exp. Ther.*, **177**, 1–12.

PERTOVAARA, A., KEMPPAINEN, P. & KAUPPILA, T. (1991). The effects of medetomidine, an α_2-adrenoceptor agonist, and cocaine on the tooth pulp-evoked jaw-opening reflex in cat. *Pharmacol. Biochem. Behav.*, **38**, 287–292.

PINEDA, J., UGEDO, L. & GARCIA-SEVILLA, J.A. (1993). Stimulatory effects of clonidine, cirazoline and rilmenidine on locus coeruleus noradrenergic neurones: possible involvement of imidazoline-preferring receptors. *Naunyn-Schmied. Arch. Pharmacol.*, **348**, 134–140.

RAJAONA, J., DALLEL, R. & WODA, A. (1986). Is electrical stimulation of the rat incisor an appropriate experimental nociceptive stimulus? *Exp. Neurol.*, **93**, 291–299.

RATKOWSKY, D. (1983). Comparing parameter estimates from more than one data set. In *Nonlinear Regression Modelling: A Unified and Practical Approach*. pp. 135–152. New York, U.S.A.: Dekker.

SESSLE, B.J. (1981). Initiation, regulation and significance of jaw muscle reflexes. In *Oral-Facial Sensory and Motor Functions*. ed. Kawamura, Y. & Dubner, R., pp. 187–203. Tokyo: Quintessence.

SESSLE, B.J. & HU, J. (1981). Raphe-induced suppression of the jaw-opening reflex and single neurons in trigeminal subnucleus oralis, and influence of naloxone and subnucleus caudalis. *Pain*, **10**, 19–36.

STARKE, K., MONTEL, H., GAYK, W. & MERKER, R. (1974). Comparison of the effects of clonidine on pre- and postsynaptic adrenoceptors in the rabbit pulmonary artery. *Naunyn-Schmied. Arch. Pharmacol.*, **285**, 133–150.

TAL, M., SHARAV, Y. & DEVOR, M. (1981). Modulation of the jaw-opening reflex by peripheral electrical stimulation. *Exp. Neurol.*, **74**, 907–919.

TANAKA, H. & TODA, K. (1982). Inhibition of the tooth pulp-evoked jaw opening reflex by stimulation of raphe nuclei in the rat. *Exp. Neurol.*, **77**, 102–112.

TODA, K. (1982a). Peripheral nerve stimulation for producing the suppressive effect on the tooth pulp-evoked jaw opening reflex in rat: relation between stimulus intensity and degree of suppression. *Exp. Neurol.*, **76**, 309–317.

TODA, K. (1982b). Peripheral afferent nerve impulses for conveying electroacupuncture effects on the jaw opening reflex evoked by tooth pulp stimulation in rat. *Am. J. Chinese Med.*, **9**, 319–325.

TODA, K., IRIKI, A. & TANAKA, H. (1981). Jaw opening reflex affected by electroacupuncture in rat. *Neurosci. Lett.*, **25**, 161–166.

VORNOV, J.J. & SUTIN, J. (1983). Brainstem projections to the normal and noradrenergically hiperinnervated trigeminal motor nucleus. *J. Comp. Neurol.*, **214**, 198–208.

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The effects of propofol and enflurane on single calcium channel currents of guinea-pig isolated ventricular myocytes

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1 The effects of the anaesthetics, propofol (100 μM) and enflurane (3%, 1.46 mM), on single L type calcium channel currents were investigated in single myocytes isolated from guinea-pig ventricles. Channel activity was recorded from membrane patches by use of the 'cell-attached' patch-clamp technique (pipette solution containing 110 mM $BaCl_2$, 5 μM Bay K 8644, 5 μM HEPES, pH 7.4; temperature 36°C).

2 Channel conductance was calculated from the slope of the relationship between single channel current and membrane potential during step depolarizations to activate the channel over a range of approximately − 20 to + 20 mV. Neither propofol (6 cells) nor enflurane (7 cells) caused any significant reduction in channel conductance.

3 Both propofol (7 cells) and enflurane (9 cells) decreased the probability of the channel being open during depolarizations to + 10 mV (measured from histograms of the fraction of time spent by the channel at different current levels, taking areas under the Gaussian curves fitted to the open and closed components of the distributions to represent the proportion of time spent in the two states).

4 A fraction of the current traces showed no detectable channel openings in response to step depolarizations to + 10 mV. Both propofol and enflurane significantly increased the fraction of silent traces.

5 Transitions across a threshold halfway between the open and closed levels were used to define periods spent in the open and closed states. Both propofol (7 cells) and enflurane (9 cells) reduced the mean open times and increased the mean closed times of the calcium channel.

6 Histograms were plotted showing the distributions of times spent by the channels in the open and closed states. Two exponentials were fitted to the open and closed time distributions. Both propofol (7 cells) and enflurane (9 cells) shortened both time constants fitted to the open times and lengthened both time constants fitted to the closed times.

7 It is concluded that both propofol and enflurane appear to alter the kinetics of opening and closing of calcium channels to favour shut channels without altering channel conductance. This effect would be expected to result in a reduction of the macroscopic calcium current and thus contribute to the negative inotropic action of these anaesthetics.

Keywords: Propofol; enflurane; general anaesthetics; calcium channel currents; isolated ventricular myocytes

Introduction

Many general anaesthetics depress myocardial contractility, an effect which may in certain circumstances prove harmful. This depression of contractility may arise, at least in part, from a reduction of the inward calcium current into cardiac cells (Ikemoto *et al.*, 1985; Terrar & Victory, 1988a,b; Puttick & Terrar, 1989; 1992; Hirota *et al.*, 1989; Niggli *et al.*, 1989; Bosnjak *et al.*, 1991). This would be expected to reduce the cytosolic calcium transient (both directly because calcium entering would be reduced and indirectly since calcium-induced calcium release from the sarcoplasmic reticulum would be expected to be less); this in turn would reduce contraction.

The aim of this work was to investigate possible mechanisms for reductions in macroscopic calcium current by examining actions of anaesthetics on the single calcium channel activity which underlies this current. Two different anaesthetics were chosen: the intravenous agent, propofol (2,4-diisopropylphenol) and the inhalational halogenated anaesthetic, enflurane. Preliminary observations have been presented to the British Pharmacological Society (Puttick & Terrar, 1991).

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Methods

Cells were isolated from guinea-pig ventricles by collagenase digestion (Powell *et al.*, 1981; Mitchell *et al.*, 1983). Aliquots of cell suspension were mounted on the surface of an agar-coated coverslip in a perspex organ bath. The surperfusing solution contained (mM): NaCl 105, KCl 20, $NaHCO_3$ 14.5, KH_2PO_4 1.18, $MgCl_2$ 1.18, $CaCl_2$ 2.5 and glucose 11.1. All solutions were bubbled with 95% O_2: 5% CO_2 (pH 7.4) and maintained at 36°C. The high potassium solution (following Shibasaki, 1987) was chosen because the membrane potential was expected to be less variable (at approximately − 40 mV) between cells than would be the case in normal potassium; this is particularly important in cell attached recording (see below) where the pipette potential is under experimental control but where transmembrane potential is the sum of the cell resting potential and the pipette potential. In one series of experiments, the resting membrane potential of guinea-pig ventricular myocytes in our extracellular solution was measured by breaking through the cell membrane with vigorous suction. After the membrane was broken through the cells were current clamped and the mean resting membrane potential was 40.0 ± 0.6 mV ($n = 11$).

A tap at the inflow to the bath permitted rapid changeover to anaesthetic-containing solution. Measurements in the presence of anaesthetic were made at least 3 min after changeover to anaesthetic-containing solution when the

actions appeared to have reached a steady state. Recovery was slow or incomplete in some experiments (possibly as a consequence of accumulation of anaesthetic within the patch pipette); observations in the absence of anaesthetic showed that no significant changes in calcium channel activity occurred over the duration of these experiments (e.g. in the absence of drugs, values before and after a time equivalent to drug exposure were: mean open time 6.0 ± 1.0 and 6.2 ± 0.8 ms; mean closed time 20.8 ± 4.5 and 20.4 ± 3.0 ms; probability of being in the open state 0.21 ± 0.05 and 0.21 ± 0.05; all changes not significant, $P > 0.05$, $n = 6$ cells).

Patch electrodes were pulled from borosilicate capillary tubes (Clark Electromedical) on a vertical electrode puller (Narishige PE-2) by a two step process. Electrodes were backfilled with a solution containing (mM): $BaCl_2$ 110, Bay K 8644 0.005, HEPES 5 (pH 7.4). The high concentration of barium ions was used to maximize the signal to noise ratio and to eliminate potassium currents (see e.g. Cavalie *et al.*, 1986); the dihydropyridine calcium channel agonist (Bay K 8644) increased the tendency for long openings and therefore facilitated single channel recording (see Kokubun & Reuter, 1984; Hess *et al.*, 1984). Capacitance noise was kept to a minimum by maintaining the solution in the electrode at a low level and coating electrodes with 'Repelcoat' (Sigma) which reduced the tendency for fluid films (with additional noise associated with the increased capacitance) to form on the electrode above the solution bathing the cells. Resistances of the patch electrodes were between 3 and 6 MΩ. Seal resistances were between 5 and 20 GΩ.

Currents were recorded from membrane patches in the cell attached configuration (Hamill *et al.*, 1984) with a L/M-EPC 7 patch-clamp amplifier (List-Medical-Electronic: Darmstadt, Germany). Current records were filtered at 1 kHz (eight pole Bessel filter) and stored together with potential records on videotape using a Sony pulse code modulation unit coupled to a Panasonic video recorder. Calcium currents were evoked by 400 ms step depolarizations (applied to the patch membrane by making the pipette potential negative with respect to the bath; frequency 0.5 Hz). When slope conductance was to be measured, step potentials were 20 to 60 mV depolarized from the resting potential (and thus approximately -20 to $+20$ mV across the membrane). Measurements of open channel probability and of open and closed times were made for steps of 50 mV (approximately $+10$ mV). Cells showing more than one open channel current level (indicating more than one channel contributing to activity of the membrane patch) were rejected for this analysis. Channels were thought to be L type calcium channels since T type channels would be expected to be inactivated at a membrane potential of -40 mV, and because their conductance was approximately 30 pS with 110 mM external Ba^{2+} ions (see e.g. Cavalie *et al.*, 1986); in addition,

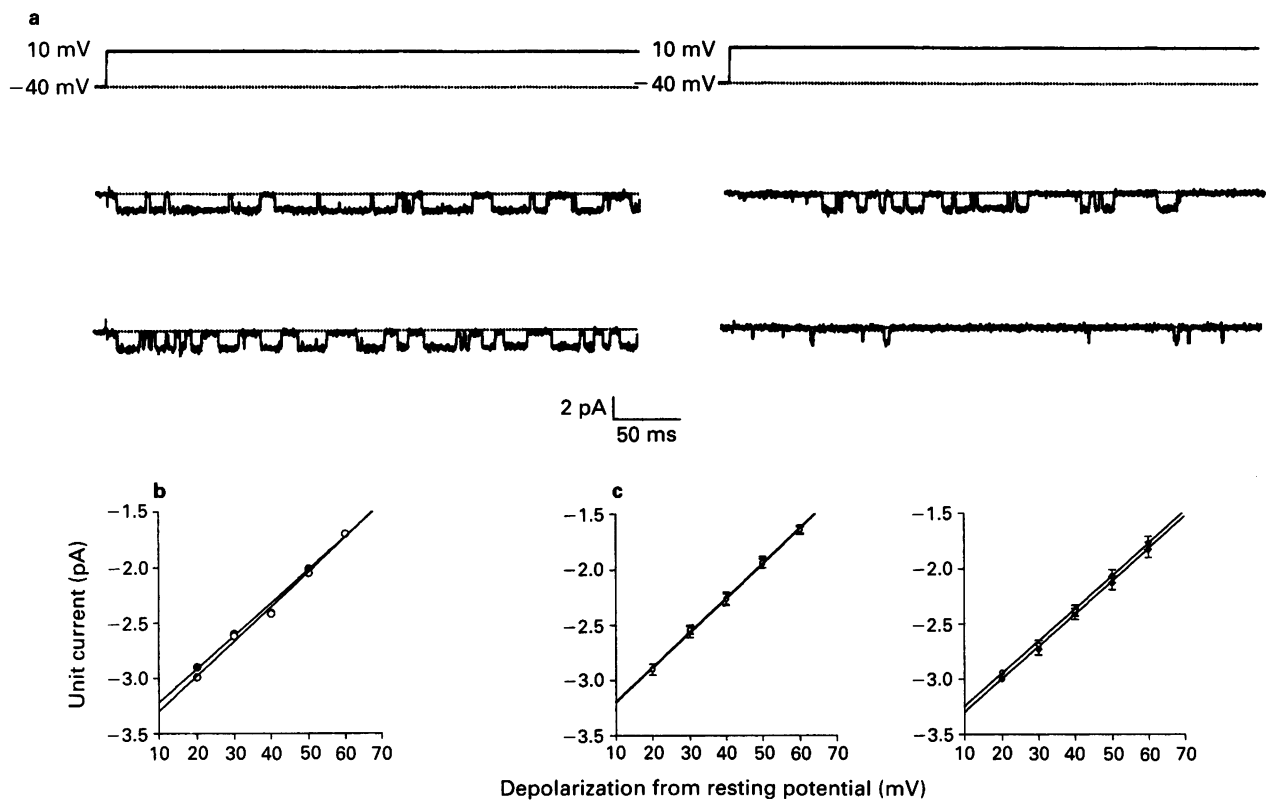


Figure 1 (a) Single calcium channel currents carried by barium ions (middle and lower traces) were evoked in response to a depolarization (duration 400 ms) of 50 mV (upper traces) in the absence (left) and presence (right) of 100 μM propofol. The cell membrane potential was assumed to be approximately -40 mV under these conditions (extracellular potassium concentration 21.18 mM, see Methods). Components of leakage and capacitance currents have been subtracted and inward currents (channel open) are shown as downward deflections. The baseline level (i.e. channel shut) is indicated by the dotted line. The single channel record was filtered at 1 kHz. (b) Open calcium channel current-voltage relation from one cell in the presence (○) and absence (●) of 100 μM propofol. Unitary barium currents were evoked by 400 ms step depolarizations from -40 mV to -20, -10, 0, $+10$, $+20$ mV. The lines were fitted by regression from -20 to $+20$ mV and yielded a conductance of 32 pS (control) and 30 pS (propofol). The regression coefficients were 0.996 (control) and 0.995 (propofol). (c) Open calcium channel current-voltage relations from two groups of cells exposed to propofol (left) and enflurane (right). Points (○ for controls; ● for anaesthetics) represent the mean ± s.e.mean obtained from 6 (left) and 7 (right) membrane patches. Note that the points for propofol data are not shown because they are too close to the control to be shown separately. The conductances yielded by the regression lines were 31 pS both in the absence and presence of propofol and 30 pS and 29 pS in the absence and presence of enflurane respectively. The regression coefficient was 0.999 for all the lines.

single calcium channel currents were abolished by exposure to the L-type calcium channel blocker, nisoldipine (2 μM) in three cells where this was tested (Nilius *et al.*, 1985).

Current signals were digitized using a Data Translation DT2801A data acquisition card and analysed with software kindly provided by Dr J. Dempster of the University of Strathclyde (using VCAN for subtraction of capacitance and 'leakage' current components and PAT for single channel analysis). Translations across a threshold set halfway between closed and open current levels were used to define the start and end of channel openings and closings. The number of events included in the histograms ranged from 152 to over 2000. Student's paired t test was used to compare data before and after administration of propofol or enflurane (taking $P < 0.05$ to indicate significance). Observations are shown as mean ± standard error (s.e.).

Data from an earlier series of experiments where increased recording noise necessitated more severe filtering (300 Hz) are also included at the end of the Results section. Conditions for these experiments were broadly similar to those described above except that the concentration of Bay K 8644 was 1 μM and analysis of channel opening and closing was carried out for depolarizing of 40 mV (in other words at a transmembrane potential of approximately 0 mV rather than + 10 mV).

Drugs

Propofol (ICI) was used as the clinically available preparation at a final concentration of 100 μM. In experiments with enflurane (Abbott), the solution was pre-equilibrated for at least 45 min with an Enfluratec vaporiser (see Terrar & Victory, 1988a,b; Puttick & Terrar, 1992). The enflurane concentration in the bath was assayed by infrared spectrophotometry (Speden, 1963); 3% enflurane corresponded to a bath concentration of 1.46 ± 0.09 mM. The concentrations of propofol and enflurane was chosen on the basis of the dose-response curves for these drugs on whole-cell calcium currents recorded from a previous series of experiments in guinea-pig ventricular cells (Puttick & Terrar, 1992). Bay K

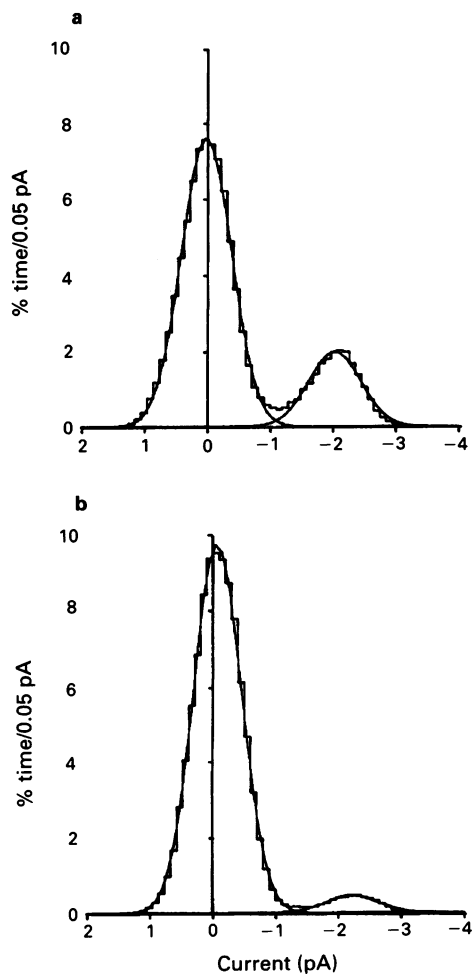


Figure 2 Histograms showing the distribution of calcium channel amplitude in the absence (a) and presence of 100 μM propofol (b). Current is plotted on the X-axis with the percentage of time the signal is found at each level (bin with 0.05 pA) on the Y-axis. The two peaks represent baseline (zero current level) and open channel unitary current level (at − 2.1 pA) respectively. Gaussian curves have been fitted to the data and the relative area under each curve represents the proportion of time spent in each state. The histograms are compiled from records obtained from one guinea-pig isolated ventricular cell. Similar effects of propofol were observed in 6 other isolated ventricular myocytes.

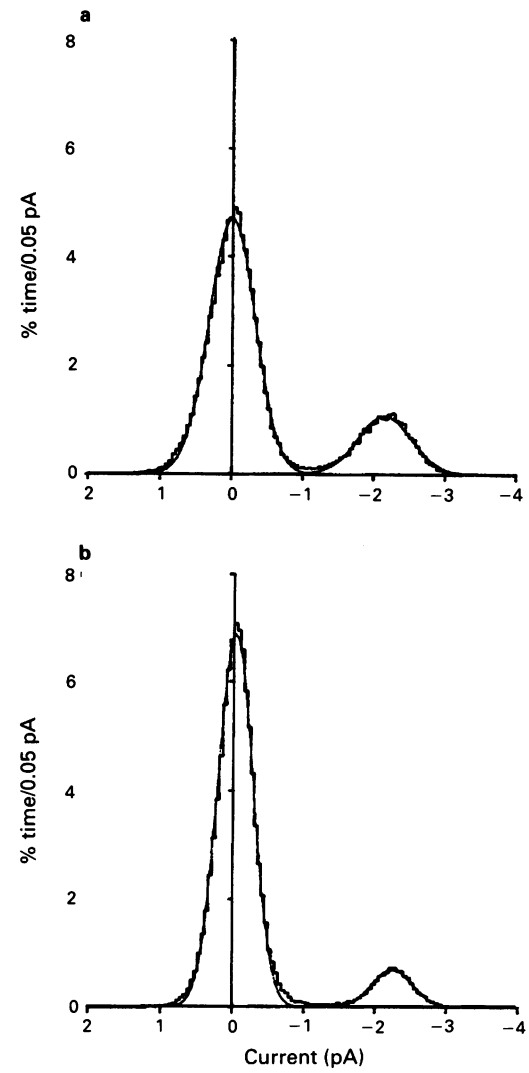


Figure 3 Histograms showing the distribution of calcium channel amplitude in the absence (a) and presence of 3% enflurane (b). Current is plotted on the X-axis with the percentage of time the signal is found at each level (bin width 0.05 pA) on the Y-axis. The two peaks represent baseline (zero current level) and open channel unitary current level (at − 2.1 pA) respectively. Gaussian curves have been fitted to the data and the relative area under each curve represents the proportion of time spent in each state. The histograms are compiled from records obtained from one guinea-pig isolated ventricular cell. Similar effects of enflurane were observed in 8 other isolated ventricular myocytes.

8644 (methyl-1,4-dihydro-2, 6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) was obtained from Calbiochem.

Results

Figure 1a shows sample records of unitary currents carried by Ba^{2+} ions through calcium channels in response to step depolarization of 50 mV from the resting potential (approximately − 40 mV in high potassium solution; see Methods). It appears from these records that during exposure to propofol (100 μM) the channel spent less time open and more time closed while the current amplitude when the channel was open showed little or no change. Channel conductance was measured from the slope of the relationship between single channel current and membrane potential during step depolarizations (over the range 20 to 60 mV depolarized with respect to the cell resting potential, or approximately − 20 to + 20 mV). This relationship is shown for a single cell in Figure 1b. Conductances measured from plots of this kind were 29.7 ± 0.8 pS in the absence and 30.5 ± 1.0 pS in the presence of 100 μM propofol ($n = 6$ cells; no significant difference, $P > 0.05$). The lack of effect of propofol on single channel conductance is further illustrated in Figure 1c which shows regression lines for the pooled data from the six cells.

When similar experiments were carried out with enflurane (3%), it was again found that the anaesthetic appeared not to influence channel conductance (30.2 ± 1.5 pS in the absence and 28.8 ± 1.3 pS in the presence of enflurane; $n = 7$; $P > 0.05$). A plot of the pooled data is shown in Figure 1c.

The effects of anaesthetics on probability of channel opening were investigated in more detail by plotting histograms of the fraction of time spent by the channel at different current levels, as shown in Figure 2. The two peaks in the distribution represent two states of the channel: closed at 0 pA and open at approximately − 2 pA. Gaussian curves were fitted to the two components of the histogram and the areas beneath them taken to represent the proportions of time spent in the closed and open states. In the presence of propofol the proportion of time spent by the channel in the open state was reduced, with a corresponding increase in the proportion of time spent in the closed state. As described in detail above, exposure to propofol did not lead to any consistent change in the size of the single channel current (shown by the difference between the two peaks of the histogram on the current axis). Similar observations were made in seven cells. The data are summarised in Table 1 where it can be seen that propofol caused a significant reduction in P_O, the probability of the channel being open ($P < 0.05$).

While channel opening was observed in response to the majority of step depolarizations, a fraction of the current traces were silent (without channel openings). The fraction of silent traces during step depolarizations was significantly increased on exposure to propofol, again consistent with a decrease in open probability (Table 1a).

When cells were exposed to 3% enflurane, similar changes as those described for propofol were seen in histograms of fraction of time spent by the channel at different current levels (Figure 3). Again P_O, the probability of the channel being open, was significantly reduced in the nine cells exposed to enflurane (Table 1a). The fraction of silent traces was also significantly decreased by enflurane (Table 1a).

Individual openings and closings of the channel were investigated and the means of all open and closed times were calculated for each cell. Openings and closings were defined by transitions across the threshold halfway between the closed and open current levels (see Methods). These data are summarised in Table 1a. It can be seen that both propofol and enflurane significantly decreased mean open time and increased mean closed time. These mean values included the silent traces (taken as 400 ms closed times).

While it is assumed in the preceding analysis that the channel exists in distinct open and closed states, the above measurements do not depend on assumptions concerning the precise number of open and closed states. Further analysis was carried out by plotting histograms of open and closed times as shown in Figure 4. It is apparent from the histograms that during exposure to propofol there was an increase in the proportion of short open times (contrast Figures 4a and b) and an increase in the proportion of long closed times (contrast Figures 4c and d). Two exponentials could be fitted to the distributions of open and closed times consistent with the possibility of two open and two closed states under these conditions (see e.g. Hess et al., 1984; Cavalie et al., 1986). Measurements of fast and slow time constants for exponentials fitted to the open and closed time histograms are shown in Table 1b. Exposure to propofol significantly decreased both time constants for channel opening and increased both time constants for channel closing.

A similar analysis for 3% enflurane is shown in Figure 5.

Table 1 Effects of propofol (100 μM) and enflurane (3%) on the kinetics and probability of openings of single 'L' type calcium channels

A

	n	*Open-time* (ms)	*Close-time* (ms)	P_O	*Fraction of silent traces*
Control	9	6.4 ± 0.7	14.6 ± 2.2	0.23 ± 0.03	0.17 ± 0.03
Enflurane	9	4.6 ± 0.5*	24.9 ± 2.5*	0.14 ± 0.02*	0.37 ± 0.05*
Control	7	5.2 ± 0.7	14.7 ± 1.6	0.20 ± 0.02	0.14 ± 0.03
Propofol	7	3.2 ± 0.4*	30.6 ± 4.5*	0.09 ± 0.01*	0.37 ± 0.07*

B

		Open state		*Closed state*	
	n	τ_{fast} (ms)	τ_{slow} (ms)	τ_{fast} (ms)	τ_{slow} (ms)
Control	9	2.8 ± 0.6	10.4 ± 1.2	2.2 ± 0.5	11.2 ± 1.5
Enflurane	9	1.3 ± 0.4*	7.3 ± 0.8*	5.8 ± 1.0*	52.9 ± 16.8*
Control	7	1.6 ± 0.3	10.4 ± 1.8	1.4 ± 0.3	12.4 ± 2.3
Propofol	7	1.0 ± 0.2*	7.6 ± 1.5*	4.1 ± 1.3*	36.6 ± 10.1*

The Table shows the effects of propofol and enflurane. (**A**) Mean open-times, mean closed-times, the mean probability of opening (P_O) and the fraction of silent traces. (**B**) mean time constants (τ) of the double exponential curves fitting the open- and closed-time distributions. Unitary currents were evoked by a 50 mV depolarizing pulse (400 ms) from the membrane potential of approximately − 40 mV. The pipette solution contained 110 mM barium chloride, 5 μM Bay K 8644 and 5 mM HEPES. Values before and after administration of propofol or enflurane are expressed as mean ± s.e.mean. *$P < 0.05$ compared to control. n is the number of cells.

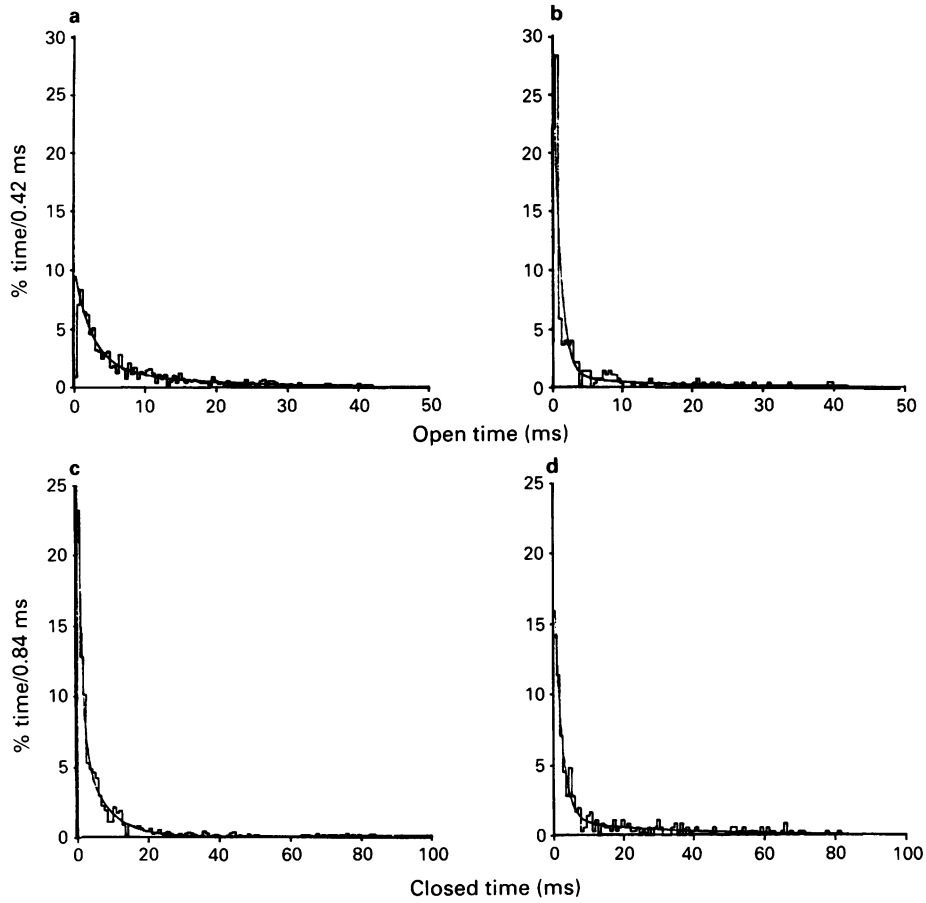


Figure 4 The histograms show the distribution of the open (a and b) and closed (c and d) channel dwell times in the absence (a and c) and presence (b and d) of 100 μM propofol. Open or closed times (ms) are plotted on the X-axis and the percentage of time the channel spent in each time bin (0.42 or 0.84 ms) was plotted on the Y-axis. Average times are (a) 7.8 ms, (b) 3.6 ms, (c) 15.9 ms and (d) 38.5 ms. Double exponentials were fitted to each distribution. The associated time constants for fast and slow components are (a) 2.5 ms (fast), 12.3 ms (slow), (b) 0.9 ms (fast), 11.1 ms (slow), (c) 1.1 ms (fast), 7.0 ms (slow), and (d) 2.0 ms (fast), 30.1 ms (slow). The histograms were compiled from records obtained from one guinea-pig isolated ventricular cell. Similar results were observed in 6 other ventricular myocytes.

Again it appears that the proportions of short open times and of long closed times were increased by enflurane (contrast Figure 5b with 5a, and 5d with 5c). The time constants for channel opening were significantly decreased and the time constants for channel closing significantly increased in the presence of enflurane (Table 1b).

Further support for the observations described above is provided by measurements from an earlier series of experiments in which the current noise was greater, necessitating filtering at 300 Hz (see Methods). Thus, short events would be under-represented compared with the above data filtered at 1 kHz. Propofol (100 μM, 6 cells) decreased open probability from 0.24 ± 0.05 to 0.13 ± 0.14, reduced mean open time from 10.4 ± 2.1 to 7.1 ± 1.8 ms and increased mean closed time from 31.5 ± 7.1 to 61.9 ± 14 ms (all changes significant, $P < 0.05$). Enflurane (3%, 4 cells) decreased open probability from 0.29 ± 0.07 to 0.09 ± 0.04 and increased closed time from 26.1 ± 7.2 to 70.2 ± 13.6 ms ($P < 0.05$); the decrease in mean open time from 9.3 ± 0.9 to 5.7 ± 0.5 ms did not reach significance with this small sample. Open and closed time histograms were broadly similar to those shown in Figures 4 and 5; single exponentials were fitted at times greater than 3 ms, accepting that a faster component may well have been missed. With this analysis the time constants of the fitted exponentials were: propofol reduced open state time constant from 6.6 ± 1.0 to 4.2 ± 0.09 ms and increased closed state time constant from 13.1 ± 1.1 to 32.0 ± 9.6 ms; enflurane reduced open state time constant from 7.4 ± 1.1 to 4.3 ± 1.1 ms and increased closed state time constant from 15.1 ± 3.7 to 60.2 ± 12.1 ms (all changes significant, $P < 0.05$). Thus, all changes found in this earlier series of experiments were consistent with the observations reported above for the lower noise recordings filtered at 1 kHz.

Discussion

It is interesting that two anaesthetics with markedly different structures and properties, the intravenous agent, propofol and the inhalation agent, enflurane, both consistently reduced the probability of the calcium channel being open during step depolarizations but had little or no effect on channel conductance. The reduction in open channel probability seemed to reflect both a shortening of the mean time for which the channels remained open and a lengthening of the mean time for which the channels remained closed.

The behaviour of L type calcium channels in cardiac muscle has been modelled with two closed states (e.g. Cavalie *et al.*, 1986). Two open states have been suggested to occur in the presence of Bay K 8644 (Hess *et al.*, 1984). The data presented here are consistent with such models since two exponential components could be fitted to the distributions of both closed and open times in the absence of anaesthetics. It would be of interest to know whether the anaesthetics propofol and enflurane simply altered the probability of transitions between pre-existing states or whether they might

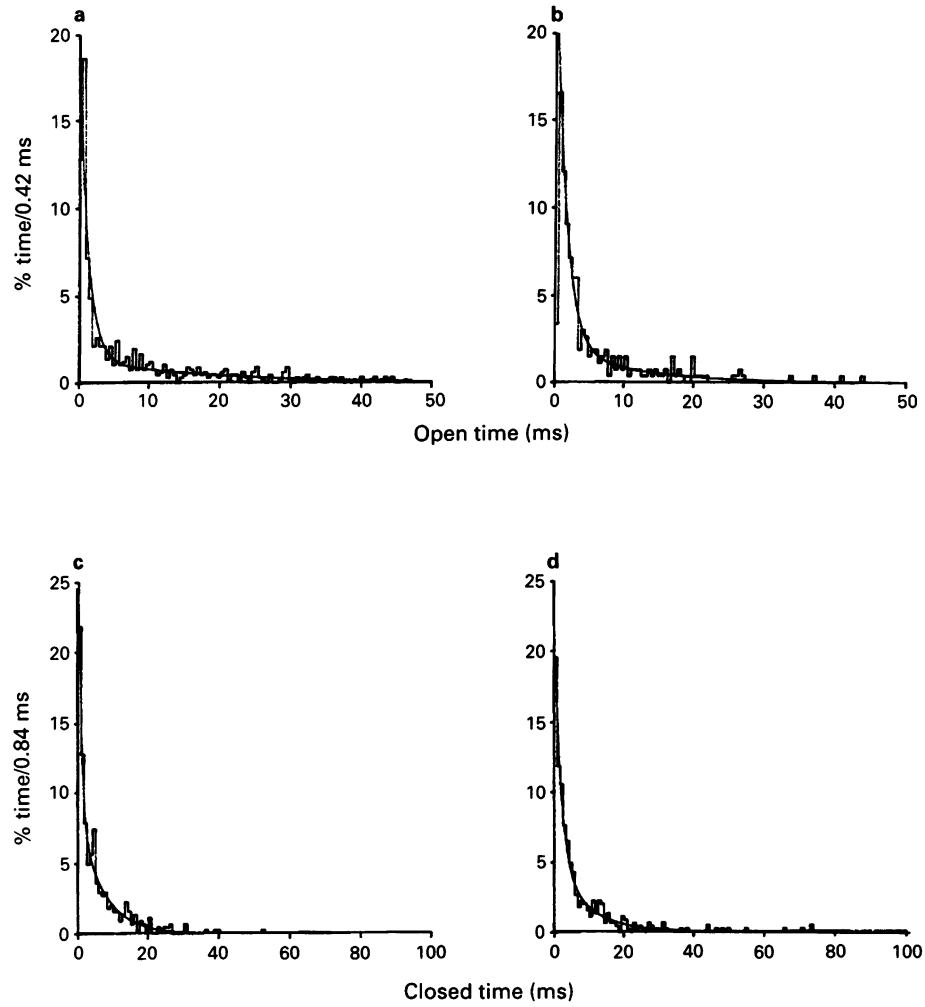


Figure 5 The histograms show the distribution of the open (a and b) and closed (c and d) channel dwell times in the absence (a and c) and presence (b and d) of 3% enflurane. Open or closed times (ms) are plotted on the X-axis and the percentage of time the channel spent in each time bin (0.42 or 0.84 ms) was plotted on the Y-axis. Average times are (a) 11.1 ms, (b) 5.9 ms, (c) 10.9 ms and (d) 13.2 ms. Double exponentials were fitted to each distribution. The associated time constants for fast and slow components are (a) 1.6 ms (fast), 11.9 ms (slow). (b) 0.3 ms (fast), 8.4 ms (slow), (c) 0.8 ms (fast), 6.9 ms (slow) and (d) 1.9 ms (fast), 9.4 ms (slow). The histograms were compiled from records obtained from one guinea-pig isolated ventricular cell. Similar results were observed in 8 other ventricular myocytes.

introduce additional anaesthetic-associated alternative states. It is clear that the shapes of the open and closed time distributions were altered by propofol and enflurane and that if two exponentials were fitted, both time constants for the open time distributions were shortened and both time constants for the closed time distributions were lengthened. However, in view of the difficulty of separating three or more exponential components when their time constants are not widely spread, the possibility that an additional component might be fitted in the presence of anaesthetic cannot be excluded.

It should also be noted that the fraction of silent traces (which represent closed times as large as or greater than the 400 ms duration of the depolarizing pulse and therefore too long to appear on the plots of the closed time distributions) was increased during exposure both to propofol and to enflurane. The appearance of these long shut periods is often attributed to shifting between 'modes' of channel opening and closing (e.g. Hess *et al.*, 1984). Following this analysis, part of the decrease in probability of the channel being open would be the result of anaesthetics leading to calcium channels spending more time in mode zero (as has been observed with calcium channel antagonists such as nitrendipine, Hess *et al.*, 1984; but see Lacerda & Brown, 1989, for an alternative analysis). These changes induced by the anaesthetics were in addition to the altered characteristics in modes where channel opening can be observed (as in Figures 4 and 5 where there was an increase in the fraction of shut times over the range 20 to 80 ms in the presence of anaesthetic).

In conclusion, it appears that both propofol and enflurane applied in the solution bathing cardiac ventricular cells can gain access to the region of calcium channels located beneath a cell-attached micropipette to decrease the probability that the channels will be open during step depolarizations without exerting an appreciable effect on channel conductance. The observations are consistent with the hypothesis that propofol and enflurane altered the kinetics of opening and closing to favour shut states. However, the data do not permit discrimination between the possibilities that anaesthetics may bind directly to the channels or alter the lipid environment of the channels to exert their effects. The observed alterations in single channel activity would, however, be expected to lead to reduction of whole-cell calcium currents, as observed (Puttick & Terrar, 1989; 1992; Bosnjak *et al.*, 1991) and therefore to contribute to the negative inotropic actions of these anaesthetics.

References

BOSNJAK, Z.J., SUPAN, F.D. & RUSCH, N.J. (1991). The effects of halothane, enflurane and isoflurane on calcium current in isolated canine ventricular cells. *Anesthesiology*, **74**, 340–345.

CAVALIE, A., PELZER, D. & TRAUTWEIN, W. (1986). Fast and slow gating behavior of single calcium channels in cardiac cells. Relation to activation and inactivation of calcium channel current. *Pflügers Arch.*, **406**, 241–258.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH,, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.*, **391**, 85–100.

HESS, P., LANSMAN, J.B. & TSIEN, R.W. (1984). Different modes of Ca channel gating behaviour favoured by dihydropyridine Ca agonists and antagonists. *Nature*, **311**, 538–544.

HIROTA, K., ITO, Y., MATSUDA, A. & MOMOSE, Y. (1989). Effects of halothane on membrane ionic currents in guinea-pig atrial and ventricular myocytes. *Acta Anaesthesiol. Scand.*, **33**, 239–244.

IKEMOTO, Y., YATANI, A., ARIMURA, H. & YOSHITAKE, J. (1985). Reduction of the slow inward current of isolated rat ventricular cells by thiamylal and halothane. *Acta Anaesthesiol. Scand.*, **29**, 583–586.

KOKUBUN, S. & REUTER, H. (1984). Dihydropyridine derivatives prolong the open state of calcium channels in cultured cardiac cells. *Proc. Natl. Acad. Sci. USA*, **81**, 4824–4827.

LACERDA, A.E. & BROWN, A.M. (1989). Nonmodal gating of cardiac calcium channels as revealed by dihydropyridines. *J. Gen. Physiol.*, **93**, 1243–1273.

MITCHELL, M.R., POWELL, T., TERRAR, D.A. & TWIST, V.W. (1983). Characteristics of the second inward current in cells isolated from rat ventricular muscle. *Proc. R. Soc. B.*, **219**, 447–469.

NIGGLI, E., RUDISULI, A., MAURER, P. & WEINGART, R. (1989). Effects of general anesthetics on current flow across membranes in guinea-pig myocytes. *Am. J. Physiol.*, **256**, C273–C281.

NILIUS, B., HESS, P., LANSMAN, J.B. & TSIEN, R.W. (1985). A novel type of cardiac calcium channel in ventricular cells. *Nature*, **316**, 443–446.

POWELL, T., TERRAR, D.A. & TWIST, V.W. (1980). Electrical properties of individual cells isolated from adult rat ventricular myocardium. *J. Physiol.*, **302**, 131–153.

PUTTICK, R.M. & TERRAR, D.A. (1989). Effects of propofol on membrane currents and contraction in single myocytes isolated from guinea-pig ventricles. *Br. J. Pharmacol.*, **98**, 742P.

PUTTICK, R.M. & TERRAR, D.A. (1991). Effects of propofol and enflurane on single calcium channel currents of guinea-pig isolated ventricular myocytes. *Br. J. Pharmacol.*, **102**, 100P.

PUTTICK, R.M. & TERRAR, D.A. (1992). Effects of propofol and enflurane on action potentials, membrane currents and contraction of guinea-pig isolated ventricular myocytes. *Br. J. Pharmacol.*, **107**, 559–565.

SHIBASAKI, T. (1987). Conductance and kinetics of delayed rectifier potassium channels in nodal cells of the rabbit heart. *J. Physiol.*, **387**, 227–250.

SPEDEN, R. (1963). Kinetics and mechanisms of action of some volatile anaesthetics. *D.Phil thesis, Oxford.*

TERRAR, D.A. & VICTORY, J.G.G. (1988a). Effects of halothane on membrane currents associated with contraction in single myocytes isolated from guinea-pig ventricle. *Br. J. Pharmacol.*, **94**, 500–508.

TERRAR, D.A. & VICTORY, J.G.G. (1988b). Isoflurane depresses membrane currents associated with contraction in myocytes isolated from guinea-pig ventricle. *Anesthesiology*, **69**, 742–749.

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Receptor for catecholamines responding to catechol which potentiates voltage-dependent calcium current in single cells from guinea-pig taenia caeci

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1 Single isolated cells were obtained from the taenia of the guinea-pig's caecum by enzymic digestion and held under voltage clamp. The effects of various catecholamines, sympathomimetics and related compounds were tested for their ability to potentiate the voltage-dependent calcium current (I_{Ca}) evoked in these cells by a depolarizing step.

2 I_{Ca} was potentiated by up to 60% by isoprenaline, adrenaline, and noradrenaline which were equipotent. The EC_{50} for isoprenaline was about 40 nM.

3 The racemic mixtures of the optical isomers of isoprenaline, adrenaline, and noradrenaline, and (+)-isoprenaline, were equipotent with the (−)-isomers of these drugs. Dopamine, L-dopa, and catechol were equipotent with these catecholamines.

4 Removal or substitution of one or more of the hydroxy groups of the catechol moiety, as in phenylephrine, salbutamol, procaterol, methoxamine, terbutaline, BRL 37344, ICI 215001 or tyramine substantially reduced efficacy and/or potency.

5 The adrenoceptor blockers propranolol, phentolamine, dihydroergotamine, atenolol, CGP 20712A and ICI 118551, or the dopamine receptor blockers, haloperidol or flupenthixol, did not block the potentiating action of catechol or the catecholamines.

6 The receptor activated by catecholamines to increase I_{Ca} we suggest should be called a C-receptor in view of its sensitivity to catechol. It may arise by enzymic modification of a conventional adrenoceptor but its transduction also involves a novel mechanism which might indicate that it is present in the muscle cells before enzyme treatment.

Keywords: Ca channel; smooth muscle; catecholamines; adrenoceptor; C-receptor; catechol; taenia caeci

Introduction

The structure of the voltage-dependent calcium channel of smooth muscles shows considerable similarities to that of the cardiac channel (Biel *et al.*, 1990; Koch *et al.*, 1990). However, β_1-adrenoceptor activation in heart increases the force of cardiac contraction and increases the calcium current (I_{Ca}) by activation of an adenosine 3':5'-cyclic monophosphate (cylic AMP)-dependent kinase and phosphorylation of the channel (Kameyama *et al.*, 1985; 1986) whereas in smooth muscle β_2-adrenoceptor activation causes relaxation and generally hyperpolarization (see Bülbring & Tomita, 1987). Despite this, β-adrenoceptor activation in tracheal smooth muscle (Welling *et al.*, 1991; 1992) and in smooth muscle cells from pig coronary artery (Fukumitsu *et al.*, 1990) has been reported to increase I_{Ca} as β_1-receptor activation does in heart.

Although cyclic AMP-dependent phosphorylation of calcium channels is believed to be responsible for the increase in I_{Ca} in heart, we have found activators of adenyl cyclase, cyclic AMP, or its analogues, did not mimic the potentiation of I_{Ca} produced by isoprenaline in smooth muscle cells from the guinea-pig taenia caeci (Muraki *et al.*, 1993). This result is similar to that of Ohya *et al.* (1987) who found that cyclic AMP did not potentiate I_{Ca} in cells from the longitudinal smooth muscle of rabbit jejunums in contrast to results in pig coronary artery smooth muscle where forskolin potentiated I_{Ca} (Fukumitsu *et al.*, 1990). We have also found that potentiation of I_{Ca} in guinea-pig taenia cells did not appear to be mediated by a G-protein, contrary to results obtained in tracheal smooth muscle by Welling *et al.* (1991; 1992) or to be mediated by activation of protein kinase C which has been implicated in the increase in I_{Ca} produced by muscarinic and and other receptor activation in amphibian gastric smooth muscle cells (Vivaudou *et al.*, 1988).

The actions of noradrenaline (NA), adrenaline (Ad) and other sympathomimetics on smooth and cardiac muscles have been intensively investigated since the pioneer work of Barger & Dale (1910). Around the time of identification of NA as the main, or as an important, transmitter at sympathetic nerve endings (von Euler, 1946) and Ad as the main hormone released from the adrenal medulla (Bülbring, 1949; Bülbring & Burn, 1949) the subdivision of adrenoceptors into α- and β-subtypes on the basis of the relative potencies of some sympathomimetic amines was proposed (Ahlquist, 1948). The characteristics of the molecule which confer ability to activate presently known adrenoceptors are the 3:4 dihydroxybenzene moiety and the aliphatic side chain of two carbon atoms (β and α) which terminates in an amino group (Barlow, 1964). β-Hydroxylation further intensifies activity but catechol itself is without sympathomimetic activity (Barger & Dale, 1910). Since Ahlquist's division into two groups, adrenoceptors have been further subdivided into α_1 and α_2 (Dubocovitch & Langer, 1974; Drew & Whiting, 1979) and β_1, β_2 and 'atypical' β_3 varieties (Lands *et al.*, 1967; Wilson *et al.*, 1984; Bond & Clarke, 1987; Hollenga & Zaagsma, 1989).

We noticed that NA, Ad and isoprenaline (Iso) were all able to increase I_{Ca} in guinea-pig taenia single cells under voltage clamp (Muraki *et al.*, 1993). NA and Ad also increased I_{Ca} in smooth muscle cells from rabbit ear artery (Benham & Tsien, 1988). In cultured aortic smooth muscle (Serebryakov & Takeda, 1992) NA or Ad decreased I_{Ca}. In both these last two tissues the receptors through which NA and Ad produced these effects were not blocked by α- or by β-adrenoceptor blockers. It was therefore decided to characterize the adrenoceptors which mediated the increase in I_{Ca} in cells of the taenia caeci of the guinea-pig. In this tissue, Ad or NA inhibit contraction and relax the muscle (Burnstock, 1958; Bülbring & Tomita, 1969) through actions on both α-

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(Bülbring & Tomita, 1969) and β-adrenoceptors (Bülbring & den Hertog, 1980). Single cells were used because we wished to examine the possibility that activation of adrenoceptors modulated I_{Ca} in these cells, and because when adrenoceptor stimulants are applied to a whole tissue, actions on one type of mechanism may be obscured by other actions on other mechanisms especially if only tension is recorded.

Methods

Preparation of cells

Guinea-pigs of either sex, weighing 200–400 g were killed by stunning and exsanguination and small pieces of taenia from the caecum were dissected. After fat and connective tissues were removed under a binocular microscope, 10–20 mm × 0.5–1.0 mm pieces of taenia were immersed in Ca^{2+} and Mg^{2+}-free physiological salt solution (Ca^{2+}-Mg^{2+} free solution for 10 min. They were then incubated for 15 min in the Ca^{2+}–Mg^{2+} free solution which contained 1 mg ml^{-1} collagenase and 0.5 mg ml^{-1} pronase using a similar method to that previously described (Inoue & Isenberg, 1990; Pacaud & Bolton, 1991). After that, the solution was changed to enzyme-free fresh Ca^{2+}-Mg^{2+} free solution and the pieces agitated gently. Then cells were spun down at 1000 r.p.m. for 2 min, resuspended in low-Ca^{2+} physiological salt solution which contained 0.55 mM Ca^{2+}, and stored at 4°C until use on the same day.

Membrane current measurements

Membrane currents were recorded with standard whole-cell voltage-clamp methods (Hamill *et al.*, 1981) using a List-EPC 5 patch clamp amplifier using pipettes which had 3 to 5 MΩ resistance. No correction for leakage currents was made. Voltage steps were produced by a command generated by a BBC microcomputer linked through a CED 1401 interface (Cambridge Electronic Devices, Cambridge, U.K.). Current signals were stored on FM tape (Racal) and later captured on a BBC micro computer using the CED 1401 and our own capturing program for precise analysis. All experiments were carried out at 22 ± 2°C.

Solutions

Experiments were conducted in physiological salt solution (PSS) containing (mM): NaCl 137, KCl 6, $MgCl_2$ 1.2, $CaCl_2$ 2.2, glucose 10 and HEPES 10. Ca^{2+} and Mg^{2+}-free solution for dispersing cells contained (mM) NaCl 128, KCl 6, glucose 10 and HEPES 10. The pH of these solutions was adjusted to 7.4 using NaOH. The composition of standard pipette solution was (mM): CsCl 140, $MgCl_2$ 1, ATP-2Na 2, GTP-3Na 0.3, HEPES 10 and EGTA 4 (titrated to pH 7.4 with CsOH).

Drugs

(±)- and (−)-adrenaline, catechol, L-β-3,4-dihydroxyphenylalanine (L-dopa), dihydroergotamine methane sulphonate, dopamine hydrochloride, isoetharine mesylate, haloperidol, (±)-isoprenaline hydrochloride, (−)- and (+)-isoprenaline bitartrate, methoxamine hydrochloride, (−)- and (±)-noradrenaline hydrochloride, phentolamine hydrochloride, (−)-phenylephrine hydrochloride, procaterol hydrochloride, (−)-propranolol hydrochloride, salbutamol hemisulphate, terbutaline hemisulphate, tyramine hydrochloride (all from Sigma). 1-[2-((3-Carbomoyl-4-hydroxy)phenoxy)ethylamino]-3-[4- (1-methyl-4-trifluoromethyl-2-imidazolyl) phenoxy]-propan-2-ol methane sulphonate (CGP 20712A, Ciba Geigy), 4-[2-[(2-hydroxy-2-(3-chlorophenyl) ethyl) amino] propyl]-phenoxyacetic acid (BRL 37344, SmithKline Beecham), erythro-DL-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol hydrochloride (ICI 118551, Cambridge Research Biochemicals), ICI 215001 (M 215001, metabolite of D7114, ICI-Zeneca) (Holloway *et al.*, 1991), *cis*(z)-flupenthixol hydrochloride (Research Biochemicals).

Results

Cells were held at − 50 mV and depolarized for 200 ms to 0 mV every 20 s. This evoked an inward current carried by calcium ions (I_{Ca}) which was not significantly contaminated by potassium currents due to the use of caesium chloride instead of potassium chloride in the pipette solution. The peak size of the evoked inward current was measured. Drugs were applied for short periods until any change in the peak size of the current had stabilized; the effect of a drug was sometimes expressed as a change in the size of the current relative to the size of the current immediately before drug application. Because current size often increased without drug application for several minutes after beginning recording in the whole-cell mode, the first response to drug application was sometimes less reliable.

It was found that NA, Ad or isoprenaline (Iso) increased the size of the voltage-dependent current and although this effect varied in size in different cells a 60% increase was not uncommon (Figure 1a,b). Because the potentiation could be produced by Iso it was suspected that the effect was mediated by β-receptors but an unexpected finding was that β-receptor antagonists such as propranolol up to 10 μM had no inhibitory effect (Figure 3a). Therefore, further experiments were made in order to characterize the adrenoceptor involved.

It was observed that the racemates of NA, Ad and Iso were about equipotent (Figure 1a-c). This observation immediately suggested that the adrenoceptor involved was not either an α- or a β-subtype as NA is invariably much more potent at α-receptors than Iso and *vice-versa* at β-receptors (Barlow, 1964). The EC_{50} for (−)-Iso was 41 nM ($n = 1-13$) (Figure 1e,f) and for (±)-Iso it was 42 nM ; for NA and Ad it was 24 nM ($n = 1-5$) and 58 nM ($n = 1-7$) respectively.

Those catecholamines with a β-OH group can exist as two isomers (−) and (+). At α and at β-receptors the (−)-isomer of Ad and NA is usually 10–100 times more potent than the (+)-isomer and at β-receptors in the case of (−)- and (+)-isoprenaline, this ratio is even greater (Barlow, 1964). The importance of the β-OH group for activity suggests that it is involved in binding at α- and at β-receptors. However, (−)-, (+)- and (±)-isoprenaline (Figure 2a), (−)- and (±)-noradrenaline, and (−)- and (±)-adrenaline were equipotent; in these experiments equal concentrations of the catecholamines were applied successively to the same cell and the ratio of the increases in Ca current calculated. No ratio was significantly different from unity (Table 1) (Figure 1c). This result suggested that the β-OH group was unimportant for binding at this receptor. This was confirmed when experiments showed that dopamine which lacks a β-hydroxy group was equipotent with the other catcholamines examined (Figure 1d) (Table 1).

The structure-activity relationship was more systematically investigated; (−)-phenylephrine, and (±)-methoxamine, normally agonists at α-adrenoceptors (Barlow, 1964) were of low efficacy and/or were more than a thousand times less potent than NA (Figure 1b,c). (±)-Salbutamol had a tenth to a hundredth of the potency, and procaterol about a thirtieth to a thousandth of the potency of NA or Iso (Figure 2e and Table 1), although relative potency was not reliable as these drugs were partial agonists (Figure 2b,e and Table 1). (±)-Salbutamol has about a sixth of the potency of (±)-Iso at β_2-adrenoceptors but negligible activity at β_1-adrenoceptors (Brittain *et al.*, 1973). (±)-Salbutamol is interesting because the 3-OH is replaced by 3-CH_2OH. However, (±)-procaterol is more potent than (±)-salbutamol at β_1 and β_2 receptors (Yabuuchi, 1977) whereas in the present experiments the opposite was found.

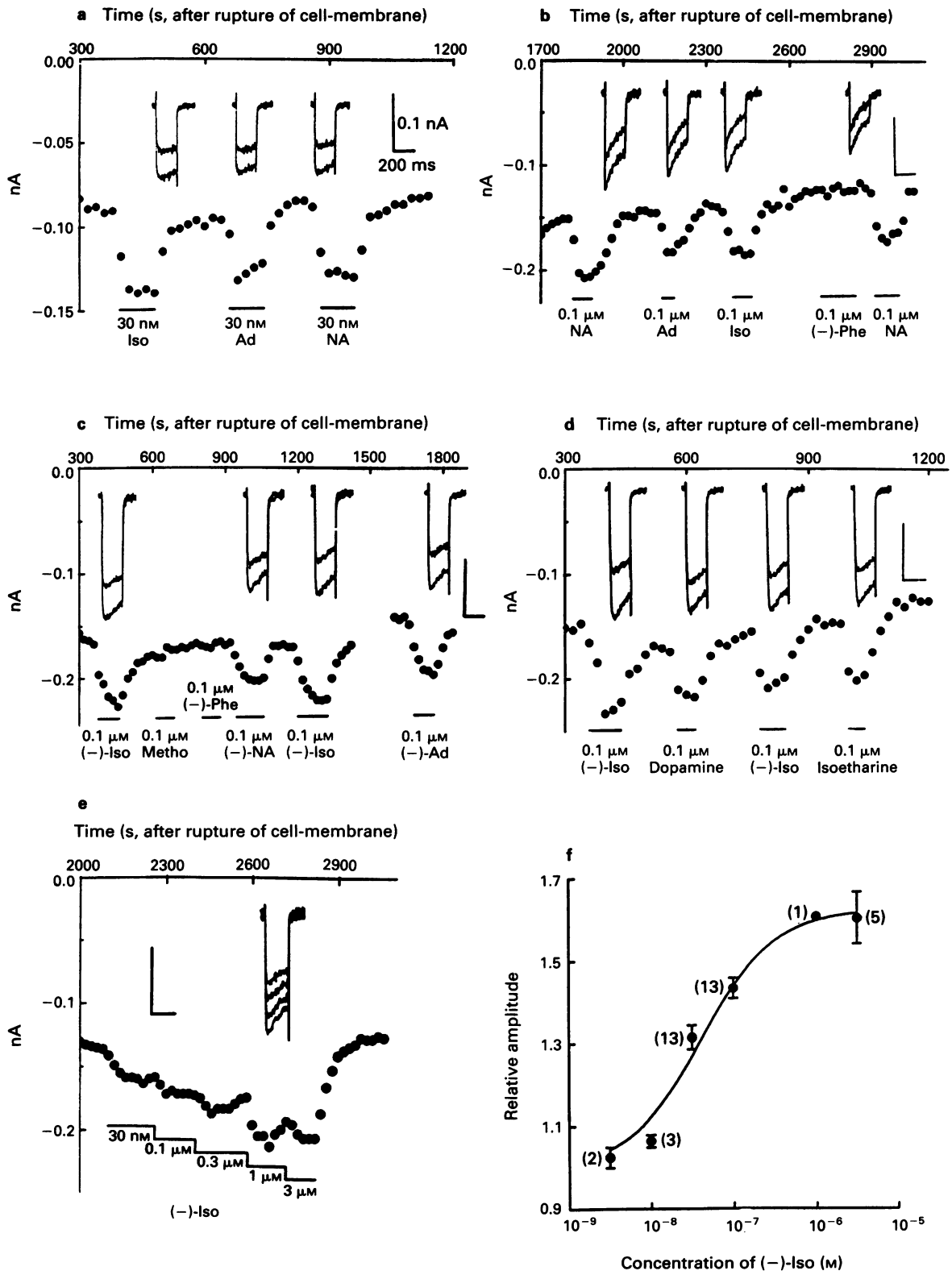


Figure 1 Effects of several sympathomimetic amines on I_{Ca} in single isolated smooth muscle cells. Voltage-clamped cells were depolarized every 20 s from − 50 to 0 mV for 200 ms. (±)-Isoprenaline (Iso), (±)-adrenaline, (Ad) and (±)-noradrenaline (NA) potentiated Ca current in the absence (**a**) and presence (**b**) of 10 μM phentolamine. (−)-Iso, (−)-Ad, (−)-NA, dopamine and (±)-isoetharine also potentiated Ca current with almost the same potency in the presence of 3 μM phentolamine (**c**, **d**). However, (−)-phenylephrine ((−)-Phe) and methoxamine (Metho) had no effect. (**e**) Increase of Ca current by cumulative application of (−)-Iso in the presence of 3 μM phentolamine. Insets show superimposed current traces before and at the peak of the effect of the drug in each panel. In 4th inset of Figure 1b, 3 traces which indicate control, (−)-phenylephrine and NA treated were superimposed. Currents traces in (**e**) are control and in the presence of 30 nM, 0.3 and 3 μM (−)-Iso. (**f**) Concentration-response of (−)-Iso on Ca current. The data were fitted to a curve drawn from $I_{\text{-Iso}}/I_{\text{cont}} = 1 + [A/(1 + K_D/[(-)\text{-Iso}])]$ where I_{cont} and $I_{\text{-Iso}}$ are relative current amplitudes before and during application of (−)-Iso, [(−)-Iso] is the concentration of (−)-Iso and K_D its apparent dissociation constant. The Hill coefficient was assumed to be one and the maximum relative increase in I_{Ca}, A = 0.615. K_D was calculated to be 41 nM . Drugs were prepared immediately before use and were applied externally by replacement of external solution by drug-containing one.

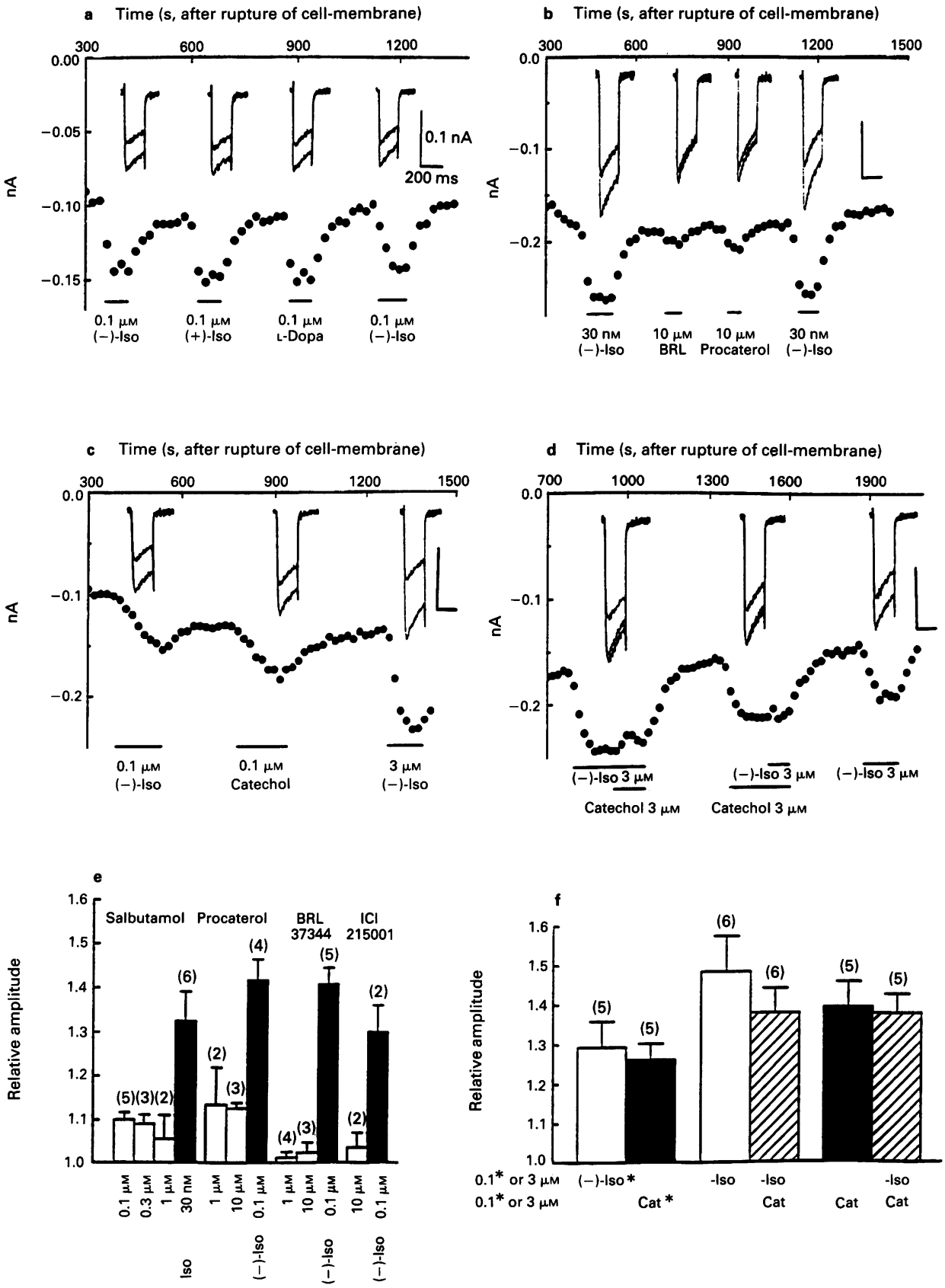


Figure 2 Effects of some sympathomimetic amines, β_2 (salbutamol and procaterol) and β_3 agonists (BRL 37344 and ICI 215001) and catechol itself on I_{Ca}. (−)-Isoprenaline ((−)-Iso), (+)-Iso, L-Dopa and catechol (**a**, **c**, **d**) equally potentiated I_{Ca}. However, BRL 37344 (BRL) and ICI 215001 had a very small effect on I_{Ca} (**b**,**e**). Salbutamol and procaterol had some effects on I_{Ca} but the potentiation was smaller than that produced by Iso in the same cells (**b**,**e**). Insets show superimposed current traces before and during application of drugs. In first and second traces of (d), current traces before and during application of 3 μM (−)-Iso, or 3 μM catechol, and in the presence of both 3 μM (−)-Iso and 3 μM catechol, were superimposed; (**e**) Summarises results of application of salbutamol, procaterol, BRL 37344 and ICI 215001 to the indicated number of cells (open columns); in each case (−)-Iso was applied at the concentration indicated to the same cells (solid columns). (**f**) Summarised the results of experiments of this type on the indicated number of cells where 0.1 or 3 μM concentrations of catechol (Cat) or (−)-Iso were used. Phentolamine (3 μM) was present in (a), (b), (c) and (d).

Some other compounds tried had very low efficacy and/or low potency. (±)-Terbutaline which resembles Iso except that 4-OH is replaced by 5-OH was more than a 1000 times less potent than (−)-Iso, emphasising the importance of the 3-OH, 4-OH catechol nucleus for activity. BRL 37344 (12) and ICI 215001 (M 215001), the active acid metabolite of ICI D7114 (Growcott *et al.*, 1992), both β_3-receptor agonists, were virtually inactive (Figure 2b and 2e).

The N-substitute on the molecule was clearly not important for activity at this receptor either since Iso, Ad and NA (− or ± forms) were equipotent (Table 1). Further, (±)-isoetharine which resembles Iso except for an α-ethyl group was also equipotent (Table 1) (Figure 1d). These results taken with those concerning the β-OH group suggested that the receptor was indifferent to the nature of the aliphatic tail. This was confirmed as L-Dopa, which has an α-COOH and

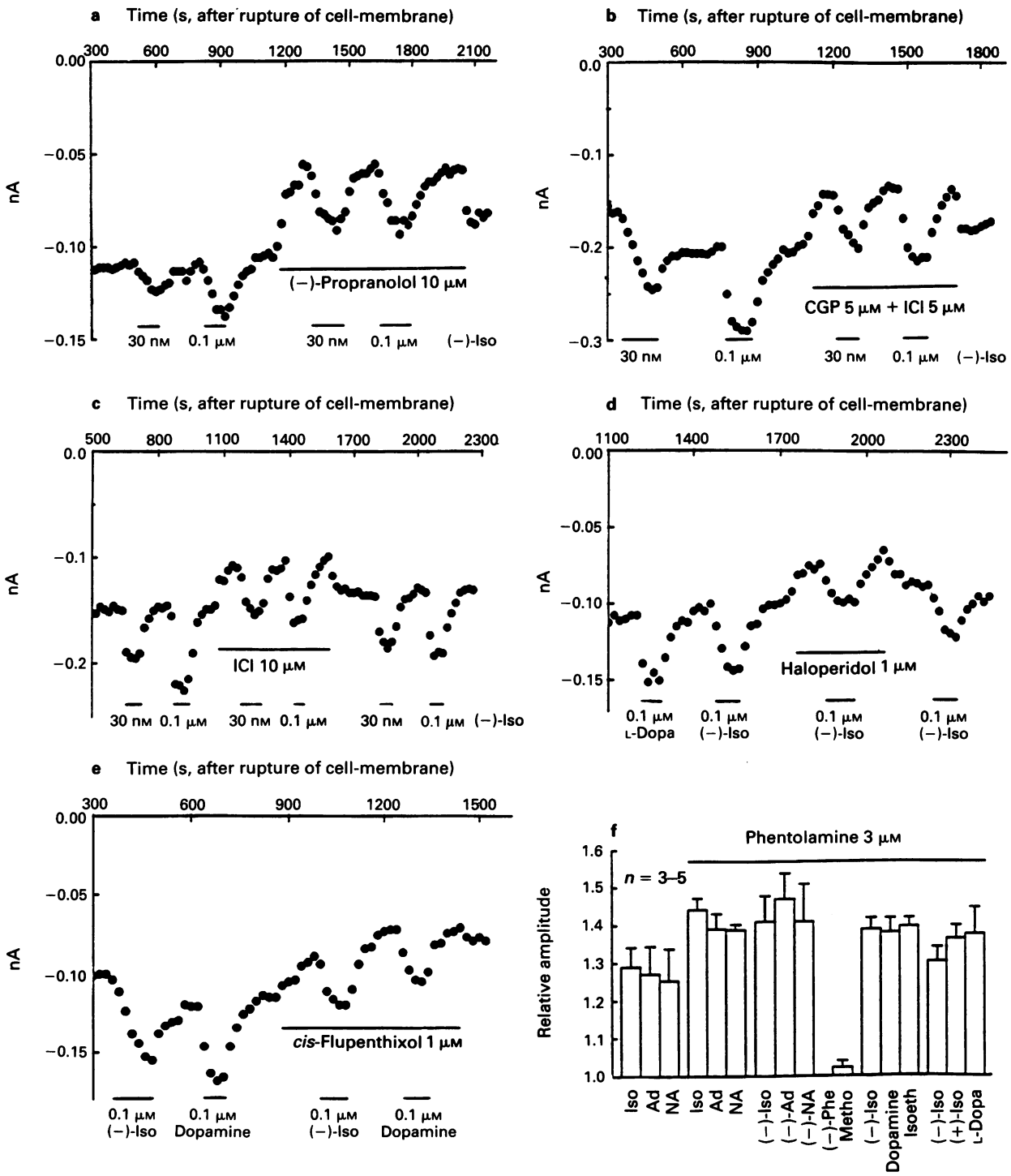
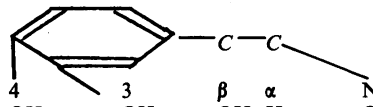


Figure 3 Effects of β-adrenoceptor and dopamine antagonists on potentiation of I_{Ca} by (−)-isoprenaline ((−)-Iso) and dopamine in the presence of α-adrenoceptor blocker phentolamine 3 μM; 10 μM β-adrenoceptor blocker (−)-propranolol, 5 μM β_1-adrenoceptor blocker CGP 20712A (CGP) with 5 μM β_2-adrenoceptor blocker ICI 118551 (ICI), 10 μM ICI 118551, and dopamine receptor blockers haloperidol (1 μM) and flupenthixol (1 μM) had no effect on potentiation of I_{Ca} by (−)-Iso or dopamine when expressed as percentage or fractional increase in basal current. (**f**) Summarizes the effects on I_{Ca} of 0.1 μM of various sympathomimetic amines mostly in 3 μM phentolamine. Same set of cells (between gaps) were used to compare the potencies. (−)-Phe, phenylephrine; Metho, methoxamine; Isoeth, isoetharine.

Table 1 Relative potencies of various sympathomimetics and related compounds on voltage-dependent Ca current



4	3	β	α	NH			*Relative potency*	*Highest conc. used* μM	*No. of cells*
OH	OH	OH	H	$CH(CH_3)_2$	(−)	Isoprenaline β	1.00	3	>30
					(±)	Isoprenaline β	1.00	3	5
					(+)	Isoprenaline β	1.2 ± 0.11		5
OH	OH	OH	H	CH_3	(−)	Adrenaline αβ	1.1 ± 0.11	0.3	3
					(±)	Adrenaline αβ	0.91 ± 0.06	3	5
OH	OH	OH	H	H	(−)	Noradrenaline $\alpha\beta_1$	0.91 ± 0.13	1	3
					(±)	Noradrenaline $\alpha\beta_1$	0.88 ± 0.03	3	3
OH	OH	H	H	H		Dopamine α	1.0 ± 0.09	0.1	9
OH	OH	OH	CH_2CH_3	$CH(CH_3)_2$	(±)	Isoetharine β	1.0 ± 0.07	0.1	6
OH	OH	H	COOH	H		L-Dopa -	1.2 ± 0.18	0.1	4
OH	OH	–	–	–		Catechol -	0.96 ± 0.11	3	5
H	OH	OH	H	CH_3	(−)	Phenylephrine α	>>0.001	3	4
$5OCH_3$	$2OCH_3$	OH	CH_3	H	(±)	Methoxamine α	>>0.001	3	4
5OH	OH	OH	H	$C(CH_3)_3$	(±)	Terbutaline β_2	>>0.001	3	3
OH	CH_2OH	OH	H	$C(CH_3)_3$	(±)	Salbutamol β_2	0.1–0.01§	1	6
OH	Ring	OH	C_2H_5	$CH(CH_3)_2$	(±)	Procaterol β_2	0.03–0.001§	10	4
H	Cl	OH	H	Complex	(±)	BRL 37344 β_3	0.0003	10	5
H	H	Complex				ICI 215001 β_3	0.0003	10	2
OH	H	H	H	H		Tyramine -	>>0.001	3	2

Substances marked § were partial agonists (maximum response less than 25–30% of maximum response to Iso). Other compounds of low potency may also be partial agonists but high enough concentrations were not tested and their relative potencies were calculated by extrapolating the concentration-effect curve and assuming it was parallel with the curve for (−)-Iso or (±)-Iso. The potency of catechol or drugs containing a catechol nucleus was obtained by application of equal concentrations (usually 30 or 100 nM) to the same cell of the drug and (−)-Iso or (±)-Iso (which were assumed equipotent as EC_{50}s were 41.8 and 41.3 nM, see text). The relative sizes of the responses were then calculated for the indicated number of cells; none proved significantly different from unity at $P<0.05$. The structure of the drug, and the adrenoceptor at which it has a major action are also shown.

no β-hydroxy group, was also equipotent with the catecholamines (Table 1, Figure 2a).

It was therefore predicted that catechol (3,4,dihydroxybenzene) should be active at this receptor. This was confirmed; freshly made solutions (catechol is somewhat unstable) indicated that catechol and catecholamines were equipotent (Figure 2c,d). Furthermore, when maximum potentiation of the current was produced by 3 μM (−)-Iso, 3 μM catechol had no effect; in the same way 3 μM (−)-Iso was without effect if 3 μM catechol had potentiated the current (Figure 2d,f).

Various adrenoceptor blocking agents were applied to test for their antagonism at this receptor. All of these were without effect if the potentiation by Iso or other catecholamine was expressed as a percentage or fractional increase in basal current size before application of the agonist. When expressed in this way, high concentrations of antagonists had no effect on the increase in size of the current produced by (−)-Iso but generally depressed basal calcium current size themselves. (−)-Propranolol up to 10 μM was without significant antagonism; this drug has a high affinity for type β_1- and type β_2-receptors, its pA_2 being in the range 8–9 (Arch *et al.*, 1984; Wilson *et al.*, 1984) although its affinity for β_3-receptors is somewhat less, pA_2 about 7 (Arch *et al.*, 1984; Bond & Clarke, 1988). Atenolol, a β_1-selective antagonist with a pA_2 of 6 to 7 (O'Donnell & Wanstall, 1983), the β_1-selective antagonst CGP 20712A with a pA_2 greater than 9 (Kaumann, 1986), and the antagonist ICI 118551 with a pA_2 for β_2-receptors in the range 8–10 (O'Donnell & Wanstall, 1983; 1985) in concentrations up to 10 μM did not significantly antagonize the effect of (−)-Iso or (−)-NA on I_{Ca} (Figure 3a,b,c). Phentolamine and dihydroergotamine are generally regarded as blockers of both α_1 and α_2 receptors with pA_2 values in the range 7–8 (Barlow, 1964; Borowski *et al.*, 1977). These agents showed some tendency at concentrations of 3–10 μM to potentiate the increase in the Ca current produced by catecholamines (Figure 3f) and responses were often obtained in their presence (Figures 1b–e, 2a–d, 3a–e). The α-agonist, phenylephrine (10 μM), applied when I_{Ca} was already potentiated by 3 μM Iso, depressed I_{Ca}; carbachol (10 μM) applied under similar conditions also depressed I_{Ca} (not shown).

The increase in Ca current produced by dopamine or by Iso was also unaffected by the dopamine receptor antagonists haloperidol (1 μM) and flupenthixol (1 μM) (Figure 3d,e). At D_1 and D_2 dopamine receptors their pA_2 is normally in the range 7–8 (Hilditch & Drew, 1985). At higher concentrations than 1 μM these two antagonists produced strong depression of the voltage-dependent Ca current themselves and 3 μM haloperidol virtually abolished it.

The effects of Iso, NA and catechol on tension in small strips of taenia in normal solution or when contracted by raising the potassium concentration to 20 or 40 mM were examined. Phentolamine (3 μM), propranolol (3 μM) and atropine (3 μM) were added to the bathing solution. Since these blockers in combination did not block C-receptors, any effects of Iso on tension might be mediated by these receptors. In normal solution, taenia strips showed spontaneous contractions of several g amplitude at a frequency of about 1 every 2 min, from time to time contractions spontaneously ceased. In the presence of blockers, Iso (10^{-7} M; four preparations, eight applications) or, NA ($1-3\times10^{-7}$ M; four preparations, five applications) did not alter the pattern of activity or increase the tension developed during spontaneous contractions. In preparations contracted in high-K solutions, Iso ($1-3\times10^{-7}$ M; seven preparations, 18 applications) NA ($0.3-1\times10^{-6}$ M; four preparations, six applications) or catechol ($0.3-1\times10^{-6}$ M; three preparations, four applications) did not increase tension and sometimes a small fall was observed, presumably due to incomplete blockade of α- and β-receptors. Thus, no increases in tension, indicative of increased entry of calcium through voltage-dependent channels were seen when any of the three catechols were added to preparations where α- and β-adrenoceptors and muscarinic receptors were blocked.

Discussion

The lack of effect of any of the antagonists indicate that the receptor which mediates the increase in the voltage-dependent calcium current in response to catecholamines is not an α- or a β-adrenoceptor nor is it a D_1 or D_2 dopamine receptor. This adrenoceptor showed unusual structure-activity relationships in that the presence of a catechol nucleus was essential for full activity and altering the position of one of the -OH groups on the benzene ring, or removing one or both of these, produced drastic reductions in potency and/or efficacy. However, this receptor was indifferent to the presence of the ethylamine side chain and so catechol was equipotent with L-dopa, dopamine, Ad, Iso and NA. The β-OH group was inessential and (−)- and (+)-isomers of Iso and (−) and (±)-isomers of NA and Ad were equipotent. An amino group was also without effect. In these latter two respects, this receptor differs from α- and β-adrenoceptors where the presence of a (−)-β-OH group substantially increases potency; at the β-receptor, N-substitution with isopropyl substantially increases potency. The sensitivity to catechol would suggest that it should be called a C-receptor.

The C-receptor shows several similarities to and some differences from the adrenoceptors, activation of which modulate I_{Ca} in various enzymatically dissociated smooth muscle cells. In smooth muscle cells of rabbit ear artery, NA and Ad were equipotent in increasing I_{Ca} although Iso was ineffective; propranolol, prazosin and phentolamine were ineffective as blockers as in taenia (Benham & Tsien, 1988). However, in smooth muscle cells from rabbit ear artery both NA and Iso have been reported by other investigators to reduce I_{Ca}; the effects of NA and Iso were found to be unaffected by propranolol and phentolamine respectively (Droogmans *et al.*, 1987). In smooth muscle cells of pig coronary artery, NA and Iso were equipotent but the potentiation they produced was blocked by propranolol but not by phentolamine (Fukumitsu *et al.*, 1990). The reduction of I_{Ca} produced by NA in cultured aortic cells was unaffected by prazosin, phentolamine or propranolol (Serebryakov & Takeda, 1992). In rat portal vein single smooth muscle cells, the action of NA to increase I_{Ca} was blocked by phentolamine (Pacaud *et al.*, 1987) and in guinea-pig vas deferens the reduction of I_{Ca} produced by NA or phenylephrine was blocked by prazosin and less well by yohimbine (Imaizumi *et al.*, 1991). In bovine tracheal smooth muscle cells, Iso increased internal free calcium activity (measured by fura 2-AM) and this effect was blocked by propranolol (0.1 μM) but not by prazosin or yohimbine (at 10 μM) (Felbel *et al.*, 1988). The action of Iso was on calcium influx into the cell, which was blocked by nitrendipine or methoxyverapamil (D600); the calcium influx presumably corresponds to I_{Ca} studied in single cells by electrophysiological techniques (Welling *et al.*, 1991; 1992). However, the action of Iso in raising intracellular calcium activity is not confined to single, enzyme-digested, cells as it has been observed in strips of tracheal smooth muscle (Takuwa *et al.*, 1988).

It is tempting to suggest that as adrenoceptors of unusual pharmacology have been described in single enzymically dissociated cells, that the enzyme treatment has modified the adrenoceptors involved. This impression might be reinforced by our failure to detect any effects of Iso on tension in strips of taenia when α- and β-adrenoceptors were blocked. The actions of isoprenaline and other catecholamines have been studied on the electrical activity of the guinea-pig taenia by means of both microelectrode recording and sucrose-gap techniques. Catecholamines through an action on β-receptors (blocked by propranolol) inhibit the generation of the pacemaker potential (Bülbring & Tomita, 1969) and cause weak hyperpolarization (Bülbring & den Hortog, 1980). These effects are associated with inhibition of contractile activity, or relaxation, never contraction or increase in the force of contraction; α-receptor activation causes hyperpolarization and relaxation also (Bülbring & Tomita, 1987). However, Iso can raise internal calcium activity (Ca_i) in tracheal smooth muscle strips as well as reducing the rise in Ca_i engendered by carbachol and this effect is mimicked by forskolin (Takuwa *et al.*, 1988). In the basilar artery of young rats, Iso elicits a depolarization instead of its usual hyperpolarization and this is a β-receptor effect; also NA and on occasions Iso can elicit a depolarization resistant to both α- and β-adrenoceptor blockers (Byrne *et al.*, 1985). Responses evoked by 'γ-receptors' in rat basilar artery segments required high (mM) concentrations of catecholamines, but like the responses evoked in our present experiments, NA, Ad, and dopamine had similar potency and isomers of NA and Ad were equiactive (Hirst *et al.*, 1982). The low concentrations of catecholamines which were effective (EC_{50} about 40 nM) in the present experiments are in contrast to those required to activate the γ-receptor of rat basilar artery which are four orders of magnitude higher (Hirst *et al.*, 1982). Thus receptors for catecholamines have been described on whole tissues which have unusual pharmacology and which may be related to the C-receptor described here. It is conceivable that the C-receptor exists in the whole tissue but its effects at the level of tension generation are for some reason not detectable.

If the C-receptor represented an α- or a β-adrenoceptor altered by collagenase and pronase treatment then it raises questions concerning the nature of the receptor binding site. The modification would represent presumably the removal of some enzyme-sensitive portion of the receptor molecule either directly or indirectly resulting in changes in the molecular architecture of the binding site. It would raise the possibility that receptors, like ion channels (e.g. Hescheler & Trautwein, 1988; Obejero-Pas *et al.*, 1991) might be deliberately modified by enzymic treatment which might allow investigation of the parts of the receptor involved in ligand binding, or perhaps even enzymic alteration of the structure-activity relationships of identified receptors with a view to making them susceptible to novel drugs or ligands.

It is curious, that, if the C-receptor should be a modified β-receptor, that the affinity for propranolol should be so severely reduced since it implies that binding of the naphthalene group to the catechol site is weak such that loss of the hydroxyethylamine binding site(s) results in very low affinity. Nevertheless, the catechol binding site alone was sufficient for retention of considerable affinity for Iso and other catechols since they were reasonably potent. An alternative possibility is that enzymic modification of the molecule causes a binding site for catechols to be revealed, or even to be created, by exposing some juxtaposition of charged groups previously inaccessible within the molecule. A surprising fact, however, is that the activated receptor retains (or perhaps gains) the ability to alter calcium channel function. Unlike a variety of other receptors activation of which alters I_{Ca} in smooth muscles (see Muraki *et al.*, 1993 for references) C-receptor activation was linked to changes in calcium channel activity by a mechanism which did not seem to involve a G-protein, cyclic AMP/adenyl cyclase, or protein kinase C (Muraki *et al.*, 1993) i.e. not only is the C-receptor novel but its transduction mechanism is also. It may be that the C-receptor is an integral part of the voltage-dependent calcium channel but, if so, it seems a separate site from that at which Bay K 8644, a dihydropyridine, binds to increase I_{Ca} because the latter increased I_{Ca} in the occasional cell which was unresponsive to catecholamine (Muraki *et al.*, 1993).

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References

AHLQUIST, R.P. (1948). A study of the adrenotropic receptors. *Am. J. Physiol.*, **153**, 586–600.

ARCH, J.R.S., AINSWORTH, A.T., CAWTHORNE, M.A., PIERCY, V., SENNITT, M.V., THODY, V.E., WILSON, C. & WILSON, S. (1984). A typical β-adrenoceptor on brown adipocytes as target for anti-obesity drugs. *Nature*, **309**, 163–168.

BARGER, G. & DALE, H.H. (1910). Chemical structure and sympathomimetic action of amines. *J. Physiol.*, **41**, 19–59.

BARLOW, R.B. (1964). *Introduction to Chemical Pharmacology*. 2nd Ed. London: Methuen & Co.

BENHAM, C.D. & TSIEN, R.W. (1988). Noradrenaline modulation of calcium channels in single smooth muscle cells from rabbit ear artery. *J. Physiol.*, **404**, 767–784.

BIEL, M., RUTH, P., BOSSE, E., HULLIN, R., STÜHMER, W., FLOCKERZI, V. & HOFMANN, F. (1990). Primary structure and functional expression of a high voltage activated calcium channel from rabbit lung. *FEBS Lett.*, **269**, 409–412.

BOND, R.A. & CLARKE, D.E. (1987). A response to isoprenaline unrelated to α- and β-adrenoceptor agonism. *Br. J. Pharmacol.*, **91**, 683–686.

BOND, R.A. & CLARKE, D.E. (1988). Agonist and antagonist characterization of a putative adrenoceptor with distinct pharmacological properties from the α- and β-subtypes. *Br. J. Pharmacol.*, **95**, 723–734.

BOROWSKI, E., STARKE, K., EHRL, H. & ENDO, T. (1977). A comparison of pre- and post-synaptic effects of α-adrenolytic drugs in the pulmonary artery of the rabbit. *Neuroscience*, **2**, 285–296.

BRITTAIN, R.T., FARMER, J.B. & MARSHALL, R.J. (1973). Some observations on the β-adrenoceptor agonist properties of the isomers of salbutamol. *Br. J. Pharmacol.*, **48**, 144–147.

BÜLBRING, E. (1949). The methylation of noradrenaline by minced suprarenal tissue. *Br. J. Pharmacol.*, **4**, 234–244.

BÜLBRING, E. & BURN, J.H. (1949). Liberation of noradrenaline from adrenal medulla by splanchnic stimulation. *Nature*, **163**, 217.

BÜLBRING, E. & DEN HERTOG, A. (1980). The action of isoprenaline on the smooth muscle of the guinea-pig taenia coli. *J. Physiol.*, **304**, 277–296.

BÜLBRING, E. & TOMITA, T. (1969). Increase of membrane conductance by adrenaline in the smooth muscle of guinea-pig taenia coli. *Proc. R. Soc. B.*, **172**, 103–119.

BÜLBRING, E. & TOMITA, T. (1987). Catecholamine action on smooth muscle. *Pharmacol. Rev.*, **39**, 49–96.

BURNSTOCK, G. (1958). The action of adrenaline on excitability and membrane potential in the taenia coli of the guinea-pig and the effect of DNP on this action and on the action of acetylcholine. *J. Physiol.*, **143**, 183–194.

BYRNE, N.G., HIRST, G.D.S. & LARGE, W.A. (1985). Electrophysiological analysis of the nature of adrenoceptors in the rat basilar artery during development. *Br. J. Pharmacol.*, **86**, 217–227.

DROOGMANS, G., DECLERCK, I. & CASTEELS, R. (1987). Effect of adrenergic agonists on Ca^{2+}-channel currents in single vascular smooth muscle cells. *Pflügers Archiv.*, **409**, 7–12.

DREW, G.M. & WHITING, S.B. (1979). Evidence for two distinct types of postsynaptic α-adrenoceptor in vascular smooth muscle *in vivo*. *Br. J. Pharmacol.*, **67**, 207–215.

DUBOCOVICH, M.L. & LANGER, S.Z. 91974). Negative feed-back regulation of noradrenaline release by nerve stimulation in the perfused cat's spleen: differences in potency of phenoxybenzamine in blocking the pre- and post-synaptic adrenergic receptors. *J. Physiol.*, **237**, 505–519.

FELBEL, J., TROCKUR, B., ECKER, T., LANDGRAF, W. & HOFMANN, F. (1988). Regulation of cytosolic calcium by cAMP and cGMP in freshly isolated smooth muscle cells from bovine trachea. *J. Biol. Chem.*, **263**, 16764–16771.

FUKUMITSU, T., HAYASHI, H., TOKUNO, H. & TOMITA, T. (1990). Increase in calcium channel current by β-adrenoceptor agonists in single smooth muscle cells isolated from porcine coronary artery. *Br. J. Pharmacol.*, **100**, 593–599.

GROWCOTT, J.W., WILSON, C., HOLLOWAY, B. & MAINWARING, S. (1991). Evaluation of ICI D7114, a putative stimulant of brown adipocytes, on histamine pre-contracted guinea-pig ileum. *Br. J. Pharmacol.*, **104**, 155P.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv.*, **391**, 85–100.

HESCHELER, J. & TRAUTWEIN, W. (1988). Modification of L-type calcium current by intracellularly applied trypsin in guinea-pig ventricular myocytes. *J. Physiol.*, **404**, 259–274.

HILDITCH, A. & DREW, G.M. (1985). Peripheral dopamine receptor subtypes – a closer look. *Trends Pharmacol. Sci.*, **6**, 396–400.

HIRST, G.D.S., NEILD, T.O. & SILVERBERG, G.D. (1982). Noradrenaline receptors on the rat basilar artery. *J. Physiol.*, **328**, 351–360.

HOLLOWAY, B.R., HOWE, R., RAO, B.S., STRIBLING, D., MAYERS, M., BRISCOE, M.G. & JACKSON, J.M. (1991). ICI D7114 a novel selective β-adrenoceptor agonist selectively stimulates brown fat and increases whole-body oxygen consumption. *Br. J. Pharmacol.*, **104**, 97–104.

HOLLENGA, C.H. & ZAAGSMA, J. (1989). Direct evidence of the atypical nature of functional β-adrenoceptors in rat adipocytes. *Br. J. Pharmacol.*, **98**, 1420–1424.

IMAIZUMI, Y., TAKEDA, M., MURAKI, K. & WATANABE, M. (1991). Mechanisms of NE-induced reduction of Ca current in single smooth muscle cells from guinea-pig vas deferens. *Am. J. Physiol.*, **260**, C17–C25.

INOUE, R. & ISENBERG, G. (1990). Effect of membrane potential on acetylcholine-induced inward current in guinea-pig ileum. *J. Physiol.*, **424**, 57–71.

KAMEYAMA, M., HESCHELER, J., HOFMANN, F. & TRAUTWEIN, W. (1986). Modulation of Ca current during the phosphorylation cycle in the guinea pig heart. *Pflügers Archiv.*, **407**, 123–128.

KAMEYAMA, M., HOFMANN, F. & TRAUTWEIN, W. (1985). On the mechanism of beta-adrenergic regulation of the Ca channel in the guinea-pig heart. *Pflügers Archiv.*, **405**, 285–293.

KAUMANN, A.J. (1986). The β_1-adrenoceptor antagonist CGP 20712A unmasks β_2-adrenoceptors activated by (−)-adrenaline in rat sinoatrial node. *Naunyn-Schmied. Arch. Pharmacol.*, **332**, 406–409.

KOCH, W.J., ELLINOR, P.T. & SCHWARTZ, A. (1990). cDNA cloning of a dihydropyridine-sensitive calcium channel from rat aorta. *J. Biol. Chem.*, **265**, 17786–17791.

LANDS, A.M., ARNOLD, A., MCAULIFF, J.P., LUDUENA, F.P. & BROWN, T.G. Jr. (1967). Differentiation of receptor systems activated by sympathomimetic amines. *Nature*, **214**, 597–598.

MURAKI, K., BOLTON, T.B., IMAIZUMI, Y. & WATANABE, M. (1993). Effect of isoprenaline on Ca^{2+} channel current in single smooth muscle cells isolated from taenia of the guinea-pig caecum. *J. Physiol.*, **471**, 563–582.

OBEJERO-PAZ, C.A., JONES, S.W. & SCARPA, A. (1991). Calcium currents in the A7r5 smooth muscle-derived cell line. *J. Gen. Physiol.*, **98**, 1127–1140.

O'DONNELL, S.R. & WANSTALL, J.C. (1985). Responses to the β_2-selective agonist procaterol of vascular and atrial preparations with different functional β-adrenoceptor populations. *Br. J. Pharmacol.*, **84**, 227–235.

O'DONNELL, S.R. & WANSTALL, J.C. (1983). Relaxation of cat trachea by β-adrenoceptor agonists can be mediated by both β_1- and β_2-adrenoceptors and potentiated by inhibitors of extraneuronal uptake. *Br. J. Pharmacol.*, **78**, 417–424.

OHYA, Y., KITAMURA, K. & KURIYAMA, H. (1987). Modulation of ionic currents in single smooth muscle balls of the rabbit intestine by intracellularly perfused ATP and cyclic AMP. *Pflügers Archiv.*, **408**, 465–473.

PACAUD, P. & BOLTON, T.B. (1991). Relation between muscarinic receptor cationic current and internal calcium in guinea-pig jejunal smooth muscle cells. *J. Physiol.*, **441**, 477–499.

PACAUD, P., LOIRAND, G., MIRONNEAU, C. & MIRONNEAU, J. (1987). Opposing effects of noradrenaline on the two classes of voltage-dependent calcium channels of single vascular smooth muscle cells in short-term primary culture. *Pflügers Arch.*, **410**, 557–559.

SEREBRYAKOV, V. & TAKEDA, K. (1992). Voltage-dependent calcium current and the effects of adrenergic modulation in rat aortic smooth muscle cells. *Phil. Trans. R. Soc. B.*, **337**, 37–47.

TAKUWA, Y., TAKUWA, N. & RASMUSSEN, H. (1988). The effects of isoproterenol on intracellular calcium concentration. *J. Biol. Chem.*, **263**, 762–768.

VIVAUDOU, M.B., CLAPP, L.H., WALSH, J.V. Jr. & SINGER, J.J. (1988). Regulation of one type of Ca^{2+} current in smooth muscle cells by diacylglycerol and acetylcholine. *FASEB J.*, **2**, 2497–2504.

VON EULER, U.S. (1946). A specific sympathomimetic ergone in adrenergic nerve fibres (Sympathin) and its relations to adrenaline and nor-adrenaline. *Acta Physiol. Scand.*, **12**, 73–97.

WELLING, A., FELBEL, J., PEPER, K. & HOFMANN, F. (1991). β-adrenergic receptor stimulates L-type calcium current in adult smooth muscle cells. *Blood Vessels*, **28**, 154–158.

WELLING, A., FELBEL, J., PEPER, K. & HOFMANN, F. (1992). Hormonal regulation of calcium current in freshly isolated airway smooth muscle cells. *Am. J. Physiol.*, **262**, L351–L359.

WILSON, C., WILSON, S., PIERCY, V., SENNITT, M.V. & ARCH, J.R.S. (1984). The rat lipolytic β-adrenoceptor, studies using novel β-adrenoceptor agonists. *Eur. J. Pharmacol.*, **100**, 309–319.

YABUUCHI, Y. (1977). The β-adrenoceptor stimulant properties of OPC-2009 on guinea-pig isolated tracheal, right atrial and left atrial preparations. *Br. J. Pharmacol.*, **61**, 513–521.

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Mechanical and biochemical responses to endothelin-1 and endothelin-3 in bovine bronchial smooth muscle

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1 In this study, mechanical responses to endothelin-1 and endothelin-3 were examined in bovine bronchial smooth muscle. In addition, the involvement of phosphatidylinositol 4,5-bisphosphate hydrolysis (PIP_2) in the responses to these peptides was assessed by measurement of inositol (1,4,5) trisphosphate ($I(1,4,5)P_3$) production using a specific mass assay.

2 ET-1 evoked contractions of bovine bronchi which were concentration-dependent and initiated at between 10^{-9} M and 10^{-8} M. ET-1-evoked responses were unaffected by slight elevation of tone with potassium chloride (3×10^{-2} M), methacholine (10^{-6} M) or U46619 (10^{-7} M).

3 Contractions to ET-1 were not altered by pre-incubation with atropine (10^{-5} M), indomethacin (10^{-5} M), nifedipine (10^{-5} M), phosphoramidon (3.67×10^{-5} M) or by removal of the epithelium.

4 ET-3 evoked small contractions which were not concentration-dependent. In the presence of phosphoramidon (3.67×10^{-5} M) however, concentration-dependent contractions were obtained to ET-3 which were unaffected by atropine (10^{-5} M) or by removal of the epithelium, but were significantly attenuated by indomethacin (10^{-5} M). Nifedipine (10^{-5} M) virtually abolished this response.

5 Both ET-1 and ET-3 (in the presence of phosphoramidon)-evoked contractions were significantly enhanced by the presence of the phorbol ester phorbol 12,13-dibutyrate (10^{-8} M). Neither ET-1-, nor ET-3-mediated responses were antagonized by the protein kinase C (PKC) inhibitor, Ro 31-8220 (3×10^{-9}–3×10^{-8} M).

6 ET-1 (3×10^{-7} M) evoked a biphasic rise in levels of $I(1,4,5)P_3$ which was unaltered by pre-incubation with atropine, whilst ET-3 (10^{-10}–3×10^{-7} M) failed to alter levels of $I(1,4,5)P_3$ at any time point examined, even in the presence of phosphoramidon (3.67×10^{-5} M).

7 These results suggest that, in bovine bronchial smooth muscle, ET-1 does not evoke contraction via cyclo-oxygenase metabolites, does not evoke release of the neurotransmitter substance acetylcholine, or require calcium influx via dihydropyridine-sensitive channels. ET-1 evokes $I(1,4,5)P_3$ production, but stimulation of protein kinase C may not be critical for the associated contraction. In contrast, ET-3-evoked contractions are partly mediated by cyclo-oxygenase metabolites. ET-3 does not stimulate PIP_2 hydrolysis, nor activate PKC, but may, either directly or as a requirement of intermediates released in response to ET-3, rely upon extracellular calcium.

Keywords: Endothelin-1; endothelin-3; phosphatidylinositol hydrolysis; bovine bronchi; protein kinase C

Introduction

Endothelin-1 (ET-1) is a potent vasoactive peptide produced from endothelial cells (Yanigasawa *et al.*, 1988). Other cells however, including porcine and canine tracheal epithelial cells (Black *et al.*, 1989) and human bronchial cells (Mattoli *et al.*, 1990) also produce ET-1. Although less potent in bronchial compared to vascular tissues, it is nevertheless the most potent bronchoconstrictor substance yet isolated, producing prolonged contractions in airways, both *in vivo* and *in vitro* (McKay *et al.*, 1992). In addition, acute pulmonary alveolar hypoxia increases lung ET-1 levels in conscious rats (Shirakami *et al.*, 1991), while elevated endothelin levels have been detected in patients with acute severe asthma (Nomura *et al.*, 1989). Accordingly, a role may exist for ET-1 in airway diseases, in particular in asthma, a condition characterized by hyperreactivity of airway smooth muscle.

The mechanisms by which ET-1 acts are incompletely understood. One possible pathway is the hydrolysis of the membrane phospholipid phosphatidylinositol 4,5,-bisphosphate (PIP_2). This biochemical pathway is thought to be an important signal-transduction mechanism coupling receptor occupation and contraction in response to a variety of spasmogens in airway smooth muscle (e.g. cholinoceptor agonists, Grandordy *et al.*, 1986; histamine, van Amsterdam *et al.*, 1990).

The products of this hydrolytic reaction are the second messengers inositol (1,4,5) trisphosphate ($I(1,4,5)P_3$), which releases intracellular stored calcium (Burgess *et al.*, 1983) and diacylglycerol (DAG), which stimulates protein kinase C (PKC, Nishizuka, 1983). It has been suggested (Rasmussen & Barrett, 1984) that the two second messengers produced from PIP_2 hydrolysis may exhibit differences in the time course of their action. The $I(1,4,5)P_3/Ca^{2+}$ system may be responsible for an initial transient response while the DAG/protein kinase C system underlies the sustained phase; the sum of these responses being a combined integrated response. Abnormal increases in intracellular calcium levels (Barnes *et al.*, 1992) or inappropriate activation of protein kinase C (Obianime *et al.*, 1989) may be important in asthma.

ET-1-stimulated PIP_2 hydrolysis has been demonstrated in vascular smooth muscle cells (Marsden *et al.*, 1989) and in human bronchial smooth muscle cells (Mattoli *et al.*, 1991). In these studies, however, measurement of PIP_2 hydrolysis was by assessment of accumulation of a number of inositol phosphates after incubation with [^{3}H]-inositol. Interpretation of such data is complicated and, in particular, it does not give any indication of the degree to which inositol monophosphate levels are derived from $I(1,4,5)P_3$ rather than from

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other inositol polyphosphates or, indeed, from possible hydrolysis of phosphatidylinositol. More accurate results are obtained using specific assays for I(1,4,5)P_3, which has the additional advantage of permitting the use of fresh, unradiolabelled tissue.

In this present study, to assess the importance of endothelins in airway disease, we examined several aspects of the mechanical and biochemical responses evoked by ET-1 and its isoform endothelin-3 (ET-3) in bovine bronchial smooth muscle.

We have attempted, using mechanical measurements, to establish the underlying reasons for potency differences between bronchial and vascular tissues and to provide some evidence for the type of endothelin receptor present in these bronchi.

The importance of PIP_2 hydrolysis in bovine bronchial smooth muscle has been examined by assessment of changes in levels of I(1,4,5)P_3 in response to ET-1 and ET-3. In addition, evidence for the importance to the contraction of PKC has been obtained by manipulation of kinase activity with phorbol esters, which mimic DAG and accordingly stimulate PKC (Castagna *et al.*, 1982) and Ro 31-8220, which inhibits this kinase (compound 3, Davis *et al.*, 1989).

Methods

Tissue collection and preparation

Bovine bronchial tissues were obtained from cattle within 30 min of slaughter. Tissues were dissected free of connective tissue and fat in oxygenated Krebs-Henseleit solution of the following composition (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2 and glucose 11.1

Measurement of contractile responses

Contractile responses were measured from rings of bronchial smooth muscle (3–5 mm external diameter) in vertical organ baths (10 ml) at 37 ± 0.5°C in oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit solution. Resting tension (2 g) was applied by inserting two platinum wires into the lumen. One wire was anchored and the other attached to a force displacement transducer (Grass FT03T). Concentration-response curves (CRCs) were constructed to ET-1 and ET-3. Drugs were added directly to the organ bath and antagonists were added 15 min before agonists. Phorbol 12,13-dibutyrate was added 5 min prior to addition of the endothelins. Where appropriate, removal of the epithelium was achieved by gentle rubbing of the intimal surface.

Preparation of samples for I(1,4,5)P_3 assay

The effects of ET-1 and ET-3 on levels of I(1,4,5)P_3 were examined in bovine bronchial smooth muscle by a modification of the method of Palmer & Wakelam (1990). Small ring segments (3–5 mm external diameter) of bronchial tissue were placed in 0.9 ml fresh Krebs-Henseleit solution at 37 ± 0.5°C, gassed with 95% O_2, 5% CO_2, pH 7.4 in 3.5 ml test tubes. After equilibrating for 40 min, tissues were stimulated with ET-1 or ET-3 (each 10^{-10}–3×10^{-7} M) for the required time (5, 10, 20, 30, 60, 120 and 300 s) and the reaction stopped by addition of 0.1 ml ice-cold perchloric acid (PCA) 20% (4°C). Preliminary experiments indicated that recovery with this technique was 79–84% ($n = 4$). In addition, preliminary results demonstrated that statistically similar results were obtained when samples were prepared by terminating the incubation in liquid nitrogen ($n = 6$). Antagonists, where appropriate were added 15 min before agonists. Experiments were conducted in duplicate. Tissues were homogenized (hand held glass homogenizer) in 2% PCA (4°C) and a 200 µl sample of homogenate taken for estimation of protein by the method of Peterson (1977). The remainder of the homogenate was treated for removal of perchloric acid by addition of potassium hydroxide (1.5 M) in HEPES (60 mM) and a minimal amount of universal indicator. Samples of the treated homogenate were taken for assay of I(1,4,5)P_3 levels by the method of Palmer & Wakelam (1990).

Materials

The following chemicals were used; atropine sulphate (Sigma), endothelin-1 (Novabiochem), endothelin-3 (Novabiochem), D-*myo*-inositol (1,4,5) trisphosphate potassium salt (Calbiochem), indomethacin (Sigma) D-*myo*-[^{3}H]-inositol (1,4,5) trisphosphate potassium salt (20–60 Ci mmol^{-1}), methacholine chloride (Sigma), nifedipine (Sigma), phorbol 12,13-dibutyrate (Sigma), N-(a-rhamnopyranosyloxyhydroxy phosphinyl)-L-leucyl-L-tryptophan (phosphoramidon, Sigma), potassium chloride (Analar), Ro 31-8220 (a gift from Roche Products Ltd. (UK)), U46619 ((15S)-hydroxy-11α,, 9α-(epoxymethane) prosta-5z,13E-dienoic acid, a gift from Upjohn). Concentration in the text refers to the salts, with the exception of nifedipine and phorbol 12,13-dibutyrate which are expressed as the base. Stock solutions of drugs were prepared in distilled water except for indomethacin, nifedipine, phorbol 12,13-dibutyrate and Ro 31-8220 which were dissolved in ethanol. In each case, subsequent dilutions were made in Krebs-Henseleit solution. Where appropriate, control CRCs included an equivalent concentration of ethanol.

Analysis of results

Unfortunately, due to financial restraints, a maximum response to ET-1 could not be obtained and a pD_2 value could not be calculated. In this present study therefore, results have been expressed in terms of the negative log concentration of the $EC_{200\,mg}$ (− log $EC_{200\,mg}$; the concentration effecting a 200 mg wt. contraction). This system of expressing results is similar to that used by MacLean *et al.* (1992).

Results are expressed as mean ± s.e.mean. Statistical significance between data samples was tested by two-way analysis of variance. Significance between $EC_{200\,mg}$ values was calculated by Student's t test. A probability level of $P < 0.05$ was considered significant. Number of observations (n) refers to the number of animals used.

Results

Endothelin-1

Contractile-responses ET-1 evoked a concentration-dependent contraction of bovine bronchi (control − log $EC_{200\,mg}$; 7.05 ± 0.13, $n = 12$. This compares with an equivalent − log $EC_{200\,mg}$ of 6.44 ± 0.4 for methacholine in the same tissue). Contraction was initiated at between 10^{-9} M and 10^{-8} M.

The contraction evoked by ET-1 in bovine bronchi was unaffected by pre-incubation with KCl (Figure 1a, 3×10^{-2} M), U46619 (Figure 1b, 10^{-7} M), or methacholine (Figure 1c, 10^{-6} M). Each of these agents evoked small (10–20% of own maximum contractions at the concentrations used; e.g. 10^{-6} M methacholine evokes a contraction of 0.29 ± 0.04 g, cf. maximum response 2.4 ± 0.27 g, $n = 14$). The steady-state contraction evoked was considered to be the baseline for subsequent ET-1-evoked contractions. ET-1-evoked contractions were unaffected by atropine (Figure 1d, 10^{-5} M), indomethacin (Figure 2a, 10^{-5} M), nifedipine (Figure 2b, 10^{-5} M), phosphoramidon (Figure 2c, 3.67×10^{-5} M) or by removal of the epithelium (Figure 2d, $n = 6$ in each case). The phorbol ester, phorbol 12,13-dibutyrate (PDBu, 10^{-8} M), however evoked a significant leftward shift of the CRC ($P < 0.001$, $n = 6$), which was abolished by the presence of Ro 31-8220 (3×10^{-8} M, Figure 3a, $n = 6$), while Ro 31-8220 (Figure 3b, 3×10^{-8} M, $n = 6$) failed to alter significantly the response to ET-1.

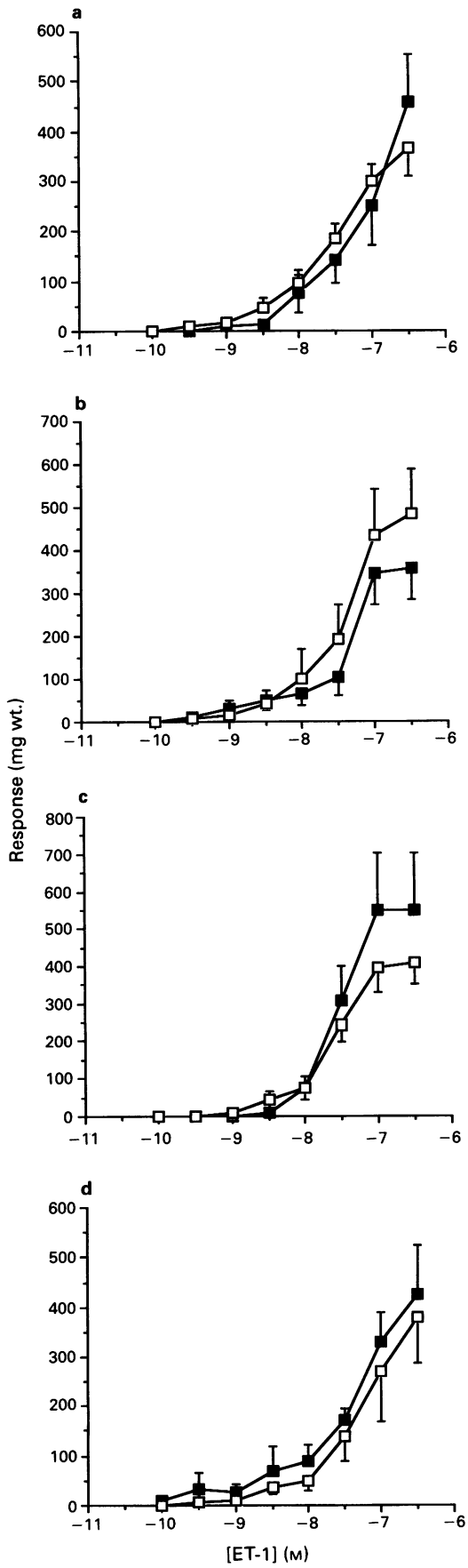


Figure 1 Concentration-response curves to endothelin-1 (ET-1), alone (■) or in the presence of (a) potassium chloride (□, 3×10^{-2} M), (b) U46619 (□, 10^{-7} M), (c) methacholine (□, 10^{-6} M) or (d) atropine (□, 10^{-5} M), in bovine bronchial rings. None of these compounds evoked any significant changes in control responses to ET-1. Number of observations (n) = 6 in each case.

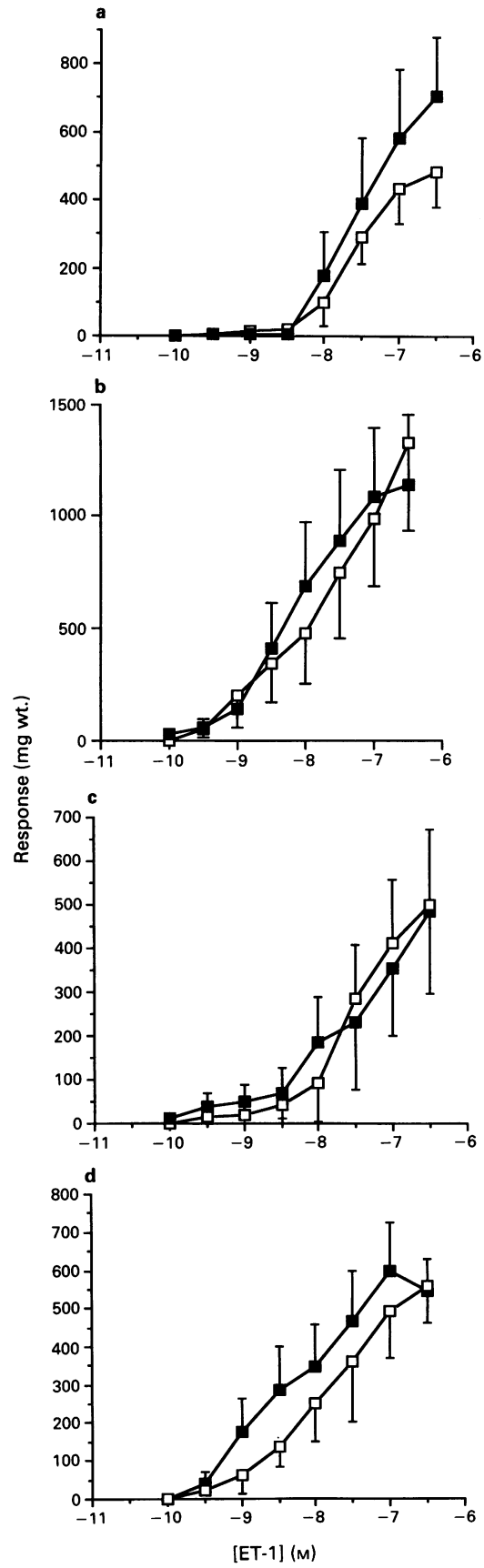


Figure 2 Concentration-response curves to endothelin-1 (ET-1), alone (■) or in the presence of (a) indomethacin (□, 10^{-5} M), (b) nifedipine (□, 10^{-5} M), (c) phosphoramidon (□, 3.57×10^{-5} M) or (d) after removal of the epithelium by gentle rubbing (□), in bovine bronchial rings. Indomethacin, nifedipine and phosphoramidon each failed to alter control responses to ET-1. Removal of the epithelium likewise failed to alter these responses to ET-1. Number of observations (n) = 6 in each case.

I(1,4,5)P_3 levels In bovine bronchial smooth muscle, ET-1 evoked a biphasic rise in I(1,4,5)P_3 levels (Figure 4). There was an initial rise, which was maximal at around 10 s, followed by a second rise peaking at 60 s. In response to 3×10^{-7} M ET-1, the mean maximum increase at 10 s was 306% ($P<0.001$), while at 60 s the increase amounted to 242% ($P<0.001$, $n=8$). The initial rise preceded, while the later rise occurred approximately coincidentally with, the time for initiation of contraction. The pattern of changes in I(1,4,5)P_3 levels evoked by ET-1 resembled that seen in response to methcholine in this tissue (Nally *et al.*, 1992), where rises at 10 s and 60 s of 461% and 345% respectively were observed in response to methacholine 10^{-3} M. Nevertheless, neither the intitial, nor the later rise in I(1,4,5)P_3 levels evoked by ET-1 was altered by pre-incubation with atropine (10^{-5} M, $n=6$).

Endothelin-3

Contractile responses In untreated bovine tissues, endothelin-3 (ET-3, $10^{-10}-3 \times 10^{-7}$ M) evoked small (0.25–0.5 g wt.) contractions which were not concentration-dependent ($n=6$). In the presence of phosphoramidon (3.67×10^{-5} M) however, the response to ET-3 became concentration-dependent (– log $EC_{200\,mg}$; 8.28 ± 0.26) and similar or greater in magnitude and potency to that seen with ET-1 in this tissue (Figure 5). Subsequent experiments using ET-3 were therefore all conducted in the presence of phosphoramidon. Under these conditions, the contractions evoked by ET-3 were unaffected by pre-incubation with atropine (10^{-5} M, $n=6$) or by removal of the epithelium. PDBu (10^{-8} M) evoked a significant leftward shift of control (plus phosphoramidon) responses to ET-3 (Figure 6a, PDBu – log $EC_{200\,mg}$, 9.7 ± 0.09, $P<0.001$), which was reversed by the additional presence of Ro 31-8220 (3×10^{-8} M, Figure 6a). Ro 31-8220 (Figure 6b, $3 \times 10^{-9}-3 \times 10^{-8}$ M) did not alter responses to ET-3 in the presence of phosphoramidon. Indomethacin (Figure 7a, 10^{-5} M) significantly ($P<0.001$) attenuated the responses evoked by ET-3 under these conditions. Three of six tissues did not evoke a response of 200 mg wt in the presence of phosphoramidon and indomethacin and accordingly the – log $EC_{200\,mg}$ could not be calculated. Responses to ET-3, in the presence of phosphoramidon, were virtually abolished by pre-incubation with nifedipine (Figure 7b, 10^{-5} M), such that the – log $EC_{200\,mg}$ could not be obtained.

I(1,4,5)P_3 levels ET-3 ($10^{-10}-3 \times 10^{-7}$ M) failed to alter levels of I(1,4,5)P_3 in bovine bronchial smooth muscle, at any time point examined (5–300 s, $n=8$), either in the presence (Figure 8, 3.67×10^{-5} M) or absence of phosphoramidon.

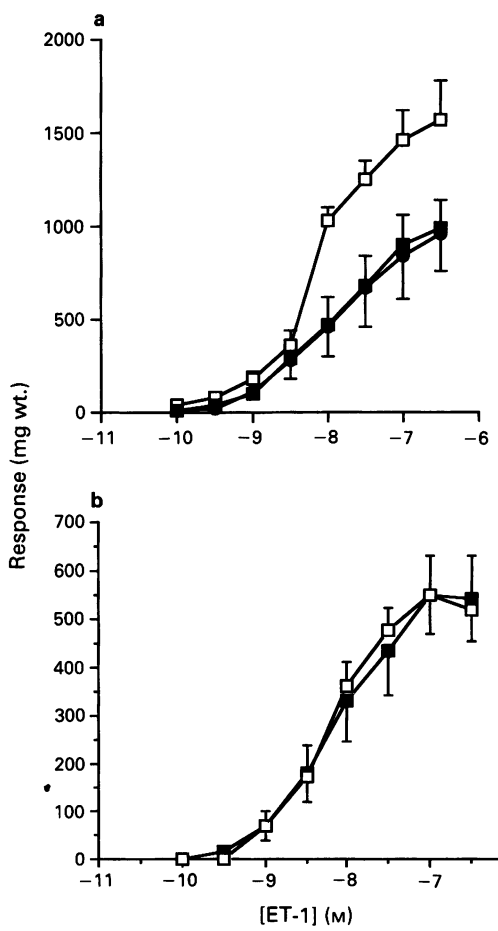


Figure 3 Concentration-response curves (CRCs) to endothelin-1 (ET-1) (■, $n=12$), in the presence of (a) phorbol 12,13-dibutyrate, (PDBu, 10^{-8} M) alone (□, $n=6$) or PDBu (10^{-8} M) and Ro 31-8220 (3×10^{-8} M) (●, $n=6$) in combination and (b) to endothelin-1 (ET-1) (●, $n=6$) in the presence of Ro 31-8220 (□, 3×10^{-8} M, $n=6$) alone. PDBu evoked a significant leftward shift, which was abolished in the additional presence of Ro 31-8220, while Ro 31-8220 alone failed to evoke any significant alteration, of the CRC to ET-1.

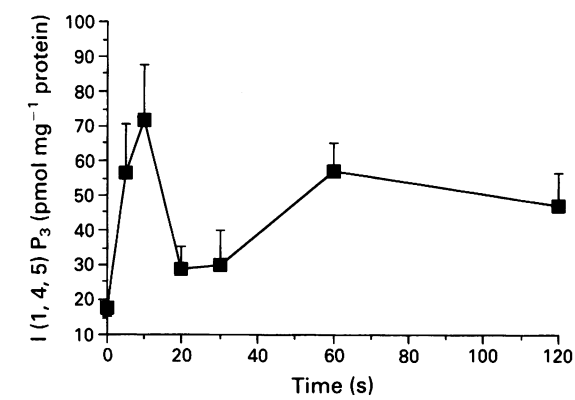


Figure 4 Time-dependent changes in levels of inositol (1,4,5)-trisphosphate (I(1,4,5)P_3) in response to endothelin-1 (3×10^{-7} M), measured as pmol mg^{-1} protein. There was an initial rise, maximal at 10 s, followed by a return to baseline by 20 s and a second rise in I(1,4,5)P_3 levels at 60 s. Each of these rises was significant with respect to zero time control values. Number of observations (n) = 10 in each case.

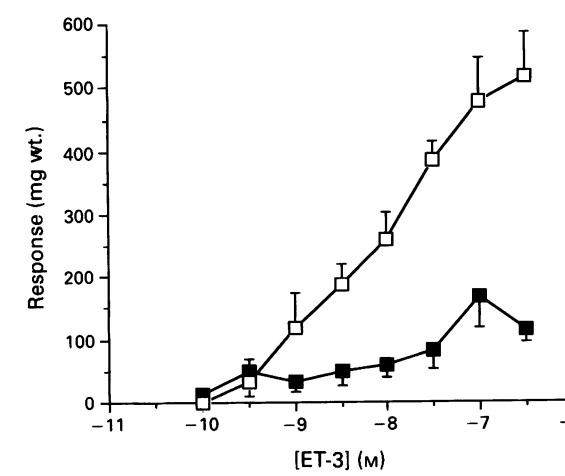


Figure 5 Concentration-response curves (CRCs) to endothelin-3 (ET-3), in the presence (□) and absence (■) of phosphoramidon (3.67×10^{-5} M). In the presence of phosphoramidon, the response to ET-3 became concentration-dependent. Number of observations (n) = 6 in each case.

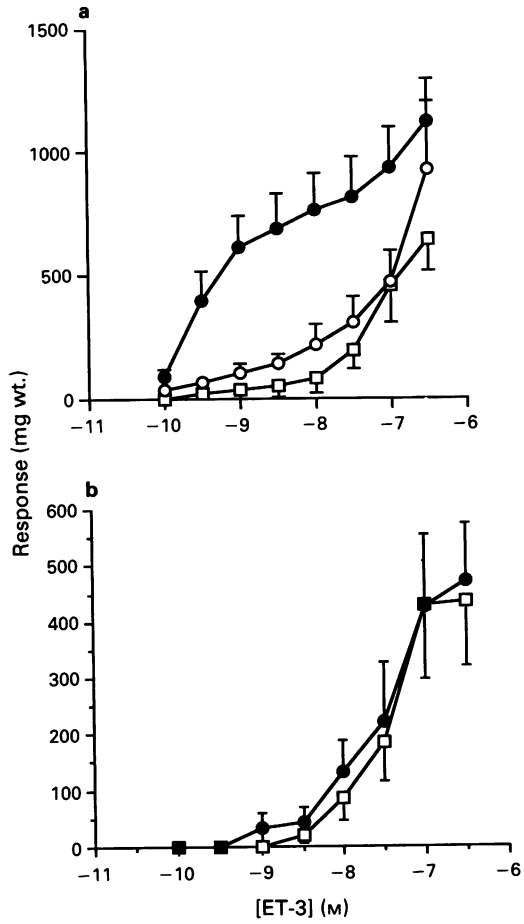


Figure 6 Concentration-response curves (CRCs) to endothelin-3 (ET-3, □, $n = 12$), alone and in the presence of (a) phorbol 12,13-dibutyrate, (PDBu, 10^{-8} M, ●, $n = 6$) as well as (○) a combination of PDBu (10^{-8} M) and Ro 31-8220 (3×10^{-8} M) ($n = 6$) and (b) CRCs to ET-3 (□, $n = 6$) in the presence of Ro 31-8220 (●, 3×10^{-8} M, $n = 6$). PDBu evoked a significant leftward shift of the CRC to ET-3, which was attenuated by the additional presence of Ro 31-8220. Ro 31-8220 alone evoked no significant change in the contractions to ET-3. Each of these experiments involving ET-3 was conducted in the presence of phosphoramidon (3.67×10^{-5} M) throughout.

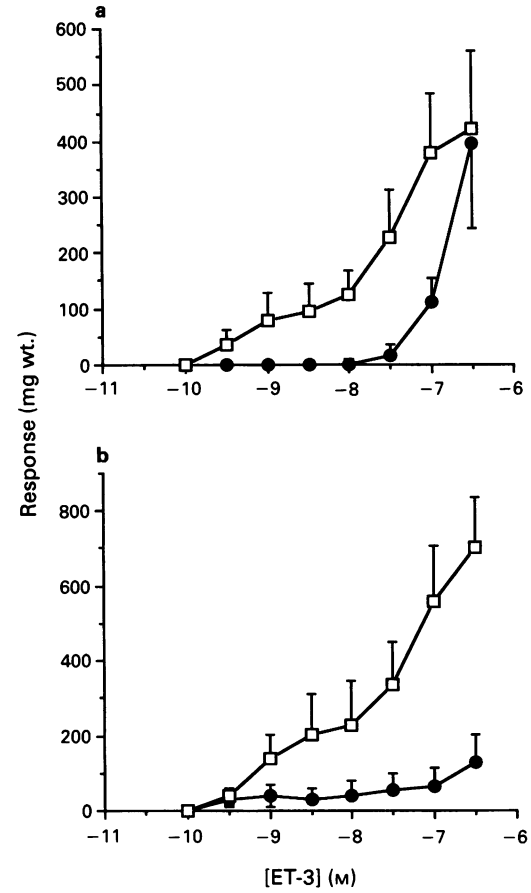


Figure 7 Concentration-response curves (CRCs) to endothelin-3 (ET-3), in the presence of phosphoramidon alone (□) and in the presence of (a) phosphoramidon (3.67×10^{-5} M) and indomethacin (●, 10^{-5} M) and (b) phosphoramidon (3.67×10^{-5} M) and nifedipine (●, 10^{-5} M). Indomethacin and nifedipine each had a significant attenuating effect on responses to ET-3 in the presence of phosphoramidon. Number of observations (n) = 6 in each case.

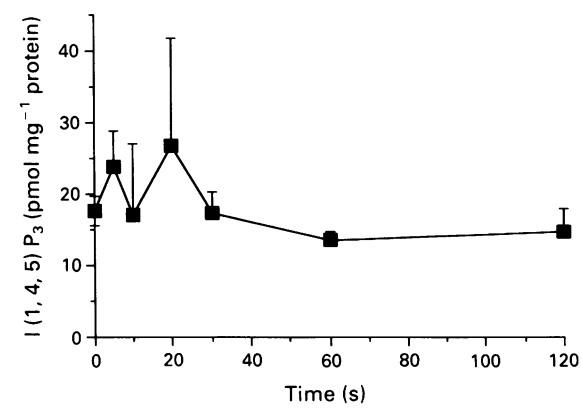


Figure 8 Time-dependent changes in levels of inositol (1,4,5)-trisphosphate (I(1,4,5)P$_3$) in response to endothelin-3 (ET-3; 10^{-6} M) in the presence of phosphoramidon (3.67×10^{-5} M), measured as pmol mg^{-1} protein. ET-3 failed to evoke any significant rise in I(1,4,5)P$_3$ levels at any time point measured. Number of observations (n) = 10 in each case.

Discussion

The potency of ET-1 in stimulating contraction in bovine bronchial smooth muscle was lower than that observed in vascular smooth muscle (e.g. rabbit pulmonary artery, MacLean & McGrath, 1991). This low potency does not reflect a lack of tone *in vitro*, since pre-contraction with other spasmogens, such as KCl, MCh or U46619 failed to alter the CRC to ET-1. ET-1 does not act by the release of the neurotransmitter substance acetylcholine, since atropine did not alter the response, nor does it act by release of prostaglandins since indomethacin was also ineffective. It is unlikely that ET-1 acts by release of agents from the epithelium since the response to ET-1 was unaffected by epithelium removal.

It seems unlikely from the present study that the lower potency of ET-1 in bovine bronchi compared to vascular tissue is due to ET-1 acting via an ET$_B$-type receptor in this tissue. If this were the case then it would be expected that the response to ET-1 would resemble that of ET-3. This does not however appear to be the case.

Early evidence had shown that, in contrast to ET-1, no concentration-dependent response to ET-3 could be shown. However, many of the mechanical differences between ET-1 and ET-3 appeared to be due to the breakdown of ET-3 under the influence of neutral endopeptidase since the presence of the endopeptidase inhibitor, phosphoramidon, resulted in a concentration-dependent response to ET-3 in bovine tissue. This is in keeping with the substantial enhancement of ET-3 responses recently demonstrated in both human and rabbit bronchus (McKay *et al.*, 1992).

Differences do however exist between the responses to ET-1 and ET-3. In bovine tissue, ET-3, but not ET-1 responses are inhibited by indomethacin and hence are partly evoked by the generation of cyclo-oxygenase products. The lack of effect of indomethacin on ET-1 responses is in contrast to, for example, the guinea-pig, where responses to this peptide in bronchi (Filep et al., 1991), trachea (White et al., 1991) and lung (Pons et al., 1991) were to a greater or lesser extent mediated via the formation of cyclo-oxygenase metabolites. In human bronchi however, (Advenier et al., 1990; McKay et al., 1991) indomethacin is without effect on ET-1 stimulated responses and this may reflect a species difference.

ET-1 responses were also unaltered by nifedipine, a dihydropyridine which antagonizes voltage-dependent calcium channels. This is similar to results obtained in human bronchial tissue, (McKay et al., 1991) where the contractions evoked by this agonist were unaffected by verapamil. In contrast, ET-3-stimulated responses were virtually abolished by nifedipine and contraction to ET-3 would therefore appear to rely on an influx of external calcium. In the present study we also examined the effect of stimulating or inhibiting PKC with, respectively, PDBu or Ro 31-8220. It was not possible during these or subsequent experiments to exceed 10^{-8} M PDBu since concentrations greater than this evoked a large contraction, masking the contractions evoked by the endothelins. The effects of the protein kinase C inhibitor, Ro 31-8220, suggest that contractions generated by ET-1 do not rely heavily upon PKC activitation, since this inhibitor failed to attenuate the ET-1-evoked response in bovine bronchial smooth muscle. The leftward shift evoked by PDBu indicates an additive effect between PKC activation and the signals evoked by ET-1.

Contractions to ET-3 are also unlikely to utilise PKC activation, since, even in the presence of phosphoramidon, contractions were unaffected by Ro 31-8220 although they were enhanced by PDBu. The ability of PDBu to shift CRCs leftward is not evidence *per se* that ET-1 or ET-3 act via PKC activation since, in several tissues, it has been suggested that a maximum physiological response can result from the synergistic action of an increase in $[Ca^{2+}]_i$ concentration and the formation of diacylglycerol (Yamanishi et al., 1983; Nishizuka, 1984), here mimicked by PDBu. Accordingly, the inability of Ro 31-8220 to inhibit these responses suggests rather that PKC activation is not critical to the mechanical response to either endothelin.

Evidence for ET-1 and ET-3 acting via different signal-transduction mechanisms and hence different receptors has been obtained from the measurement of $I(1,4,5)P_3$. ET-1 stimulated a substantial rise in $I(1,4,5)P_3$ levels in bovine tissue whilst, even in the presence of phosphoramidon, ET-3 evoked no such response. The substantial changes in $I(1,4,5)P_3$ levels evoked by ET-1 within the first few seconds following stimulation, precede increases in isometric force development. The rapid time course for production of $I(1,4,5)P_3$ is accordingly in keeping with a receptor-activated response initiating smooth muscle contraction.

We have therefore demonstrated that, while ET-1 elevates $I(,1,4,5)P_3$ levels, PKC activation does not appear to be crucial to the associated contraction. This suggests that both 'limbs' of the PIP_2 hydrolysis pathway are not necessarily utilised as signals mediating the response to the activation of receptors by ET-1. A similar pattern is seen in rat pancratic acinar cell line (Gallacher et al., 1990) in response to another polypeptide, bombesin, suggesting that it is not possible to assume, from $I(1,4,5)P_3$ production, that PKC activation is important to a particular response in any given system.

The biphasic nature of the $I(1,4,5)P_3$ response to ET-1 suggests that this peptide did not fully desensitize and differs from the time course of formation of $I(1,4,5)P_3$ in response to ET-1 in other systems. In brain capillary endothelial cells for example (Vigne et al., 1990), the response to ET-1 was transient, while a sustained elevation of $I(1,4,5)P_3$ levels was observed in Rat-1 fibroblasts in response to this peptide (Plevin et al., 1991); this latter result demonstrating another system where desensitization does not appear to occur. It is important to recognise that a mass assay, as opposed to an accumulation assay, cannot take into account potential changes in the metabolism, rather than the generation of $I(1,4,5)P_3$. Indeed, the fall in $I(1,4,5)P_3$ levels observed at 20 and 30 s is probably due to increased activity of the 5-phosphatase and 3-kinase responsible for $I(1,4,5)P_3$ metabolism. Such changes in metabolism of $I(1,4,5)P_3$ have indeed been implicated in regulation of levels of this second messenger in bovine tracheal smooth muscle in response to carbachol (Chilvers et al., 1991). Thus the biphasic $I(1,4,5)P_3$ response is due to a change in the equilibrium between $I(1,4,5)P_3$ generation and metabolism. It is probable that the major regulating aspect is, however, the phospholipase C. Although the pattern of the changes in IP_3 levels evoked by ET-1 resembles that observed for MCh in this tissue (Nally et al., 1992), pre-incubation with atropine did not alter the IP_3 response to ET-1, confirming our earlier conclusion that ET-1 did not act via release of acetylcholine.

In conclusion, these results give further evidence of the possible importance of ET-1 in airway smooth muscle. It appears that, in this tissue, the actions of ET-1 are not via acetylcholine release or by generation of cyclo-oxygenase products and occur via a different receptor from that utilised by ET-3. This receptor, when activated by ET-1, stimulates hydrolysis of PIP_2, with subsequent production of $I(1,4,5)P_3$. Neither PKC activation nor influx of extracellular calcium through dihydropyridine-sensitive calcium channels is a prerequisite for contraction to ET-1.

Addition of ET-3 does not rely on activation of protein kinase C, nor does stimulation of PIP_2 hydrolysis occur in response to this peptide. Instead ET-3 relies upon ingress of extracellular calcium through dihydropyridine-sensitive, voltage-operated channels, for production of contraction. In addition, a proportion of the contraction evoked by ET-3 appears to be mediated by cyclo-oxygenase metabolites. It is unclear if the stimulatory effect of ET-3 involves generation of arachidonic acid for ecosanoid production, perhaps through Ca^{2+}-stimulated phospholipase A_2 activation or if it is a stimulation of cyclooxygenase *per se*.

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References

ADVENIER, C., SARRIA, B., PUYBASSET, L. & LAGENTE, V. (1990). Contractile activity of three endothelins (Et-1, Et-2 and Et-3) on the human isolated bronchus. *Br. J. Pharmacol.*, **100**, 168–172.

BARNES, P.J., RODGER, I.W. & THOMSON, N.C. (1992). Pathogenesis of asthma. In *Asthma: Basic Mechanisms and Clinical Management*. ed. Barnes, P.J., Rodger, I.W. & Thomson, N.C. pp. 391–407. London: Academic Press.

BLACK, P.N., GHATEI, K., TAKAHASHI, D., BRETHERTON-WATT, D., KRAUSZ, T., DOLLERY, C.T. & BLOOM, S.R. (1989). Formation of endothelin by cultured airway epithelial cells. *FEBS Lett.*, **225**, 129–132.

BURGESS, G.M., MCKINNEY, J.S., FABIATO, A., LESLEY, B.A. & PUTNEY, J.W.Jr. (1983). Calcium pools in saponin-permeabilized guinea pig hepatocytes. *J. Biol. Chem.*, **258**, 15336–15345.

CASTAGNA, M., TAKAI, Y., KAIBUCHI, K., SANO, K., KIKKAWA, U. & NISHIZUKA, Y. (1982). Direct activation of calcium-activated phospholipid-dependent protein kinase by tumour-promoting phorbol esters. *J. Biol. Chem.*, **257**, 7847–7851.

CHILVERS, E.R., BATTY, I.H, CHALLISS, R.A.J. & BARNES, P.J. (1991). Determination of mass changes in phosphatidylinositol 4,5-bisphosphate and evidence for agonist-stimulated metabolism of inositol 1,4,5-trisphosphate in airway smooth muscle. *Biochem. J.*, **275**, 373–379.

DAVIS, P.D., HILL, C.H., KEECH, E., LAWTON, G., NIXON, J.S., SEDGWICK, J., WESTMACOTT, D. & WILKINSON, S.E. (1989). Potent selective inhibitors of protein kinase C. *FEBS Letts*, **259**, 61–63.

FILEP, J.G., BATTISTINI, B. & SIROIS, P. (1991). Pharmacological modulation of endothelin-induced contraction of guinea-pig isolated airways and thromboxane release. *Br. J. Pharmacol.*, **103**, 1633–1640.

GALLACHER, D.V., HANLEY, M.R., PETERSEN, O.H., ROBERTS, M.L., SQUIRE-POLLARD, L.G. & YULE, D.L. (1990). Substance P and Bombesin elevate cytosolic Ca^{2+} by different molecular mechanisms in a rat pancreatic acinar cell line. *J. Physiol.*, **426**, 193–207.

GRANDORDY, B.M., CUSS, F.M., SAMPSON, A.S., PALMER, J.B. & BARNES, P.J. (1986). Phosphatidylinositol response to cholinergic agonists in airway smooth muscle; relationship to contraction and muscarinic receptor occupancy. *J. Pharmacol. Exp. Ther.*, **238**, 273–279.

MCKAY, K.O., BLACK, J.L. & ARMOUR, C.L. (1991). The mechanism of action of endothelin in human lung. *Br. J. Pharmacol.*, **102**, 422–428.

MCKAY, K.O., BLACK, J.L. & ARMOUR, C.L. (1992). Phosphoramidon potentiates the contractile response to endothelin-3, but not endothelin-1 in isolated airway tissue. *Br. J. Pharmacol.*, **105**, 929–932.

MACLEAN, M.R. & MCGRATH, J.C. (1991). Endothelin-1 induced vasoconstriction and hypoxia in the rabbit pulmonary artery. *Br. J. Pharmacol.*, **104**, 419P.

MACLEAN, M.R., TEMPLETON, A.G.B. & MCGRATH, J.C. (1992). The influence of endothelin-1 on human foeto-placental blood vessels: a comparison with 5-hydroxydopamine. *Br. J. Pharmacol.*, **106**, 937–941.

MARSDEN, P.A., DANTHULURI, N.R., BRENNER, B.M., BALLERMANN, B.J. & BROCK, T.A. (1989). Endothelin action on vascular smooth muscle involves inositol trisphosphate and calcium mobilization. *Biochem, Biophys. Res. Commun.*, **158**, 1–85.

MATTOLI, S., MEZZETTI, M., RIVA, G., ALLEGRA, L. & FASOLI, A. (1990). Specific binding of Endothelin on human bronchial smooth muscle cells in culture and secretion of endothelin-like material from bronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol.*, **3**, 145–151.

MATTOLI, S., SOLOPERTO, M, MEZZETTI, M. & FASOLI, A. (1991). Mechanisms of calcium mobilization and phosphoinositide hydrolysis in human bronchial smooth muscle cells by endothelin-1. *Am. J. Respir. Cell Mol. Biol.*, **5**, 424–430.

NALLY, J.E., MCCALL, R., YOUNG, L.C., MCGRATH, J.C., WAKELAM, M.J.O. & THOMSON, N.C. (1992). Stimulated phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis in human and bovine bronchial smooth muscle. *Br. J. Pharmacol.*, **106**, 41P.

NISHIZUKA, Y. (1983). Phospholipid degeneration and signal translation for protein phosphorylation. *Trends Biochem. Sci.*, **8**, 13–16.

NISHIZUKA, Y. (1984). The role of protein kinase C in cell surface signal-transduction and tumour promotion. *Nature*, **308**, 693–698.

NOMURA, A., UCHIDA, Y., KAMEYAMA, M., SAOTOME, M., OKI, K. & HASEGAWA, S. (1989). Endothelin and bronchial asthma. *Lancet*, **ii**, 747–748.

OBIANIME, A.W., HIRST, S.J. & DALE, M.M. (1989). The effect of smooth muscle relaxants working through different transduction mechanisms on the phorbol dibutyrate-induced contraction of the guinea-pig lung parenchymal strip: possible relevance for asthma. *Pulm. Pharmacol.*, **2**, 191–200.

PALMER, S. & WAKELAM, M.J.O. (1990). Mass measurement of inositol 1,4,5-trisphosphate using a specific binding assay. In *Methods in Inositide Research*. ed. Irvine, F. pp. 127–134. New York: Raven Press.

PETERSON, G.L. (1977). A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal. Biochem.*, **83**, 346–356.

PLEVIN, R., MACNULTY, E.E., PALMER, S. & WAKELAM, M.J.O. (1991). Differences in the regulation of endothelin-1 and lysophosphatidic acid-stimulated Ins(1,4,5)P_3 formation in rat-1 fibroblasts. *Biochem. J.*, **280**, 609–615.

PONS, F., LOQUET, I., TOUVAY, P.R., CHABRIER, P.-E., MENCIA-HUERTA, J.M. & BRAQUET, P. (1991). Comparison of the bronchopulmonary and pressor activities of endothelin isoforms Et-1, Et-2 and Et-3 and characterization of their binding sites in guinea-pig lung. *Am. Rev. Respir. Dis.*, **143**, 294–300.

RASMUSSEN, H. & BARRETT, P.Q. (1984). Calcium messenger system: an integrated view. *Physiol. Rev.*, **64**, 938–984.

SHIRAKAMI, G., NAKAO, K., SAITO, Y., MAGARIBUCHI, T., JOUGASAKI, M., MUKOYAMA, M., ARAI, H., HOSODA, K., SUGA, S.-I., OGAWA, Y., YAMADA, T., MORI, K. & IMURA, H. (1991). Acute pulmonary alveolar hypoxia increases lung and plasma endothelin-1 levels in conscious rats. *Life Sci.*, **48**, 969–976.

VAN AMSTERDAM, R.G., MEURS, H., TEN BERGE, R.E.J., VENNINGA, C.M., BROUWER, F. & ZAAGSMA, J. (1990). Role of phosphoinositide metabolism in human bronchial smooth muscle contraction and in functional antagonism by beta-adrenoceptor agonists. *Am. Rev. Respir. Dis.*, **142**, 1124–1128.

VIGNE, P., MARSAULT, R., BREITTMAYER, J.-P. & FRELIN, C. (1990). Endothelin stimulates phosphatidylinositol hydrolysis and DNA synthesis in brain capillary endothelial cells. *Biochem. J.*, **226**, 415–420.

WHITE, S.R., HATHAWAY, D.P., UMANS, J.G., TALLET, J., ABRAHAMS, C. & LEFF, A.R. (1991). Epithelial modulation of airway smooth muscle response to endothelin-1. *Am. Rev. Respir. Dis.*, **144**, 373–378.

YAMANISHI, J., TAKAI, Y., KAIBUCHI, K., SANO, K., CASTAGNAS, M. & NISHIZUKI, Y. (1983). Synergistic functions of phorbol esters and calcium in serotonin release from human platelets. *Biochem. Biophys. Res. Commun.*, **112**, 778–786.

YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, S., KOBAYASHI, M., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **332**, 411–415.

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The pharmacological properties of Y-23684, a benzodiazepine receptor partial agonist

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1 The pharmacological properties of a benzodiazepine receptor (BZR) partial agonist, Y-23684 were investigated in comparison with those of diazepam, a conventional BZR full agonist.

2 Y-23684 and diazepam showed high and selective affinity for the BZR with K_i values of 41 and 5.8 nM, respectively.

3 In contrast to diazepam, variability was noted in the anticonvulsive potency of Y-23684 depending on convulsants (bicuculline, pentylenetetrazol and maximal electrical shock). Y-23684 produced the most potent protective effect against bicuculline in rats and mice with ED_{50}s of 1.3 and 1.2 mg kg^{-1}, respectively.

4 In rat conflict models (Geller-Seifter and water-lick tests), Y-23684 produced an antipunishment action at doses 2–4 times lower than diazepam. In contrast to diazepam, Y-23684 did not affect unpunished responding up to 50 mg kg^{-1} in the Geller-Seifter test.

5 In other rat models of anxiety (social interaction and elevated plus-maze tests), Y-23684 was as efficacious as and ten fold more potent than diazepam. In a mouse model of anxiety (exploration (light/dark box) test), Y-23684 was as efficacious and two fold less potent as diazepam. In these paradigms, Y-23684 showed a selective anxiolytic profile over a wide dose-range without loss of efficacy and sedative action.

6 The impairment of motor coordination (rotarod) and potentiation of CNS depressants (ethanol and hexobarbitone) by Y-23684 was much weaker than that of diazepam.

7 These results suggest that Y-23684 would be a potent and selective anxiolytic agent in man with less side-effects than conventional BZ-anxiolytics.

Keywords: Y-23684; benzodiazepine receptor partial agonist; diazepam; Geller-Seifter test; water-lick test; social interaction test; elevated plus-maze test; exploration test

Introduction

Since the discovery of specific binding sites in mammalian brain for benzodiazepines (BZs) (Möhler & Okada, 1977; Squires & Braestrup, 1977), numerous pharmacological analyses have permitted great progress in the field of BZ research. The history of BZs as the standard treatment for anxiety disorders during the past three decades indicates that they have been highly effective agents. However, considerable concern has been expressed regarding their ability to produce a series of undesirable features (i.e. sedation, muscle relaxation, amnesia, interactions with alcohol/barbiturates and dependency-liability).

A huge variety of novel agents interacting with the BZR have been synthesized over the past 10 years in order to understand the molecular mechanisms by which these agents produce their biological responses, and ultimately to develop non-sedative 'anxioselective' agents. These advances enabled two hypotheses to be proposed. One is BZR multiplicity, two receptor subtypes, $BZ(omega)_1$ and $BZ(omega)_2$, exist in different brain areas, subserving different physiological functions. The introduction of BZ_1-selective imidazopyridines, zolpidem and alpidem (Depoortere *et al.*, 1986; Zivkovic *et al.*, 1990), has fuelled the debate about the functional consequences of receptor multiplicity (Doble & Martin, 1992). An alternative approach is the development of BZR partial agonists (Petersen, 1987; Müller, 1988; Haefely *et al.*, 1990). It is now generally accepted that central BZR is an integral part of the $GABA_A$ receptor channel, a hetero-oligometric glycoprotein complex (Jenck *et al.*, 1992). Experimental evidence indicates that BZR ligands can be classified with different efficacy on a continuum from full agonists to inverse agonists, according to their modulatory effects on GABAergic transmission. Partial agonists are of interest because of their lower positive intrinsic efficacy, between those of full agonists represented by diazepam and the zero intrinsic efficacy of pure antagonists such as flumazenil, which might be sufficient to maintain the anxiolytic and anticonvulsant responses, but insufficient to induce unwanted side-effects seen with conventional full agonists. The triazopyridazine, CL 218,872 (Lippa & Crichett, 1979), the imidazobenzodiazepinone, bretazenil (Martin *et al.*, 1988), the β-carboline, abecarnil (Stephens *et al.*, 1990) and other classes of compounds have to date been claimed to be BZR partial agonists, even though some of them also discriminate between BZ_1 and BZ_2 sites. The results in animal studies with these drugs have demonstrated their distinguishable 'anxioselective' behavioural profile from full agonists to confirm the plausibility of the BZR partial agonist theory.

Based on this hypothesis, the functional properties of a series of condensed pyridazinone derivatives have been studied in our laboratories with a view to designing new anxiolytics (Nakao *et al.*, 1990; 1991a,b; 1992). Among them, Y-23684, (±)-2-(4-chlorophenyl)-5,6-dihydro-[1]benzothiepino-[5,4-c]pyridazin-3(2H)-one 7-oxide (Figure 1, molecular wt.: 356.83), showed a BZR partial agonistic property that possessed a lower intrinsic efficacy than diazepam on the BZR/GABA-chloride channel complex in an electrophysiological assay, which was similar to CL 218,872 and bretazenil (Yakushiji *et al.*, 1990). In the present paper, we describe the preclinical pharmacological profile of Y-23684 in comparison with diazepam.

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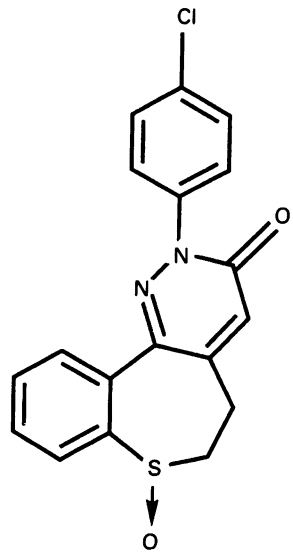


Figure 1 Chemical structure of Y-23684.

Methods

Animals

Wistar rats (170–250 g), Wistar-King A rats (350–450 g), Lister hooded rats (270–350 g), ddY mice (20–24 g) and BKW mice (30–38 g) were obtained from Seiwa Experimental Animals, Ltd. (Fukuoka, Japan). Wistar and Lister hooded rats were housed in groups of 5; ddY and BKW mice were housed in groups of 10–20. They were allowed food and water *ad libitum*. Wistar-King A rats were housed individually, and were food deprived to 85% of their free feeding body weight. Unless stated otherwise, animals were kept in environmentally controlled conditions (06 h 00 min–18 h 00 min lights on; 24 ± 1°C). All behavioural assessments were carried out between 09 h 00 min and 18 h 00 min.

Drugs

Y-23684 and diazepam were synthesized at our medicinal chemistry department. They were suspended in 0.5% methylcellulose (MC) solution, and administered orally (p.o.) in a volume of 1 or 2 ml kg^{-1} for rats and 10 ml kg^{-1} for mice. Unless stated otherwise, Y-23684 and diazepam were administered 3 and 1 h before each test in rats, respectively. In mouse studies, both compounds were administered 1 h before each test. The pretreatment time in both species for each compound was fixed to attain the maximum plasma concentration based upon pharmacokinetic studies (unpublished observations). All control groups were treated with an equal volume of vehicle. (+)-Bicuculline (Sigma) was dissolved in 0.1 N HCl. Pentylenetetrazol (Aldrich) and ethanol (Ishizu Seiyaku) were dissolved in distilled water. Hexobarbitone (Teikoku Kagaku) was suspended in 0.5% MC solution.

BZ binding

A binding study of [^{3}H]-diazepam to crude synaptosomal membranes of rat cerebral cortex was performed, based on the method described previously (Möhler & Okada, 1977). A total volume of 1.0 ml consisted of 900 µl of crude synaptosomal membranes containing approximately 1 mg of protein, 50 µl of [^{3}H]-diazepam (final concentration: 2 nM) and compounds dissolved in 50 µl of ethanol incubated at 0°C for 10 min. Membrane bound radioactivity was determined by filtration through Whatman GF/B filters followed by liquid scintillation counting. Nonspecific binding was determined in the presence of 1 µM unlabelled diazepam.

Radioligand binding to other neurotransmitter receptors

The affinity of the test compounds for other neurotransmitter receptors was assayed with rat brain homogenates. All the studies summarized in Table 1 were performed based on methods described previously; $GABA_A$ receptors (Beaumont *et al.*, 1978), dopamine D_2 receptors (Creese *et al.*, 1977), α_1-adrenoceptors (Greengrass & Bremner, 1979), α_2-adrenoceptors (U'Prichard *et al.*, 1979), 5-HT_{1A} receptors (Hall *et al.*, 1985), 5-HT_2 receptors (Peroutka & Snyder, 1979) and muscarinic cholinoceptors (Yamamura & Snyder, 1974).

Anticonvulsant studies

Male Wistar rats in groups of 7–21 or male ddY mice in groups of 7–14 were used. The animals were challenged with bicuculline (0.6 mg kg^{-1}, i.v.: Lippa & Regan, 1977; Rastogi & Ticku, 1986), pentylenetetrazol (150 mg kg^{-1}, s.c. for mice and 100 mg kg^{-1}, i.p. for rats: Tsumagari *et al.*, 1978) or maximal electrical shock (MES, AC 2000 V, 12.5 mA, 0.2 s for mice) delivered through bilateral corneal electrodes. The criteria used to indicate convulsant responses were tonic extension (hindlimb for mice and forelimb for rats) in bicuculline- and MES-seizures, and lethality in pentylenetetrazol-seizures. The anticonvulsant potency of Y-23684 and diazepam were estimated as ED_{50} values and their 95% confidence limits.

Geller-Seifter conflict test

The procedure was based on the method described previously (Geller & Seifter, 1960). Food-deprived male Wistar-King A rats were trained to press a lever for milk reinforcement in operant chambers. The schedule consisted of alternations of four 10 min fixed ratio (FR) 20 unpunished periods and four 5 min FR1 punished periods signalled by a white noise. During the punished periods, a 300 ms foot-shock was given in association with milk delivery. To study anticonflict effects, the intensity of foot-shock (400–800 µA) was adjusted for each rat to suppress total lever presses during the four punished periods to under 10. Y-23684 and diazepam were administered 2.5 and 0.5 h before the start of tests, respectively. In this test, the pretreatment time for each compound was modified from other rat studies so that the maximum plasma concentration could be obtained in the middle of a 60 min period.

Water-lick conflict test

The procedure was modified from the method described previously (Vogel *et al.*, 1971). Male Wistar rats were deprived of water for 72 h, and were placed in the test chamber and allowed to drink from the water spout. Licking was automatically accompanied by a 100 V, 0.2–0.3 mA, 300 ms electrical stimulus across the grid floor and spout every twenty licks. After the rat received the first electrical stimulus, the number of stimuli was recorded automatically during the subsequent 3 min test. All rats were naive to the test chamber.

Social interaction test

The procedure was essentially as described by File (1980). The test apparatus used for the detection of rat social interaction behaviour and exploratory behaviour consisted of an open-topped perspex box (51 × 51 × 20 cm(H)) with 17 cm square areas marked on the floor of the box. The light intensity of the arena floor was 1200 lux, and that of the experimental sound-proof room at the floor level was

100 lux. Male Lister hooded rats were allowed at least 4 h for adaptation to the new environment. Effects of the test compounds were determined by treating each pair of rats, which were naive to the test arena.

The test was started by placing each pair of rats, from separate housing cages, in opposite corners of the arena, and their behaviours were observed over a 10 min period by video recording, leaving them undisturbed. All behavioural assessments were made from the recordings, with the observer unaware of the original treatment. Social interactions between the rats were determined by timing sniffing of the partner, climbing over or crawling under the partner, genital investigation of the partner and following of the partner. Additionally, exploratory locomotion was measured as the number of line crossings marked on the floor of the test arena.

Elevated plus-maze test

The elevated plus-maze test is based on the hypothesis that exposure to an elevated and open maze alley leads to an approach-avoidance conflict that is considerably stronger than that evoked by exposures to a closed alley (Chopin & Briley, 1987). The procedure is valid in various rat strains, heights of alley or walls of the closed arms, judgement of entries (whole arms or end sections) and lighting conditions (Handley & Mithani, 1984; Pellow *et al.*, 1985; Meert & Janssen, 1989; Costall *et al.*, 1989b; Singh *et al.*, 1991). Since these studies suggested that a change of these factors should affect the intensity of aversive environmental stimuli, we further modified the experimental conditions to demonstrate a higher environmental aversion.

Naive male Lister hooded rats were used. Behavioural testing was conducted between 13 h 00 min and 17 h 00 min in a darkened sound-proof room illuminated with dim red lights (5 lux at the floor level), and animals were allowed at least 4 h for adaptation to the new environment. The apparatus consisted of a plus-shaped maze constructed of perspex, elevated 70 cm from the floor. It comprised two closed arms which had transparent sides and an end 10 cm high and two open arms. Both types of arms (45 × 10(W) cm) were marked into two equal sections, and the furthermost (end) sections of open arms were illuminated brightly (1200 lux) with white bulb lights. Individual rats were placed on the centre square of the apparatus facing an open arm and observed over the 10 min test period by video recording, leaving the rats undisturbed. All behavioural assessments were made from the recordings with the observer unaware of the original treatment, and the time spent on the end sections of open arms was noted.

Exploration (light/dark box) test

The procedure was performed based on the method as described previously (Costall *et al.*, 1989a). Naive BKW mice which had been kept for at least 2 weeks under a reversed 12 h light/dark cycle with lights off at 07 h 00 min were used, and behavioural testing was conducted between 13 h 00 min and 17 h 00 min (dark period) in a darkened sound-proof room illuminated with dim red lights (5 lux at the floor level). Animals were allowed at least 4 h for adaptation to the new environment.

The apparatus used for the detection of changes in exploratory behaviours consisted of an open-topped metal box (45 × 45 × 27 cm(H)) lined into 9 cm squares on the floor, two-fifth painted black and illuminated under a dim red light (less than 5 lux at the centre of the arena) and partitioned from the remainder of the box which was brightly illuminated (1000 lux). An opening 7.5 × 7.5 cm located at the floor level in the centre of the partition allowed access between the two compartments. At the start of testing, individual mice were placed in the centre of the brightly lit area of the test box.

The behaviour of mice was recorded over a 5 min period by video recording, leaving them undisturbed. All behavioural assessments were made from the recordings with the observer unaware of the original treatment, and three behaviours were noted: (i) the time spent in the light section (s), (ii) the number of line crossings in the light and dark sections and (iii) the number of exploratory rearings in both sections.

Motor incoordination: rotarod

Male ddY mice and male Wistar rats in groups of 10 were used. Untrained mice were placed on the rotarod (2.8 cm in diameter, 11 r.p.m.). In rat studies, only rats trained previously to stay on the rotarod (5 cm in diameter, 5 r.p.m.) for more than 3 min were used. In both species, the number of animals which dropped off the rotarod within 1 min was determined to estimate ED_{50} values and their 95% confidence limits.

Interactions with alcohol and barbiturates

Male ddY and male Wistar rats in groups of 7–14 were used. The ability of the test compounds to potentiate the action of a subnarcotic dose of ethanol (30% at 10 ml kg^{-1}, i.p. for mice and 25% at 100 ml kg^{-1}, i.p. for rats) or that of hexobarbitone (40 mg kg^{-1}, i.p. for mice and 60 mg kg^{-1}, i.p. for rats) were examined using the loss of righting reflex as a behavioural index. The number of animals which lost the righting reflex for more than 10 s was determined, and ED_{50} values and their 95% confidence limits were estimated.

Statistics

Mean ± s.e.mean were calculated and presented for each treated group. Statistical differences between two groups were made by Aspin-Welch's t-test (Welch, 1949) in the Geller-Seifter test, single factor ANOVA followed by the LSD test in the water-lick conflict test, single factor ANOVA followed by Dunnett's t-test in the social interaction and exploration test, and Kruskal-Wallis ANOVA followed by the Mann-Whitney U-test (two-tailed) in the elevated plus-maze test. In each case the accepted level of significance was $P<0.05$. The ED_{50} values and their 95% confidence limits were determined by probit analyses.

Results

Radioligand binding to BZ and other neurotransmitter receptors

In membranes prepared from rat cerebral cortex, Y-23684 displaced [^{3}H]-diazepam in a concentration-dependent manner. Drug competition studies demonstrated that Y-23684 displayed a high affinity for the ^{3}H-labelled sites (K_i = 41 nM). Under the same experimental conditions, diazepam exhibited a higher affinity (K_i = 5.8 nM) than Y-23684. However, Y-23684 and diazepam did not displace appreciably the appropriate tracers labelling $GABA_A$ receptors, dopamine D_2 receptors, α_1-adrenoceptors, α_2-adrenoceptors, 5-HT_{1A} receptors, 5-HT_2 receptors nor muscarinic cholinoceptors even at a final concentration of 100 μM (Table 1).

Anticonvulsant potency of the test compounds

The administration of Y-23684 and diazepam dose-dependently blocked convulsions triggered by bicuculline, pentylenetetrazol and MES in rats and mice (Table 2). However, these seizures were blocked in a divergent manner by Y-23684. Tonic extensions induced by bicuculline were antagonized by Y-23684 with an ED_{50} of 1.3 mg kg^{-1} in rats and 1.2 mg kg^{-1} in mice, whereas those triggered by MES

Table 1 Affinity of Y-23684 and diazepam for various neurotransmitter receptors in rat brain

Receptor	*^{3}H-ligand*	*Index* (nM)	*Brain tissue*	*Y-23684*	*Diazepam*
Benzodiazepine	Diazepam	Ki	Cerebral cortex	41	5.8
$GABA_A$	Muscimol	IC_{50}	Cerebral cortex	>100000	>100000
Dopamine D_2	Spiperone	IC_{50}	Striatum	>100000	>100000
Noradrenaline α_1	Prazosin	IC_{50}	Cerebral cortex	>100000	>100000
Noradrenaline α_2	Clonidine	IC_{50}	Cerebral cortex	>100000	>100000
5-HT_{1A}	8-OH-DPAT[a]	IC_{50}	Hippocampus	>100000	>100000
5-HT_2	Spiperone	IC_{50}	Cerebral cortex	>100000	>100000
Muscarine	QNB[b]	IC_{50}	Cerebral cortex	>100000	>100000

[a]8-OH-DPAT: 8-hydroxy-2-(di-n-propylamino)tetraline.
[b]QNB: 3-quinuclidyl benzilate.

Table 2 Anticonvulsive potency of Y-23684 and diazepam against bicuculline-, pentylenetetrazol- and maximal electrical shock (MES)-induced seizures

		ED_{50} (95% confidence limits) mg kg^{-1}, p.o.	
Convulsants	*Animals*	*Y-23684*	*Diazepam*
Bicuculline	Rats	1.3 (0.7–2.1)	13.3 (9.9–18.0)
	Mice	1.2 (0.8–1.9)	0.4 (0.2–0.5)
Pentylenetetrazol	Rats	15.6 (11.4–21.0)	12.8 (8.8–19.2)
	Mice	11.7 (7.4–17.0)	1.1 (0.8–1.6)
MES	Mice	20.5 (13.6–29.8)	1.7 (1.2–2.3)

Y-23684 was administered orally 3 h and 1 h before the test in rats and mice, respectively. Diazepam was administered orally 1 h before the test in both species. Results are shown as the ED_{50} values and their 95% confidence limits to prevent each convulsive response determined by probit analysis with 7–21 animals per group.

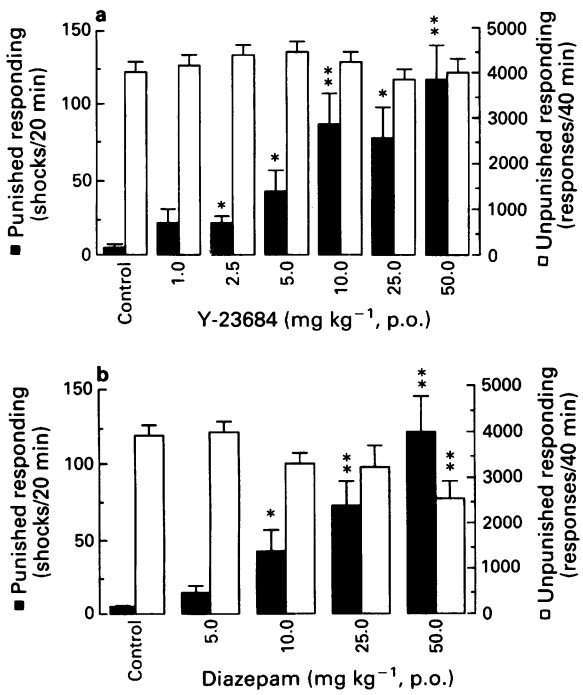


Figure 2 Effects of Y-23684 and diazepam in the rat Geller-Seifter test. Y-23684 and diazepam were administered orally 2.5 and 0.5 h before the test, respectively. Closed and open columns indicate punished and unpunished responding, respectively. Results are shown as the mean (with s.e.) of 10–11 animals per group. Significantly different from each control group $*P<0.05$; $**P<0.01$ (Aspin-Welch's t test).

were antagonized with an ED_{50} of 20.5 mg kg^{-1} in mice. Y-23684 also protected against lethality triggered by pentylenetetrazol with an ED_{50} of 15.6 mg kg^{-1} in rats and 11.7 mg kg^{-1} in mice. In contrast, the anticonvulsant profile of diazepam was characterized by nonselective antagonism of seizures triggered by these convulsants. In mice, convulsions induced by bicuculline, MES and pentylenetetrazol were antagonized with ED_{50}s of 0.4, 1.7 and 1.1 mg kg^{-1}, respectively. In rats, diazepam also blocked convulsions induced by bicuculline and pentylenetetrazol in a similar manner with ED_{50}s of 13.3 and 12.8 mg kg^{-1}, respectively.

Effects of the test compounds in the Geller-Seifter and water-lick conflict tests

In the Geller-Seifter test, Y-23684 and diazepam produced dose-dependent increases in punished responding, with significant effects occurring at 2.5 mg kg^{-1} for Y-23684 and 10 mg kg^{-1} for diazepam (Figure 2). Unpunished responding remained stable under Y-23684 (1–50 mg kg^{-1}), whereas diazepam at 50 mg kg^{-1} caused a large decrease in unpunished responding.

In the water-lick test, significant increases in licking were noted following the administration of Y-23684 (5–50 mg kg^{-1}) and diazepam (10 and 25 mg kg^{-1}) (Figure 3). However, the magnitude of the increase induced by Y-23684 was somehow less than that by diazepam.

Effects of the test compounds on social interaction behaviour

The administration of Y-23684 and diazepam dose-dependently increased social interaction with MEDs of 0.1 and 1.0 mg kg^{-1}, respectively (Figure 4). The effect of Y-23684 on social interaction was as efficacious as that of diazepam. There were no significant changes in locomotor activity throughout the dose-range of Y-23684, although diazepam at 25 mg kg^{-1}, (25 fold more than its MED on social interaction) produced an obvious sedative action that resulted in reduced interaction and line crossings.

Effects of the test compounds in the elevated plus-maze test

In the elevated plus-maze test, Y-23684 and diazepam increased the time spent on the end sections of open arms in a dose-related manner, with significant effects occurring at 0.05 mg kg^{-1} for Y-23684 and 0.5 mg kg^{-1} for diazepam (Figure 5).

Effects of the test compounds in the exploration (light/dark box) test

Y-23684 and diazepam produced an increase in exploratory behaviour (line crossings and exploratory rearings) in the light section of the test box accompanied by a decrease in exploratory behaviour in the dark section; also there was an increase in the amount of time spent in the light section (Figures 6 and 7). These changes attained significance for

three indices at 1.0 mg kg^{-1} of Y-23684 and 0.5 mg kg^{-1} of diazepam. However, at 10.0 mg kg^{-1} of diazepam, there appeared to be some evidence for the development of sedation indicated by a non-specific decrease in the behaviours measured, whereas Y-23684 did not show any similar signs up to 50 mg kg^{-1}.

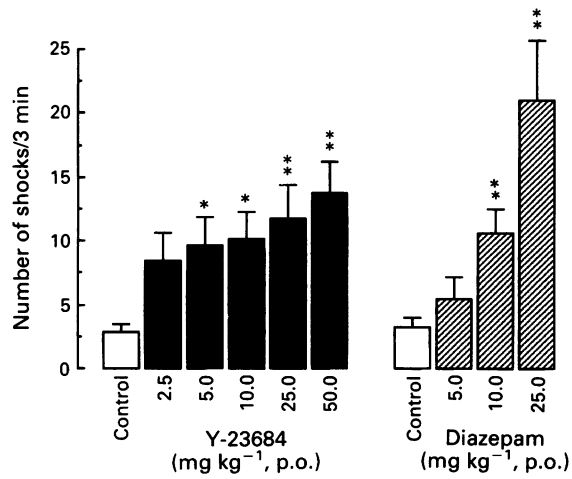


Figure 3 Effects of Y-23684 and diazepam in the water-lick conflict test. Y-23684 and diazepam were administered orally 3 and 1 h before the test, respectively. Results are shown as the mean with s.e. of 10–20 animals per group. Significantly different from each control group. *$P<0.05$; **$P<0.01$ (ANOVA followed by the LSD test).

Effects of the test compounds on rotarod performance and interaction with CNS depressants

Table 3 summarizes the potency of the test compounds in impairing rotarod performance and the loss of righting reflex after treatment with ethanol and hexobarbitone. In these tests, Y-23684 showed far less potent activity than diazepam in both rats and mice.

Discussion

In *in vitro* binding studies, Y-23684 showed a high and selective affinity for the BZR in the rat brain in spite of its non-BZ structure. Furthermore, it was reported previously that the potentiation of the GABA response by Y-23684 was less marked than that of conventional BZ-anxiolytics in frog sensory neurones *in vitro*, indicating its partial agonistic property on the BZR (Yakushiji *et al.*, 1990). The present paper describes the behavioural profile of Y-23684 in both various models of anxiety and in tests assessing adverse properties associated with classical BZs in comparison with those of diazepam.

The most frequently used paradigms for assessing the potential therapeutic efficacy of BZR agonists are anticonvulsant and anticonflict tests (Haefely *et al.*, 1990). In the seizure models in rodents presented here, the anticonvulsant profile of Y-23684 depended upon the convulsant: diazepam did not show such variation. Similar phenomena have been demonstrated previously with bretazenil and abecarnil which also possess partial agonistic properties on the BZR (Martin

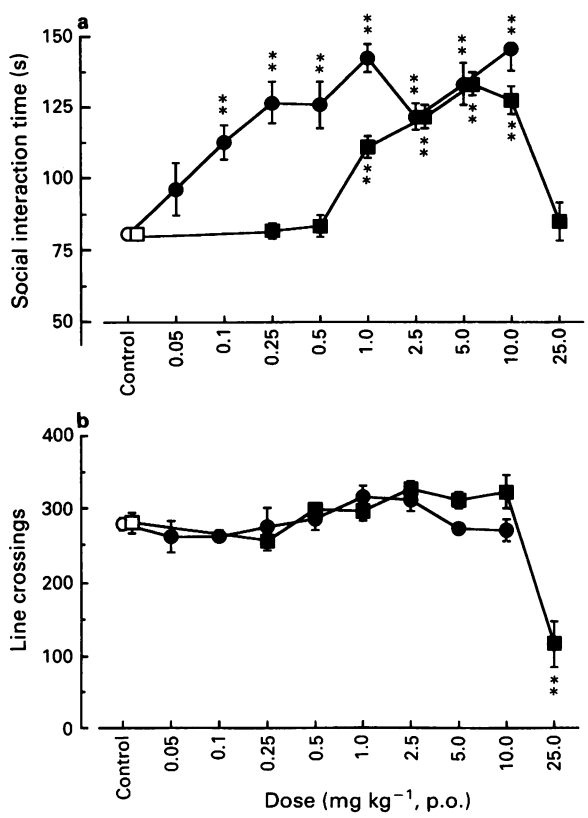


Figure 4 Effects of Y-23684 and diazepam on rat social interaction behaviour. Y-23684 and diazepam were administered orally 3 and 1 h before the test, respectively. Points indicate mean ± s.e. of 5–10 pairs of animals per group treated with Y-23684 (●) and diazepam (■), respectively. Time of social interaction behaviour (a) and the number of line crossings (b) were measured. Significantly different from each control group. *$P<0.05$; **$P<0.01$ (ANOVA followed by Dunnett's t test).

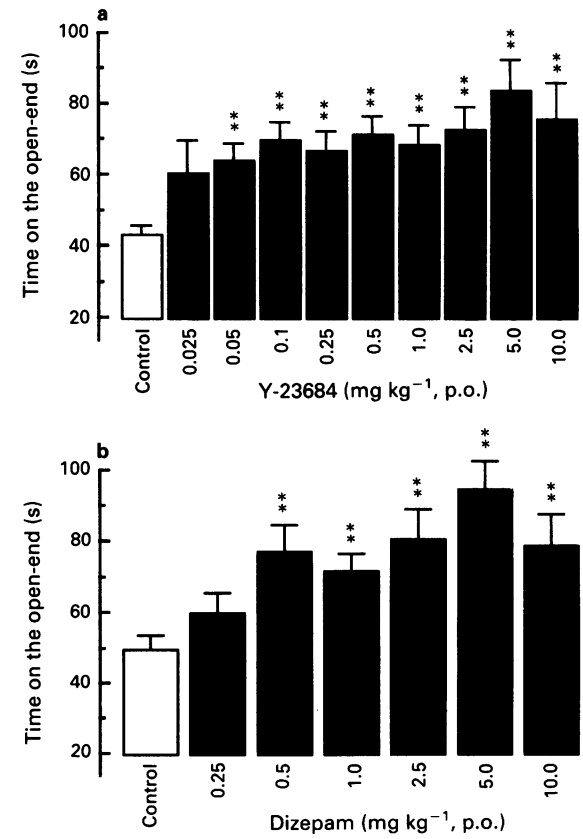


Figure 5 Effects of Y-23684 (a) and diazepam (b) in the elevated plus-maze test. The time on the open-end was measured. Y-23684 and diazepam were administered orally 3 and 1 h before the test, respectively. Results are shown as the mean with s.e. of 16–24 animals per group. Significantly different from each control group. *$P<0.05$; **$P<0.01$ (Kruskal-Wallis ANOVA followed by Mann-Whitney's U-test).

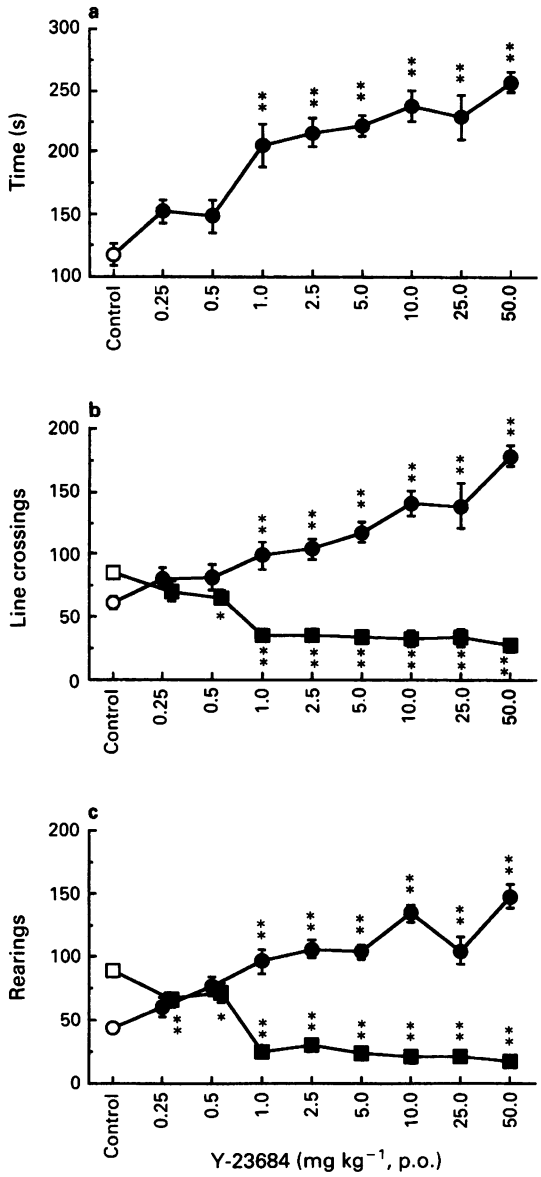


Figure 6 Effects of Y-23684 in the mouse exploration (light/dark box) test. Y-23684 was administered orally 1 h before the test. Values in the light (●) and dark (■) compartments are shown. Time spent in the light compartment (a), the numbers of line crossings (b) and the number of exploratory rearings (c) were measured. Results are shown as the mean ± s.e. of 8–24 animals per group. Significantly different from each control group. $*P<0.05$; $**P<0.01$ (ANOVA followed by Dunnett's t test).

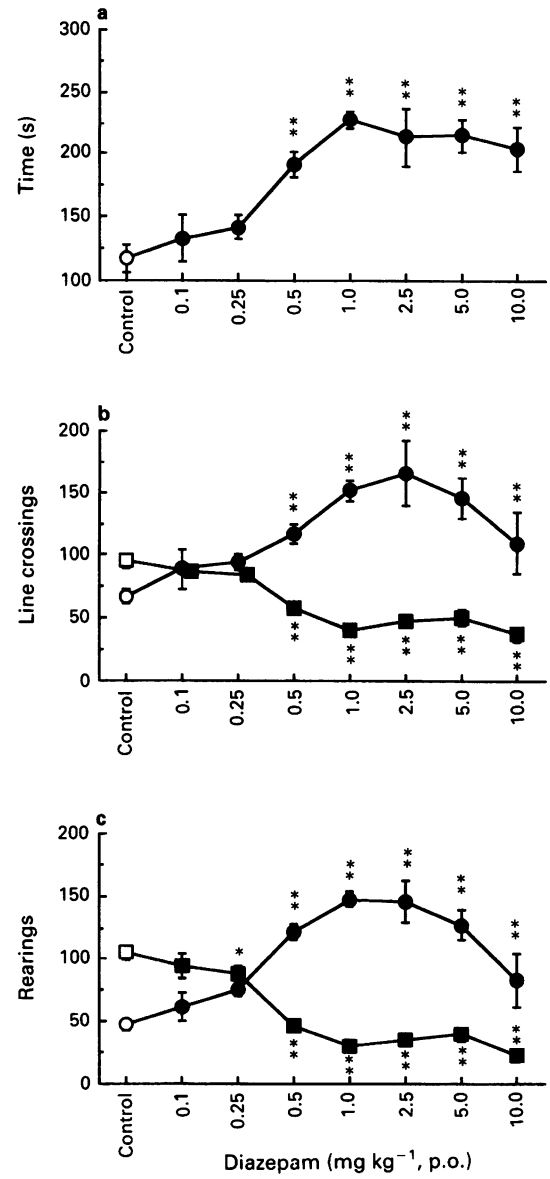


Figure 7 Effects of diazepam in the mouse exploration (light/dark box) test. Diazepam was administered orally 1 h before the test. Values in the light (●) and dark (■) compartments are indicated. Time spent in the light compartment (a), the numbers of line crossings (b) and the number of exploratory rearings (c) were measured. Results are shown as the mean ± s.e. of 8–16 animals per group. Significantly different from each control group: $*P<0.05$; $**P<0.01$ (ANOVA followed by Dunnett's t test).

et al., 1988; Turski *et al.*, 1990). The differences between Y-23684 and diazepam may account for the possibility that they and/or convulsants act on heterogenic domains of the BZR/GABA-chloride channel ionophore complexes in different ways. This possibility appears to be supported in part by previous findings that bicuculline is a $GABA_A$ receptor antagonist whilst pentylenetetrazol acts on the BZR and/or the *t*-butylbicyclophosphorothionate (TBPS) binding site on the complexes (Rehavi *et al.*, 1982; Bowery *et al.*, 1983; Ramanjaneyulu & Ticku, 1984). One might argue that the compounds show variations in the BZR occupancy in different brain areas mediating each convulsive response and/or its spreading. These inferences should be proved with biochemical and electrophysiological techniques.

Y-23684 showed antipunishment properties in the rat Geller-Seifter and water-lick conflict tests; it was effective at doses 2 to 4 times lower than diazepam. In contrast to diazepam, Y-23684 did not alter unpunished responding in the Geller-Seifter paradigm at any of the doses tested. On the other hand, it induced a consistent effect in the water-lick conflict test, but its activity was weaker than diazepam. The reason for this difference in activity between Y-23684 and diazepam is unclear. However, since these classical conflict paradigms were originally optimized for conventional BZ-anxiolytics (BZR full agonists), what is considered to be an 'antipunishment' activity may be composed of any combination of antipunishment, sedative, myorelaxant, amnesic or anticonvulsant elements (Singh *et al.*, 1991). In addition, as food or water-deprived rats were exposed to conflict situations involving rewards and punishment in these paradigms, motivational changes produced by the test compounds may interfere with detection of their antipunishment activity. Previous studies have demonstrated the ability of diazepam to increase food or water-intake under non-conflict situations

Table 3 Effect of Y-23684 and diazepam on rotarod performance and potentiation of ethanol- and hexobarbitone-induced loss of righting reflexes

Tests	Animals	ED_{50} (95% confidence limits) mg kg^{-1}, p.o. Y-23684	Diazepam
Rotarod	Rats	$\geqslant 100$ (5/10)[a]	8.8 (6.5–12.2)
	Mice	100.1 (68.6–147.8)	1.7 (1.2–2.6)
Ethanol potentiation	Rats	>250 (5/14)[a]	6.1 (3.9–10.0)
	Mice	$\geqslant 500$ (7/14)[a]	3.4 (2.3–4.6)
Hexobarbitone potentiation	Rats	$\geqslant 250$ (7/14)[a]	12.4 (8.7–20.0)
	Mice	80.3 (44.1–138.7)	2.6 (1.4–3.9)

Y-23684 was administered orally 3 h and 1 h before the test in rats and mice, respectively. Diazepam was administered orally 1 h before the test in both species. Results are shown as the ED_{50} values and their 95% confidence limits to impair rotarod performance and potentiate loss of the righting reflex after administration of ethanol or hexobarbitone determined by probit analysis with 7–14 animals per group. [a]If an ED_{50} could not been obtained throughout the tested doses, each result at the indicated maximum dose is shown as (the number of animals which showed positive effects/the number of animals used).

that leads to motivational changes (Johnson, 1978; Shimizu *et al.*, 1987). In contrast, we failed to detect any changes in free drinking with Y-23684 when measuring the volume of water consumed from a spout during a 3 min test session under a non-conflict situation in a pilot study (data not shown). Therefore, we conclude that Y-23684, which displays little hyperdipsic effect, should show a different profile from diazepam in the water-lick conflict test. This view is supported by the evidence obtained in other models of anxiety which did not involve water- or food-deprivation and electrical shocks as discussed below.

Recently, not only conventional conflict models but behavioural suppression induced by exposing animals to aversive environmental stimuli have been used extensively to assess the anxiolytic profile of known clinically active anxiolytic agents (Chopin & Briley, 1987; File, 1991). However, there have as yet, been few systematic comparisons of the anxiolytic actions of BZR full and partial agonists in these paradigms.

Firstly, Y-23684 and diazepam increased social interaction relative to controls under conditions of maximal environmental suppression (high light, unfamiliar condition) in the present study. There were no increases in locomotion in groups displaying enhanced social interaction, suggesting that the two measures are independent. This would rule out the possibility that the increased social interaction was the result of a direct stimulatory action of these compounds (Higgins *et al.*, 1988; 1992). Importantly, the increased social interaction induced by Y-23684 was maintained over a wide dose range (100 fold) without loss in efficacy and sedative action, highlighting a distinctive 'anxioselective' anxiolytic property of Y-23684.

Secondly, on the elevated maze used in the present paradigm, the tendency of rats, which had been housed under dim red lights, to explore their environments conflicted with the inherent tendency of these rats to avoid brightly lit and the end parts of the open arms. The present result is consistent with the idea that Y-23684 and diazepam possess anxiolytic activity during this aversive state.

Thus, in these two rat models of anxiety, Y-23684 was as efficacious and ten fold more potent than diazepam. Furthermore, it is important to mention that Y-23684 and diazepam displayed anxiolytic activity at lower doses in these paradigms than those in classical conflict models, Geller-Seifter and water-lick tests.

Finally, the measurement of changes in exploration of mice as measured in a light and dark test box would appear to offer a particularly simple model of anxiety (Crawley & Goodwin, 1980), the method being based on the observation that whilst rodents tend to explore a novel environment, open fields appear to have aversive properties which may inhibit their exploratory behaviour (Costall *et al.*, 1989a). As a consequence, Y-23684 and diazepam were shown to reinstate suppressed behaviours. However, Y-23684 did not cause any sedative effects in contrast to diazepam. Again in this study, the magnitude of the anxiolytic effect of Y-23684 was not less than that of diazepam. The fact that diazepam showed a more potent anxiolytic action than Y-23684 in this paradigm can be explained by an *ex vivo* [^{3}H]-diazepam binding study in mice. The inhibition of specific binding by orally administered diazepam was more potent than that by Y-23684, whereas this trend was reversed in rats (unpublished observations).

This 'anxioselective' characteristic of Y-23684 is supported in tests assessing unwanted side-effects. Impairment in the rotarod test and potentiation of CNS depressants by Y-23684 were 11–147 times weaker than diazepam. This shows a marked separation of the anxiolytic and undesired side-effects of Y-23684 in contrast to diazepam.

An important mechanistic explanation of the anxioselective profile of Y-23684 comes from a recent finding in an electrophysiological study with mammalian CNS neurones (Yakushiji *et al.*, 1993). In that study, each BZR subtype-density contributed little to efficacy of the augmentation of the GABA-induced chloride currents by Y-23684 in acutely dissociated rat cerebeller Purkinje (CPJ) neurones (BZ_1 subtype predominant) and spinal ventral horn (SVH) neurones (BZ_2 subtype predominant), whereas the augmentation by diazepam depended upon the BZ_2 receptor-density. Indeed, the augmentation of the GABA response by Y-23684 was observed to be as efficacious in CPJ neurones but to be less in SVH neurones than diazepam which displayed the more marked enhancement in the latter neurones. This characterization relates to lower intrinsic activities of Y-23684 than diazepam, a conventional BZR full agonist, at the BZ_2 receptors even at their highly 'reserved' phase (Hoyer & Boddeke, 1993). Although the functional specificity of BZR multiplicity is unsettled, one possible way of distinguishing the anxioselective profile of Y-23684 from diazepam arises from the difference in intrinsic activities at the BZ_2 sites.

In conclusion, the present studies demonstrate that Y-23684, a BZR partial agonist, produced potent anxiolytic effects in several rodent models of anxiety. Furthermore, Y-23684 was shown to improve the side-effect profile seen in a conventional BZ, diazepam. Therefore, Y-23684 is predicted to be a potent anxioselective anxiolytic agent that exhibits considerable advantages over conventional BZs in man.

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References

BEAUMONT, K., CHILTON, W.S., YAMAMURA, H.I. & ENNA, S.J. (1978). Muscimol binding in rat brain: association with synaptic GABA receptor. *Brain Res.*, **148**, 153–162.

BOWERY, N.G., HILL, D.R. & HUDSON, A.L. (1983). Characteristics of $GABA_B$ receptor binding sites on rat whole brain synaptic membranes. *Br. J. Pharmacol.*, **78**, 191–206.

CHOPIN, P. & BRILEY, M. (1987). Animal models of anxiety: the effect of compounds that modify 5-HT neurotransmission. *Trends Pharmacol. Sci.*, **8**, 383–388.

COSTALL, B., JONES, B.J., KELLY, M.E., NAYLOR, R.J. & TOMKINS, D.M. (1989a). Exploration of mice in a black and white test box: validation as a model of anxiety. *Pharmacol. Biochem. Behav.*, **32**, 777–785.

COSTALL, B., KELLY, M.E. & TOMKINS, D.M. (1989b). Use of the elevated plus maze to assess anxiolytic potential in the rat. *Br. J. Pharmacol.*, **96**, 312P.

CRAWLEY, J.N. & GOODWYN, F.K. (1980). Preliminary report of a simple animal behaviour model for the anxiolytic effects of benzodiazepines. *Pharmacol. Biochem. Behav.*, **13**, 167–170.

CREESE, L., SCHNEIDER, R. & SNYDER, S.H. (1977). ^{3}H-Spiroperidol labels dopamine receptors in pituitary and brain. *Eur. J. Pharmacol.*, **46**, 377–381.

DEPOORTERE, H., ZIVKOVIC, B., LLOYD, K.G., SANGER, D.J., PERRAULT, G., LANGER, S.Z. & BARTHOLINI, G. (1986). Zolpidem, a novel nonbenzodiazepine hypnotic. I. Neuropharmacological and behavioral effects. *J. Pharmacol. Exp. Ther.*, **237**, 649–658.

DOBLE, A. & MARTIN, I.L. (1992). Multiple benzodiazepine receptors: no reason for anxiety. *Trends Pharmacol. Sci.*, **13**, 76–81.

FILE, S.E. (1980). The use of social interaction as a method for detecting anxiolytic activity of chlordiazepoxide-like drugs. *J. Neurosci. Methods*, **2**, 219–238.

FILE, S.E. (1991). Animal models of anxiety. In *Biological Psychiatry*, Vol. 2 ed. Racagni, G., Brunello, N. & Fukuda, T. pp. 596–599. Amsterdam: Elsevier Science Publication B.V.

GELLER, I. & SEIFTER, J. (1960). The effect of meprobamate, barbiturates, d-amphetamine and promazine on experimentally induced conflict in the rat. *Psychopharmacologia (Berlin)*, **1**, 482–492.

GREENGRASS, P. & BREMNER, R. (1979). Binding characteristics of ^{3}H-prazosin to rat brain α-adrenergic receptors. *Eur. J. Pharmacol.*, **55**, 323–326.

HAEFELY, W.E., MARTIN, J.R. & SCHOCH, P. (1990). Novel anxiolytics that act as partial agonist at benzodiazepine receptors. *Trends Pharmacol. Sci.*, **11**, 452–456.

HALL, M.D., MESTIKAWY, S.E., EMERRIT, M.B., PICHAT, L., HAMON, M. & GOZLAN, H. (1985). [^{3}H]-hydroxy-2-(di-n-propylamino)tetralin binding to pre- and postsynaptic 5-hydroxytryptamine sites in various regions of the rat brain. *J. Neurochem.*, **44**, 1685–1696.

HANDLEY, S.L. & MITHANI, S. (1984). Effects of alpha-adrenoceptor agonists and antagonists in a maze-exploration model of 'fear'-motivated behaviour. *Naunyn-Schmied. Arch. Pharmacol.*, **327**, 1–5.

HIGGINS, G.A., BRADBURY, A.J, JONES, B.J. & OAKLEY, N.R. (1988). Behavioural and biochemical consequences following activation of 5-HT$_1$-like and GABA receptors in the dorsal raphe nucleus of the rat. *Neuropharmacol.*, **277**, 993–1001.

HIGGINS, G.A., JONES, B.J. & OAKLEY, N.R. (1992). Effect of 5-HT$_{1A}$ receptor agonists in two models of anxiety after dorsal raphe injection. *Psychopharmacol.*, **106**, 261–267.

HOYER, D. & BODDEKE, H.W.G.M. (1993). Partial agonists, full agonists, antagonists: dilemmas of definition. *Trends Pharmacol. Sci.*, **14**, 270–275.

JENCK, F., MOREAU, J.-L., BONETTI, E.P., MARTIN, J.R. & HAEFELY, W.E. (1992). Ro19-8022, a nonbenzodiazepine partial agonist at benzodiazepine receptors: neuropharmacological profile of a potential anxiolytic. *J. Pharmacol. Exp. Ther.*, **262**, 1121–1127.

JOHNSON, D.N. (1978). Effect of diazepam on food consumption in rats. *Psychopharmacologia (Berlin)*, **56**, 111–112.

LIPPA, A.S. & CRICHETT, D.J. (1979). Benzodiazepine receptors. *Pharmacol. Biochem. Behav.*, **10**, 831–843.

LIPPA, A.S. & REGAN, B. (1977). Additional studies on the importance of glycine and GABA in mediating the actions of benzodiazepines. *Life Sci.*, **21**, 1779–1784.

MARTIN, J.R., PIERI, L., BONETTI, E.P., SHAFFNER, R., BURKARD, W.P., CUMIN, R. & HAEFELY, W.E. (1988). Ro16-6028: a novel anxiolytic acting as a partial agonist at the benzodiazepine receptor. *Pharmacopsychiat.*, **21**, 360–362.

MEERT, T.F. & JANSSEN, P.A.J. (1989). Psychopharmacology of ritanserin: comparison with chlordiazepoxide. *Drug. Develop. Res.*, **18**, 119–144.

MÖHLER, H. & OKADA, T. (1977). Properties of ^{3}H-diazepam binding to benzodiazepine receptors in rat cerebral cortex. *Life Sci.*, **20**, 2101–2110.

MÜLLER, W.E. (1988). New trends in benzodiazepine research. *Drugs Today*, **24**, 649–663.

NAKAO, T., OBATA, M., KAWAKAMI, M., MARUBAYASHI, N., MORITA, K. & TAHARA, T. (1990). Selective oxidation of 2-(4-chlorophenyl)-4a,5-dihydro-2H-(1)benzothieopyrano[4,3-c]pyridazin-3(4H)-one to sulfoxide. *Chem. Pharmacol. Bull.*, **38**, 3182–3183.

NAKAO, T., OBATA, M., KAWAKAMI, M., MORITA, K., TANAKA, H., MORIMOTO, Y., TAKEHARA, S., YAKUSHIJI, T. & TAHARA, T. (1991a). Studies on the synthesis of condensed pyridazine derivatives. IV. Synthesis and anxiolytic activity of 2-aryl-5,6-dihydro-(1)benzothiepino[5,4-c]pyridazin-3(2H)-ones and related compounds. *Chem. Pharmacol. Bull.*, **39**, 2556–2563.

NAKAO, T., OBATA, M., YAMAGUCHI, Y., MARUBAYASHI, N., IKEDA, K. & MORIMOTO, Y. (1992). Synthesis and biological activities of optical isomers of 2-(4-chlorophenyl)-5,6-dihydro-(1)benzothiepino[5,4-c]pyridazin-3(2H)-one 7 oxide. *Chem. Pharmacol. Bull.*, **40**, 117–121.

NAKAO, T., OBATA, M., YAMAGUCHI, Y. & TAHARA, T. (1991b). Synthesis of 2-aryl-5,6-dihydro-(1)benzothiepino[5,4-c]pyridazin-3(2H)one by a novel dehydrogenation reaction. *Chem. Pharmacol. Bull.*, **39**, 524–526.

PELLOW, S., CHOPIN, P., FILE, S.E. & BRILEY, M. (1985). Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J. Neurosci. Methods*, **14**, 149–167.

PEROUTKA, S.J. & SNYDER, S.H. (1979). Multiple serotonin receptors: differential binding of [^{3}H]5-hydroxytryptamine, [^{3}H]lysergic acid dietylamide and [^{3}H]spiroperidol. *Mol. Pharmacol.*, **16**, 687–699.

PETERSEN, E.N. (1987). Benzodiazepine receptor pharmacology: new vistas. *Drugs Future*, **12**, 1043–1053.

RAMANJANEYULU, R. & TICKU, M.K. (1984). Interactions of pentamethylenetetrazole and trazole analogues with the picrotoxin site of the benzodiazepine-GABA receptor-ionophore complex. *Eur. J. Pharmacol.*, **98**, 337–345.

RASTOGI, S.K. & TICKU, M.K. (1986). Anticonvulsant profile of drugs which facilitate gabanergic transmission on convulsions mediated by a gabanergic mechanism. *Neuropharmacol.*, **25**, 175–185.

REHAVI, M., SKOLNICK, P. & PAUL, S.M. (1982). Effects of tetrazole derivatives on [^{3}H]diazepam binding in vitro: correlation with anticonvulsant potency. *Eur. J. Pharmacol.*, **78**, 353–356.

SHIMIZU, H., HIROSE, A., TATSUNO, T., NAKAMURA, M. & KATSUBE, J. (1987). Pharmacological properties of SM-3997: a new anxioselective anxiolytic candidate. *Jpn. J. Pharmacol.*, **45**, 493–500.

SINGH, L., FIELD, M.J., HUGHES, J., MENZIES, R., OLES, R.J., VASS, C.A. & WOODRUFF, G.N. (1991). The behavioural properties of CI-988, a selective choecystokinin$_B$ receptor antagonist. *Br. J Pharmacol.*, **104**, 239–245.

SQUIRES, R.F. & BRAESTRUP, C. (1977). Benzodiazepine receptors in rat brain. *Nature*, **266**, 732–734.

STEPHENS, D.N., SCHNEIDER, H.H., KEHR, W., ANDREWS, J.S., RETTIG, K.-J., TURSKI, L., SCHMIECHEN, R., TURNER, J.D., JENSEN, L.H., PETERSEN, E.N., HONORE, T. & BOND HANSEN, J. (1990). Abecarnil, a metabolically stable, anxioselective β-carboline acting at benzodiazepine receptors. *J. Pharmacol. Exp. Ther.*, **253**, 334–343.

TSUMAGARI, T., NAKAJIMA, A., FUKUDA, T., SHUTO, S., KENJO, T., MORIMOTO, Y. & TAKIGAWA, Y. (1978). Pharmacological properties of 6-(o-chlorophenyl)-8-ethyl-1-methyl-4H-s-triazolo-[3,4-c]thieno[2,3-e][1,4] diazepine (Y-7131), a new anti-anxiety drug. *Arzneim. Forsch.*, **28**, 1158–1164.

TURSKI, L., STEPHENS, D.N., JENSEN, L.H., PETERSEN, E.N., MELDRUM, B.S., PATEL, S., BONDO HANSEN, J., LOSCHER, W., SCHNEIDER, H.H. & SCHMIECHEN, R. (1990). Anticonvulsant action of β-carboline abecarnil: studies in rodents and baboon, *Papio papio*. *J. Pharmacol. Exp. Ther.*, **253**, 344–352.

U'PRICHARD, D.C., BECHTEL, W.D., ROUOT, B.M. & SNYDER, S.H. (1979). Multiple apparent alpha-noradrenergic receptor binding sites in rat brain: effect of 6-hydroxydopamine. *Mol. Pharmacol.*, **16**, 47–60.

VOGEL, J.R., BEER, B. & CLODY, D.C. (1971). A simple and reliable conflict procedure for testing anti-anxiety agents. *Psychopharmacologia (Berlin)*, **21**, 1–12.

WELCH, B.L. (1949). Further note on Mrs Aspin's tables and on certain approximations to the tabled function. *Biometrika*, **36**, 293–296.

YAKUSHIJI, T., MORIMOTO, Y., FUKUDA, T., SETOGUCHI, M. & AKAIKE, N. (1990). Characteristics of augmentation of Y-23684 on the GABA-induced response to frog isolated sensory neurones. *17th C.I.N.P. Congress in Kyoto.*

YAKUSHIJI, T., SHIRASAKI, T., MUNAKATA, M., HIRATA, A. & AKAIKE, N. (1993). Differential properties of type I and type II benzodiazepine receptors in mammalian CNS neurones. *Br. J. Pharmacol.*, **109**, 819–825.

YAMAMURA, H.I. & SNYDER, S.H. (1974). Muscarinic cholinergic receptor binding in the longitudinal muscle of guinea-pig ileum with [^{3}H]quinuclidyl benzilate. *Mol. Pharmacol.*, **10**, 861–867.

ZIVKOVIC, B., MOREL, E., JOLY, D., PERRAULT, G., SANGER, D.J. & LLOYD, K.G. (1990). Pharmacological and behavioral profile of alpidem as an anxiolytic. *Pharmacopsychiat.*, **23**, 108–113.

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IgE-receptor activated chloride uptake in relation to histamine secretion from rat mast cells

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1 Antigen-stimulated histamine secretion from rat peritoneal mast cells was inhibited when extracellular chloride was replaced by either isethionate or gluconate anions, but the histamine release still remained quite substantial.

2 Rat peritoneal mast cells take up ^{36}Cl and the uptake reaches a steady state after 60 min incubation with the isotope. At steady state, the intracellular chloride level in the cells was calculated to be 29 ± 11.5 mM.

3 The chloride uptake in mast cells was exponential with a rate constant of 0.036 min^{-1} in resting cells. When the cells were stimulated with antigen, and rate constant for chloride uptake increased to 0.90 min^{-1}: an increase of 25 fold. Under identical experimental conditions histamine release increased 3 fold.

4 The rate of chloride uptake in either resting cells or in antigen-stimulated cells was not changed when the extracellular medium was nominally calcium-free but histamine release was almost completely inhibited in the absence of extracellular calcium.

5 The putative chloride channel blocker DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid) 0.3 to 30 μM, produced a concentration-related inhibition of antigen-stimulated histamine secretion but DIDS (30 μM) did not inhibit the antigen-stimulated increase of chloride uptake.

6 The cyclic AMP analogue, dibutyryl cyclic AMP (1 mM) produced a delayed increase in chloride uptake in resting mast cells but neither dibutyryl cyclic AMP nor 8-bromo cyclic AMP *per se* induced any histamine secretion.

7 Ouabain (1 mM) which inhibits the Na^+/K^+ ATPase in rat peritoneal mast cells, failed to affect the uptake of chloride in resting mast cells.

8 The Na/K/2Cl-cotransport inhibitor, furosemide (0.7 mM), slowed the unstimulated chloride uptake in resting mast cells and abolished the increased antigen-induced chloride uptake when added together with antigen. In contrast, spontaneous and antigen-induced histamine release were unaffected by the presence of furosemide. However, when furosemide was added to the cell suspension 5 min before stimulation, furosemide was without effect on the antigen-induced chloride uptake.

9 In addition to the chloride uptake mediated by chloride channels which may be related to the mechanism of histamine secretion, crosslinking of the high affinity membrane receptors for IgE is followed by a fast chloride uptake that is likely to occur through a furosemide-sensitive Na/K/2Cl-cotransporter.

Keywords: Mast cells; chloride transport; histamine secretion; IgE receptors

Introduction

Secretion from mast cells stimulated by crosslinking of the high affinity membrane receptors for IgE is dependent on extracellular calcium, although a small part of the secretion still occurs when extracellular calcium is removed (Foreman & Mongar, 1972). In addition, elevation of intracellular calcium is capable of activating the secretory process of these cells (Foreman *et al.*, 1973). Further, the rise in intracellular calcium in rat mast cells and rat basophilic leukaemia cells which follows crosslinking of the high affinity receptor for IgE is largely dependent upon extracellular calcium (Beaven *et al.*, 1984; White *et al.*, 1984). The evidence would suggest an important role for extracellular calcium in secretion from mast cells or rat basophilic leukaemia cells in response to crosslinking of the high affinity receptor for IgE. Early attempts to identify the putative calcium channel opened by crosslinking IgE receptors failed (Lindau & Fernandez, 1986).

This apparent paradox concerning the role of calcium has recently been resolved by the discovery of a membrane calcium current in the mast cell which is activated by release of calcium from intracellular stores (Hoth & Penner, 1992).

Previous studies in mast cells by Matthews and coworkers (1989a,b) have revealed a stimulus-induced, delayed chloride current which may also be activated by adenosine 3':5'-cyclic-monophosphate (cyclic AMP) or GTP-γ-S. This chloride current was large and would tend to drive the mast cell membrane potential towards E_{Cl} (Matthews *et al.*, 1989b). The authors assume that E_{Cl} in the mast cell is about -35 mV and that the unstimulated membrane potential is near zero mV, whereby the membrane potential would be held in the range where there is a hyperpolarization-driven calcium influx (Matthews *et al.*, 1989a,b). However, the studies on the chloride conductance did not measure secretion and the stimuli applied to the cells (compound 48/80 and substance P) are known to cause secretion which is independent of extracellular calcium (Foreman & Mongar, 1973; Sagi-Eisenberg *et al.*, 1985).

The aim of the study was to examine the role of extracellular chloride in histamine secretion stimulated by the crosslinking of high affinity IgE receptors and to examine the effect of such stimulation on the membrane permeability to chloride.

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Methods

Isolation of mast cells

Male Sprague-Dawley (specific pathogen-free) rats weighing 250 to 420 g, were used for experiments on unstimulated cells, whereas male Wistar (specific pathogen-free) rats weighing 300 to 420 g, were used for experiments with antigen- or anti-IgE-stimulated cells. Rats were killed by bleeding from the carotid arteries after asphyxiation in CO_2.

Mixed peritoneal cells were collected by injecting through a small incision in the abdominal wall a physiological salt solution buffered with 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid). The cell suspension was removed from the peritoneal cavity and kept at 4°C. The remaining part of the cell isolation was carried out at 4°C. After centrifugation at 220 *g* for 10 min, the supernatant was discarded and the cells were resuspended in a small volume (200–300 µl) of physiological salt solution and transferred to an isotonic solution of Percoll (SG = 1.017) buffered with 20 mM HEPES. A continuous gradient of Percoll was created by centrifugation at 10,000 *g* for 20 min. The mast cells were concentrated in the lower third of the gradient, whereas the peritoneal leucocytes and macrophages were located in the upper third. The mast cells were harvested and washed three times to remove the remaining Percoll. The cells were then suspended in physiological salt solution containing bovine serum albumin, 1 mg ml^{-1}, and glucose, 1 mg ml^{-1}, pH 7.4 at room temperature.

A Coulter counter was used to count the number of cells and inspection of a stained (toluidine blue) smear was used to determine the fraction of mast cells in the cell suspension (97.9% ± 0.5%, $n = 35$).

Sensitization of mast cells

Rats were sensitized by injection of 1 ml pertussis vaccine and 0.25 ml of a solution of ovalbumin (20 mg ml^{-1}) the first day, reinjected the following day with 0.5 ml incomplete Freunds adjuvant and 0.25 ml ovalbumin solution (20 mg ml^{-1}), and the third day, injected with 0.25 ml ovalbumin solution (20 mg ml^{-1}) alone. The sensitized rats were used 3 to 4 weeks after the sensitization was completed. Sensitized mast cells were stimulated with ovalbumin, 1 mg ml^{-1}. This rather high concentration was chosen based on dose-response experiments in the *absence* of phosphatidylserine.

Incubation procedure

Mast cells pooled from 5 rats were resuspended to a cell density of 0.8 to 3.4×10^6 cells ml^{-1}, depending on the experimental protocol. The cells were equilibrated at 37°C for 60 min in either calcium-containing or calcium-free physiological salt solutions. In some experiments the cells were preincubated with ouabain or 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) for 10 min or furosemide for 5 min before ^{36}Cl was added.

Measurement of ^{36}Cl uptake

Triplicate samples of cells were incubated in a medium containing the normal concentration of chloride, 143 mM (see below), in addition to the radioactive tracer. The specific activity of ^{36}Cl was 17.8 kBq µmol^{-1}, and the concentration was between 7.2 to 8.3 mM, leading to a total chloride concentration of 150.2 to 151.3 mM in the medium. The incubation of the cells with ^{36}Cl lasted for 1 to 120 min and was performed at 37°C. The incubation was terminated by transferring 100 µl of cell suspension to an Eppendorf tube containing 100 µl of silicone oil (37°C), followed by centrifugation at 16,000 *g* for 25 s at room temperature. The tubes were frozen at − 50°C, and the tip containing the cell pellet was cut off and placed into a tube with 100 µl of NaOH 48 mM, mixed vigorously for 1 min, and then 1.8 ml Ecoscint scintillation counting medium were added before mixing and counting. The samples were counted in a liquid scintillation spectrometer using the preset window for ^{32}P. The counting efficiency was greater than 90%. For the calculation of the cellular uptake of ^{36}Cl, the counts for each sample were corrected for space, which amounted to 128 ± 33 nl/10^6 cells. This space value was determined with the extracellular markers ^{14}C-inulin and $^{35}SO_4$ and provide a reliable estimate of ^{36}Cl associated with the cell pellet which is not uptake into the cells but reflects ^{36}Cl trapped in the extracellular space. Based upon efficiency determinations, c.p.m. were converted into d.p.m. and the specific activity of chloride in the extracellular medium was used to calculate the cellular uptake of ^{36}Cl in nmol/10^6 cells.

Measurement of histamine release

Mast cells, in a final volume of 0.5 ml, were incubated in duplicate at 37°C for 10 min with secretagogues (and without: spontaneous release) under the same experimental conditions as described for the associated ^{36}Cl uptake experiments. The incubation was terminated by the addition of 1.5 ml of ice-cold physiological salt solution to the samples. After centrifugation at 220 *g* for 10 min at 4°C, the supernatants were collected for determination of the amount of histamine released. The cell pellets were suspended in 250 µl of a physiological salt solution and boiled for 3 min. After addition of 1.75 ml of physiological salt solution and centrifugation the supernatant was collected for determination of the residual histamine. This was performed by the fluorometric method (Shore *et al.*, 1959) omitting the extraction procedure. The histamine release was expressed as a percentage of the total histamine content of the cells. The anti-IgE- and antigen-induced histamine release were adjusted for the spontaneous histamine release.

Solutions

The physiological salt solution used in these experiments had the following composition in mM: NaCl 137, KCl 4, $MgSO_4$ 1, $CaCl_2$ 1, HEPES 20. The solution contained bovine serum albumin, 1 mg ml^{-1}, and glucose, 1 mg ml^{-1}. Changes in the concentrations of calcium in the physiological salt solution were balanced by changes in the concentration of NaCl in order to maintain isotonicity. The pH of the solution was adjusted to 7.4. In those experiments in which extracellular chloride concentration was varied, sodium chloride was substituted with equimolar amounts of either sodium isethionate or sodium gluconate. The activity of Ca^{2+} in solutions containing isethionate or gluconate was checked by means of a calcium electrode and in the case of solutions containing gluconate, the total calcium concentration was adjusted to give the desired calcium ion activity by adding small volumes of calcium chloride solution, 1 M.

Data presentation

The data are presented as means ± s.e.mean.

Materials

Bovine serum albumin, HEPES, 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid disodium salt (DIDS), sodium D-gluconate, furosemide and ouabain were supplied by Sigma Chemical Company, St. Louis, U.S.A.; sodium isethionate by Fluka, Gillingham, Dorset, U.K.; Percoll by Pharmacia Fine Chemicals, Uppsala, Sweden; silicone oil (AR 200/50 Wacker, density 1.04) by Serva, Heidelberg, Germany. ^{36}Cl was obtained from Amersham, Buckinghamshire, U.K., and the scintillation solution, Ecoscint, was from BN Plastic, Helsinge, Denmark. Pertussis vaccine and Freunds adjuvant were obtained from Statens Serum Institut, Copenhagen, Denmark. All other chemicals were of analytical grade.

Results

Relationship between extracellular chloride and histamine release

The removal of extracellular chloride inhibited antigen-stimulated histamine secretion. The extracellular calcium concentration-effect relationship was shifted to the right and the maximum depressed when chloride was replaced by isethionate or gluconate (Figure 1).

Chloride uptake

Figure 2a shows that in a physiological solution containing calcium, resting mast cells took up chloride. The rate of chloride uptake was not changed when the extracellular medium was nominally calcium-free. Equilibration of intracellular and extracellular ^{36}Cl was 50% complete in about 15 min and complete after 60 min. In six different and unrelated experiments, incubation of mast cells with ^{36}Cl lasted for 120 min, during which the steady state value remained constant (data not shown). The specific activity of intracellular ^{36}Cl at steady state gives an intracellular chloride concentration of 29 ± 11.5 mM ($n = 9$). In resting mast cells, spontaneous histamine release in the presence of extracellular calcium was $5.3 \pm 0.9\%$ ($n = 4$) and in the absence of calcium it was $6.4 \pm 3.6\%$ ($n = 4$).

Stimulation of sensitized rat mast cells with antigen (ovalbumin, 1 mg ml^{-1}) in the presence of calcium greatly increased the initial rate of chloride uptake, and again, this was not changed when the extracellular medium was made nominally calcium-free (Figure 2a). In these experiments, antigen and ^{36}Cl were added simultaneously to cells suspended in physiological salt solution which contained calcium (1 mM) or was nominally calcium-free. The histamine release after stimulation with antigen amounted to $17 \pm 1\%$ in the presence of calcium ($n = 4$) and $8.3 \pm 2.5\%$ in its absence ($n = 4$) after 10 min of incubation after which histamine release is complete (Johansen & Chakravarty, 1975).

The data in Figure 2a were fitted to the exponential $y = a(1\text{-}e^{-kt})$ using an iterative method. In calcium-containing media, the estimated rate constants for chloride uptake were 0.036 ± 0.015 min^{-1} in resting cells and 0.90 ± 0.1 min^{-1} in antigen-stimulated mast cells. Thus, antigen stimulation of the mast cells produced a 25 fold increase in chloride uptake. Estimates of a, the maximum value for chloride uptake, were 45 ± 15 nmol/10^6 cells in resting cells and 38 ± 1 nmol/10^6 cells in antigen-stimulated cells.

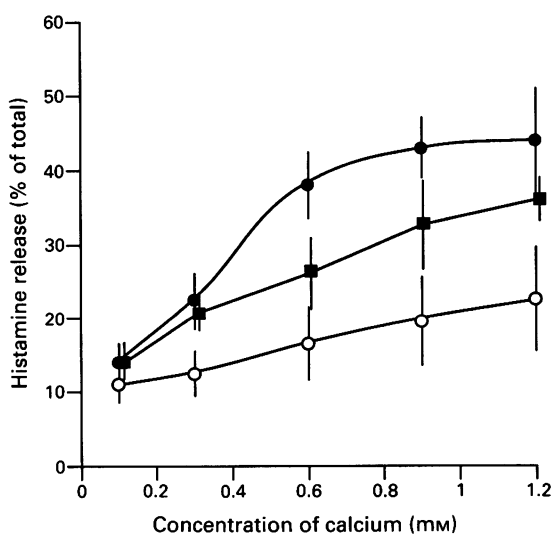


Figure 1 Concentration-response relationships for calcium and histamine secretion from rat mast cells stimulated by anti-IgE. Normal physiological salt solution (●); physiological salt solution in which sodium chloride was replaced by sodium isethionate (○) or gluconate (■). Each point is the mean ± s.e.mean from 6 experiments.

The action of dibutyryl cyclic AMP on ^{36}Cl uptake

Since previous studies (Matthews *et al.*, 1989b) have shown that the chloride conductance of mast cells may be activated by intracellular cyclic AMP, the effect of membrane-permeable analogues of cyclic AMP, applied extracellularly, was studied. Dibutyryl cyclic AMP, 0.1 μM to 3 mM, did not induce any histamine secretion above 3% of total. The range of histamine release in the dose-response experiments was 1.0–2.2% ($n = 33$). Similar data were obtained with the cyclic AMP analogue, 8-bromo cyclic AMP (range 0.8–3.6%, $n = 33$). Addition of dibutyryl cyclic AMP, 1 mM did not increase the rate of ^{36}Cl uptake. The rate constant in the absence of cyclic AMP was 0.084 ± 0.006 min^{-1} and in the presence of dibutyryl cyclic AMP (1 mM) was 0.034 ± 0.003 min^{-1}. However, the estimated maximum chloride uptake was increased from 31 ± 1 nmol/10^6 cell min^{-1} in the absence of dibutyryl cyclic AMP to 64 ± 4 nmol/10^6 cells min^{-1} in the presence of dibutyryl cyclic AMP.

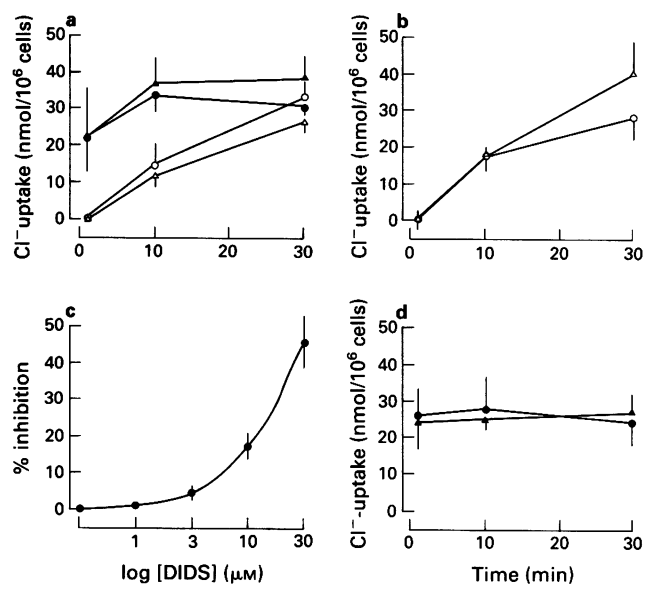


Figure 2 (a) Time course of chloride uptake by rat mast cells. Unstimulated cells in the absence of extracellular calcium (○), unstimulated cells in the presence of extracellular calcium, 1 mM (△); cells stimulated with ovalbumin, 1 mg ml^{-1} in the absence of extracellular calcium (●); cells stimulated with ovalbumin, 1 mg ml^{-1} in the presence of extracellular calcium, 1 mM (▲). Each point is the mean ± s.e.mean from 4 experiments. Resting cells (open symbols) were obtained from non-sensitized animals, whereas antigen-stimulated cells (closed symbols) were obtained from sensitized animals. (b) Effect of dibutyryl cyclic AMP on the rate of chloride uptake by rat mast cells. Unstimulated cells in the absence of dibutyryl cyclic AMP (○); unstimulated cells in the presence of dibutyryl cyclic AMP, 1 mM (△). Each point is the mean ± s.e.mean from 3 experiments. (c) Concentration-response curve for the inhibition of anti-IgE-stimulated histamine release by 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS). Cells were preincubated with DIDS for 10 min at 37°C before stimulation with anti-IgE. Each point is the mean ± s.e.mean from 6 experiments and in these experiments the anti-IgE-stimulated histamine release in the absence of DIDS was 55.5 ± 4.7%. (d) Effect of DIDS on the rate of chloride uptake by sensitized rat mast cells. Cells stimulated with ovalbumin, 1 mg ml^{-1} in the absence of DIDS (●); cells stimulated with ovalbumin, 1 mg ml^{-1} in the presence of DIDS, 30 μM (▲). Each point is the mean ± s.e.mean from 4 experiments. The cells were preincubated with DIDS for 10 min before addition of ^{36}Cl and antigen.

Effect of DIDS on histamine release and ^{36}Cl uptake

The putative chloride channel blocker DIDS, 0.3 to 30 μM, caused a concentration-dependent inhibition of antigen-stimulated histamine secretion from rat mast cells (Figure 2c). However, DIDS had no effect on chloride uptake in antigen-stimulated mast cells (Figure 2d). The cells were preincubated with DIDS, 30 μM, for 10 min before addition of ^{36}Cl and antigen. The initial rate of chloride uptake in the first minute after antigen-stimulation of the cells was 27 ± 7.5 nmol/10^6 cells min^{-1} in the absence of DIDS ($n = 4$) and 24.5 ± 7.5 nmol/10^6 cells min^{-1} in the presence of DIDS, 30 μM ($n = 4$). Further, DIDS, 30 μM, did not change the steady state value of chloride uptake.

The effect of ouabain on ^{36}Cl uptake

Rat mast cells have a Na^+/K^+ ATPase which is sensitive to relatively high ouabain concentrations (Knudsen *et al.*, 1993). Since ouabain is likely to cause sodium accumulation by mast cells, the possibility that sodium accumulation might result in a secondary change in chloride uptake was examined. The cells were preincubated with ouabain, 1 mM, in physiological salt solution containing calcium, 1 mM for 10 min before addition of ^{36}Cl but the chloride uptake was unaffected by this treatment (Figure 3a).

The effect of furosemide on ^{36}Cl uptake

Furosemide was used to investigate the role of a Na/K/2Cl cotransport. When furosemide (0.7 mM) was added to unstimulated mast cells, a small delay in obtaining steady state was observed (Figure 3b). Under identical experimental conditions, the spontaneous histamine release was $7.9 \pm 1.1\%$ in the absence of furosemide ($n = 4$) and $6.6 \pm 1.1\%$ in its presence ($n = 4$).

This experiment was repeated on antigen-stimulated sensitized mast cells, where furosemide, antigen and ^{36}Cl were added simultaneously to the cell suspension. Under these conditions, furosemide was able to abolish the chloride uptake induced by stimulation with antigen (Figure 3c). In these experiments, the antigen-induced histamine release was $30.8 \pm 3.6\%$ in the absence of furosemide ($n = 4$) and $51.9 \pm 3.7\%$ in its presence ($n = 4$).

When furosemide was added to sensitized mast cells 5 min before addition of antigen and ^{36}Cl, however, the inhibitory effect of furosemide had almost disappeared (Figure 3d). The antigen-induced histamine release in the presence of furosemide was $34.7 \pm 2.5\%$ ($n = 5$), whereas the unstimulated histamine release in the presence of furosemide was $8.0 \pm 1\%$ ($n = 5$).

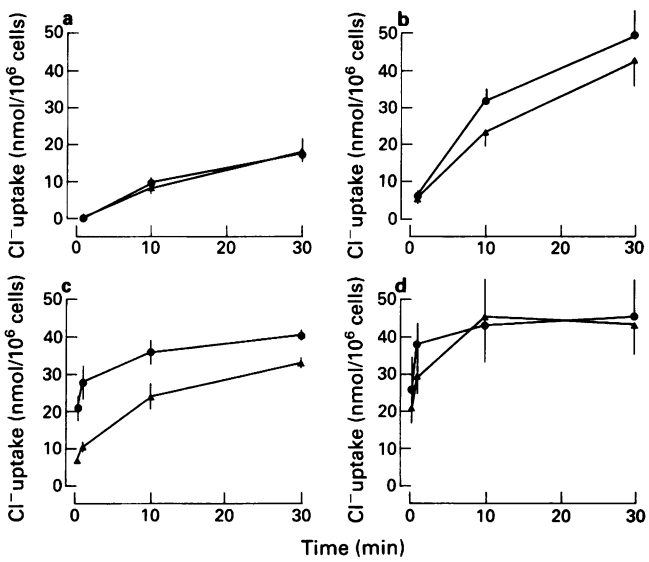


Figure 3 (a) Effect of ouabain on the rate of chloride uptake by rat mast cells. Unstimulated cells in the absence of ouabain (●); unstimulated cells in the presence of ouabain, 1 mM (▲). The cells were preincubated with ouabain for 10 min before addition of ^{36}Cl. Each point is the mean ± s.e.mean from 4 experiments. (b) Effect of furosemide on the rate of chloride uptake by rat mast cells. Unstimulated cells in the absence of furosemide (●); unstimulated cells in the presence of furosemide, 0.7 mM (▲). Each point is the mean ± s.e.mean from 4 experiments. (c) Effect of furosemide on the rate of chloride uptake by sensitized rat mast cells. Furosemide, antigen and ^{36}Cl were added simultaneously to the cell suspension. Cells stimulated with ovalbumin, 1 mg ml^{-1} in the absence of furosemide (●); cells stimulated with ovalbumin, 1 mg ml^{-1} in the presence of furosemide, 0.7 mM (▲). Each point is the mean ± s.e. mean from 4 experiments. (d) Effect of furosemide on the rate of chloride uptake by sensitized rat mast cells. Cells stimulated with ovalbumin, 1 mg ml^{-1} in the absence of furosemide (●); cells stimulated with ovalbumin, 1 mg ml^{-1} in the presence of furosemide, 0.7 mM added 5 min before stimulation with antigen (▲). Each point is the mean ± s.e.mean from 5 experiments.

Discussion

In the electrophysiological studies of Matthews *et al.* (1989b) an outward membrane current (corresponding to influx of chloride) was observed in rat peritoneal mast cells when they were stimulated with substance P or compound 48/80. They also demonstrated the existence of a hyperpolarization resulting from chloride influx into the cell which created a driving force to facilitate the entry into the cytosol of calcium required to initiate histamine secretion (Matthews *et al.*, 1989a).

We have shown that removal of extracellular chloride shifts the calcium concentration-effect curve for antigen-stimulated histamine secretion to the right and depresses its maximum. This observation is consistent with the hypothesis that chloride-mediated hyperpolarization of the cell acts as a driving force for calcium that is required to enter the cell to cause secretion. It seems unlikely that the inhibition of secretion seen when chloride was replaced in the extracellular medium was due to the chemical nature of the substituting anion since both gluconate and isethionate produced similar effects.

We have also shown that when rat mast cells are stimulated with antigen, there is a large increase in the rate of chloride uptake into the cell. Extracellular calcium had no effect on either the resting uptake of chloride or on the antigen-stimulated uptake. Removal of extracellular calcium produced a marked reduction of antigen-stimulated histamine secretion but did not change the chloride uptake, implying that chloride uptake is not a consequence of secretion.

Matthews *et al.* (1989b) were able to mimic the substance P- or compound 48/80-induced chloride conductance in rat mast cells by the intracellular application of cyclic AMP, although the effect was slower than that produced by substance P or compound 48/80. Using the dibutyryl analogue of cyclic AMP, we have shown that this compound produce a small and slow increase in chloride uptake, and it is clear that the effect is by no means as marked as that caused by antigen-stimulation. It is possible that the level of intracellular cyclic AMP achieved with the analogue is insufficient because of metabolism or diffusion limitations. However, both of these explanations seem unlikely because dibutyryl cyclic AMP inhibits histamine secretion (Kaliner & Austen, 1974) and hence presumably gains access to the intracellular compartment. The antigen-stimulated increase in chloride permeability that we observe is large and may represent mechanisms in addition to the opening of the chloride channels described by Matthews *et al.* (1989b). Whilst cyclic AMP activates the chloride channels, it may not affect other mechanisms of increased chloride permeability.

The chloride conductance described in rat peritoneal mast

cells by Matthews *et al.* (1989b) was shown to be blocked by DIDS. We have shown that DIDS inhibits antigen-induced histamine secretion at concentrations similar to those used to block chloride channels, although the inhibition was incomplete even at the higher concentrations. We were, however, unable to demonstrate any effect of DIDS on the antigen-induced increase of chloride uptake, and this uptake may represent a DIDS-insensitive mechanism of increased permeability to chloride, other than the DIDS-sensitive chloride channels described by Matthews and coworkers (1989b).

Besides these chloride channels, the Na^+/K^+ ATPase is the only other electrolyte transporter that has been characterized in mast cells. Ouabain, 1 mM, has been shown to inhibit the Na^+/K^+ ATPase in rat peritoneal mast cells (Knudsen *et al.*, 1993). In the present study, inhibition of the Na^+/K^+ ATPase with ouabain did not change the rate of chloride uptake.

The chloride uptake that we measure is probably different from the chloride current described by Matthews *et al.* (1989b) because of the apparently contradictory results with cyclic AMP and DIDS as discussed above, and because the delayed chloride current was only fully developed 6 min after stimulation (Matthews *et al.*, 1989b), whereas the antigen-induced increase in chloride uptake in the present study was near maximal 1 min after stimulation. Since this rapid chloride uptake was not detected in the electrophysiological studies, it may represent chloride uptake via electroneutral transporters such as the Na/K/2Cl cotransporter or the Cl^-/HCO_3^--exchanger, described in other cell types. Experiments with the Na/K/2Cl-cotransport inhibitor, furosemide, confirmed that the antigen-induced uptake of chloride may be due to the activity of the cotransporter, since the increase in chloride uptake was almost abolished in the presence of furosemide. In addition to this cotransporter, our data have revealed that other chloride transporters may also be operating in the early events after stimulation, since furosemide added 5 min before stimulation was without notable effect on the rapid antigen-induced increased chloride uptake. A possible candidate for this chloride transport is the Cl^-/HCO_3^--exchanger, which is at present under investigation in our laboratory.

The antigen-induced increased chloride uptake is not directly related to histamine release, since the absence of extracellular calcium or the presence of DIDS inhibited antigen-induced histamine release without affecting the chloride uptake. Furthermore, furosemide added simultaneously with antigen inhibited the increase in chloride uptake without inhibiting histamine release. Chloride uptake by the Na/K/2Cl cotransporter would not be involved in membrane hyperpolarization, as it is electroneutral. Our data so far do not point to any possible functions of this chloride uptake in the signal-transduction mechanism. It coincides with the large increase in the activity of the Na^+/K^+ pump that follows a secretory response (Knudsen *et al.*, 1993). It may be that these observations reflect an increased activity of an electroneutral chloride transport that contributes to compensatory ion transport necessary for the cell to recover during histamine secretion.

In conclusion, crosslinking of the high affinity membrane receptors for IgE initiates a large and fast transport of chloride into the mast cell which occurs independently of the secretory response and is thus not directly related to the signal-transducing mechanism. The inhibition of the fast chloride uptake by furosemide points to an increased activity of the Na/K/2Cl cotransporter. However, inward movement of chloride through chloride channels may be part of the mechanism controlling histamine secretion since removal of extracellular chloride or addition of DIDS inhibits histamine secretion.

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References

BEAVEN, M.A., ROGERS, J., MOORE, J.P., HESKETH, T.R., SMITH, G.A. & METCALFE, J.C. (1984). The mechanism of the calcium signal and correlation with histamine release in 2H3 cells. *J. Biol. Chem.*, **259**, 7129–7136.

FOREMAN, J.C. & MONGAR, J.L. (1972). The role of alkaline earth ions in anaphylactic histamine secretion. *J. Physiol.*, **224**, 753–769.

FOREMAN, J.C. & MONGAR, J.L. (1973). The action of lanthanum and manganese on anaphylactic histamine secretion. *Br. J. Pharmacol.*, **48**, 527–537.

FOREMAN, J.C., MONGAR, J.L. & GOMPERTS, B.D. (1973). Calcium ionophores and movement of calcium ions following the physiological stimulus to a secretory process. *Nature*, **245**, 249–251.

HOTH, M. & PENNER, R. (1992). Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature*, **355**, 353–356.

JOHANSEN, T. & CHAKRAVARTY, N. (1975). The utilization of adenosine triphosphate in rat mast cells during histamine release induced by anaphylactic reaction and compound 48/80. *Naunyn-Schmied. Arch. Pharmacol.*, **288**, 243–260.

KALINER, M. & AUSTEN, K.F. (1974). Cyclic AMP, ATP and reversed anaphylactic histamine release from rat mast cells. *J. Immunol.*, **112**, 664–674.

KNUDSEN, T., FERJAN, I. & JOHANSEN, T. (1993). Activation of the Na^+/K^+-pump in rat peritoneal mast cells following histamine release: a possible role in cell recovery. *Br. J. Pharmacol.*, **108**, 120–125.

LINDAU, M. & FERNANDEZ, J.M. (1986). IgE-mediated degranulation of mast cells does not require opening of ion channels. *Nature*, **319**, 150–153.

MATTHEWS, G., NEHER, E. & PENNER, R. (1989a). Second messenger-activated calcium influx in rat peritoneal mast cells. *J. Physiol.*, **418**, 105–130.

MATTHEWS, G., NEHER, E. & PENNER, R. (1989b). Chloride conductance activated by external agonists and internal messengers in rat peritoneal mast cells. *J. Physiol.*, **418**, 131–144.

SAGI-EISENBERG, R., FOREMAN, J.C. & SHELLY, R. (1985). Histamine release induced by histone and phorbol ester from rat peritoneal mast cells. *Eur. J. Pharmacol.*, **113**, 11–17.

SHORE, P.A., BURKHALTER, A. & COHN, V.H. (1959). A method for the fluorometric assay of histamine in tissues. *J. Pharmacol. Exp. Ther.*, **127**, 182–186.

WHITE, J.R., ISHIZAKA, T., ISHIZAKA, K. & SHA'AFI, R. (1984). Direct demonstration of increased intracellular concentration of free calcium as measured by quin-2 in stimulated rat peritoneal mast cell. *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 3978–3982.

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Reduction of endothelial function with age in the mesenteric arterial bed of the normotensive rat

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1 Age-related changes in endothelial vasodilator function were studied in an *in vitro* preparation of the mesenteric arterial bed removed from male, normotensive, Wistar rats.

2 Animals were killed at 2, 12 or 22 months of age, the superior mesenteric artery was cannulated and the gut removed. The mesenteric arterial bed was perfused at a constant flow rate of 4 ml min^{-1} and perfusion pressure was taken as an index of arteriolar tone.

3 The muscarinic agonist, carbachol, antagonized noradrenaline-induced vasoconstriction in the presence, but not in the absence, of endothelium. This cholinoceptor agonist-induced release of endothelial-derived relaxing factor (EDRF) was impaired in 22 month old rats.

4 Noradrenaline-induced vasoconstriction increased following removal of endothelium suggesting that agonist-induced release of EDRF attenuates vasoconstrictor responses to noradrenaline measured in the presence of endothelium.

5 Removal of endothelium had less effect on noradrenaline-induced vasoconstriction in old rats suggesting once again that agonist-induced release of EDRF is impaired in old rats.

6 The noradrenaline dose-response curve established in the presence of endothelium was shifted to the left in 22 month old rats.

7 In conclusion, aging in the rat appears to lead to a reduction in endothelial vasodilator function in a resistance vessel.

Keywords: Aging; endothelium; noradrenaline; rat; mesenteric arterial bed.

Introduction

Vasoconstrictor mechanisms in response to noradrenaline are impaired with age (Tuttle, 1966; Elliott *et al.*, 1982; Simpkins *et al.*, 1983; Soltis *et al.*, 1984; Fouda & Atkinson, 1986; Hyland *et al.*, 1987; Wanstall & O'Donnell, 1988; Johnson & Wray, 1990). Furthermore several (Shimizu & Toda, 1986; Luscher *et al.*, 1993) but not all (Hynes & Duckles, 1987) reports suggest that agonist induced release of endothelium-derived relaxing factor (EDRF) also decreases with age. This parallel fall in noradrenaline and EDRF responsiveness is paradoxical. Noradrenaline-induced release of EDRF attenuates vasoconstriction to noradrenaline (Miller *et al.*, 1987), so an age-related decrease in EDRF release could counterbalance any age-related decrease in the sensitivity to noradrenaline. If this were to be the case the vascular responsiveness to noradrenaline would be apparently unaltered. This is suggested by reports from several groups (Frolkis *et al.*, 1975; Scott & Reid, 1982; Hyland & Docherty, 1985; Hynes & Duckles, 1987). Furthermore, several reviews have stressed that the results on the evolution of α-adrenoceptor vasoconstrictor mechanisms with age are inconsistent (Fleisch, 1980; Kelly & O'Malley, 1984; Bennett & Gardiner, 1988; Vanhoutte, 1988). We would suggest that part of this inconsistency lies in the functional state of the endothelium of the preparations in which vasoconstriction in response to noradrenaline is measured.

Our objective was, therefore, to investigate whether responses to noradrenaline and agonist-induced release of EDRF evolve in a similar fashion in aging rats. The mesenteric arterial bed was used as an *in vitro* model of a resistance vessel network. Preparations were removed from 2-, 12- and 22-month old, male normotensive, Wistar rats. Vasoconstrictor responses to noradrenaline were recorded in the presence and absence of endothelium. Endothelium was removed by perfusing the arterial bed with air for a short period. Pharmacomechanical responses produced by noradrenaline were compared to those produced electromechanically by depolarizing solutions of potassium chloride.

Vasodilator endothelial function was evaluated from the degree of antagonism by carbachol of vasoconstriction in response to noradrenaline. In the mesenteric arterial bed of the rat, carbachol antagonizes noradrenaline-induced vasoconstriction via a mechanism which is dependent on EDRF release, but independent of prostaglandin release (Randall & Hiley, 1988). Responses to carbachol were compared to those evoked by sodium nitroprusside, which vasodilates via a mechanism which does not rely upon release of EDRF (Murphy *et al.*, 1990).

Methods

Animals

Experiments were performed in outbred, Wistar, male rats (IOPS AF/Han, Iffa-Credo, L'Arbresle, France) of 2, 12 or 22 months of age, kept under standard conditions in the animal house for ≥ 1 week before experiments were performed.

The mesenteric arterial bed preparation

Animals were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.). The superior mesenteric artery was cannulated and the gut removed (McGregor, 1965; Henrion *et al.*, 1991). The mesenteric arterial bed was perfused at 4 ml min^{-1} with modified Krebs bicarbonate solution (mM: NaCl 118, KCl 4.7, $MgCl_2.6H_2O$ 1.2, NaH_2PO_4 1.0, $CaCl_2.2H_2O$ 2.6, $NaHCO_3$ 25 and glucose 11.1; 37°C; pH 7.35–7.45), oxygenated with a 95% O_2 and 5% CO_2

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mixture. A bubble trap system, with a flow rate of 0.2 ml min^{-1}, removed any air bubbles in the perfusate.

The flow rate was fixed at 4 ml min^{-1}, a rate we have previously used for mesenteric arterial bed preparations from adult Wistar rats (Henrion *et al.*, 1991). Arteriolar vasoconstriction produced an increase in perfusion pressure (mmHg) which was measured with a strain gauge transducer (Beckman, Palo Alto, CA, U.S.A.), placed in the perfusion circuit just before the mesenteric arterial bed. Mean perfusion pressure was recorded after electronic integration of the pulsatile pressure signal. All pressure values given were corrected by subtraction of the pressure generated by the tubing of the perfusion system (= 4 to 6 mmHg). Baseline perfusion pressure was between 9 and 12 mmHg.

After a 15 min stabilization period, noradrenaline was injected (0.6 μg in a bolus of 0.33 ml, during 20 s) into the perfusate, at 5 min intervals from 15 to 35 min (see Table 1), by which time a reproducible vasoconstrictor response was obtained. Protocols were based on the repeated injection of a bolus of noradrenaline every 5 min. This allowed us to check that the reactivity of each individual preparation had returned to normal following each intervention.

Preliminary experiment in 12-month old rats: evolution of noradrenaline responses in the presence and absence of endothelium

This experiment was performed in 12-month old rats only. Following equilibration and stabilization of responses (see above), noradrenaline was injected every 5 min up to the 160th min ($n = 6$). A second group followed a similar protocol except that following equilibration and stabilization, endothelium was removed by passing air bubbles through the perfusion system (0.2 ml min^{-1} for 5 min), by inversing the direction of flow in the bubble trap system. Noradrenaline was then injected every 5 min up to the 160th min ($n = 6$).

General protocol

This was split up into 5 consecutive stages (Table 1): (i) attenuation by carbachol of noradrenaline-induced vasoconstriction, (ii) noradrenaline dose-response curve, (iii) removal of endothelium, (iv) potassium chloride dose-response curve, and (v) attenuation by sodium nitroprusside of noradrenaline-induced vasoconstriction. At the end of each of the 5 stages, the vasoconstrictor response to the noradrenaline bolus was measured.

Responses in the presence of endothelium

Firstly, the mesenteric bed was perfused for 5 min with carbachol (10^{-9} M), at the end of which time a noradrenaline bolus was injected in the presence of carbachol. The protocol was repeated with stepwise increases in the concentration of carbachol up to 10^{-4} M. The antagonism by carbachol of noradrenaline-induced contraction, is expressed as maximal (%) and IC_{50} i.e., the concentration of carbachol required to reduce the vasoconstriction induced by noradrenaline in the absence of carbachol, by half (following (logit [response])/(log_{10} [dose] transformation)). The midrange sensitivity is estimated from the IC_{50} values for antagonists and the ED_{50} values for agonists, i.e., if IC_{50} or ED_{50} increase then midrange sensitivity is said to decrease and *vice versa*.

Secondly, a noradrenaline dose-response curve was constructed by injecting noradrenaline (0.33 ml in 20 s, 56 ng to 56 μg) into the perfusion system every 5 min. Responses to noradrenaline are expressed as: (i) maximal increases in perfusion pressure (perfusion pressure, mmHg), and (ii) midrange sensitivity, estimated from ED_{50}, i.e., the dose required to produce an increase in perfusion pressure equivalent to 50% of the maximal value (following (logit [response])/(log_{10} [dose] transformation)).

Thirdly, following perfusion with Krebs bicarbonate solution for 15 min, the endothelium was removed. The absence of endothelium was checked by perfusing carbachol (10^{-5} M) for 5 min then injecting a noradrenaline bolus. Carbachol failed to attenuate noradrenaline-induced vasoconstriction in arterial beds without endothelium in all groups (results not shown).

Responses in the absence of endothelium

Fourthly, a dose-response curve for potassium chloride was constructed by injecting increasing concentrations of potassium chloride (1–5 mg, 0.33 ml in 20 s) every 5 min. Responses to potassium chloride are expressed as maximum and EC_{50}.

Fifthly, sodium nitroprusside (10^{-9} M) was perfused for 5 min, at the end of which time noradrenaline was injected. The protocol was repeated with stepwise increases in the concentration of sodium nitroprusside up to 10^{-4} M. Antagonism by sodium nitroprusside of vasoconstriction to noradrenaline is expressed as maximum and IC_{50}.

There were 15 rats in each age group.

Drugs used

Carbachol chloride, and sodium nitroprusside were purchased from Sigma, St. Louis, MO, U.S.A. Noradrenaline bitartrate was dissolved in 0.1 mM ascorbic acid. Concentrations or doses are given as base. Sodium pentobarbitone was purchased from Sanofi SA, Paris, France. Noradrenaline bitartrate and other chemicals were purchased from Merck AG, Darmstadt, Germany.

Statistics

Results are expressed as means ± s.e.mean. Experiments were performed according to a randomized block design. Significant differences between means were determined either by ANOVA and Scheffe test, or Student's paired t test. Results from linear regression ANOVA are given as origin = a, slope = b, correlation coefficient = r. The probability level chosen was $P < 0.05$ (NS = not significant).

Table 1 Experimental protocol

Time (min)	*Operation*
0–15	Equilibration
15–35	Stabilization of responses to 5 challenges with NA (0.6 μg)
40–65	Physiological antagonism of NA-induced vasoconstriction by carbachol
70–100	NA dose-response curve
110	Removal of endothelium
115–135	Potassium chloride dose-response curve
135–160	Physiological antagonism of NA-induced vasoconstriction by sodium nitroprusside

NA = noradrenaline

Results

Rats weighed 295 ± 2 g at 2 months, 513 ± 6 g at 12 months and 625 ± 15 g at 22 months of age.

Preliminary experiment in 12-month old rats: evolution of noradrenaline responses in the presence and absence of endothelium

Responses in the presence of endothelium were stable whereas those determined in the absence of endothelium decreased linearly with time (Figure 1).

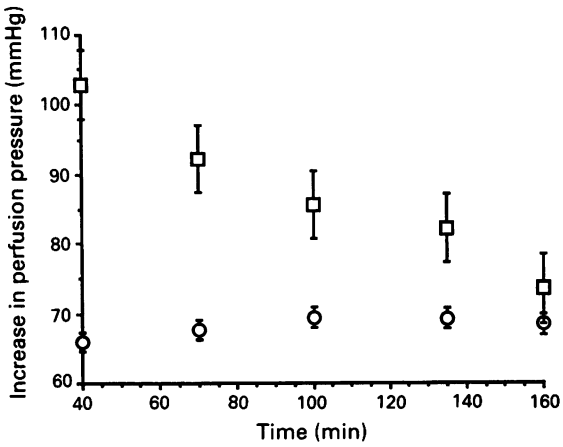


Figure 1 Evolution of noradrenaline responses in the presence (○) and absence (□) of endothelium, in mesenteric arterial bed preparations removed from 12-month old Wistar rats ($n = 6$ per group). Noradrenaline (0.6 μg) was injected every 5 min but results are given for times 40, 70, 100, 135 and 160 min only (see Table 1). In the absence of endothelium (□) there was a significant fall in responses (Y) with time (X): a = 110, b = − 0.22, $r = 0.981$, $P < 0.05$.

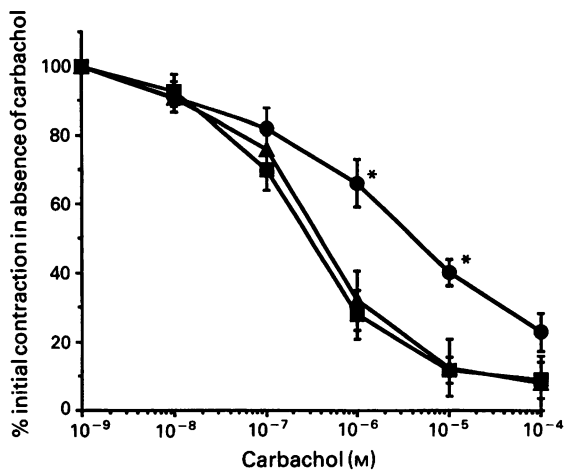


Figure 2 Effect of age on physiological antagonism by carbachol of noradrenaline-induced vasoconstriction in the mesenteric arterial bed (in the presence of endothelium) from 2-month old (■), 12-month old (▲) and 22-month old rats (●). *$P < 0.05$ versus 2-month old rats (■). Initial vasoconstrictor responses to a noradrenaline bolus (0.6 μg) in the absence of carbachol were 47 ± 4, 59 ± 8 and 86 ± 6 mmHg in 2-, 12- and 22-month old rats, respectively.

Responses in the presence of endothelium

Carbachol ($\leq 10^{-4}$ M) had no effect on baseline perfusion pressure (results not shown), but antagonized vasoconstriction to noradrenaline in a dose-related manner (Figure 2). IC_{50} and maxima for carbachol were 0.21 ± 0.05 μM and 90 ± 6%, 0.42 ± 0.05 μM and 93 ± 2%, and 5.00 ± 0.12 μM ($P < 0.05$ versus 2-month old) and 78 ± 3% in 2-, 12- and 22-month old rats.

Noradrenaline EC_{50} was lower in 22-month old rats (0.3 ± 0.1 μg) than in 2-month old rats (1.0 ± 0.2 μg, $P < 0.05$, Figure 3). Maximal responses were similar in all groups (2-month old: 158 ± 12 mmHg).

Removal of endothelium and responses to single bolus injections of noradrenaline

Responses to noradrenaline increased in 2- (54 ± 5 and 90 ± 6 mmHg, paired t test, $P < 0.05$) and 12-month old rats

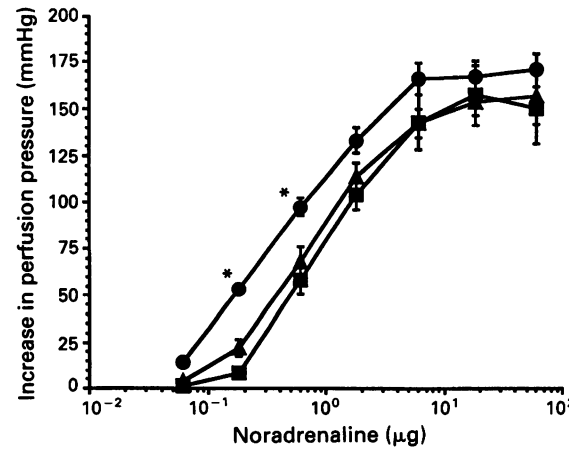


Figure 3 Noradrenaline dose-response curves in mesenteric arterial beds (in the presence of endothelium) from 2-month old (■), 12-month old (▲) and 22-month old rats (●). *$P < 0.05$ versus 2-month old rats (■); $n = 15$ per group.

(65 ± 6 and 95 ± 4 mmHg, paired t test, $P < 0.05$) but not in 22-month old rats (89 ± 6 and 110 ± 7 mmHg, paired t test, NS), following removal of endothelium.

Responses in the absence of endothelium

There was a slight decrease in midrange sensitivity to potassium chloride in 12- (2.13 ± 0.03 mg, $P < 0.05$) and 22-month old rats (2.23 ± 0.03 mg, $P < 0.05$) compared to 2-month old rats (1.95 ± 0.03 mg). Maximal responses were similar (2-month old: 110 ± 6 mmHg).

Sodium nitroprusside had no effect on basal perfusion pressure (results not shown), but antagonized vasoconstriction to noradrenaline in a dose-related manner. Vasoconstrictor responses to noradrenaline (0.6 μg) before sodium nitroprusside were 93 ± 6, 96 ± 4 and 94 ± 5 mmHg in 2-, 12- and 22-month old rats. IC_{50} values for sodium nitroprusside were similar in all groups (2-month old 0.55 ± 0.20 μM), but maximal responses were significantly reduced in 12- (74 ± 2%, $P < 0.05$) and 22-month old rats (73 ± 4%, $P < 0.05$) compared to 2-month old rats (93 ± 4%).

Discussion

We describe several lines of evidence which suggest that endothelial vasodilator function is impaired in the mesenteric arterial bed of 22-month old rats. Firstly, physiological antagonism by carbachol of noradrenaline-induced vasoconstriction was less marked in mesenteric arterial beds from 22-month old rats. Secondly, antagonism by the endothelium of the vasoconstrictor response to a bolus of noradrenaline decreased in 22-month old rats. An experimental consequence of the latter was that the dose-response curve for noradrenaline, constructed in the presence of endothelium, was shifted to the left. This occurred in spite of the fact that the underlying vasoreactivity to noradrenaline, measured in the absence of endothelium, was unchanged, as far as could be judged from responses to a bolus administration. Furthermore, electromechanical vasoconstrictor responses (elicited by potassium depolarization) decreased. Thus when vasoconstrictor responses to noradrenaline were measured in the *in vitro* preparation of the rat mesenteric arterial bed, before removal of the endothelium, an apparent age-related increase in sensitivity to noradrenaline was observed.

Our working hypothesis is based upon the fact that the removal of endothelium had a major effect on responses in 2- and 12-month old rats and that there was no time-dependent

increase in the response to a bolus injection of noradrenaline. We checked this in 12-month old rats. Responses in the presence of endothelium were stable throughout the 160 min of the protocol. In the absence of endothelium responses to a repeated bolus administration of noradrenaline were seen to decrease, not increase, with time.

Published reports on the effect of maturation and/or age on vasoconstrictor responses to noradrenaline are contradictory (see Introduction). We would suggest that a partial explanation of this lies in the functional and/or structural state of the endothelium in the different preparations used. In addition, in human isolated vessels, endothelial function may be impaired by age-related vascular pathologies, such as hypertension and atherosclerosis, which confound studies on vascular disease-free aging in man (Bennett & Gardiner, 1988). These considerations would appear to be of less importance in the present study. Blood pressure does not increase with age in the rat strain we used (Lartaud *et al.*, 1993) and the rat has a natural resistance to the development of age-related vascular pathologies seen in man such as atherosclerosis (Gill *et al.*, 1989) and arteriosclerosis following calcium overload (Atkinson, 1992).

Our observation that vasodilator endothelial function decreases with age confirms previous reports (see Introduction). Before considering possible explanations of this, it should be noted that in this and other protocols, the initial response (before carbachol) to a bolus of noradrenaline in 22-month old rats was higher than in younger rats and such differences in vasoconstrictor responses to noradrenaline could have influenced the degree of physiological antagonism by carbachol. The explanation generally given for the age-related decrease in vasodilator endothelial function is based on age-related changes in the function of the endothelial cell. Other functional changes may be involved as in this work and others (Moritoki *et al.*, 1988), there was an age-related attenuation of responses to sodium nitroprusside. This suggests that age-related changes may also occur at the level of guanylate cyclase or a later step. Functional changes may not be the only ones involved. Although on the basis of the bioassay type of protocol used in our experiments, it is difficult to draw conclusions as to the structural or functional origin of the age-related changes in endothelial function, it is possible that the decrease in endothelial function observed was of structural origin. Several studies have shown that aging is associated with intimal thickening and an increase in collagen in the subendothelial layer (Soltis *et al.*, 1984). This could lead to the formation of a barrier, between the endothelium and the smooth muscle cells of the media, which becomes less and less permeable to the passage of endothelial dilator factor(s), with time.

In our experiments the same flow rate was used in mesenteric arterial bed preparations from rats of different body weights. This technique has been adopted in other aging studies using perfused preparations such as the tail artery and the mesenteric arterial bed (Hynes & Duckles, 1987). It should be pointed out that a vascular network with a smaller initial average diameter when perfused at the same constant flow rate will have a greater proportional increase in pressure with the same absolute change in radius than a vascular network with a larger initial average diameter. This could modify maximal responses but in our experiments such responses were generally similar. We observed parallel changes in dose-response curves for carbachol and noradrenaline. We would suggest that this can be taken as evidence of age-related vascular changes which are independent of any methodological artefact linked to the flow rate used. Furthermore we administered the agonist (noradrenaline) in the presence of the physiological antagonist (carbachol or sodium nitroprusside). In other studies the antagonist was added after precontraction with the agonist (Hynes & Duckles, 1987). It should be recalled that in studies of antagonism, the tissue is generally exposed to the antagonist before adding the agonist. Finally, differences in responses to a single bolus of noradrenaline could be due to changes in altered potency and/or in altered maximal response. In our experiments maximal responses to noradrenaline were similar in all three age groups.

In conclusion, when considering possible *in vivo* consequences of these *in vitro* observations, it could be argued that if a diminution of endothelial vasodilator function with age is a common feature of most vascular beds then, in the absence of any compensatory changes in the renal pressure/volume relationship, old Wistar rats should be hypertensive. Although some reports (Bunag & Teravainen, 1991) suggest that Wistar rats may become moderately hypertensive with age, our own studies on rats of the same strain and sex, from the same breeder, show that Wistar rats do not become hypertensive with age (Lartaud *et al.*, 1993). This could mean that: (i) age-related changes in the mesenteric arterial bed are not representative of changes in other vessels; (ii) age-related changes in agonist-induced EDRF mechanisms do not reflect changes in other EDRF mechanisms such as those stimulated by flow or shear stress; (iii) the age-related change in EDRF function is compensated by other functional and/or structural changes in the endothelium or in other organs (such as the kidney).

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References

ATKINSON, J. (1992). Vascular calcium overload. Physiological and pharmacological consequences. *Drugs*, **44** (suppl. 1), 111–118.

BENNETT, T. & GARDINER, S.M. (1988). Physiological aspects of the aging cardiovascular system. *J. Cardiovasc. Pharmacol.*, **12** (suppl. 8), S1–S7.

BUNAG, R.D. & TERAVAINEN, T.L. (1991). Tail-cuff detection of systolic hypertension in different strains of ageing rats. *Mech. Age. Dev.*, **59**, 197–213.

ELLIOTT, H.L., SUMMER, D.J., McLEAN, K. & REID, J.L. (1982). Effect of age on the responsiveness of vascular alpha-adrenoceptors in man. *J. Cardiovasc. Pharmacol.*, **4**, 388–392.

FLEISCH, J.H. (1980). Age-related changes in the sensitivity of blood vessels to drugs. *Pharmacol. Ther.*, **8**, 477–487.

FOUDA, A.K. & ATKINSON, J. (1986). Sensitivity to noradrenaline and electrical stimulation decreases with age in the rat tail artery. *Naunyn-Schmied. Arch. Pharmacol.*, **334**, 37–39.

FROLKIS, V.V., BEZRUKOV, V.V. & SHEVCHUK, V.G. (1975). Hemodynamics and its regulation in old age. *Exp. Geront.*, **10**, 251–271.

GILL, T.J., SMITH, G.J., WISSLER, R.W. & KUNZ, H.W. (1989). The rat as an experimental animal. *Science*, **245**, 269–276.

HENRION, D., CHILLON, J.M., CAPDEVILLE-ATKINSON, C., VINCENAUX-FEUGIER, M. & ATKINSON, J. (1991). Chronic treatment with the angiotensin I converting enzyme inhibitor, perindopril, protects *in vitro* carbachol-induced vasorelaxation in a rat model of vascular calcium overload. *Br. J. Pharmacol.*, **104**, 966–972.

HYLAND, L. & DOCHERTY, J.R. (1985). An investigation of age-related changes in pre- and postjunctional alpha-adrenoceptors in human saphenous vein. *Eur. J. Pharmacol.*, **114**, 361–364.

HYLAND, L., WARNOCK, P. & DOCHERTY, J.R. (1987). Age-related alterations in α_1- and β-adrenoceptors mediated responsiveness of rat aorta. *Naunyn-Schmied. Arch. Pharmacol.*, **335**, 50–53.

HYNES, M.R. & DUCKLES, S.P. (1987). Effect of increasing age on the endothelium-mediated relaxation of rat blood vessels *in vitro*. *J. Pharmacol. Exp. Ther.*, **241**, 387–392.

JOHNSON, M.D. & WRAY, A. (1990). Alpha$_1$ adrenergic receptor function in senescent Fischer 344 rat aorta. *Life Sci.*, **46**, 359–366.

KELLY, J. & O'MALLEY, K. (1984). Adrenoceptor function and ageing. *Clin. Sci.*, **66**, 509–515.

LARTAUD, I., BRAY-DES-BOSCS, L., CHILLON, J.M., ATKINSON, J. & CAPDEVILLE-ATKINSON, C. (1993). *In vivo* cerebrovascular reactivity in Wistar and Fischer 344 rat strains during aging. *Am. J. Physiol.*, **264**, H851–H858.

LUSCHER, T.F., DOHI, Y. & TSCHUDI, M. (1992). Endothelium-dependent regulation of resistance arteries: alterations with aging and hypertension. *J. Cardiovasc. Pharmacol.*, **19** (suppl. 5), S34–S42.

MCGREGOR, D.D. (1965). The effect of sympathetic nerve stimulation on vasoconstrictor responses in perfused blood vessels of the rat. *J. Physiol.*, **177**, 21–30.

MILLER, R.C., SCHINI, V. & SCHAFFTER, P. (1987). Modulation by the endothelium of agonist-induced contractions by vascular smooth muscle. In *Endothelium-derived Vasoactive Factors*, ed. Vanhoutte, P.M. pp. 1–12. New York: Humana.

MORITOKI, H., TANIOKA, A., MAESHIBA, Y., IWAMOTO, T., ISHIDA, Y. & ARAKI, H. (1988). Age-associated decrease in histamine-induced vasodilatation may be due to reduction of cyclic GMP formation. *Br. J. Pharmacol.*, **95**, 1015–1022.

MURPHY, J., LAVIE, C.J. & BRESNAHAN, D. (1990). Nitroprusside. In *Cardiovascular Drug Therapy*, ed. Messerli. F.H. pp. 861–869. Philadelphia: Saunders.

RANDALL, M.D. & HILEY, C.R. (1988). Detergent and methylene blue affect endothelium-dependent vasorelaxation and pressure/flow relations in rat blood perfused mesenteric arterial bed. *Br. J. Pharmacol.*, **95**, 1081–1088.

SCOTT, P.J.W. & REID, J.L. (1982). The effect of age on the responses of human isolated arteries to noradrenaline. *Br. J. Clin. Pharmacol.*, **13**, 237–239.

SHIMIZU. I. & TODA, N. (1986). Alterations with age of the response to vasodilator agents in isolated mesenteric arteries of the beagle. *Br. J. Pharmacol.*, **89**, 769–778.

SIMPKINS, J.W., FIELD, F.P. & REES, R.J. (1983). Age-related decline in adrenergic responsiveness of the kidney, heart and aorta of male rats. *Neurobiol. Aging*, **4**, 233–238.

SOLTIS, E.E., WEBB, R.C. & BOHR, D.F. (1984). The vasculature in hypertension and aging. In *Blood Pressure Regulation and Aging*, ed. Horan, M.J., Steinberg, G.M., Dunbar, J.B. & Hadley, E.C. pp. 141–156. Washington: National Institutes of Health.

TUTTLE, R.S. (1966). Age-related changes in the sensitivity of rat aortic strips to norepinephrine and associated chemical and structural alterations. *J. Gerontology*, **21**, 510–516.

VANHOUTTE, P.M. (1988). Aging and vascular responsiveness. *J. Cardiovasc. Pharmacol.*, **12**, S11–S18.

WANSTALL, J.C. & O'DONNELL, S.R. (1988). Inhibition of norepinephrine contraction by diltiazem on aorta and pulmonary artery of young and aged rats: influence of alpha-adrenoceptor reserve. *J. Pharmacol. Exp. Ther.*, **245**, 1016–1020.

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Replacement of salt by a novel potassium- and magnesium-enriched salt alternative improves the cardiovascular effects of ramipril

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1 The influence of salt (sodium chloride; NaCl) (an additional 6% in the diet) and that of a novel sodium-reduced, potassium-, magnesium-, and L-lysine-enriched salt alternative on the cardiovascular effects of ramipril was studied in stroke-prone spontaneously hypertensive rats in a 6-week study. The intake of sodium chloride was adjusted to the same level by adding the salt alternative at a 1.75 times higher amount than regular salt.

2 Salt produced a marked rise in blood pressure and induced cardiac hypertrophy and significant mortality, while the salt alternative neither increased blood pressure nor caused any mortality and produced less cardiac hypertrophy than salt.

3 Ramipril treatment at a daily dose of 3 mg kg^{-1} normalized blood pressure and prevented the development of cardiac hypertrophy of rats on control diet. These effects of ramipril were blocked by the addition of salt but were only slightly attenuated by the addition of the salt alternative. The mortality in the salt group was prevented by ramipril.

4 Responses of mesenteric arterial rings *in vitro* were examined at the end of the study. Salt, but not the salt alternative, increased vascular contractile responses to noradrenaline. Ramipril treatment improved the arterial relaxation responses to acetylcholine and to sodium nitroprusside. The vascular relaxation enhancing effect of ramipril was blocked by salt but only slightly attenuated by the salt alternative.

5 Ramipril treatment did not significantly increase plasma renin activity in the presence or in the absence of salt supplementation. The salt alternative did not cause hyperkalaemia, either alone or in combination with ramipril treatment.

6 Both salt supplementations, irrespective of ramipril treatment, induced a six to eight fold increase in the urinary excretion of calcium. There was an expected 90 to 140% rise in the urinary excretion of magnesium and 200% rise in the urinary excretion of potassium in the salt alternative group. Salt also produced an approximately 50% increase in magnesuria.

7 Our findings suggest that replacement of salt by the potassium-, magnesium- and L-lysine-enriched salt alternative improves the cardiovascular effects of ramipril. In the present study the beneficial effect was related to the increased intakes of potassium and/or magnesium and L-lysine from the salt alternative because the amount of sodium chloride was the same.

Keywords: Stroke-prone spontaneously hypertensive rats; cardiac hypertrophy; angiotensin-converting enzyme inhibition; ramipril; arterial smooth muscle; sodium; potassium; magnesium; calcium

Introduction

Angiotensin-converting enzyme inhibitors (ACEIs) belong in many countries to the first-line drugs in the treatment of arterial hypertension and congestive heart failure. Unfortunately, the high sodium chloride (salt) intake, typical for most industrialized societies (Law *et al.*, 1991a), interferes with the therapeutic effects of ACEIs. Therefore, salt restriction or natriuretic diuretics are frequently used to improve the therapeutic efficacy of ACEIs (Waeber *et al.*, 1990).

Even without drug treatment, reduction in the intake of sodium chloride has an antihypertensive effect (Jula *et al.*, 1990; Frost *et al.*, 1991; Law *et al.*, 1991a,b). Moreover, recent evidence indicates that potassium supplementation produces natriuresis (Young *et al.*, 1976), lowers blood pressure (Cappucio & MacGregor, 1991) and protects against stroke (Khaw & Barrett-Connor, 1986; Tobian, 1988). Increased intake of magnesium may also, in some cases, reduce blood pressure and produce a variety of other favourable cardiovascular and metabolic effects (Sjögren *et al.*, 1989; Altura & Altura, 1990; Karppanen, 1990; Lind *et al.*, 1991). In agreement with these observations we have recently found that, unlike normal salt, a novel potassium-, magnesium-, and L-lysine-enriched salt alternative, even in high doses did not raise blood pressure and produced little if any left ventricular hypertrophy in the spontaneously hypertensive rats (SHR) (Mervaala *et al.*, 1992). In experimental hypertension, the endothelium-dependent relaxation of vascular smooth muscle is impaired (Konishi & Su, 1983; Lüscher *et al.*, 1987; Tesfamariam & Halperin, 1988). Moreover, high sodium chloride diet and severe hypertension in the stroke-prone SHR induce injury to endothelial cells (Tobian, 1988), and a high potassium diet protects against the salt-induced endothelial dysfunction (Sugimoto *et al.*, 1988). ACEI treatment improves the endothelium-dependent vascular relaxation in SHR by increasing the production of endothelium-derived relaxing factor(s) and by preventing the de-

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gradation of bradykinin liberated from the endothelial cells (Clozel *et al.*, 1990; Wiemer *et al.*, 1991; Arvola *et al.*, 1993). In experimental and clinical studies, ACEIs reduce cardiac hypertrophy in antihypertensive doses (Waeber *et al.*, 1990). Ramipril is able to prevent the left ventricular hypertrophy even in non-antihypertensive doses in rats made hypertensive by aortic ligation (Linz *et al.*, 1992).

In the present six-week study we compared the influence of the novel potassium- and magnesium-enriched salt alternative with that of normal salt on the cardiovascular effects of the ACEI ramipril in stroke-prone SHR. The effects of long-term angiotensin-converting enzyme inhibition and those of long-term salt supplementations on arterial functions were estimated by responses of the mesenteric arterial rings *in vitro*. In addition, we tried to elucidate the safety profile of the salt alternative by measuring the plasma electrolyte levels. In particular, we were interested in examining whether the moderate increase in the intake of potassium from the salt alternative, in combination with the ramipril treatment, increases the risk of hyperkalaemia.

Methods

Experimental animals and diets

Sixty inbred male stroke-prone spontaneously hypertensive rats (SHRSP), originally developed by Okamoto *et al.* (1974), were used. The rats from our breeding were housed five animals in a cage in an animal laboratory (illuminated from 06 h 30 min to 18 h 30 min, room temperature 22–24°C). At the beginning of the study, the nine-week-old rats were randomized into six subgroups to receive different diet and drug regimens for six weeks. The NaCl-enriched diet was produced by adding 6.0 g of NaCl to 94.0 g of the standard rat chow (Finnewos Aqua, Helsinki, Finland). The salt alternative enriched diet was produced by adding 10.5 g of the commercially available salt alternative Pansalt, (Oriola Oy, Espoo, Finland), to 89.5 g of the chow. This salt alternative has the following composition: NaCl 57%, KCl 28%, $MgSO_4 \cdot 7H_2O$ 12%, L-lysine hydrochloride 2% and anticaking agents ($MgCO_3$, SiO_2) 1%. Hence, sodium chloride was adjusted to the same level in the NaCl-enriched and in the salt alternative-enriched chows. The contents of sodium, potassium, magnesium and other nutrients in the different diets are given in Table 1. The rats had free access to tap water and chow. Ramipril was added to the pellets (35 mg ramipril kg^{-1} dry weight of the pellets) to produce an approximate daily dose of 3 mg kg^{-1} body weight. Systolic blood pressure of the pretrained rats was measured weekly by a tail cuff method (Blood pressure recorder, model no. 8002e, W + W electronics Inc., Basel, Switzerland) as described in detail earlier (Mervaala *et al.*, 1992). The animals were examined daily for survival and signs of stroke throughout the study. Assessment of stroke was based on the presence of evident and stable hemiplegia, akinesia, lethargy, and hyporesponsiveness according to the symptomatological classification described by Yamori *et al.* (1982). During the fifth week of the experiment the rats were housed individually in metabolic cages. Food intake was recorded and urine was collected over a 24-h period. After the six-week experimental period, the rats were anaesthetized with sodium pentobarbitone (65 mg kg^{-1}, i.p.), the carotid artery was cannulated and blood samples were taken into two chilled tubes on ice using EDTA (4.5 mM) and heparin (100 units ml^{-1}) as anticoagulants. The hearts were excised, great vessels, atria and the free wall of the right ventricle were dissected and the left ventricular mass (LVM) was measured. The left ventricular

Table 1 Contents of sodium, potassium, magnesium and other nutrients in the different diets

	Control diet	*Salt (NaCl) diet*	*Salt alternative diet*
Mineral element			
Sodium	0.3*	2.6*	2.6*
Potassium	0.8*	0.8*	2.3*
Magnesium	0.2*	0.2*	0.3*
Other nutrients (contents common for all diets)			
Mineral elements			
Calcium	1.0*		
Phosphorus	0.75*		
Iron	178 mg kg^{-1}		
Zinc	115 mg kg^{-1}		
Manganese	89 mg kg^{-1}		
Copper	18 mg kg^{-1}		
Iodine	2 mg kg^{-1}		
Cobalt	1 mg kg^{-1}		
Vitamins			
A	12000 iu kg^{-1}		
D_3	1500 iu kg^{-1}		
E	63 mg kg^{-1}		
K_1	0.25 mg kg^{-1}		
K_3	10 mg kg^{-1}		
B_1	3 mg kg^{-1}		
B_2	12 mg kg^{-1}		
B_6	5 mg kg^{-1}		
B_{12}	0.02 mg kg^{-1}		
Calcium pantothenate	10 mg kg^{-1}		
Niacin	40 mg kg^{-1}		
Choline chloride	1000 mg kg^{-1}		
Major constituents			
Water	11.7*		
Crude fat	5.3*		
Crude protein	21.0*		
Fibre	2.8*		
Carbohydrate	52.2*		
Ash	7.0*		
Metabolizable energy	13.0 MJ kg^{-1}		

*Values are expressed as % (g 100 g^{-1}) of the dry weight of the pellets.

wet weight-to-body weight ratio was calculated as an index of left ventricular hypertrophy. The superior mesenteric artery was carefully excised and cleaned of adherent connective tissue. The experimental design of the study was approved by the Animal Experimentation Committee of the University of Helsinki, Finland.

Mesenteric arterial responses in vitro

A modification of the method described by Arvola *et al.* (1993) was applied. Briefly, a 3-mm-long standard section of the mesenteric artery, 0.3 cm distal from the mesenteric artery-aorta junction, was cut. The ring was placed between stainless steel hooks and mounted in an organ bath chamber in physiological salt solution (PSS) (pH 7.4) of the following composition (mM): NaCl 119.0, $NaHCO_3$ 25.0, glucose 11.1, $CaCl_2$ 1.6, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, and aerated with 95% O_2 and 5% CO_2. The ring was equilibrated for 20 min at + 37°C with a resting tension of 1.0 g. The force of contraction was measured with an isometric force-displacement transducer and registered on a polygraph (FTO3C transducer, Model 7C8 Polygraph; Grass Instrument Co., Quincy, Ma., U.S.A.). The presence of intact endothelium in vascular preparations was confirmed by observing a relaxation response to 1 μM acetylcholine (ACh) in 1 μM noradrenaline (NA) precontracted rings. The contractile concentration-curves to NA and to KCl, and the relaxation concentration-curves to cumulative ACh and sodium nitroprusside (SNP) were determined.

Determination of plasma renin activity

Plasma renin activity was determined by use of a radioimmunoassay (RIA) of angiotensin I, modified for rat plasma (Tikkanen *et al.*, 1980).

Determination of electrolyte concentrations

The concentrations of the elements, sodium, potassium, phosphorus, magnesium, calcium and zinc in urine were determined by using Baird PS-4 inductively-coupled plasma emission spectrometer (Baird Co, Bedford, MA, U.S.A.) as described in detail elsewhere (Laakso *et al.*, 1991). The intra-assay imprecision for the elements was better than 3% and the inter-assay imprecision was not more than 5%. For magnesium the intra-assay imprecision was 1.5% and the inter-assay imprecision was 0.5%. The mean levels obtained for the NIST (National Institute of Standards, Washington, U.S.A.) 1577b reference material were within 3.5% of NIST certified values. The concentrations of sodium and potassium in plasma were determined by a standard ion selective electrode method employing a Cobas Fara II random access analyzer (F. Hoffman-La Roche, Basel, Switzerland) according to the instructions of the manufacturer. The concentration of magnesium in plasma was determined with a spectroscopic method using metallochromic dye, Camalgite (Sherwood Medical, St Louis, MO., U.S.A.), and the Cobas Fara II. Interassay imprecisions were 0.5%, 0.65% and 1.5%, for plasma sodium, potassium and magnesium respectively.

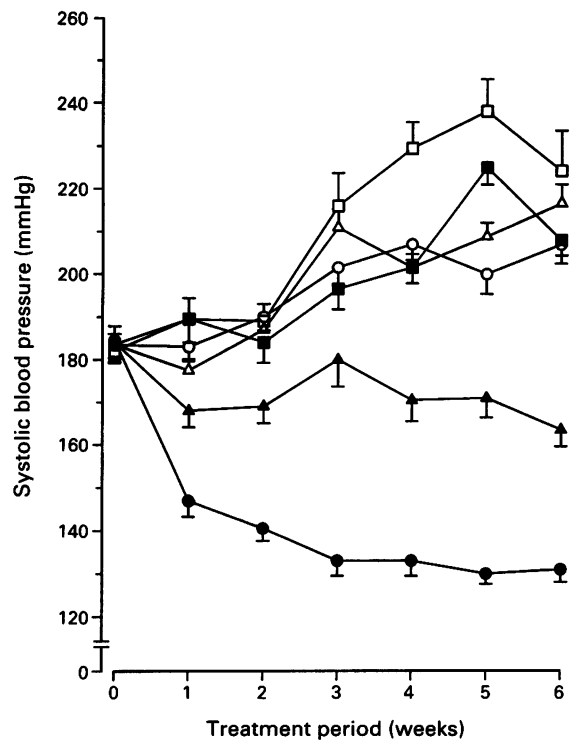


Figure 1 Line plots show blood pressure in stroke-prone spontaneously hypertensive rats (SHRSP) during different diet and drug regimens: (○) controls on normal diet ($n = 10$); (□) supplementation with salt (NaCl) ($n = 7-10$); (△) supplementation with the salt alternative ($n = 10$); (●) ramipril during control diet ($n = 10$); (■) ramipril during NaCl-enriched diet ($n = 10$); (▲) ramipril during salt alternative-enriched diet ($n = 10$). Repeated analysis of variance between-subjects effects, $P < 0.0001$; within-subject effects, $P < 0.0001$; time-group interaction, $P < 0.0001$. NaCl-enriched diet produced a rise in blood pressure ($P < 0.01$ to 0.001 versus all other groups). Ramipril treatment (3 mg kg^{-1} day^{-1}) during control and salt alternative-enriched diets significantly decreased blood pressure ($P < 0.001$ versus all other groups). Symbols indicate means with s.e.mean.

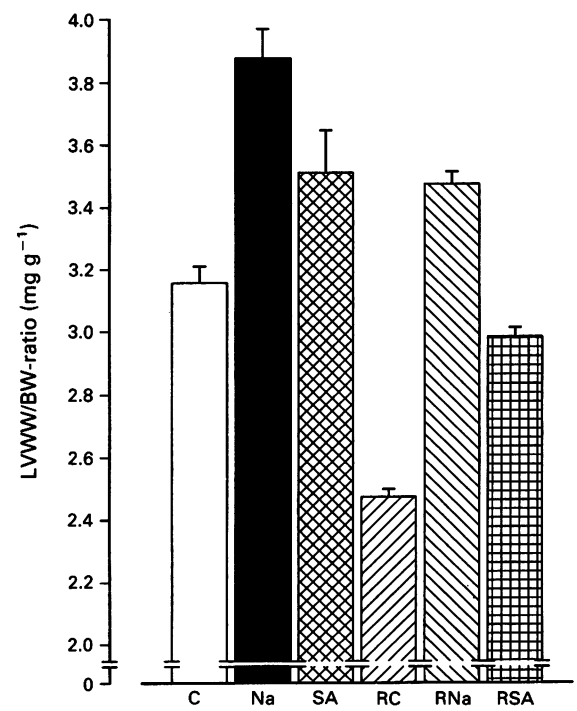


Figure 2 Graph showing left ventricular hypertrophy index expressed as left ventricular wet weight (LVWW) -to- body weight (BW) ratio, of stroke-prone spontaneously hypertensive rats after six weeks on the different diet and drug regimens. C, controls on normal diet ($n = 10$); Na, supplementation with salt (NaCl) ($n = 7$); SA, supplementation with the salt alternative ($n = 10$); RC, ramipril during control diet ($n = 10$); RNa, ramipril during NaCl-enriched diet ($n = 10$); RSA, ramipril during salt alternative-enriched diet ($n = 10$). Analysis of variance $P < 0.0001$. NaCl produced a marked left ventricular hypertrophy ($P < 0.01$ versus the salt alternative group and ramipril-treated NaCl group; $P < 0.001$ versus all other groups). Ramipril treatment (3 mg kg^{-1} day^{-1}) during control diet prevented the development of left ventricular hypertrophy ($P < 0.001$ versus control group). Columns indicate means with s.e.mean.

Drugs

The following drugs were used: ramipril (Astra Hässle AB, Mölndal, Sweden), sodium pentobarbitone (Ph.Eur., The University Pharmacy, Helsinki, Finland), heparin (Leiras Oy, Turku, Finland), acetylcholine chloride, noradrenaline bitartrate (Sigma Chemical Co., St. Louis, MO., U.S.A.), and sodium nitroprusside (SNP) (F. Hoffman-La Roche AG., Basel, Switzerland). The stock solutions of the compounds used in *in vitro* studies were dissolved in distilled water. All solutions were freshly prepared before daily use and protected from light.

Statistical analysis

Statistical analysis was carried out by one-way analysis of variance (ANOVA) supported by the Tukey's test. Data for multiple observations over time were analyzed by two-way ANOVA with repeated measures for overall treatment effect, and Tukey's test was used for multiple pairwise comparisons of treatment groups at different times. The area under the curve (AUC) (blood pressure versus time) of each individual rat in different treatment groups was also calculated mathematically by the methods outlined by Matthews *et al.* (1990). The AUCs were then tested by ANOVA, supported by Tukey's test. Statistical analysis of the mortality during the 42-day follow-up period was calculated by 2 × 2 Table using the Fisher's exact test (2-tail). Differences between means that had $P<0.05$ were considered significant. The data were analyzed with SAS statistical Software (SAS Institute Inc., Cary, NC., U.S.A.). The results are expressed as means ± s.e.mean.

Results

Death-rate and stroke

Three of the 10 SHRSP which received the NaCl-enriched diet died during the experiment. All those three salt-loaded SHRSP suffered before death from clinically diagnosed stroke. By contrast, none of the SHRSP receiving the salt alternative-enriched diet died or suffered from stroke. In the group receiving ramipril treatment, no deaths occurred and there was no evidence of stroke in the SHRSP supplemented either with salt or the salt alternative. The mortality in the NaCl group without ramipril treatment was significant as compared to the other groups (mortality within 42 days, 2 × 2 Table, NaCl group versus other groups combined, Fisher exact two-tail test, $P = 0.0035$).

Blood pressure and left ventricular mass

Enrichment of the diet with salt produced a marked further elevation of blood pressure as compared to the age-related increase in blood pressure of the SHRSP receiving control diet (Figure 1). The rats receiving the diet which was en-

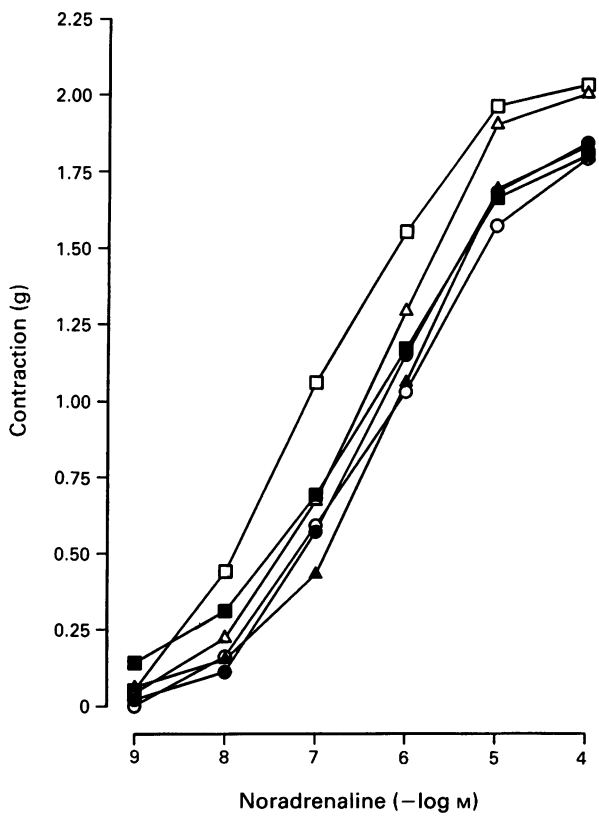


Figure 3 Contractile responses to noradrenaline of isolated endothelium-intact mesenteric arterial rings from the stroke-prone spontaneously hypertensive rats after six weeks on the different diet and drug regimens: (○) controls on normal diet; (□) supplementation with salt (NaCl); (△) supplementation with the salt alternative; (●) ramipril during control diet; (■) ramipril during NaCl-enriched diet; (▲) ramipril during salt alternative-enriched diet. Repeated analysis of variance between-subjects effects, $P = 0.027$; within-subject effects, $P<0.0001$; interaction, $P = 0.0399$. NaCl supplementation increased vascular response to noradrenaline ($P<0.05$ versus control group and ramipril-treated groups during control and the salt alternative-enriched diets). Symbols indicate means, $n = 5-6$ in each group.

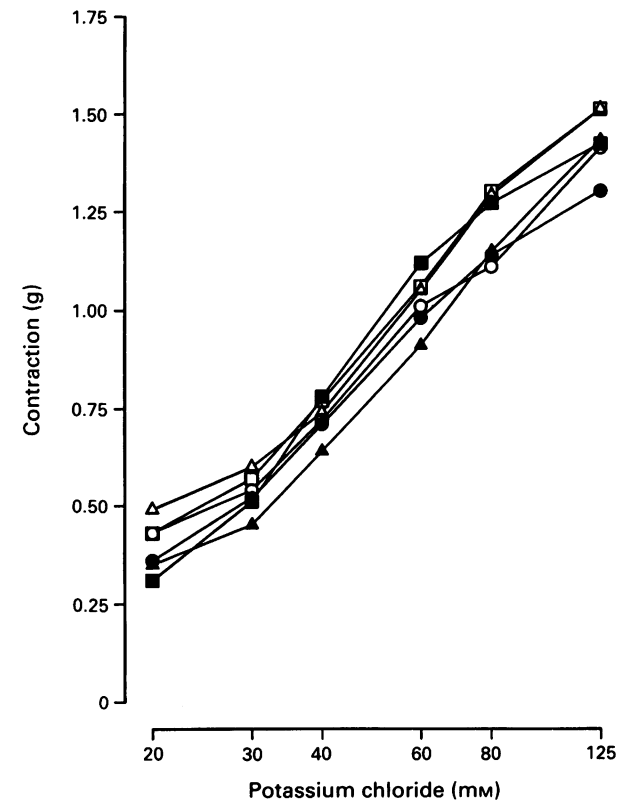


Figure 4 Contractile responses to KCl of isolated endothelium-intact mesenteric arterial rings from the stroke-prone spontaneously hypertensive rats after six weeks on the different diet and drug regimens: (○) controls on normal diet; (□) supplementation with salt (NaCl); (△) supplementation with the salt alternative; (●) ramipril during control diet; (■) ramipril during NaCl-enriched diet; (▲) ramipril during salt alternative-enriched diet. Repeated analysis of variance between-subjects effects, $P = 0.9093$; within-subject effects, $P<0.0001$; interaction, $P = 0.6795$. Symbols indicate means, $n = 5-6$ in each group.

riched with the salt alternative showed no significant increase in blood pressure when compared to the control SHRSP. Ramipril treatment normalized blood pressure during control diet, markedly lowered blood pressure during the salt alternative-enriched diet, but did not significantly affect the rise of blood pressure during the NaCl-enriched diet (Figure 1).

Salt produced left ventricular hypertrophy as indicated by a marked increase in the left ventricular wet weight-to-body weight ratio (Figure 2). In the salt alternative supplemented rats, the left ventricular hypertrophy index was increased when compared to the control group but lower as compared to the NaCl group. Ramipril treatment during control diet prevented the development of left ventricular hypertrophy. Ramipril treatment only partially blocked the left ventricular hypertrophy in the NaCl group. By contrast, in the ramipril-treated SHRSP of the salt alternative group the left ventricular hypertrophy index did not differ from that in the control SHRSP.

Mesenteric arterial responses in vivo

The vascular contractile responses to noradrenaline were increased in the NaCl group (Figure 3). No changes between the different groups were found in the vascular contractile responses to potassium chloride (Figure 4). Ramipril treatment during control and the salt alternative-enriched diets enhanced the vascular relaxation responses to acetylcholine (Figure 5). In these groups, the first significant relaxation response to ACh was found at 10 nM ACh, while in the other groups equal relaxation was obtained at about ten fold higher ACh concentration. The maximal relaxation response to ACh (at 1–10 μM ACh concentration) was significantly greater in the ramipril-treated groups during control and the salt alternative-enriched diets (about 65–75% from maximum) as compared to those of the other groups (about 25–35% from maximum) ($P<0.05$–0.001). Ramipril treatment during control and the salt alternative-enriched diets enhanced the vascular relaxation responses to sodium nitroprusside (Figure 6). The dose-response curves of these groups were shifted to the left, and the 90% relaxation response to SNP was obtained at 0.1 μM SNP concentration. The equal relaxation was obtained at about 10 to 100 fold higher SNP concentrations in the other groups. There was no difference between different groups in the maximal relaxation response to SNP. If only ramipril-treated groups during control and the salt alternative-enriched diets were included in the statistical analysis, a slight but insignificant difference in the vascular relaxation responses to SNP was found (repeated ANOVA, $P = 0.0995$).

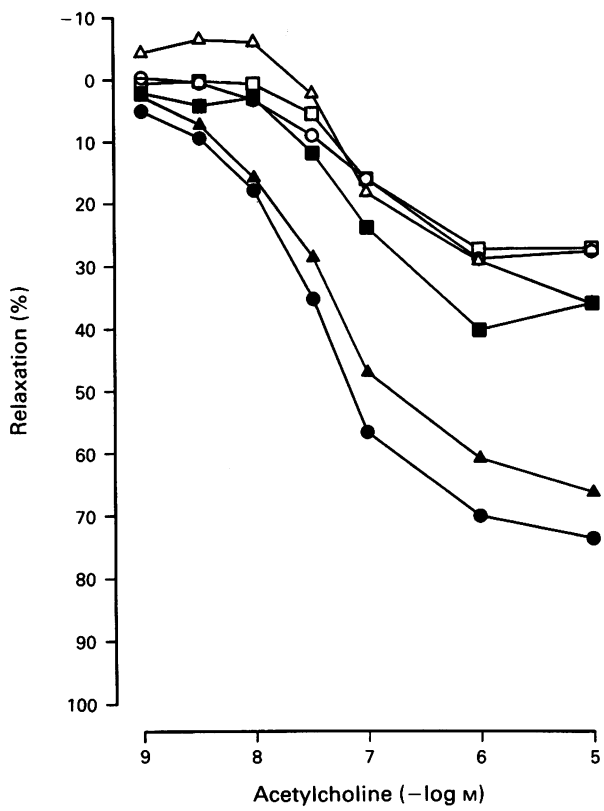


Figure 5 Cumulative relaxation responses to acetylcholine of isolated endothelium-intact mesenteric arterial rings from the stroke-prone spontaneously hypertensive rats after six weeks on the different diet and drug regimens: (○) controls on normal diet; (□) supplementation with salt (NaCl); (△) supplementation with the salt alternative; (●) ramipril during control diet; (■) ramipril during NaCl-enriched diet; (▲) ramipril during salt alternative-enriched diet. Repeated analysis of variance between-subjects effects, $P = 0.0018$; within-subject effects, $P<0.0001$; interaction, $P<0.001$. Ramipril treatment improved relaxation responses to acetylcholine during control diet ($P<0.01$ versus all ramipril-untreated groups) and during the salt alternative-enriched diet ($P<0.05$ versus ramipril-untreated groups during NaCl- and salt alternative-enriched diets; $P<0.05$–0.01 versus control group at acetylcholine concentrations 10^{-6} M and 10^{-5} M). Symbols indicate means, $n = 5$–6 in each group.

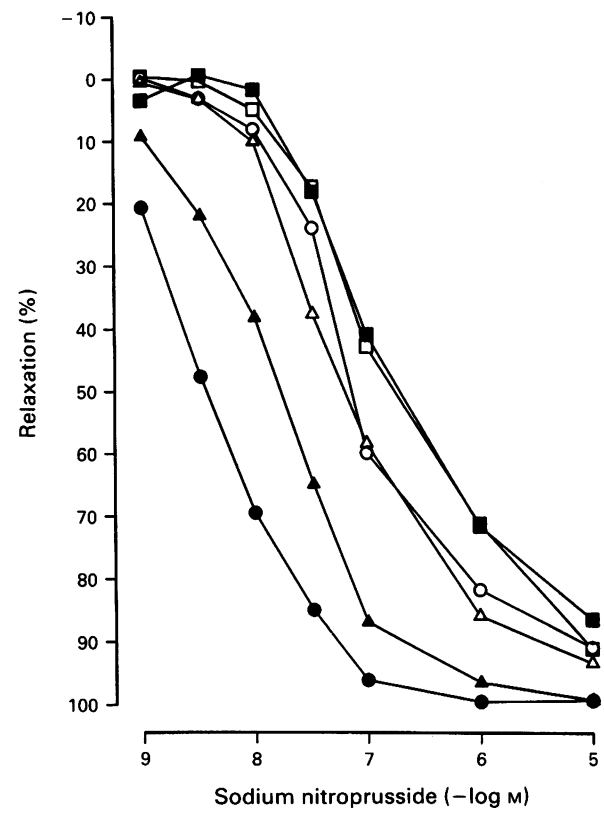


Figure 6 Cumulative relaxation responses to sodium nitroprusside of isolated endothelium-intact mesenteric arterial rings from the stroke-prone spontaneously hypertensive rats after six weeks on the different diet and drug regimens: (○) controls on normal diet; (□) supplementation with salt (NaCl); (△) supplementation with the salt alternative; (●) ramipril during control diet; (■) ramipril during NaCl-enriched diet; (▲) ramipril during salt alternative-enriched diet. Repeated analysis of variance between-subjects effects, $P<0.0001$; within-subject effects, $P<0.0001$; interaction, $P<0.0001$. Ramipril treatment improved relaxation responses to sodium nitroprusside during control diet ($P<0.001$ versus all ramipril-untreated groups and ramipril group during NaCl-enriched diet) and during the salt alternative-enriched diet ($P<0.05$–0.01 versus control group and NaCl groups with and without ramipril treatment). Symbols indicate means, $n = 5$–6 in each group.

Metabolic variables and indicators

At the end of the follow-up period, the body weights of NaCl- and salt alternative-supplemented rats were slightly decreased (repeated ANOVA between-subjects effects $P = 0.0041$; within-subject effects $P < 0.0001$, time-group interaction $P < 0.0001$; NaCl group versus control group and all ramipril-treated groups, $P < 0.05$; salt alternative group versus ramipril group on control diet, $P < 0.05$). There were no significant differences between the groups in the food intake after four weeks on the different diet and drug regimens (Table 2). The ramipril group on control diet and all groups with salt supplements, in the presence or in the absence of ramipril, had an increased diuresis. The calculated average daily dose of ramipril was 2.8 ± 0.1, 2.6 ± 0.1 and 3.0 ± 0.2 mg kg^{-1} body weight in the ramipril-treated rats on control, NaCl-enriched and the salt alternative-enriched diets, respectively ($P = 0.2048$).

Urine electrolytes

The 24 h urinary excretion of sodium was increased in all groups with salt supplements when compared to the control diet groups (Table 2). Potassium excretion was increased in the salt alternative-supplemented rats both with and without ramipril. The excretion of magnesium was markedly increased in the salt alternative groups, and it was significantly increased even in the NaCl groups. There was a marked rise in the urinary excretion of calcium in all groups with salt supplements when compared to the control diet groups. The level of phosphorus in urine was increased in NaCl supplemented rats both with and without ramipril. The level of zinc in urine was markedly increased in the NaCl and in the salt alternative groups without ramipril treatment.

Plasma electrolytes and renin activity

There were no significant differences between the different groups in the plasma levels of sodium, potassium and magnesium, or in plasma renin activity (Table 3).

Discussion

An increased intake of salt (sodium chloride; NaCl) produced a marked further elevation of blood pressure and increase in left ventricular hypertrophy as compared to the age-related increases in control SHRSP. The increased intake of sodium chloride also produced significant mortality of SHRSP during the six-week experiment. These effects of NaCl are in agreement with previous findings in SHR and SHRSP (Oparil *et al.*, 1988; Tobian, 1988; Karppanen, 1989; Mervaala *et al.*, 1992).

By contrast, the salt alternative, when added at 1.75 times higher amounts to produce the same dietary level of sodium chloride, neither caused any mortality nor induced any significant rise in blood pressure. It also caused significantly less left ventricular hypertrophy than NaCl. These results confirm our recent findings (Mervaala *et al.*, 1992). In our previous study the salt alternative prolonged the life span of SHR nearly three fold as compared with the animals receiving NaCl (Karppanen, 1989).

Previous studies have suggested that the detrimental effects of a high intake of sodium chloride could have been mediated mainly by elevated blood volume and cardiac output and by elevated sympathetic nervous system activity (Müntzel & Drüeke, 1992). Normalization of sodium and water balance and sympathetic nervous activity appeared to explain, at least in part, the protection against the harmful effects of the increased intake of sodium chloride by the increased intakes of potassium and/or magnesium and L-lysine from the salt alternative (Karppanen, 1989; Mervaala *et al.*, 1992).

In experimental and clinical studies ACEIs have been demonstrated to be effective in the treatment of cardiac hypertrophy when used at antihypertensive doses (Waeber *et al.*, 1990). In the present study, consistent with previous work (Unger *et al.*, 1984), chronic treatment of SHRSP with ramipril at an approximate daily dose of 3 mg kg^{-1} normalized blood pressure and prevented the development of left ventricular hypertrophy during a relatively low-sodium control diet. Reduction of myocardial tissue angiotensin II by

Table 2 Twenty-four hour food consumption, urine volume and urinary excretion rates of various mineral elements of stroke-prone spontaneously hypertensive rats after four weeks on the different diet and drug regimens

	C	*Na*	*SA*	*RC*	*RNa*	*RSA*	*ANOVA*	*Difference between groups at* $P < 0.05$
Food intake ($g\,d^{-1}$)	21.5 ± 0.9	21.5 ± 1.6	24.0 ± 1.3	21.4 ± 0.8	19.3 ± 1.2	21.5 ± 0.5	0.1473	–
Urine								
Volume ($ml\,d^{-1}$)	19.9 ± 2.5	76.8 ± 6.6	80.6 ± 4.7	43.3 ± 3.9	67.2 ± 2.7	56.7 ± 2.4	<0.0001	C vs. all other groups RC vs. Na, SA and RNa RSA vs. Na and SA
Sodium ($mmol\,d^{-1}$)	1.4 ± 0.1	17.2 ± 0.8	18.9 ± 1.0	1.9 ± 0.05	20.4 ± 0.5	16.4 ± 0.7	<0.0001	C and RC vs. Na, SA, RNa and RSA RNa vs. Na and RSA
Potassium ($mmol\,d^{-1}$)	3.1 ± 0.1	3.0 ± 0.2	9.2 ± 0.4	2.9 ± 0.1	3.0 ± 0.1	8.2 ± 0.4	<0.0001	C, Na, RC and RNa vs. SA and RSA
Magnesium ($mmol\,d^{-1}$)	0.25 ± 0.02	0.39 ± 0.02	0.60 ± 0.02	0.29 ± 0.03	0.44 ± 0.02	0.56 ± 0.02	<0.0001	C and RC vs. Na, SA, RNa and RSA SA and RSA vs. Na and RNa
Phosphorus ($mmol\,d^{-1}$)	0.85 ± 0.06	1.29 ± 0.05	1.02 ± 0.04	0.82 ± 0.03	1.37 ± 0.03	0.94 ± 0.06	<0.0001	Na and RNa vs. C, SA, RC and RSA SA vs. RC
Calcium ($mmol\,d^{-1}$)	0.041 ± 0.005	0.28 ± 0.02	0.32 ± 0.02	0.032 ± 0.002	0.29 ± 0.01	0.24 ± 0.02	<0.0001	C and RC vs. Na, SA, RNa and RSA RSA vs. NA and RNa
Zinc ($\mu mol\,d^{-1}$)	0.21 ± 0.02	0.63 ± 0.08	0.63 ± 0.07	0.46 ± 0.14	0.50 ± 0.07	0.38 ± 0.07	0.0046	C vs. Na and SA

The control group (C, $n = 10$) and the ramipril group (RC, $n = 10$; ramipril mixed in the chow to produce an approximate daily dose of 3 mg kg^{-1}) received standard rat chow (Na 0.3%). The chow of the high sodium chloride group (Na, $n = 10$) and that of the high sodium chloride + ramipril group (RNa, $n = 10$; ramipril as above) was supplemented with salt (6% sodium chloride). The chow of the salt alternative group (SA, $n = 10$) and that of the salt alternative + ramipril group (RSA, $n = 10$; ramipril as above) was supplemented with 10.5% of the salt alternative (Pansalt).

Table 3 The plasma levels of sodium, potassium and magnesium and plasma renin activity of stroke-prone spontaneously hypertensive rats after six weeks on the different diet and drug regimens

	C	*Na*	*SA*	*RC*	*RNa*	*RSA*	*ANOVA*	*Difference between groups at* $P<0.05$
Plasma								
Sodium (mmol l^{-1})	136.4 ± 1.2	134.4 ± 1.7	132.6 ± 2.2	133.3 ± 1.9	133.3 ± 1.6	136.7 ± 1.7	0.4070	–
Potassium (mmol l^{-1})	3.42 ± 0.08	3.53 ± 0.17	3.51 ± 0.08	3.62 ± 0.10	3.56 ± 0.12	3.72 ± 0.18	0.5967	–
Magnesium (mmol l^{-1})	0.69 ± 0.01	0.74 ± 0.08	0.77 ± 0.03	0.65 ± 0.02	0.72 ± 0.04	0.71 ± 0.01	0.2711	–
Renin activity (ng Ang I ml^{-1} h^{-1})	8.33 ± 1.3	16.6 ± 4.8	10.7 ± 2.9	14.6 ± 2.7	11.8 ± 2.6	13.2 ± 2.6	0.3962	–

The control group (C, $n = 10$) and the ramipril group (RC, $n = 10$; ramipril mixed in the chow to produce an approximate daily dose of 3 mg kg^{-1}) received standard rat chow (Na 0.3%). The chow of the high sodium chloride group (Na, $n = 10$) and that of the high sodium chloride + ramipril group (RNa, $n = 10$; ramipril as above) was supplemented with salt (6% sodium chloride). The chow of the salt alternative group (SA, $n = 10$) and that of the salt alternative + ramipril group (RSA, $n = 10$; ramipril as above) was supplemented with 10.5% of the salt alternative (Pansalt).

chronic treatment with ACEI in SHR has been reported (Nagano *et al.*, 1991). There is much evidence to suggest that angiotensin II is involved in the production of myocardial (Pfeffer *et al.*, 1982; Hall & Karlberg, 1986; Clozel *et al.*, 1989; Childs *et al.*, 1990; Schunkert *et al.*, 1990; Brilla *et al.*, 1991; Lindpaintner & Ganten, 1991) and vascular wall hypertrophy (Kato *et al.*, 1991). ACE inhibition increases the levels of bradykinin which appears to give protection against the development of cardiovascular hypertrophy (Wiemer *et al.*, 1991).

Ramipril treatment did not produce any significant lowering in blood pressure, and it only partially protected against the development of left ventricular hypertrophy, in SHRSP receiving the NaCl-enriched diet. However, in spite of the lack of effect on these cardiovascular variables, ramipril protected against stroke and mortality. In agreement with our observation, Stier *et al.* (1989) also found that, during a high intake of sodium chloride, ACEI treatment decreased SHRSP mortality. The mechanism of this protective effect of ACEI is not clear.

In spite of the high intake of sodium chloride, ramipril produced a remarkable antihypertensive effect in SHRSP receiving the salt alternative-enriched diet, and it protected against stroke and mortality. In addition, during the salt alternative-enriched diet ramipril protected against the development of sodium chloride-induced cardiac hypertrophy. Hence, increased intakes of potassium and/or magnesium and L-lysine during a high intake of sodium chloride improved the beneficial effects of ramipril. It has been suggested that ramipril treatment might increase the risk of hyperkalaemia, at least in patients taking supplemental potassium or a potassium-sparing diuretic (Waeber *et al.*, 1990). However, the present salt alternative did not cause any tendency to hyperkalaemia, either alone or in combination with ramipril treatment.

The inhibitory effect of a high intake of sodium chloride on the antihypertensive effect of ACEI is consistent with previous observations (Fernandez *et al.*, 1988; Waeber *et al.*, 1990). It has been suggested that the resistance towards the effects of ACEI results from salt-induced inhibition in the activity of the renin-angiotensin-aldosterone system (Waeber *et al.*, 1990). In the present study increased intake of potassium and/or magnesium and L-lysine from the salt alternative counteracted the inhibitory effect of high intake of sodium chloride on the cardiovascular effects of ramipril. However, neither in the NaCl nor in the salt alternative supplemented SHRSP did the increased intake of sodium chloride affect plasma renin activity (PRA). Furthermore, in the presence of ramipril treatment PRA was only slightly and insignificantly increased. Hence, PRA does not appear to explain the marked improvement of the effects of ramipril by the salt alternative in comparison to NaCl.

Previous studies have shown that, in experimental hypertension, the endothelium-dependent relaxation of vascular smooth muscle is impaired (Konishi & Su, 1983; Lüscher *et al.*, 1987; Tesfamariam & Halperin, 1988). Arvola *et al.* (1993) recently demonstrated that quinapril treatment improved the endothelium-dependent vascular relaxation of SHR. The ACEI treatment has been shown to improve the endothelium-dependent vascular relaxation in SHR by increasing the production of endothelium-derived relaxing factor(s) and by preventing the degradation of bradykinin liberated from the endothelial cells (Clozel *et al.*, 1990; Wiemer *et al.*, 1991; Arvola *et al.*, 1993). In the present study the effects of long-term angiotensin-converting enzyme inhibition and those of long-term salt supplementations on arterial functions were estimated by responses of the mesenteric arterial rings *in vitro*. Supplementation with NaCl, but not that with the salt alternative, increased vascular contractile responses to noradrenaline. Ramipril treatment improved the arterial relaxation responses to acetylcholine as well as to sodium nitroprusside, suggesting the involvement of both endothelium-dependent and endothelium-independent mechanisms. This could partly result from normalization of blood pressure in the ramipril-treated SHR and thus from better preservation of the endothelial functions during the six-week study. Consistent with our findings Arvola *et al.* (1993) have shown that, in SHR, ACE treatment improves the vascular relaxation responses to three different types of vasorelaxants (to acetylcholine, nitroprusside and isoprenaline). They have suggested that the augmentation of the endothelium-dependent and endothelium-independent vascular relaxation responses by ACEI treatment can be explained, at least in part, by improved cellular calcium regulation. In the present study the vascular relaxation enhancing effects of ramipril were blocked by NaCl supplementation. However, during the salt alternative-enriched diet the vascular relaxation enhancing effects of ramipril were only slightly attenuated. The observed changes in vascular reactivity may partly explain the hypertensive effect of sodium chloride and the beneficial effects of potassium, magnesium and L-lysine.

Supplementation of SHRSP with NaCl or the salt alternative produced a six to eight fold increase in the urinary calcium excretion, both in the absence and in the presence of ramipril treatment. This finding confirms the results of previous studies demonstrating a rise in urinary calcium excretion with increasing intake of sodium chloride both in man and animals (Shortt & Flynn, 1990). Since the calciuric effect was similar in the NaCl and the salt alternative supplemented groups, it neither explains the markedly different cardiovas-

cular effects of NaCl and the salt alternative, nor the remarkable difference in the ramipril-induced cardiovascular effects between the groups. However, our finding lends further support to the suggestion that a high intake of sodium chloride may contribute to the development of osteoporosis (Shortt & Flynn, 1990). The increase in urinary excretion of phosphorus by increased intake of sodium chloride may explain the fall in serum phosphate levels, observed by Altura & Altura (1991–1992) in rats with malignant salt-induced hypertension. Interestingly, rats given magnesium exhibited restoration of phosphate to normal levels (Altura & Altura, 1991–1992). In agreement with this, in our study the excretion of phosphorus was not significantly increased in the rats receiving the magnesium-containing salt alternative. Consistently with previous studies (Wallach & Verch, 1986) a high intake of sodium chloride increased the urinary excretion of magnesium.

In conclusion, the present sodium-reduced, potassium- and magnesium-enriched salt alternative was virtually devoid of the hypertensive and cardiac hypertrophy producing effects, typical of NaCl. Replacement of salt in the diet by the salt alternative remarkably improved the cardiovascular effects of ramipril. This effect was due to increased intake of potassium and/or magnesium and L-lysine. The present salt alternative with moderate content of potassium did not produce any tendency to hyperkalaemia, even when combined with ACEI treatment.

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References

ALTURA, B.M. & ALTURA, B.T. (1990). Role of magnesium in the pathogenesis of hypertension: Relationship to its actions on cardiac and vascular smooth muscle. In *Hypertension: Pathophysiology, Diagnosis and Management*. ed. Laragh, J.H. & Brenner, B.M. pp. 1003–1025. New York: Raven Press.

ALTURA, B.M. & ALTURA, B.T. (1991–1992). Cardiovascular risk factors and magnesium: relationship to atherosclerosis, ischemic heart disease and hypertension. *Magnes. Trace Elem.*, **10**, 182–192.

ARVOLA, P., RUSKOAHO, H., WUORELA, H., PEKKI, A., VAPAATALO, H. & PÖRSTI, I. (1993). Quinapril treatment and arterial smooth muscle responses in spontaneously hypertensive rats. *Br. J. Pharmacol.*, **108**, 980–990.

BRILLA, C.G., JANICKI, J.S. & WEBER, K.T. (1991). Cardioreparative effects of lisinopril in rats with genetic hypertension and left ventricular hypertrophy. *Circulation*, **83**, 1771–1779.

CAPPUCCIO, F.P. & MACGREGOR, G.A. (1991). Does potassium supplementation lower blood pressure? A meta-analysis of published trials. *J. Hypertens*, **9**, 465–473.

CHILDS, T.J., ADAMS, M.A. & MAK, A.S. (1990). Regression of cardiac hypertrophy in spontaneously hypertensive rats by enalapril and the expression of contractile proteins. *Hypertension*, **16**, 662–668.

CLOZEL, J.-P., KUHN, H. & HEFTI, F. (1989). Effects of chronic ACE inhibition on cardiac hypertrophy and coronary vascular reserve in spontaneously hypertensive rats with developed hypertension. *J. Hypertens.*, **7**, 267–275.

CLOZEL, M., KUHN, H. & HEFTI, F. (1990). Effects of angiotensin converting enzyme inhibitors and of hydralazine on endothelial function in hypertensive rats. *Hypertension*, **16**, 532–540.

FERNANDEZ, D., BOLLI, P., SNEDDEN, W., VASDEV, S. & FERNANDEZ, P.G. (1988). Modulation of left ventricular hypertrophy by dietary salt and inhibition of angiotensin converting enzyme. *J. Hypertens.*, **6** (suppl 4), S145–S147.

FROST, C.D., LAW, M.R. & WALD, N.J. (1991). By how much does dietary salt reduction lower blood pressure? II- Analysis of observational data within populations. *Br. Med. J.*, **302**, 815–818.

HALL, C. & KARLBERG, B.E. (1986). Plasma concentration of angiotensin II and aldosterone during acute left ventricular failure in the dog. *Res. Exp. Med.*, **186**, 387–395.

JULA, A., RÖNNEMAA, T., RASTAS, M., KARVETTI, R.L. & MÄKI, J. (1990). Long-term nonpharmacological treatment for mild to moderate hypertension. *J. Int. Med.*, **227**, 413–421.

KARPPANEN, H. (1989). New oral salt in treatment of high blood pressure. *Magnesium*, **8**, 274–287.

KARPPANEN, H. (1990). Epidemiologic evidence for considering magnesium deficiency as a risk factor for cardiovascular diseases. *Magnesium Bull.*, **12**, 80–86.

KATO, H., SUZUKI, H., TAJIMA, S., OGATA, Y., TOMINAGA, T., SATO, A. & SARUTA, T. (1991). Angiotensin II stimulates collagen synthesis in cultured vascular smooth muscle cells. *J. Hypertens.*, **9**, 17–22.

KHAW, K.-T. & BARRETT-CONNOR, E. (1986). Dietary potassium and stroke-associated mortality – a 12 year prospective population based study. *J. Hypertens.*, **4**, 794–798.

KONISHI, M. & SU, C. (1983). Role of endothelium in dilator responses of spontaneously hypertensive rat arteries. *Hypertension*, **5**, 881–886.

LAAKSO, J.T., TIKKANEN, H. & MICHELSSON, J.-E. (1991). Element concentrations in normal and immobilization-induced necrotic rabbit muscles. *Trace Elem. Med.*, **8**, 34–42.

LAW, M.R., FROST, C.D. & WALD, N.J. (1991a). By how much does dietary salt reduction lower blood pressure? I- Analysis of observational data among populations. *Br. Med. J.*, **302**, 811–815.

LAW, M.R., FROST, C.D. & WALD, N.J. (1991b). By how much does dietary salt reduction lower blood pressure? III- Analysis of data from trials of salt reduction. *Br. Med. J.*, **302**, 819–824.

LIND, L., LITHELL, H., POLLARE, T. & LJUNGHALL, S. (1991). Blood pressure response during long-term treatment with magnesium is dependent on magnesium status: A double-blind placebo-controlled study in essential hypertension and in subjects with high-normal blood pressure. *Am. J. Hypertens.*, **4**, 674–679.

LINDPAINTNER, K. & GANTEN, D. (1991). The cardiac renin-angiotensin system. An appraisal of present experimental and clinical evidence. *Circ. Res.*, **68**, 905–921.

LINZ, W., SCHAPER, J., WIEMER, G., ALBUS, U. & SCHÖLKENS, B.A. (1992). Ramipril prevents left ventricular hypertrophy with myocardial fibrosis without blood pressure reduction: a one year study in rats. *Br. J. Pharmacol.*, **107**, 970–975.

LÜSCHER, T.F., RAIJ, L. & VANHOUTTE, P.M. (1987). Endothelium-dependent vascular responses in normotensive and hypertensive Dahl rats. *Hypertension*, **9**, 157–163.

MATTHEWS, J.N.S., ALTMAN, D.G., CAMPBELL, M.J. & ROYSTON, P. (1990). Analysis of serial measurements in medical research. *Br. Med. J.*, **300**, 230–235.

MERVAALA, E.M.A., HIMBERG, J.-J., LAAKSO, J., TUOMAINEN, P. & KARPPANEN, H. (1992). Beneficial effects of a potassium- and magnesium-enriched salt alternative. *Hypertension*, **19**, 535–540.

MÜNTZEL, M. & DRÜEKE, T. (1992). A comprehensive review of the salt and blood pressure relationship. *Am. J. Hypertens.*, **5**, 1s–42s.

NAGANO, M., HIGAKI, J., MIKAMI, H., NAKAMURA, M., HIGASHIMORI, K., KATAHIRA, K., TABUCHI, Y., MORIGUCHI, A., NAKAMURA, F. & OKIHARA, T. (1991). Converting enzyme inhibitors regressed cardiac hypertrophy and reduced tissue angiotensin II in spontaneously hypertensive rats. *J. Hypertens.*, **9**, 595–599.

OKAMOTO, K., YAMORI, Y. & NAGAOKA, A. (1974). Establishment of the stroke-prone spontaneously hypertensive rat (SHR). *Circ. Res.*, **34/35** (Suppl I), 143–153.

OPARIL, S., MENG, Q.C., CHEN, Y.-F., YANG, R.-H., JIN, H. & WYSS, J.M. (1988). Genetic basis of NaCl-sensitive hypertension. *J. Cardiovasc. Pharmacol.*, **12**, S56–S69.

PFEFFER, J.M., PFEFFER, M.A., MIRSKY, I. & BRAUNWALD, E. (1982). Regression of left ventricular hypertrophy and prevention of left ventricular dysfunction by captopril in the spontaneously hypertensive rats. *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 3310–3314.

SCHUNKERT, H., DZAU, V.J., TANG, S.S., HIRSCH, A.T., APSTEIN, C.S. & LORELL, B.H. (1990). Increased rat cardiac angiotensin converting enzyme activity and mRNA expression in pressure overload left ventricular hypertrophy. Effects on coronary resistance, contractility and relaxation. *J. Clin. Invest.*, **86,** 1913–1920.

SHORTT, C. & FLYNN, A. (1990). Sodium-calcium interrelationships with specific reference to osteoporosis. *Nutr. Res. Rev.*, **3,** 101–115.

SJÖGREN, A., EDVINSSON, L. & FALLGREN, B. (1989). Magnesium deficiency in coronary artery disease and cardiac arrhythmias. *J. Int. Med.*, **226,** 213–222.

STIER, Jr., C.T., BENTER, I.F., AHMAD, S., ZUO, H., SELIG, N., ROETHEL, S., LEVINE, S. & ITSKOVITZ, H.D. (1989). Enalapril prevents stroke and kidney dysfunction in salt-loaded stroke-prone spontaneously hypertensive rats. *Hypertension*, **13,** 115–121.

SUGIMOTO, T., TOBIAN, L. & GANGULI, M.C. (1988). High potassium diets protect against dysfunction of endothelial cells in stroke-prone spontaneously hypertensive rats. *Hypertension*, **11,** 579–585.

TESFAMARIAM, B. & HALPERIN, W. (1988). Endothelium-dependent and endothelium-independent vasodilatation in resistance arteries from hypertensive rats. *Hypertension*, **11,** 440–444.

TIKKANEN, I., FYHRQUIST, F. & PUUTULA-RÄSÄNEN, L. (1980). Enzyme inhibitors for renin assay in rat plasma. *Clin. Sci.*, **59,** 381–383.

TOBIAN, L. (1988). Potassium and sodium in hypertension: The Volhard Lecture. *J. Hypertens.*, **6** (suppl 4), 12–24.

UNGER, T., FLECK, T., GANTEN, D., LANG, R.E. & RETTIG, F. (1984). 2-(N- (S)-1-ethoxycarbonyl-3-phenylpropyl-L-alanyl)-(1S, 3S,5S)-2-azabicyclo(3.3.0) octane-3-carboxylic acid (Hoe 498): antihypertensive action and persistent inhibition of tissue converting enzyme activity in spontaneously hypertensive rats. *Arzneim.-Forsch./Drug Res.*, **34,** 1426–1430.

WAEBER, B., NUSSBERGER, J. & BRUNNER, H.R. (1990). Angiotensin-converting-enzyme inhibitors in hypertension. In *Hypertension: Pathophysiology, Diagnosis and Management*. ed. Brenner, B.M. & Laragh, J.H. pp. 2209–2232. New York: Raven Press.

WALLACH, S. & VERCH, R.L. (1986). Tissue magnesium in spontaneously hypertensive rats. *Magnesium*, **5,** 33–38.

WIEMER, G., SCHÖLKENS, B.A., BECKER, R.H.A. & BUSSE, R. (1991). Ramiprilat enhances endothelial autacoid formation by inhibiting breakdown of endothelium-derived bradykinin. *Hypertension*, **18,** 558–563.

YAMORI, Y., HORIE, R., AKIGUCHI, I., KIHARA, M., NARA, Y. & LOVENBERG, W. (1982). Symptomatological classification in the development of stroke in stroke-prone spontaneously hypertensive rats. *Jpn. Circ. J.*, **46,** 274–283.

YOUNG, D.B., MCCAA, R.E., PAN, Y.-J. & GUYTON, A.C. (1976). The natriuretic and hypotensive effects of potassium. *Circ. Res.*, **38,** 84–89.

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Pulmonary effects of type V cyclic GMP specific phosphodiesterase inhibition in the anaesthetized guinea-pig

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1 We have investigated the bronchodilator potential of type V phosphodiesterase (PDE V) inhibitors in anaesthetized ventilated guinea-pigs using the potent and selective PDE V inhibitor, SK&F 96231. We have compared its activity to that of salbutamol, the PDE III inhibitors, siguazodan and SK&F 95654 and to the PDE IV inhibitor rolipram.

2 Administered as an i.v. infusion SK&F 96231 (0.6 and 1 mg kg^{-1} min^{-1}, i.v.) caused a slowly developing inhibition of histamine (100 nmol kg^{-1}, i.v.)-induced bronchoconstriction and elevated tracheal cyclic GMP levels in the anaesthetized guinea-pig. SK&F 96231 (0.1 and 0.3 mg kg^{-1} min^{-1}, i.v.) was without effect on histamine-induced bronchoconstriction. In the presence of a sub-threshold infusion of SNP (0.1 μmol kg^{-1} min^{-1}, i.v.) there was a marked enhancement of SK&F 96231-induced inhibition of histamine responses such that at infusion rates that were ineffective alone, SK&F 96231 caused a $>50\%$ inhibition of histamine responses. The stimulation of tracheal cyclic GMP accumulation by SK&F 96231 was also potentiated.

3 Administered directly into the airway, SK&F 96231 (300 μg in 5 mg lactose carrier) was largely without effect on histamine-induced bronchoconstriction ($4.9 \pm 1.9\%$ inhibition). In the presence of SNP (0.1 μmol kg^{-1} min^{-1}, i.v.) or isosorbide dinitrate (200 μg administered by insufflation into the trachea) there was a marked potentiation of the inhibitory activity of SK&F 96231 ($40 \pm 4\%$ and $62 \pm 1.8\%$ respectively).

4 Salbutamol and rolipram (3–300 μg by insufflation) caused a dose-related inhibition of histamine responses with a maximum of $91 \pm 2\%$ and $59 \pm 10\%$ respectively. The PDE III inhibitor, siguazodan, was without effect on histamine responses but they were reduced ($27.7 \pm 4.8\%$ at 300 μg) by SK&F 95654. There was a marked enhancement of the inhibitory activity of rolipram in the presence of SK&F 95654.

5 We conclude that SK&F 96231 has weak anti-spasmogenic activity in the guinea-pig *in vivo*, we suggest that this is primarily a consequence of a low endogenous guanylate cyclase activity in the airway. The potentiation of the anti-spasmogenic activity of SK&F 96231 by SNP suggests that a combination of PDE V inhibitor and guanylate cyclase agonist might provide significant bronchodilator activity.

6 We have established that PDE IV inhibitors are bronchodilators when administered directly into the airway of anaesthetized guinea-pigs but that PDE III inhibitors are only weakly active. The marked enhancement of the inhibitory activity of rolipram by the PDE III inhibitor, SK&F 95654, indicates that inhibitors of both PDE III and PDE IV might offer greater potential as bronchodilators than inhibitors of either isoenzyme alone.

Keywords: Cyclic nucleotide phosphodiesterases; selective PDE inhibition; SK&F 96231; rolipram; siguazodan; SK&F 95654; SNP; bronchodilatation; anti-spasmogenic activity

Introduction

Adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) play an important role in the regulation of airway tone, elevations in either being causally associated with smooth muscle relaxation (Iscii & Murad, 1989; Murray, 1990; Torphy & Undem, 1991).

Intracellular concentrations of cyclic nucleotides are determined by their relative rates of formation, via agonist-induced stimulation of adenylate and guanylate cyclase, and hydrolysis by phosphodiesterase enzymes (PDE). Multiple PDE isoenzymes have been isolated from a variety of tissues and based on their kinetic characteristics, substrate specificity, sensitivity to endogenous regulators and susceptibility to inhibition by selective inhibitors have been divided into five distinct families (PDE I–V; Beavo, 1988; Beavo & Reifsnyder, 1990). Of these PDE I, II and III can utilise both cyclic AMP and cyclic GMP as substrate, PDE IV is a cyclic AMP-specific PDE and PDE V a cyclic GMP-specific PDE (Beavo, 1989; Beavo & Reifsnyder, 1990). Although their relative proportions vary depending on species and airway generation, members of all five families have been detected in smooth muscle from canine (Trophy & Cieslinski, 1990), bovine (Shahid *et al.*, 1991), guinea-pig (Takagi *et al.*, 1992; Turner *et al.*, unpublished observations) and human (de Boer *et al.*, 1992; Giembycz *et al.*, 1992; Cortijo *et al.*, 1993) airway.

The bronchodilator activity and anti-asthma potential of xanthines is attributed, at least in part, to non-selective inhibition of multiple PDE activities (Torphy & Undem, 1991). More recently, however, interest has focused on the potential of isoenzyme-selective PDE inhibitors as bronchodilator and anti-inflammatory agents. Selective inhibitors of the PDE III and IV isoenzymes are effective relaxants of guinea-pig trachea (Harris *et al.*, 1989; Tomkinson *et al.*, 1993), canine trachealis (Torphy *et al.*, 1988; Torphy *et al.*, 1991), bovine trachea (PDE III inhibitors are only weakly active, Shahid *et al.*, 1991) and human bronchus (de Boer *et*

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al., 1992; Cortijo *et al.*, 1993) *in vitro* and have bronchodilator activity in the guinea-pig (Harris *et al.*, 1989; Cortijo *et al.*, 1993; Howell *et al.*, 1993) and dog (Heaslip *et al.*, 1991) *in vivo*. In contrast despite the presence of PDE V in the airway of all species so far studied, the PDE V inhibitor zaprinast alone is without effect on canine tracheal tone (Torphy *et al.*, 1991) and at concentrations that retain selectivity for PDE V, is a relatively ineffective relaxant of bovine trachea (Hall *et al.*, 1990; Shahid *et al.*, 1991) and human bronchus (de Boer *et al.*, 1992) *in vitro*. Nevertheless it is generally accepted that increases in intracellular cyclic GMP are causally related to relaxation of airway smooth muscle (Polson *et al.*, 1985; Torphy & Undem, 1991). In this regard both sodium nitroprusside and atrial naturetic peptide (ANP) increase cyclic GMP levels and potently relax bovine and canine tracheal smooth muscle (Lorenz & Wells, 1983; Torphy *et al.*, 1985; Ishii & Murad, 1989). Furthermore, zaprinast potentiates both sodium nitroprusside-induced relaxation of canine trachealis and the associated increase in cyclic GMP accumulation (Torphy *et al.*, 1991).

We have previously reported that SK&F 96231 (1,7.-dihydro-2-(2-propoxyphenyl)-6H-purin-6-one) is a potent and selective inhibitor of the cyclic GMP PDE V (Murray *et al.*, 1991). SK&F 96231 relaxes guinea-pig trachea precontracted with K^+, histamine or the thromboxane-mimetic, U46619 and causes a slow time-dependent increase in the cyclic GMP content of guinea-pig lung strips, *in vitro* (Murray *et al.*, 1991). *In vivo*, SK&F 96231 has been shown to produce a modest, short-lived inhibition of U44619-induced bronchoconstriction in anaesthetized guinea-pigs and is approximately 4 fold more potent than zaprinast in this regard (Murray *et al.*, 1991).

In the present study we have used SK&F 96231 to examine the role of PDE V in mediating bronchodilatation in the anaesthetized guinea-pig, a widely employed model of human asthma. In addition we have compared its activity to that of the PDE III inhibitors, siguazodan or SK&F 95654 and the PDE IV inhibitor, rolipram.

Methods

In vivo *inhibition of histamine-induced bronchoconstriction in anaesthetized guinea-pigs*

Male Dunkin-Hartley guinea-pigs (450–570 g) were anaesthetized with sodium pentobarbitone (Sagatal, 50–60 mg kg^{-1}, i.p.). A carotid artery was cannulated for measurement of blood pressure and a jugular vein for administration of test substances. The trachea was cannulated and the animals mechanically ventilated with room air using a Palmer pump set at a stroke volume of 1 ml 100 g^{-1} and a respiratory rate of 50–55 inflations min^{-1}. Pulmonary inflation pressure (PIP) was measured with a Druck PDCR 75 pressure transducer attached to a side arm off the tracheal cannula. After a 15 min equilibration period, histamine 100 nmol kg^{-1}, i.v. was administered at 5 min intervals. When a reproducible increase in PIP had been established, SK&F 96231 or vehicle was administered by constant i.v. infusion (6 ml h^{-1}) for 30 min. In a further series of experiments SK&F 96231, rolipram, siguazodan, SK&F 95654, SNP, isosorbide dinitrate or vehicle were administered directly into the airway. In the latter studies agents were administered as dry powders in 5 mg lactose carrier; animals were temporarily disconnected from the ventilator and the PDE inhibitors introduced into the airway by insufflation into the trachea.

In some experiments, the effects of a sub-threshold dose of SNP on the SK&F 96231 response was investigated; in these studies a reproducible increase in PIP to histamine was established and SNP infused (10^{-7} mol kg^{-1} min^{-1}, i.v.) for 15 min prior to infusion of SK&F 96231 + SNP for 30 min, or administration of SK&F 96231 into the airway.

Histamine-induced bronchoconstriction was assessed as the difference between the basal PIP and the maximum increase in PIP caused by histamine, each animal serving as its own control.

In a separate series of experiments, tracheae were removed at time intervals following infusion of SK&F 96231, SNP or their combination and snap frozen in liquid nitrogen and stored at − 70°C prior to measurement of cyclic GMP levels. Cyclic nucleotides were extracted by addition of 1 ml cold 0.3 M perchloric acid to the frozen tissues; tissues were then finely chopped, in the perchloric acid, and homogenized (Polytron PTA7KI probe 4 × 10 s burst, setting 8). Following centrifugation, acid was removed from the supernatant by extraction with a 50/50 (v/v) mixture of tri-n-octylamine and freon. Cyclic GMP levels were estimated with a commercially available (Amersham) radioimmunoassay kit.

Protein estimations were determined by the Bio-Rad protein assay based on the method described by Bradford (1976).

Guinea-pig isolated trachea

Male Dunkin-Hartley guinea-pigs weighing from 350–450 g were killed by cervical dislocation. Each trachea was rapidly excised, placed in oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit solution (composition in mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2SO_4 1.2, D-glucose 5.5, $CaCl_2$ 2.5) at room temperature and trimmed free of adherent fat and connective tissue.

Each trachea was cut into rings containing 3 adjacent cartilage plates, the rings were opened opposite the trachealis and suspended in 25 ml organ baths containing Krebs solution at 37°C, under an initial load of 2.5 g. Following a 60 min equilibration period, tissues were primed for contractile activity by addition of 1 μM carbachol (CCh). This procedure was repeated 3 times after washing. Sustained contractions were elicited by addition of 0.1 or 1.0 μM CCh. When the increase in tension had stabilized, the relaxant activity of SK&F 96231 and sodium nitroprusside (SNP) were investigated by their cumulative addition to the organ bath. Changes in tension were measured with a dynamometer UF1 isometric transducer coupled to an Lectromed MT8P chart recorder. Relaxations were expressed as a percentage of the maximum relaxation induced by 10^{-4} M SNP. EC_{50} values are expressed as geometric means with 95% confidence limits.

Data analysis

The data are expressed as mean ± s.e.mean and were compared by Mann Whitney U test for unpaired observations. A two-tailed probability of <0.05 was considered significant. $t_{\frac{1}{2}}$ values for PDE inhibitors and salbutamol represent the time to 50% recovery of histamine responses.

Materials

All drugs and chemicals were obtained from Sigma or BDH Chemicals (both of Poole, Dorset). The cyclic GMP radioimmunoassay kit was from Amersham International (Amersham, Buckinghamshire). SK&F 96231 (1,7-dihydro-2-(2-propoxyphenyl)-6H purin-6-one), siguazodan (SK&F 94836 2-cyano-1-methyl-3-[4-(4-methyl-6-oxo-1,4,5,6-tetrahydropyridazin-3-yl)phenyl]guanidine, SK&F 95654 (**R**,**S**-4,5-dihydro-6-[4-(1,4-dihydro-4-oxopyriiny-1-yl)phenyl]-5-methyl-3(2H)-pyridazinone) and rolipram(4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidinone) were synthesized by Dr W.J. Coates of the Department of Medicinal Chemistry, SmithKline Beecham, The Frythe, Welwyn.

Results

Anti-spasmogenic activity of SK&F 96231 in vivo

SK&F 96231 administered as an i.v. infusion at 0.6 and 1.0 mg kg^{-1} min^{-1} caused a slowly developing inhibition of histamine (100 nmol kg^{-1}, i.v.)-induced bronchoconstriction which reached a maximum (ca 40% and 90% respectively) 30 min after commencing the infusion. SK&F 96231 infused at 0.1 and 0.3 mg kg^{-1} min^{-1} was without effect on histamine-induced bronchoconstriction (Figure 1a). In the presence of SNP (0.1 μmol kg^{-1} min^{-1}) which alone was without effect on the histamine-induced increase in PIP, there was a pronounced enhancement of the anti-spasmogenic activity of

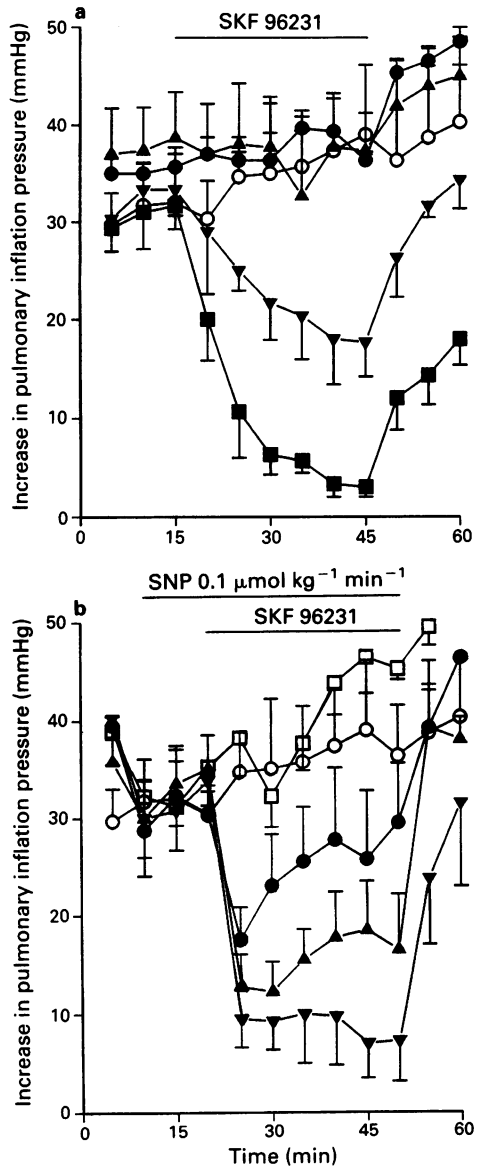


Figure 1 Effect of SK&F 96231 infused i.v. in anaesthetized ventilated guinea-pigs on histamine-induced bronchoconstriction. The increase in pulmonary inflation pressure to histamine (100 nmol kg^{-1}, i.v.) was measured at a tidal volume of 1 ml 100 g^{-1} and a ventilation rate of 50–55 breaths min^{-1}. Histamine responses were obtained at 5 min intervals. SK&F 96231 alone (a) or in the presence of 0.1 μmol kg^{-1} min^{-1} sodium nitroprusside (SNP) (b) was infused over 30 min at a rate of 0.1 (●), 0.3 (▲), 0.6 (▼) or 1 (■) mg $kg^{-1}min^{-1}$; (○) responses to histamine in vehicle control animals and; (□) effect of SNP alone. SNP was infused for 15 min prior to commencing infusion of SK&F 96231. Each point is the mean ± s.e.mean of 3 experiments.

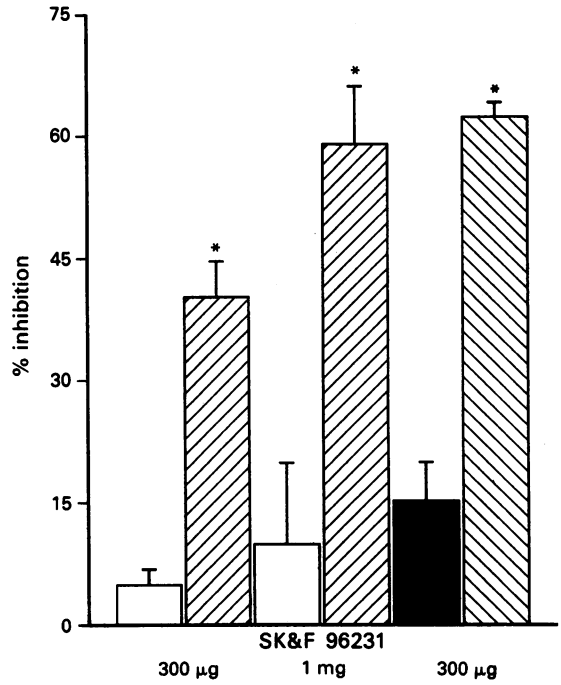


Figure 2 Inhibition of histamine-induced (100 nmol kg^{-1}, i.v.) bronchoconstriction in anaesthetized guinea-pigs by SK&F 96231 administered by insufflation into the airway. Open columns are responses to SK&F 96231 (300 μg or 1 mg, $n = 5$) alone and ▨ in the presence of SNP (0.1 μmol kg^{-1} min^{-1}, i.v., $n = 5$) or ▧ isosorbide dinitrate (200 μg by insufflation, $n = 3$). The solid column is the response to isosorbide alone ($n = 4$). SK&F 96231 was administered in 5 mg lactose carrier, which alone caused a small potentiation of the response to histamine (5.1 ± 3.2%, $n = 5$). The SNP infusion also caused a small increase in the bronchoconstriction elicited by histamine (7.4 ± 5.9%, $n = 5$). $^*P < 0.05$ compared to SK&F 96231 alone.

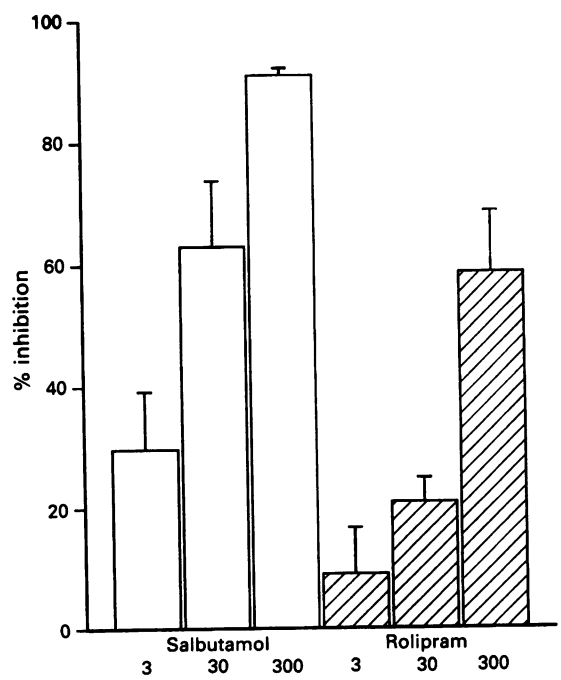


Figure 3 The effect of salbutamol and rolipram (both 3, 30 and 300 μg), administered by insufflation into the airway, on histamine-induced (100 nmol kg^{-1}, i.v.) bronchoconstriction in the anaesthetized guinea-pig. Compounds were administered directly into the airway in 5 mg lactose carrier. Results are expressed as % inhibition of the histamine response ($n = 3$).

SK&F 96231 (Figure 1b). Under these latter conditions the inhibition of histamine-induced bronchoconstriction was rapid in onset, achieving a maximum within 5 min of starting the SK&F 96231 infusion; in addition doses which previously were devoid of inhibitory activity were found to cause > 50% inhibition of histamine bronchoconstriction in the presence of SNP.

Administered directly into the airway, SK&F 96231 (300 μg or 1 mg) caused a small, short-lived (4.9 ± 1.9%, $t_{\frac{1}{2}}$ 6.6 ± 1.9 min and 9.9 ± 10%, $t_{\frac{1}{2}}$ 4.7 ± 2.3 min respectively) inhibition of the increase in pulmonary inflation pressure elicited by bolus injection of histamine (100 nmol kg^{-1}, i.v.). SK&F 96231 however significantly inhibited the histamine-induced bronchoconstriction (40 ± 4% at 300 μg and 59 ± 7% at 1 mg, $n = 5$, $P < 0.05$) in the presence of SNP (infused i.v. at a dose that alone was without effect on the histamine induced bronchoconstriction:- 0.1 μmol kg^{-1} min^{-1}). There was however no effect of SNP on the duration of the inhibition.

In contrast to the potentiation of the antispasmogenic activity of SK&F 96231 by SNP, when the latter was administered by i.v. infusion, there was no potentiation of the effects of SK&F 96231 by SNP (300 μg) when both were administered locally. In contrast whilst isosorbide dinitrate (200 μg) administered directly into the airway caused only a 15 ± 5% inhibition of the responses to histamine its combination with SK&F 96231 (300 μg) reduced the histamine-induced bronchoconstriction by 62 ± 1.8% (Figure 2), the duration of the inhibition however was not affected.

Salbutamol and rolipram (3–300 μg) caused dose-related inhibitions of histamine-induced bronchoconstriction (Figure 3, Table 1); at 300 μg, histamine responses were reduced by 91 ± 2% and 59 ± 10% respectively. The PDE III inhibitor, siguazodan (300 μg) was without effect on histamine responses but they were reduced 28 ± 5% by the PDE III inhibitor, SK&F 95654 (300 μg). There was a marked potentiation of the effects of rolipram in the presence of SK&F 95654 (Figure 4, Table 1).

Mean resting blood pressure in these experiments was 43.3 ± 1.4 mmHg systolic, 28.0 ± 1.14 diastolic ($n = 55$). Administration of salbutamol, SK&F 96231, SK&F 95654 and rolipram (or the combination of the latter two) into the airways was accompanied by a small (up to 10 mmHg), transient fall in blood pressure associated with disconnection of the animal from the ventilator. On recomencement of ventilation this was followed by a return to pre-dosing levels ($n = 23$) or by a small (11.5 ± 1.5 mmHg) but sustained increase in blood pressure ($n = 16$). The pressor response however was unrelated to either compound or dose, suggesting that it was a nonspecific event related to the dosing. Infusion

Table 1 Duration of the anti-spasmogenic activity of salbutamol, rolipram and SK&F 95654 when given directly into the airway of anaesthetized guinea-pigs

	Inhibition of histamine induced bronchoconstriction ($t_{\frac{1}{2}}$ min)		
	3 μg	30 μg	300 μg
Salbutamol	8.7 ± 1.7	17 ± 2.9	> 30 min
Rolipram	4.7 ± 2.3	8.7 ± 1.7	13.7 ± 4.4
SK&F 95654		3.7 ± 1.7	7.0 ± 0.0
Rolipram (30 μg) + SK&F 95654	12 ± 0.0	15.3 ± 3.3	23.7 ± 1.7

Histamine (100 nmol kg^{-1}, i.v.) was administered at 5 min intervals. When a reproducible increase in pulmonary inflation pressure had been established, compounds were administered directly into the airway, by insufflation on 5 mg lactose carrier, and their effect on histamine responses followed for the following 30 min. Duration of action ($t_{\frac{1}{2}}$) is expressed as the time to 50% recovery of the increase in pulmonary inflation pressure induced by histamine. The magnitude of the inhibition of histamine responses are as shown in Figures 3 and 4 ($n = 3$ in all cases).

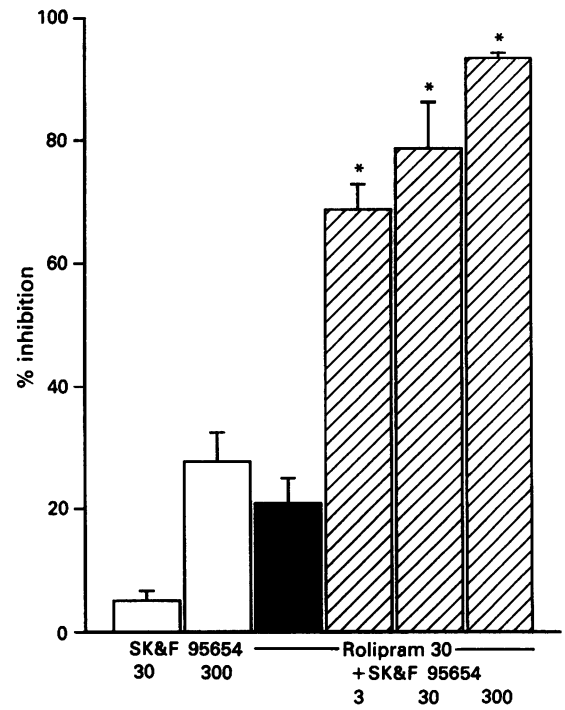


Figure 4 Potentiation of the inhibition of histamine-induced (100 nmol kg^{-1}, i.v.) bronchoconstriction by rolipram in the presence of SK&F 95654. Rolipram (30 μg) was administered by insufflation into the airway alone (solid column, $n = 3$) or in combination with SK&F 95654 (3, 30, 300 μg, hatched columns, $n = 3$). Open columns are the inhibitory effects of SK&F 95654 administered alone ($n = 3$). *$P < 0.05$ compared to rolipram alone.

Table 2 Effect of SK&F 96231 on *in vitro* tracheal cyclic GMP levels

		Cyclic GMP (pmol mg^{-1} protein, 0.1 μM CCh)	*Cyclic GMP* (pmol mg^{-1} protein, 1 μM CCh)
Carbachol alone		0.71 ± 0.12*	0.65 ± 0.01*
+ SK&F 96231	1 μM	0.62 ± 0.07	0.64 ± 0.08
	10 μM	0.88 ± 0.07	0.61 ± 0.14
	100 μM	1.49 ± 0.28†	1.39 ± 0.11†

Guinea-pig tracheal tubes were incubated, free floating in Krebs-Henseleit at 37°C, containing either 0.1 μM or 1.0 μM carbachol (CCh) alone or in combination with SK&F 96231 (1–100 μM), for 15 min. Tissues were frozen by immersion in liquid nitrogen prior to assay for cyclic GMP levels by radioimmunoassay (Amersham). Results are the mean ± s.e.mean of 3 observations. Unstimulated levels of cyclic GMP in control tissues were 0.37 ± 0.08 pmol mg^{-1} protein. *$P < 0.05$ compared to unstimulated levels; †$P < 0.05$ compared to carbachol alone.

of SNP however caused a sustained 24.7 ± 1.55 mmHg fall in systolic blood pressure. Neither SK&F 96231 nor isosorbide dinitrate alone had any effect on resting blood pressure, when administered directly into the airways, their combination, however, caused a transient fall (14.7 ± 1.76 mmHg).

Effect of SK&F 96231 infusion on tracheal cyclic GMP levels in vitro *and* in vivo

Previous observations indicate that SK&F 96231 elevates total cyclic GMP levels in guinea-pig lung strips *in vitro* (Murray *et al.*, 1991). Since interpretation of these data is complicated by the highly vascularized nature of this preparation, we have carried out studies to determine if SK&F 96231 also increases cyclic GMP levels in tracheal tissue *in vitro* and *in vivo*. The results are summarized in Tables 2 and 3. In the isolated trachea, cyclic GMP levels were elevated above control (in the presence of carbachol) only at concentrations of SK&F 96231 that caused maximum relaxations of carbachol elevated tone. When administered by i.v. infusion in the anaesthetized guinea-pig, SK&F 96231 caused a dose- and time-dependent increase in tracheal cyclic GMP levels that paralleled the time-course of inhibition of histamine-induced contractions. Table 4 shows the results of a study of the interaction of SK&F 96231 and SNP. When administered alone there was no effect of SNP (0.1 μmol kg^{-1} min^{-1}) on either the histamine responses or on tracheal cyclic GMP levels (measured at 5 min post-dose). Similarly SK&F 96231 (0.6 mg kg^{-1} min^{-1}) by itself did not alter histamine-induced bronchoconstriction but caused a small non-significant increase in cyclic GMP content. In contrast, the combination of SK&F 96231 and SNP at the same doses caused a 68% inhibition of histamine-induced bronchoconstriction and a 4 fold increase in tracheal cyclic GMP content.

Table 3 Time-course of SK&F 96231-induced cyclic GMP accumulation in anaesthetized guinea-pigs

	Cyclic GMP (pmol mg^{-1} protein)		
SK&F 96231	5 min	15 min	30 min
0.6 mg kg^{-1} min^{-1}	0.33	0.45	0.74
1.0 mg kg^{-1} min^{-1}	0.56	1.07	1.17

SK&F 96231 was infused i.v. for 30 min at 0.6 or 1.0 mg kg^{-1} min^{-1}. Bronchoconstriction was induced by i.v. injection of histamine (100 nmol kg^{-1}) at 5 min intervals beginning 15 min prior to infusion of SK&F 96231, as in the functional studies. Tracheae were removed at time intervals during the infusion, snap frozen in liquid nitrogen and assayed for cyclic GMP levels by radioimmunoassay. Assays were performed in triplicate and values are the mean of data from two separate experiments. Cyclic GMP levels in animals not receiving SK&F 96231 were 0.19 ± 0.032 pmol mg^{-1} protein ($n = 3$).

Relaxant activity of SK&F 96231 in guinea-pig isolated trachea

SK&F 96231 (0.1–100 μM) and SNP (0.01–10 μM) caused concentration-related relaxation of tissues precontracted with 0.1 μM CCh, EC_{50}'s 10.5 μM, (95% confidence limits 6.79 to 16.22 μM) and 0.33 μM, (95% confidence limits 0.23 to 0.46 μM) respectively; the spasmolytic activity of both SK&F 96231 and SNP was reduced in tissues precontracted with 1 μM CCh (Figure 5). The functional antagonism of the

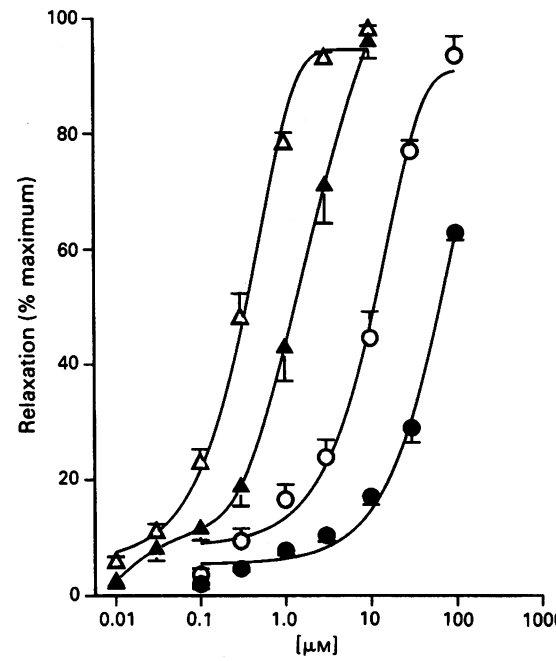


Figure 5 Functional antagonism of the spasmolytic activity of sodium nitroprusside (SNP) (Δ) and SK&F 96231 (○). Tissues were pre-contracted with 0.1 μM carbachol (open symbols) or 1.0 μM carbachol (closed symbols). When the increase in tension had reached a plateau, concentration-response curves to SNP or SK&F 96231 were constructed by their cumulative addition to the bath. Each point is the mean ± s.e.mean of 5 or 6 observations.

Table 4 Effect of SK&F 96231 alone and in combination with sodium nitroprusside (SNP) on histamine induced bronchoconstriction and tracheal cyclic GMP levels in anaesthetized guinea-pigs

	Increase in pulmonary inflation pressure (mmHg)	*Tracheal cyclic GMP* (pmol mg^{-1} protein)
Control	30 ± 3.9	0.19 ± 0.03
SNP (0.1 μmol kg^{-1} min^{-1})	38 ± 3.9	0.24 ± 0.03
SK&F 96231 (0.6 mg kg^{-1} min^{-1})	29 ± 6.4	0.33 ± 0.12
SNP (0.1 μmol kg^{-1} min^{-1}) + SK&F 96231 (0.6 mg kg^{-1} min^{-1})	9.5 ± 2.9*	0.68 ± 0.22*

Bronchoconstriction was induced by i.v. injection of histamine (100 nmol kg^{-1}) at 5 min intervals. SNP was infused for 15 min prior to infusion of SK&F 96231 as in the functional studies. Effects of agents on the increase in pulmonary inflation pressure were measured 5 min after commencing the infusion of SK&F 96231. In separate experiments tracheae were removed at this time point, frozen in liquid nitrogen and assayed for cyclic GMP levels by radioimmunoassay. Assays were performed in triplicate and values are the mean of data from three separate experiments. *$P < 0.05$ compared to control.

relaxant activity of SK&F 96231 was not accompanied by a concomitant reduction in tracheal cyclic GMP levels (Table 2). The spasmolytic activity of SK&F 96231 was unaffected by epithelium removal or by 10 μM methylene blue.

Discussion

Both SNP and SK&F 96231 elevate cyclic GMP levels in airway smooth muscle *in vitro* (Katsuki & Murad, 1977; Torphy *et al.*, 1985; Ishii & Murad, 1989; Murray *et al.*, 1991); however, whilst SNP stimulates the soluble guanylate cyclase directly (Waldman & Murad, 1987) the ability of PDE V inhibitors to elevate smooth muscle cyclic GMP levels, *in vivo*, might be expected to be dependent on the level of endogenous guanylate cyclase activity.

Consistent with this hypothesis we have shown that SK&F 96231, infused i.v. at high doses, causes a slowly developing inhibition of histamine-induced bronchoconstriction which was accompanied by a parallel increase in tracheal cyclic GMP content. We consider therefore that the effects of SK&F 96231 on airway tone *in vivo* are likely to be a consequence of cyclic GMP accumulation. This conclusion is supported by the observation that the SK&F 96231 inhibition of histamine-induced bronchoconstriction and increase in tracheal cyclic GMP content was significantly enhanced in the presence of a sub-threshold infusion of SNP. Moreover at infusion rates of SK&F 96231 and SNP which alone were devoid of any inhibitory activity, their combination elicited a marked inhibition of histamine responses. Similarly, administered directly into the airway SK&F 96231 was largely without effect on the bronchoconstriction to histamine. This is consistent with its poor anti-spasmogenic activity when administered by i.v. bolus injection (32% inhibition of histamine induced bronchoconstriction at 1 mg kg^{-1} i.v; unpublished observation). When coupled with a subthreshold i.v. infusion of SNP however, SK&F 96231 administered directly into the airways caused significant inhibition (40% at 300 μg, 62% at 1 mg). In addition, although SNP failed to potentiate the effects of SK&F 96231 when both agents were delivered by the inhaled route the less labile nitrovasodilator, isosorbide dinitrate, significantly potentiated its bronchorelaxant activity. This combined action, of agents that stimulate guanylate cyclase directly and SK&F 96231, particularly at doses, systemically or locally, that alone are without any anti-spasmogenic activity strongly suggests that even at mechanically silent doses of SK&F 96231, PDE V activity is inhibited. That this is only uncovered in the presence of guanylate cyclase stimulation therefore suggests that the anti-spasmogenic and spasmolytic activity of PDE V inhibitors, *in vivo*, is limited by guanylate cyclase activity.

Isoenzyme profiling of guinea-pig trachea (Turner, unpublished information; Tagaki *et al.*, 1992) has shown that it contains at least two cyclic GMP hydrolytic activities, the Ca^{2+}-calmodulin stimulated PDE I and the cyclic GMP-specific PDE V. We cannot rule out therefore the possibility that the poor spasmolytic activity of SK&F 96321 is due to hydrolysis of cyclic GMP by PDE's other than PDE V. However, since coupling SK&F 96231 with an exogenous stimulus to guanylate cyclase activity uncovered significant inhibition of bronchoconstriction, we consider it more likely that it is a consequence of low endogenous guanylate cyclase activity in the airway, perhaps due to the absence of an endogenous agonist.

In vitro studies also showed that the SK&F 96231- and SNP-induced relaxations of guinea-pig trachea were functionally antagonized by high concentrations of carbachol (but not histamine, data not shown). Functional antagonism of relaxant responses by high concentrations of muscarinic agonists have previously been reported in canine and bovine tracheal smooth muscle for agents which elevate cyclic AMP:- prostaglandin E_2, isoprenaline, forskolin, SK&F 94836, AH21-132; but not cyclic GMP (Torphy *et al.*, 1983; 1985; 1988; Giembycz & Barnes, 1991). The results of the present study demonstrated that, unlike canine trachea where the concentration of muscarinic agonist is reported to have little effect on the mechanical responses to SNP or 8-bromo cyclic GMP (Torphy *et al.*, 1985), in the guinea-pig trachea, the spasmolytic effects of SK&F 96231 and SNP are modified by the degree of muscarinic tone. In contrast the increase in intracellular cyclic GMP, stimulated by SK&F 96231, was unaffected; indeed, consistent with previous findings in canine tracheal smooth muscle (Katsuyama *et al.*, 1990) carbachol alone stimulated a 2 fold increase in tracheal cyclic GMP levels. The attenuation of the spasmolytic activity of SK&F 96231 by high concentrations of carbachol therefore cannot be explained by a muscarinic receptor modulation of guanylate cyclase activity or phosphodiesterase activation. Torphy *et al.* (1988) have reported that methacholine suppresses the ability of SK&F 94836 to activate the cyclic AMP-dependent protein kinase (Torphy *et al.*, 1985; 1988). Our present results suggest that the functional antagonism of the relaxant activity of agents that elevate cyclic GMP may similarly operate at or beyond the level of the cyclic GMP-dependent protein kinase.

We have also shown that when administered directly into the airway, rolipram inhibits histamine-induced bronchoconstriction demonstrating that selective PDE IV inhibitors can act as bronchodilators when inhaled. The bronchodilator activity of rolipram however was only 60% of that to an equivalent dose of salbutamol (300 μg) and was of a shorter duration. In contrast Underwood *et al.* (1993) have reported that rolipram fails to affect significantly histamine-induced contraction of guinea-pig isolated trachea at concentrations up to 1 μM. Furthermore, the same authors showed that rolipram only reduced histamine-induced bronchoconstriction at high doses (3–10 mg kg^{-1}, i.v; Underwood *et al.*, 1993). Similarly, rolipram (3 mg kg^{-1}, i.v.) has been reported to elicit only a weak inhibition of the bronchoconstriction induced by aerosolized histamine (Howell *et al.*, 1993). Our present study therefore indicates that the anti-spasmogenic potency of rolipram, when delivered directly into the airway is approximately 30 fold greater than when it is given i.v.

The results of the present study also suggest that PDE III inhibitors, when administered into the airway are relatively ineffective in inhibiting histamine-induced bronchoconstriction. Nevertheless, the combination of rolipram and the PDE III inhibitor SK&F 95654, at doses that alone had only minimal effects on histamine responses, caused a marked inhibition of histamine-induced bronchoconstriction. This combined action of inhaled PDE III and IV inhibitors has, to our knowledge, not previously been reported *in vivo* but is in agreement with their reported interaction in guinea-pig isolated trachea (Harris *et al.*, 1989). The results suggest that both PDE III and PDE IV are involved in hydrolysis of cyclic AMP in the guinea-pig airway, *in vivo*, but that when only one isoenzyme is inhibited its ability to elevate intracellular cyclic AMP levels and thence to cause bronchodilatation is restricted by cyclic AMP hydrolysis through other PDE isoforms. The data suggest therefore that inhibitors of both PDE III and PDE IV may offer greater potential as bronchodilators in asthma than inhibitors of either isoenzyme alone.

In conclusion PDE V inhibitors alone are relatively weak inhibitors of histamine-induced bronchoconstriction in the anaesthetized guinea-pig. The enhancement of the inhibition of bronchoconstriction by coupling SK&F 96231 with either SNP or isosorbide dinitrate suggests that the poor activity of SK&F 96231 alone is secondary to low guanylate cyclase activity in the airway. The combination of PDE V inhibitor and guanylate cyclase agonist might therefore provide significant bronchodilator potential in asthma.

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References

BEAVO, J.A. (1989). Multiple isoenzymes of cyclic Nucleotide Phosphodiesterase. In *Advances in Second Messenger and Phosphoprotein Research*, Vol 22. ed. Greengard, P. & Robison, G.A. New York: Raven Press.

BEAVO, J.A. & REIFSNYDER, (1990). Primary sequence of cyclic nucleotide phosphodiesterase isoenzymes and the design of selective inhibitors. *Trends Pharmacol. Sci.*, **11**, 150–155.

DE BOER, J., PHILPOTT, A.J., VAN AMSTERDAM, R.G.M., SHAHID, M., ZAAGSMA, J. & NICHOLSON, C.D. (1992). Human bronchial cyclic nucleotide phosphodiesterase isoenzymes: biochemical and pharmacological analysis using selective inhibitors. *Br. J. Pharmacol.*, **106**, 1028–1034.

BRADFORD, M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein using the principal of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

CORTIJO, J., BOU, J., BELETA, I., CARDELUS, I., LLENAS, J., MORCILLO, E. & GRISTWOOD, R.W. (1993). Investigation into the role of phosphodiesterase IV in bronchorelaxation, including studies with human bronchus. *Br. J. Pharmacol.*, **108**, 562–568.

GIEMBYCZ, M.A. & BARNES, P.J. (1991). Selective inhibition of a high affinity type IV cyclic AMP phosphodiesterase in bovine trachealis by AH21-132. Relevance to the spasmolytic and antispasmogenic actions of AH21-132 in intact tissues. *Biochem. Pharmacol.*, **42**, 663–677.

GIEMBYCZ, M.A., BELVISI, M.G., MIURA, M., PERKINS, R.S., KELLY, J., TADJKARIMI, S., YACOUB, M. & BARNES, P.J. (1992). Soluble cyclic nucleotide phosphodiesterase isoenzymes from human tracheal smooth muscle. *Br. J. Pharmacol.*, **107**, 52P.

HALL, I.P., DONALDSON, J. & HILL, S.J. (1990). Modulation of carbachol-induced inositol phosphate formation in bovine tracheal smooth muscle by cyclic AMP phosphodiesterase inhibitors. *Biochem. Pharmacol.*, **39**, 1357–1363.

HARRIS, A.L., CONNELL, M.J., FERGUSON, E.W., WALLACE, A.M., GORDON, R.J., PAGANI, E.D. & SILVER, P.J. (1989). Role of low Km cyclic AMP phosphodiesterase inhibition in tracheal relaxation and bronchoconstriction in the guinea-pig. *J. Pharmacol. Exp. Ther.*, **251**, 199–206.

HEASLIP, R.J., BUCKLEY, S.K., SICKLES, B.D. & GRIMES, D. (1991). Bronchial vs. cardiovascular activities of selective phosphodiesterase inhibitors in the anaesthetized beta-blocked dog. *J. Pharmacol. Exp. Ther.*, **257**, 741–747.

HOWELL, R.E., SICKELS, B.D. & WOEPELL, S.L. (1993). Pulmonary antillergic and bronchodilator effects of isoenzyme-selective phosphodiesterase inhibitors in guinea-pig. *J. Pharmacol. Exp. Ther.*, **264**, 609–615.

ISHII, K. & MURAD, F. (1989). ANP relaxes bovine tracheal smooth muscle and increases cGMP. *Am. J. Physiol.*, **256**, C495–C500.

KATSUKI, S. & MURAD, F. (1977). Regulation of adenosine cyclic 3′,5′-monophosphate and guanosine cyclic 3′,5′-monophosphate levels and contractility in bovine tracheal smooth muscle. *Mol. Pharmacol.*, **13**, 330–341.

KATSUYAMA, H., SUZUKI, S. & NISHIYE, E. (1990). Actions of second messengers synthesized by various spasmogenic agents and their relation to mechanical responses in dog tracheal smooth muscle. *Br. J. Pharmacol.*, **100**, 41–48.

LORENZ, K.L. & WELLS, J.N. (1982). Potentiation of the effects of sodium nitroprusside and of isoproterenol by selective phosphodiesterase inhibitors. *Mol. Pharmacol.*, **23**, 424–430.

MURRAY, K.J. (1990). Cyclic AMP and mechanisms of vasodilatation. *Pharmacol. Ther.*, **47**, 329–345.

MURRAY, K.J., EDEN, R.J., ENGLAND, P.J., DOLAN, J., GRIMSDITCH, D.C., STUTCHBURY, C.A., PATEL, B., REEVES, M.L., WORBY, A., TORPHY, T.J., WOOD, L.M., WARRINGTON, B.H. & COATES, W.J. (1991). Potential use of selective phosphodiesterase inhibition in the treatment of asthma. In *New Drugs for Asthma Therapy*. pp.27–46. Basel: Birkhanser Verlag.

POLSON, J.B., KRAZANOWSKI, J.J. & SVETIVANYI, A. (1985). Correlation between inhibition of a cyclic GMP phosphodiesterase and relaxation of canine tracheal smooth muscle. *Biochem. Pharmacol.*, **34**, 1875–1879.

SHAHID, M., VAN AMSTERDAM, R.G.M., DE BOER, J., TEN BERGE, R.E., NICHOLSON, C.D. & ZAAGSMA, J. (1991). The presence of five cyclic nucleotide phosphodiesterase isoenzyme activities in bovine tracheal smooth muscle and the functional effects of selective inhibitors. *Br. J. Pharmacol.*, **104**, 471–477.

TAKAGI, K., OGAWA, K., TANAKA, H., SATAKE, T., WATANABE, Y., CHIJWA, T. & HIKADA, H. (1992). Relaxant effects of various xanthine derivatives. Relationship to cyclic nucleotide phosphodiesterase inhibition. In *Advances in Second Messenger and Phosphoprotein Research*, Vol 25. ed. Strada, S.J. & Hikada, H. New York: Raven Press.

TOMKINSON, A., KARLSSON, J.-A. & REABURN, D. (1993). Comparison of the effects of selective inhibitors of phosphodiesterase types III and IV in airway smooth muscle with differing β-adrenoceptor subtypes. *Br. J. Pharmacol.*, **108**, 57–61.

TORPHY, T.J., BURMAN, M., HUANG, L.B.F. & TUCKER, S.S. (1988). Inhibition of the low Km cyclic AMP phosphodiesterase in intact canine trachealis by SK&F 94836: mechanical and biochemical responses. *J. Pharmacol. Exp. Ther.*, **246**, 834–850.

TORPHY, T.J. & CIESLINSKI, L.B. (1990). Characterization and selective inhibition of cyclic nucleotide phosphodiesterase isozymes in canine tracheal smooth muscle. *Mol. Pharmacol.*, **37**, 206–214.

TORPHY, T.J., RINARD, G.A., RIETOW, M.G. & MAYER, S.E. (1983). Functional antagonism in canine tracheal smooth muscle: Inhibition by methacholine of the mechanical and biochemical responses to isoprenaline. *J. Pharmacol. Exp. Ther.*, **227**, 694–699.

TORPHY, T.J. & UNDEM, B.J. (1991). Phosphodiesterase inhibitors: new opportunities for the treatment of asthma. *Thorax*, **46**, 512–523.

TORPHY, T.J., ZHENG, C., PETERSON, S.M., FISCUS, R.R., RINARD, G.A. & MAYER, S.E. (1985). Inhibitory effect of methacholine on drug-induced relaxation, cyclic AMP accumulation and cyclic AMP-dependent protein kinase activation in canine tracheal smooth muscle. *J. Pharmacol. Exp. Ther.*, **233**, 409–417.

TORPHY, T.J., ZHOU, H.-L., BURMAN, M. & HUANG, L.B. (1991). Role of cyclic nucleotide phosphodiesterase isoenzymes in intact canine trachealis. *Mol. Pharmacol.*, **39**, 376–384.

UNDERWOOD, D.C., OSBORN, R.R., NOVAK, L.B., MATHEWS, J.K., NEWSHOLME, S.J., UNDEM, B.J., HAND, J.M. & TORPHY, T.J. (1993). Inhibition of antigen-induced bronchoconstriction and eosinophil infiltration in the guinea-pig by the cyclic AMP-specific phosphodiesterase inhibitor, rolipram. *J. Pharmacol. Exp. Ther.*, **266**, 306–313.

WALDMAN, S.A. & MURAD, F. (1987). Cyclic GMP synthesis and function. *Pharmacol. Rev.*, **39**, 163–196.

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Effect of a 5-lipoxygenase inhibitor, ZM 230487, on cutaneous allergic inflammation in the guinea-pig

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1 Leukotrienes have potent biological effects *in vitro* and *in vivo* and are found in tissue and in biological fluids in various pathological conditions including allergic diseases. Leukotriene B_4 (LTB_4) is a potent stimulus for eosinophil accumulation and activation and there is much interest in determining its importance in mediating the accumulation of eosinophils at sites of allergic inflammation *in vivo*. In this study, we investigated the effects of a potent 5-lipoxygenase inhibitor, ZM 230487, on the accumulation of eosinophils and on local oedema formation in cutaneous inflammation in the guinea-pig.

2 The i.d. injection of increasing concentrations of arachidonic acid (AA) led to a dose-dependent accumulation of ^{111}In-eosinophils but oedema formation was only significant at the top dose of AA tested (3×10^{-8} mol per site). Co-injection of ZM 230487 with AA inhibited ^{111}In-eosinophil accumulation up to 99% but the small oedema response to AA was only partially inhibited. AA-induced oedema formation was only effectively inhibited when a combination of a PAF antagonist, an antihistamine and ZM 230487 was used.

3 Local administration of the cyclo-oxygenase inhibitor, ibuprofen, partially inhibited AA-induced oedema formation suggesting that vasodilator prostaglandins may be released following i.d. injection of AA. AA-induced ^{111}In-eosinophil accumulation was also partially inhibited by ibuprofen.

4 PAF-induced ^{111}In-eosinophil accumulation was partially suppressed by local administration of ZM 230487. In contrast, LTB_4-induced ^{111}In-eosinophil accumulation was enhanced by ZM 230487. These data suggest that locally-released leukotrienes may modulate mediator-induced eosinophil accumulation. ZM 230487 had no effect on PAF-or LTB_4-induced oedema formation.

5 ZM 230487 significantly inhibited the accumulation of ^{111}In-eosinophils, but did not affect local oedema formation, in a passive cutaneous anaphylaxis (PCA) reaction. However, the PAF antagonist WEB 2086 either alone or in combination with ZM 230487 had no effect on ^{111}In-eosinophil accumulation or oedema formation in the PCA reaction.

6 In conclusion, it appears that a product of 5-lipoxygenase, probably LTB_4, is important for the accumulation of ^{111}In-eosinophils, but not local oedema formation, in the PCA reaction in guinea-pig skin. These data support a major role for LTB_4 in allergic inflammation in the guinea-pig and make this animal (and the PCA model) suitable for studying the effects of inhibitors of leukotriene synthesis or action *in vivo*.

Keywords: Eosinophils; leukotrienes; 5-lipoxygenase inhibitors; oedema formation; passive cutaneous anaphylaxis; allergy

Introduction

Leukotrienes are oxidation products of arachidonic acid synthesized via the 5-lipoxygenase pathway (Piacentini & Kaliner, 1991). These eicosanoids not only have potent inflammatory effects in man and experimental animals but also affect bronchial and vascular smooth muscle tone (Bray, 1986; Batt, 1992). Elevated levels of leukotrienes or their metabolites have been detected in tissue or fluids of different pathological conditions (Bray, 1986; Busse & Gaddy, 1991; Salmon & Garland, 1991). For example, the urinary excretion of leukotriene E_4 (LTE_4, a metabolite of LTD_4) and bronchoalveolar lavage fluid levels of LTC_4 was enhanced after allergen challenge in allergic human subjects (Taylor *et al.*, 1989; Wenzel *et al.*, 1990). These levels of leukotrienes appeared to correlate with the severity of the bronchoconstrictor response (Wenzel *et al.*, 1990; Salmon & Garland, 1991). 20-OH-LTB_4, a metabolite of LTB_4, has been found in sputum of patients with asthma (Lam *et al.*, 1988) and synovial fluid from patients with rheumatoid arthritis (Davidson *et al.*, 1983). In addition, biologically active levels of LTB_4-like material were found in antigen-treated skin of allergic subjects (Barr *et al.*, 1984). Because of the widespread presence of leukotrienes in pathological conditions and their potent biological effects, great efforts have been made to identify drugs which can inhibit leukotriene synthesis (5-lipoxygenase inhibitors) or drugs which may interfere with their action (LTD_4 and LTB_4 antagonists) (McMillan & Walker, 1992). It is hoped that these drugs will be a major improvement in the treatment of chronic inflammatory human diseases.

In asthma and other related diseases, it has been suggested that eosinophils and eosinophil products play an important role in the lesion of the airways and development of bronchial hyperresponsiveness (Djukanovic *et al.*, 1990). Leukotrienes, specially LTB_4, have been shown to be potent eosinophil chemoattractants both *in vivo* (Faccioli *et al.*, 1991) and *in vitro* (Czarnetzki & Mertensmeier, 1985; Sehmi *et al.*, 1991). Interestingly, human eosinophils also release LTC_4 which may contribute to bronchoconstriction in airway disease (Kauffman *et al.*, 1987).

In the guinea-pig, the intradermal injection of different chemotactic agents leads to accumulation of eosinophils in skin sites (Faccioli *et al.*, 1991; Collins *et al.*, 1993; Teixeira *et al.*, 1993a). In this model, LTB_4 is potent in inducing the accumulation of ^{111}In-eosinophils but causes very little increase in microvascular permeability and subsequent oedema formation (Faccioli *et al.*, 1991). The injection of antigen in sites previously sensitized with an IgG_1-rich sera also leads to accumulation of ^{111}In-eosinophils and to oedema formation in a passive cutaneous anaphylaxis (PCA) reaction (Weg *et al.*, 1992; Teixeira *et al.*, 1993a,b). Oedema formation in a PCA reaction is due to the release and action of histamine

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and to newly synthesized LTD_4 and PAF (Weg *et al.*, 1991). However, the mediators responsible for the accumulation of ^{111}In-eosinophils in the same reaction do not seem to depend on PAF (as assessed by use of the PAF antagonist WEB 2086) or leukotrienes (as assessed by use of the weak leukotriene synthesis inhibitor PF 5901) (Weg *et al.*, 1992). While the endogenous mediators responsible for inducing ^{111}In-eosinophil accumulation in the PCA reaction are unknown, it has recently been established that the mechanism of their accumulation involves the leukocyte cell adhesion molecules CD18 and VLA-4 (Weg *et al.*, 1993; Teixeira *et al.*, 1994a). In view of the potent action of exogenous LTB_4 in inducing ^{111}In-eosinophil accumulation in the guinea-pig, we have re-evaluated, by using the potent 5-lipoxygenase inhibitor ZM 230487 (formerly ICI 230487), the role of this mediator in contributing to the accumulation of ^{111}In-eosinophils in a PCA reaction in guinea-pig skin.

Methods

Preparation of zymosan-activated plasma

Zymosan-activated plasma (ZAP) was used as a source of guinea-pig C5a des-Arg. ZAP was prepared by incubating heparinized (10 iu ml^{-1}) plasma obtained from naive guinea-pigs (Harlan Porcellus, Oxon, 350–400 g) with zymosan (5 mg ml^{-1}) for 30 min at 37°C. Zymosan was then removed by centrifugation (2 × 10 min at 3000 *g*). The activated plasma was desalted with a PD-10 Sephadex G-25M column and stored in aliquots at − 20°C.

Preparation of passive cutaneous anaphylaxis sera and reactions

Details of the preparation of IgG_1-rich sera are described elsewhere (Weg *et al.*, 1991). Briefly, male guinea-pigs (Harlan Porcellus, 350–400 g) were immunized with bovine gamma-globulin (BGG) in Freund's complete adjuvant (0.2 mg BGG 0.2 ml^{-1} of adjuvant s.c.). These animals received a boost of antigen in Freund's incomplete adjuvant on day 21 and the serum prepared on day 30. Recipient animals received an injection of 50 µl of a 1/50 dilution of the antiserum i.d., followed 16–20 h later by the i.d. injection of antigen (BGG, 0.1 or 1.0 µg per site).

Measurement of local oedema formation and ^{111}In-eosinophil accumulation in guinea-pig skin

Radiolabelled eosinophil infiltration and oedema formation were measured simultaneously at sites of cutaneous inflammation. ^{125}I-labelled human serum albumin (^{125}I-HSA, ~5 µCi) was added to ^{111}In-labelled eosinophils (purified and radiolabelled as previously described by Faccioli *et al.*, 1991; Teixeira *et al.*, 1993a) and injected i.v. (5 × 10^6 cells per animal) into recipient guinea-pigs (350–400 g) anaesthetized with Hypnorm (0.15 ml, i.m.). After 5 min, inflammatory stimuli or antigen were injected i.d. in 0.1 ml volumes into the dorsal shaved skin. ZM 230487 or ibuprofen were mixed with the stimuli or saline before the i.d. injections. Each animal received a duplicate of each treatment following a randomized injection plan and the inflammatory response (^{111}In-labelled eosinophil accumulation and oedema formation) was assessed after 1 h. This time point was chosen because previous experiments showed that the majority of oedema formation induced by a local injection of arachidonic acid and the greatest portion of the ^{111}In-eosinophil accumulation in a PCA reaction occur during the first 60 min after i.d. injection of antigen (Weg *et al.*, 1992; Teixeira *et al.*, unpublished observations). At this time, a blood sample was obtained by cardiac puncture, the animals were killed by an overdose of sodium pentobarbitone, the dorsal skin was removed, cleaned free of excess blood and the sites punched out with a 17 mm punch. The samples were counted in an automatic 5-head gamma-counter (Canberra Packard Ltd, Pangbourne, Berks) and the counts were cross-channel corrected for the two isotopes.

Eosinophil numbers in the skin sites were expressed as the number of ^{111}In-eosinophil per skin site and oedema formation as the ratio of ^{125}I counts of the skin sample divided by the ^{125}I counts in 1 µl of plasma.

Reagents

The following compounds were purchased from Sigma Chemical Company (Poole, Dorset): arachidonic acid (AA), bovine gamma globulin (BGG), dimethyl sulphoxide (DMSO), sodium nitroprusside (SNP) and zymosan. Hanks solutions, HEPES buffer and horse serum were purchased from Life Technologies Ltd (Paisley, Scotland). Percoll was purchased from Pharmacia (Milton Keynes, Bucks). PAF and calcitonin-gene related peptide (CGRP) from Bachem (Saffron Walden, Essex) and LTB_4 from Cascade Biochem Ltd (Reading, Berks). ^{125}I-HSA and $^{111}InCl_3$ were obtained from Amersham International plc (Amersham, Bucks). ZM 230487 (1-ethyl-6-[fluoro-5-(4-methoxy-3, 4, 5, 6-tetrahydro-2H-pyran-4-yl) phenoxy] methyl-quinol-2-one) was a kind gift from Zeneca Pharmaceuticals (Macclesfield, Cheshire) and ibuprofen a gift from Boots Chemical Company plc (Nottingham, Notts). WEB 2086 (3-[4-(2-chlorophenyl)-9-methyl-6H-thienol[3,2-β][1,-2,4]-triazolo-[4,3,a][1,4]-diazepin-2-yl]-1-(4-morpholimyl)-1-propanone) was donated by Boehringer-Ingelheim KG (Ingelheim am Rhein, Germany). ZM 230487 was dissolved in 100% DMSO and further diluted in saline. The final concentration of DMSO in the injection fluid was never greater than 1%. All other drugs were dissolved in saline.

Statistics

Data were analysed by two-way analysis of variance on normally distributed data. P values were assigned using the Newman-Keuls procedure and values of $P < 0.05$ were considered statistically significant. Percentage inhibition was calculated after subtracting background values.

Results

Effect of ZM 230487 on inflammation induced by arachidonic acid

In the guinea-pig skin, i.d. injection of AA induced a dose-dependent accumulation of ^{111}In-eosinophils (Figure 1a). Significant oedema formation was induced only at the top dose of AA tested (3 × 10^{-8} mol per site, Figure 1b). Whereas ^{111}In-eosinophil accumulation was markedly greater than saline (Figure 1a), oedema formation was only around 1.5 times greater (Figure 1b). Figure 2 shows the inhibitory effects of ZM 230487 on the ^{111}In-eosinophil accumulation and oedema formation induced by AA (3 × 10^{-8} mol per site). AA-induced ^{111}In-eosinophil accumulation was abolished by ZM 230487 with an IC_{50} of approximately 2 × 10^{-10} mol per site (Figure 2a). The small level of oedema formation induced by AA was slightly enhanced at the lower doses of ZM 230487 but inhibited at doses greater than 10^{-10} mol per site although the degree of inhibition was never greater than 40% (Figure 2b). Since inhibition of AA-induced inflammation by ZM 230487 was maximum at 10^{-8} mol per site, this dose was chosen to be used in further experiments. ZM 230487 possessed no significant inflammatory properties itself.

The effects of ibuprofen, a cyclo-oxygenase inhibitor, on AA-induced inflammation are shown in Figure 3, for comparison with ZM 230487. Ibuprofen (10^{-7} mol per site) partially inhibited ^{111}In-eosinophil accumulation and oedema formation induced by AA (3 × 10^{-8} mol per site). In the

same experiments, ZM 230487 virtually abolished the accumulation of ^{111}In-eosinophils (Figure 3a) but did not significantly reduce oedema formation induced by AA (Figure 3b).

The mechanisms of AA-induced local oedema formation were examined in further experiments. We tested the effects of a PAF antagonist (WEB 2086) and an anti-histamine (mepyramine) used alone or in combination with ZM 230487 against AA-induced oedema formation. Administration of ZM 230487 or mepyramine (2.5×10^{-8} mol per site) partially inhibited AA-induced oedema formation while WEB 2086 (10^{-7} mol per site) was without effect (Figure 4). These doses of WEB 2086 and mepyramine effectively inhibited oedema formation induced by PAF (10^{-9} mol per site) and histamine (2.5×10^{-8} mol per site), respectively (data not shown). However, the combination of ZM 230487, WEB 2086 and mepyramine effectively suppressed AA-induced oedema formation by 76% (Figure 4) while the combination of any two of the antagonists/inhibitor did not show similar inhibitory effects (data not shown).

Effect of ZM 230487 on other inflammatory mediators

ZM 230487 was tested against ^{111}In-eosinophil accumulation and oedema formation induced by PAF (10^{-9} mol per site), LTB_4 (5×10^{-10} mol per site) and ZAP (30% in saline). PAF-induced ^{111}In-eosinophil accumulation was slightly but significantly inhibited by ZM 230487 while LTB_4-induced ^{111}In-eosinophil accumulation was significantly enhanced by the compound (Figure 5a). ZM 230487 had no effect on ZAP-induced responses (Figure 5). In the same experiments, AA-induced ^{111}In-eosinophil accumulation was abrogated and oedema formation partially suppressed by ZM 230487 (Figure 5). As shown in Figure 5b, ZM 230487 had no effect on oedema formation induced by PAF or LTB_4.

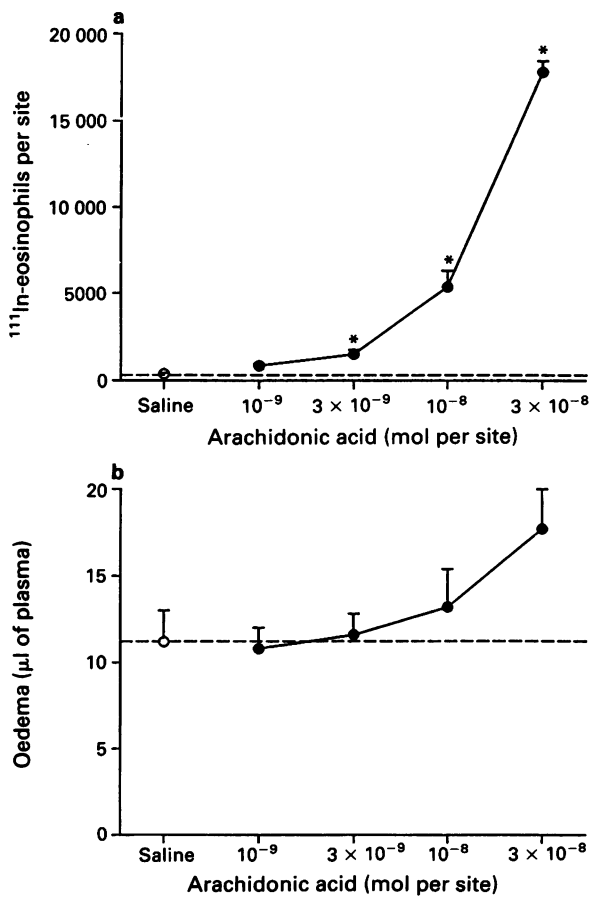


Figure 1 Local inflammation induced by i.d. injection of arachidonic acid in guinea-pig skin. ^{111}In-eosinophil accumulation (a) and oedema formation (b) were measured over 1 h. The dashed line across the graphs represents background values in sites injected with saline. Results are mean ± s.e.mean of 4 guinea-pigs.

Effect of ZM 230487 on local inflammation in a passive cutaneous anaphylaxis reaction

The injection of antigen (BGG) in sites previously sensitized with a IgG_1-rich anti-serum (anti-BGG) leads to a dose-dependent ^{111}In-eosinophil accumulation and oedema formation in a PCA reaction (Weg *et al.*, 1992; Teixeira *et al.*, 1993a) (Figure 6). ZM 230487, when co-injected with 0.1 μg and 1.0 μg of antigen per site, significantly inhibited the accumulation of ^{111}In-eosinophils by 72% and 44%, respectively (Figure 6a). Oedema formation measured in the same sites was not affected (Figure 6b). Again in these experiments, ZM 230487 abrogated AA-induced ^{111}In-eosinophil accumulation (Figure 6a) but had little effect on AA-induced oedema formation (Figure 6b).

Weg *et al.* (1992) have shown that WEB 2086, when tested alone, had no effect on oedema formation or ^{111}In-eosinophil accumulation in a PCA reaction. In the present study, we also examined the effect of WEB 2086. As shown in Figure 7,

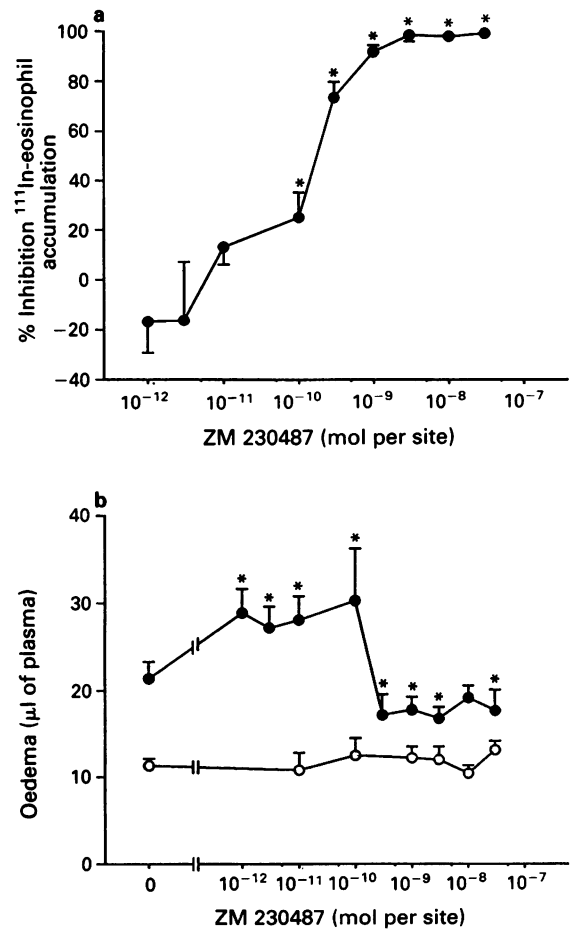


Figure 2 Effect of ZM 230487 on arachidonic acid-induced ^{111}In-eosinophil accumulation (a) and oedema formation (b) in guinea-pig skin. Arachidonic acid was used at a dose of 3×10^{-8} mol per site and ^{111}In-eosinophil accumulation and oedema formation were measured over 1 h. Percentage inhibition of ^{111}In-eosinophil accumulation was calculated by subtracting values in the presence of ZM 230487 alone. There were 11880 ± 2331, 305 ± 47 and 273 ± 41 ^{111}In-eosinophils accumulating per skin site after injection of arachidonic acid, saline and ZM 230487 (10^{-8} mol per site), respectively. Local oedema formation was measured in sites injected with ZM 230487 alone (○) or with arachidonic acid (●). Results are mean ± s.e. mean of 8 guinea-pigs. $^*P < 0.01$ when compared to injection of AA alone.

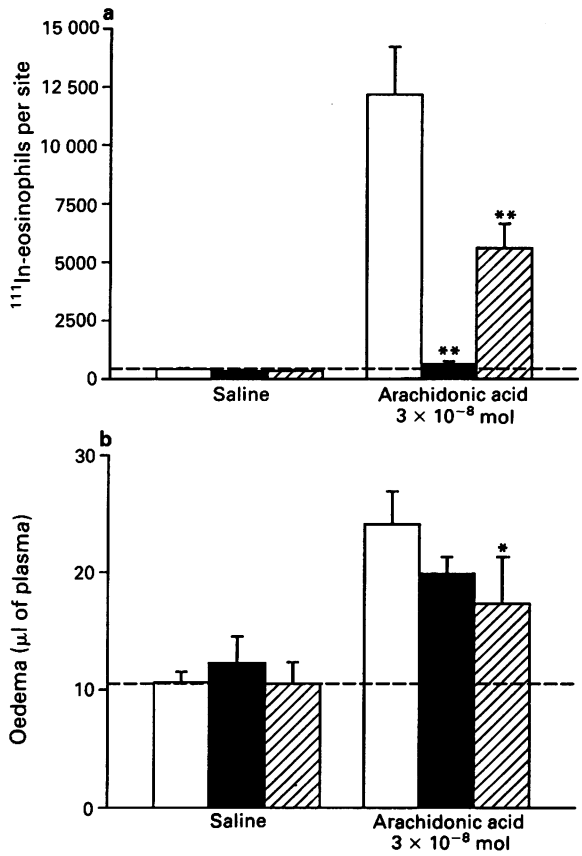


Figure 3 Effect of ZM 230487 and ibuprofen on arachidonic acid-induced ^{111}In-eosinophil accumulation (a) and oedema formation (b) in guinea-pig skin. Saline or arachidonic acid (3×10^{-8} mol per site) were injected alone (open columns) or with ZM 230487 at 10^{-8} mol per site (solid columns) or ibuprofen at 10^{-7} mol per site (hatched columns). ^{111}In-eosinophil accumulation and oedema formation were measured over 1 h. The dashed line across the graphs represent the background values in sites injected with saline. Results are mean ± s.e.mean of 8 guinea-pigs. $*P<0.05$; $**P<0.01$.

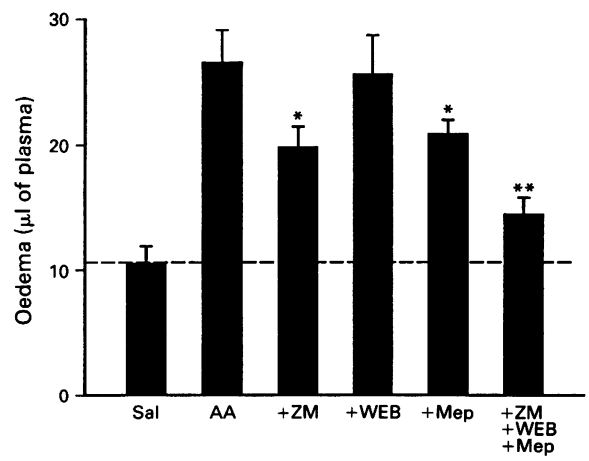


Figure 4 Effect of ZM 230487, WEB 2086 and mepyramine on arachidonic acid-induced oedema formation in guinea-pig skin. Arachidonic acid (AA) was used at a dose of 3×10^{-8} mol per site, ZM 230487 (ZM) at 10^{-8} mol per site, WEB 2086 (WEB) at 10^{-7} mol per site and mepyramine (Mep) at 2.5×10^{-8} mol per site. Drugs were mixed prior to i.d. injection and oedema formation measured over 1 h. The dashed line across the graph represents the background values in sites injected with saline. Results are mean ± s.e.mean of 4 guinea-pigs. $*P<0.01$ when compared to AA-induced oedema formation and $**P<0.01$ when compared to AA plus any other treatment.

WEB 2086 abrogated PAF-induced ^{111}In-eosinophil accumulation but had no effect on the PCA reaction when tested alone or when tested in combination with ZM 230487.

Discussion

The increased prevalence of asthma and other inflammatory diseases even with the anti-inflammatory therapy available at present warrants a search for more effective treatment (Piacentini & Kaliner, 1991; Salmon & Garland, 1991). The potent biological effects of leukotrienes together with their widespread presence in different pathological conditions make these mediators of considerable interest. The protective effects of LTD_4 antagonists against allergen-induced bronchoconstriction in man (O'Shaughnessy *et al.*, 1993) and in exercise-induced asthma (Makker *et al.*, 1993) further support the importance of leukotrienes in the pathogenesis of asthma (Cheng, 1992). It is hoped that the development of drugs that inhibit 5-lipoxygenase and thus reduce the synthesis not only of peptidoleukotrienes (LTD_4, LTC_4) but also of LTB_4 will add substantially to the protective effects of LTD_4 antagonists since 5-lipoxygenase inhibitors may also have anti-inflammatory effects. Special interest has been given to a series of non-redox inhibitors of 5-lipoxygenase because of their efficacy both *in vitro* and *in vivo* and the

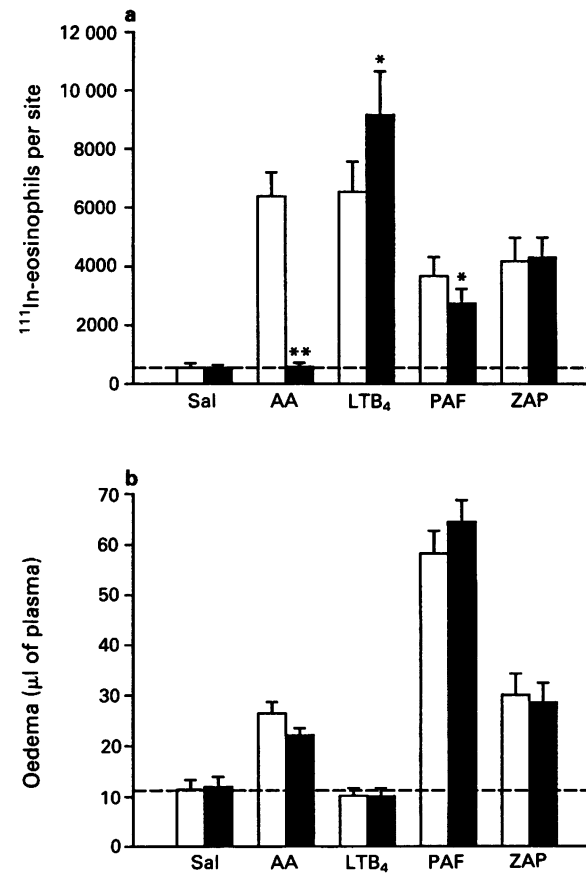


Figure 5 Effect of ZM 230487 on ^{111}In-eosinophil accumulation (a) and oedema formation (b) induced by arachidonic acid (AA), PAF, leukotriene B_4 (LTB_4) and zymosan-activated plasma (ZAP) in guinea-pig skin. AA (3×10^{-8} mol per site), PAF (10^{-9} mol per site), LTB_4 (5×10^{-10} mol per site) and ZAP (30% in saline) were injected alone (open columns) or with ZM 230487 (10^{-8} mol per site, solid columns). ^{111}In-eosinophil accumulation and oedema formation were measured over 1 h. The dashed line across the graphs represents the background values in sites injected with saline. Results are mean ± s.e.mean of 8 guinea-pigs. $*P<0.05$; $**P<0.01$.

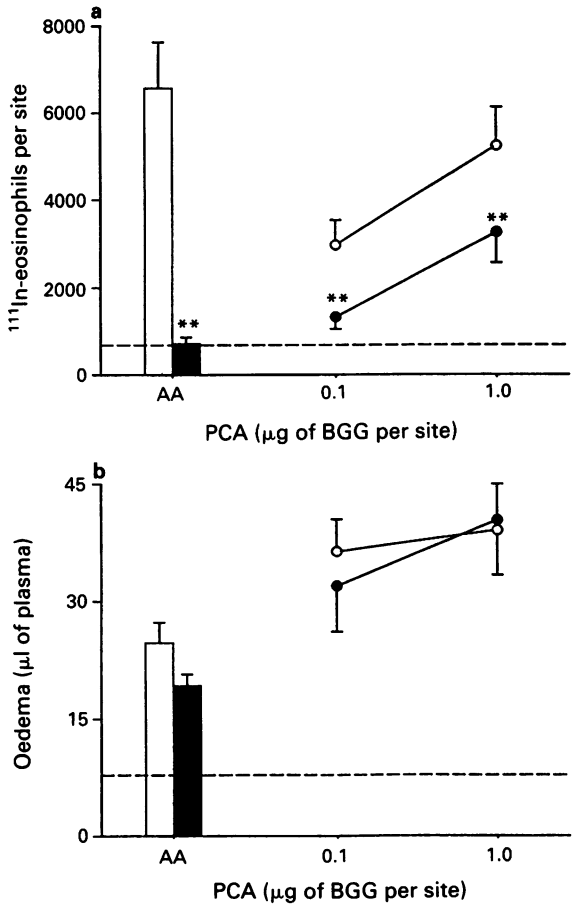


Figure 6 Effect of ZM 230487 on ^{111}In-eosinophil accumulation (a) and oedema formation (b) in a passive cutaneous anaphylaxis (PCA) reaction. Arachidonic acid (AA, 3×10^{-8} mol per site) and bovine gamma-globulin (BGG, 0.1 and 1 μg per site in sites previously sensitized with anti-BGG) were injected alone (open symbols and columns) or with ZM 230487 (10^{-8} mol per site, solid symbols and column). ^{111}In-eosinophil accumulation and oedema formation were measured over 1 h. The dashed line across the graphs represents the background values in sites injected with saline. Results are mean ± s.e.mean of 8 guinea-pigs. $^{**}P<0.01$.

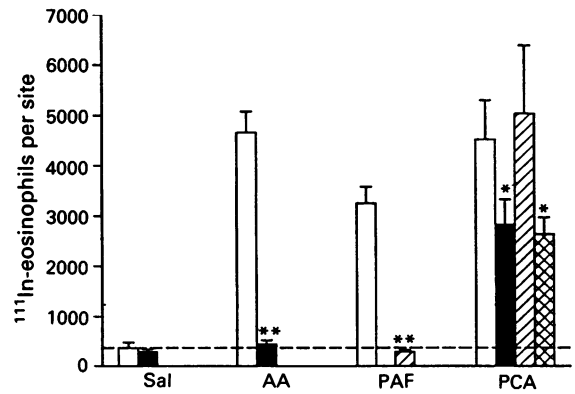


Figure 7 Effect of ZM 230487 alone or in combination with WEB 2086 on the ^{111}In-eosinophil accumulation in a passive cutaneous anaphylaxis (PCA) reaction. Arachidonic acid (AA, 3×10^{-8} mol per site), PAF (10^{-9} mol per site) and bovine gamma-globulin (BGG 1.0 μg per site in sites previously sensitized with anti-BGG) were injected alone (open columns), with ZM 230487 (10^{-8} mol per site, solid columns), with WEB 2086 (10^{-7} mol per site, hatched columns) or with ZM 230487 and WEB 2086 (cross-hatched column). ^{111}In-eosinophil accumulation was measured over 1 h. The dashed line across the graph represents background values in sites injected with saline. Results are mean ± s.e.mean of 4 guinea-pigs. $^{*}P<0.05$; $^{**}P<0.01$.

stereospecificity observed with methoxyalkyl thiazole analogues (McMillan *et al.*, 1991; McMillan & Walker, 1992). A related series, methoxy tetrahydropyrans, includes ZD 2138 (formerly ICI D2138; McMillan *et al.*, 1992) which is undergoing clinical evaluation and a close achiral analogue, ZM 230487 (Crawley *et al.*, 1993), which was employed in these studies.

In rabbit skin, intradermal injection of high doses of AA ($>10^{-8}$ mol per site) causes an inflammatory response (oedema formation and neutrophil accumulation) which is dependent on the formation of LTB_4 and PGE_2 (Aked & Foster, 1987) and can be modulated pharmacologically with 5-lipoxygenase inhibitors (Aked *et al.*, 1986; McMillan *et al.*, 1992). In the guinea-pig skin, i.d. injection of AA caused a dose-dependent accumulation of ^{111}In-eosinophils. In contrast to the rabbit skin (Aked *et al.*, 1986), oedema formation was only of mild intensity and only significant at the top dose of AA tested (3×10^{-8} mol per site). Ibuprofen partially inhibited oedema formation suggesting that vasodilator prostaglandins may be important in this reaction (Williams & Peck, 1977). The effects of ZM 230487 were more complicated since they partially inhibited oedema formation at higher doses ($>10^{-10}$ mol per site) but potentiated at lower doses. Indeed the interaction between leukotrienes in guinea-pig skin is complicated since they can have vasoconstrictor effects in addition to permeability-increasing effects in the cutaneous microcirculation (Hellewell & Williams, 1988). In this species, LTD_4 causes potent increase in microvascular permeability (Ueno *et al.*, 1981) whereas LTC_4 is a more potent vasoconstrictor (Hellewell & Williams, 1988). It is possible that at lower doses, ZM 230487 is interfering with the action of any LTC_4 (vasoconstrictor) generated. Vasoconstrictors or drugs which inhibit vasodilatation are known to inhibit inflammation in rabbit and guinea-pig skin (Brain *et al.*, 1989; Teixeira *et al.*, 1993b). It was surprising that AA-induced oedema formation was not abolished by ZM 230487 since in the same skin sites ^{111}In-eosinophil accumulation was completely blocked. We found that AA-induced oedema formation could be effectively inhibited when a combination of a PAF antagonist, an anti-histamine and ZM 230487 was used (Figure 4). This observation is identical to that found in earlier studies in our laboratory in which a combination of similar mediator antagonists was also necessary to abrogate oedema formation in a PCA reaction in guinea-pig skin (Weg *et al.*, 1991).

Accumulation of ^{111}In-eosinophils was abrogated by ZM 230487 suggesting that endogenous leukotrienes are important for AA-induced ^{111}In-eosinophil accumulation. Some workers have described chemoattractant properties for peptidoleukotrienes (in particular LTD_4) in guinea-pig conjunctiva (Spada *et al.*, 1986) and lung (Crean *et al.*, 1989) but studies in our department (Weg, unpublished observations) could not detect any ^{111}In-eosinophil accumulation in response to i.d. injection of doses of LTD_4 similar to or higher than those of LTB_4 in guinea-pig skin. In contrast, LTB_4 is a potent agonist for ^{111}In-eosinophil accumulation in the model (Faccioli *et al.*, 1991). Thus, it is probable that LTB_4 is the main leukotriene responsible for ^{111}In-eosinophil accumulation in guinea-pig skin induced by AA but further experiments using a specific LTB_4 antagonist are necessary to confirm this idea. We have used the LTB_4 antagonist LY 255,883 but it did not produce consistent inhibition of LTB_4-induced ^{111}In-eosinophil accumulation in guinea-pig skin (data not shown). The possible cellular sources of leukotrienes in our system have not been investigated but they could be the mast cell or even migrating neutrophils. Guinea-pig eosinophils also release LTB_4 as their main leukotriene product (Hirata *et al.*, 1990).

Ibuprofen, a cyclo-oxygenase inhibitor, partially inhibited ^{111}In-eosinophil accumulation induced by AA. This inhibition was not reversed by PGE_1 suggesting that suppression of vasodilator prostaglandins was not the mechanism by which ibuprofen inhibited AA-induced ^{111}In-eosinophil accumula-

tion (data not shown). However, the demonstration that PGE_1 alone may inhibit ^{111}In-eosinophil accumulation induced by different stimuli when injected in guinea-pig skin (Teixeira *et al.*, 1993a) complicates interpretation of these experiments. Nevertheless other vasodilators (CGRP and SNP) that do not share the inhibitory effect of PGE_1 on ^{111}In-eosinophil accumulation (Teixeira *et al.*, 1993a) were unable to reverse the effects of ibuprofen on AA-induced ^{111}In-eosinophil accumulation, despite all three vasodilators greatly potentiating AA-induced oedema formation even in the presence of ibuprofen (data not shown). It is possible that ibuprofen is partially inhibiting 5-lipoxygenase (5-LO) in guinea-pig skin. For example it has an IC_{50} of 69.5 μM on LTB_4 generation by rat leukocytes (Bray, 1986). Alternatively, it may be having some non-specific inhibitory effects on leukocyte function, as previously described (Nielson & Webster, 1987), which inhibits their accumulation. Another interesting possibility is that ibuprofen may be inhibiting local production of prostaglandin D_2 (PGD_2). Indeed, PGD_2 is able to elicit accumulation of eosinophils in dog trachea (Emery *et al.*, 1989). However, in guinea-pig skin, we have no evidence for the release of PGD_2 after i.d. injection of AA and this mediator (PGD_2) only weakly induced the accumulation of ^{111}In-eosinophils (data not shown).

PAF is a potent eosinophil chemoattractant and activator (Wardlaw *et al.*, 1986; Kroegel *et al.*, 1991). Nevertheless, guinea-pig eosinophils seem to be less responsive to PAF than to LTB_4 (Coeffier *et al.*, 1991; Faccioli *et al.*, 1991) (See Figure 5). Interestingly, PAF-induced ^{111}In-eosinophil accumulation was partially inhibited by ZM 230487. Indeed, PAF is capable of releasing leukotrienes in different experimental conditions and this may account for some of its effects (Voelkel *et al.*, 1982; Anderson & Fennessy, 1988; Kidney *et al.*, 1993). Another interesting finding was the small but consistent enhancement of LTB_4-induced ^{111}In-eosinophil accumulation by ZM 230487. We have not addressed this finding in greater detail but it is possible that ZM 230487 is inhibiting the action of vasoconstrictor leukotrienes (LTC_4 potently vasoconstricts in guinea-pig skin; Hellewell & Williams, 1988). If this is true, locally produced LTC_4 may be modulating the inflammatory effects of cutaneously-injected LTB_4.

The migration of inflammatory cells into lungs of sensitized subjects challenged with antigen may be important in the development of late phase response (Pradalier, 1993). Even though the microcirculation of the skin and bronchial tree may be different, the study of the mechanisms involved in this migration and its pharmacological modulation may give insights into pathological events in the lung. In a PCA reaction in guinea-pig skin, ZM 230487 significantly inhibited ^{111}In-eosinophil accumulation but had no effect on oedema formation. This suggests an important role for leukotrienes as agonists of ^{111}In-eosinophil accumulation in this reaction. The addition of a potent PAF antagonist had no further inhibitory effect on the accumulation of ^{111}In-eosinophils. Future studies will be aimed at characterizing the other mediator(s) generated in the PCA reaction that may lead to ^{111}In-eosinophil accumulation, including the possibility of mast cell-derived cytokines.

Our group has previously shown that the 5-LO inhibitor, PF 5901, was ineffective on inflammation induced in a guinea-pig PCA reaction (Weg *et al.*, 1992). The lesser potency of PF 5901 compared with ZM 230487 may explain these findings, suggesting that almost complete inhibition of 5-LO is necessary to be able to measure an inhibitory effect in the PCA reaction. A similar conclusion has been drawn from clinical studies with 5-LO inhibitors and LTD_4 antagonists in man (see Hui & Barnes, 1993 for review). Efficacy in acute bronchospasm was only apparent when inhibition of leukotriene synthesis or action was complete. Alternatively, extensive metabolism of PF 5901 *in vivo* and its high capacity for binding to proteins may prevent it from reaching its site of action in sufficient concentration in guinea-pig skin (Musser & Kreft, 1992).

Thus it seems that a product of 5-LO, probably LTB_4, is important for the accumulation of ^{111}In-eosinophils in a PCA reaction in guinea-pig. This, taken together with the results of others (Richards *et al.*, 1991; Sehmi *et al.*, 1991; Hidi *et al.*, 1992; Ishida *et al.*, 1993), support a major role for LTB_4 in the guinea-pig and makes this animal (and this PCA model) suitable for studying the effects of inhibitors of leukotriene synthesis or action *in vivo*.

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References

AKED, D.M. & FOSTER, S.J. (1987). Leukotriene B_4 and prostaglandin E_2 mediate the inflammatory response of rabbit skin to intradermal arachidonic acid. *Br. J. Pharmacol.*, **92**, 545–552.

AKED, D.M., FOSTER, S.J., HOWARTH, A., MCCORMICK, M.E. & POTTS, H.C. (1986). The inflammatory response of rabbit skin to topical arachidonic acid and its pharmacological modulation. *Br. J. Pharmacol.*, **89**, 431–438.

ANDERSON, G.P. & FENNESSY, M.R. (1988). Lipoxygenase metabolites as mediators of platelet-activating factor-induced increased airways responsiveness to histamine in guinea-pig. *Agents Actions*, **S28**, 195–199.

BARR, R.M., BRAIN, S.D., CAMP, R.D.R., CILLIERS, J., GREAVES, M.W., MALLET, A.I. & MISCH, K. (1984). Levels of arachidonic acid and its metabolites in the skin in human allergic and irritant dermatitis. *Br. J. Dermatol.*, **111**, 23–28.

BATT, D.G. (1992). 5-lipoxygenase inhibitors and their anti-inflammatory activities. In *Progress in Medicinal Chemistry*, Vol. 29, ed. Ellis, G.P. & Luscombe, D.K. pp. 1–63. Amsterdam: Elsevier Science Publishers B.V.

BRAIN, S.D., CROSSMAN, D.C., BUCKLEY, T.L. & WILLIAMS, T.J. (1989). Endothelin-1: Demonstration of potent effects on the microcirculation of humans and other species. *J. Cardiovasc. Pharmacol.*, **13** (Suppl 5), S147–S149.

BRAY, M.A. (1986). Leukotrienes in inflammation. *Agents Actions*, **19**, 87–99.

BUSSE, W.W. & GADDY, J.N. (1991). The role of leukotriene antagonists and inhibitors in the treatment of airway disease. *Am. Rev. Respir. Dis.*, **143**, S103–S107.

CHENG, J.B. (1992). Early efficacy data with a newer generation of LTD_4 antagonists in antiasthma trials: early promise for a single mediator antagonist. *Pulm. Pharmacol.*, **5**, 77–80.

COEFFIER, E., JOSEPH, D. & VARGAFTIG, B.B. (1991). LTB_4, a potent chemotactic factor for purified guinea-pig eosinophils: interference of PAF-acether antagonists. *Int. J. Immunopharmacol.*, **13**, 273–280.

COLLINS, P.D., WEG, V.B., FACCIOLI, L.H., WATSON, M.L., MOQBEL, R. & WILLIAMS, T.J. (1993). Eosinophil accumulation induced by human interleukin-8 in the guinea-pig in vivo. *Immunology*, **79**, 312–318.

CRAWLEY, G.C., BRIGGS, M.T., DOWELL, R.I., EDWARDS, P.N., HAMILTON, P.M., KINGSTON, J.F., OLDHAM, K., WATERSON, D. & WHALLEY, D.P. (1993). 4-methoxy-2-methyltetrahydropyrans: Chiral leukotriene biosynthesis inhibitors, related to ICI D2138, which display enantioselectivity. *J. Med. Chem.*, **36**, 295–296.

CREAN, G.L., SHAY, A.K. & CONNOLLY, T.P. (1989). Recruitment of eosinophils by LTD_4 aerosol in the guinea-pig lung. *Am. Rev. Respir. Dis.*, **139**, A484.

CZARNETZKI, B.M. & MERTENSMEIER, R. (1985). In vitro and in vivo chemotaxis of guinea-pig leukocytes toward leukotreine B_4 and its ω-oxidation products. *Prostaglandins*, **30**, 5–11.

DAVIDSON, E.M., RAE, S.A. & SMITH, M.J.H. (1983). Leukotriene B4, a mediator of inflammation present in synovial fluid in rheumatoid arthritis. *Ann. Rheum. Dis.*, **42**, 677–679.

DJUKANOVIC, R., ROCHE, W.R., WILSON, J.W., BEASLEY, C.R.W., TWENTYMAN, O.P., HOWARTH, P.H. & HOLGATE, S.T. (1990). Mucosal inflammation in asthma. *Am. Rev. Respir. Dis.*, **142,** 434–457.

EMERY, D.L., DJOKIC, T.D., GRAF, P.D. & NADEL, J.A. (1989). Prostaglandin D2 causes accumulation of eosinophils in the lumen of the dog trachea. *J. Appl. Physiol.*, **67,** 959–962.

FACCIOLI, L.H., NOURSHARGH, S., MOQBEL, R., WILLIAMS, F.M., SEHMI, R., KAY, A.B. & WILLIAMS, T.J. (1991). The accumulation of ^{111}In-eosinophils induced by inflammatory mediators in vivo. *Immunology*, **73,** 222–227.

HELLEWELL, P.G. & WILLIAMS, T.J. (1988). Interactions between eicosanoids and other mediators of inflammation. In *Eicosanoids in Inflammatory Conditions of the Lung, Skin and Joint.* ed. Church, M.K. & Robinson, C. pp. 43–66. Lancaster: MTP.

HIDI, R., COEFFIER, E. & VARGAFTIG, B.B. (1992). Formation of LTB_4 by fMLP-stimulated alveolar macrophages accounts for eosinophil migration in vitro. *J. Leuk. Biol.*, **51,** 425–431.

HIRATA, K., MAGHNI, K., BORGEAT, P. & SIROIS, P. (1990). Guinea pig alveolar eosinophils and macrophages produce leukotriene B_4 but no peptido-leukotriene. *J. Immunol.*, **144,** 1880–1885.

HUI, K.P. & BARNES, N.C. (1993). Inhibition of leukotriene synthesis: New therapy in asthma? *Pulm. Pharmacol.*, **6,** 3–9.

ISHIDA, K., THOMSON, R.J. & SCHELLENBERG, R.R. (1993). Role of leukotrienes in airway hyperresponsiveness in guinea-pigs. *Br. J. Pharmacol.*, **108,** 700–704.

KAUFFMAN, H.F., VAN DER BELT, B., DE MONCHY, J.G.R., BOELENS, H., KOETER, G.H. & DE VRIES, K. (1987). Leukotriene C_4 production by normal-density and low-density eosinophils of atopic individuals and other patients with eosinophils. *J. Allergy Clin. Immunol.*, **79,** 611–619.

KIDNEY, J.C., RIDGE, S.M., CHUNG, K.F. & BARNES, P.J. (1993). Inhibition of platelet-activating factor-induced bronchoconstriction by the leukotriene D4 receptor antagonist ICI 204,219. *Am. Rev. Respir. Dis.*, **147,** 215–217.

KROEGEL, C., CHILVERS, E.R., GLEMBYCZ, M.A., CHALLISS, R.A.J. & BARNES, P.J. (1991). Platelet-activing factor stimulates a rapid accumulation of inositol (1,4,5)trisphosphate in guinea pig eosinophils: relationship to calcium mobilization and degranulation. *J. Allergy Clin. Immunol.*, **88,** 114–124.

LAM, S., CHAN, H., LERICHE, J.C., CHAN-YEUNG, M. & SALARI, H. (1988). Release of leukotrienes in patients with bronchial asthma. *J. Allergy Clin. Immunol.*, **81,** 711–717.

MAKKER, H.K., LAU, L.C., THOMSON, H.W., BINKS, S.M. & HOLGATE, S.T. (1993). The protective effect of inhaled leukotriene D_4 receptor antagonist ICI 204,219 against exercise-induced asthma. *Am. Rev. Respir. Dis.*, **147,** 1413–1418.

MCMILLAN, R.M., BIRD, T.G.C., CRAWLEY, G.C., EDWARDS, M.P., GIRODEAU, J.-M., KINGSTON, J.F. & FOSTER, S.J. (1991). Methoxyalkyl thiazoles: a novel series of potent, orally active and enantioselective inhibitors of 5-lipoxygenase. *Agents Actions*, **34,** 110–112.

MCMILLAN, R.M., SPRUCE, K.E., CRAWLEY, G.C., WALKER, E.R.H. & FOSTER, S.J. (1992). Pre-clinical pharmacology of ICI D2138, a potent orally-active non-redox inhibitor of 5-lipoxygenase. *Br. J. Pharmacol.*, **107,** 1042–1047.

MCMILLAN, R.M. & WALKER, E.R.H. (1992). Designing therapeutically effective 5-lipoxygenase inhibitors. *Trends Pharmacol. Sci.*, **13,** 323–330.

MUSSER, J.H. & KREFT, A.F. (1992). 5-lipoxygenase: properties, pharmacology, and the quinolinyl(bridged)aryl class of inhibitors. *J. Med. Chem.*, **35,** 2503–2524.

NIELSON, V.G. & WEBSTER, R.O. (1987). Inhibition of human polymorphonuclear leukocyte functions by ibuprofen. *Immunopharmacology*, **13,** 61–71.

O'SHAUGHNESSY, K.M., TAYLOR, I.K., O'CONNOR, B., O'CONNELL, F., THOMSON, H. & DOLLERY, C.T. (1993). Potent leukotriene D_4 receptor antagonist ICI 204,219 given by the inhaled route inhibits the early but not the late phase of allergen-induced bronchoconstriction. *Am. Rev. Respir. Dis.*, **147,** 1431–1435.

PIACENTINI, G.L. & KALINER, M.A. (1991). The potential roles of leukotrienes in bronchial asthma. *Am. Rev. Respir. Dis.*, **143,** S96–S99.

PRADALIER, A. (1993). Late-phase reaction in asthma: basic mechanisms. *Int. Arch. Allergy Immunol.*, **101,** 322–325.

RICHARDS, I.M., SUN, F.F., TAYLOR, B.M., SHIELDS, S.K., GRIFFIN, R.L., MORRIS, J., WISHKA, D.G., SMITH, H.W., JOHNSON, R.A. & DUNN, C.J. (1991). Contribution of leukotriene B_4 to airway inflammation and the effect of antagonists. *Ann. N.Y. Acad. Sci.*, **629,** 274–287.

SALMON, J.A. & GARLAND, L.G. (1991). Leukotrienes antagonists and inhibitors of leukotriene biosynthesis as potential therapeutic agents. *Prog. Drug Res.*, **37,** 9–90.

SEHMI, R., CROMWELL, O., TAYLOR, G.W. & KAY, A.B. (1991). Identification of guinea-pig eosinophil chemotactic factor of anaphylaxis as leukotriene B_4 and 8(S)-Dihydroxy-5,9,11,13(Z,E, Z,E)-Eicosatetraenoic acid. *J. Immunol.*, **147,** 2276–2283.

SPADA, C.S., WOODWARD, D.F., HAWLEY, S.B. & NIEVES, A.L. (1986). Leukotrienes cause eosinophil migration into conjunctival tissue. *Prostaglandins*, **31,** 795–809.

TAYLOR, G.W., TAYLOR, I., BLACK, P., MALTBY, N.H., TURNER, N., FULLER, R.W. & DOLLERY, C.T. (1989). Urinary leukotriene E_4 after antigen challenge and in acute asthma and allergic rhinitis. *Lancet*, **ii,** 584–588.

TEIXEIRA, M.M., REYNIA, S., ROBINSON, M., WILLIAMS, T.J., WILLIAMS, F.M. & HELLEWELL, P.G. (1994). Role of CD11/CD18 in mediating cutaneous inflammation in the guinea pig. *Br. J. Pharmacol.*, **111,** 81P.

TEIXEIRA, M.M., WILLIAMS, T.J. & HELLEWELL, P.G. (1993a). E-type prostaglandins enhance local oedema formation and neutrophil accumulation but suppress eosinophil accumulation in guinea pig skin. *Br. J. Pharmacol.*, **110,** 416–422.

TEIXEIRA, M.M., WILLIAMS, T.J. & HELLEWELL, P.G. (1993b). Role of prostaglandins and nitric oxide in acute inflammatory reactions in guinea pig skin. *Br. J. Pharmacol.*, **110,** 1515–1521.

UENO, A., TANAKA, K., KATORI, M., HAYASHI, M. & ARAI, Y. (1981). Species difference in increased vascular permeability by synthetic leukotrienes C_4 and D_4. *Prostaglandins*, **21,** 637–648.

VOELKEL, N.F., WORTHEN, S., REEVES, J.T., HENSON, P.M. & MURPHY, R.C. (1982). Nonimmunological production of leukotrienes induced by platelet-activating factor. *Science*, **218,** 286–288.

WARDLAW, A.J., MOQBEL, R., CROMWELL, O. & KAY, A.B. (1986). Platelet-activating factor. A potent chemotactic and chemokinetic factor for human eosinophils. *J. Clin. Invest.*, **78,** 1701–1706.

WEG, V.B., WATSON, M.L., CORDEIRO, R.S.B. & WILLIAMS, T.J. (1991). Histamine, leukotriene D_4 and platelet activating factor in guinea-pig passive cutaneous anaphylaxis. *Eur. J. Pharmacol.*, **204,** 157–163.

WEG, V.B., WATSON, M.L., FACCIOLI, L.H. & WILLIAMS, T.J. (1992). [^{111}In]-eosinophil accumulation during passive cutaneous anaphylaxis in the guinea pig. *Br. J. Pharmacol.*, **105,** 127P.

WEG, V.B., WILLIAMS, T.J., LOBB, R.R. & NOURSHARGH, S. (1993). A monoclonal antibody recognising very late activation antigen-4 (VLA-4) inhibits eosinophil accumulation in vivo. *J. Exp. Med.*, **177,** 561–566.

WENZEL, S.E., LARSEN, G.L., JOHNSTON, K., VOELKEL, N.F. & WESTCOTT, J.Y. (1990). Elevated levels of leukotriene C_4 in bronchoalveolar lavage fluid from atopic asthmatics after endobronchial allergen challenge. *Am. Rev. Respir. Dis.*, **142,** 112–119.

WILLIAMS, T.J. & PECK, M.J. (1977). Role of prostaglandin-mediated vasodilatation in inflammation. *Nature*, **270,** 530–532.

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Activation of the sheep cardiac sarcoplasmic reticulum Ca^{2+}-release channel by analogues of sulmazole

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1 The effect of sulmazole and several structurally related analogues on cardiac sarcoplasmic reticulum (SR) Ca^{2+}-release channel gating and on [^{3}H]-ryanodine binding to isolated SR membrane vesicles has been investigated.

2 The optical isomers, (+)- and (−)-sulmazole, increased the open probability (P_o) of single Ca^{2+}-release channels incorporated into phospholipid bilayers held under voltage clamp by increasing the frequency and duration of open events. The respective EC_{50}s were 423 μM and 465 μM at 10 μM activating cytosolic Ca^{2+} and the Hill coefficients for activation were approximately two, suggesting that at least two molecules of either enantiomer are required to bind for channel activation.

3 Similarly the related enantiomers, (+)- and (−)-isomazole, which differ from sulmazole in the position of the pyridine nitrogen (4,5b for sulmazole; 4,5c for isomazole), were approximately as potent as each other and as potent as the isomers of sulmazole with EC_{50}s of approximately 445 μM.

4 In contrast, EMD 46512 and EMD 41000, which are sulmazole and isomazole analogues respectively, each with the methylsulphinyl oxygen removed, increased single-channel P_o with EC_{50}s of 42 μM and 40 μM. The open and closed lifetime distributions were similar to those of the less potent analogues and the Hill coefficients were the same, suggesting that these compounds act at the sulmazole site on the Ca^{2+}-release channel.

5 All of the compounds tested were able to increase the P_o of channels in the absence of activating Ca^{2+} but were less potent than in the presence of Ca^{2+}. The drugs were effective only when added to the cytosolic face of the channel. None of the drugs could fully activate the channel in the absence of Ca^{2+}, partly due to only one drug molecule binding in the absence of Ca^{2+}, which is in contrast to the situation when activating Ca^{2+} is present. This suggests a synergistic action of these drugs and Ca^{2+} in Ca^{2+}-release channel activation.

6 EMD 46512 and EMD 41000 increased [^{3}H]-ryanodine binding to HSR vesicles with Hill coefficients of approximately two and EC_{50}s of 25 μM and 28 μM, respectively, at 10 μM Ca^{2+}. These drugs also increased [^{3}H]-ryanodine binding to HSR vesicles at pM Ca^{2+} but with Hill slopes of only one and EC_{50}s of 112 and 133 μM for EMD 46152 and EMD 41000, respectively. In addition, maximal binding was reduced at pM Ca^{2+} in comparison to 10 μM Ca^{2+}.

7 These data show that analogues of sulmazole increase the P_o of the cardiac SR Ca^{2+}-release channel and this occurs as the result of an increase in the frequency and duration of open events. They also demonstrate that the activation of the channel by these drugs is not stereoselective and therefore the configuration of the oxygen atom or methyl group attached to the sulphur atom does not affect their ability to elicit their effect. Similarly, the results show that the nitrogen in the 4, 5b or 4, 5c position of the pyridine ring does not affect Ca^{2+}-dependent or Ca^{2+}-independent activation of the Ca^{2+}-release channel. However, removal of the methylsulphinyl oxygen in sulmazole and isomazole results in two drugs which display a ten fold increase in potency over their respective parent compound in the activation of the Ca^{2+}-release channel. It is apparent that minor modifications of the sulmazole or isomazole molecules around the terminal sulphur atom dramatically affect potency but not maximal attainable effect, suggesting that the area around the sulphur atom may be critically involved in channel activation.

Keywords: Sulmazole analogues; sarcoplasmic reticulum; cardiac Ca^{2+}-release channel; caffeine; structure-activity relationships

Introduction

The main pathway for the release of intracellular Ca^{2+} in cardiac muscle cells is the sarcoplasmic reticulum (SR) Ca^{2+}-release channel. The open probability (P_o) of the Ca^{2+}-release channel is modulated primarily by the concentration of Ca^{2+} on the cytosolic face of the channel. It is also affected by various secondary agents such as adenosine and ATP (Smith *et al.*, 1985; McGarry & Williams, 1994), caffeine (Rousseau *et al.*, 1986; Sitsapesan & Williams, 1990) and anthraquinones (Holmberg & Williams, 1990b; Pessah & Zimanyi, 1991), some of which may act independently of activating Ca^{2+}. Ryanodine and ruthenium red act to modify single-channel conductance and to cause channel closure, respectively (Meissner & Henderson, 1987). It has recently been demonstrated that therapeutically relevant concentrations of cardiac glycosides increase the P_o of the cardiac Ca^{2+}-release channel in a Ca^{2+}-dependent manner, an effect which may underlie part of the positive inotropic effect of these drugs, providing further evidence for the role of the Ca^{2+}-release channel in excitation-contraction coupling (McGarry & Williams, 1993a).

Williams & Holmberg (1990) have shown that the caffeine analogue, (+)-sulmazole, activates the Ca^{2+}-release channel by both a Ca^{2+}-dependent and a Ca^{2+}-independent mechanism and that there is a synergistic action of Ca^{2+} and sulmazole on single-channel activity. Sulmazole isomers and several structurally related compounds (including caffeine) appear to exert similar effects in the cell. They increase the sensitivity of the contractile proteins to Ca^{2+} (Blinks & Endoh, 1984), inhibit type III phosphodiesterase and inhibit

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the sarcolemmal Na^+/K^+ ATPase (van Meel *et al.*, 1988; Honerjager *et al.*, 1989). Sulmazole has also been shown to be an adenosine A_1 antagonist and to be a functional antagonist of an inhibitory G-protein (Parsons *et al.*, 1988). All of these effects may contribute to the cardiotonic actions of these drugs.

Van Meel *et al.* (1988) showed that (+)- and (−)-sulmazole were equipotent in their ability to inhibit adenosine 3':5'-cyclic monophosphate (cyclic AMP) and guanosine 3':5'-cyclic monophosphate (cyclic GMP) phosphodiesterase (PDE) but that the (+)-isomer was a more potent Ca^{2+}-sensitizing agent, a more potent inhibitor of the Na^+/K^+ ATPase and a more potent inotropic compound (see Figure 1 for structures). They concluded that the increased myofibrillar sensitivity to Ca^{2+} induced by (+)-sulmazole underlay its increased inotropic effect. This would appear to suggest that the configuration of the methyl and oxygen groups around the sulphur atom of sulmazole mediate the increased potency of the compound over its (−)-enantiomer.

The effects on cardiac function of some of the sulmazole analogues used in this study were examined by Honerjager *et al.* (1989). In their study, they found that the position of the pyridine nitrogen (4,5b in sulmazole and EMD 46512 and 4,5c in isomazole and EMD 4100), was crucial for inhibition of the sarcolemmal Na^+/K^+ ATPase, since EMD 41000 and isomazole had little inhibitory activity. The presence of a methylsulphinyl oxygen also affected the ability of each compound to inhibit this enzyme, as sulmazole was approximately forty times less potent than its reduced analogue EMD 46512. The pyridine nitrogen position also affected the inhibition of type III PDE, as isomazole and EMD 41000 were approximately two and three times as potent as EMD 46512 and sulmazole, respectively. The reduced analogues were slightly more potent inhibitors of type III PDE than their respective oxidised parent compounds. Not surprisingly, the reduced analogue EMD 46512 was found to be a more potent cardiotonic drug than sulmazole.

The effects of these compounds on the cardiac SR Ca^{2+}-release channel, with the exception of (+)-sulmazole, have not been directly investigated. An investigation of their action on the channel may help elucidate the structural features of compounds that are responsible for activity at the caffeine/sulmazole site on the release channel and may also shed light on a new action of these drugs in cardiac muscle cells.

A preliminary account of this work has been presented to the British Pharmacological Society (McGarry & Williams, 1993b).

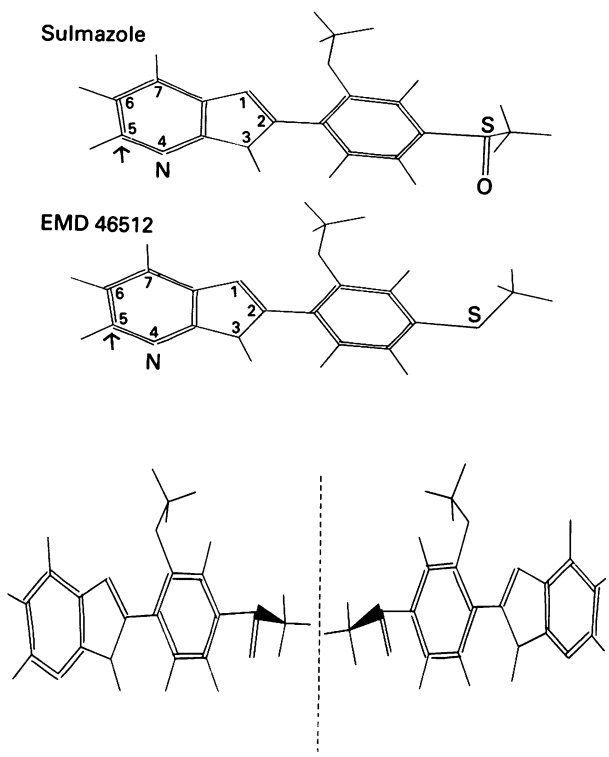


Figure 1 The structures of sulmazole and its reduced analogue, EMD 46512. The position of the chiral sulphur atom is highlighted in sulmazole. The difference between sulmazole and isomazole is the position of the pyridine nitrogen. Its position in isomazole and its reduced analogue EMD 41000 is shown by the arrow. The benzimidazole numbering system is used. In the lower part of the figure, the position of the asymmetry in sulmazole is shown.

Methods

Preparation of SR membrane vesicles

The membrane preparation was as described by Sitsapesan & Williams (1990). Fresh sheep hearts were obtained from the abattoir and transported to the laboratory in cold modified cardioplegic solution (Tomlins *et al.*, 1986). Approximately 100 g of left ventricle and septum were homogenized in a solution containing 300 mM sucrose, 20 mM potassium piperazine-N'N'-*bis*-ethanesulphonic acid (PIPES) and 1 mM phenylmethanesulphonyl chloride (PMSF), pH 7.4. The homogenate was centrifuged at 8000 g_{av} in a Sorvall GSA rotor for 20 min at 4°C. The supernatant was centrifuged at 100,000 g_{av} in a Sorvall T647.5 rotor for 40 min. The resulting membrane pellet was resuspended in a solution containing (mM): KCl 400, $MgCl_2$ 0.5, $CaCl_2$ 0.5, 1,2-di(2-aminoethoxy)ethane-N,N,N',N'-tetraacetic acid (EGTA) 0.5, PIPES 25 and 10% w/v sucrose, pH 7.0. The membrane suspension was layered onto identical salt solutions containing 20, 30 and 40% w/v sucrose and centrifuged at 100,000 g_{av} for 120 min in a Sorvall AH-629 rotor at 4°C. Heavy SR (HSR) membrane vesicles collecting at the 30–40% interface were diluted into 400 mM KCl and pelleted by centrifugation at 100,000 g_{av} for 40 min in a Sorvall T647.5 rotor before resuspension in a solution containing 400 mM sucrose, 5 mM N'-2-hydroxyethylpiperazine-N'-2-sulphonic acid (HEPES) titrated to pH 7.4 with tris(hydroxymethyl)-methylamine (Tris). Membrane vesicles were snap frozen in liquid nitrogen and stored at − 80°C.

Single-channel methods

Lipid bilayers containing phosphatidylethanolamine in decane (30 mg ml^{-1}) were formed across a 200 μm diameter hole in the partition between two fluid-filled styrene copolymer chambers, referred to as *cis* and *trans*. The *trans* chamber was held at ground and the *cis* chamber clamped at holding potentials relative to this. Current flow through the bilayer was measured with an operational amplifier as a current-voltage converter (Miller, 1982). Initially, both chambers contained 50 mM choline chloride, 10 mM HEPES and 5 mM $CaCl_2$, with the pH adjusted to 7.4 with Tris. HSR vesicles were added to the *cis* chamber. The chamber was stirred and the choline chloride concentration increased to give a 7:1 gradient *cis-trans* to promote vesicle fusion with the bilayer, fusion being marked by the appearance of Cl^--selective channels (Smith *et al.*, 1985). SR vesicles incorporated into the bilayer in a fixed orientation such that the cytosolic face of the Ca^{2+}-release channel was directed towards the *cis* chamber and the luminal face to the *trans* chamber. Following vesicle fusion, the *cis* and *trans* chambers were perfused with solution allowing resolution of only Ca^{2+}-release channel currents. The *cis* chamber was perfused with 250 mM HEPES, 125 mM Tris, pH 7.4; and the *trans* with 250 mM glutamic acid, 10 mM HEPES, with the pH adjusted to 7.4 with $Ca(OH)_2$, giving a $[Ca^{2+}]$ in the *trans* chamber of

67 mM. The concentration of Ca^{2+} in the *cis* chamber was buffered to the desired level by the addition of $CaCl_2$ and EGTA. The concentrations of each were calculated using EQCAL (Biosoft, Cambridge). The concentrations of free Ca^{2+} used in this study (10 μM and 80 pM) were attained by the addition of 1 mM EGTA and 1.005 mM or 0.15 μM $CaCl_2$, respectively, to the cytosolic face of the channel. Experiments were performed at room temperature (22 ± 1°C).

Data acquisition, analysis and display

Single-channel data were displayed on an oscilloscope and recorded on video tape. Current recordings were replayed, low-pass filtered with an 8-pole Bessel filter (Frequency Devices 902) at 1 kHz and digitised at 2 kHz using an AT based system, Satori (Intracel, Cambridge). Channel P_o and open and closed lifetimes were determined by 50% threshold analysis at a holding potential of 0 mV. Single-channel P_o was determined from $P_o = t_{open}/(t_{open} + t_{closed})$ where t_{open} and t_{closed} are the mean open and closed lifetime durations, respectively. Digitized single-channel traces were transferred from Satori as plot files into CorelDraw 4 (Corel Corporation) and labelled for display. Lifetimes, accumulated from 3 min of steady state recordings, were stored in sequential files and displayed in non-cumulative histograms. Individual lifetimes were fitted to a probability density function using the method of maximum likelihood (Colquhoun & Sigworth, 1983). Lifetimes lasting less than 1 ms were incompletely resolved under these data acquisition conditions and were therefore excluded from the fitting procedures. A missed events correction was applied (Colquhoun & Sigworth, 1983) and a likelihood ratio test was used to compare fits to double and triple exponentials (Blatz & Magleby, 1986).

Single-channel conductance was obtained from the slope of the linear regression lines drawn through single-channel current amplitude data monitored at holding potentials within the range (+ 40 to − 40 mV). Reversal potentials were obtained from extrapolations of these lines. The $Ca^{2+}/Tris^+$ permeability ratio was calculated from the equation given by Fatt & Ginsborg (1958).

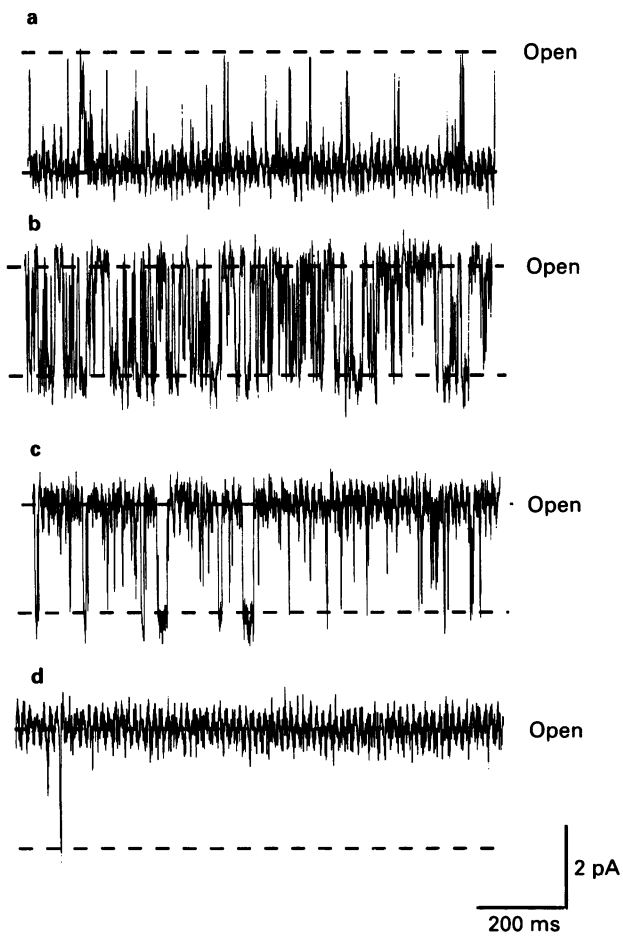


Figure 2 A single cardiac sarcoplasmic reticulum Ca^{2+}-release channel held at 0 mV. The channel was activated by 10 μM Ca^{2+} added to the cytosolic face of the channel (a). Single-channel P_o determined from 3 min of continuous recordings was increased on addition of 0.25 (b), 1 (c) and 3 mM (d) (−)-sulmazole: P_o increased to 0.353, 0.786 and 0.989 respectively. A full dose-response curve for all the data revealed an EC_{50} of 465 μM ($n = 4$). Single-channel P_o was calculated as described in the Methods section. Single-channel conductance was unaffected by addition of the drug. Channel open level is shown above each respective portion of trace.

Binding assay

Binding of [^{3}H]-ryanodine to HSR membranes was conducted essentially as described by Holmberg & Williams (1990a). For equilibrium binding, HSR membranes (100 μg protein) were incubated with 1 nM [^{3}H]-ryanodine (76 Ci $mmol^{-1}$) for 90 min at 37°C in a solution containing 1 M KCl and 25 mM PIPES, pH 7.4, in a final volume of 1 ml. The appropriate analogue was added at the beginning of the assay. Non-specific binding was determined by the addition of 1 μM unlabelled ryanodine. Binding was terminated by the addition of 5 ml ice cold binding buffer followed by immediate filtration through a Whatman GF/B filter pre-soaked in binding solution. The filter was then washed with three further 5 ml volumes of buffer before being placed in 10 ml Packard MV scintillant and the remaining radioactivity on the filter counted in a liquid scintillation counter.

The data points from each single-channel and binding experiment for each drug were averaged and are displayed graphically as the mean ± standard error of the mean. The data were fitted to a four parameter logistic equation using INPLOT4 (GraphPad Software):

$$Y = A + \frac{(B-A)}{1 + (10^c/10^x)^d}$$

where A is the bottom of the curve; B is the maximum, constrained to 1; c is the log EC_{50}; d is the Hill slope; and x is the ligand concentration.

Molecular modelling of drug structures

The structures of the different sulmazole analogues and also caffeine were modelled using the molecular modelling package HyperChem (release 3 for Microsoft Windows), from Autodesk Inc. on a Viglen 486 DX33. The structures were built and minimized in the MM+ force field using the Fletcher-Reeves algorithm (conjugate gradient method). The minimized structures were compared to those obtained using the Polak-Ribiere algorithm, to ensure a minimum had been reached, and in all cases were found to be identical.

Drugs

(+)- and (−)-sulmazole were gifts from Boehringer Ingelheim and were dissolved in distilled water. (+)- and (−)-isomazole were generously donated by E. Merck Pharmaceuticals and were also soluble in distilled water. EMD 46512 and EMD 41000, also donated by E. Merck, were dissolved in dimethylsuphoxide (DMSO). The concentration of DMSO never exceeded 2% v/v and this did not affect either the single-channel or binding experiments. Ryanodine was purchased from Cambridge Bioscience (U.S.A.) and [^{3}H]-ryanodine (76 Ci $mmol^{-1}$) from NEN (Stevenage, Herts). Phosphatidylethanolamine was from Avanti Polar Lipids, Alabama, U.S.A.

Results

Lack of stereoselectivity at the sulmazole activation site: (+)- and (−)-sulmazole

The P_o of single cardiac SR Ca^{2+}-release channels incorporated into lipid bilayers was increased dose-dependently by both (+)- and (−)-sulmazole. Figure 2 shows the activation of a single Ca^{2+}-release channel by increasing concentrations of (−)-sulmazole at 10 μM activating Ca^{2+}. The increase in P_o was seen only when the drugs were added to the cytosolic face of the channel. Analysis of the open and closed lifetime durations of these single-channels shows that the increase in P_o induced by (−)-sulmazole (250 μM–3 mM) occurred by an increase in the frequency and duration of open events. The increase in P_o at 0.25 mM and 0.5 mM was mainly due to the increased frequency of channel opening. This is demonstrated in Figure 3, which shows the open and closed lifetime durations of a single Ca^{2+}-release channel activated by 0.5 mM (−)-sulmazole. At 10 μM Ca^{2+}, the gating of the channel is best described by at least two open and three closed states. After addition of 0.5 mM (−)-sulmazole, the gating is best described by three open and two closed states. Occasionally, however, gating may be best described by three open and three closed states.

The activation of the channel by (+)-sulmazole has been described by Williams & Holmberg (1990). This enantiomer was also able to increase the P_o of single channels and this also occurred by an increase in the frequency and duration of open events. The EC_{50}s for activation of the Ca^{2+}-release channel were 423 μM ($n = 4$) for (+)-sulmazole (see Figure 5) and 465 μM ($n = 4$) for (−)-sulmazole. The Hill slope for the activation was approximately two for both drugs, suggesting that at least two molecules bind to the Ca^{2+}-release channel. Both enantiomers displayed the ability to cause almost maximal activation of the channel in the presence of submaximally activating Ca^{2+} (10 μM) and the responses showed no signs of desensitization or fade, even over periods in excess of 20 min.

The position of the nitrogen in the pyridine ring is unimportant for activation of the Ca^{2+}-release channel

The structurally-related compounds (+)- and (−)-isomazole, which differ from the sulmazole isomers in the position of the pyridine nitrogen (4,5b in sulmazole; 4,5c in isomazole), also dose-dependently increased the P_o of single Ca^{2+}-release channels in the presence of 10 μM activating Ca^{2+}. Figure 4 shows the increase in single-channel P_o induced by (+)-isomazole. In common with the sulmazole compounds, the activation occurred when the drugs were added to the cytosolic face of the channel protein. The increase in P_o caused by both drugs was achieved by increasing the frequency and duration of open events and yielded EC_{50}s of 445 μM ($n = 4$) for both enantiomers. The sigmoid dose-response curves also had Hill slopes of two.

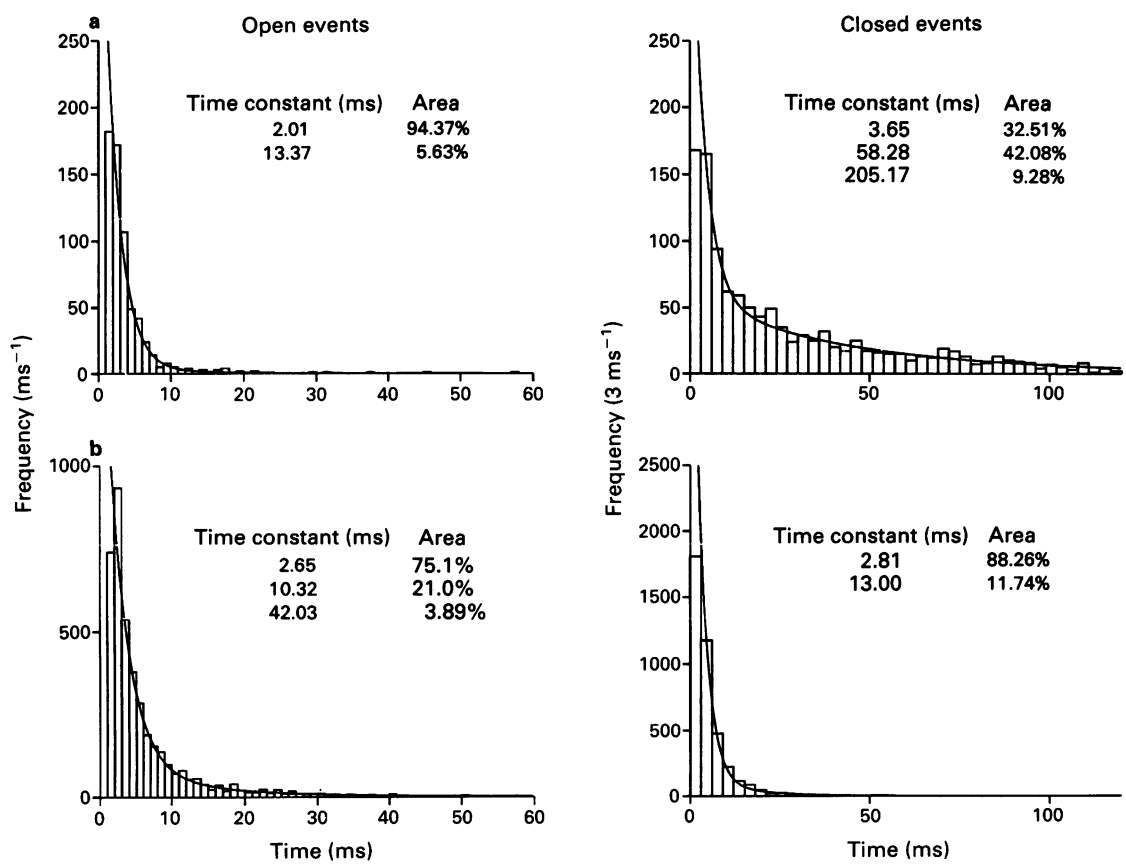


Figure 3 Open and closed lifetime histograms, with probability density functions obtained by maximum likelihood fitting to individual lifetimes for a single sheep cardiac sarcoplasmic reticulum (SR) Ca^{2+}-release channel activated by (a) 10 μM Ca^{2+} and (b) 10 μM Ca^{2+} + 500 μM (−)-sulmazole. Lifetimes, obtained from 3 min recordings, are displayed in non-cumulative histograms. Probability density functions, obtained by the method of maximum likelihood are drawn according to: $f(t) = a_1(1/\tau_1)\exp(-t/\tau_1) + \ldots + a_n(1/\tau_n)\exp(-t/\tau_n)$, using Efit (Intracel). Open and closed lifetimes at 10 μM Ca^{2+} were best fitted by two and three exponentials, respectively, indicative of at least two open and three closed states. After addition of 500 μM (−)-sulmazole, the open and closed lifetime durations were best described by three open and two closed states. The areas and time constants are shown for each respective exponential component.

Removal of the methylsulphinyl oxygen of sulmazole or isomazole increases potency by ten fold

In both sulmazole and isomazole isomers, the terminal sulphur atom is connected by a double bond to an oxygen. Removal of this oxygen atom creates two reduced compounds: the sulmazole analogue, EMD 46512 and the isomazole congener, EMD 41000. EMD 46512 increases the P_o of single SR Ca^{2+}-release channels by a similar mechanism to its parent compounds (+)- and (−)-sulmazole. However, the reduced compound is approximately ten times as potent in comparison (Figure 5). EMD 46512 is able almost to activate fully the channel at 10 μM Ca^{2+}, with an EC_{50} of 42 μM and a Hill slope of two ($n = 3$), by increasing the frequency and duration of open events (Figure 6).

Similarly, the reduced isomazole analogue, EMD 41000, was as potent as EMD 46512, with an EC_{50} of 40 μM ($n = 5$). In comparison to its parent compounds it is also approximately ten times as potent (Figure 7).

None of the sulmazole congeners affected the single Ca^{2+}-release channel conductance at 10 μM Ca^{2+} (90 ± 3 pS (± s.e. mean), $n = 7$) or the $Ca^{2+}/Tris^+$ permeability ratio (12.8 ± 0.2, $n = 7$). These values are similar to those obtained by Williams & Holmberg (1990). The P_o of channels activated by any of the sulmazole analogues in the presence of activating Ca^{2+} was decreased by the addition of ruthenium red or Mg^{2+} (not shown).

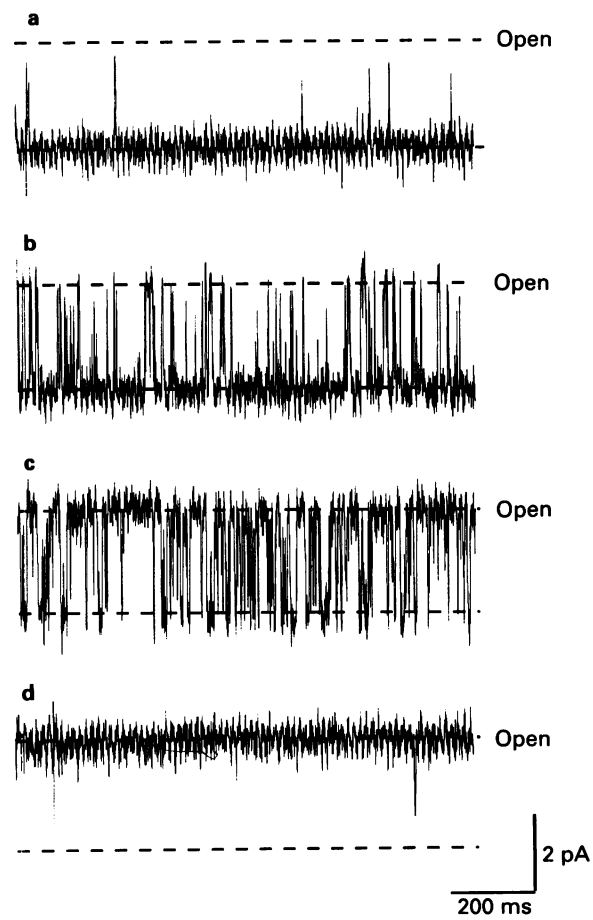


Figure 4 The increase in single-channel P_o induced by addition of *cis*-(+)-isomazole at 10 μM activating Ca^{2+}. At 10 μM Ca^{2+} the P_o was 0.031 (a). This was increased to 0.267, 0.701 and 0.991 on addition of 0.25 (b), 1 (c) and 3 mM (d) (+)-isomazole to the *cis* chamber. The holding potential was 0 mV. The P_o was calculated from 3 min of steady state recording of single-channel activation. The EC_{50} obtained for this compound was 445 μM ($n = 4$). The channel open level is indicated above each portion of data.

Ca^{2+}-independent activation of the Ca^{2+}-release channel by sulmazole analogues

All of the sulmazole analogues tested were able to increase the open probability of SR Ca^{2+}-release channels at sub-activating Ca^{2+} concentrations. In the effective absence of cytosolic Ca^{2+} (pM Ca^{2+}) the Ca^{2+}-release channel is closed. Figure 8 shows the increase in P_o when 100 μM EMD 46512 was added to the cytosolic face of a single-channel in the presence of 80 pM Ca^{2+}. The P_o increased from zero to 0.56, at 10 μM Ca^{2+}, this concentration of EMD 46512 would maximally activate the channel. None of the compounds tested was able to cause full activation of the channel, even at up to 3 mM. The results show that there is a synergistic action of the sulmazole analogues and Ca^{2+} in increasing the P_o of single Ca^{2+}-release channels, in agreement with the results obtained by Williams & Holmberg (1990) for (+)-sulmazole.

Interestingly, single channels activated by the sulmazole congeners at pM Ca^{2+} were still modified to a characteristic sub-conductance state by addition of 200 nM ryanodine to the cytosolic face of the channel protein (not shown). This would appear to suggest that the binding of ryanodine to the Ca^{2+}-release channel may occur independently of Ca^{2+}, merely requiring the channel to be in an open state. This was also found by Williams & Holmberg (1990).

Effect of EMD 46512 and EMD 41000 on [^{3}H]-ryanodine binding to SR membranes

[^{3}H]-ryanodine binding is often used as a marker for Ca^{2+}-release channel open probability (Chu *et al.*, 1990; Holmberg & Williams, 1990a). Williams & Holmberg (1990) have shown that (+)-sulmazole increases the binding of [^{3}H]-ryanodine to HSR membranes. This is achieved by increasing the affinity of the Ca^{2+}-release channel for ryanodine.

EMD 46512 and EMD 41000 increased [^{3}H]-ryanodine binding to HSR membranes with EC_{50}s of 25 μM and 25.12 μM, respectively, similar to the values for the increase in the P_o of the Ca^{2+}-release channel by these drugs (Figure 9). Both compounds increase equilibrium binding of 1 nM [^{3}H]-ryanodine from approximately 1 pmol $^3H\ mg^{-1}$ protein to 2 pmol $^3H\ mg^{-1}$ protein ($n = 5$) with Hill slopes of approximately

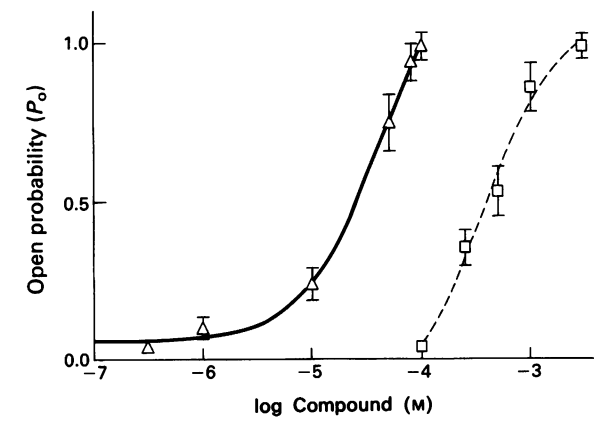


Figure 5 Comparison of the dose-response curves for (+)-sulmazole (□) and its reduced analogue EMD 46512 (△). The EC_{50} for each compound was 423 μM ($n = 4$) and 42 μM ($n = 3$), respectively. The Hill slopes for the activation were 1.98 and 2.04 for EMD 46512 and (+)-sulmazole. Both compounds were able to activate almost maximally the Ca^{2+}-release channel but with different potencies. Each data point was obtained from a single sarcoplasmic reticulum SR Ca^{2+}-release channel activated in the presence of 10 μM Ca^{2+} by either compound and held at 0 mV. Data points are shown ± s.e. mean. The curves were fitted to the data with INPLOT (GraphPad Software).

two for both drugs. This is also in agreement with the Hill slope for the increase of channel P_o by these drugs at 10 μM Ca^{2+}.

Ca^{2+}-independent increase in [^{3}H]-ryanodine binding to HSR membranes by EMD 46512 and EMD 41000

As these drugs are able to increase the P_o of single Ca^{2+}-release channels in the effective absence of activating Ca^{2+}, their effect on equilibrium binding of [^{3}H]-ryanodine to HSR membranes in pM Ca^{2+} was investigated. At 80 pM Ca^{2+}, [^{3}H]-ryanodine binding is almost undetectable. However, pretreatment of membranes with 1 μM–2 mM EMD 46512 dose-dependently increased binding to 111.36 ± 4.51 fmol ^{3}H mg^{-1} protein (n = 5) (Figure 10). This yielded an EC_{50} of 112.1 μM and an accompanying Hill coefficient of 0.96. Values of 139.5 μM and 0.83 μM (n = 5) were obtained for EMD 41000.

Site of action in relation to caffeine

The action of caffeine on cardiac SR Ca^{2+}-release channel P_o has been studied previously by Rousseau & Meissner (1989) and Sitsapesan & Williams (1990). Their results showed that the mechanism by which caffeine increases single-channel P_o is similar to that demonstrated here and by Williams & Holmberg (1990) for sulmazole and its analogues. As sulmazole and isomazole exert similar effects on contractile proteins, PDEs and on Ca^{2+}-release channel P_o to caffeine, it may be concluded that they act at the same sites on the release channel as caffeine. Figure 11 demonstrates the superimposition of the energy minimized structures of (+)-sulmazole and caffeine. It clearly shows that the imidazopyridine group of sulmazole is almost exactly superimposable on the corresponding purine ring system of the caffeine molecule.

Discussion

The increase in the P_o of single Ca^{2+}-release channels caused by (+)- and (−)-sulmazole demonstrates that there is no stereoselectivity at this activation site on the channel protein, as both enantiomers are approximately equally potent. Both compounds have similar EC_{50}s and activate the channel with Hill coefficients of approximately two, suggesting that at least two molecules of either compound are required to bind to the Ca^{2+}-release channel to cause full activation in the presence of 10 μM activating Ca^{2+}. The drugs increased P_o by increasing the frequency and duration of open events. However, at lower concentrations, although the open lifetime durations were slightly increased, the single-channel P_o was increased mainly by an increase in the frequency of channel opening. This is broadly in accord with the data of Williams & Holmberg (1990).

The observation that there is no stereoselectivity at the sulmazole site on the Ca^{2+}-release channel is supported by the effects of the analogues, (+)- and (−)-isomazole. These drugs are equipotent in increasing the P_o of the channel at 10 μM Ca^{2+}, with EC_{50}s of 445 μM. They are also approximately as potent as (+)- and (−)-sulmazole. As the sul-

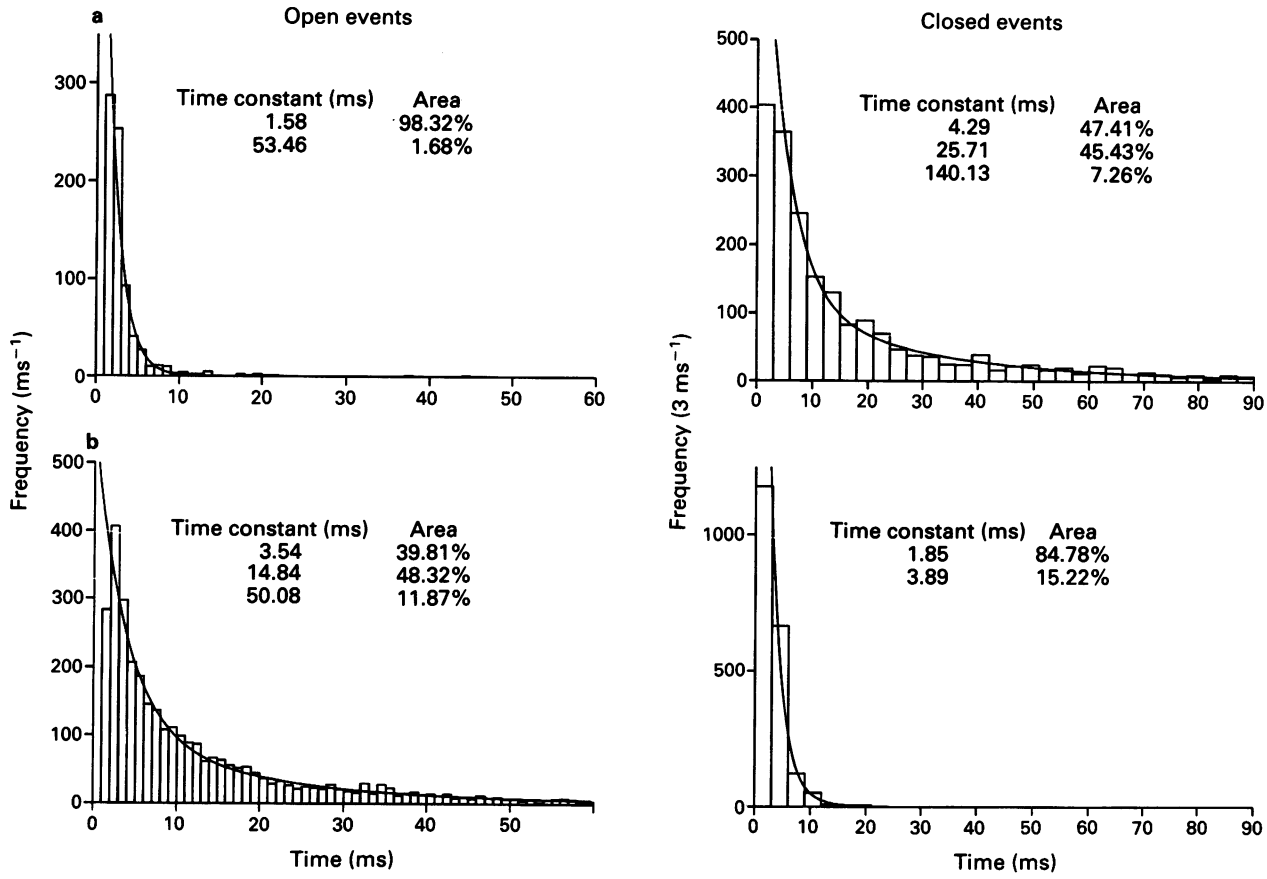


Figure 6 Open and closed lifetime durations of a single cardiac sarcoplasmic reticulum Ca^{2+}-release channel activated by 10 μM Ca^{2+} (a) and 10 μM Ca^{2+} + 80 μM EMD 46512 (b). Lifetime durations are displayed in non-cumulative histograms. The probability density functions, obtained by the method of maximum likelihood, are fitted using Efit (Intracel) according to $f(t) = a_1(1/\tau_1)\exp(-t/\tau_1) + \ldots + a_n(1/\tau_n)\exp(-t/\tau_n)$. At 10 μM Ca^{2+}, open and closed lifetime durations are best described by two open and three closed states; in the presence of 80 μM EMD 46512, these are best described by three open and two closed states, reflecting that EMD 46512 increases P_o by increasing the frequency of opening as well as the duration of open events.

mazole and isomazole enantiomers are equipotent, activate the Ca^{2+}-release channel with Hill coefficients of approximately two and increase single-channel P_o by similar mechanisms it seems unlikely that these two sets of drugs exert their effects by binding to distinct sites on the Ca^{2+}-release channel. It is therefore reasonable to conclude that they bind to the sulmazole sites.

The data obtained with both these sets of isomers also demonstrate that the position of the nitrogen in the pyridine ring, either in the 4,5b or 4,5c position, does not affect the potency of these drugs in activating the Ca^{2+}-release channel. The change in the position of the pyridine nitrogen changes the electron density in the ring system. As this has no effect, this may suggest that the nitrogen atom in the 4,5b or 4,5c positions in the pyridine ring in these compounds does not play a significant role in the binding of these compounds to their sites on the channel protein.

That there is no stereoselectivity at the sulmazole activation site together with our demonstration that the position of the pyridine nitrogen is unimportant for the activation of the Ca^{2+}-release channel by these analogues is in contrast to the situation with the sensitization of the myofilaments to Ca^{2+} and the inhibition of the Na^+/K^+ ATPase and type III PDE by these compounds. Van Meel *et al.* (1988) showed that (+)-sulmazole was a more potent Ca^{2+}-sensitizing agent than its (−)-enantiomer. Also, Honerjager *et al.* (1989) found that sulmazole compounds were more potent inhibitors of the ATPase and that isomazole compounds were slightly more potent PDE inhibitors. It could have been suggested that these analogues were binding to type III PDE which are known to be present in SR membranes (Kauffman *et al.*, 1989; Lugnier *et al.*, 1993), thereby modulating Ca^{2+}-release channel P_o. However, as the pharmacology of these compounds in activating the SR Ca^{2+}-release channel is different

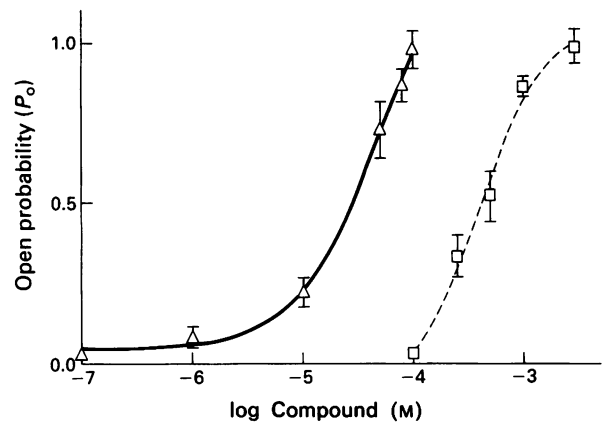


Figure 7 Comparison of the dose-response curves for (+)-isomazole (□) and its reduced analogue EMD 41000 (△). Each point is the mean ± s.e.mean from four (isomazole) or five (EMD 41000) different experiments. In each experiment the channels were activated by these compounds in the presence of 10 μM Ca^{2+} and were voltage clamped at 0 mV. The EC_{50}s for (+)-isomazole and EMD 41000 were 445 μM and 40 μM respectively. The sigmoidal curves were fitted to the data by INPLOT (GraphPad Software).

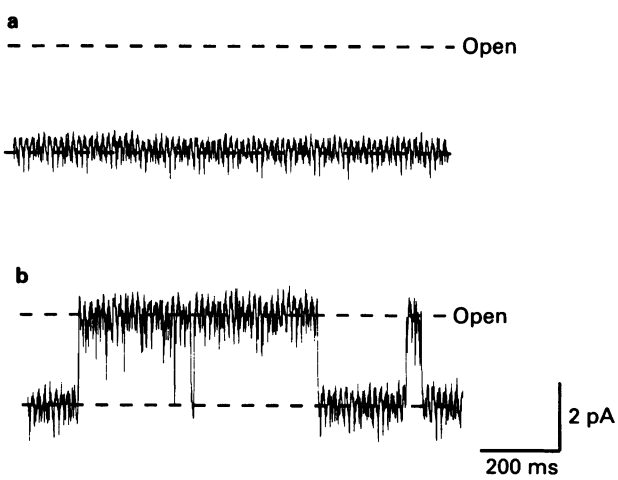


Figure 8 A single cardiac sarcoplasmic reticulum Ca^{2+}-release channel held at 0 mV at sub-activating (80 pM) Ca^{2+}. At this concentration, the channel is closed (a). On addition of 100 μM EMD 46512 to the cytosolic face of the channel, P_o increased from 0 to 0.560 (b), showing that these compounds are able to increase the P_o of the channel independently of activating Ca^{2+} and therefore must do so by binding to a closed conformation of the channel protein.

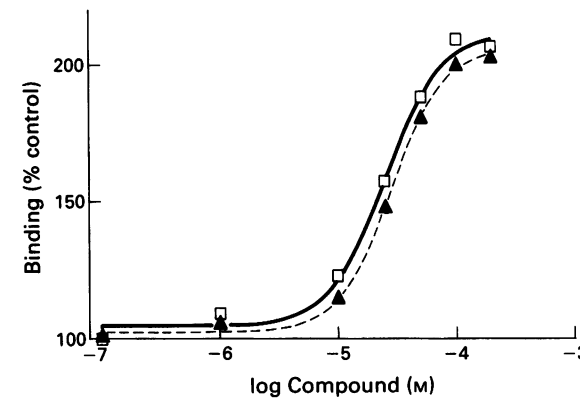


Figure 9 Stimulation of [^{3}H]-ryanodine binding to cardiac sarcoplasmic reticulum vesicles by EMD 46512 (□) and EMD 41000 (▲) at 10 μM Ca^{2+}. Incubation of vesicles with 1 nM [^{3}H]-ryanodine yielded specific equilibrium binding with Ca^{2+} of 1.117 ± 0.187 ($n = 6$) pmol $^3H\ mg^{-1}$ protein. Both EMD 46512 and EMD 41000 caused an approximate doubling of specific binding. The EC_{50}s for activation were 25 and 28.2 μM, respectively. Each point is the mean of six different experiments. The standard errors are omitted for clarity. The Hill coefficients were 1.84 and 2.00 for EMD 46512 and EMD 41000. The curves were fitted using INPLOT (GraphPad Software).

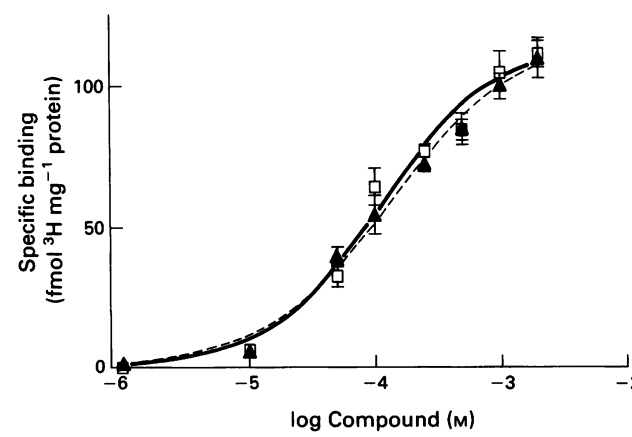


Figure 10 Stimulation of [^{3}H]-ryanodine binding by EMD 46152 (□) and EMD 41000 (▲) at pM Ca^{2+}. At pM Ca^{2+}, specific binding was almost undetectable. However, addition of these analogues increased specific binding to approximately 112 fmol $^3H\ mg^{-1}$ protein, significantly less than that obtained at 10 μM Ca^{2+}. The EC_{50}s obtained from the fitted sigmoid curves for EMD 46512 and EMD 41000 were 112.1 μM and 139.5 μM. Each point is the mean ± s.e. mean of five experiments. The Hill slopes were 0.96 and 0.83 respectively, suggesting that only one molecule of either drug binds to the closed state of the channel protein to increase [^{3}H]-ryanodine binding.

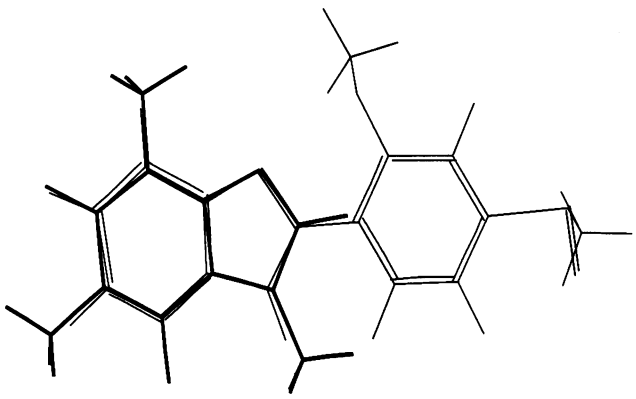


Figure 11 The superimposition of the minimized structure of caffeine and sulmazole. The structures of both compounds were minimized using both the Fletcher-Reeves and Polak-Ribeire algorithms in the MM+ force field, to ensure a minimum had been reached for each, using HyperChem (Autodesk Inc.). The structures were superimposed using the programme. Double bonds in the caffeine ring system have been omitted for clarity. The imidazopyridine ring of sulmazole (and the rest of the analogues tested here) was almost exactly superimposable on the purine ring of caffeine. This structural homology and that the ring systems adopt similar low energy conformations may indicate that they bind to common sites on the cardiac SR Ca^{2+}-release channel.

from that in the inhibition of the ATPase or PDE, it is apparent that these drugs are binding to sites, probably on the Ca^{2+}-release channel, distinct from either ATPase or PDE.

Reduction of either sulmazole or isomazole, thereby removing the methylsulphinyl oxygen, produces EMD 46512 and EMD 41000, which are approximately ten times as potent as their parent compounds in activating the Ca^{2+}-release channel. They increase P_o by increasing the frequency and duration of open events and the Hill slopes for the activation are two, suggesting that two molecules of either reduced compound are also required to bind to the channel for full activation at 10 μM Ca^{2+}.

The compounds tested here displayed the ability to increase [^{3}H]-ryanodine binding to cardiac HSR membranes at 10 μM Ca^{2+}. EMD 46512 and EMD 41000 increased equilibrium binding to approximately twice the level in the absence of either drug. The EC_{50}s were 25 μM and 25.12 μM, close to those values obtained from single-channel experiments. The Hill slopes for the sigmoidal binding curves were two, also in agreement with the single-channel data and providing further confirmation that two molecules of either drug bind to the Ca^{2+}-release channel in the presence of activating Ca^{2+}.

All of the analogues are able to increase the P_o of single Ca^{2+}-release channels in the effective absence of activating cytosolic Ca^{2+}, however full activation (P_o approximately 1) could not be attained, in agreement with the earlier findings of Williams & Holmberg (1990). The compounds also increased [^{3}H]-ryanodine binding in the effective absence of activating Ca^{2+} (80 pM). EMD 46512 and EMD 41000 increased specific binding from almost zero to approximately 112 fmol ^{3}H mg^{-1} protein, with EC_{50}s of 112 μM and 139 μM, respectively. The value for maximal binding is significantly less than that obtained in the presence of 10 μM Ca^{2+} and the EC_{50}s are significantly increased. Hence, both the potency and efficacy of these compounds are much reduced in the absence of Ca^{2+}.

Interestingly, the Hill slope for the increase in binding in the absence of Ca^{2+} was approximately one for both compounds. These data suggest that only one molecule of either isomer may bind to the closed conformation of the channel and that maximal [^{3}H]-ryanodine binding cannot be realised under these conditions. Further, it suggests that Ca^{2+} may stimulate the binding of a second molecule of EMD 46512 or 41000. When two drug molecules are bound to the channel protein and Ca^{2+} occupies its activation sites, maximal [^{3}H]-ryanodine binding may then occur. Hence the presence of activating Ca^{2+} increases the potency and efficacy of these drugs partly by stimulating the binding of a second drug molecule to the channel.

Extrapolating this to the single-channel data, suggests that when one molecule of one of these drugs binds to the channel, maximal P_o may not be attained and a different pattern of gating occurs. When Ca^{2+} is present, the binding of a second drug molecule is stimulated, and the presence of two molecules of activating drug together with Ca^{2+} allows full channel activation. This shows that there is a Ca^{2+}-dependent and a Ca^{2+}-independent action of these drugs to increase the P_o of the Ca^{2+}-release channel.

The differences in the structures of the sulmazole and isomazole enantiomers reside in the orientation of the double-bonded oxygen atom and the methyl group around the terminal sulphur atom, the sulphur being a chiral centre. As the enantiomers are mirror images of each other, it can be estimated that the oxygen in each protrudes 0.825Å above or below the plane of the molecule (which is flat) and the carbon of the methyl group protrudes 1.715Å above or below the plane. The reduced analogues, EMD 46512 and EMD 41000, are devoid of a double bonded oxygen attached to the sulphur and the remaining methyl group reorientates into the plane of the molecule. As these drugs are approximately ten times as potent as their respective parent compounds, it is possible that the presence of this oxygen in sulmazole and isomazole hinders the binding of the compounds to the Ca^{2+}-release channel. The lack of the physical bulk or lack of charge due to the oxygen may thus allow the reduced analogues better access to their sites, resulting in increased potency of the reduced compounds. However, it is also a possibility that the protruding methyl group in sulmazole or isomazole may similarly hinder proper access to binding sites and contribute to the lack of potency of these drugs in comparison to the reduced analogues. Regardless of this uncertainty, it is apparent that minor modifications around the sulphur atom dramatically affect potency, suggesting that the area around the sulphur may be critically involved in the channel activation. Further modifications in the area around this atom may result in drugs with greater ability to activate the Ca^{2+}-release channel or even antagonists of the compounds studied here. Notwithstanding this, it is not unreasonable to suggest that modifications at other points in the molecule may also affect the potency of the compound.

Modelling of the structures of the analogues used in this study and caffeine show that the imidazopyridine ring of the sulmazole analogues can be directly superimposed on the purine ring of caffeine. As these compounds exert similar effects by similar mechanisms (albeit with different potencies) on the SR Ca^{2+}-release channel, it is possible that they act by binding to the same sites on the channel protein. As the caffeine site on the channel has been demonstrated to be distinct from the adenosine sites (McGarry & Williams, 1993a) this suggests that adenosine and sulmazole also act at different sites on the channel. If the sulmazole analogues and caffeine act at the same sites on the Ca^{2+}-release channel then this suggests that although substituents in the area around the sulphur atom of the sulmazole compounds may influence potency, the imidazopyridine ring of the drugs is also involved in their binding to the protein.

In conclusion, this study shows that several analogues of sulmazole increase the P_o of the cardiac SR Ca^{2+}-release channel. The increase in P_o occurs by an increase in the frequency and duration of open events. The data show that there is no stereoselectivity at the sulmazole site and therefore, the positions of the oxygen and methyl groups attached to the sulphur atom of sulmazole and isomazole do not affect activity. Similarly, the position of the pyridine nitrogen, 4,5b or 4,5c positions, does not affect potency of the drugs. However, removal of the methylsulphinyl oxygen increases po-

tency by approximately ten fold. These results also suggest that activation of the Ca^{2+}-release channel by these compounds in the absence of Ca^{2+} occurs by only one drug molecule binding to the channel. Full activation occurs in the presence of Ca^{2+} partly due to Ca^{2+} stimulating the binding of a second drug molecule. Modelling the structures of the drugs suggests that they act at the same activation sites as caffeine on the Ca^{2+}-release channel.

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References

BLATZ, A.L. & MAGLEBY, K.L. (1986). Quantitative description of three modes of activity of fast chloride channels from rat skeletal muscle. *J. Physiol.*, **378**, 615–634.

BLINKS, J.R. & ENDOH, M. (1984). Sulmazole (AR-L 115 BS) alters the relation between Ca^{2+} and tension in living canine ventricular muscle. *J. Physiol.*, **353**, 63P.

COLQUHOUN, D. & SIGWORTH, F. (1983). Fitting and statistical analysis of single-channel records. In *Single-Channel Recording*. ed. Sakmann, B. & Neher, E. pp. 191–263. New York: Plenum Press.

FATT, P. & GINSBORG, B.L. (1958). The ionic requirements for the production of action potentials in crustacean muscle fibres. *J. Physiol.*, **142**, 516–543.

HOLMBERG, S.R.M. & WILLIAMS, A.J. (1990a). The cardiac sarcoplasmic reticulum calcium-release channel: modulation of ryanodine binding and single-channel activity. *Biochim. Biophys. Acta*, **1022**, 187–193.

HOLMBERG, S.R.M. & WILLIAMS, A.J. (1990b). Patterns of interaction between anthraquinone drugs and the calcium-release channel from cardiac sarcoplasmic reticulum. *Circ. Res.*, **67**, 272–283.

HONERJAGER, P., KLOCKOW, M., SCHONSTEINER, G. & JONAS, R. (1989). Imidazopyridine: roles of pyridine nitrogen position and methylsulfinyl oxygen for in vitro positive inotropic mechanism and chronotropic activity. *J. Cardiovasc. Pharmacol.*, **13**, 673–681.

KAUFFMANN, R.F., UTTERBACK, B.G. & ROBERTSON, D.W. (1989). Characterisation and pharmacological relevance of high affinity binding sites for [^{3}H]LY186126, a cardiotonic phosphodiesterase inhibitor, in canine cardiac membranes. *Circ. Res.*, **65**, 154–163.

LUGNIER, C., MULLER, B., LE BEC, A., BEAUDRY, C. & ROUSSEAU, E. (1993). Characterisation of indolidan- and rolipram-sensitive cyclic nucleotide phosphodiesterase in canine and human cardiac microsomal fractions. *J. Pharmacol. Exp. Ther.*, **265**, 1142–1151.

MCGARRY, S.J. & WILLIAMS, A.J. (1994). Adenosine discriminates between the caffeine and the adenine nucleotide sites on the sheep cardiac sarcoplasmic reticulum Ca^{2+}-release channel. *J. Memb. Biol.*, (in press).

MCGARRY, S.J. & WILLIAMS, A.J. (1993a). Digoxin activates sarcoplasmic reticulum Ca^{2+}-release channels: a possible role in cardiac inotropy. *Br. J. Pharmacol.*, **108**, 1043–1050.

MCGARRY, S.J. & WILLIAMS, A.J. (1993b). Activation of the sheep cardiac Ca^{2+}-release channel by analogues of sulmazole. *Br. J. Pharmacol.*, **108**, 150P.

MEISSNER, G. (1986). Ryanodine activation and inhibition of the Ca^{2+}-release channel of sarcoplasmic reticulum. *J. Biol. Chem.*, **261**, 6300–6306.

MEISSNER, G. & HENDERSON, J.S. (1987). Rapid Ca^{2+} release from cardiac sarcoplasmic reticulum vesicles is dependent on Ca^{2+} and is modulated by Mg^{2+}, adenine nucleotides and calmodulin. *J. Biol. Chem.*, **262**, 3065–3073.

MILLER, C. (1982). Open-state structure of single chloride channels from Torpedo electroplax. *Phil. Trans. R. Soc.*, **B299**, 401–411.

PARSONS, W.J., RAMKUMAR, V. & STILES, G. (1988). The new cardiotonic agent sulmazole is an A_1 adenosine receptor antagonist and functionally blockes the inhibitory regulator, G_i. *Mol. Pharmacol.*, **33**, 441–448.

PESSAH, I. & ZIMANYI, I. (1991). Characterisation of multiple [^{3}H] ryanodine binding sites on the Ca^{2+}-release channel of sarcoplasmic reticulum from skeletal and cardiac muscle: evidence for a sequential mechanism in ryanodine action. *Mol. Pharmacol.*, **39**, 679–689.

ROUSSEAU, E. & MEISSNER, G. (1989). Single cardiac sarcoplasmic reticulum Ca^{2+}-release channel: activation by caffeine. *Am. J. Physiol.*, **256**, H328–H333.

SITSAPESAN, R. & WILLIAMS, A.J. (1990). Mechanisms of caffeine activation of single calcium-release channels of sheep cardiac sarcoplasmic reticulum. *J. Physiol.*, **423**, 425–439.

SMITH, J.S., CORONADO, R. & MEISSNER, G. (1985). Sarcoplasmic reticulum contains adenine nucleotide-activated calcium channels. *Nature*, **316**, 446–449.

TOMLINS, B., HARDING, S.E., KIRBY, M.S., POOLE-WILSON, P.A. & WILLIAMS, A.J. (1986). Contamination of a cardiac sarcolemmal preparation with endothelial plasma membrane. *Biochim. Biophys. Acta*, **856**, 137–143.

VAN MEEL, J.C.A., ZIMMERMANN, R., DIEDEREN, W., ERDMAN, E. & MRWA, U. (1988). Increase in calcium sensitivity of cardiac myofibrils contributes to the cardiotonic action of sulmazole. *Biochem. Pharmacol.*, **37**, 213–220.

WILLIAMS, A.J. & HOLMBERG, S.R.M. (1990). Sulmazole (AR-L-115 BS) activates the sheep cardiac muscle sarcoplasmic reticulum calcium-release channel in the presence and absence of calcium. *J. Memb. Biol.*, **115**, 167–178.

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NMDA antagonists increase recovery of evoked potentials from slices of rat olfactory cortex after anoxia

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1 The role of glutamate in producing tissue damage during cerebral anoxia was investigated in brain slices using antagonists to the NMDA and AMPA receptor types.

2 Tissue function was assessed by field recordings of the synaptically evoked potentials elicited by stimulating the main afferent input to the olfactory cortex, the lateral olfactory tract. Anoxia was produced by bathing the slice in glucose-free solution equilibrated with 95% N_2/5% CO_2.

3 The amount of recovery of the evoked potential was inversely dependent on the period of anoxia and temperature: at 24°C, 15 min of anoxia followed by reoxygenation produced a 14.6 ± 4.1% recovery whereas there was no recovery at 35°C.

4 Dizocilpine and ketamine had no effect on synaptic transmission in oxygenated media but following anoxia they produced an increased recovery of the responses: from 14.6 ± 4.1% to 48.3 ± 7.8% for dizocilpine (10 μM) and 21.6 ± 7.7% to 87.2 ± 7.1% for ketamine (200 μM); the tissue endurance to anoxia was increased by around 5 min.

5 Blockade of the AMPA receptors did not influence recovery in spite of the depressed synpatic transmission. A similar synaptic attenuation produced by lignocaine provided some increase in post-anoxic recovery.

6 The NMDA receptor antagonist, AP5, antagonized NMDA at 50 μM by 3.7 fold and at 200 μM by 15 fold but only 200 μM increased post-anoxic recovery. This suggests that a substantial degree of NMDA antagonist is required before anoxic tissue damage due to NMDA receptor activation can be nullified. The antagonist to the glycine binding site, 7-chlorokynurenic acid also increased recovery.

7 These *in vitro* experiments confirm the idea that NMDA receptor activation makes a substantial contribution to cerebral tissue damage and that this can be reduced by a substantial blockade of these receptors.

Keywords: Glutamate; NMDA; anoxia; brain; neuroprotection; rat; olfactory cortex; dizocilpine

Introduction

A variety of changes take place in tissues during oxygen deprivation. The central nervous system is particularly vulnerable to ischaemia partly because of glutamate release (Benveniste *et al.*, 1984; Rothman, 1984; Drejer *et al.*, 1985; Sanchez-Prieto & Gonzalez, 1988; Nicholls & Attwell, 1990) which causes cell death (Choi, 1988; Frandsen *et al.*, 1989; Meldrum & Garthwaite, 1990). Under pathological conditions, glutamate acts on several receptor systems. For cell pathology, the most important of these is the N-methyl-D-aspartate receptor type (NMDA) since it is coupled to voltage-dependent channels that are Ca^{2+}permeant (Garthwaite & Garthwaite, 1986; McDermott *et al.*, 1986; Choi, 1987; Michaels *et al.*, 1990) producing Ca^{2+} loading of the cell. This loading in turn activates several vigorous Ca-activated enzymes including phospholipase, proteases and nucleases leading to cell death.

There has been an assessment of several strategies that might provide some protection of neural tissue during ischaemia and one of these is based on NMDA antagonists. Several studies have found NMDA antagonists to offer some protection against ischaemia/anoxia (Park *et al.*, 1988; Kochlar *et al.*, 1988; Bielenberg, 1989) whilst in others, the protection has been small or absent (Perkins *et al.*, 1988; Aitkin *et al.*, 1988; Fleischer *et al.*, 1989; Sterz *et al.*, 1989; Lanter *et al.*, 1990; McDonald *et al.*, 1990). Most studies on the pharmacology of neuroprotection have been *in vivo*. Such studies are best suited to the assessment of substances that might have immediate clinical application. However, there are a large number of variables than can contribute to the tissue damage so the mechanism of action of protective agents is more difficult to analyse. For example, in cerebral ischaemia, tissue damage might result from oxygen lack directly or from accumulated metabolites. Furthermore, since most *in vivo* experiments have been conducted over several days, the post-ischaemic changes might be secondary to gliosis or alterations in the blood brain barrier (Giulian, 1993). Some of the problems encountered *in vivo* are circumvented in experiments with isolated tissues. Although most studies have concentrated on the microscopic changes after glutamate agonist insult some have looked to the effects of anoxia on evoked potentials in the hippocampus (Clark & Rothman, 1987; Rader & Lanthorn, 1989; Papas *et al.*, 1993). In the present study, we have assessed the effects of anoxia by following the changes in electrical activity in slices of rat olfactory cortex maintained *in vitro*. Two goals were in mind (a) to elucidate the mechanisms underlying the anoxia-induced cell death, and (b) to find substances that might provide tissues with some protection during anoxia. We show that some NMDA antagonists produce a substantial increase in the recovery of tissues following short periods of anoxia. A preliminary account of this work has been published (Scholfield & Yassin, 1992).

Methods

Female Sprague-Dawley rats of 200–300 *g* weight were decapitated and their brains removed. The brain was bisected along the mid-line and pial surface slices of olfactory cortex were cut to a thickness of either 350 μm for studies at 35°C or 450 μm for 24°C. The time between decapitation and placement of the slices in oxygenated bathing solution at 24°C was 3 min, a period too short to produce permanent

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changes in tissue responsiveness to electrical stimulation (see results). The two olfactory cortex slices were each subdivided along the axonal radiations into three, such that each had a length of lateral olfactory tract (LOT). The slices were placed in a 50 ml bath of Krebs solution contained within a sintered glass Buchner funnel. 95% O_2/5% CO_2 was supplied to the bottom of the funnel allowing the slices to be continually agitated in the bathing medium.

Individual slices were placed on a nylon mesh within a recording chamber (effective volume of 0.5 ml) and superfused with Krebs solution equilibrated with 95% O_2/5% CO_2 at 5 ml min^{-1} by recirculation from a 50 ml reservoir. The connections were through silicon rubber tubing the length of which was minimized to avoid gaseous de-equilibration. The gas mixture was also blown over the surface of the recording chamber to avoid gaseous exchange with air. The reservoir was water-jacketed and the flow line immediately adjacent to the recording chamber contained a heat exchanger to maintain the recording chamber at either 24° or 35°C.

A pair of stimulating electrodes was placed on the LOT and a recording pipette placed on the pial surface 2 mm away from the stimulating electrodes. The electrode was connected to a preamplifier and the waveforms were digitized, stored and analysed by computer. The LOT was continually stimulated with 10 V, 0.2 ms pulses at 0.05 Hz and each response stored.

The tissue was allowed to equilibrate for 30 min in the bath before the collection of records. Antagonist was added to the reservoir and recirculated for 30 min. The recirculating solution was then changed for glucose-free solution equilibrated with 95% N_2/5% CO_2. This nitrogen gas mixture was also blown over the surface of the recording chamber. After the period of anoxia, the bathing solution was changed back to normal solution equilibrated with 95% O_2/5% CO_2. Most experiments were performed at 24 ± 1°C (maximum range) but we also looked at some effects at a temperature nearer to that *in vivo* (38°C in the rat). However, at this temperature, the responses become unstable (Scholfield, 1980) presumably because of greater oxygen demands. Therefore to avoid the possibility of metabolic insufficiency and 'sensitization' to anoxia, experiments were conducted at 35°C rather than 38°C. Test substances were present for 30 min before and during the anoxia period. They were not present during the washout period. The concentration of NMDA antagonist was assessed in another series of experiments. The amplitude of the evoked potential was monitored during the application of NMDA at 5–1000 μM in normal solution and after 30 min equilibration in combination with various antagonists.

Between animals, a substantial amount of variability was observed. To reduce the effects of variability, a control experiment was performed in normal solution on one slice and then the test antagonist was tested on another slice from the same animal. The next pair of slices, the test experiment preceded the control. Some slices were used within 2 h of cutting whereas others were used up to 6 h after preparation. There was no time-dependent difference in control effects of anoxia. Each slice was subjected to one period of anoxia only. In some experiments, a range of anoxic periods was used. For most experiments, one time period was used to produce a 5–30% control recovery, a level chosen to allow for any enhancement in the recovery of test slices. The peak amplitudes of the synaptic responses were measured and the amount of recovery expressed as a proportion of the response immediately before the anoxic period. Test and control experiments were compared by Student's paired t test (two tailed). All values are means ± s.e. of mean and significance was taken as $P < 0.05$.

The Krebs solution had the following composition (mM): Na^+ 144, K^+ 5.0, Ca^{2+} 2.5, Mg^{2+} 1.3, Cl^- 128, HCO_3^- 25, D-glucose 2.0 and it was equilibrated with 95% O_2/5% CO_2. The rather low glucose concentration was necessary because previous work (Donaghy & Scholfield, 1990) had shown a delayed response to anoxia when slices were stored with 11 mM. During anoxia, glucose was removed since it had been shown also that responses could be sustained in glucose. The drugs used were from the following sources: dizocilpine (MK801) Merck, Sharp and Dohme, Harlow, UK; 5,7-dinitroquinoxaline-2,3-dione (DNQX), DL-2-amino-5-phosphopentanoic acid (AP5) and 7-chlorokynurenic acid, Tocris Neuramin, Bristol, UK; 2-[2-chlorophenyl]-2-[methylamino]-cyclohexanone (ketamine), Sigma, Poole, UK; lignocaine, Astra Laboratories, Watford, UK.

Results

Stimulation of the LOT elicited a surface negative waveform which is the result of the release of acidic amino-acid (Collins, 1979) acting on α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) type receptors (Collins & Buckley, 1989). NMDA receptors are present in olfactory cortex (Tacconi *et al.*, 1993) and they may also be involved in synaptic transmission (Collins, 1991) but in our experiments, this component did not contribute to the evoked potential (see below). When recordings were made from an area of the slice close to the LOT, the synaptic potential was preceded by a shorter transient response due to the activity of the axons underlying the recording electrode. This fibre response is not apparent in Figure 1 but can be seen with both preparations in Figure 2. The fibre potential is close to the stimulus artefact but it can be distinguished by comparing the response labelled 'anoxia' in Figure 2 where all the biologically generated responses had been lost. Without any experimental intervention, all the responses were maintained constant over a period of several hours. (Scholfield, 1980).

Effect of anoxia

When the bathing solution was changed to one equilibrated with 95%N_2/5%CO_2 there was a rapid and complete loss of both the pre- and postsynaptic responses (Figures 1 and 2). The response started to decline when the new solution arrived at the slice and this was normally complete within 4–6 min (Figure 3). When oxygenation was restored, the response recovered over a period of 30 min. In 5 slices, we followed the responses for a further 5 h after anoxia but there was no further recovery. Therefore, routinely, we studied only the initial 30 min recovery period. In some experiments, anoxia was tested at 24° and 35°C. After 15 min of anoxia the recovery was 14.6 ± 4.1% and 3.5 ± 1.4% respectively.

With some responses, a postsynaptic population spike could be resolved and the relation between this and the excitatory postsynaptic potential (e.p.s.p.) can be used to gain insight into the changes in membrane potential (Scholfield, 1980; Richards, 1972). During the onset of anoxia, there was a large depression of the e.p.s.p. with little change in the population spike. If the e.p.s.p. was reduced by decreasing the stimulus voltage, the e.p.s.p. and population spike were reduced in sympathy. These observations indicate that anoxia caused postsynaptic depolarization.

At 35°C, slices were exposed to varying periods of anoxia between 5 and 15 min and the amount of recovery is shown in Figure 4. For short periods (5 min), recovery was complete but diminished with longer periods such that little recovery was seen after 12 min.

Effect of NMDA

Application of NMDA (5–50 μM) produced a depression in the evoked potential. The following NMDA antagonists (Kemp *et al.*, 1987) were tested for their ability to reduce the action of NMDA: AP5 (50 and 200 μM), 7-chlorokynurenic acid (50 μM), dizocilpine (10 μM) and ketamine (200 μM). NMDA was not blocked immediately by dizocilpine or ketamine but antagonism developed over a period of 30 min

whereas AP5 and 7-chlorokynurenate showed a full effect within 10 min of addition. The concentrations of NMDA which produced a 50% depression of the evoked response in the presence of the above antagonists are shown in Table 1. All the substances tested produced a substantial antagonism of NMDA and AP5 was dose-dependent.

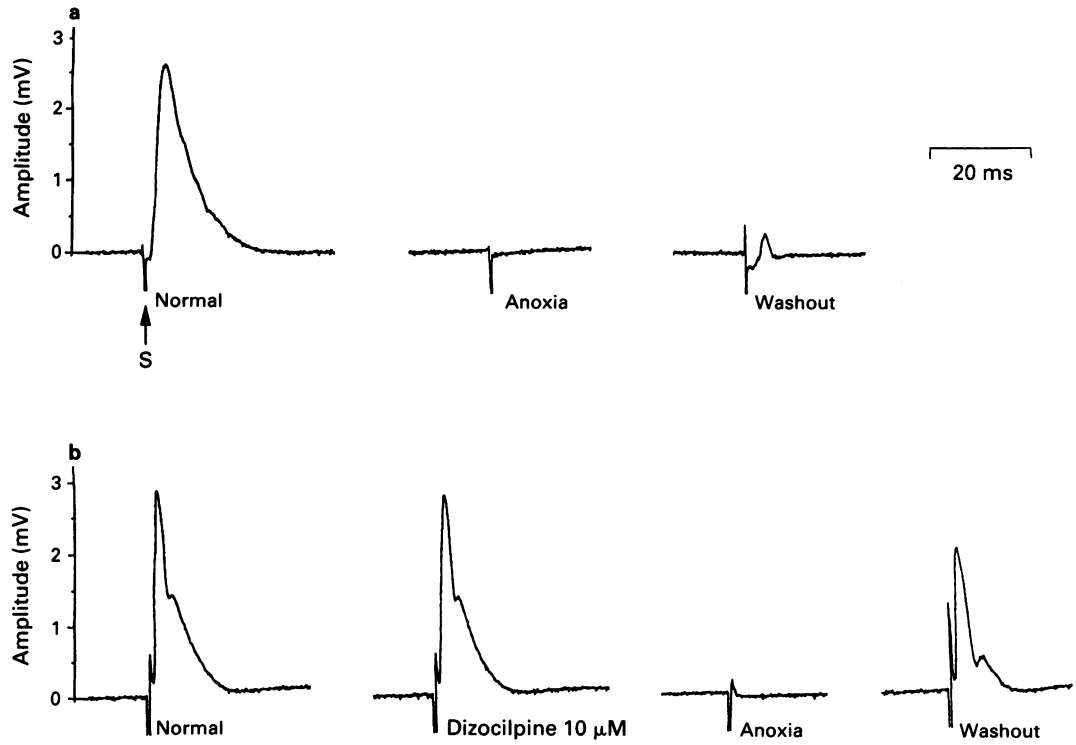


Figure 1 Single synaptically evoked potentials recorded from slices of rat olfactory cortex. In the upper traces (a), the slices was bathed in normal solution ('normal') followed 15 min in solution equilibrated with 95% N_2/5% CO_2 ('Anoxia') followed by normally oxygenated solution ('washout'). A time-course for a similar experiment is seen in Figure 3a. In the lower traces (b), another slice from the same animal was bathed in dizocilpine (10 μM) for 30 min before the period of anoxia and during the anoxia. 'S' indicates the point of stimulus application. On these traces, negative is upwards.

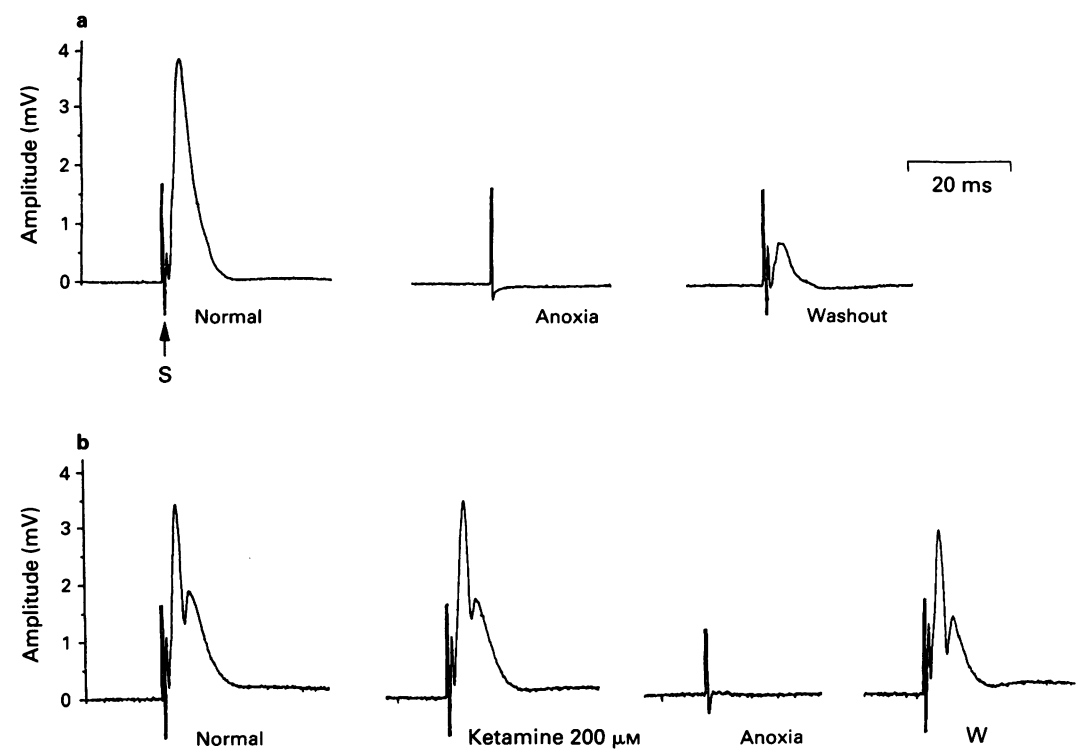


Figure 2 An experiment on evoked responses from another pair of slices comparing the effect of normal solution and incubation with ketamine (200 μM). In this experiment, the presysnaptic action is seen as a transient between the stimulus artifact (arrow labelled 'S') and the synaptic response. This transient has disappeared from the records labelled 'Anoxia'.

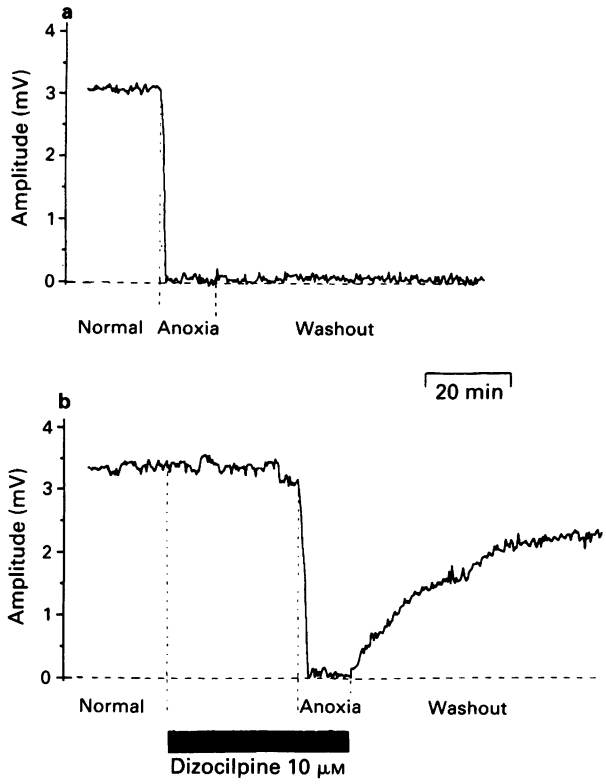


Figure 3 Time-courses for changes in the amplitude of the evoked response. The preparation was stimulated at 0.05 Hz and for each response, the peak amplitude of the evoked potential was measured and plotted on the time-course as a single point. Each point was joined to produce the continuous trace shown. In (a) the slice was bathed successively in normal (oxygenated) solution, in solution equilibrated with N_2/CO_2 (12 min) and then re-oxygenated ('washout'). (b) Shows a time-course of a slice from the same animal but with a preincubation period of 30 min in 10 μM dizocilpine.

Effect of dizocilpine

Application of 10 μM dizocilpine in normal oxygenated solution had no effect on the synaptic response (Figures 1 and 3) confirming that any involvement of NMDA receptors in this pathway is small (Collins, 1991). The presence of dizocilpine made no difference to onset and the decline response during anoxia. However, the speed of recovery and level of recovery attained were substantially augmented by this agent (Figure 1 and 3). Thus at 24°C, a 15 min period of anoxia produced a recovery in normal solution which was 14.6% compared to 48.3% in 10 μM dizocilpine (Table 2). The same effect was seen at 35°C for 12 min of anoxia (6.3% recovery in normal solution and 86.9% in dizocilpine).

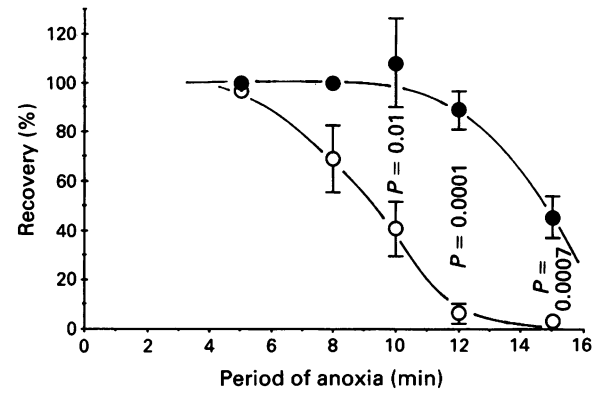


Figure 4 Effect of varying the period of anoxia (abscissa scale) on the recovery of the evoked potential (ordinate scale) in normal solution (○) and in the presence of 10 μM dizocilpine (●). The ordinate scale is the ratio of the amplitude of the evoked potential 30 min after the period of anoxia to that before the anoxia. The lines were drawn by 'eye'.

Table 1 Concentrations of N-methyl-D-aspartate (NMDA) which produced a 50% depression of the evoked potential

Antagonist	*ED_{50} for NMDA (μM) Normal*	*Test*	*Ratio*	n	P-*value*
AP5 50 μM	21.3 ± 2.9	71.2 ± 4.7	3.7 ± 0.3	6	<0.0001
AP5 200 μM	23.8 ± 3.2	336 ± 27	15.5 ± 2.4	6	<0.0001
7-Chlorokynurenate 50 μM	18.0 ± 1.1	565 ± 55	32.3 ± 3.7	6	<0.0001
Dizocilpine 10 μM	18.4 ± 1.7	>1000	>54	6	<0.0001
Ketamine 200 μM	18.4 ± 1.7	262 ± 13	16.3 ± 1.3	4	<0.0001

The ratios are the values in antagonist ('Test') divided by that in normal solution ('Normal') for each slice. The *n*-values are the numbers of pairs of slices and the *P*-value calculated using the two-tailed *t* test.

Table 2 Effect of glutamate antagonists on recovery after a period of anoxia

Experiment	*% recovery control*	*% recovery test*	*Change*	n	P-*value*
Lignocaine	8.1 ± 2.2	26.5 ± 5.6	18.4 ± 4.3	12	0.0012
DNQX	29.4 ± 5.3	36.5 ± 4.7	7.0 ± 5.2	10	0.21
AP5, 50 μM	24.9 ± 5.0	34.1 ± 3.4	9.2 ± 5.4	12	0.12
AP5, 200 μM	27.2 ± 3.9	75.3 ± 7.0	48.0 ± 7.2	8	<0.0001
7-Chlorokynurenate	20.2 ± 4.5	80.3 ± 6.9	60.1 ± 8.6	8	<0.0001
Dizocilpine	14.6 ± 4.1	48.3 ± 7.8	33.3 ± 6.5	12	0.003
Dizocilpine at 35°C	6.3 ± 4.2	86.9 ± 10.0	80.6 ± 6.7	12	0.0001
Ketamine	21.6 ± 7.7	87.2 ± 7.1	65.7 ± 9.2	12	0.0009

The change was calculated by subtracting the % recovery in normal solution (control) from that in the experimental solution (test) for paired slices from the same animals and averaging these. The *P* values were calculated from the paired two tailed *t* test for the control and test groups. All experiments were done at 24°C with a period of anoxia of 15 min except those at 35°C where the period of anoxia was 12 min.

In one group of experiments, slices were exposed to varying periods of anoxia. For all periods of anoxia tested, there was an enhanced recovery in the presence of dizocilpine (Figure 4). Thus in effect, the endurance of the tissue was increased by 5–6 min. As in normal solution, there was no further change in the amplitude of the response after 30 min in the recovery solution.

Effect of ketamine

Ketamine at 200 μM had no effect on synaptic transmission (Figure 2): higher concentrations were not tested since they depress axonal responses (McGivern & Scholfield, 1990). Nor did ketamine influence the depression produced by anoxia but like dizocilpine, the rate and the amount of recovery attained were substantially increased (Table 2).

Effect of AP5

Both dizocilpine and ketamine block the channels associated with the NMDA receptor whereas AP5 was tested as a substance that competes with the NMDA binding site. It was tested at two concentrations, 50 and 200 μM. The lower concentration had no effect on responses in normoxic and anoxic conditions nor was the recovery significantly affected by its presence (Table 2). AP5 200 μM had two clear effects: (a) alone it caused a depression of the response from 1.45 ± 0.23 to 1.24 ± 0.22 mV (a $16 \pm 1\%$ depression, $n = 14$); (b) following anoxia, it permitted a greater recovery in the response Table 2).

Effect of 7-chlorokynurenic acid

7-Chlorokynurenic acid is an antagonist at the glycine binding site (Kemp *et al.*, 1988). Under normoxic conditions, it produced a depressant effect on its own (Figure 5) which was concentration-dependent between 20–200 μM. To test its effect on recovery, 50 μM was used and this depressed the evoked potentials from 2.35 ± 0.20 to 1.34 ± 0.12 mV (a depression of 42.3%: $n = 14$). 7-Chlorokynurenic acid (50 μM) also permitted a substantial increase in the recovery after anoxia (Table 2).

Effect of DNQX

Neurotoxicity may also arise from non-NMDA receptors (Koh *et al.*, 1990). Therefore an antagonist to the AMPA receptor (DNQX) was also tested. At a concentration of 10 μM it depressed the synaptically mediated potential confirming the role of AMPA receptors in normal transmission (Figure 6). After equilibration for 30 min with this antagonist, anoxia completely abolished the residual response. The DNQX was washed out at the same time as reoxygenation and the response recovered to a level similar to that in slices without DNQX pretreatment (Figure 6; Table 2).

Effect of other agents

In some experiments above, there was a depression of the response by the test agent (e.g., DNQX and 7-chlorokynurenic acid) before the period of anoxia. We therefore tested the effect of a relatively non-selective depressant agent, lignocaine. As shown in Table 2, there was a small but significant enhancement in the recovery provided by this agent.

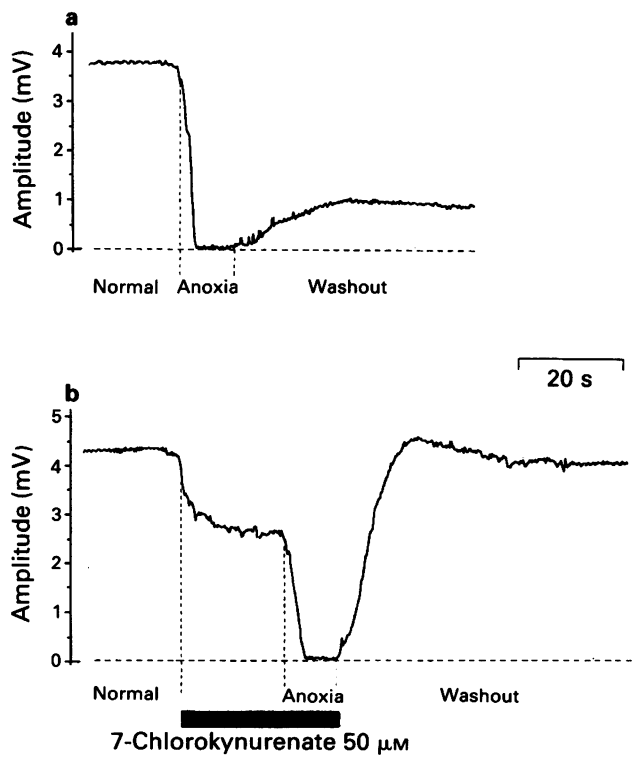


Figure 5 A similar time course to Figure 3 during the application of 50 μM 7-chlorokynurenic acid present during the period indicated by the solid bar. Note that there is some depression of response on adding the 7-chlorokynurenic acid.

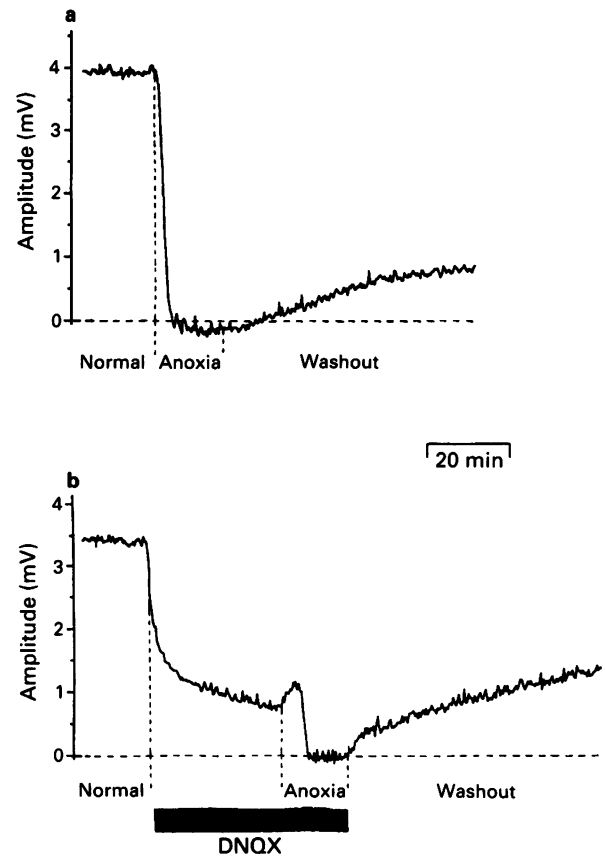


Figure 6 Another time-course using 5,7-dinitroquinoxaline-2,3-dione (DNQX, 10 μM) as the test substance. Note that this agent on its own also depressed the synaptic response. The DNQX was washed out at the end of the period of anoxia. Without the intervening period of anoxia, the response would normally recover completely from the DNQX.

Discussion

Tissue anoxia produces a complex series of events and in the present study, we have looked at one of these: short-term changes in synaptic function. This is particularly vulnerable to anoxia because the presynaptic nerve terminals are very fine ($<0.2\,\mu m$ in olfactory cortex, Gracey & Scholfield, 1990). Cessation of ATP production will cause a run down of ionic gradients at a rate, dependent on the surface area to volume ratio and on activity. Thus ionic run-down would be rapid in fine terminals and rather slower in postsynaptic neurones. Thus the cell bodies may survive anoxia, but in the long term (*in vivo* studies) the effective deafferentation may induce their auto-degeneration.

In olfactory cortex, transmission is glutamate-mediated (see results) and the axonal depolarization caused glutamate to be released from the nerve terminals (see Introduction). The sustained rise in extracellular glutamate stimulates NMDA receptors and since the associated channels are depolarization-dependent, any depolarization due to metabolic insufficiency will accentuate NMDA channel opening.

Several glutamate blockers were tested but none of them influenced the time course for the loss of excitability during the onset of anoxia. This would suggest that synaptic function is not lost as a consequence of any NMDA- or AMPA-mediated process.

Recovery was substantially enhanced by dizocilpine, ketamine, the higher AP5 concentration and 7-chlorokynurenic acid. This would suggest that NMDA receptors are activated during anoxia and that these contribute to tissue damage.

The lower AP5 concentration provided little protection although it did produce some antagonism of NMDA. The stronger protective action of dizocilpine, ketamine and $200\,\mu M$ AP5 reflect the greater effectiveness of these as NMDA channel blockers at the concentrations used. This implies that a substantial blockade of NMDA receptor/channel is required before any protective effect is obtained. This requirement for a high degree of block may explain why some studies have failed to show any effect of NMDA antagonists with anoxia and ischaemia.

On the other hand, AMPA receptor blockade by DNQX did not aid recovery. This indicates that AMPA receptors are not important in priming the depolarization for NMDA channel opening and it would suggest that depolarization results from other causes.

Although the NMDA channel antagonists were the most effective protectants, the degree of protection only extended the endurance of the tissue by around 5 min. Clearly, NMDA receptor activation is only one of probably a multiplicity of degradative functions in neural tissue. However, the present results do confirm the idea that NMDA receptors play an important part in the pathogenesis of cerebral anoxia.

References

AITKIN, P.G., BALESTRINO, M. & SOMJEN, G.C. (1988). NMDA antagonists: lack of protective effect against hypoxic damage in CA1 region of hippocampal slices. *Neurosci. Letts.*, **89**, 187–192.

BENVENISTE, H., DREJER, J., SCHOUSBOE, A. & DIEMER, N.H. (1984). Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *J. Neurochem.*, **43**, 1369–1374.

BIELENBERG, G.W. (1989). Pre- or postischaemic treatment with NMDA antagonists reduces infarct size after MCA occlusion in rat. *J. Cereb. Blood Flow Metab.*, **9**, S298.

CHOI, D.W. (1987). Ionic dependence of glutamate neurotoxicity. *J. Neurosci.*, **7**, 369–379.

CHOI, D.W. (1988). Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischaemic damage. *Trends Neurosci.*, **11**, 465–469.

CLARK, G.D. & ROTHMAN, S.M. (1987). Blockade of excitatory amino acid receptors protects anoxia hippocampal slices. *Neuroscience*, **21**, 665–671.

COLLINS, G.G.S. (1979). Evidence of a neurotransmitter role for aspartate and g-aminobutyric acid in the rat olfactory cortex. *J. Physiol.*, **291**, 51–60.

COLLINS, G.C.S. (1991). Pharmacological evidence that NMDA receptors contribute to mono- and di-synaptic potentials in slices of mouse olfactory cortex. *Neuropharmacol.*, **30**, 547–555.

COLLINS, G.G.S. & BUCKLEY, K.S. (1989). Antagonism of monosynaptic excitations in the mouse olfactory cortex slice by 6,7 dinitroquinoxaline-2,3-dione. *Neuropharmacol.*, **10**, 1123–1128.

DONAGHY, K. & SCHOLFIELD, C.N. (1990). Adenosine-induced enhanced recovery of evoked responses after anoxia. *Neuroreport*, **1**, 123–125.

DREJER, J., BENVENISTE, H., DIEMER, N.H. & SCHOUSBOE, A. (1985). Cellular origin of ischaemia-induced glutamate release from brain tissue in vivo and in vitro. *J. Neurochem.*, **45**, 145–151.

FLEISCHER, J.E., TATEISHI, A., DRUMMON, J.C., SCHELLER, M.S., GRAFE, M.R., ZORNOW, M.H., SHEARMAN, G.T. & SHAPIRO, H.M. (1989). MK-801, an excitatory amino-acid antagonist, does not improve neurologic outcome following cardiac arrest in cats. *J. Cereb. Blood Flow Metab.*, **9**, 795–804.

FRANDSEN, A., DREJER, J. & SCHOUSBOE, A. (1989). Direct evidence that excitotoxicity in cultured neurons is mediated via N-methyl-D-aspartate (NMDA) as well as non-NMDA receptors. *J. Neurochem.*, **53**, 297–299.

GARTHWAITE, G. & GARTHWAITE, J. (1986). Neurotoxicity of excitatory amino-acid receptor agonists in rat cerebellar slices: Dependent on calcium concentration. *Neurosci. Letts.*, **66**, 193–198.

GIULIAN, D. (1993) Reactive glia as rivals in regulating neuronal survival. *Glia*, **7**, 102–110.

GRACEY, A. & SCHOLFIELD, C.N. (1990). Studies on unmyelinated axons and *en passant* synapses in the olfactory cortex. *Exp. Brain Res.*, **80**, 436–440.

KEMP, J.A., FOSTER, A.C. & WONG, E.H.F. (1987). Non-competitive antagonists of excitatory amino acid receptors. *Trends Neurosci.*, **10**, 294–298.

KEMP, J.A., FOSTER, A.C., LEESON, P.D., PRIESTLEY, T., TRIDGETT, R., IVERSEN, L.L. & WOODRUFF, G.N. (1988). 7-Chlorokynurenic acid is a selective antagonist at the glycine modulatory site of the N-methyl-D-aspartate receptor complex. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 6547–6550.

KOCHHAR, A., ZIVIN, J.A., LYDEN, P.D. & MAZZARELLA, V. (1988). Glutamate antagonist therapy reduces neurologic deficits produced by focal central nervous system ischaemia. *Arch Neurol.*, **45**, 148–153.

KOH, J.Y., GOLDBERG, M.P., HARTLEY, D.M. & CHOI, D.W. (1990). Non-NMDA receptor-mediated neurotoxicity in cortical culture. *J. Neurosci.*, **10**, 693–705.

LANIER, W.L., PERKINS, W.J., KARLSSON, B.R., MILDE, J.H., SCHEITHAUER, B.W., SHEARMAN, G.T. & MICHENFELDER, J.D. (1990). The effects of dizocilpine maleate (MK-801), an antagonist of the N-Methyl-D-aspartate receptor, on neurologic recovery and the pathology following complete cerebral ischaemia in primates. *J. Cereb. Blood Flow Metab.*, **10**, 252–261.

MACDERMOTT, A.B., MAYER, M.L., WESTBROOK, G.L., SMITH, S.J. & BARKER, J.L. (1986). NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurones. *Nature*, **321**, 519–522.

MCDONALD, J.W., SILVERSTEIN, F.S. & JOHNSTON, M.V. (1990). MK-801 pretreatment enhances N-methyl-D-aspartate mediated brain injury and increases brain N-methyl-D-aspartate recognition site binding in rats. *Neurosci.*, **38**, 103–113.

MCGIVERN, J. & SCHOLFIELD, C.N. (1990). General anaesthetics and field currents in unclamped, unmyelinated axons of rat olfactory cortex. *Br. J. Pharmacol.*, **101**, 217–223.

MELDRUM, B. & GARTHWAITE, J. (1990). Excitatory amino acid neurotoxicity and neurodegenerative disease. *Trends Pharmacol. Sci.*, **11**, 379–387.

MICHAELS, R.L. & ROTHMAN, S.M. (1990). Glutamate neurotoxicity in vitro: antagonist pharmacology and intracellular calcium concentrations. *J. Neurosci.*, **10**, 283–292.

NICHOLLS, D. & ATTWELL, D. (1990). The release and uptake of excitatory amino acids. *Trends Neurosci.*, **11**, 462–468.

PAPAS, S., CREPEL, V. & BEN ARI, Y. (1993). The NMDA receptor contributes to anoxic aglyemic induced irreversible inhibition of synaptic transmission. *Brain Res.*, **607**, 54–60.

PARK, C.K., NEHLS, D.G., GRAHAM, D.I., TEASDALE, G.M. & MCCULLOCH, J. (1988). The glutamate antagonist MK-801 reduces focal ischaemic brain damage in the rat. *Ann. Neurol.*, **24**, 543–551.

PERKINS, W.J., LANIER, W.L., SCHEITHAUER, B.W., MILDE, J.H. & MICHENFELDER, J.D. (1988). Effect of an excitatory amino acid antagonist (MK-801) on neurologic outcome in a canine model of complete cerebral ischaemia. *Anesthesiol.*, **69**, A5.

RADER, R.K. & LANTHORN, T.H. (1989). Experimental ischemia induces a persistent depolarisation blocked by decreased calcium and NMDA antagonists. *Neurosci. Lett.*, **99**, 125–130.

RICHARDS, C.D. (1972). Potentiation and depression of synaptic transmission in the olfactory cortex of the guinea-pig. *J. Physiol.*, **222**, 209–231.

ROTHMAN, S. (1984). Synaptic release of excitatory amino acid neurotransmitter mediates anoxia neuronal death. *J. Neurosci.*, **4**, 1884–1891.

SANCHEZ-PRIETO, J. & GONZALEZ, P. (1988). Occurrence of a large Ca2 + -independent release of glutamate during anoxia in isolated nerve terminals (synaptosomes). *J. Neurochem.*, **50**, 1322–1324.

SCHOLFIELD, C.N. (1980). Intracellular and extracellular recordings in the isolated olfactory cortex slice and some problems associated with assessing drug action. In *Electrophysiology of Isolated Mammalian CNS Preparations*, ed. Kerkut, G.A. & Weal, H. pp. 133–151. London: Academic Press.

SCHOLFIELD, C.N. & YASSIN, M. (1992). Glutamate antagonists and anoxia in slices of rat olfactory cortex. *J. Physiol.*, **459**, 285P.

STERZ, F., LEONOV, SAFAR, P., RADOVSKY, A., STEZOSKI, S.W., REICH, H., SHEARMAN, G.T. & GREBER, T.F. (1989). Effect of excitatory amino acid receptor blocker MK-801 on overall, neurolgic and morphologic outcome after prolonged cardiac arrest in dogs. *Anesthesiol.*, **71**, 907–918.

TACCONI, S., RATTI, E., MARIEN, M.R., GAVIRAGHI, G. & BOWERY, N.G. (1993). Inhibition of [^{3}H]-(+)-MK801 binding to rat brain sections by CPP and 7-chlorokynurenic acid: an autoradiographic analysis. *Br. J. Pharmacol.*, **108**, 577–582.

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Bradykinin binding sites in healthy and carcinomatous human lung

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1 Direct ligand binding techniques have been used to compare bradykinin receptors in squamous- or adeno-carcinoma and healthy lung membranes removed from patients during operations.

2 The binding of [^{3}H]-bradykinin to healthy lung membrane is time-dependent and saturable with a K_D value of 1.08 ± 0.18 nM and a B_{max} value of 46.1 ± 3.1 fmol mg^{-1} protein ($n = 10$). In squamous-carcinoma tissue ($n = 8$) the same amount of receptors are present, $B_{max} = 52.2 \pm 3.3$ fmol mg^{-1} protein ($P = 0.22$) but the K_D value is significantly higher 2.57 ± 0.40 nM ($P = 0.004$). Similar measurements were obtained for adeno-carcinoma tissue ($n = 3$), $K_D = 2.80 \pm 0.29$ mM ($P = 0.001$) and $B_{max} = 49.8 \pm 2.1$ fmol mg^{-1} protein ($P = 0.56$).

3 In both healthy and squamous-carcinoma preparations, bradykinin analogues displace [^{3}H]-bradykinin binding with the following relative order of potency: Hoe 140 > bradykinin > kallidin > D-Arg^0[Hyp^3,D-Phe^7]bradykinin >>> des-Arg^9-bradykinin. Of the analogues used, bradykinin and D-Arg^0[Hyp^3,D-Phe^7]bradykinin appear to be able to differentiate the bradykinin receptors present in both preparations.

4 It is concluded that bradykinin receptors present in healthy and carcinomatous human lung are of the B_2 type.

Keywords: Bradykinin; bradykinin binding sites; human lung; tumours; B_2 receptor

Introduction

Evidence that bradykinin (BK) and related kinins are involved in various pulmonary diseases has accumulated during the past 30 years (for review see Trifilieff *et al.*, 1993). In human lung, kinin interacts with specific receptors which have been mapped by autoradiography techniques (Mak & Barnes, 1991), but the lack of effect by BK on healthy human lung has hampered the characterization of the subtype of BK receptors present in this tissue. On the other hand, multiple neuropeptides, including kinins, have been proposed as acting as autocrine and paracrine growth factors for lung cancer, in particular in the case of neuroendocrine bronchial cancer as an example of small cell lung cancer (SCLC) (Woll & Rozengurt, 1989; Sethi & Rozengurt, 1991). The possible role of kinins in cancer remains speculative but some results obtained to date are of great interest in defining a role for kinin in this pathology. BK stimulates DNA synthesis in human fibroblasts (Owen & Villereal, 1983) and human endometrial stroma cells (Endo *et al.*, 1991). A mitogenic effect of kinins has also been reported on human embryonic lung fibroblasts (Goldstein & Wall, 1984), and SCLC cells (Sethi & Rozengurt, 1991). In human tumour ascites (Maeda *et al.*, 1988) and in plasma from patients with various cancers (Matsumura *et al.*, 1991), the kinin level has been reported to be significantly increased. Moreover, exposure of human SCLC to BK and other neuropeptides has been shown to lead to an increase in intracellular calcium (Woll & Rozengurt, 1989) and proliferation of these cells (Sethi & Rozengurt, 1991). Recently, BK has been reported to be the most potent peptide in increasing intracellular calcium on a number of SCLC or non-SCLC (Bunn *et al.*, 1992). From these observations, it has been proposed that BK may function as a paracrine growth factor for SCLC or large cell lung cancer (Bunn *et al.*, 1990; 1992; Sethi & Rozengurt, 1991).

Two classes of kinin receptors, B_1 and B_2, have been proposed on the basis of the bioassay potencies of BK analogues (Regoli & Barabé, 1980). Des-Arg^9-BK, a natural metabolite of BK, has high affinity for B_1 receptors, whilst BK has a low affinity. B_1 receptors are found mainly in vascular tissues and are specifically inhibited by des-Arg^9-[Leu^8]BK. B_2 receptors have a high affinity for BK whilst des-Arg^9-BK and des-Arg^9-[Leu^8]BK are mainly inactive. B_2 receptors are widely distributed in mammals and are strongly inhibited by the new antagonist Hoe 140 (D-Arg^0[Hyp^3,Thi^5, D-Tic^7,Oic^8]BK) (Hock *et al.*, 1991). Moreover, the recent cloning and sequencing of the gene coding for a B_2 receptor from rat (Mc Eachern *et al.*, 1991) and from man (Hess *et al.*, 1992) has led to the prediction of a seven transmembrane G-protein coupled receptor. Nothing is known about binding properties of BK in healthy human lung and since alterations of binding sites have been reported for other physiological receptors in human lung tumours (Morin *et al.*, 1987), we decided to investigate the binding of BK in membrane preparations of healthy and carcinomatous human lung.

Methods

Lung preparation

Human lung and tumour samples were obtained after thoracotomy of patients undergoing surgery for lung carcinoma, and were transported, on ice, to the laboratory within 20 to 40 min. All patients had received droperidol (20 mg), thiopentone (250 mg), fentanyl (0.5 mg), atropine (0.5 mg) and curare (10 mg). The histological studies showed that eight of the tumours were well differentiated squamous-carcinoma, the three other were adenocarcinomas. For membrane preparations the following buffer was used: 25 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid, pH 6.8 containing 1 mM 1,10-phenanthroline (buffer A). All membranes were prepared as described previously (Trifilieff *et al.*, 1992) with minor modifications. Briefly, tissues were minced and homogenized in 0.25 mM ice-cold sucrose. After

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centrifugation at 2500 *g* for 10 min at 4°C, the pellets were resuspended, homogenized and resedimented at the same speed. These two supernatants were pooled and diluted with three volumes of buffer A. The mixtures were then centrifuged twice at 50,000 *g* for 10 min at 4°C. The resulting pellets were resuspended in 10 ml of assay buffer per g of tissue for BK binding studies. Assay buffer is buffer A with 140 μg ml^{-1} bacitracin, 10 μM captopril, 1 mM dithiothreitol and 0.1% bovine serum albumin added.

Radioligand binding assay

[^{3}H]-BK binding was carried out as described previously (Trifilieff *et al.*, 1992). Competition and kinetic experiments were performed with 0.5 to 0.7 nM and 0.4 nM [^{3}H]-BK, respectively. Saturation experiments were performed with increasing concentrations of radioligand (0.06 to 3.5 nM). Each experiment was performed for 90 min at 25°C in triplicate. All the data were analysed as described previously (Gies *et al.*, 1989). Student's *t* test (two-tailed) for unpaired data was used to determine the significance of differences in K_D and B_{max} values between the healthy and carcinomatous preparations.

Materials

[^{3}H]-BK (74.7 to 110 Ci mmol^{-1}) was obtained from New England Nuclear (Boston, MA, U.S.A.). Des-Arg^9-BK was purchased from Bachem (Bubendorf, Switzerland). Captopril was a gift from Squibb Pharmaceuticals (Epernon, France), Hoe 140 (D-Arg^0[Hyp^3,Thi^5,D-Tic^7,Oic^8]BK) was kindly provided by Hoechst AG (Frankfurt, Germany). Unlabelled BK, kallidin, D-Arg^0[Hyp^3,D-Phe^7]BK and all other reagents were from Sigma Chemical Co. (St Louis, MO, U.S.A.).

Results

For both tissue sources (healthy and carcinomatous), we verified that binding of [^{3}H]-BK was linear over a broad range of membrane particulates concentration (89 to 785 μg of protein per assay). Binding assays were therefore conducted with 240 to 270 μg of protein for healthy tissue and 650 to 700 μg of protein for cancer tissue. In these conditions, non-specific binding of [^{3}H]-BK (0.6 nM) was 50 and 60% in healthy and carcinomatous tissues, respectively.

The [^{3}H]-BK binding to healthy lung membranes was observed to be time-dependent ($n = 2$, Figure 1). Binding reached a maximum after 20 to 30 min and remained steady up to 150 min. After 90 min of association, dissociation was initiated by addition of 1 μM unlabelled BK and resulted in a rapid reduction of specifically bound [^{3}H]-BK: 50% of dissociation occurred in 4 min (4.3–4.1). The association rate constant (K_{obs}) was 0.28 min^{-1} (0.31–0.25) and the dissociation rate constant (K_{-1}) was 0.16 min^{-1} (0.16–0.17). The value of the association rate constant (K_{+1}) calculated from the equation $K_{+1} = (K_{obs} - K_{-1})/[\text{radioligand}]$ was 2.98 10^8 min^{-1} M^{-1} (3.49 10^8–2.47 10^8). Finally, the equilibrium dissociation constant $K_D = K_{-1}/K_{+1}$ could be calculated from these kinetic parameters, giving value of 0.57 nM (0.46–0.68). Scatchard analysis of saturation studies (Figure 2) indicated the presence of a homogeneous class of binding sites with a dissociation constant (K_D) of 0.73 ± 0.18 nM and a density of binding sites (B_{max}) of 37.7 ± 6.2 fmol mg^{-1} protein ($n = 3$). The Hill coefficient was determined to be 0.90 ± 0.05 and confirms the existence of one population of binding sites.

Due to the limited amount of human tissue, we used the method described by De Blasi *et al.* (1989) to determine the binding parameters for the tumour membranes and seven of the healthy membrane preparations (Table 1). In order to improve this method, we have compared the results obtained by Scatchard analysis of saturation studies (parameters above) and those obtained by analysis of competition studies proposed by De Blasi *et al.* (1989) ($K_D = 1.23 \pm 0.23$ nM, $B_{max} = 49.6 \pm 2.4$ fmol mg^{-1} protein, $n = 7$). As shown, similar values were obtained with both methods. This result allows us to calculate binding parameters for tumour samples using competition experiments. The mean values for all the healthy membrane preparations were $K_D = 1.08 \pm 0.18$ nM and $B_{max} = 46.1 \pm 3.1$ fmol mg^{-1} protein ($n = 10$).

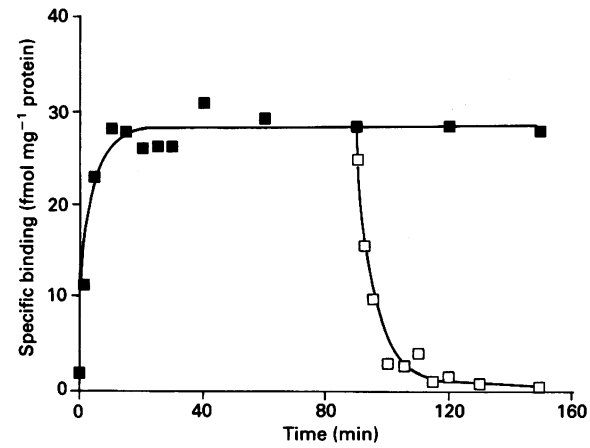


Figure 1 Kinetics of [^{3}H]-bradykinin binding to healthy lung membranes. Association (■) and dissociation (□) kinetics were examined at 25°C. When equilibrium was reached (90 min), the dissociation was started by addition of 1 μM unlabelled bradykinin (arrow). Points are the mean of triplicate measurements in one representative experiment.

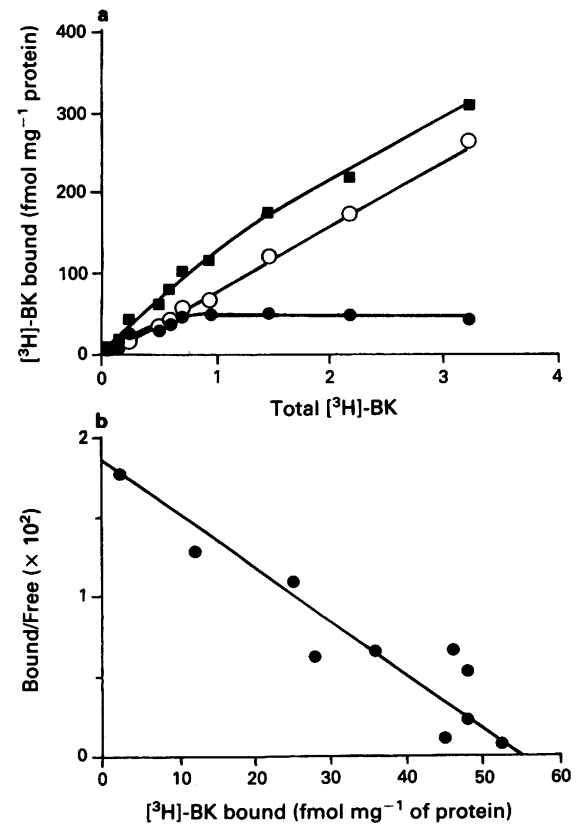


Figure 2 (a) Saturation binding of [^{3}H]-bradykinin to healthy human lung membranes. Specific binding (●) was calculated by subtracting non-specific binding (○) determined in the presence of 1 μM of unlabelled bradykinin from total binding (■). (b) Scatchard plot of the binding data. Points are the mean of triplicate measurements in one representative experiment.

For squamous-carcinoma membranes (Table 1, $n = 8$) no significant change in the B_{max} value was observed when compared with healthy membranes (52.2 ± 3.3 fmol mg^{-1} protein, $P = 0.22$), but the K_D value is significantly higher (2.57 ± 0.40 nM, $P = 0.004$). Similarly, the K_D value for adeno-carcinoma membranes ($n = 3$) was significantly higher when compared with healthy membranes (2.80 ± 0.29 nM, $P = 0.001$), but the B_{max} value remains in the same range (49.8 ± 2.1 fmol mg^{-1} protein, $P = 0.56$).

In order to define the pharmacological subtype of the BK binding sites present on healthy and tumour preparations, the affinities of B_1 and B_2 ligands were studied in competition experiments (Figure 3). In both healthy and squamous-carcinoma preparations, the natural B_1 agonist des-Arg^9-BK did not displace the specific [^{3}H]-BK binding even at the highest dose tested (10^{-5} M). For the other analogues tested, we found for both preparations the same relative order of potency in displacing [^{3}H]-BK binding: Hoe 140 > BK > kallidin > D-Arg^0[Hyp^3,D-Phe^7]BK. The affinities in healthy and squamous-carcinoma preparations are compared in Table 2. Based on their affinities, Hoe 140 and kallidin did not differentiate the BK binding sites present in both preparations, whereas BK and D-Arg^0[Hyp^3,D-Phe^7]BK are able to distinguish binding sites in both preparations. Because of the small amount of tissue available for adeno-carcinoma tumour membrane preparations, we could not analyse all the bradykinin analogues tested for healthy and squamous-carcinoma membranes. We chose the B_1 agonist, (des-Arg^9-BK), and the new more potent B_2 antagonist, Hoe 140. As observed with squamous-carcinoma membranes, des-Arg^9-BK did not displace [^{3}H]-BK binding, whereas Hoe 140 displaced completely the [^{3}H]-BK binding with an affinity similar to that of bradykinin (Table 2).

Again, when compared with healthy membranes, Hoe 140 did not differentiate the bradykinin binding sites present in either preparation. The specific [^{3}H]-BK-binding was unaffected by various unrelated peptides such as substance P, bombesin or calcitonin gene-related peptide (each at 10^{-6} M) with the three membrane preparations (data not shown).

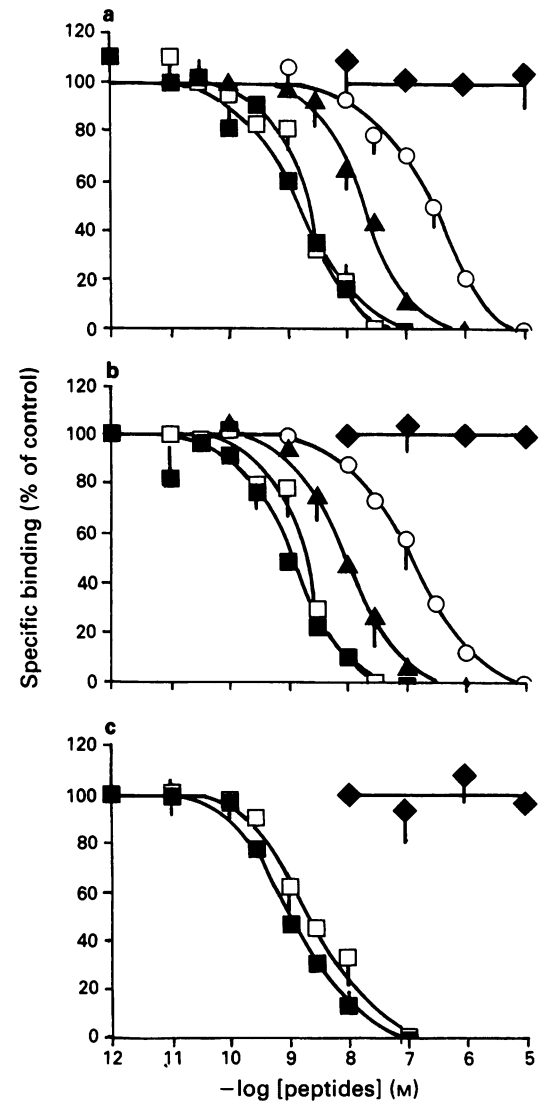


Figure 3 Competitive inhibition of specific binding of [^{3}H]-bradykinin in healthy (a), squamous-carcinoma (b) and adeno-carcinoma membranes (c). [^{3}H]-bradykinin (0.5 to 0.7 nM) was incubated for 90 min at 25°C with increasing concentrations of (■) Hoe 140, (□) unlabelled bradykinin, (▲) kallidin, (○) D-Arg^0[Hyp^3,D-Phe^7] bradykinin or (◆) des-Arg^9-bradykinin. Each point represents the mean ± s.e. mean of the number of experiments indicated in Table 2.

Table 1 Binding parameters of [^{3}H]-bradykinin sites in healthy and carcinomatous lung membranes

	K_D (nM)		B_{max} (fmol mg^{-1} protein)	
Subjects	*Healthy*	*Tumour*	*Healthy*	*Tumour*
Squamous 1	0.87*	3.00	52.5*	36.7
Squamous 2	1.02*	3.81	33.2*	44.4
Squamous 3	0.76	1.83	55.4	46.7
Squamous 4	0.56	3.03	52.9	56.9
Squamous 5	2.26	0.84	54.1	69.2
Squamous 6	1.93	3.11	52.9	50.2
Squamous 7	–	3.94	–	58.3
Squamous 8	–	0.99	–	55.1
Adeno 1	0.88	2.10	36.7	44.9
Adeno 2	0.82	3.00	43.4	51.1
Adeno 3	–	3.29	–	53.3
Healthy 1	0.29*	–	27.4*	–
Healthy 2	1.40	–	52.1	–

Parameters were determined using competition experiments by the method of De Blasi *et al.* (1989) or saturation experiments (*).

Table 2 Comparison of the potency of kinin analogues for competition in [^{3}H]-bradykinin binding to healthy and carcinomatous lung membranes

	Healthy		*Squamous-carcinoma*		*Adeno-carcinoma*	
	K_i (nM)	(n)	K_i (nM)	(n)	K_i (nM)	(n)
Hoe 140	0.73 ± 0.21	(4)	1.23 ± 0.43	(5)	0.56 ± 0.14	(3)
Bradykinin (BK)	1.03 ± 0.17	(7)	2.39 ± 0.43*	(8)	2.42 ± 0.22*	(3)
Kallidin	18.37 ± 1.03	(3)	15.41 ± 1.19	(3)	–	–
D-Arg^0[Hyp^3,D-Phe^7]BK	265.82 ± 12.67	(3)	82.65 ± 4.15*	(3)	–	–
Des-Arg^9-BK	Inactive	(3)	Inactive	(3)	Inactive	(3)

*$P < 0.005$ when compared with healthy membranes (Student's t test for unpaired data, two tailed).

Discussion

Here we describe for the first time the determination of [^{3}H]-BK-binding constants in healthy and carcinomatous human lung. In a previous study (Trifilieff *et al.*, 1991) we showed the presence of two [^{3}H]-BK binding sites in the guinea-pig lung, such a situation was not observed in the human lung. This could suggest a species variation or may be due to the relative high level of non-specific binding observed in human lung, as has been reported elsewhere (Mak & Barnes, 1991), which prevented the detection of a second binding site. In squamous- and adeno-carcinoma membranes, the same amount of BK receptors are present, whereas the equilibrium dissociation constant of [^{3}H]-BK is significantly higher compared with the value for healthy membranes. Among the BK analogues used in competition experiments, only the B_2 receptor antagonist, D-Arg^0[Hyp^3,D-Phe^7]BK was able to differentiate the BK binding sites present in healthy and squamous-carcinoma membranes. The two others, Hoe 140 and kallidin, possessed similar affinities in both tissue sources. In the same way, Hoe 140 did not differentiate the BK receptor present in adeno-carcinoma membranes when compared with healthy membranes. Only for two of the eleven samples studied (squamous 5 and probably squamous 8), did BK appear to have a greater affinity for its receptor on carcinomatous tissue rather than its receptor on healthy tissue. However, when taking all the K_D values together, BK had a significantly higher affinity for the healthy receptor than for the tumour receptor. Moreover when other compounds were used (B_2 antagonists and kallidin), in squamous 5 and 8, the affinity values for the BK receptor were in a similar order to the other samples.

We observed a change in affinity values for BK and D-Arg^0[Hyp^3,D-Phe^7]BK between healthy and tumour tissue. A similar magnitude of alteration (two fold) was also observed for [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) between healthy carcinomatous human lung (Morin *et al.*, 1987) and the authors suggested that it might be related to a heterogeneity of muscarinic receptors in both tissues. In a previous study, we reported such a heterogeneity of BK receptors in the guinea-pig lung using saturation and kinetic experiments (Trifilieff *et al.*, 1991). However, in the human lung, neither saturation nor kinetic studies were able to reveal two binding sites, but, as mentioned above, this could be due to the relatively high level of non-specific binding in this tissue. It is of importance to note that BK and D-Arg^0[Hyp^3,D-Phe^7]BK were able to differentiate the two binding sites in guinea-pig lung whereas kallidin was not (Trifilieff *et al.*, 1991). A similar behaviour for these compounds was observed between healthy and carcinomatous human lung; therefore, we cannot exclude the possibility of a heterogeneity of BK receptors in these tissues. However, the differences observed between healthy and carcinomatous lung remain rather small. The biological significance of such a difference could be related to an increase in the kinin concentration in tumour cells inducing a desensitization of the BK receptors. In asthmatic subjects, tachyphylaxis of the kinin receptor has been reported following repeated inhalations of BK (Polosa *et al.*, 1990). As an increase in kinin levels has been observed in various cancers (Maeda *et al.*, 1988; Matsumara *et al.*, 1991), the change in the K_D values of BK could be related to a desensitization of the kinin receptor in tumour samples. Alternatively, a structural modification of the BK receptor could also be involved. Whatever the case, if the change in affinity values observed in the present study had a biological significance, we believe that this might reflect a modification of the membrane environment or a difference in the coupling efficiency of the receptors rather than a structural difference in the BK receptors. Indeed, this hypothesis fits well with the work of Eggericks *et al.* (1992) who have shown that a human BK B_2 receptor gene, when expressed in different eukaryotic systems, encodes a receptor which can be differentiated by BK analogues. In fact, it maybe of interest to continue this work with other tumour samples of different histological types to ascertain whether or not there are differences in [^{3}H]-BK binding.

The failure of the B_1 agonist, des-Arg^9-BK to displace [^{3}H]-BK binding in healthy and tumour membrane preparations demonstrates the absence of B_1 receptors in these preparations. On the other hand, the affinity sequence for the other BK analogues allowed us to classify these binding sites as B_2 (Figure 3). These results are in accordance with the presence of B_2 receptors in healthy human lung as suggested by Hess *et al.* (1992) who found the highest level of B_2 receptor mRNA in kidney, uterus and lung.

Since only minor bronchoconstrictor effects for BK have been reported in healthy human subjects, one can speculate as to the physiological role of the B_2 receptors described in our study. Besides its bronchocontractile properties, BK is a well known vasoactive peptide, and has been shown to increase vascular permeability in guinea-pig airways (Saria *et al.*, 1983; Rogers *et al.*, 1990). Moreover, an autoradiographic study of human lung has revealed a high density of BK binding sites on bronchial and pulmonary blood vessels (Mak & Barnes, 1991). As in the guinea-pig, BK could also increase vascular leakage in human airways.

In conclusion, we have shown the presence of a high affinity BK B_2 receptor in healthy and carcinomatous human lung. The physiological role of these receptors remains to be determined, but the recent development of potent BK receptor antagonists, such as Hoe 140, may be useful for its elucidation.

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References

BUNN, P.A. Jr., CHAN, D., DIENHART, D.G., TOLLEY, R., TAGANA, M. & JEWETT, P.B. (1992). Neuropeptides signal transduction in lung cancer: clinical implications of bradykinin sensitivity and overall heterogeneity. *Cancer Res.*, **52**, 24–31.

BUNN, P.A. Jr., DIENHART, D.G., CHAN, D., PUCK, T.T., TAGAWA, M., JEWETT, P.B. & BRAUNSCHWEIGER, E. (1990). Neuropeptides stimulation of calcium flux in human lung cancer cells: Delineation of alternative pathways. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 2162–2166.

DE BLASI, A., O'REILLY, K. & MOTULSKY, H.J. (1989). Calculating receptor number from binding experiments using some compound as radioligand and competitor. *Trends Pharmacol. Sci.*, **10**, 227–229.

EGGERICKS, D., RASPE, E., BERTRAND, D., VASSART, G. & PARMENTIER, M. (1992). Molecular cloning, functional expression and pharmacological characterization of a human bradykinin B_2 receptor gene. *Biochem. Biophys. Res. Commun.*, **187**, 1306–1313.

ENDO, T., FUKUE, H., KANAYA, M., MIZUNUMA, M., FUJII, M., YAMAMOTO, H., TANAKA, S. & HASHIMOTO, M. (1991). Bombesin and bradykinin increase inositol phosphates and cytosolic free Ca^{2+}, and stimulate DNA synthesis in human endometrial stromal cells. *J. Endocrinol.*, **131**, 313–318.

GIES, J.P., BERTRAND, C., VANDERHEYDEN, P., WAELDELE, F., DUMONT, P., PAULI, P. & LANDRY, Y. (1989). Characterization of muscarinic receptors in human, guinea pig and rat lung. *J. Pharmacol. Exp. Ther.*, **250**, 309–315.

GOLDSTEIN, R.H. & WALL, M. (1984). Activation of protein formation and cell division by bradykinin and des-Arg^9-bradykinin. *J. Biol. Chem.*, **259**, 9263–9268.

HESS, J.F., BORWSKI, J.A., YOUNG, G.S., STRADER, C.D. & RANSOM, R.W. (1992). Cloning and pharmacological characterization of a human bradykinin (BK-2) receptor. *Biochem. Biophys. Res. Commun.*, **184**, 260–268.

HOCK, F.J., WIRTH, K., ALBUS, U., LINZ, W., GERHARDS, H.J., WIEMER, G., HENKE, S.T., BREIPHOHL, G., KÖNIG, W., KNOLLE, J. & SCHÖLKENS, B.A. (1991). Hoe 140 a new potent and long acting bradykinin-antagonist: *in vitro* studies. *Br. J. Pharmacol.*, **102**, 769–773.

MAEDA, H., MATSUMURA, Y. & KATO, H. (1988). Purification and identification of [Hydroxyprolyl^3]bradykinin in ascitic fluid from a patient with gastric cancer. *J. Biol Chem.*, **263**, 16051–16054.

MAK, J.C.W. & BARNES, P.J. (1991). Autoradiographic visualization of bradykinin receptors in human and guinea-pig lung. *Eur. J. Pharmacol.*, **194**, 37–43.

MATSUMURA, Y., MARUO, K., KIMURA, M., YAMAMOTO, T., KONNO, T. & MAEDA, H. (1991). Kinin-generating cascade in advanced cancer patients and *in vitro* study. *Jpn. J. Cancer Res.*, **82**, 732–741.

McEACHERN, A.E., SHELTON, E.R, BHAKTA, S., OBERNOLTE, R., BACH, C., ZUPPAN, P., FUJISAKI, J., ALDRICH, R.W. & JARNAGIN, K. (1991). Expression cloning of a rat B_2 bradykinin receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 7724–7728.

MORIN, D., ZINI, R., LANGE, F., LANGE, J. & TILLEMENT, J.-P. (1987). Alterations of beta-adrenergic, muscarinic cholinergic receptors and imipramine binding sites in human lung tumours. *Int. J. Clin. Pharmacol. Ther. Toxicol.*, **25**, 605–608.

OWEN, N.E. & VILLEREAL, M.L. (1983). Lys-bradykinin stimulates Na^+ influx and DNA synthesis in cultured human fibroblasts. *Cell*, **32**, 979–985.

POLOSA, R., LAI, C.K.W., ROBINSON, C. & HOLGATE, S.T. (1990). The influence of cyclooxygenase inhibition on the loss of bronchoconstriction response to repeated challenge in asthma. *Eur. Respir. J.*, **3**, 914–921.

REGOLI, D. & BARABÉ, J. (1980). Pharmacology of bradykinin and related peptides. *Pharmacol. Rev.*, **32**, 1–46.

ROGERS, D.F., DIJK, S. & BARNES, P.J. (1990). Bradykinin-induced plasma exudation in guinea-pig airways: involvement of platelet activating factor. *Br. J. Pharmacol.*, **101**, 739–745.

SARIA, A., LUNDBERG, J.M., SKOFITSCH, G. & LEMBECK, F. (1983). Vascular protein leakage in various tissues induced by substance P, capsaicin, bradykinin, serotonin, histamine and by antigen challenge. *Naunyn-Schmied. Arch. Pharmacol.*, **324**, 212–218.

SETHI, T. & ROZENGURT, E. (1991). Multiple neuropeptides stimulate clonal growth of small cell lung cancer: effect of bradykinin, vasopressin, cholecystokinin, gelanin and neurotensin. *Cancer Res.*, **51**, 3621–3623.

TRIFILIEFF, A., DA SILVA, A. & GIES, J.P. (1993). Kinins and respiratory tract diseases. *Eur. Respir. J.*, **6**, 576–587.

TRIFILIEFF, A., DA SILVA, A., LANDRY, Y. & GIES, J.P. (1992). Effect of Hoe 140, a new B_2 non-competitive antagonist, on guinea pig tracheal receptors. *J. Pharmacol. Exp. Ther.*, **263**, 1377–1382.

TRIFILIEFF, A., HADDAD, E.-B., LANDRY, Y. & GIES, J.P. (1991). Evidence for two high-affinity bradykinin binding sites in the guinea-pig lung. *Eur. J. Pharmacol. (Mol. Pharmacol. Sect.)*, **207**, 129–134.

WOLL, P.J. & ROZENGURT, E. (1989). Multiple neuropeptides mobilise calcium in small cell lung cancer: effects of vasopressin, bradykinin, cholecystokinin, galanin and neurotensin. *Biochem. Biophys. Res. Commun.*, **164**, 66–73.

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Ischaemia/reperfusion selectively attenuates coronary vasodilatation to an adenosine A_2- but not to an A_1-agonist in the dog

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1 The effects of myocardial ischaemia/reperfusion were tested on the coronary vasorelaxant responses to agonists selective for the A_1 and A_2 adenosine receptor subtypes in the dog. The left anterior descending (LAD) coronary artery was occluded distal to the first diagonal branch. The occlusion was maintained for 1 h, followed by 1 h of reperfusion.

2 In the first series of experiments, LAD and circumflex arteries were excised and contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Ischaemia/reperfusion did not significantly alter the vasorelaxation produced by either sodium nitroprusside (endothelium-independent) or acetylcholine (endothelium-dependent). The A_1 selective agonist, cyclopentyladenosine (CPA), produced coronary vasorelaxation in both normally perfused vessels and vessels subjected to ischaemia/reperfusion. In contrast, the relaxation produced by the A_2-selective agonist N6-{2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl) ethyl} adenosine (DPMA) was significantly attenuated by ischaemia/reperfusion (14 fold shift in EC_{50}).

3 In the second series of experiments, coronary blood flow was increased by administration of the A_1 and A_2 agonists before and after ischaemia/reperfusion of the LAD in anaesthetized dogs. Both compounds dose-dependently increased coronary blood flow. The slopes of the dose-response functions to CPA or DPMA were not significantly altered in the normally perfused circumflex vascular bed. Similarly, the CPA dose-response function in the LAD was unaltered by ischaemia/reperfusion. However, the slope of the coronary vasodilator response to the A_2 agonist was significantly reduced following ischaemia/reperfusion of the LAD.

4 We conclude that ischaemia/reperfusion reduces responsiveness to an adenosine A_2 receptor subtype agonist, but not an A_1 receptor subtype agonist. These data confirm the independent nature of A_1- and A_2-mediated coronary vasodilatation.

Keywords: Cyclopentyladenosine; DPMA; endothelium; ischaemia; reperfusion; adenosine receptors; coronary blood flow; haemodynamics; vascular resistance

Introduction

Adenosine has been proposed as an important metabolic regulator of coronary blood flow (Berne, 1963). Purinoceptors have been classified as P_1 or P_2 receptors. Adenosine exhibits an almost complete selectivity for the P_1 receptor which can be further differentiated into A_1, A_2, and A_3 receptor subtypes (Van Calker *et al.*, 1979; Londos *et al.*, 1983; Zhou *et al.*, 1993). Selective agonists for A_1 and A_2 receptor sub-types have revealed that A_1 receptors mediate negative chronotropic and renal vasoconstrictor effects, while A_2 agonists primarily dilate the mesenteric and cerebral vascular beds (for review see Olsson & Pearson, 1990). We have been exploring the coronary vasorelaxant/vasodilator effects of an A_1 selective agonist, cyclopentyladenosine (CPA) and an A_2 selective agonist, N6-{2-(3,5-dimethoxyphenyl)-2- (2-methylphenyl)ethyl} adenosine (DPMA) (Cox *et al.*, 1991; Merkel *et al.*, 1992).

In addition to producing myocardial infarction, occlusion and reperfusion of a coronary artery produces changes in the remaining viable myoctyes, including depression of contractility, electrophysiological changes, and alteration of metabolic processes (Heyndrickx *et al.*, 1975; Jennings *et al.*, 1985; Kloner *et al.*, 1983; Bolli *et al.*, 1988). In clinical situations, this reperfusion injury can occur even when the ischaemic insult may not be so severe as to produce myocardial infarction, such as during percutaneous transluminal angioplasty (Cowley *et al.*, 1984). Recent evidence has suggested that a further effect of ischaemia/reperfusion is to alter reactivity in coronary vascular smooth muscle (Bolli *et al.*, 1990; Pearson *et al.*, 1990; Quillen *et al.*, 1990).

Adenosine and adenosine agonists have been shown to limit infarct size in several models of ischaemia/reperfusion (Lasley *et al.*, 1990; Norton *et al.*, 1992; Toombs *et al.*, 1992; Thornton *et al.*, 1992). In addition, ischaemia has been shown to inhibit the binding of a mixed adenosine agonist to an adenosine receptor identified as the A_2 receptor subtype in rat isolated working hearts subjected to global ischaemia (Zucchi *et al.*, 1992). Further, A_1 and A_2 receptors have been shown to desensitize by independent mechanisms in a vascular smooth muscle cell line (Ramkumar *et al.*, 1991). The present study was designed to define whether physiological stimuli (coronary occlusion followed by reperfusion) applied *in vivo* could produce a functional impairment of A_1- or A_2-mediated vasorelaxation. These experiments were conducted both on isolated coronary vessels and entirely *in vivo*. Further, we defined whether the vasorelaxant responses to endothelium-independent (sodium nitroprusside) or endothelium-dependent (acetylcholine, ACh) agents were altered by the 1 h ischaemia/1 h reperfusion protocol.

Methods

All experiments were conducted in accordance with a protocol approved by the Rhône-Poulenc Rorer Animal Care and Use Committee and conformed to the NIH Guide for the Care and Use of Laboratory Animals. Purpose-bred mongrel dogs (13.0–14.3 kg) were anaesthetized with sodium pentobarbitone (35 mg kg^{-1}, Abbott Laboratories, North Chicago, IL, U.S.A.). Animals were intubated and ventilated with room air. Body temperature was monitored and main-

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tained throughout the experiment. Femoral arterial and venous catheters were implanted and 0.9% saline (0.5 ml min^{-1}, i.v.) was infused throughout the experiment. Electrocardiogram electrodes were placed on the four extremities, with a fifth electrode placed on the ventral midline opposite the apex of the heart. All haemodynamic and electrocardiographic parameters were displayed on a Grass Model 7 polygraph. The chest was opened at the fifth intercostal space and a pericardial cradle established. The LAD coronary artery was isolated, and a micrometer- or screw-driven occluder was placed distal to the first diagonal branch. Generation or release of occlusions took place incrementally over a 10 min period. Ischaemia was maintained for 1 h, followed by a 1 h reperfusion period. The five lead electrocardiogram was analyzed after 5, 15, 30, and 60 min of ischaemia or reperfusion. No pharmacological interventions were used to minimize arrhythmias.

In vitro

At the end of the reperfusion period the animal was exsanguinated and the heart was removed immediately and placed in warm, oxygenated (37°C; 95% O_2, 5% CO_2) Krebs Henseleit buffer of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 10.0 (pH 7.4). Starting approximatly 10 mm distal to the occlusion site, the LAD artery was carefully dissected out, cleaned of fat, blood and adhering tissue and cut into rings 2–3 mm wide. The left circumflex coronary artery was prepared similarly and used as control. Previous studies have shown no difference in vascular reactivity between these two arteries (Suba & Roth, 1987; Van Benthuysen *et al.*, 1987; Pearson *et al.*, 1990). Rings were transferred to water-jacketed tissue baths (10 ml) containing Krebs buffer and were mounted on L-shaped wire hooks between a stainless steel rod and a force transducer (Grass, FT-03) and changes in isometric tension were displayed on a Grass Model 7 Polygraph. Data were electronically captured using a Po-Ne-Mah acquisition system. The optimal lengths (L_o) for rings from the LAD and the circumflex were determined to be 2 g using 80 mM KCl (Ku, 1982). During the equilibration period, buffer was changed every 5 min for the first 15 min, and at 15 min intervals thereafter. Subsequently, tissues were stimulated twice with 36 mM KCl and then with 10 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). After isometric tension had reached a steady state, cumulative doses of vasorelaxants were added to the baths. Subsequent concentrations were added only after tension had reached a new steady state. Each ring was exposed to one vasorelaxant only. Tension was expressed as percentage of maximal $PGF_{2\alpha}$-induced tension.

In vivo

In a second series of experiments, animals were instrumented as described above. In addition, the circumflex and LAD coronary arteries were instrumented with electromagnetic flow probes (Gould Statham Model 2202). A Millar pressure tip transducer was inserted into the left ventricle. Finally, pairs of sonomicrometry crystals (Triton Technology) were implanted in both the normally perfused and ischaemic myocardium. Animals with segment shortening of less than 5.0% during the control period were not included in the analysis. In addition, one animal did not become ischaemic despite complete occlusion of the LAD, and was also not included in the group data (individual data for this animal are provided in results section). Data were displayed on a Grass Model 7 polygraph and captured using a Po-Ne-Mah HD-4 computerized acquisition system.

The protocol involved intravenous administration of 3, 10, and 30 μg kg^{-1} of CPA, followed by 3, 10, and 30 μg kg^{-1} of DPMA. Higher doses were not tested due to the risk of adversely affecting the data by lengthening the protocol and/or downregulating the receptors with exogenous agonist. Intravenous (versus intracoronary) administration ensured that plasma levels of drug were equivalent in all branches of the circumflex and LAD vascular bed. All parameters were allowed to return to, or very near, baseline prior to administering the subsequent dose of adenosine agonist. All values illustrate the response at the time of peak change in coronary blood flow. The entire dosing protocol (for both CPA and DPMA) averaged 48.5 ± 4.5 min for the six animals. The LAD coronary artery was completely occluded over a 10 min period. The occlusion was maintained over 1 h then released over a 10 min period. The region was reperfused for 1 h and CPA and DPMA were again administered as described above.

An additional four animals were instrumented and drugs administered as described above except that the occluder was not tightened (sham ischaemia).

Drugs

Sodium nitroprusside, ACh, and prostaglandin $F_{2\alpha}$ were purchased from Sigma (St. Louis, MO, U.S.A.). Cyclopentyladenosine was produced by Research Biochemicals Inc. (Natick, MA, U.S.A.). CGS15943 (9-chloro-2-(2-furyl)[1,2,4] triazolo [1,5-c] quinazolin-5-amine) was a gift from Ciba Geigy (Summit, NJ, U.S.A.). DPMA [N6-{2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)ethyl} adenosine] was synthesized by Dr A. Spada and N. Setzer of the Department of Medicinal Chemistry, Rhône-Poulenc Rorer. For the *in vitro* studies, adenosine agonists were dissolved in dimethyl sulphoxide (DMSO, Sigma, St. Louis MO, U.S.A.) and serially diluted with Krebs buffer. Final DMSO concentration in the bath did not exceed 0.1%. For the *in vivo* studies, adenosine agonists and antagonists were initially dissolved in DMSO (Fisher Scientific, Fairlawn, NJ, U.S.A.). The final solution was 5% DMSO, 47.5% polyethylene glycol 200 (J.T. Baker, Inc. Phillipsberg, NJ, U.S.A.), and 47.5% normal (0.9%) saline (Abbot Laboratories). Solutions were then serially diluted with 0.9% saline.

Statistical analysis

In vitro EC_{50} values were determined from individual dose-response curves with a computerized curve fitting programme (RS/1, BBN Software Products Corp. Cambridge, MA, U.S.A.). Negative logarithmic transformations were conducted on the control and ischaemia/reperfusion EC_{50} values (− log M). These − log EC_{50} values were then compared by Student's two-tailed paired *t* test. The full dose-response functions were analyzed by two-way analysis of variance. The responses to individual doses with and without ischaemia/reperfusion were compared by Student's two-tailed *t* test.

In vivo Changes in baseline haemodynamics (Table 1) were compared by a repeated measures analysis of variance (followed by a Newman Keul's *post-hoc* test). Drug responses either before or after ischaemia/reperfusion were compared to vehicle also using a repeated measures analysis of variance. Slope analysis was conducted using the slopes of individual dose-response functions. Statistical analysis of the data using CPA demonstrated that the three doses used were on the linear aspect of the dose-response function under all four conditions ($R^2 = 0.986 \pm 0.005$, $n = 4$ (LAD and circumflex; before and after ischaemia/reperfusion)). Similarly, statistical analysis of the data obtained with DPMA also confirmed that these three doses were on the linear aspect of the dose-response function under all four conditions ($R^2 = 0.911 \pm 0.028$; $n = 4$ (LAD and circumflex, before and after ischaemia/reperfusion)). Student's paired *t* test was used for specific comparisons where appropriate. Values are means ± the standard error of the mean (s.e.mean). Significance was set at $P \leqslant 0.05$ for all tests.

Results

Arterial pressure prior to ischaemia was 119 ± 3 mmHg and heart rate was 171 ± 6 beats min^{-1} ($n = 7$). After ischaemia/reperfusion, neither arterial pressure (113 ± 6 mmHg) nor heart rate (161 ± 12 beats min^{-1}) were significantly altered. All seven hearts exhibited an elevated or depressed S-T segment and cyanosis. In five of seven dogs, heart rates were highly irregular during the reperfusion period. During the reperfusion period, three animals developed ventricular tachycardia, followed by ventricular fibrillation. Animals regained a largely sinus rhythm after defibrillation. During ventricular fibrillation, blood pressure was reduced to 20 ± 4 mmHg for 1.6 ± 0.3 min ($n = 3$). Reactivity of the isolated arteries from these animals was not markedly different. For example, the $-\log EC_{50}$ for vasorelaxation to ACh ($n = 6$ total) was 6.27 ± 0.19 ($n = 3$) in arteries from non-defibrillated hearts while the $-\log EC_{50}$ was 6.44 ± 0.26 ($n = 3$) in arteries from defibrillated hearts.

Vasorelaxation in vitro

$PGF_{2\alpha}$-contracted dog coronary arterial rings were relaxed by both the endothelium-independent dilator sodium nitroprusside and the endothelium-dependent agent ACh. For sodium nitroprusside (Figure 1a), vasorelaxation was not significantly different in rings from control and reperfused arteries ($-\log EC_{50}$ 7.01 ± 0.28 versus 7.41 ± 0.18; $n = 5$). For ACh (Figure 1b), vasorelaxation again was not significantly different ($-\log EC_{50}$ 6.35 ± 0.15 versus 6.42 ± 0.25 μM; $n = 6$). While ischaemia/reperfusion appeared to attenuate the relaxation to ACh at higher doses, this trend was not statistically significant even at the highest dose ($P = 0.07$).

In addition, $PGF_{2\alpha}$-contracted dog coronary rings from control and reperfused arteries were relaxed dose-dependently by the adenosine A_1-receptor agonist, CPA and the A_2-receptor agonist, DPMA (Figure 1c,d). For CPA, the vasorelaxation in control and ischaemia/reperfusion arteries was not significantly different ($-\log EC_{50}$ 5.69 ± 0.36 versus 5.73 ± 0.25; $n = 6$). In contrast, DPMA-induced vasorelaxation was significantly attenuated after ischaemia/reperfusion, with a log unit increase in the concentration necessary for half-maximal vasorelaxation ($-\log EC_{50}$ 6.57 ± 0.16 versus 5.64 ± 0.28; $n = 6$, $P \leqslant 0.01$).

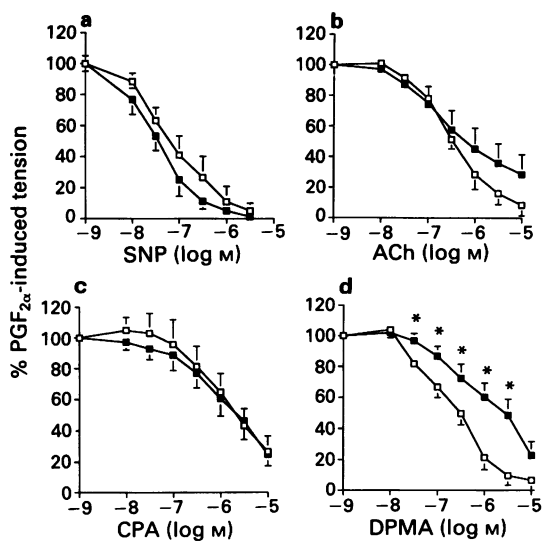


Figure 1 Concentration-response curves to the endothelium-independent vasorelaxant, sodium nitroprusside (a, $n = 5$), the endothelium-dependent vasorelaxant acetylcholine (b, $n = 6$), the A_1 selective agonist, cyclopentyladenosine (c, $n = 6$) and the A_2-selective agonist, DPMA (d, $n = 6$) in control (□) and ischaemia/reperfused (■) arteries. Control maximal isometric tension was 2.02 ± 0.44, 2.06 ± 0.82, 1.15 ± 0.46, and 1.76 ± 0.82 g for a,b,c and d, respectively. In vessels subjected to ischaemia/reperfusion, maximal isometric tension was 1.80 ± 0.54, 4.31 ± 0.98, 1.42 ± 0.52, and 1.99 ± 0.59 g for groups a,b,c and d, respectively. Note that only the vasorelaxation to the A_2-selective agonist is significantly affected by ischaemia/reperfusion.

Table 1 Effects of 1 h of ischaemia and 1 h of reperfusion on baseline haemodynamics

	Control	*Ischaemia*	*Reperfusion*
MAP (mmHg)	106 ± 6	107 ± 4	102 ± 4
HR (b.p.m.)	180 ± 12	193 ± 13	185 ± 13
dP/dt_{50} (mmHg s^{-1})	1757 ± 234	1882 ± 228	1751 ± 240
Circ BF (ml min^{-1})	23 ± 4	32 ± 2	25 ± 3
LAD BF (ml min^{-1})	15 ± 4	1 ± 2*	17 ± 5
SS Circ (%)	9.1 ± 1.2	8.6 ± 1.6	7.2 ± 1.1
SS LAD (%)	7.5 ± 0.7	− 3.6 ± 3.5*	− 2.1 ± 1.9*

Shown are mean arterial pressure (MAP), heart rate (HR), rate of change in left ventricular pressure (dP/dt_{50}), circumflex blood flow (Circ BF), left anterior descending arterial blood flow (LAD BF), and % segmental shortening (SS). Data are shown as mean ± standard error ($n = 6$). *indicates significantly different from control.

Maximal isometric tension

Maximal $PGF_{2\alpha}$-induced tension developed was significantly increased in rings from arteries subjected to ischaemia/reperfusion compared to controls ($P < 0.05$). Rings from control arteries developed 1.79 ± 0.64 g ($n = 7$) of tension; this value increased significantly after ischaemia/reperfusion to 2.57 ± 0.48 g, ($n = 7$). Isometric tension development to 36 mM KCl was not different between the two groups (4.55 ± 0.79 g control; 5.01 ± 0.70 g ischaemia/reperfusion, $n = 7$).

Vasodilatation in vivo

Table 1 illustrates the control values for mean arterial pressure, heart rate, $dP/dt50$ (rate of pressure rise at 50 mmHg), LAD blood flow, circumflex blood flow, and percent segmental shortening in both the circumflex and LAD vascular beds. In addition, Table 1 lists these parameters following 1 h of ischaemia, and again following 1 h of reperfusion. Segmental shortening in the myocardium perfused by the circumflex was not significantly affected by ischaemia/reperfusion of the LAD. In contrast, the myocardium perfused by the LAD was dysfunctional following the 1 h of ischaemia (Table 1). This region of the myocardium remained dysfunctional (as evidenced by the negative segmental shortening values) for the remainder of the protocol. This index of ischaemia could detect physiologically significant levels of collateral blood flow, as is discussed in detail in a subsequent paragraph.

The A_1 and A_2 agonists produced minimal effects on systemic haemodynamics at the point of maximal coronary vasodilatation. Table 2 lists changes in arterial pressure, heart rate, $dP/dt50$, and segmental shortening produced by vehicle, CPA and DPMA both before and after ischaemia/reperfusion. The A_1 agonist, CPA, did not significantly lower arterial pressure, either before or after ischaemia/reperfusion. The A_2 agonist, DPMA (at 30.0 μg kg^{-1}), lowered arterial pressure slightly compared to the response obtained with vehicle before but not after ischaemia/reperfusion. Heart rate was not significantly altered by either drug at the doses used. The value for $dP/dt50$ was modestly increased by the highest dose of CPA before ischaemia/reperfusion. DPMA did not alter $dP/dt50$ at any dose tested either before or after ischaemia/reperfusion. The highest dose of CPA did increase % segmental shortening in the circumflex region both before and after ischaemia/reperfusion. DPMA did not alter % segmental shortening in the circumflex field at any dose tested. In the LAD, CPA again increased % segmental

shortening at the highest dose tested, and DPMA again had no effect.

The A_1 agonist, CPA, produced a dose-dependent increase in blood flow in the normally perfused circumflex coronary artery before and after ischaemia/reperfusion of the LAD. Circumflex blood flow was significantly increased at 10.0 and 30.0 $\mu g\ kg^{-1}$ of CPA both before and after ischaemia/reperfusion (Figure 2). Ischaemia/reperfusion did significantly en-

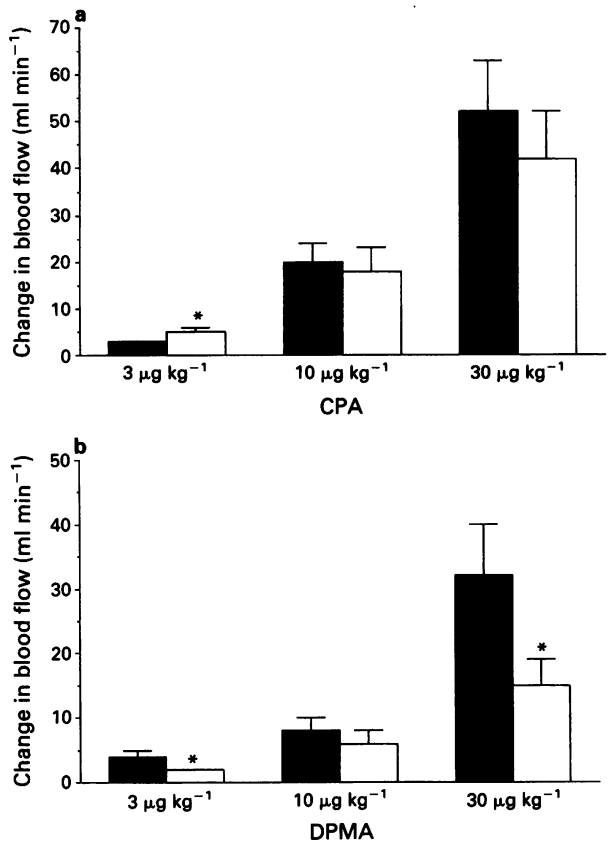


Figure 2 Effects of the A_1 agonist cyclopentyladenosine (a) and the A_2 agonist DPMA (b) on circumflex blood flow before (solid columns) and after (open columns) ischaemia/reperfusion of the left anterior descending (LAD) coronary artery. The adenosine agonists were administered intravenously at 3, 10, and 30 $\mu g\ kg^{-1}$ both before and after a 1 h ischaemia/1 h reperfusion protocol. Note that in the normally perfused circumflex coronary artery, the vasodilatation to cyclopentyladenosine is enhanced at the lowest dose and is unaltered at the two higher doses. In contrast, the vasodilator response to the A_2 agonist, DPMA, is significantly attenuated at the lowest and highest dose. $*P \leqslant 0.05$.

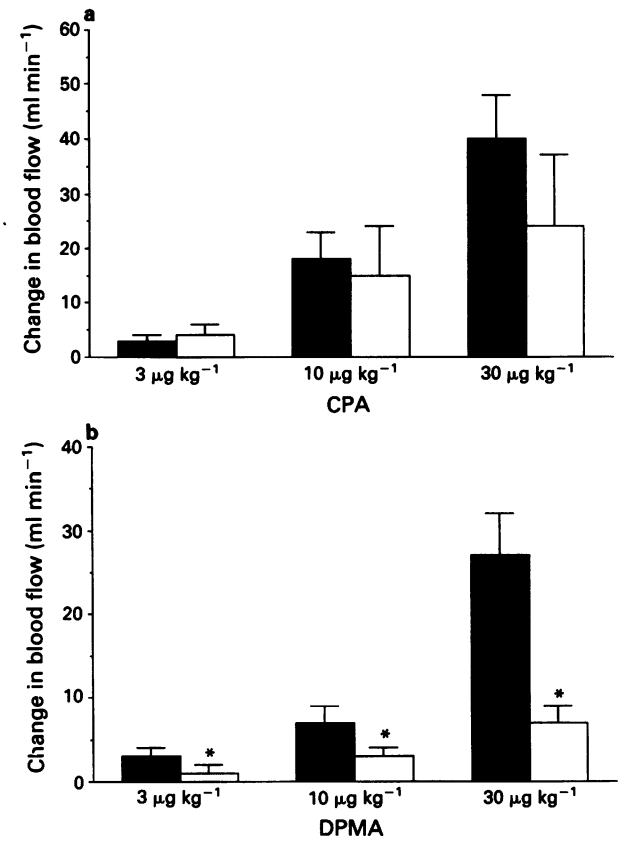


Figure 3 Effects of the A_1 agonist cyclopentyladenosine (a) and an A_2 agonist DPMA (b) on left anterior descending (LAD) coronary blood flow before (solid columns) and after (open columns) ischaemia/reperfusion. The adenosine agonists were administered intravenously at 3, 10, and 30 $\mu g\ kg^{-1}$ before and after a 1 h ischaemia/1 h reperfusion protocol. Note that the increase in blood flow produced by A_1 agonist tends to be reduced by ischaemia/reperfusion only at the highest dose while the response to the A_2 agonist is significantly attenuated at all doses. $*P \leqslant 0.05$.

Table 2 Change in haemodynamics and myocardial function produced by administration of vehicle compared with the A_1 agonist, CPA and the A_2 agonist, DPMA before and after ischaemia/reperfusion

		CPA			*DPMA*		
	Vehicle	*3 μg kg⁻¹*	*10 μg kg⁻*	*30 μg kg⁻¹*	*3 μg kg⁻¹*	*10 μg kg⁻¹*	*30 μg kg⁻¹*
MAP (mmHg)							
Pre	2 ± 1	1 ± 1	1 ± 2	− 2 ± 2	2 ± 1	0 ± 1	− 2 ± 1*
Post		− 1 ± 3	8 ± 4†	2 ± 3	2 ± 2	1 ± 3	− 2 ± 2
HR (b.p.m.)							
Pre	1 ± 1	− 2 ± 1	− 1 ± 1	− 9 ± 6	1 ± 3	5 ± 2	7 ± 2
Post		1 ± 3	0 ± 5	− 14 ± 6	0 ± 1	3 ± 1	0 ± 2†
dP/dt_{50} (mmHg s^{-1})							
Pre	11 ± 39	24 ± 30	56 ± 53	243 ± 58*	59 ± 24	361 ± 292	288 ± 28
Post		22 ± 46	144 ± 62	246 ± 204	10 ± 17	96 ± 47	58 ± 33†
SS Circ (%)							
Pre	− 0.7 ± 1.6	0.1 ± 0.2	0.2 ± 0.9	1.9 ± 1.1	0.1 ± 0.4	0.5 ± 0.4	1.2 ± 0.9
Post		1.0 ± 1.0	1.1 ± 1.4	3.8 ± 1.2	0.6 ± 0.4	0.4 ± 0.7	1.1 ± 0.4
SS LAD (%)							
Pre	− 0.3 ± 0.7	− 0.4 ± 0.4	− 0.1 ± 0.7	1.6 ± 0.4	0.4 ± 0.3	0.8 ± 0.3	0.9 ± 0.6
Post		3.2 ± 2.2	0.1 ± 0.2	1.0 ± 0.9	0.1 ± 0.3	0.3 ± 0.1	0.8 ± 0.3

Shown are mean arterial pressure (MAP), heart rate (HR), rate of change in left ventricular pressure (dP/dt_{50}), and percentage segmental shortening (% SS) in the Circumflex (Circ) and left anterior descending (LAD) coronary arteries. Significant difference from vehicle: $*P < 0.05$. Significant differences pre- versus post ischaemia/reperfusion: $\dagger P < 0.05$.

hance the increase, albeit modestly, to the lowest dose of CPA (Figure 2). Circumflex blood flow was significantly increased by the 30.0 $\mu g\,kg^{-1}$ dose of DPMA before ischaemia/reperfusion, and by 3.0, 10.0 and 30.0 $\mu g\,kg^{-1}$ after ischaemia/reperfusion (Table 1). The change in circumflex blood flow was attenuated by ischaemia/reperfusion of the LAD at both the lowest and the highest dose of DPMA (Figure 2).

CPA also increased coronary blood flow in the LAD before ischaemia/reperfusion, at the 10.0 and 30.0 $\mu g\,kg^{-1}$ doses, and after ischaemia/reperfusion at the 30.0 $\mu g\,kg^{-1}$ dose (Table 1). The changes in LAD blood flow were not significantly different before versus after ischaemia/reperfusion (Figure 3). The A_2 agonist, DPMA, also dose-dependently increased LAD blood flow before ischaemia/reperfusion (Table 1). However, the A_2 agonist did not significantly increase LAD blood flow following ischaemia/reperfusion, also shown in Table 1. Figure 3 illustrates the attenuated increases in LAD blood flow at all three doses of DPMA after ischaemia/reperfusion.

Slope analysis was conducted on the individual dose-response functions. Only the slope of the DPMA vasodilatation function in the LAD was altered by the ischaemia/reperfusion protocol.

Presence or absence of physiologically significant collateral blood flow was assessed functionally. In one animal, complete occlusion of the LAD did not produce myocardial dysfunction in the region monitored by the LAD sonomicrometry crystals (control = + 14.4%, ischaemia = + 9.4%, reperfusion = + 9.4%). As predicted, the A_2-mediated increase in LAD blood flow was not affected by the ischaemia/reperfusion protocol. For example, 30 $\mu g\,kg^{-1}$ of DPMA produced a 26 ml min^{-1} increase before ischaemia/reperfusion, and a 30 ml min^{-1} increase after ischaemia/reperfusion. In addition, DPMA increased LAD blood flow equally before and after the sham ischaemia/reperfusion protocol. For example, 10.0 $\mu g\,kg^{-1}$ of DPMA increased LAD blood flow by 13 ± 8 ml min^{-1} before sham ischaemia/reperfusion, and by 12 ± 6 ml min^{-1} after sham ischaemia/reperfusion ($n = 4$). These data further demonstrate the stability of DPMA in solution over the duration of the protocol.

Discussion and conclusions

In these experiments, we clearly demonstrate that both an adenosine A_1 and an adenosine A_2 receptor agonist can produce coronary vasorelaxation in the dog, *in vitro* as well as *in vivo*. Further, we found that ischaemia/reperfusion differentially attenuates coronary vasorelaxant responses to the A_2 receptor agonist while not affecting the vasorelaxant response to the A_1 receptor agonist. This differential effect is not due to endothelial damage, since the response to the endothelium-dependent vasorelaxant ACh is unaffected by ischaemia/reperfusion. Vascular smooth muscle also appears unaffected, since the response to the endothelium-independent vasorelaxant, sodium nitroprusside, is not altered.

Although our data indicate that maximal tension development to $PGF_{2\alpha}$ was significantly higher in rings from the ischaemic group compared to those from the control group, individual analysis revealed that only one of the four groups (CPA) displayed significantly higher vasoconstriction to $PGF_{2\alpha}$. The reason for this difference remains unclear since the rings from all four groups are derived from adjacent sections of the coronary arteries. However, analysis of the CPA data on an absolute gram (rather than a percentage) basis resulted in no significant difference in the dose-response curves. We therefore conclude that these intergroup differences have no influence on the interpretation of the results.

For the *in vitro* studies, it should be noted that the ischaemic vessels are taken 10 mm distal to the occluder. This calibre section of a conduit artery should not be subject to a pressure gradient allowing for collateral flow into the lumen of the vessel. This fact, coupled with the electrocardiographic data demonstrating S-T segment alterations and arrhythmias, clearly demonstrates that this region of the LAD was subjected to ischaemia. For the *in vivo* studies, we could clearly determine if collateral flow was present in physiologically significant amounts by examining the segmental shortening values in the ischaemic zone. As noted in the results section, one animal was excluded, based on insufficient reductions in segmental shortening following ischaemia/reperfusion. It should also be noted that the coronary vasodilatation produced by DPMA was not attenuated in this animal.

In the *in vivo* ischaemia/reperfusion studies, the protocol produces results which are commensurate with the increased complexity of the model. While the circumflex vascular bed was not subjected to ischaemia/reperfusion, the vasodilator responses to both the A_1 and A_2 agonist were altered at specific doses. The increase in coronary blood flow with CPA was enhanced at the lowest dose, and the vasodilator response to DPMA attenuated at the lowest and highest doses. However, slope analysis demonstrated no significant change in slope for either dose-response curve. We would hypothesize that the attenuated response to DPMA at the 3.0 and 30.0 $\mu g\,kg^{-1}$ doses is a response to the increased endogenous adenosine in the circumflex vascular bed. This adenosine may either originate from the ischaemic, dysfunctional LAD region, or be a result of the initial compensatory increase in myocardial work conducted by the circumflex region to maintain total cardiac function in spite of the dysfunctional myocardium in the LAD region. In support of this hypothesis, note the increase in baseline circumflex coronary blood flow (Table 1) following 1 h of ischaemia of the LAD (from 23 ± 4 to 32 ± 2 ml min^{-1}, $P < 0.05$, Student's paired t test).

Ischaemia/reperfusion of the LAD clearly attenuates the vasodilator response to the A_2 agonist, DPMA. The increase in coronary blood flow produced by DPMA is significantly reduced at all three doses. In addition, slope analysis revealed a significant reduction in slope for the three point dose-response function. In contrast, the increases in coronary blood flow produced by CPA were not significantly affected. It should be noted that a trend ($P = 0.06$) exists for the increase in coronary blood flow in response to the highest dose of CPA to be attenuated. We would hypothesize that CPA, at higher doses, may produce increases in coronary blood flow through activation of A_2 receptors, as well as A_1 receptors. However, this effect does not appear to occur at the lower two doses, and slope analysis reveals no change in the slope of the dose-response function.

In the present study, we have chosen to administer the A_1 and A_2 agonists intravenously, thus ensuring that plasma levels of drug were equivalent in all branches of the circumflex and LAD vascular beds. In addition, note that the doses used did not alter significantly mean arterial pressure or heart rate at the time of the peak increase in coronary blood flow. These data are in agreement with our previous results (Cox *et al.*, 1991) and that obtained with related A_1 and A_2 agonists (Gerencer *et al.*, 1992).

In previous studies, we have demonstrated that the vasorelaxant response of both the A_1 agonist, CPA and the A_2 agonist, DPMA can be completely blocked by the selective adenosine antagonist CGS15943 both *in vitro* in the dog (Cox *et al.*, 1991) and *in vitro* in the swine (Merkel *et al.*, 1992). CGS15943 is an antagonist selective for adenosine receptors but does not differentiate between A_1 and A_2 receptor subtypes (Jarvis *et al.*, 1991). These data are important since we have previously shown that coronary vasorelaxant responses to the A_1 agonist, CPA but not the A_2 agonist, DPMA, can be attenuated by the potassium channel blocker, glibenclamide in the swine (Merkel *et al.*, 1992). It should also be noted that low doses of CPA produce vasoconstriction in isolated coronary arteries from swine, but not from dog. The present study confirms the lack of A_1-mediated vasoconstriction in the dog, both *in vitro* and *in vivo*. Thus a species difference occurs with the constrictor, but not the dilato-

properties of CPA. Together, these data clearly demonstrate that the vasorelaxant actions of CPA are due to activation of a CGS15943-sensitive receptor. However, the glibenclamide and ischaemia/reperfusion data demonstrate that these vasorelaxant/vasodilator responses are not simply due to activation of A_2 receptors. The role of differing second messenger systems in separate species is not currently known.

Other investigators have examined the effect of ischaemia/reperfusion on the coronary vasorelaxant response to purinoceptor agonists. In one study, dog circumflex arteries were subjected to several levels of ischaemia/reperfusion. In this study, relaxation of conduit arteries in response to ACh, ADP, bradykinin, A23187, nitroglycerin and adenosine was not altered by a 1 h ischaemia/1 h reperfusion protocol. However, ischaemia/reperfusion did significantly blunt the EC_{50} to ACh and ADP in coronary microvessels (110–220 μm). However, adenosine was not tested in the microvessel model (Quillen *et al.*, 1990). In another study, the vasorelaxant effects of ADP on coronary arteries were blunted by a 1 h ischaemia/1 h reperfusion protocol (Pearson *et al.*, 1990). In the latter study, it would appear that the ischaemia may have been more severe, damaging the endothelium. In the study of Quillen *et al.* (1990), and consistent with our data, the endothelium is functional following our 1 h ischaemia/1 h reperfusion protocol. In the present study, there was a trend ($P = 0.07$) toward a blunted vasorelaxant response to ACh only at the higher doses. Despite the evidence for intact endothelium-dependent and independent vasorelaxation, the A_2 receptor agonist is functionally less active throughout the majority of the dose-response curve. This attenuated response occurred at doses where endothelium-dependent responses are clearly intact.

Thus, our data suggest that ischaemia/reperfusion selectively decreases A_2 receptor number, alters the coupling of A_2 receptors to its second messenger system, or alters the functioning of the second messenger system. While our data cannot differentiate between these possible mechanisms, we have clearly demonstrated a functional impairment of A_2, but not A_1, adenosine receptor-mediated coronary vasorelaxation. This effect may be in response to the elevated local adenosine concentrations which occur during ischaemia (Deussen *et al.*, 1988). This theory is supported by the findings of Ramkumar *et al.* (1991) who demonstrated that the downregulation of A_2 receptors by A_2 agonists occurred more rapidly ($t_{\frac{1}{2}} = 45$ min) than downregulation of A_1 receptors by A_1 agonists ($t_{\frac{1}{2}} = 8$ h) in a smooth muscle cell line (DDT MF-2). Therefore, one could speculate that ischaemia/reperfusion selectively decreases A_2 receptors, possibly by the accumulation of endogenous adenosine.

Our previous studies have shown that A_1-receptor-mediated vasorelaxation is linked to potassium channels (Merkel *et al.*, 1992). Additionally, potassium channel activators, such as aprikalim, have been shown to reduce ischaemia/reperfusion-mediated myocardial injury (Grover *et al.*, 1990; Auchampach *et al.*, 1991). Thus, by reducing adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-(A_2) mediated vasodilatation in favor of an 'anti-ischaemic' potassium channel (A_1)-mediated vasodilatation, a greater mass of the myocardium may potentially be spared. These data support the development of selective adenosine agonists to treat ischaemia clinically followed by reperfusion.

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References

AUCHAMPACH, J.A., MARUYAMA, M., CAVERO, I. & GROSS, G.J. (1991). The new K^+ channel opener RP52891 reduces experimental infarct size in dogs in the absence of systemic haemodynamic changes. *J. Pharmacol. Exp. Ther.*, **259**, 961–967.

BERNE, R.M. (1963). Cardiac nucleosides in hypoxia: possible role in regulation of coronary blood flow. *Am. J. Physiol.*, **204**, 317–322.

BOLLI, R., TRIANA, J.F. & JEROUDI, M.O. (1990). Prolonged impairment of coronary vasodilation after reversible ischaemia: evidence for microvascular 'stunning'. *Circ. Res.*, **67**, 332–343.

BOLLI, R., ZHU, W.X., THORNBY, J.I., O'NEILL, P.G. & ROBERTS, R. (1988). Time-course and determinants of recovery of function after reversible ischaemia in conscious dogs. *Am. J. Physiol.*, **254**, H102–H114.

COWLEY, M.J., DORROS, G., KELSEY, S., VANRADEN, M. & DETRE, K. (1984). Acute coronary events associated with percutaneous transluminal angioplasty. *Am. J. Cardiol.*, **53**, 12c–16c.

COX, B.F., LAPPE, R.W., SHELDON, J.H., PERRONE, M.H. & ROTH, R.A. (1991). Adenosine 1 (A_1) receptor agonist produces coronary vasodilation independent of bradycardia in the dog. *FASEB J.*, **5**, A494.

DEUSSEN, A., BORST, M., KROLL, K. & SCHRADER, J. (1988). Formation of S-adenosylhomocysteine in the heart. II: a sensitive index for regional myocardial underperfusion. *Circ. Res.*, **63**, 250–261.

GERENCER, R.Z., FINEGAN, B.A. & CLANACHAN, A.S. (1992). Cardiovascular selectivity of adenosine receptor agonists in anaesthetized dogs. *Br. J. Pharmacol.*, **107**, 1048–1056.

GROVER, G.J., DSWONCZYK, S. & SLEPH, P.G. (1990). Reduction of ischaemic reperfusion damage in isolated rat hearts by the potassium channel opener RP52891. *Eur. J. Pharmacol.*, **191**, 11–18.

HEYNDRICKX, G.R., MILLARD, R.W., MCRITCHIE, R.J. & VATNER, S.F. (1975). Regional myocardial function and electrophysiological alterations after brief coronary artery occlusion in conscious dogs. *J. Clin. Invest.*, **56**, 978–985.

JARVIS, M.F., WILLIAMS, M., DO, U.H. & SILLS, M.H. (1991). Characterization of the binding of a novel nonxanthine adenosine antagonist radioligand [3H]CGS15943 to multiple affinity states of the adenosine A_1 receptor in the rat cortex. *Mol. Pharmacol.*, **39**, 49–54.

JENNINGS, R.B., SCHAPER, J., HILL, M.L., STEENBERGEN, Jr. C. & REIMER, K.A. (1985). Effect of reperfusion late in the phase of reversible ischaemic injury: changes in cell volume, electrolytes, metabolites, and ultrastructure. *Circ. Res.*, **56**, 262–278.

KLONER, R.A., ELLIS, S.G., LANGE, R. & BRAUNWALD, E. (1983). Studies of experimental coronary artery reperfusion: effects on infarct size, myocardial function, biochemistry, ultrastructural and microvascular damage. *Circulation*, **68**, I8–I15.

KU, D.D. (1982). Coronary vascular reactivity after acute myocardial ischaemia. *Science*, **218**, 576–578.

LASLEY, R.D., RHEE, J.W., VAN WYLEN, G.L. & MENTZER, R.M. (1990). Adenosine A_1 receptor mediated protection of the globally ischaemic isolated rat heart. *J. Mol. Cell Cardiol.*, **22**, 39–47.

LONDOS, C., WOLFF, J. & COOPER, D.M.F. (1983). Adenosine receptors and adenylate cyclase interactions. In *Regulatory Function of Adenosine* ed. Berne, R.M., Rall, T.W. & Rubio, R. pp. 17–32. Boston MA; Nijhoff.

MERKEL, L.A., LAPPE, R.W., RIVERA, L.M., COX, B.F. & PERRONE, M.H. (1992). Demonstration of vasorelaxant activity with an A_1-selective adenosine agonist in porcine coronary arteries: involvement of potassium channels. *J. Pharmacol. Exp. Ther.*, **260**, 437–443.

NORTON, E.D., JACKSON, E.K., TURNER, M.B, VIRMANI, R. & FORMAN, M.B. (1992). The effects of intravenous infusions of selective adenosine A_1-receptor and A_2-receptor agonists on myocardial reperfusion injury. *Am. J. Heart.*, **123**, 332–338.

OLSSON, R.A. & PEARSON, J.D. (1990). Cardiovascular purinoceptors. *Physiol. Rev.*, **70**, 761–845.

PEARSON, P.J., SCHAFF, H.V. & VANHOUTTE, P.M. (1990). Acute impairment of endothelium-dependent relaxations to aggregating platelets following reperfusion injury in dog coronary arteries. *Circ. Res.*, **67**, 385–393.

QUILLEN, J.E., SELLKE, F.W., BROOKS, L.A. & HARRISON, D.G. (1990). Ischaemia-reperfusion impairs endothelium-dependent relaxation of coronary microvessels but does not affect large arteries. *Circulation*, **82**, 586–594.

RAMKUMAR, V., OLAH, M.E., JACOBSON, K.A. & STILES, G.L. (1991). Distinct pathways of desensitization of A_1 and A_2-adenosine receptors in DDT_1 MF-2 cells. *Mol. Pharmacol.*, **40**, 639–647.

SUBA, E.A. & ROTH, B.L. (1987). Prostaglandins active phosphoinositide metabolism in rat aorta. *Eur. J. Pharmacol.*, **136**, 325–332.

THORNTON, J.D., LIU, G.S., OLSSON, R.A. & DOWNEY, J.M. (1992). Intravenous pretreatment with A_1-selective adenosine analogues protects the heart against infarction. *Circulation*, **85**, 659–665.

TOOMBS, C.F., MCGEE, D.S., JOHNSON, W.E. & VINTEN-JOHANSEN, J. (1992). Myocardial protective effects of adenosine – infarct size reduction with pretreatment and continued receptor stimulation during ischemia. *Circulation*, **86**, 986–994.

VAN BENTHUYSEN, K.M., MCMURTRY, I.F. & HORWITZ, C.D. (1987). Reperfusion after acute coronary occlusion in dogs impairs endothelium-dependent relaxation to acetylcholine and augments contractile reactivity *in vitro*. *J. Clin. Invest.*, **79**, 265–274.

VAN CALKER, D., MULLER, M. & HAMPRECHT, B. (1979). Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured cells. *J. Neurochem.*, **33**, 999–1005.

ZHOU, Q., LI, Y., OLAH, M., JOHNSON, R., STILES, G. & CIVELLI, O. (1992). Molecular cloning and characterization of an adenosine receptor: the A_3 receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 7432–7436.

ZUCCHI, R., RONCA-TESTONI, S., GALBANI, P., YU, G., MARIANI, M. & RONCA, G. (1992). Cardiac A_2 adenosine receptors – influence of ischaemia. *Cardiovasc. Res.*, **26**, 549–554.

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Characterization of 5-hydroxytryptamine receptors mediating mucosal secretion in guinea-pig ileum

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1 The receptor subtypes through which 5-hydroxytryptamine (5-HT) increases electrolyte secretion across the mucosa of guinea-pig ileum were studied.

2 Flat sheep preparations of guinea-pig mucosa plus submucosa were placed in Ussing chambers and the short circuit current (I_{SC}), an index of net electrogenic electrolyte transport across the mucosa, was measured under voltage clamp conditions.

3 Low concentrations of 5-HT (10–300 nM) evoked monophasic increases in I_{SC} which were significantly reduced by hyoscine (100 nM), tetrodotoxin (TTX, 300 nM) and the 5-HT_2 receptor antagonist, ketanserin (3–300 nM).

4 Higher concentrations of 5-HT (1–10 μM) produced biphasic responses which were reduced by hyoscine (100 nM), TTX (300 nM), ketanserin (3–300 nM) and also by the 5-HT_3 receptor antagonists, granisetron (1 μM) and ICS 205-930 (100 nM).

5 2-Methyl-5-HT (1–100 μM) and α-methyl-5-HT (30 nM–30 μM), agonists at 5-HT_3 and 5-HT_2 receptors respectively, also evoked I_{SC} increases. These responses were reduced by hyoscine (100 nM) and abolished by TTX (300 nM) and the respective receptor antagonists, granisetron (1 μM) and ketanserin (30 nM).

6 The 5-HT_4 receptor antagonist, SDZ 205-557 (300 nM) had no effect on the response to 5-HT.

7 The TTX-resistant response to 5-HT was not affected by 5-$HT_{2,3\text{ or }4}$ receptor antagonists.

8 These results indicate that 5-HT mediates secretion partly by an action on 5-HT_3 receptors located on cholinergic and noncholinergic secretomotor neurones, partly by an action on higher affinity '5-HT_2-like' receptors predominantly on noncholinergic neurones, and partly by a direct action on the epithelium.

Keywords: 5-Hydroxytryptamine; mucosal transport; secretomotor neurones; 5-HT_2 receptors; 5-HT_3 receptors; intestine

Introduction

5-Hydroxytryptamine (5-HT) produces a wide variety of effects on central and peripheral neurones. This variety is due in part to the presence of a number of 5-HT receptor subtypes (Bradley *et al.*, 1986; Watson & Girdlestone, 1993). 5-HT promotes mucosal secretion of water and electrolytes in the intestine of many species, including the rat (Hardcastle *et al.*, 1981), guinea-pig (Cooke & Carey, 1985) and rabbit (Donowitz *et al.*, 1977). In the guinea-pig ileum this secretory response appears to be mediated via neurones in the submucous plexus (Keast *et al.*, 1985; Cooke & Carey, 1985). Flux chamber experiments using isolated mucosa plus submucosa preparations have shown that 5-HT evokes a biphasic increase in short circuit current (I_{SC}) which has been attributed to an increase in chloride secretion (Cooke & Carey, 1985).

Multiple sites exist in the gut for the potential action of 5-HT. Receptors for the amine have been localized on cell bodies of neurones in the submucous plexus (Hirst & Silinksy, 1975; Surprenant & Crist, 1988). Pharmacological data suggest these receptors exist on both cholinergic and noncholinergic secretomotor neurones (Keast *et al.*, 1985). 5-HT may also have a small direct secremotor effect on the mucosal epithelium (Zimmerman & Binder, 1984), although ligand binding studies have been unable to locate epithelial receptors for 5-HT (Gaginella *et al.*, 1983).

Characterization of the receptor subtypes mediating secretion has been limited by a lack of selective pharmacological tools. Hendriks and colleagues (1989) found evidence for 5-HT_3 receptors on cholinergic secretomotor neurones, but were unable to classify the receptors on the noncholinergic neurones. However, the recent development of more selective 5-HT drugs has led us to re-investigate this problem.

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Methods

Tissue preparation

Guinea-pigs of either sex weighing between 150 and 300 g were stunned by a blow to the head and then killed by severing the carotid arteries and spinal cord. A 10 cm segment of ileum was taken approximately 10–30 cm from the ileo-caecal junction and placed in physiological saline. The segment was opened along the mesenteric border and pinned flat with the mucosal surface down. The longitudinal and circular muscle layers and myenteric plexus were removed and the remaining mucosa plus submucous plexus preparation was divided into flat 1 cm^2 sheets and mounted in Ussing flux chambers (window area 0.38 cm^2). Four preparations were made from adjacent segments of intestine from each animal. One served as an internal control (i.e. for construction of a concentration-effect curve in the absence of the drugs being tested), the other three were used to test different combinations of drugs. In the data outlined below, the number of preparations used refers to the number of animals.

The tissues were bathed on both sides with physiological saline at 37°C containing (in mM): NaCl 118, $NaHCO_3$ 25, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, NaH_2PO_4 1.0, and gassed with 95% O_2, 5% CO_2. Glucose (11 mM) was added to the serosal bathing solution and mannitol (11 mM) to the mucosal side to prevent coupled sodium absorption of glucose which may have affected the measurement of secretion.

Electrical measurements

The potential difference across the tissue was measured by Krebs-agar bridges mounted on either side of the preparation and connected to calomel reference electrodes. This potential difference was clamped to zero, with a suitable compensation for fluid resistance, by a voltage clamp (Physiological Instruments Voltage-Current Clamp model VCC600); the current required to do this, termed the short-circuit current (I_{SC}; Ussing & Zerahn, 1950), was recorded as an index of net electrogenic electrolyte transport. The responses were recorded directly on to a CODAS data acquisition programme (DATAQ).

Experimental protocol

The tissue was left to equilibrate for at least 1 h or until stable baseline I_{SC} responses were achieved. Baseline I_{SC} values were similar to those described in previous studies using identical Ussing chambers (Keast *et al.*, 1985; Hendriks *et al.*, 1989). All drugs were added to the serosal bathing solution. Repeated doses of 5-HT (1 μM) were added to the baths at 30 min intervals (up to 4 repetitions) until it was ascertained that the response to this concentration of 5-HT was consistent and could serve as a control. This concentration was chosen because it had previously been shown to evoke a submaximal response without desensitizing the tissue using a similar protocol (Hendriks *et al.*, 1989). No significant differences were observed in control I_{SC} values either within or between groups of experiments.

Non-cumulative concentration-effect curves were performed by adding increasing concentrations of agonists at 30 min intervals. Agonists were left in contact with the tissue for 2.5 min before washout. In some experiments, and in previous studies (Keast *et al.*, 1985; Hendriks *et al.*, 1989), a small but significant increase in the tissue response to 5-HT was observed during the time (up to 5 h) needed for these experiments. When used, antagonists were added 15 min prior to each addition of an agonist.

Analysis of results

The maximum amplitudes of the I_{SC} responses to the agonists were expressed as percentages of the amplitude of the initial stable 5-HT 1 μM control response. When the response was biphasic (see Figure 1), the amplitude of the larger phase was measured although this sometimes changed from the first to the second phase with drug treatment. Although this may have introduced some distortions with high concentrations of 5-HT, most experiments were carried out under conditions where the responses were monophasic. Concentration-effect curves for the agonists in the presence and absence of antagonists were presented graphically and compared by two-way analysis of variance.

Drugs

The following drugs were used: 5-hydroxytryptamine creatinine sulphate complex (5-HT), hyoscine hydrobromide, tetrodotoxin (TTX; Sigma); 2-methyl-5-hydroxytryptamine maleate, α-methyl-5-hydroxytryptamine maleate, cyproheptadine hydrochloride, ketanserin (+)-tartrate (Research Biochemicals Incorporated); ICS 205-930 (tropisetron), granisetron (BRL 43694, SmithKline Beecham); SDZ 205-557 (2-methoxy-4-amino-5-chloro-benzoic acid 2-(diethylamino) ethyl ester; gift from SmithKline Beecham). All drugs were dissolved in physiological saline except ICS 205-930 which was dissolved in 0.1 M hydrochloric acid. The vehicle was shown to have no effect on secretion or the response to drugs.

Results

Effects of 5-HT on I_{SC}

As previously described by Keast *et al.* (1985), 5-HT produced a concentration-dependent rise in I_{SC} with a maximum increase of 97.5 ± 2.6 μA cm^{-2} and an apparent EC_{50} of 0.7 μM ($n = 8$). Low concentrations of 5-HT (10–300 nM) evoked a monophasic increase in I_{SC} whereas higher concentrations (1–30 μM) produced a biphasic response composed of an initial rapid transient rise in I_{SC}, followed by a slower more maintained phase that was similar to the response evoked by lower 5-HT concentrations (Figure 1).

Concentration-effect curves were established for 5-HT, 2-methyl-5-HT (a relatively selective 5-HT_3 receptor agonist; Mawe *et al.*, 1986) and α-methyl-5-HT (an agonist with some selectivity for 5-HT_2 receptors; Richardson *et al.*, 1985). The effects of various selective receptor antagonists on these concentration-effect relationships were investigated to determine the roles of the different receptor subtypes.

Effect of hyoscine on the response to 5-HT

The muscarinic receptor antagonist, hyoscine (100 nM), abolished the first phase of the response to high concentrations of 5-HT (>300 nM), and reduced, but did not abolish the second component (see also Keast *et al.*, 1985; Hendriks *et al.*, 1989). The response evoked by lower 5-HT concentrations was also decreased. Overall, the effect of hyoscine on the I_{SC} response was significant ($P < 0.005$, $n = 4$, Figure 2a).

5-HT_3 receptors

The selective 5-HT_3 receptor antagonist, granisetron (1 μM; Sanger & Nelson, 1989) decreased the amplitudes of both phases of the I_{SC} response evoked by high concentrations of 5-HT (>300 nM); the decrease in the maximum amplitude of the response was significant ($P < 0.005$, $n = 4$). The second phase was apparently reduced ($P < 0.05$, $n = 4$), but this may have been solely due to the decrease in the overlapping first phase. The response to lower concentrations of 5-HT was unaffected by granisetron (Figures 1 and 2a).

2-Methyl-5-HT had no effect on secretion at concentrations below 1 μM, but evoked a biphasic increase in I_{SC} at higher concentrations (Figure 1), with an EC_{50} of 6.6 μM ($n = 4$). This response was significantly decreased by granisetron (1 μM; $P < 0.0001$, $n = 4$), and abolished by TTX (300 nM; $P < 0.0001$, $n = 4$; Figure 2b). Hyoscine (100 nM) abolished the first phase of the response to 2-methyl-5-HT but had little effect on the second component. The maximum amplitude of the I_{SC} response was reduced by an average of 64% (Figures 1 and 2b; $P < 0.0001$, $n = 4$). The initial com-

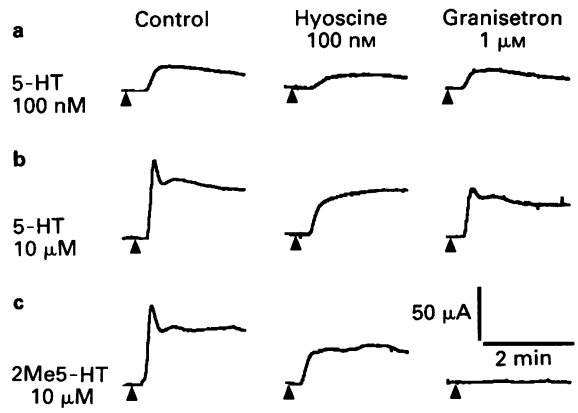


Figure 1 Representative records illustrating control I_{SC} responses to 5-hydroxytryptamine (5-HT) and 2-methyl-5-HT, and responses in the presence of hyoscine (1 μM) and granisetron (1 μM): (a) 5-HT 100 nM; (b) 5-HT 10 μM; (c) 2-methyl-5-HT 10 μM; (▲) indicate the times of addition of agonists.

ponent of the response to 2-methyl-5-HT, like that to high concentrations of 5-HT, appears to be due to the action of acetylcholine on muscarinic receptors on the mucosal epithelium (Keast *et al.*, 1985). ICS 205-930, an antagonist at both 5-HT_3 and 5-HT_4 receptors, also reduced the response evoked by 2-methyl-5-HT (data not shown) when used at 100 nM, a concentration expected to block only 5-HT_3 receptors (Craig & Clarke, 1990).

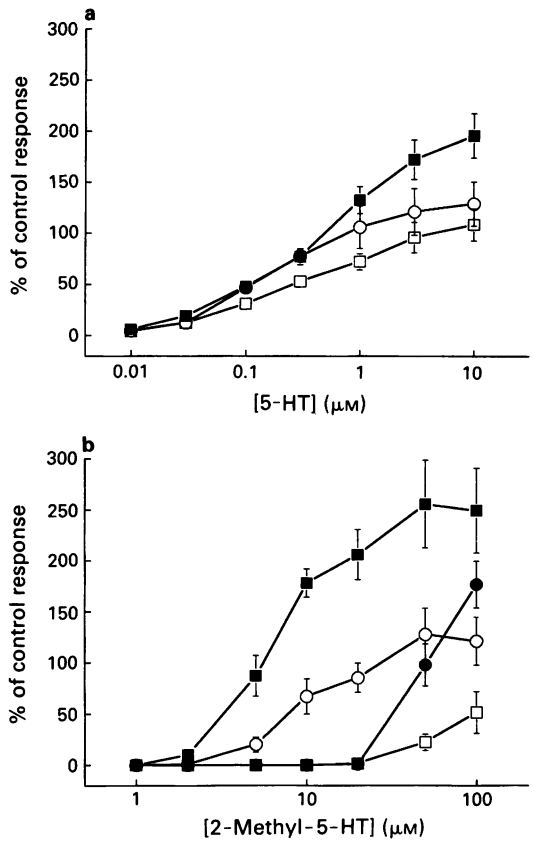


Figure 2 Concentration-effect curves for (a) 5-hydroxytryptamine (5-HT) alone (■) and in the presence of granisetron (1 μM, ○) or hyoscine (100 nM, □); and (b) 2-methyl-5-HT alone (■) and in the presence of granisetron (1 μM, ○), hyoscine (100 nM, □) or tetrodotoxin (300 nM, ●). Results were expressed as mean ± s.e.mean; $n = 4$.

5-HT_2 receptors

The 5-HT_2 receptor antagonist, ketanserin (3–300 nM), produced a significant depression of the responses evoked by all but the highest concentrations of 5-HT ($P < 0.05$, $n = 5$, Figure 3). It did not appear to abolish either component of the biphasic response to 5-HT. The rightward shift in the concentration-effect curve was similar in magnitude in the presence of three concentrations of ketanserin tested (3, 30 and 300 nM). This suggested that the effects of low concentrations of 5-HT might be mediated via 5-HT_2 receptors.

Results similar to those seen with ketanserin were obtained with cyproheptadine (100 nM) which depressed responses to all concentrations of 5-HT (not illustrated). However, this concentration of cyproheptadine also depressed responses to carbachol suggesting that its effects on high concentrations of 5-HT may have been mediated, at least in part, via an action on muscarinic receptors on the mucosa.

α-Methyl-5-HT, a predominantly 5-HT_2 receptor agonist, evoked a monophasic increase in I_{SC} with a similar time course to the second phase of the response to 5-HT. This response was almost completely abolished by ketanserin (30 nM; $P < 0.0001$; $n = 4$). In the presence of hyoscine (100 nM), the amplitude of the I_{SC} increase evoked by α-methyl-5-HT was reduced by approximately 30% (Figure 4). These experiments were performed in the presence of ICS 205-930 (3 μM) to block any residual effect of the agonist at 5-HT_3 or 5-HT_4 receptors.

5-HT_4 receptors

The 5-HT_4 receptor antagonist, SDZ 205-557 (300 nM) (Buchheit *et al.*, 1991), had no significant effect on the response to 5-HT ($n = 4$; data not shown). Furthermore, a substantial TTX-sensitive component of the response persisted even in the presence of 3 μM ICS 205-930, ($n = 4$; data not shown; see also Hendriks *et al.*, 1989) a concentration expected to block both 5-HT_3 and 5-HT_4 receptors (Craig & Clarke, 1990).

Effects of TTX on the 5-HT response

Addition of TTX (300 nM) to the serosal bathing solution decreased the baseline I_{SC} by approximately 30% ($n = 4$). In the presence of tetrodotoxin (300 nM), 5-HT evoked a slow, well maintained monophasic response. The TTX-resistant response could not be further reduced by granisetron (1 μM) or ketanserin (30 nM; Figure 5).

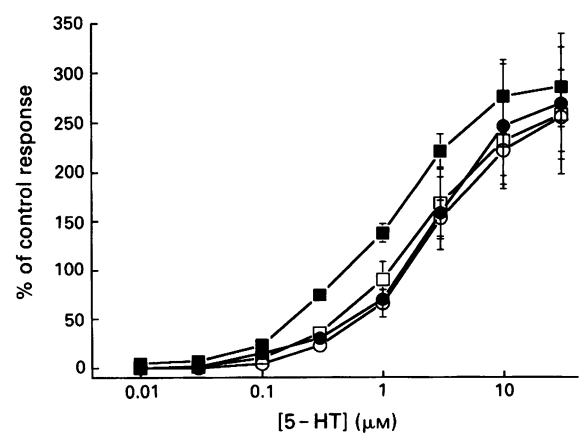


Figure 3 Effect of 5-HT_2 receptor antagonism: concentration-effect curves for 5-hydroxytryptamine (5-HT) in the absence (■) and presence of ketanserin (3 nM □, 30 nM ●, 300 nM ○). Results are expressed as mean ± s.e.mean; $n = 5$.

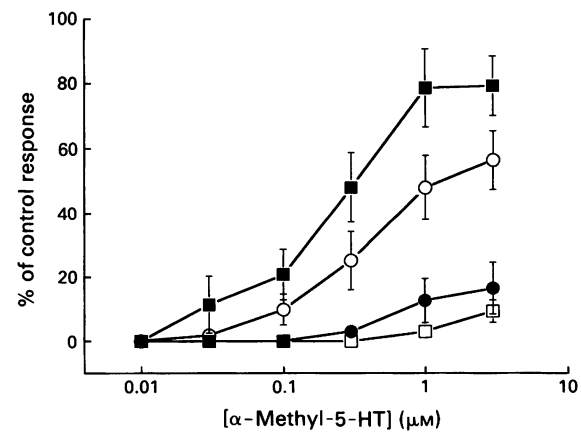


Figure 4 Concentration-effect curves for α-methyl-5-HT in the absence (■) and presence of ketanserin (30 nM, ●), hyoscine (1 μM, ○) or tetrodotoxin (300 nM, □). All curves were performed in the presence of ICS 205-930 (3 μM). Results are shown as means ± s.e.mean; $n = 4$.

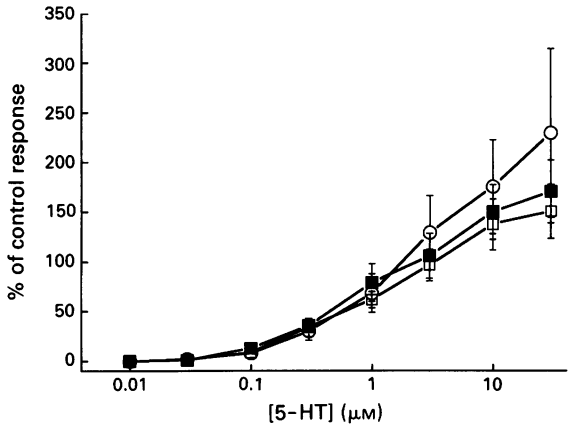


Figure 5 The effect of 5-HT_2 and 5-HT_3 receptor antagonists on the tetrodotoxin (TTX)-resistant response to 5-hydroxytryptamine (5-HT). Concentration-effect curves for 5-HT in the presence of TTX alone (300 nM, ■), TTX and ketanserin (30 nM, ○) or TTX and granisetron (1 μM, □). Results are expressed as means ± s.e.mean; $n = 4$.

Discussion

These results confirm that the primary effect of 5-HT in the promotion of intestinal secretion is to excite secretomotor neurones of the submucous plexus. The neurally mediated secretory response to 5-HT appears to be evoked via at least two distinct receptor subtypes.

Both cholinergic and noncholinergic secretomotor neurones were shown to be excited by 5-HT acting on 5-HT_3 receptors, a result consistent with those obtained by Surprenant & Crist (1988), who found that virtually all submucous neurones were depolarized by 5-HT acting on 5-HT_3 receptors. This contrasts with the conclusion of Hendriks *et al.* (1989), who found that the hyoscine-resistant component of the I_{SC} response to 5-HT (1 μM) was not further reduced by ICS 205-930, apparently demonstrating the presence of these receptors on cholinergic neurones only. However, it is possible that the noncholinergic secretomotor neurones were maximally stimulated via another receptor subtype, thereby masking the presence of 5-HT_3 receptors on these neurones. The use of the 5-HT_3 receptor agonist, 2-methyl-5-HT, in the present study has permitted a more direct test of the presence of this receptor.

The 5-HT_3 receptor antagonist, granisetron, decreased the response to 5-HT only at concentrations of 5-HT greater than 1 μM. This suggests that the 5-HT_3 receptor has a relatively low affinity for 5-HT, which is consistent with studies performed in other preparations (e.g. Buchheit *et al.*, 1985). 5-HT_3 receptor antagonists did not alter the 5-HT response. Thus it is unlikely that they play a significant role in the response of the preparation to 5-HT.

An intriguing result of the present study was the identification on some secretomotor neurones of a higher affinity 5-HT receptor which appears to belong to the '5-HT_2-like' receptor subtype. The response to 5-HT was substantially reduced by the 5-HT_2 antagonist, ketanserin. In addition, α-methyl-5-HT evoked a relatively large increase in I_{SC} which was almost completely blocked by ketanserin. Hysocine slightly reduced the response to α-methyl-5-HT. However, a substantial response persisted, indicating the existence of a population of '5-HT_2-like' receptors on both cholinergic and noncholinergic neurones. Figure 3 indicates that the effects of ketanserin were maximal with 3–30 nM of the antagonist. This suggests that the effects of 5-HT may be mediated via the 5-HT_{2A} receptor subtype (Humphrey *et al.*, 1993).

5-HT_2 receptors have been implicated in the secretory process in rat ileum, colon and jejunum (Hardcastle *et al.*, 1981; Moriarty *et al.*, 1987; Siriwardena *et al.*, 1991). Surprenant & Crist (1988) found that in addition to its effects at 5-HT_3 receptors, 5-HT evoked a slower, higher affinity depolarization in a subset of submucous neurones, which they were unable to attribute to a known receptor subtype. The high affinity receptor described by Surprenant & Crist (1988) may correspond to that detected in the present study, especially as they were able to detect only two excitatory effects of 5-HT on submucous neurones. However, Surprenant & Crist (1988) were unable to block the response to 5-HT with ketanserin, in direct contrast with the present study. Also, Hendriks *et al.* (1989) found no effect of cyproheptadine on the response to 5-HT that was resistant to ICS 205-930. Baird & Cuthbert (1987) found a small but non-significant effect of ketanserin on the increase in I_{SC} evoked by 5-HT. The present study suggests that the pharmacology of the electrophysiological effects of 5-HT on secretomotor neurones should be re-investigated.

A substantial response to 5-HT persisted in the presence of TTX which could not be attributed to an action at 5-HT_2, 5-HT_3 or 5-HT_4 receptors. As similar concentrations of TTX have been shown to block effectively action potential-dependent transmission from enteric neurones (Cooke & Carey, 1985), the residual response was probably due to a direct effect of 5-HT on the mucosal epithelium. Scott *et al.* (1992) examined the profile of a number of 5-HT_4 receptor agonists in the presence of TTX and concluded that a part of the TTX-resistant responses may be mediated via 5-HT_4 receptors. However, in the present study, the 5-HT_4 antagonist SDZ 205-557 (Buchheit *et al.*, 1991) had no effect on the response to 5-HT. Also, at concentrations of ICS 205-930 whch would be expected to block 5-HT_4 receptors there was no reduction in the response to low 5-HT concentrations. Thus it appears that this non-neural component of the 5-HT response may be mediated via an, as yet, unidentified receptor subtype. Experiments to characterize this receptor are currently underway.

In summary, the results provide evidence that mucosal secretion in response to 5-HT in the guinea-pig small intestine is mediated partly by an action on low affinity 5-HT_3 receptors present on both cholinergic and noncholinergic secretomotor neurones, partly by an action at high affinity '5-HT_2-like' receptors located predominantly on noncholinergic neurones, and partly by a direct action on the epithelium through receptors that have yet to be identified.

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References

BAIRD, A.W. & CUTHBERT, A.W. (1987). Neuronal involvment in type 1 hypersensitivity reactions in gut epithelia. *Br. J. Pharmacol.*, **92**, 647–655.

BRADLEY, P.B., ENGEL, G., FENUIK, W., FOZARD, J.R., HUMPHREY, P.P.A., MIDDLEMISS, D.N., MYLECHARANE, E.J., RICHARDSON, B.P. & SAXENA, P.R. (1986). Proposals for the classification and nomenclature of functional receptors for 5-hydroxytryptamine. *Neuropharmacol.*, **25**, 563–576.

BUCHHEIT, K.H., ENGEL, G., MUTSCHLER, E. & RICHARDSON, B. (1985). Study of the contractile effect of 5-hydroxytryptamine (5-HT) in the isolated longitudinal muscle strip from guinea-pig ileum. *Naunyn-Schmied. Arch. Pharmacol.*, **329**, 36–41.

BUCHHEIT, K.H., GAMSE, R. & PFFANKUCHE, H.J. (1991). SDZ 205-557, a selective antagonist at 5-HT_4 receptors in the isolated guinea-pig ileum. *Eur. J. Pharmacol.*, **200**, 373–374.

COOKE, H.J. & CAREY, H.V. (1985). Pharmacological analysis of 5-hydroxytryptamine actions on guinea-pig ileal mucosa. *Eur. J. Pharmacol.*, **111,** 329–337.

CRAIG, D.A. & CLARKE, D.E. (1990). Pharmacological characterisation of a neuronal receptor for 5-hydroxytryptamine in guinea-pig ileum with properties similar to the 5-hydroxytryptamine$_4$ receptor. *J. Pharmacol. Exp. Ther.*, **252,** 1378–1386.

DONOWITZ, M., CHARNEY, A.N. & HEFFERNAN, J.M. (1977). Effect of serotonin treatment on intestinal transport in the rabbit. *Am. J. Physiol.*, **232,** E85–E94.

GAGINELLA, T.S., RIMELA, T.J. & WIETECHA, M. (1983). Studies on rat intestinal epithelial cell receptors for serotonin and opiates. *J. Physiol.*, **335,** 101–111.

HARDCASTLE, J., HARDCASTLE, P.T. & REDFERN, J.S. (1981). Action of 5-hydroxytryptamine on ion transport in the rat. *J. Physiol.*, **320,** 41–55.

HENDRIKS, R., BORNSTEIN, J.C. & FURNESS, J.B. (1989). Evidence for two types of 5-hydroxytryptamine receptor on secretomotor neurons of the guinea-pig ileum. *Naunyn-Schmied. Arch. Pharmacol.*, **339,** 409–414.

HIRST, G.D.S. & SILINSKY, E.M. (1975). Some effects of 5-hydroxytryptamine, dopamine and noradrenaline on neurons in the submucous plexus of the guinea-pig small intestine. *J. Physiol.*, **251,** 817–832.

HUMPHREY, P.P.A., HARTIG, P. & HOYER, D. (1993). A proposed new nomenclature for 5-HT receptors. *Trends Pharmacol. Sci.*, **14,** 233–236.

KEAST, J.R., FURNESS, J.B. & COSTA, M. (1985). Investigations of nerve populations influencing ion transport that can be stimulated electrically, by serotonin and by a nicotinic agonist. *Naunyn-Schmied. Arch. Pharmacol.*, **331,** 260–266.

LEYSEN, J.E., NIEMEGEERS, C.J.E., VAN NEUTEN, J.M. & LADURON, P.M. (1982). [^{3}H]Ketanserin (R 41 468), a selective ^{3}H-ligand for serotonin$_2$ receptor binding sites. *Mol. Pharmacol.*, **21,** 301–314.

MAWE, G.M., BRANCHEK, T.A. & GERSHON, M.D. (1986). Peripheral neural serotonin receptors: identification and characterisation with specific antagonists and agonists. *Proc. Natl. Acad. Sci. U.S.A.*, **83,** 9799–9803.

MORIARTY, K.J., HIGGS, M.B., WOODFORD, M., WARHURST, G. & TURNBERG, L.A. (1987). Inhibition of the effect of serotonin on rat ileal transport by cisapride: evidence in favour of the involvement of 5-HT$_2$ receptors. *Gut*, **28,** 844–848.

RICHARDSON, B.P., ENGEL, G., DONATSCH, P. & STADLER, P.A. (1985). Identification of serotonin M-receptor subtypes and their specific blockade by a new class of drugs. *Nature*, **316,** 126–131.

SANGER, G.J. & NELSON, D.R. (1987). Selective and functional 5-hydroxytryptamine$_3$ receptor antagonism by BRL 43694 (granisetron). *Eur. J. Pharmacol.*, **159,** 113–124.

SCOTT, C.M., BUNCE, K.T. & SPRAGGS, C.F. (1992). Investigation of the 5-hydroxytryptamine receptor mediating the 'maintained' short-circuit response in guinea-pig ileal mucosa. *Br. J. Pharmacol.*, **106,** 877–882.

SIRIWARDENA, A., BOOKER, C., PRATT, J. & KELLUM, J.M. (1991). Pathways of serotonin-induced electrolyte in rat distal colon. *Surgery*, **110,** 411–418.

SURPRENANT, A. & CRIST, J. (1988). Electrophysiological characterisation of functionally distinct 5-hydroxytryptamine receptors on guinea-pig submucous plexus. *Neuroscience*, **24,** 283–295.

USSING, H.H. & ZERAHN, K. (1950). Active transport of sodium as the source of electrical current in the short-circuited isolated frog skin. *Acta Physiol. Scand.*, **23,** 110–127.

WATSON, S. & GIRDLESTONE, D. (1993). 1993 receptor nomenclature supplement. *Trends Pharmacol. Sci.*, **14.**

ZIMMERMAN, T.W. & BINDER, H.J. (1984). Serotonin-induced alteration of colonic electrolyte transport in the rat. *Gastroenterol.*, **86,** 310–317.

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Interactions between indomethacin, noradrenaline and vasodilators in the fetal rabbit ductus arteriosus

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1 Interactions between indomethacin, noradrenaline and vasodilators were studied in rings of ductus arteriosus isolated from fetal rabbits. The effect of incubation with prostaglandin E_2 (PGE_2) on the noradrenaline concentration-contraction response curve was studied in the presence and absence of indomethacin. Also, the ductus was pre-contracted with 10 μM noradrenaline and concentration-relaxation response curves (CRRC) to PGE_2, cicaprost, cromakalim and forskolin were obtained in the presence and absence of indomethacin.

2 In the absence of indomethacin, PGE_2 (from 1 nM to 100 nM) decreased the pEC_{50} to noradrenaline to a maximum of 0.4 to 0.5 log units (i.e. an approximately three fold increase in EC_{50} [M]). In the presence of 1 μM indomethacin, PGE_2 (0.1 nM to 100 nM) decreased the pEC_{50} to noradrenaline by 2.39 log units (i.e. a 245 fold increase in EC_{50}). By comparing the control pEC_{50} to noradrenaline with the relationship between the pEC_{50} to noradrenaline and [PGE_2] in 1 μM indomethacin, the effect of endogenous PGE_2 synthesized in the vessel wall was estimated as being equivalent to a bath concentration of approximately 1 nM exogenous PGE_2.

3 When the vessel was pre-contracted with 10 μM noradrenaline, indomethacin had no effect on the CRRC to PGE_2 but did alter the CRRC to other vasodilators. The sensitivity of the vessel to cicaprost, cromakalim and forskolin was decreased in 1 μM indomethacin compared with control. Forskolin caused complete relaxation in the presence and absence of indomethacin. Indomethacin decreased the maximum response to cromakalim but increased the maximum response to cicaprost. PGE_2, 0.3 nM, partially reversed the effect of indomethacin on the sensitivity of the vessel to forskolin.

4 We conclude that under varying experimental conditions, indomethacin increased the sensitivity of the ductus to the effects of PGE_2 but decreased its sensitivity to other vasodilators. Both effects can be explained by elimination of endogenous PGE_2. However, indomethacin increased the maximum response to cicaprost, which cannot be explained by elimination of endogenous PGE_2 but may be due to elimination of endogenous prostacyclin.

Keywords: Fetus; ductus arteriosus; indomethacin; prostaglandin E_2; cicaprost; cromakalim; forskolin; congenital heart disease; prostacyclin

Introduction

The ductus arteriosus is a fetal blood vessel which extends between the pulmonary artery and the aorta (Heymann & Rudolph, 1975). It shunts deoxygenated blood away from the pulmonary circulation to the descending aorta and thence to the placenta where gaseous exchange takes place. In man, functional closure of the ductus takes place in the first few days of life (Drayton & Skidmore, 1987). Later, the lumen is obliterated by necrosis of the vessel wall, with formation of a fibrous remnant, the *ligamentum arteriosum*. Patency of the vessel *in utero* is an active state. The smooth muscle of the ductus has intrinsic tone which is inhibited by the profound dilator effect of prostaglandin E_2 (PGE_2) (Clyman, 1987). Circulating concentrations of PGE_2 are 1–2 nM in the fetal lamb (Clyman *et al.*, 1980b), which is well within the range which affects the ductus. PGE_2 is also produced in the vessel's wall (Coceani *et al.*, 1986): indomethacin contracts the isolated ductus (Coceani *et al.*, 1975) and increases its sensitivity to noradrenaline (Smith & McGrath, 1988).

Physiological closure of the ductus in the neonate is at least partly due to elimination of the effects of PGE_2. Circulating concentrations of PGE_2 fall to less than 10% of fetal levels within hours of birth (Clyman *et al.*, 1980b). The oxygen tension to which the ductus is exposed increases soon after birth (Heymann & Rudolph, 1975). Increasing oxygen tension decreases the sensitivity of the ductus to the dilator effects of PGE_2 (Coceani *et al.*, 1975; Smith & McGrath, 1991; 1993).

Failure of the ductus to close in early neonatal life, patent ductus arteriosus (PDA), is common in premature infants and can lead to heart failure (see Gersony, 1986, for review). Elucidation of the role of PGE_2 in the physiological control of the vessel led directly to the successful use of indomethacin to treat PDA. Re-opening of the ductus after indomethacin, however, is common and the reason for this is unclear. Conversely, in some neonates with certain forms of congenital heart disease, the post-natal circulation is dependent on a patent ductus (ductus-dependent circulation). These infants are treated with PGE_1 to prolong ductal patency until definitive surgery can be performed (see Gersony, 1986, for review).

Indomethacin increases the sensitivity of the ductus arteriosus to PGE_2, which has been postulated to be due to an effect of the drug's action on the PGE_2 'target site' (Coceani *et al.*, 1975). Increased sensitivity to PGE_2 might explain the tendency of the ductus to re-open after administration of indomethacin in the treatment of PDA. Elucidation of the mechanism of this effect might facilitate the development of drugs with greater efficacy in PDA. Furthermore, PGE_1 was selected for use in ductus-dependent circulation on the basis of comparison with other prostaglandins in the presence of indomethacin, both *in vitro* (Clyman *et al.*, 1978; Coceani *et al.*, 1978) and *in vivo* (Friedman *et al.*, 1983). If indomethacin increases ductal sensitivity to PGE_2, this may have exaggerated the sensitivity of the ductus to PGE_2 relative to other prostaglandins.

The aim of this study was to characterize the effects of indomethacin on the response of the ductus to PGE_2 and to other vasodilators.

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Methods

Fetal New Zealand White rabbits at 28 days gestation (term 31 days) were delivered by Caesarean section (after the mother had been killed) and decapitated prior to the onset of respiration. One or two rings of ductus arteriosus (1–1.5 mm in length) were obtained from each fetus and each ring was suspended between two stainless steel hooks, one stable, the other connected to an isometric tension transducer (Grass, model FT 03C). The hooks were passed through the narrow vessel lumen, denuding it of endothelium, as indicated by light microscopy. The mounted vessels were placed in 10 ml organ baths containing physiological salt solution (PSS) of the following composition (mM): NaCl 119, KCl 4.7, $MgSO_4$ 1.0, $KHPO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0 and glucose 11.0; with 24 μM Na_2EDTA. The signal from the transducer was relayed to a bridge conditioner (Fylde FE 492 BBS) and thence to a chart recorder (Linseis 7208).

The vessels were maintained at 36.5–37°C. The baths were bubbled with 15% O_2, 5% CO_2, and remainder N_2 resulting in a Po_2 of 100–110 mmHg, which is consistent with post-natal arterial oxygen tension. Temperature and oxygen tension were measured continuously in an additional organ bath. The oxygen measuring system (O_2 electrode and O_2 meter, Strathkelvin Instruments, model 781) was zeroed in a 0.01 M solution of sodium borate saturated with sodium sulphite. The gain was set at 36.5 to 37°C bubbling with room air, after correction for atmospheric and water vapour pressure and assuming ambient oxygen of 20.93%.

Effects of PGE_2 on the response to noradrenaline

The vessel was stretched to 0.2 g tension when set up (Smith & McGrath, 1991), incubated for 70 min before the addition of indomethacin and the PSS was changed twice during this period. Although the degree of stretch was slightly sub-optimal for contractile responses of the vessel in the presence of indomethacin (maximum responses to noradrenaline were 70% of optimal), higher degrees of stretch resulted in greater variation in the sensitivity to noradrenaline over the course of the experiment (Smith & McGrath, unpublished data). Where indomethacin was used, the vessel was incubated in a concentration of 1 μM of the drug for at least 30 min prior to addition of PGE_2. Where the drug was not used, an identical time protocol was employed. The vessel was exposed to a given concentration of PGE_2 for 10 min prior to addition of noradrenaline. Concentration-contraction response curves (CCRC) to noradrenaline were obtained cumulatively. The response at a given concentration was allowed to reach a plateau prior to obtaining the next. The PSS was changed at least 3 times after each CCRC. A period of at least 45 min was allowed between CCRC and no more than 5 were obtained from each vessel.

In the absence of indomethacin, a control response was obtained and subsequent responses in the presence of exogenous PGE_2 were compared to it. In the presence of indomethacin a control response was obtained in 1 nM PGE_2 and subsequent responses in a given concentration of PGE_2 were compared to it. Consecutive time controls were obtained for 5 responses (1) in the absence of indomethacin (2) in 1 μM indomethacin and 1 nM PGE_2. Where the time controls indicated that the responses were stable, paired statistical comparison was made between the test and control response. Where time controls indicated change in response parameters with time, un-paired statistical comparison was made between the extent of the change between the test and control response and the appropriate change in time controls.

Concentration-relaxation response curves

The vessel was stretched to 0.6 *g* tension when set up and this was re-set 1 h later (Smith & McGrath, 1993). The vessel was incubated for 90 min before the addition of any drugs and the PSS was changed twice in that time. After the vessel had been incubated in 1 μM propranolol (with or without 1 μM indomethacin) for 30 min, to eliminate any effects of noradrenaline mediated through β-adrenoceptors, it was contracted with 10 μM noradrenaline. The contraction was stable (after an initial peak) and was sustained for at least 80 min. Concentration-relaxation response curves (CRRC) to a given vasodilator were obtained cumulatively and commenced 20 min after addition of noradrenaline. The effect of a given concentration of drug was allowed to reach a maximum before addition of the next. The PSS was changed at least 4 times after a CRRC and at least 1 h was allowed between CRRC. The effect of indomethacin was elucidated by comparing the response in 1 μM indomethacin with the response in the absence of the drug, comparing the responses of different vessels using identical time protocols.

Quantification of response

The sensitivity of the vessel to agonists was quantified by the pEC_{50}, i.e. the $-\log_{10}$ of the EC_{50}, where the EC_{50} is the interpolated molar concentration of the drug causing 50% of its own eventual maximum response (either contractile or relaxant). The maximum contractile response (MCR) of noradrenaline was expressed as the increase in tone above changes induced by indomethacin and PGE_2. For vasodilators, the magnitude of the relaxation at any given concentration and the maximum relaxant response (MRR) were expressed as a % of the maximum relaxation as determined by addition of 100 nM PGE_2 and 30 μM papaverine at the end of every CRRC, which resulted in loss of all active tone as indicated by a fall below baseline and no further response to 1 μM forskolin.

Drugs

The following drugs were used: forskolin, indomethacin, noradrenaline bitartrate, papaverine hydrochloride, propranolol hydrochloride, (all Sigma); cicaprost (Schering); cromakalim (BRL 38227, Smith Kline Beecham); and, PGE_2 (U-12062, Upjohn). The stock solutions of drugs were as follows: PGE_2 was in ethanol (10 mg ml^{-1}) which was diluted to 100 μM in 0.1 M phosphate buffer; cromakalim (10 mM), and papaverine (20 mM) were in ethanol; indomethacin (10 mM) was in 10% $NaHCO_3$; noradrenaline (10 mM) was in 23 μM Na_2EDTA; forskolin (10 mM) was in dimethyl sulphoxide; cicaprost (0.5 mg ml^{-1}) and propranolol (10 mM) were in distilled water. All subsequent dilutions were made in 0.9% saline, except noradrenaline, which was diluted in 23 μM Na_2EDTA. All drugs were stored on ice for the duration of the experiment.

Statistics

All data are expressed as the mean with the standard error in parentheses (or bars in graphs). Comparison of responses was made by Student's *t* test, paired and un-paired as appropriate. Significance was assumed at the 5% level. Statistical analysis was performed on Minitab Release 8.2 for the Apple Macintosh.

Results

Time control response to noradrenaline

There was no change in the MCR to noradrenaline over 5 consecutive CCRC, either in the presence or absence of indomethacin. However, the pEC_{50} of the first CCRC to noradrenaline was higher than the subsequent responses whether indomethacin was present or not (Figure 1). In the presence of indomethacin, there was no further change in pEC_{50} (Figure 1b), whereas in its absence, the pEC_{50} of the

second CCRC was slightly elevated compared to the third and subsequent responses (Figure 1a).

Effects of PGE_2 on the response to noradrenaline

When looking at the effect of PGE_2 on the noradrenaline CCRC, the second CCRC was always used as the control response (in view of the time controls), and CCRC 3–5 were obtained in a range of $[PGE_2]$.

In the absence of indomethacin, PGE_2 of 1 nM and above decreased the pEC_{50} to noradrenaline compared with control (Figure 2a). The maximum decrease in pEC_{50} from control and correcting for the effect of time was between 0.4 to 0.5 log units (i.e. an approximately three fold increase in the EC_{50} [M]). Addition of PGE_2 between 0.1 nM and 10 nM increased the MCR (% control maximum) to noradrenaline, whereas 100 nM PGE_2 decreased it (Figure 2b).

In the presence of 1 μM indomethacin, PGE_2 from 0.1 nM to 100 nM decreased the pEC_{50} to noradrenaline covering a range of 2.39 log units (i.e. a 245 fold increase in EC_{50} [M] to noradrenaline, Figure 3a). The relationship between $[PGE_2]$ and the MCR (% control maximum) to noradrenaline was bell-shaped. Concentrations of 0.03 nM and below and 30 nM and above were associated with a decreased MCR compared with the MCR in 1 nM PGE_2 (Figure 3b). By comparing the plot of the raw data for the relationship between $[PGE_2]$ and pEC_{50} to noradrenaline in the presence of indomethacin with the 95% confidence intervals for the control pEC_{50} to noradrenaline in the absence of indomethacin, the effect of endogenous prostaglandins on the response of the vessel to noradrenaline was equal to a bath concentration of approximately 1 nM PGE_2 with indomethacin present (Figure 4).

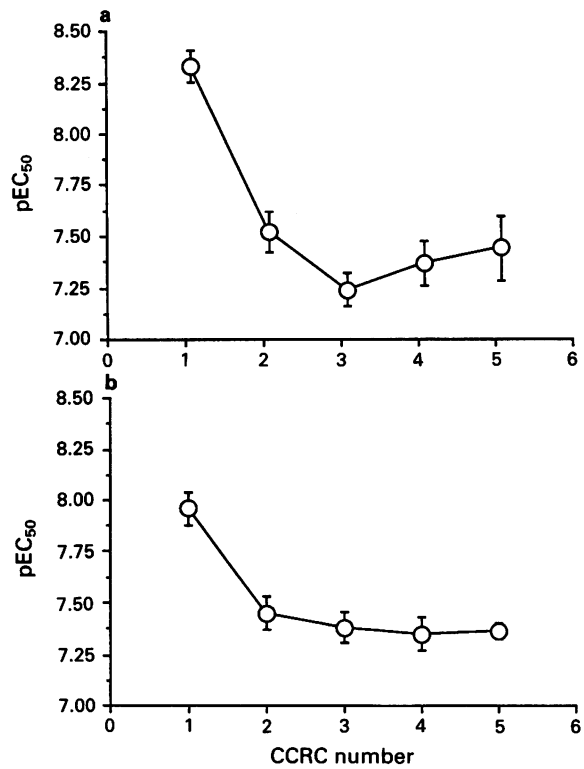


Figure 1 pEC_{50} ($-\log_{10}$ M) to noradrenaline for consecutive concentration-contraction response curves (CCRC). (a) Indomethacin absent ($n = 7-13$). The first pEC_{50} was significantly elevated compared to all the others (all $P < 0.0001$). The second pEC_{50} was elevated compared with responses 3 to 5 (mean difference 0.169 [s.e.mean 0.036], $P < 0.0001$). There was no significant difference in the pEC_{50} between responses 3–5. (b) In 1 μM indomethacin and 1 nM prostaglandin E_2 (PGE_2) ($n = 8$). The first pEC_{50} was significantly elevated compared with all the others (all $P < 0.0001$). There were no significant differences in the pEC_{50} of responses 2 to 5.

Effects of indomethacin on vasodilator CRRC

Figure 5 illustrates a typical CRRC to PGE_2 with the ductus pre-contracted with 10 μM noradrenaline in 1 μM indomethacin. The response to PGE_2 was virtually the same in the presence and absence of 1 μM indomethacin (Figure 6a).

The response of the ductus to a range of vasodilators was elucidated in the presence and absence of indomethacin, the vessel pre-contracted with 10 μM noradrenaline. Indomethacin decreased the pEC_{50} to cicaprost but increased its MRR (% maximum relaxation, Figure 6b); decreased the pEC_{50} and MRR to cromakalim (Figure 7a); and decreased the pEC_{50} to forskolin, but had no effect on the MRR (Figure 7b). There was no significant difference in the amount of active tone present with 10 μM noradrenaline comparing the presence and absence of indomethacin (mean in mN [s.e.mean]: 13.0 [0.52], $n = 28$ vs 13.5 [0.56], $n = 28$, respectively, $P = 0.55$).

Addition of 0.3 nM PGE_2 partially reversed the effect of indomethacin on the sensitivity of the ductus to forskolin (Figure 8).

Discussion

One aim of this study was to elucidate whether indomethacin has an effect on the sensitivity of the PGE_2 receptor-effector system. The evidence which suggests that it does is work by

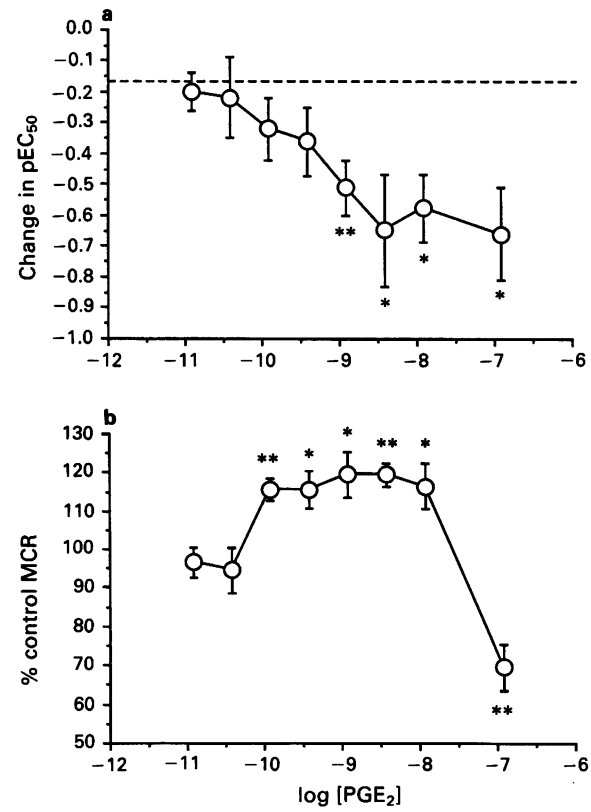


Figure 2 Effects of prostaglandin E_2 (PGE_2) on noradrenaline concentration-contraction response curves (CCRC) in the absence of indomethacin ($n = 7-12$). (a) pEC_{50} ($-\log_{10}$ M). A control response was obtained in the absence of exogenous PGE_2 (CCRC 2, same time protocol as Figure 1a). The pEC_{50} of the control response was subtracted from the pEC_{50} of subsequent responses to noradrenaline in the presence of a given $[PGE_2]$. The difference was compared with the mean difference in pEC_{50} of time controls (−0.169 [s.e.mean 0.036], see Figure 1a) which is indicated on the graph by the broken line. Student's t test $^*P < 0.05$; $^{**}P < 0.01$. (b) MCR. The MCR in the presence of a given $[PGE_2]$ is expressed as a % of the MCR of the control response. Students's paired t test, $^*P < 0.05$; $^{**}P < 0.01$.

Coceani *et al.* (1975) which demonstrated an increased response to PGE_2 following incubation in indomethacin in the presence of elevated, but not low, oxygen tension. The ductus synthesizes its own prostaglandins (Terragno *et al.*, 1977; Coceani *et al.*, 1986) which relax the vessel, as evidenced by the contraction induced by indomethacin (Coceani *et al.*, 1975). The effects of indomethacin on the ductus are thought to be mediated entirely by elimination of PGE_2 (Clyman, 1987). In the absence of indomethacin, therefore, the effect of exogenous PGE_2 on the ductus will be superimposed on the effects of PGE_2 produced by the vessel. It follows that exogenous PGE_2 will only begin to have an effect when it significantly exceeds the concentration of endogenous PGE_2 to which the vessel is exposed. Where a tissue produces a sufficient concentration of prostaglandin to have a biological effect, indomethacin will cause an apparent increase in the tissue's response by removing the contribution of endogenous prostaglandins. Coceani and colleagues (1975) did not consider this explanation for their results. We wanted to test the hypothesis that the effect of indomethacin on the sensitivity of the ductus arteriosus to PGE_2 could be explained by elimination of the effect of endogenous PGE_2.

We tested the hypothesis in two ways. Firstly we looked at the effect of PGE_2 on the pEC_{50} to noradrenaline, in the presence and absence of indomethacin. Treatment with indomethacin increases the pEC_{50} of the ductus to noradrenaline (Smith & McGrath, 1988) and PGE_2 decreases it (Smith & McGrath, 1991). It is unaffected by changes in

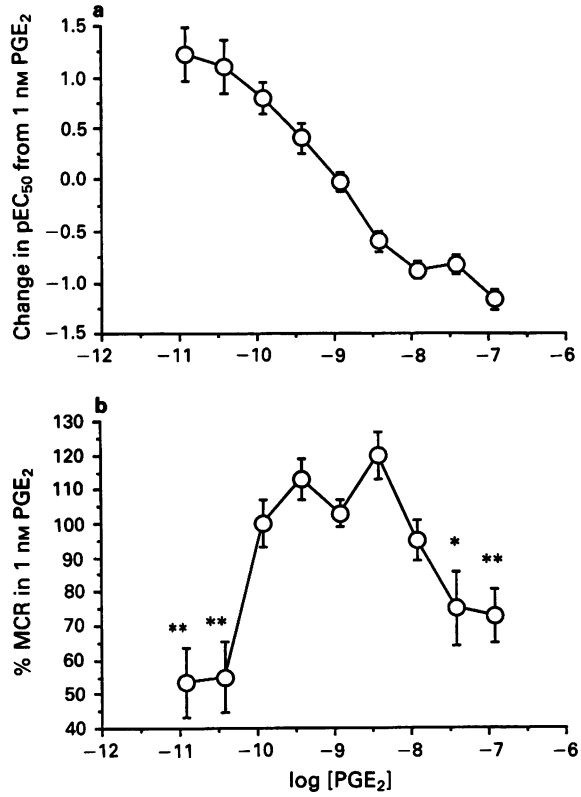


Figure 3 Effects of prostaglandin E_2 (PGE_2) on noradrenaline concentration-contraction response curves (CCRC) in the presence of 1 μM indomethacin ($n = 8-14$). (a) pEC_{50} ($-\log_{10}$ M). A control response was obtained in 1 nM PGE_2 (CCRC 2, same time protocol as Figure 1b). The pEC_{50} of the control response was subtracted from the pEC_{50} of the response in a given $[PGE_2]$ and the difference is plotted on the y axis. The range of change in pEC_{50} across the range of $[PGE_2]$ is 2.39. (b) MCR. The MCR in the presence of a given $[PGE_2]$ is expressed as a % of the MCR of the control response in 1 nM PGE_2. Students's paired t test, $^*P<0.05$; $^{**}P<0.01$.

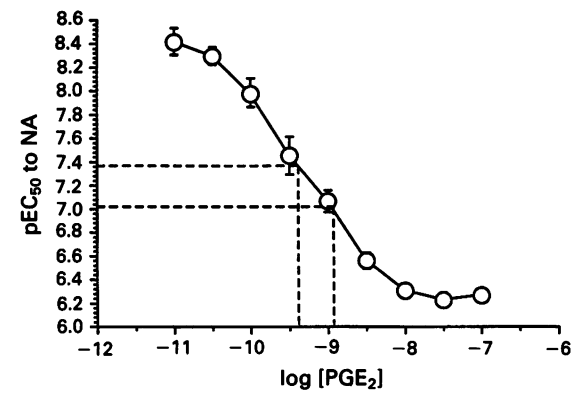


Figure 4 The pEC_{50} ($-\log_{10}$ M) to noradrenaline in 1 μM indomethacin in the presence of 0.01 to 100 nM prostaglandin E_2 (PGE_2). This graph presents the raw data from Figure 3a. The dotted lines from the y-axis represent the 95% confidence intervals of the pEC_{50} to noradrenaline in the absence of indomethacin and PGE_2 (7.01 to 7.34, $n = 32$: these data are from the control response, CCRC 2, of Figure 2a).

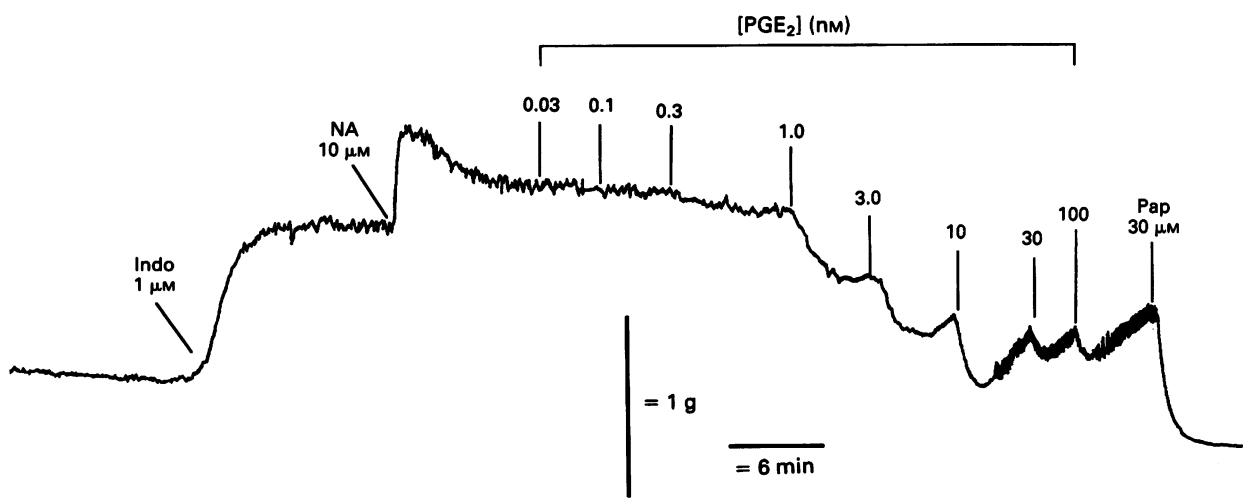


Figure 5 Typical concentration-relaxation response curves (CRRC) to prostaglandin E_2 (PGE_2). Indo 1 μM = where indomethacin was added to bath, NA 10 μM = addition of noradrenaline, Pap 30 μM = addition of papaverine. Calibration was by a 1 g weight = 9.81 mN. Time calibration bar applies from the beginning of the PGE_2 CRRC.

ductal smooth muscle tone *per se* (Smith & McGrath, 1988). It acts as a useful index, therefore, of the effects of endogenous and exogenous PGE_2. The second approach was to look at the dilator effects of PGE_2, with and without indomethacin, in the presence of a very high level of tone which exceeded the inhibitory effects of endogenous PGE_2. For this the ductus was pre-contracted with 10 μM noradrenaline.

Looking at our data on the effect of a range of [PGE_2] on the pEC_{50} to noradrenaline in the presence and absence of indomethacin, they appear to confirm that indomethacin increases ductal sensitivity to PGE_2. In the absence of indomethacin, PGE_2 (from 1 nM to 100 nM) decreased the pEC_{50} to noradrenaline to a maximum of 0.4 and 0.5 log units (i.e. an approximately three fold increase in EC_{50} [M], Figure 2). In the presence of 1 μM indomethacin, PGE_2, across the range 0.1 nM to 100 nM, decreased the pEC_{50} to noradrenaline by 2.39 log units (i.e. a 245 fold increase in EC_{50}, Figure 3).

Figure 4 illustrates the relationship between [PGE_2] and pEC_{50} to noradrenaline. By superimposing the 95% confidence intervals of the control pEC_{50} to noradrenaline on this graph, it is clear that in the absence of indomethacin, endogenous PGE_2 had a profound inhibitory effect on the vessel's pEC_{50} to noradrenaline. Indeed, a rough estimation of the effects of endogenous PGE_2 can be made in terms of equivalence to a bath [PGE_2] with indomethacin present, which we estimate to be in the region of 1 nM. In the absence of indomethacin, PGE_2 had an effect on the pEC_{50} to noradrenaline only at concentrations of 1 nM and above. Taking these data together, we conclude that the major effect, possibly exclusive effect of indomethacin to increase

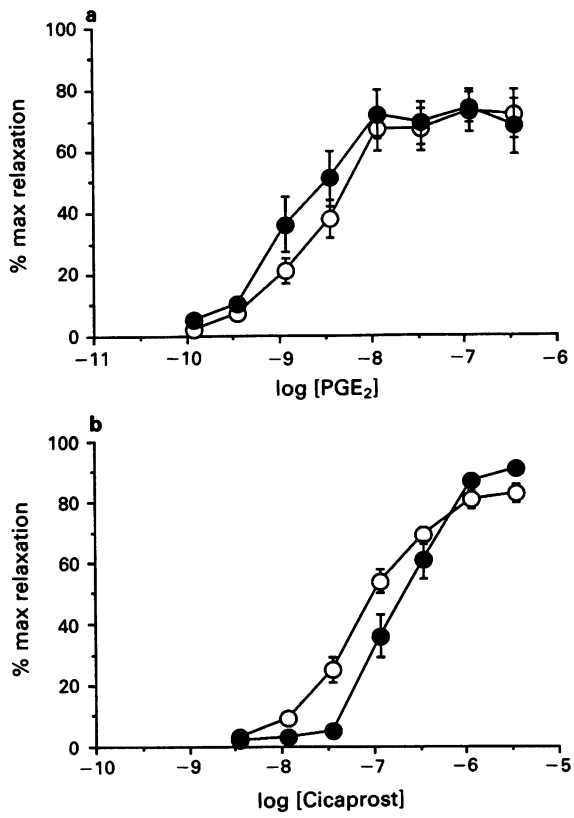


Figure 6 The effect of indomethacin on concentration-relaxation response curves to prostanoids in 10 μM noradrenaline: 1 μM indomethacin (●) and control (○). (a) Prostaglandin E_2 (PGE_2): comparing 1 μM indomethacin and control, there was no difference in either pEC_{50} ($-\log_{10}$ M, 8.71 [0.16], $n = 8$, and 8.50 [0.10], $n = 8$, respectively, $P = 0.30$) or MRR (% maximum relaxation, 76.1 [5.6] and 74.5 [5.5], respectively, $P = 0.39$). (b) Cicaprost: the pEC_{50} was significantly lower in 1 μM indomethacin compared with control (6.78 [0.10], $n = 8$, vs 7.20 [0.07], $n = 8$, respectively, $P = 0.0053$) whereas the MRR was significantly elevated in indomethacin (88.8 [1.6] and 81.3 [2.5], respectively, $P = 0.028$).

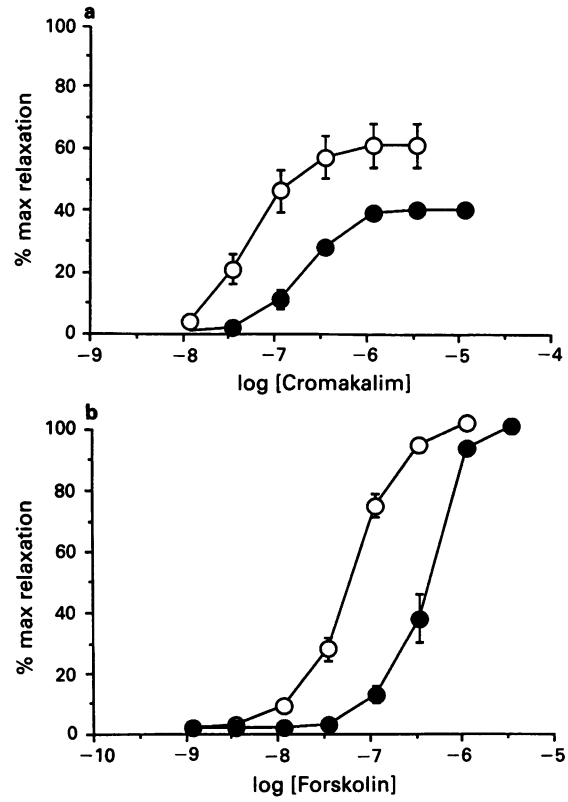


Figure 7 The effect of indomethacin on concentration-relaxation response curves to non-prostanoid vasodilators in 10 μM noradrenaline: 1 μM indomethacin (●) and control (○). (a) Cromakalim: the pEC_{50} ($-\log_{10}$ M) was significantly lower in 1 μM indomethacin compared with control (6.75 [0.04], $n = 4$ vs 7.30 [0.03], $n = 4$, respectively, $P = 0.0001$) as was the MRR (% maximum relaxation, 39.2 [1.5] and 59.8 [6.9], respectively, $P = 0.061$). (b) Forskolin: the pEC_{50} was significantly lower in 1 μM indomethacin compared with control (6.43 [0.07], $n = 8$, vs 7.25 [0.08], $n = 8$, respectively, $P = 0.0001$). Forskolin caused complete relaxation both in the presence and absence of indomethacin.

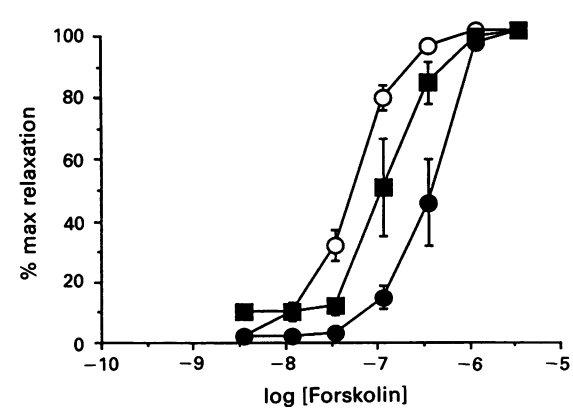


Figure 8 Partial reversal of the effect of indomethacin on forskolin by prostaglandin E_2 (PGE_2). Control response in absence of indomethacin and PGE_2 (○, $n = 8$). Subsequent response in 1 μM indomethacin (●, $n = 4$) and 1 μM indomethacin + 0.3 nM PGE_2 (■, $n = 4$). The change in pEC_{50} ($-\log_{10}$ M) from the control response was significantly less in the presence of 0.3 nM PGE_2 than in its absence (−0.47 [0.06] vs −0.78 [0.09], respectively, $P = 0.034$).

the sensitivity of the ductus to the effects of PGE_2 is explained by elimination of endogenous PGE_2.

This hypothesis was further tested by raising tone with 10 μM noradrenaline and obtaining a CRRC to PGE_2 (see Figure 5 for typical response). The absence of a major inhibitory effect of endogenous PGE_2 is indicated by the fact that the extent of precontraction was the same in the presence and absence of indomethacin. Under these circumstances, indomethacin had no effect on the sensitivity or MRR to PGE_2 (Figure 6), which provided further support for our model of the effect of indomethacin.

This hypothesis predicts that the effects of indomethacin on the sensitivity of the ductus will be increased in situations where endogenous PGE_2 synthesis is stimulated. Elevated oxygen tension massively stimulates ductal synthesis of PGE_2 (Clyman *et al.*, 1980a). Coceani *et al.* (1975), looking at similar changes in oxygen tension, found that indomethacin only increased sensitivity to PGE_2 in elevated oxygen tension. We conclude, therefore, that indomethacin potentiates the response to PGE_2 primarily by eliminating the effects of endogenous PGE_2. Any study of the effects of prostaglandin synthesis inhibitors on the response of the ductus to exogenous prostaglandins will have to take this effect into account before postulating effects of indomethacin on the PGE_2 receptor-effector mechanism.

Both in the presence and absence of indomethacin, PGE_2 had complex effects on the MCR (% control maximum) to noradrenaline. With indomethacin present (Figure 3b), low concentrations of PGE_2 (<0.1 nM) were associated with a decreased MCR to noradrenaline. This was probably due to the fact that the noradrenaline and indomethacin contractions are not additive (as indicated by the fact that the active tone present in 10 μM noradrenaline was the same in the presence and absence of 1 μM indomethacin). By eliminating some of the indomethacin-induced tone, moderate concentrations of PGE_2 (0.1 to 10 nM) actually increased the MCR. This effect was overcome by a direct inhibitory effect on the response to noradrenaline of higher concentrations of PGE_2. The effects of PGE_2 on the MCR to noradrenaline in the absence of indomethacin (Figure 2b) were more difficult to explain. PGE_2 caused a small, but significant increase in MCR across the range 0.1 to 10 nM. This may have been due, again, to elimination of tone. Alternatively, we have some evidence for the existence of a contractile receptor for PGE_2 on the ductus (Smith & McGrath, unpublished data) which might explain this stimulatory effect. Whatever the mechanism, this effect was overcome by a direct inhibitory action in high concentrations of PGE_2.

While indomethacin had no effect on the response of the ductus to PGE_2 in the presence of 10 μM noradrenaline, it did have effects on the response of the ductus to other vasodilators (Figures 6b and 7). In the absence of indomethacin, the sensitivity of the ductus to cicaprost (a stable prostacyclin mimetic [Armstrong *et al.*, 1989]), forskolin (a direct activator of adenyl cyclase [Seamon & Daly, 1981]) and cromakalim (an activator of ATP-sensitive potassium channels [Bray *et al.*, 1991]) was increased. This was probably due to synergy between these exogenous dilators and endogenous PGE_2. We tested this hypothesis by comparing the effect of indomethacin on the response to forskolin in the presence and absence of 0.3 nM PGE_2 (Figure 8). Addition of this small concentration of PGE_2 reversed somewhat the effect of indomethacin. We conclude, therefore, that the ductus was more sensitive to exogenous vasodilators, other than PGE_2, in the absence of indomethacin and that this was due to a potentiating effect of endogenous PGE_2.

Converting the data in Figures 6 and 7 into molar concentrations, PGE_2 was about 85 times more potent than cicaprost in the presence of indomethacin but only 20 times more potent than cicaprost in its absence. The greater the contribution of endogenous PGE_2, the more potent cicaprost will become when compared with PGE_2 since (1) endogenous PGE_2 will increase the vessel's sensitivity to cicaprost; and, (2) endogenous PGE_2 will decrease the vessel's sensitivity to exogenous PGE_2. The above data were obtained with 10 μM noradrenaline which minimized the effects of endogenous PGE_2. The potency of cicaprost compared with PGE_2 may be increased in situations of lesser constriction of ductal smooth muscle, as the contribution of endogenous PGE_2 will further decrease the vessel's response to exogenous PGE_2. Furthermore, given that the ductus is also exposed to circulating PGE_2 *in vivo* (indeed it has been suggested that this is a major source of PGE_2 to which the vessel is exposed [Clyman *et al.*, 1980a]) this suggests that cicaprost may be even more potent relative to PGE_2 *in vivo* than in the present study.

Previous comparisons of the relative efficacy of different prostaglandins as dilators of the ductus, both *in vitro* (Clyman *et al.*, 1978; Coceani *et al.*, 1978) and *in vivo* (Friedman *et al.*, 1983) have been in the presence of indomethacin and it was on the basis of these studies that PGE_1 was selected to be used to maintain artificially ductal patency in neonates with ductus-dependent circulation. It seems likely, therefore, that comparing the effects of different prostaglandins in the presence of indomethacin as a guide to their potency in the human infant with ductus-dependent circulation has led to two sources of error: (1) the sensitivity of the vessel to PGE_1 was overestimated as the effects of endogenous PGE_2 have been eliminated; and, (2) the sensitivity of the vessel to prostacyclin was underestimated, due to the absence of its potentiation by endogenous PGE_2. The extent of this 'error' will depend on the concentration of endogenous PGE_2 to which the smooth muscle of the ductus is exposed *in vivo*. We propose that the relative potencies of ductal vasodilators needs to be re-evaluated in better models of the physiological and clinical states.

The effect of indomethacin on the MRR (% maximum relaxation) to dilators was less straightforward. Indomethacin increased the MRR to cicaprost, decreased the MRR to cromakalim and had no effect on the MRR to forskolin. The MRR to forskolin was unchanged by indomethacin as it caused complete relaxation whether endogenous PGE_2 was present or not. The decrease in MRR to cromakalim in the presence of indomethacin was probably due to loss of synergy with endogenous PGE_2. Endogenous PGE_2 potentiated the sensitivity to cicaprost (as indicated by the effect of indomethacin). If it had also potentiated its MRR, indomethacin would have decreased rather than increased the MRR to cicaprost. The effect of indomethacin on the MRR to cicaprost cannot, therefore, be explained by eliminating the effects of endogenous PGE_2, but it could be explained by the elimination of endogenous prostacyclin. The ductus arteriosus actually synthesizes more than 10 times more prostacyclin than PGE_2 (Terragno *et al.*, 1977). If endogenous prostacyclin inhibited the contraction to 10 μM noradrenaline, indomethacin would be expected to increase the maximum relaxation to a prostacyclin-mimetic by a similar reasoning as developed above for the effect of indomethacin on the response to PGE_2.

In summary, indomethacin increased the sensitivity of the ductus arteriosus to PGE_2, decreased its sensitivity to cicaprost, cromakalim and forskolin, and decreased its maximum response to cromakalim. All these effects can be explained in terms of eliminating endogenous PGE_2. In addition, indomethacin increased the maximum response to cicaprost, which could be explained by elimination of endogenous prostacyclin, but not PGE_2.

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References

ARMSTRONG, R.A., LAWRENCE, R.A., JONES, R.L., WILSON, N.H. & COLLIER, A. (1989). Functional and ligand binding studies suggest heterogeneity of platelet prostacyclin receptors. *Br. J. Pharmacol.*, **97**, 657–668.

BRAY, K.M., WESTON, A.H., DUTY, S., NEWGREEN, D.T., LONGMORE, J., EDWARDS, G. & BROWN, T.J. (1991). Differences between the effects of cromakalim and nifedipine on agonist-induced responses in rabbit aorta. *Br. J. Pharmacol.*, **102**, 337–344.

CLYMAN, R.I. (1987). Ductus arteriosus: current theories of prenatal and postnatal regulation. *Semin. Perinatol.*, **11**, 64–71.

CLYMAN, R.I., MAURAY, F., DEMERS, L.M., RUDOLPH, A.M. & ROMAN, C. (1980a). Does oxygen regulate prostaglandin-induced relaxation in the lamb ductus arteriosus? *Prostaglandins*, **19**, 489–498.

CLYMAN, R.I., MAURAY, F., ROMAN, C. & RUDOLPH, A.M. (1978). PGE_2 is a more potent dilator of the lamb ductus arteriosus than is either PGI_2 or 6 keto $PGF_{1\alpha}$. *Prostaglandins*, **16**, 259–264.

CLYMAN, R.I., MAURAY, F., ROMAN, C., RUDOLPH, A.M. & HEYMANN, M.A. (1980b). Circulating prostaglandin E_2 concentrations and patent ductus arteriosus in fetal and neonatal lambs. *J. Pediatr.*, **97**, 455–461.

COCEANI, F., BODACH, E., WHITE, E., BISHAI, I. & OLLEY, P.M. (1978). Prostaglandin I_2 is less relaxant than prostaglandin E_2 on the lamb ductus arteriosus. *Prostaglandins*, **15**, 551–556.

COCEANI, F., HUHTANEN, D., HAMILTON, N.C., BISHAI, I. & OLLEY, P.M. (1986). Involvement of intramural prostaglandin E_2 in prenatal patency of the lamb ductus arteriosus. *Can. J. Physiol. Pharmacol.*, **64**, 737–744.

COCEANI, F., OLLEY, P.M. & BODACH, E. (1975). Lamb ductus arteriosus: effect of prostaglandin synthesis inhibitors on the muscle tone and the response to prostaglandin E_2. *Prostaglandins*, **9**, 299–308.

DRAYTON, M.R. & SKIDMORE, R. (1987). Ductus arteriosus blood flow during the first 48 hours of life. *Arch. Dis. Child.*, **62**, 1030–1034.

FRIEDMAN, W.F., PRINTZ, M.P., KIRKPATRICK, S.E. & HOSKINS, E.J. (1983). The vasoactivity of the fetal lamb ductus arteriosus studied *in utero*. *Pediatr. Res.*, **17**, 331–337.

GERSONY, W.M. (1986). Patent ductus arteriosus in the neonate. *Pediatr. Clin. North Am.*, **33**, 545–560.

HEYMANN, M.A. & RUDOLPH, A.M. (1975). Control of the ductus arteriosus. *Physiol. Rev.*, **55**, 62–78.

SEAMON, K.B. & DALY, J.W. (1981). Forskolin: a unique diterpene activator of cyclic AMP activating system. *J. Cyclic. Nucleotide Res.*, **7**, 210–224.

SMITH, G.C.S. & MCGRATH, J.C. (1988). Indomethacin, but not oxygen tension, affects the sensitivity of isolated neonatal rabbit ductus arteriosus, but not aorta, to noradrenaline. *Cardiovasc. Res.*, **22**, 910–915.

SMITH, G.C.S. & MCGRATH, J.C. (1991). Prostaglandin E_2 and fetal oxygen tension synergistically inhibit response of isolated fetal rabbit ductus arteriosus to norepinephrine. *J. Cardiovasc. Pharmacol.*, **17**, 861–866.

SMITH, G.C.S. & MCGRATH, J.C. (1993). Characterisation of the effect of oxygen tension on the response of the fetal rabbit ductus arteriosus to vasodilators. *Cardiovasc. Res.*, **27**, 2205–2211.

TERRAGNO, N.A., TERRAGNO, A., MCGIFF, J.L. & RODRIGUEZ, D.J. (1977). Synthesis of prostaglandins by the ductus arteriosus of the bovine fetus. *Prostaglandins*, **14**, 721–727.

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Coupling of a transfected human brain A_1 adenosine receptor in CHO-K1 cells to calcium mobilisation via a pertussis toxin-sensitive mechanism

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1 The presence of A_1 adenosine receptors in CHO-K1 cells transfected with the human brain A_1 sequence was confirmed by ligand binding studies using 8-cyclopentyl-[^{3}H] 1,3-dipropylxanthine ([^{3}H]-DPCPX).

2 Alterations in intracellular calcium ($[Ca^{2+}]_i$) were measured with the calcium-sensitive dye, fura-2.

3 N^6-cyclopentyladenosine (CPA), the selective A_1 agonist, and 5'-N-ethylcarboxaminoadenosine (NECA), a relatively non-selective adenosine receptor agonist, elicited rapid, biphasic increases in $[Ca^{2+}]_i$ which involved both mobilisation from intracellular stores and calcium entry.

4 The calcium response to CPA was significantly inhibited by the selective A_1 antagonist DPCPX. The non-selective adenosine receptor, xanthine amino congener (XAC), was less potent.

5 The calcium response to CPA was completely prevented by pretreatment of the cells with pertussis toxin implicating the involvement of G_i in the receptor-mediated response.

6 In summary, we present evidence for the coupling of transfected human brain A_1 adenosine receptors in CHO-K1 cells to mobilisation of $[Ca^{2+}]_i$ via a pertussis toxin-sensitive G protein.

Keywords: A_1 adenosine receptor; transfection; intracellular calcium; pertussis toxin; fura-2

Introduction

Adenosine receptors have been divided into three major subtypes (A_1, A_2 and A_3) on the basis of agonist structure-activity relationships and the use of a range of xanthine and non-xanthine antagonists (Daly *et al.*, 1986; Bruns *et al.*, 1987; Jacobson *et al.*, 1992; van Galen *et al.*, 1992; Zhou *et al.*, 1992; Carruthers & Fozzard, 1993; Gurden *et al.*, 1993). The A_2-adenosine receptor has been further divided into high and low affinity subtypes, A_{2a} and A_{2b} through the use of the A_{2a}-selective agonist, CGS 21680 (Daly *et al.*, 1983; Bruns *et al.*, 1986; Abbracchio *et al.*, 1993). A_2 adenosine receptors are positively coupled to adenylyl cyclase via a G_s-protein, while A_1 adenosine receptors are generally negatively linked to adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation via a pertussis toxin-sensitive G-protein (Van Calker *et al.*, 1978; Gerwins & Fredholm, 1992). Preliminary data obtained with cells expressing cDNA for the putative A_3-adenosine receptor indicate that this receptor may also regulate cyclic AMP formation in a negative fashion (Zhou *et al.*, 1992).

The molecuar sequences for A_1 and A_{2a} receptors have been cloned from a variety of sources, including dog (Maenhaut *et al.*, 1990; Libert *et al.*, 1991), rat (Mahan *et al.*, 1991; Chern *et al.*, 1992) and man (Furlong *et al.*, 1992; Townsend-Nicholson & Shine, 1992). Putative A_{2b} receptors have also been cloned from rat (Stehle *et al.*, 1992) and human (Pierce *et al.*, 1992) tissues although the lack of a selective ligand for the A_{2b} subtype makes conclusive identification difficult.

The A_1 adenosine receptor is thought to be a promiscuous receptor in that it is reported to couple to inhibition of adenylyl cyclase activity, modulation of ion channels and modulation of phosphoinositide turnover (van Galen *et al.*, 1992). For example, in the hamster vas deferens smooth muscle cell line, DDT_1 MF-2, A_1 receptors couple to inhibition of adenylyl cyclase activity (Gerwins *et al.*, 1990), stimulate phosphoinositide turnover (White *et al.*, 1992) and mobilise intracellular calcium ($[Ca^{2+}]_i$) (Dickenson & Hill, 1993a) via a pertussis toxin-sensitive G-protein (Dickenson & Hill, 1993b).

The human brain A_1 adenosine receptor expressed in CHO-K1 cells has been reported to inhibit forskolin-stimulated cyclic AMP accumulation (Townsend-Nicholson & Shine, 1992). The aim of this investigation was to determine whether this transfected receptor was also able to stimulate mobilisation of $[Ca^{2+}]_i$.

Methods

Cell culture

CHO-NAX2 (CHO-K1 cells transfected with the human brain A_1 adenosine receptor sequence, Townsend-Nicholson & Shine, 1992) cells were cultured in 75 cm^2 flasks in Dulbecco's Modified Eagles Medium/Nutrient Mix F12 (1:1, with glutamine) containing 10% foetal calf serum without antibiotics.

Calcium measurements

$[Ca^{2+}]_i$ was measured as previously described (Iredale *et al.*, 1992). Briefly, the monolayers from two near-confluent flasks were detached with trypsin/EDTA (trypsin, 0.5 g; EDTA 0.2 g and NaCl, 0.85 g per litre) and resuspended in a simple saline HEPES buffer (composition mM: $CaCl_2$ 2, NaCl 145, glucose 10, KCl 5, $MgSO_4$ 1 and HEPES 10, pH 7.45). This was followed by incubation with fura-2 acetoxy methyl ester (5 μM; Calbiochem/Novobiochem) at 37°C (in the presence of 5% foetal calf serum) for 20 min and for a further 5 min following a three fold dilution (to ensure maximum hydrolysis of ester to the acid form). At the end of this loading period, excess dye was removed by centrifugation, the cells resuspended in fresh buffer (without serum), and left at room temperature until use. Each time course was preceded by a rapid spin in a microcentrifuge followed by resuspension in fresh buffer (2 ml).

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All experiments were carried out using a Perkin Elmer LS 50 Spectrometer, with excitation ratioing between 340 and 380 nm, recording at 500 nm. The time course for each calcium measurement was 200 s with drugs added in 10 μl aliquots.

Calibration

At the end of each time course, ionomycin (20 μM; Calbiochem/Novabiochem) was added followed by EGTA (6.25 mM, pH greater than 8.5) in order to calculate R_{max} and R_{min}. Autofluorescence was determined in a separate cuvette following the addition of manganese (5 mM) after the ionophore ionomycin (20 μM). From these values and those obtained with fura-2 free acid, $[Ca^{2+}]_i$ was calculated according to the method of Grynkiewicz *et al.* (1985).

Pertussis toxin experiments

Culture media was removed from two confluent flasks and replaced with fresh media containing pertussis toxin (200 ng ml^{-1}). The cells were incubated for 4 h before experiments were carried out.

[^{3}H]-DPCPX binding

Ligand binding analysis in CHO-K1 cells was performed as previously described (Iredale *et al.*, 1993). Cells from 1 or 2 confluent 175 cm^2 flasks were detached with PBS/EDTA (composition mM: NaCl 138, KCl 2.7, $Na_2HPO_4.2H_2O$ 12.9, KH_2PO_4 1.5, pH 7.45). Membranes were prepared by resuspending the cells in ice-cold Tris-EDTA buffer (50 mM: 1 mM), followed by homogenization in a glass-teflon homogenizer (approximately 7 strokes) and centrifugation at 20,000 *g* for 15 min. The resulting pellet was resuspended in 500 μl of Tris-EDTA buffer. Samples (10 μl) were incubated in the absence (total binding) or presence (non-specific binding) of theophylline (5 mM) with increasing concentrations of 8-cyclopentyl-[^{3}H] 1,3-dipropylxanthine ([^{3}H]-DPCPX) in Tris-EDTA buffer containing adenosine deaminase (1 unit ml^{-1}) and Triton X-100 (0.01%) for 90 min at room temperature (assay volume of 200 μl). The incubation was stopped by rapid filtration and washing with ice-cold Tris-EDTA buffer (3 times, approximate volume 10 ml) over Whatman GF/B filters (pre-soaked for 1 h in 0.3% polyethylenemine to reduce non-specific binding) using a Brandel MR24 cell harvester. The bound [^{3}H]-DPCPX was quantified by liquid scintillation spectrometry.

An estimate of the protein content of each sample was made using the method of Bradford (1976) following digestion in NaOH (0.5 M).

Statistics and data analysis

EC_{50} and IC_{50} (concentrations of drug producing 50% of maximal stimulation or inhibition) values were obtained by computer-assisted curve fitting by use of the computer programme InPlot (GraphPad, California, U.S.A.). Curves were also fitted to radioligand binding data using the same programme. Calcium data were captured using the ICBC programme supplied by Perkin-Elmer and imported to the graphics programme Sigma-Plot (Jandel, California, U.S.A.).

Statistical significance was determined by Student's unpaired *t* test ($P<0.05$ was considered statistically significant). The *n* in the text refers to the number of separate experiments.

Materials

CHO-NAX2 cells were a generous gift from Dr Andrea Townsend-Nicholson (Garvan Institute, Sydney, Australia). Cell culture flasks were obtained from Costar with Dulbecco's Modified Eagles Medium/Nutrient Mix F12 and foetal calf serum from Biological Industries (Glasgow, U.K.). Fura-2 AM and ionomycin were from Calbiochem/Novabiochem (Nottingham, U.K.). 8-Cyclopentyl-1,3-dipropylxanthine, xanthine amino congener, 5'-N-ethylcarboxaminoadenosine, N^6-cyclopentyladenosine were supplied by RBI/Sexmat (Herts, U.K.), adenosine deaminase, theophylline, nickel chloride and Triton X-100 by Sigma (Poole, U.K.), 8-cyclopentyl-[^{3}H] 1,3-dipropylxanthine by NEN Dupont (Stevenage, U.K.) and pertussis toxin and trypsin/EDTA by Gibco (Uxbridge, U.K.).

Results

[^{3}H]-DPCPX binding

Saturation assays using increasing concentrations of [^{3}H]-DPCPX to membranes of CHO-NAX2 cells revealed a K_d of 1.2 ± 0.3 nM ($n = 3$) and a B_{max} of 200 ± 44 fmol mg^{-1} protein ($n = 3$; Figure 1).

The calcium response to N^6-cyclopentyladenosine

Addition of N^6-cyclopentyladenosine (CPA; 100 nM) to populations of fura-2-loaded CHO NAX2 cells resulted in a rapid increase in $[Ca^{2+}]_i$ of 274 ± 39 nM above basal from a resting value of 103 ± 14 nM ($n = 5$; Figure 2a). This initial rise in $[Ca^{2+}]_i$ was accompanied by a secondary, maintained response (111 ± 17 nM above basal, measured at 120 s; $n = 5$; $P<0.01$ compared with basal) which was abolished by omission of extracellular calcium or the presence of nickel ions (Ni^{2+} 1 mM; Table 1 and Figure 2). The CPA response was concentration-dependent with an EC_{50} of 15 ± 1 nM ($n = 3$; Figure 2). A similarly-sized maximal increase in $[Ca^{2+}]_i$ was observed after addition of 5'-N-ethylcarboxaminoadenosine (NECA; Figure 3b and data not shown; EC_{50} 7 ± 2 nM, $n = 3$).

Antagonism of the adenosine receptor response

In order to verify the adenosine receptor subtype involved, the effects of antagonists on the observed increases in $[Ca^{2+}]_i$ were assessed. The cells were pre-incubated with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 25 nM) and xanthine amino congener (XAC; 25 nM) for 10 min. Both agents significantly inhibited the calcium response to a sub-maximal concentration of CPA (60 nM), however DPCPX was more effective (39 ± 3%, 66 ± 6% ($n = 5$) of controls respectively; Figure 4a). Further, a full concentration-response inhibition

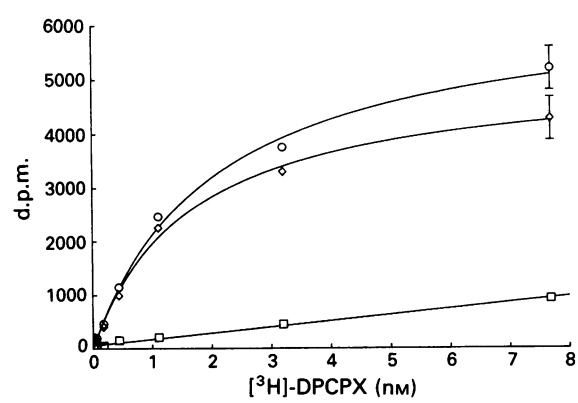


Figure 1 Binding of 8-cyclopentyl-[^{3}H] 1,3-dipropylxanthine ([^{3}H]-DPCPX) to CHO-NAX2 membranes. The graph shows saturation isotherms (total (○), non-specific (□) and specific (◇) binding shown). Data points represent single experiments carried out in quadruplicate. The experiment was carried out twice more with similar results.

curve revealed an IC_{50} for DPCPX of 26 ± 1 nM ($n = 3$) (Figure 4b). The K_d for this response, as calculated by the method of White *et al.* (1992) was 2.5 ± 0.2 nM ($n = 3$).

Pertussis toxin sensitivity

Pretreatment of the cells with pertussis toxin (200 ng ml^{-1} for 4 h) completely prevented the response to CPA (100 nM; Figure 5a; $n = 6$). However, the calcium rise following stimulation of the endogenous P_{2U} purinoceptor (Iredale & Hill, 1993) was unaffected (100 ± 1% of controls, $n = 6$; Figure 5b).

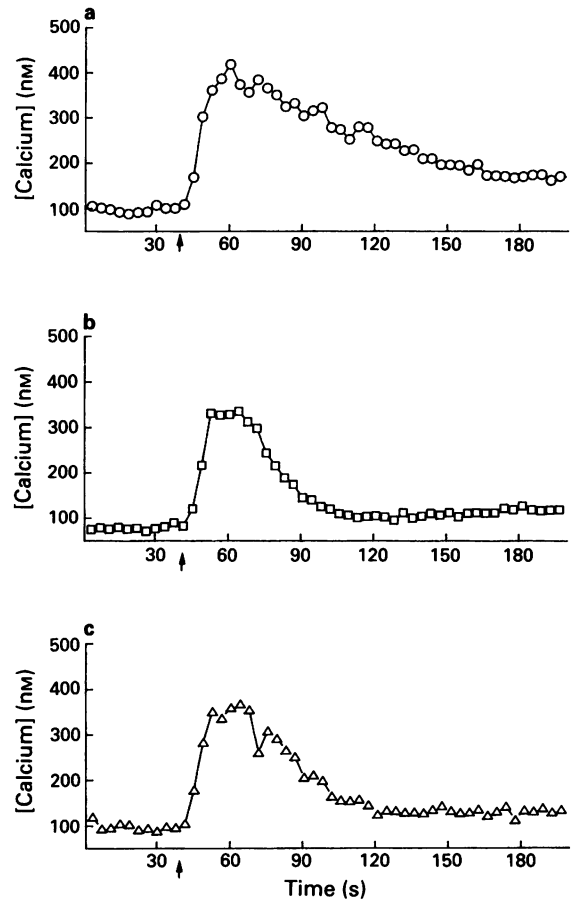


Figure 2 The effect of N^6-cyclopentyladenosine (CPA, 100 nM) on fura-2-loaded CHO-NAX2 cells in the presence (a) and absence of (b) 2 mM extracellular calcium and in the presence of the inorganic calcium channel blocker, Ni^{2+} (1 mM; c). Each graph shown is typical of three others.

Table 1 The effect of the omission of extracellular calcium and Ni^{2+} on the responses to N^6-cyclopentyladenosine (CPA)

Agonist		*Basal*	*Peak*	*120 s*
CPA	+ Ca^{2+}	103 ± 14	377 ± 51	214 ± 28**
	− Ca^{2+}	77 ± 7	320 ± 52	94 ± 8
	+ Ni^{2+}	80 ± 9	403 ± 44	101 ± 14

The data are the $[Ca^{2+}]_i$ ($n = 5$; except Ni^{2+}, $n = 4$) of cells challenged with CPA (100 nM) in the presence and absence of extracellular calcium and following pre-incubation for 5 min with the inorganic calcium channel blocker, Ni^{2+} (1 mM). Those responses still significantly above basal at 120 s are shown with asterisks (**$P < 0.01$, Student's unpaired t test).

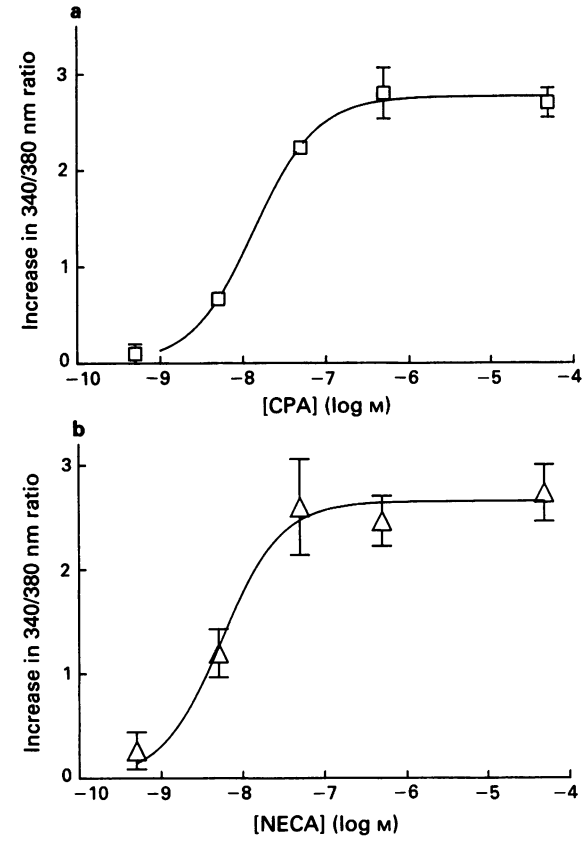


Figure 3 Concentration-response curves to N^6-cyclopentyladenosine (CPA, a) and 5'-N-ethylcarboxaminoadenosine (NECA, b). Data are means ± s.e.mean of three separate determinations.

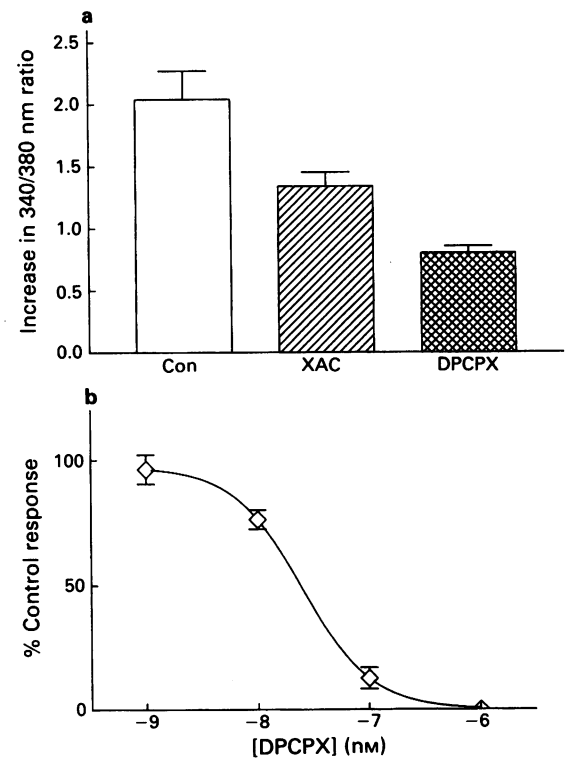


Figure 4 Panel (a) shows the effects of the adenosine antagonists, xanthine amino congener (XAC, 25 nM) and 8-cyclopentyl 1,3-dipropylxanthine (DPCPX, 25 nM), on the calcium response to N^6-cyclopentyladenosine (CPA, 60 nM). The cells were pre-incubated with each antagonist for 10 min prior to stimulation with CPA. Panel (b) shows the concentration-response relationship for DPCPX. Aliquots of cells were pre-incubated for 10 min with increasing concentrations of DPCPX and then challenged with CPA (100 nM). Data are means ± s.e.mean of three separate determinations.

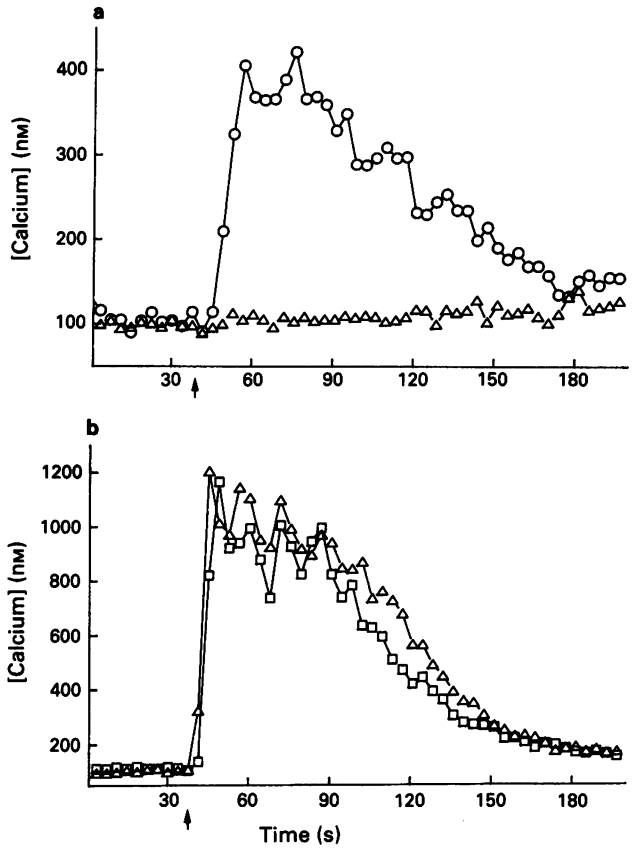


Figure 5 The effect of pertussis toxin on the calcium responses to (a) N^6-cyclopentyladenosine (CPA, 100 nM): (○) control; (△) pertussis toxin pretreated and (b) ATP (100 μM) (□) control; (△) pertussis toxin pretreated. The cells were pre-incubated with 200 ng ml^{-1} for 4 h as described in the text. Each graph shown is typical of five others.

Discussion

The present paper describes the coupling of the human brain A_1 adenosine receptor to phosphoinositide turnover and calcium mobilisation in CHO-K1 cells transfected with the A_1 adenosine receptor cDNA.

Saturation assays using the highly selective A_1 adenosine receptor radioligand, [^{3}H]-DPCPX (van Galen *et al.*, 1992) confirmed the presence of the A_1 receptor subtype in membranes derived from CHO-NAX2 cells. The determined K_d is in close agreement with the value of 0.96 nM reported by Townsend-Nicholson & Shine (1992) in the same cell line and also with the native human brain A_1 adenosine receptor (Ferkany *et al.*, 1986).

Stimulation of populations of fura-2-loaded cells revealed a concentration-dependent increase in $[Ca^{2+}]_i$ following stimulation with both CPA and NECA suggesting receptor coupling to phosphoinositide C. Both agonists showed similar potencies. The responses were biphasic, with an initial rapid increase in $[Ca^{2+}]_i$ which returned to a secondary, maintained plateau (data for NECA not shown). Results from a number of different cell lines have revealed that some agonist responses are biphasic, involving an initial transient release of calcium from intracellular stores followed by a secondary sustained influx across the plasma membranes resulting in a maintained plateau (Baird *et al.*, 1989; Jacob *et al.*, 1990). This plateau is maintained as long as the agonist occupies the receptor. The characteristics of the responses to both CPA and NECA are consistent with this theory. Furthermore, omission of calcium, or inclusion of the inorganic calcium channel blocker Ni^{2+} in the extracellular medium, prevented the formation of the plateau phase, providing further evidence that influx of calcium forms part of the overall response.

The binding data clearly indicate the presence of an A_1 adenosine receptor in CHO-NAX2 cells. The fact that the observed increases in $[Ca^{2+}]_i$ are a consequence of stimulation of this receptor can be deduced mainly from the effects of the antagonists used. The similar potency observed for CPA and NECA is a further characteristic of the human brain A_1 adenosine receptor identified by radioligand binding experiments (Ferkany *et al.*, 1986). The response to CPA (a selective A_1 receptor agonist; van Galen *et al.*, 1992) was potently inhibited following pre-incubation with DPCPX, a highly selective A_1 adenosine receptor antagonist (van Galen *et al.*, 1992). XAC, a high affinity but relatively non-selective adenosine receptor antagonist (van Galen, 1992) was less potent than DPCPX at inhibiting the CPA response (Figure 3). These data clearly suggest that the observed increase in $[Ca^{2+}]_i$ is a consequence of stimulation of the transfected A_1 adenosine receptor, most likely via stimulation of phosphoinositidase C as has been reported in DDT_1 MF2 cells (White *et al.*, 1992). In human brain slices, adenosine receptor activation fails to elicit phosphoinositide turnover (Kendall & Firth, 1990). However, the presence of adenosine analogues leads to an inhibition of histamine-stimulated [^{3}H]-inositol phosphates accumulation, similar to the phenomenon observed in mouse cerebral cortical slices (Kendall & Hill, 1988). The receptor which mediates this modulatory response appears to be an A_1 adenosine receptor. Thus, the current study implies that a transfected receptor couples to a second messenger system to which it fails to couple under native conditions. Examples of A_1 adenosine receptors which couple to phosphoinositide turnover under native conditions are relatively sparse, e.g. in guinea-pig myometrium (Schiemann & Buxton, 1991) and DDT1 MF-2 cells (White *et al.*, 1992). There are however, other instances of the promiscuity of transfected receptors (Milligan, 1993).

We have previously shown that CHO-K1 cells contain an endogenous P_{2U}-purinoceptor (Iredale & Hill, 1993) which is coupled to increases in $[Ca^{2+}]_i$ (mobilisation and entry). Pretreatment of the cells with pertussis toxin inhibited the calcium response to CPA but had no effect on the response to ATP. These data suggest that the transfected A_1 adenosine receptor is not coupled to the same G protein as the P_{2U}-purinoceptor. The lack of pertussis toxin-sensitivity of the latter receptor response suggests that it belongs to the G_q-family. It has previously been shown that the transfected A_1 adenosine receptor in CHO-NAX2 cells is negatively linked to adenylyl cyclase (Townsend-Nicholson & Shine, 1992). One possibility is, therefore, that the G protein involved is coupled to both adenylyl cyclase and phosphoinositidase C, as has been suggested for the A_1 adenosine receptor found in DDT_1 MF2 cells (Gerwins & Fredholm, 1992). This hypothesis would certainly explain the sensitivity to pertussis toxin. One interesting possibility is that the receptor-mediated calcium response might be as a consequence of G_i-derived $\beta\gamma$ subunits activating phosphoinositidase C and not due to direct coupling of the A_1 adenosine receptor to the enzyme (Dickenson & Hill, 1993b). $\beta\gamma$ subunits have been shown to stimulate phosphoinositidase C in cytosolic fractions obtained from HL-60 cells (Camps *et al.*, 1992) and therefore this remains a distinct possibility.

In summary, we have shown that the A_1 adenosine receptor transfected into CHO-K1 cells couples to phosphoinositidase C and is therefore linked to mobilisation of $[Ca^{2+}]_i$. Agonist stimulation is pertussis toxin-sensitive suggesting the involvement of G_i in the mechanism, although whether the enzyme is directly coupled or is activated by $\beta\gamma$ subunits remains to be elucidated.

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References

ABBRACCHIO, M.P., CATTABENI, F., FREDHOLM, B.B. & WILLIAMS, M. (1993). Purinoceptor nomenclature: a status report. *Drug Dev. Res.*, **28**, 207–213.

BAIRD, J.G., LAMBERT, D.G., MCBAIN, J. & NAHORSKI, S.R. (1989). Muscarinic receptors coupled to phosphoinositide hydrolysis and elevated cytosolic calcium in a human neuroblastoma cell line SK-N-SH. *Br. J. Pharmacol.*, **98**, 1328.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. *Anal. Biochem.*, **72**, 248–254.

BRUNS, R.F., FERGUS, J.H., BADGER, E.W., BRISTOL, J.A., SANTAY, L.A., HARTMAN, J.D., HAYS, S.J. & HUANG, C.C. (1987). Binding of the A_1-selective adenosine antagonist 8-cyclopentyl-1, 3-dipropylxanthine to rat membranes. *Naunyn-Schmied Arch. Pharmacol.*, **335**, 59–63.

BRUNS, R.F., LU, G.H. & PUGSLEY, T.A. (1986). Characterization of the A2 adenosine receptor labeled by [^{3}H]-NECA in rat striatal membranes. *Mol. Pharmacol.*, **29**, 331–346.

CAMPS, M., HOU, C., SIDIROPOULOS, D., STOCK, J.B., JAKOBS, K.H. & GIERSCHIK, P. (1992). Stimulation of phospholipase C by guanine-nucleotide-binding protein $\beta\gamma$ subunits. *Eur. J. Biochem.*, **206**, 821–831.

CARRUTHERS, A.M. & FOZARD, J.R. (1993). Adenosine A_3 receptors: two into one won't go. *Trends Pharmacol. Sci.*, **14**, 290–291.

CHERN, Y., KING, K., LAI, H.-L. & LAI, H.-T. (1992). Molecular cloning of a novel adenosine receptor gene from rat brain. *Biochem. Biophys. Res. Commun.*, **185**, 304–309.

DALY, J.W., BUTTS-LAMB, P. & PADGETT, W. (1983). Subclasses of adenosine receptors in the central nervous system: interaction with caffeine and related methylxanthines. *Cell. Mol. Neurobiol.*, **3**, 69–80.

DALY, J.W., PADGETT, W., THOMPSON, R.D., KUSACHI, S., BUGNI, W.J. & OLSSON, R.A. (1986). Structure-activity relationships for N^6-substituted adenosines at a brain A_1-adenosine receptor with a comparison to an A_2-adenosine receptor regulating coronary blood flow. *Biochem. Pharmacol.*, **35**, 2467–2481.

DICKENSON, J.M. & HILL, S.J. (1993a). Adenosine A_1-receptor stimulated increases in intracellular calcium in the smooth muscle cell line DDT1MF-2. *Br. J. Pharmacol.*, **108**, 85–92.

DICKENSON, J.M. & HILL, S.J. (1993b). Intracellular cross talk between receptors coupled to phospholipase C via pertussis toxin-sensitive and insensitive G-proteins in DDT_1 MF2 cells. *Br. J. Pharmacol.*, **109**, 719–724.

FERKANY, J.W., VALENTINE, H.L., STONE, G.A. & WILLIAMS, M. (1986). Adenosine A1 receptors in mammalian brain: species differences in their interactions with agonists and antagonists. *Drug Develop. Res.*, **9**, 85–93.

FURLONG, T.J., PIERCE, K.D., SELBIE, L.A. & SHINE, J. (1992). Molecular characterization of a human brain adenosine A_2 receptor. *Mol. Brain Res.*, **15**, 62–66.

GERWINS, P. & FREDHOLM, B.B. (1992). ATP and its metabolite adenosine act synergistically to mobilize intracellular calcium via the formation of inositol 1,4,5-trisphosphate in a smooth muscle cell line. *J. Biol. Chem.*, **267**, 16081–16087.

GERWINS, P., NORDSTEDT, C. & FREDHOLM, B.B. (1990). Characterization of adenosine A1 receptors in intact DDT_1 MF2 smooth muscle cells. *Mol. Pharmacol.*, **38**, 660–666.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biochem. Sci.*, **260**, 3440–3450.

GURDEN, M.F., COATES, J., ELLIS, F., EVANS, B., FOSTER, M., HORNBY, E., KENNEDY, I., MARTIN, D.P., STRONG, P., VARDEY, C.J. & WHEELDON, A. (1993). Functional characterization of three adenosine receptor types. *Br. J. Pharmacol.*, **109**, 693–698.

IREDALE, P.A., FUKUI, H. & HILL, S.J. (1993). High, stable expression of the bovine histamine H_1-receptor coupled to $[Ca^{2+}]_i$ mobilisation in CHO-K1 cells. *Biochem. Biophys. Res. Commun.*, **195**, 1153–1436.

IREDALE, P.A. & HILL, S.J. (1993). Increases in intracellular calcium via activation of an endogenous P_2-purinoceptor in cultured CHO-K1 cells. *Br. J. Pharmacol.* (in press).

IREDALE, P.A., MARTIN, K.F., HILL, S.J. & KENDALL, D.A. (1992). Agonist-induced changes in $[Ca^{2+}]_i$ in N1E-115 cells: differential effects of bradykinin and carbachol. *Eur. J. Pharmacol.*, **226**, 163–168.

JACOB, R. (1990). Agonist-stimulated divalent cation entry into single cultured human umbilical vein endothelial cells. *J. Physiol.*, **421**, 55–77.

JACOBSON, K.A., VAN GALEN, P.J.M. & WILLIAMS, M. (1992). Adenosine receptors: pharmacology, structure-activity relationships, and therapeutic potential. *J. Med. Chem.*, **35**, 407–422.

KENDALL, D.A. & FIRTH, J.L. (1990). Inositol phospholipid hydrolysis in human brain; adenosine inhibition of the response to histamine. *Br. J. Pharmacol.*, **100**, 37–40.

KENDALL, D.A. & HILL, S.J. (1988). Adenosine inhibition of histamine-stimulated inositol phospholipid hydrolysis in mouse cerebral cortex. *J. Neurochem.*, **50**, 497–502.

LIBERT, F., SCHIFFMANN, S.N., LEFORT, A., PARMENTIER, M., GERARD, C., DUMONT, J.E., VANDERHAEGHEN, J.-J. & VASSART, G. (1991). The orphan receptor cDNA RDC7 encodes an A1 adenosine receptor. *EMBO J.*, **10**, 1677–1682.

MAENHAUT, C., VAN SANDE, J., LIBERT, F., ABRAMOWICZ, M., PARMENTIER, M., VANDERHAEGEN, J.-J., DUMONT, J.E., VASSART, G. & SCHIFFMANN, S. (1990). RDC8 codes for an adenosine A_2 receptor with physiological constititutive activity. *Biochem. Biophys. Res. Commun.*, **173**, 1169–1178.

MAHAN, L.C., MCVITTIE, L.D., SMYK-RANDALL, E.M., NAKATA, H., MONSMA, F.J., GERFEN, C.R. & SIBLEY, D.R. (1991). Cloning and expression of an A_1 adenosine receptor from rat brain. *Mol. Pharmacol.*, **40**, 1–7.

MILLIGAN, G. (1993). Mechanisms of multifunctional signalling by G protein-linked receptors. *Trends Pharmacol. Sci.*, **14**, 239–244.

PIERCE, K.D., FURLONG, T.J., SELBIE, L.A. & SHINE, J. (1992). Molecular cloning and expression of an adenosine A_{2b} receptor from human brain. *Biochem. Biophys. Res. Commun.*, **187**, 86–93.

SCHIEMANN, W.P. & BUXTON, I.L. (1991). Adenosine A_1-receptor coupling to phosphoinositide metabolism in pregnant guinea pig myometrium. *Am. J. Physiol.*, **261**, E665–E672.

STEHLE, J.H., RIVKEES, S.A., LEE, J.J., WEAVER, D.R., DEEDS, J.D. & REPPERT, S.M. (1992). Molecular cloning and expression of the cDNA for a novel A_2-adenosine receptor subtype. *Mol. Endocrinol.*, **6**, 384–393.

TOWNSEND-NICHOLSON, A. & SHINE, J. (1992). Molecular cloning and characterisation of a human brain A_1-adenosine receptor cDNA. *Mol. Brain Res.*, **16**, 365–370.

VAN CALKER, D., MÜLLER, M. & HAMPRECHT, B. (1979). Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J. Neurochem.*, **33**, 999–1005.

VAN GALEN, P.J.M., STILES, G.L., MICHAELS, G. & JACOBSON, K.A. (1992). Adenosine A_1 and A_2 receptors: structure-function relationships. *Med. Res. Rev.*, **12**, 423–471.

WHITE, T.E., DICKENSON, J.M., ALEXANDER, S.P.H. & HILL, S.J. (1992). Adenosine A_1-receptor stimulation of inositol phospholipid hydrolysis and calcium mobilisation in DDT_1 MF2 cells. *Br. J. Pharmacol.*, **106**, 215–221.

ZHOU, Q.-Y., LI, C., OLAH, M.E., JOHNSON, R.A., STILES, G.L. & CIVELLI, O. (1992). Molecular cloning and characterization of an adenosine receptor: the A_3 adenosine receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 7432–7436.

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Effect of anti-fungal imidazoles on mRNA levels and enzyme activity of inducible nitric oxide synthase

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1 Experiments were performed to examine the effects of anti-fungal imidazole compounds (clotrimazole, econazole and miconazole) on the induction of nitric oxide (NO) synthase and subsequent production of NO in the cultured murine monocyte/macrophage cell line J774 using a specific cDNA probe for inducible NO synthase mRNA and by monitoring nitrite production.

2 Stimulation of J774 cells with lipopolysaccharide (LPS, 10 $\mu g\,ml^{-1}$) resulted in the induction of NO synthase activity as determined by nitrite accumulation in the culture medium (48 ± 3 nmol per 10^6 cells over 24 h). Production of nitrite was inhibited by co-incubation of cells with LPS (10 $\mu g\,ml^{-1}$) and either dexamethasone (10 μM) or N^G-monomethyl-L-arginine (L-NMMA; 0.1 mM), however, only L-NMMA was an effective inhibitor of nitrite production when added after induction of NO synthase had occurred.

3 Co-incubation of J774 cells with LPS (10 $\mu g\,ml^{-1}$) and either clotrimazole, econazole or miconazole (1–10 μM) resulted in a concentration-dependent inhibition of nitrite production over the subsequent 24 h without any evidence for a cytotoxic effect. However, addition of these imidazoles after induction of NO synthase did not inhibit nitrite production.

4 Messenger RNA for inducible NO synthase was not detected in unstimulated J774 cells. Treatment with LPS (10 $\mu g\,ml^{-1}$) for 4 h resulted in significant expression of mRNA for inducible NO synthase which was not altered in the presence of econazole (10 μM) but was reduced significantly by dexamethasone (10 μM).

5 These results demonstrate that anti-fungal imidazoles inhibit the production of nitric oxide by cultured J774 cells by a mechanism which appears to differ from that of dexamethasone and substrate-type inhibitors of NO synthase. Furthermore, the presence of mRNA for NO synthase does not indicate the presence of functionally active NO synthase.

Keywords: Macrophage; lipopolysaccharide; nitric oxide; clotrimazole; econazole; miconazole; imidazoles; anti-fungal agents; dexamethasone; inducible NO synthase

Introduction

Nitric oxide (NO) is a potent and widespread signalling molecule within the cardiovascular, nervous and immune systems (Moncada *et al.*, 1991). In certain tissues, including endothelium and brain, synthesis of NO from L-arginine is catalyzed by the action of a constitutively expressed Ca^{2+}/calmodulin-dependent NO synthase (Palmer & Moncada, 1989; Mayer *et al.*, 1989; Bredt & Snyder, 1990). The NO produced activates guanylate cyclase in target cells. However, in endothelium and many other cells that do not normally synthesize NO (including macrophages), exposure to bacterial endotoxin (lipopolysaccharide, LPS) and/or cytokines, results in expression of a distinct isoform of NO synthase which is often functionally Ca^{2+}/calmodulin-insensitive and makes large amounts of NO (Moncada *et al.*, 1991). In the quantities produced by the inducible enzyme NO is toxic to invading pathogens (Hibbs *et al.*, 1987; Marletta *et al.*, 1988) and certain host cells (Palmer *et al.*, 1992). The two broad groups of NO synthase share certain similarities. One common feature is that they are haemoproteins with homology to cytochrome P450 reductase and possess both oxidase and reductase domains (Bredt *et al.*, 1991; White & Marletta, 1992).

Inhibition of NO synthesis can be achieved in a number of ways. Substrate-based analogues of L-arginine including N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine (L-NOARG) compete with L-arginine for metabolism to NO and inhibit the activity of constitutive and inducible enzymes (Moore *et al.*, 1990; Rees *et al.*, 1989; 1990). Alternatively, the expression of the inducible enzyme may be inhibited by glucocorticoids (e.g. dexamethasone and hydrocortisone; Di Rosa *et al.*, 1990; Radomski *et al.*, 1990) which probably act by inhibiting gene expression or the transcription of inducible NO synthase messenger RNA (mRNA). In addition, by inhibiting protein synthesis (Radomski *et al.*, 1990) or production of essential co-factors such as tetrahydrobiopterin (Gross & Levi, 1992), induction of NO synthesis can be inhibited. Recently it has been reported that anti-fungal imidazoles inhibit the activity of constitutive NO synthase through an effect on Ca^{2+}/calmodulin (Wolff *et al.*, 1993a). However, these compounds also inhibit the activity of mammalian cytochrome *P*450 proteins (Rodrigues *et al.*, 1987) by binding to the haem domain. If this interaction also occurred with NO synthase, anti-fungal imidazoles would be expected to inhibit the activity of the Ca^{2+}/calmodulin-insensitive and sensitive isoforms. In the present study we have examined the effects of anti-fungal imidazoles on inducible NO synthase in the murine macrophage cell line J774 and have sought to identify the mechanism of action of inhibition.

Methods

Cell culture

Cells of the murine monocyte/macrophage line J774 were maintained in continuous culture in tissue-culture flasks (75 cm^2 growth area) in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, 4 mM L-glutamine, penicillin (100 units ml^{-1}) and streptomycin (100 $\mu g\,ml^{-1}$).

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Cells were harvested by gentle scraping and passaged every 3–6 days by dilution of a suspension of the cells 1:10 in fresh medium.

Measurement of nitrite production as an assay of NO release

Production of NO was assayed by measuring the accumulation of nitrite in the culture medium by the Griess reaction (Green *et al.*, 1982; Bogle *et al.*, 1992). J774 cells were seeded into 96-well microtitre plates (10^5 cells per well) and allowed to adhere for 2 h at 37°C following which the medium was removed and replaced with fresh medium containing lipopolysaccharide (LPS, 10 $\mu g\,ml^{-1}$) and either N^G-monomethyl-L-arginine (L-NMMA; 0.1 or 1 mM), dexamethasone (1 or 10 μM) or imidazole compounds (1–10 μM). In some experiments, cells were pre-activated with LPS (10 $\mu g\,ml^{-1}$) for 24 h following which the LPS was removed and cells treated with fresh medium containing either L-NMMA, dexamethasone or anti-fungal imidazoles. Twenty-four hours later an aliquot (100 μl) of the culture medium was removed, mixed with an equal volume of Griess reagent (final concentration: sulphanilamide 1% w/v, napthylethylenediamine dihydrochloride 0.1% w/v and orthophosphoric acid 2.5% v/v) and incubated at room temperature for 10 min. The absorbance was read at 560 nm in a Titertek Multiskan II plate reader (Flow, High Wycombe, U.K.). Nitrite concentration in the medium was determined using sodium nitrite as a standard.

Assays of cell viability

Cell viability was measured either by the ability of the cells to exclude the vital stain trypan blue (0.4% w/v in Dulbecco's phosphate buffered saline of composition, mM: NaCl 138, KCl 2.6, Na_2PO_4 8.1, KH_2PO_4 1.5, pH 7.4) as determined by visual microscopy or by assessment of the ability of the cells to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl) tetrazolium bromide; MTT; 0.5 $mg\,ml^{-1}$) into formazan over a 4 h time period according to the method of Mosmann (1983).

Production of cDNA probe for inducible macrophage NO synthase

J774 cells (10^6 cells) were treated with LPS (10 $\mu g\,ml^{-1}$) for 6 h and poly(A)$^+$-selected mRNA was isolated with a Fast-Track mRNA isolation kit according to the manufacturer's instructions (Invitrogen, San Diego, U.S.A.). The mRNA obtained was used for the synthesis of first strand cDNA using random primers with a commercial kit (First Strand, Pharmacia, Milton Keynes, U.K.). The cDNA was then used as a template in a polymerase chain reaction (conditions: 25 cycles of denaturing, 95°C, 1 min; annealing 65°C, 2 min and polymerization 72°C, 1 min), utilizing oligonucleotide primers designed from the published sequence of murine macrophage inducible NO synthase (Primer 1: base pairs 2476–2498 (sense), Primer 2: base pairs 2969–2947 (antisense); Lyons *et al.*, 1992). The derived product was purified by electrophoresis on a low-melting point agarose gel (1% w/v) and subcloned into a plasmid vector (EcoRV linearized dephosphorylated pBluescript II, Stratagene Ltd, Cambridge, U.K.). Subsequently the plasmid was transfected into competent bacteria (XL Blue-1 *E. coli*, Stratagene) and plasmid DNA prepared from bacterial cultures. The identity of the insert was confirmed by double stranded dideoxy sequencing with a kit (Pharmacia). A 493 base pair fragment was isolated by restriction enzyme digestion (BamHI/HindIII, Northumbria Biologicals, Northumberland, U.K.) and used as a probe in Northern blotting.

Total RNA isolation and analysis

J774 cells (10^6 cells in 3 cm^2 wells) were stimulated for 4 h with either medium alone, or medium plus LPS (10 $\mu g\,ml^{-1}$) or LPS in the presence of either econazole (10 μM) or dexamethasone (10 μM). Total RNA was extracted using RNAzol reagent according to the manufacturer's instructions (Biogenesis Ltd, Bournemouth, U.K.). The RNA samples (10 μg quantified from the OD_{260}) were electrophoresed on a formaldehyde-agarose gel and transferred using 20 × SSPE buffer (sodium chloride 3.6 M, sodium phosphate 0.2 M, pH 7.7; disodium EDTA 0.002 M) onto a nylon membrane (Hybond N+, Amersham, Bucks, U.K.) and fixed by exposure to u.v. radiation. The cDNA probe for inducible NO synthase was labelled with [α^{32}P]-dCTP (3000 Ci $mmol^{-1}$, ICN Flow, High Wycombe, U.K.) using a multiprime DNA labelling system (Amersham) according to the manufacturer's instructions. Pre-hybridization was performed as follows, nylon membranes were incubated at 42°C for 1 h with 5 × SSPE, formamide (50% v/v), 5 × Denhardt's solution (bovine serum albumin 0.1% w/v, Ficoll 0.1% w/v and polyvinyl pyrollidone 0.1% (w/v) and sodium dodecyl sulphate (SDS; 0.5% w/v) supplemented with 20 $\mu g\,ml^{-1}$ denatured herring sperm DNA) (Promega, Southampton, U.K.). Following this, membranes were incubated with the ^{32}P-labelled NO synthase probe for 18 h at 42°C in the above buffer minus herring sperm DNA. Buffer was removed and membranes washed twice with 50 ml of 2 × SSPE/0.1% SDS for 15 min at 42°C, then with 50 ml 1 × SSPE/0.1% SDS for 30 min at 65°C. Finally, a high stringency wash was performed with 0.1% SSPE/0.1% SDS for 15 min at 65°C. The membranes were then sealed in plastic wrap and autoradiographed at −70°C using an intensifying screen (Dupont Ltd, Herts, U.K.). As a control for consistency of loading, ribosomal RNA (18S) was quantified by ethidium bromide fluorescence and photography of the formaldehyde-agarose gel before transfer and quantitation by Northern blot analysis.

Materials

All reagents for tissue culture were obtained from either Sigma (Poole, U.K.) or Flow (High Wycombe, Bucks). LPS, phenol-extracted from *Escherichia coli* (serotype 055:B5), clotrimazole, econazole nitrate, miconazole nitrate and dexamethasone were obtained from Sigma. Imidazoles were dissolved to a stock concentration of 2 mM in 100% ethanol (final concentration 0.05–0.5%). N^G-monomethyl-L-arginine acetate was a gift from Dr S. Moncada, Wellcome Research Laboratories, Beckenham, Kent, U.K.

Data analysis and statistics

Results are shown as the mean ± s.e.mean of n experiments. Statistical analysis was performed by a one-way analysis of variance with $P<0.05$ considered statistically significant. The density of bands on Northern blots and 18S ribosomal RNA bands were quantified with a scanner attached to a computer with image analysis software.

Results

In unstimulated J774 cells, nitrite production over a 24 h period was below the level of detection using the Griess reagent ($n = 6$). Stimulation of cells with lipopolysaccharaide (LPS; 10 $\mu g\,ml^{-1}$) resulted in significant nitrite production (43 ± 3 nmol per 10^6 cells over 24 h, $n = 6$) which was inhibited by co-incubation with either L-NMMA (0.1 or 1 mM) or dexamethasone (1 or 10 μM; Figure 1a). When these agents were added to cells pre-stimulated with LPS

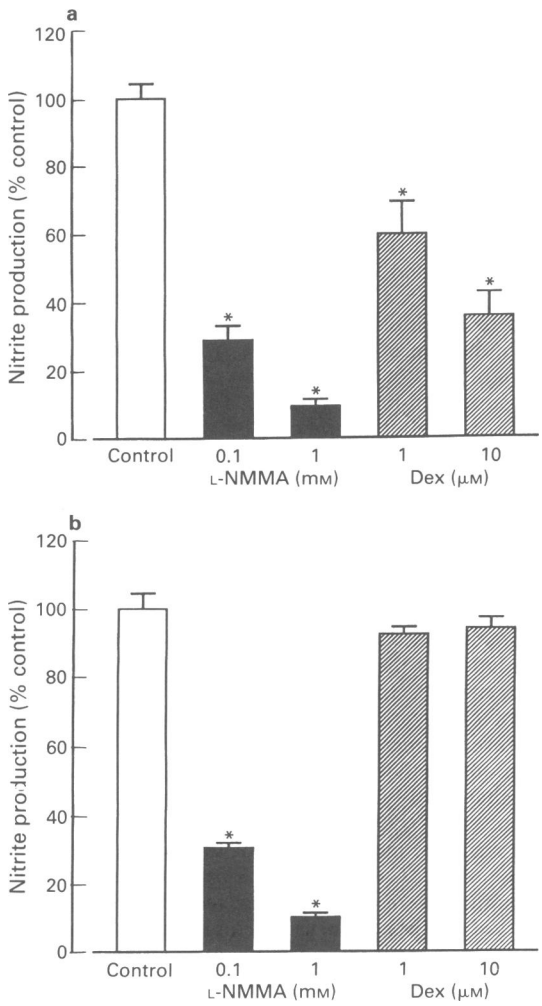


Figure 1 Effects of N^G-monomethyl-L-arginine (L-NMMA) and dexamethasone (Dex) on nitrite production in J774 cells activated with lipopolysaccharide. (a) Cells were stimulated with lipopolysaccharide (LPS, 10 μg ml^{-1}) in the presence of either L-NMMA (0.1 or 1 mM, solid columns) or dexamethasone (1 or 10 μM, hatched columns) and nitrite production was determined 24 h later. (b) Cells were stimulated with LPS (10 μg ml^{-1}) for 24 h after which LPS was removed and cells incubated for a further 24 h in either control medium, or medium containing L-NMMA (0.1 or 1 mM, solid columns) or dexamethasone (1 or 10 μM, hatched columns). Subsequently, nitrite production was measured using the Griess reaction. Values denote the mean ± s.e.mean of replicate measurements in 3 separate experiments; *$P<0.05$ unpaired t test.

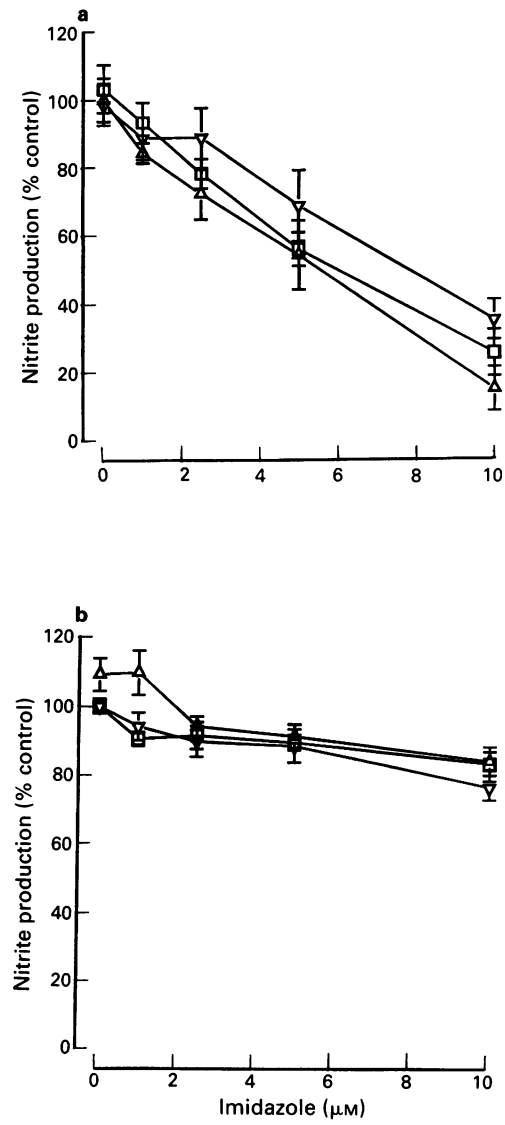


Figure 2 Effects of anti-fungal imidazoles on nitrite production by lipopolysaccharide (LPS)-stimulated J774 cells. (a) J774 cells were stimulated with LPS (10 μg ml^{-1}) for 24 h in the presence of increasing concentrations (1–10 μM) of either clotrimazole (□), econazole (△) or miconazole (▽). (b) J774 cells were pre-stimulated for 24 h with LPS (10 μg ml^{-1}) and then the LPS removed and clotrimazole (□), econazole (△) or miconazole (▽) were added for a further 24 h. Nitrite production was then measured using the Griess reagent. Values denote the mean ± s.e.mean of replicate measurements in 3 separate experiments.

(10 μg ml^{-1} for 24 h), L-NMMA but not dexamethasone caused an inhibition of nitrite production (Figure 1b).

Inhibition of nitrite production by antifungal imidazoles

Clotrimazole, econazole or miconazole (1–10 μM) incubated with J774 cells and LPS (10 μg ml^{-1}) for 24 h produced a concentration-dependent inhibition of nitrite production (Figure 2a, IC_{50} 5.3 ± 0.3, 5.0 ± 0.4 and 7.8 ± 0.7 μM respectively; $n = 3$). In contrast, when cells were pre-activated with LPS (10 μg ml^{-1}, 24 h) and subsequently incubated for 24 h with clotrimazole, econazole or miconazole (1–10 μM) no inhibition of nitrite production was observed (Figure 2b). Ethanol vehicle (0.05–0.5%) did not inhibit nitrite production whether given at the same time as or after LPS ($n = 3$, data not shown). No cytotoxic effects of imidazoles were detected over the concentration-range which inhibited nitrite production (1–10 μM) as assessed by either conversion of MTT to formazan or trypan blue exclusion ($n = 3$ in each case, data not shown).

Effects of antifungal imidazoles on inducible NO synthase mRNA levels

In unstimulated J774 cells, no inducible NO synthase mRNA was observed. However, following stimulation with LPS (10 μg ml^{-1}, 4 h) significant levels of inducible NO synthase mRNA were detected (Figure 3a) and this was associated with an increased nitrite production (Figure 3b). Incubation of cells with LPS (10 μg ml^{-1}, 4 h) in the presence of dexamethasone (10 μM) resulted in a reduced expression of inducible NO synthase mRNA and inhibition of nitrite production (Figure 3a and b). In contrast, treatment of J774 cells with LPS (10 μg ml^{-1}, 4 h) and econazole (10 μM) resulted in levels of mRNA similar to those observed in cells stimulated with LPS alone, whilst production of nitrite was almost completely abolished (Figure 3a and b).

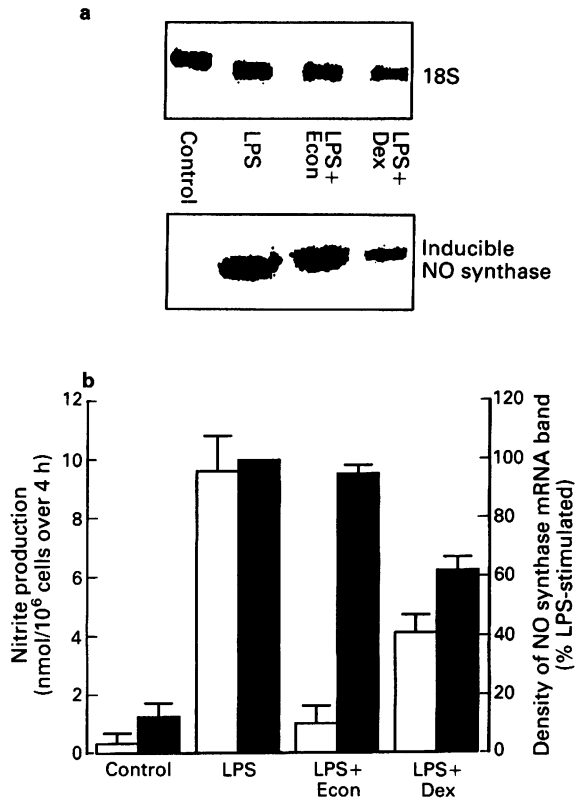


Figure 3 Effects of anti-fungal imidazoles on inducible NO synthase mRNA levels. (a) Representative Northern blot of RNA extracted from J774 cells and hybridized with a cDNA probe specific for murine inducible NO synthase. Cells were incubated for 4 h with either medium alone (control), lipopolysaccharide (LPS, 10 $\mu g\ ml^{-1}$), LPS (10 $\mu g\ ml^{-1}$) and econazole (Econ, 10 μM) or LPS (10 $\mu g\ ml^{-1}$) and dexamethasone (Dex, 10 μM). 18S ribosomal RNA (stained with ethidium bromide) is also shown as a control for consistency of loading. The figure is representative of data obtained in 3 separate experiments. (b) Nitrite production (open columns) and levels of inducible NO synthase mRNA (solid columns) in J774 cells stimulated as described above. Density of mRNA bands, determined by scanning densitometry, is shown as a percentage of that measured in cells stimulated with LPS alone. Results are the mean ± s.e.mean of 3 experiments.

Discussion

The data presented in this study demonstrate that anti-fungal imidazoles inhibit the induction of nitrite synthesis in macrophages. In concentrations similar to those achieved therapeutically (e.g. 2 μM for miconazole; Benson & Nahata, 1988), clotrimazole, econazole and miconazole inhibited nitrite production by J774 cells if added to the cells at the same time as the LPS but were ineffective in cells pre-stimulated with LPS. This profile of action is similar to that of glucocorticoids and might suggest that anti-fungal imidazoles inhibit induction of NO synthase gene expression. However, whereas treatment of J774 cells with dexamethasone resulted in inhibition of mRNA for inducible NO synthase and inhibition of nitrite production, econazole inhibited nitrite production without affecting mRNA levels. These results demonstrate that anti-fungal imidazoles and glucocorticoids inhibit induction of NO synthase activity by different mechanisms. Glucocorticoids appear to inhibit the production of NO before or at the level of transcription while anti-fungal imidazoles inhibit at a stage after mRNA production.

A recent report indicates that anti-fungal imidazoles inhibit the activity of the constitutive NO synthase and this appears to be due to binding and inactivation of the Ca^{2+}/calmodulin complex (IC_{50} <10 μM, Wolff *et al.*, 1993a,b), although at higher concentrations (mM), these drugs may also directly inactivate constitutive NO synthase by interacting with its haem moeity (Wolff *et al.*, 1993b). In the present study, anti-fungal imidazoles inhibited the activity of the inducible NO synthase if given at the same time as the stimulus for enzyme induction (LPS) but were ineffective when given after the enzyme was induced. These results are unlikely to be due to a cytotoxic effect of the compounds since this would be expected to be similar whether the drugs were added before or after induction of NO synthase. More importantly, assays of cell viability showed no adverse effects of imidazoles over the concentration-range studied. The failure of anti-fungal imidazoles to inhibit enzymic activity of the expressed inducible NO synthase is not unexpected since this enzyme type is functionally Ca^{2+}/calmodulin-independent although it is known to bind the Ca^{2+}/calmodulin complex tightly (Cho *et al.*, 1992). Similarly, although imidazoles are reported to inactivate the haem-moiety of enzymes this only occurs at concentrations 50–100 fold higher than those used in the present study (Wolff *et al.*, 1993a,b).

One possible mechanism of action is that imidazoles inhibit the translation of the inducible NO synthase mRNA into NO synthase enzyme. Interaction of imidazoles with the haem moiety of NO synthase at a vital stage of protein assembly might prevent dimerization of the enzyme which is necessary for functional activity (Baek *et al.*, 1993). Alternatively, it is known that anti-fungal imidazoles bind Ca^{2+}/calmodulin (Hegeman *et al.*, 1993) and thereby through this mechanism could prevent the incorporation of this complex into the NO synthase enzyme. Calcium itself appears to be less important in the induction of NO synthase activity; although Ca^{2+}-channel antagonists such as nifedipine inhibit induction of NO synthase in J774 cells (Szabo *et al.*, 1993), other Ca^{2+}-channel antagonists such as verapamil and diltiazem do not (Hauschildt *et al.*, 1990; Szabo *et al.*, 1993). Further experiments will be required to determine the precise mechanism by which anti-fungal imidazoles inhibit the induction of NO synthase activity in J774 cells.

The results of this study demonstrate that anti-fungal imidazoles inhibit the induction of functionally active NO synthase in J774 cells through a mechanism that differs from other known inhibitors of induction including glucocorticoids and actinomycin D which are likely to act via inhibition of gene expression and transcription respectively. The results obtained with anti-fungal imidazoles give some insight into the process of induction of NO synthase activity and coupled with previous reports suggest that Ca^{2+}/calmodulin may play an important role in regulating the activity of the inducible isoform of NO synthase. Furthermore our results indicate that it is possible to produce levels of mRNA for inducible NO synthase without production of NO. This finding has implications for studies in which the level of NO synthase mRNA are taken as an indication of functional activity. It remains to be determined how our study, and other studies demonstrating that imidazoles inhibit the activity of the constitutive NO synthase (Wolff *et al.*, 1993a) contribute to the reported anti-inflammatory (Petri *et al.*, 1986) and hypertensive effects of these and other imidazole drugs.

Note added in proof

Whilst this paper was in press, Yu & Tomasa (*Crit. Care Med.* (1993), **21**, 1635–1642) reported that ketoconazole reduced mortality in patients with septic shock.

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References

BAEK, K.J., THIEL, B.A., LUCAS, S. & STEUHR, D.J. (1993). Macrophage nitric oxide synthase subunits: characterization, and control of their assembly into a dimeric enzyme. *Endothelium*, **1** (suppl.), S11.

BENSON, J.M. & NAHATA, M.C. (1988). Clinical use of systemic anti-fungal agents. *Clin. Pharmacol.*, **7**, 424–428.

BERG, D., BUCHEL, K.H., PLEMPEL, M. & REGEL, E. (1986). Antimycotic sterol biosynthesis inhibitors. *Trends Pharmacol. Sci.*, **7**, 233–238.

BOGLE, R.G., BAYDOUN, A.R., PEARSON, J.D., MONCADA, S. & MANN, G.E. (1992). L-arginine transport is increased in macrophages generating nitric oxide. *Biochem. J.*, **284**, 15–18.

BREDT, D.S., HWANG, P.M., GLATT, C.E., LOWENSTEIN, C., REED, R.R. & SNYDER, S.S. (1991). Cloned and expressed nitric oxide synthase structurally resembles cytochrome P450 reductase. *Nature*, **351**, 714–718.

BREDT, D.S. & SNYDER, S.L. (1990). Isolation of nitric oxide synthetase, a calmodulin requiring enzyme. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 682–685.

CHO, H.J., XIE, Q.-W., CALAYCAY, J., MUMFORD, R.A., SWIDEREK, K.M., LEE, T.D. & NATHAN, C.F. (1992). Calmodulin is a subunit of nitric oxide synthase from macrophages. *J. Exp. Med.*, **176**, 599–604.

DI ROSA, M., RADOMSKI, M., CARNUCCIO, R. & MONCADA, S. (1990). Glucocorticoids inhibit the induction of nitric oxide synthase in macrophages. *Biochem. Biophys. Res. Commun.*, **172**, 1246–1252.

GREEN, L.C., WAGNER, D.A., GLOGOWSKI, K., SKIPPER, P.L., WISHNOK, J.S. & TANNENBAUM, S.R. (1982). Analysis of nitrate, nitrite and [^{15}N]nitrate in biological fluids. *Anal. Biochem.*, **126**, 131–138.

GROSS, S.S. & LEVI, R.L. (1992). Tetrahydrobiopterin synthesis: an absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle cells. *J. Biol. Chem.*, **26**, 25722–25729.

HAUSCHILDT, S., LÜCKHOFF, A., MÜLSCH, A., KOHLER, J., BESSLER, W. & BUSSE, R. (1990). Induction and activity of NO synthase in bone-marrow-derived macrophages are independent of Ca^{2+}. *Biochem. J.*, **270**, 351–356.

HEGEMANN, L., TOSO, S.M., LAHIJANI, K.I., WEBSTER, G.F. & UITTO, J. (1993). Direct interaction of antifungal azole-derivatives with calmodulin: a possible mechanism for their therapeutic activity. *J. Invest. Dermatol.*, **100**, 343–346.

HIBBS, J.B., TAINTOR, R.R. & VAVRIN, Z. (1987). Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. *Science*, **235**, 473–476.

LYONS, C.R., ORLOFF, G.J. & CUNNINGHAM, J.M. (1992). Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage cell line. *J. Biol. Chem.*, **267**, 6370–6374.

MARLETTA, M.A., YOON, P.S., IYENGAR, R., LEAF, C.D. & WISHNOK, J.S. (1988). Macrophage oxidation of L-arginine to nitrate and nitrite: nitric oxide is an intermediate. *Biochemistry*, **27**, 8706–8711.

MAYER, B., SCHMIDT, K., HUMBERT, P. & BOHME, E. (1989). Biosynthesis of endothelium-derived relaxing factor: a cytosolic enzyme in porcine aortic endothelial cells Ca^{2+}-dependently converts L-arginine into an activator of soluble guanylate cyclase. *Biochem. Biophys. Res. Commun.*, **164**, 678–685.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pharmacology and pathophysiology. *Pharmacol. Rev.*, **43**, 109–142.

MOORE, P.K., AL-SWAYEH, O.A., CHONG, N.S.W., EVANS, R. & GIBSON, A. (1990). L-N^{G}nitroarginine (L-NOARG), a novel, L-arginine reversible inhibitor of endothelium-dependent vasodilation. *Br. J. Pharmacol.*, **99**, 408–412.

MOSMANN, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, **65**, 55–63.

PALMER, R.M.J. & MONCADA, S. (1989). A novel citrulline forming enzyme implicated in the formation of nitric oxide by vascular endothelial cells. *Biochem. Biophys. Res. Commun.*, **158**, 348–352.

PALMER, R.M.J., BRIDGE, L.A., FOXWELL, N.A. & MONCADA, S. (1992). The role of nitric oxide in endothelial cell damage and its inhibition by glucocorticoids. *Br. J. Pharmacol.*, **105**, 11–12.

PETRI, H., TRONNIER, H. & HASS, P. (1986). Investigations into the anti-inflammatory effect of bifuconazole. In *Advances in Topical Antifungal Therapy*. ed. Hay, R.J. pp. 26–31. Berlin: Springer Verlag.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1990). Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 10043–10047.

REES, D.D., PALMER, R.M.J., HODSON, H.F. & MONCADA, S. (1989). A specific inhibitor of nitric oxide formation from L-arginine attentuates endothelium-dependent relaxation. *Br. J. Pharmacol.*, **96**, 418–424.

REES, D.D., SCHULZ, R., HODSON, H.F., PALMER, R.M.J. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **101**, 746–752.

RODRIGUES, A.D., GIBSON, G.G., IONNIDES, C. & PARKE, D.V. (1987). Interactions of imidazole antifungal agents with purified cytochrome P-450 protein. *Biochem. Pharmacol.*, **36**, 4277–4281.

SZABÖ, C., MITCHELL, J.A., GROSS, S.S., THIEMERMANN, C. & VANE, J.R. (1993). Nifedipine inhibits induction of nitric oxide synthase by bacterial lipopolysaccharide. *J. Pharmacol. Exp. Ther.*, **265**, 674–680.

WHITE, K.A. & MARLETTA, M.A. (1992). Nitric oxide synthase is a cytochrome P-450 type hemoprotein. *Biochemistry*, **31**, 6627–6630.

WOLFF, D.J., DATTO, G.A., SAMATOVICZ, R.A. & TEMPSICK, R.A. (1993a). Calmodulin-dependent nitric oxide synthase. Mechanism of inhibition of imidazole and phenylimidazoles. *J. Biol. Chem.*, **268**, 9425–9429.

WOLFF, D.J., DATTO, G.A. & SAMATOVICZ, R.A. (1993b). The dual mode of inhibition of calmodulin-dependent nitric oxide synthase by antifungal imidazole agents. *J. Biol. Chem.*, **268**, 9430–9436.

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Modulation of antagonist binding to histamine H_1-receptors by sodium ions and by 2-amino-2-hydroxymethyl-propan-1,3-diol HCl

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1 NaCl (100 mM) reduced the potency of (+)-N-methyl-4-methyldiphenhydramine ((+)-QMDP) as an inhibitor of the binding of [^{3}H]-mepyramine to histamine H_1-receptors on guinea-pig cerebellar membranes to a greater extent than that of mepyramine, consistent with the greater inhibitory effect of Na^+ on the binding of [^{3}H]-QMDP than on the binding of [^{3}H]-mepyramine.

2 The concentration of 2-amino-2-hydroxymethyl-propan-1,3-diol HCl (Tris, HCl) buffer, pH 7.5, present had little effect on the temelastine-insensitive binding of [^{3}H]-mepyramine, but caused a concentration-dependent inhibition of the binding of [^{3}H]-mepyramine sensitive to 1 μM temelastine (H_1-receptor binding), with an approximate IC_{50} of 75 mM, assuming that complete inhibition would have been achieved.

3 Inhibition of [^{3}H]-mepyramine binding by Na^+ was more marked in 10 mM than in 50 mM Tris HCl and was not evident in 200 mM Tris HCl.

4 The K_d for the temelastine-sensitive binding of [^{3}H]-mepyramine measured in 10 mM Tris HCl, 0.24 ± 0.01 nM, was increased by 2.2 ± 0.2 fold by 100 mM NaCl, without any significant change in the maximum binding (B_{max}). The B_{max} for [^{3}H]-mepyramine was similarly unchanged in 50 mM Tris HCl, but the K_d was increased 2.5 ± 0.2 fold.

5 The K_d for the temelastine-sensitive binding of [^{3}H]-mepyramine was also increased in 50 mM, compared with 10 mM, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid] KOH (HEPES.KOH) buffer (K_d 0.25 ± 0.02 nM in 10 mM HEPES), but the evidence for an interaction between HEPES and Na^+ was less clear.

6 The effect of 100 mM NaCl on the inhibition of [^{3}H]-mepyramine binding in 10 mM Tris HCl was examined for a range of antagonists. The decrease in potency caused by Na^+ was greatest for triprolidine, (+)-chlorpheniramine and benzilylcholine (9.6–10.3 fold increase in K_d values) but the binding of mepyramine and promethazine was much less affected (1.8 and 1.9 fold increase in K_d respectively). The K_d for temelastine was not significantly changed. In contrast to the general decrease in antagonist affinity in the presence of Na^+, the K_d for MDL 16,455A (4-[1-hydroxy-4-[4-(hydroxydiphenylmethyl)-1-piperidinyl]butyl]-α,α-dimethylbenzene acetic acid) was increased, but only by 1.5 fold.

7 It is concluded that Na^+ can act as an allosteric effector of the binding of antagonists at the histamine H_1-receptor. Tris HCl also appears to have an allosteric action at the H_1-receptor.

Keywords: Histamine H_1-receptors; [^{3}H]-mepyramine; sodium ions; Tris.HCl buffer; HEPES.KOH buffer; temelastine; terfenadine; MDL 16,455A; picumast

Introduction

G-protein-coupled receptors possess a highly conserved aspartate residue in the second transmembrane loop which appears to be associated with the modulation of agonist binding by intracellular Na^+ and H^+ (for a review see Hibert *et al.*, 1993). The most direct evidence for the involvement of this residue comes from mutation studies with the α_2-adrenoceptor (Horstman *et al.*, 1990) and the dopamine D_2-receptor (Neve *et al.*, 1991), in which substitution of the aspartate with asparagine (α_2-receptor) or alanine (D_2-receptor) abolished the regulatory action of Na^+ on ligand binding. With the histamine H_1-receptor the regulation of agonist binding by Na^+ (Chang & Snyder, 1980) presumably involves Asp-74 in the second transmembrane loop of the H_1-receptor cloned from bovine adrenal medulla (Yamashita *et al.*, 1991) or Asp-73 in the rat H_1-receptor (Fujimoto *et al.*, 1993). However, antagonist binding can also be modulated by Na^+, as originally demonstrated for opiate receptors (Pert & Snyder, 1974; Paterson *et al.*, 1986), and we have reported that Na^+ ions distinguish between the binding of two antagonist radioligands to the histamine H_1-receptor (Treherne *et al.*, 1991). We describe here a study of the action of Na^+ on the binding of a wider range of antagonists to the H_1-receptor, as reflected by the effect of Na^+ on antagonist potency in inhibiting the binding of [^{3}H]-mepyramine to guinea-pig cerebellar membranes. At the same time, in view of the reported effect of the 2-amino-2-hydroxymethyl-propan-1,3-diol (Tris) cation in obscuring the modulation by Na^+ of [^{3}H]-nitrendipine binding to L-type Ca^{2+} channels (Bolger & Skolnick, 1986), we have investigated the possible modulation of H_1-antagonist binding by Tris HCl buffers.

Methods

Preparation of cerebellar membranes

Cerebella from Dunkin-Hartley strain guinea-pigs (males, 250–600 g; Tucks, Battlebridge, Essex) were washed in 10 or 50 mM 2-amino-2-hydroxymethylpropan-1,3-diol hydrochloride (Tris-HCl) buffer, pH 7.5, or 10 or 50 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid] (HEPES) buffer, pH 7.5, containing 1 mM ethylenediaminetetracetic acid (EDTA), and then homogenized in 10 volumes of the same buffer in a teflon-glass homogeniser with a motor-driven pestle (3,000 r.p.m., 5 or 6 up and down strokes). The

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homogenate was centrifuged at 17,000 *g* for 30 min and the pellet then resuspended in buffer but without EDTA and centrifuged again at 17,000 *g* for 30 min. The resulting pellet was suspended in 10 or 50 mM HEPES or Tris buffer, pH 7.5, to give a final protein concentration of 6–10 mg ml^{-1} and was stored at − 20°C until required for use. Protein was determined essentially as described by Lowry *et al.* (1951) with bovine serum albumin used as a standard.

Measurement of the binding of [^{3}H]-mepyramine

Incubations in 10 or 50 mM HEPES or Tris-HCl buffer, pH 7.5, contained [^{3}H]-mepyramine (usually in the concentration range 0.4–0.9 nM for inhibition curves), inhibitor, 100 mM NaCl (where appropriate) and cerebellar homogenate (0.19–0.27 mg protein) in a total volume of 1.00–1.11 ml (four replicates at each inhibitor concentration, 8–16 replicates in the absence of inhibitor, spread through the experiment). For a given inhibitor, curves in the presence and absence of NaCl were always determined in the same experiment. HEPES buffers were adjusted to pH 7.5 with KOH, giving approximately 36 mM K^+ in 50 mM HEPES buffer. In experiments in which H_1-receptor binding was measured as a function of the concentration of [^{3}H]-mepyramine, non-specific binding was determined as the binding insensitive to inhibition by 1 μM temelastine, which has a low affinity for secondary, non-H_1-receptor, binding sites for [^{3}H]-mepyramine (Arias-Montaño & Young, 1993a). The extent of the non-specific binding of 0.4–0.6 nM [^{3}H]-mepyramine in 10 or 50 mM Tris-HCl buffer was usually 9–16% of total binding. Equilibration was for 60 min at 30°C and was terminated by filtration through Whatman GF/B glass fibre paper, presoaked in 0.3% (w/v) polyethylenimine for 3–5 h, using a Brandel (Gaithersburg, Md, U.S.A.) cell harvester. The filters were washed with ice-cold buffer and then transferred to scintillation insert vials containing 4.0 ml scintillator (Emulsifier-Safe, Packard) and allowed to stand at room temperature at least 2 h before determination of tritium by liquid scintillation counting.

Analysis of data

Curves of inhibition of [^{3}H]-mepyramine binding were fitted to the equation: % of uninhibited binding of [^{3}H]-mepyramine = (100-NS)/{(A/IC_{50})^n + 1} + NS where n is the Hill coefficient, A is the concentration of inhibitor, IC_{50} is the concentration of inhibitor required for 50% inhibition of the binding of [^{3}H]-mepyramine sensitive to the inhibitor, and NS is the percentage of the binding insensitive to the inhibitor, using the Harwell Library non-linear regression routine VB01A. All points were weighted according to the reciprocal of the approximate variance associated with them. Where n did not differ significantly from unity the dissociation constant of the inhibitor was calculated from the relationship: $K_d = IC_{50}/([^3H]\text{-mepyramine}]K_{d(\text{mepyramine},} + 1)$. In the special case of mepyramine versus [^{3}H]-mepyramine this simplifies to $K_d = IC_{50}$–[^{3}H]-mepyramine, assuming that the substitution of one atom of tritium for one atom of hydrogen has no significant effect on the affinity of mepyramine.

Curves of the temelastine-sensitive binding of [^{3}H]-mepyramine versus the concentration of [^{3}H]-mepyramine were fitted as above to the equation:

$$[^3H]\text{-mepyramine bound} = B_{max}.C^n/(C^n + EC_{50}{}^n)$$

where C is the concentration of radioligand, EC_{50} is the concentration giving half-maximal binding, n is the Hill coefficient and B_{max} is the maximum binding. Where the Hill coefficient was not significantly different from unity (in all experiments except one with 10 and 50 mM HEPES), the data were fitted to a hyperbola (n = 1 in the equation above) to obtain the K_d(= EC_{50}) and B_{max}.

The overall value of K_d from a number of determinations was calculated as the weighted mean ± s.e.mean from individual values and their estimated standard errors, derived from the best-fit estimates of IC_{50}. For the three incubation conditions with the largest number of determinations, the K_d of mepyramine in 10 and 50 mM Tris HCl and in 10 mM HEPES KOH, the final estimate was similar when the weighted mean was calculated from values of log(K_d) and the approximate standard error, i.e. 0.24 ± 0.01 and 0.25 ± 0.01 nM in 10 mM Tris, 0.77 ± 0.03 and 0.80 ± 0.03 nM in 50 mM Tris and 0.25 ± 0.02 and 0.29 ± 0.02 nM in 10 mM HEPES, using K_d and log (K_d), respectively.

Statistical comparison of parameters characterizing concentration-response curves in 10 and 50 mM Tris or HEPES buffer was made by fitting the curves simultaneously (using the NAG library routine E04FDF) and assessing the increase in the residual sum of squares when a parameter was constrained to be the same for both curves, by calculating the *F*-statistic (Rodbard, 1974):

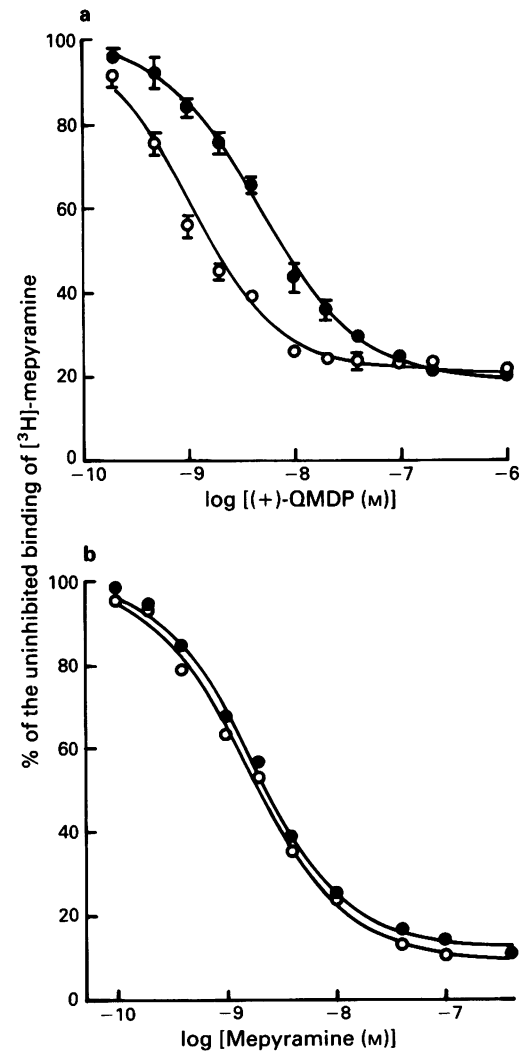


Figure 1 Effect of 100 mM NaCl on the inhibition of [^{3}H]-mepyramine binding in 50 mM Tris HCl buffer by (a) (+)-N-methyl-4-methyldiphenhydramine ((+)-QMDP) and (b) mepyramine. Values are means ± approximate s.e.mean of quadruplicate determinations from single experiments with (a) 0.63 nM and (b) 0.77 nM [^{3}H]-mepyramine. Where no error bars are shown the error was within the size of the symbol. The curves drawn are the weighted best-fit lines to a Hill equation (see Methods). Best-fit values: (a) Hill coefficient (n_H) 1.03 ± 0.11 and 0.92 ± 0.03, IC_{50} 1.1. ± 0.1 and 5.0 ± 0.2 nM; (b) n_H 0.98 ± 0.04 and 1.03 ± 0.05, IC_{50} 1.6 ± 0.1 and 1.8 ± 0.1 nM, in the absence and presence of 100 mM NaCl, respectively. In the presence of NaCl the specific binding of [^{3}H]-mepyramine was reduced by 24% in (a) and 21% in (b). The experiment with (+)-QMDP was repeated twice more and that with mepyramine three times more, with similar results. (○) No NaCl, (●) 100 mM NaCl present.

$$F = \{(SS_2 - SS_1)/(d.f._2 - d.f._1)\}/(SS_1/d.f._1)$$

where SS_2 is the sum of squares when a parameter is shared ($d.f._2$ degrees of freedom) and SS_1 the sum of squares when all parameters are allowed to float freely for each curve ($d.f._1$ degrees of freedom).

Drugs

[^{3}H]-mepyramine (21 and 26 Ci mmol^{-1}) was obtained from Amersham International. Histamine dihydrochloride, mepyramine maleate and promethazine hydrochloride were purchased from Sigma. (+)- and (−)-Chlorpheniramine maleate were gifts from Schering, hydroxyzine hydrochloride from Pfizer, icotidine trihydrochloride and temelastine from Smith, Kline and French Research, terfenadine and MDL 16,455A hydrochloride (4-[1-hydroxy-4-[4-(hydroxydiphenylmethyl)-1-piperidinyl]butyl]-α,α-dimethylbenzene acetic acid) from Marion Merrell Dow, tiagabine hydrochloride from Novo Nordisk and picumast dihydrochloride from Boehringer Mannheim. N-methyl-4-methyldiphenhydramine iodide [(+)-QMDP], pirdonium bromide and norpirdonium maleate were kindly provided by Prof. H. Timmerman, Vrije Universiteit, Amsterdam and triprolidine hydrochloride by Dr A.F. Casy, University of Bath. Benzilylcholine chloride was synthesized by Mr B. Peck.

Results

Effect of Na$^+$ on antagonist inhibition of [^{3}H]-mepyramine binding in 50 mM Tris HCl

Consistent with the effect of Na^+ in causing a greater inhibition of the binding of [^{3}H]-mepyramine to H_1-receptors in guinea-pig cerebellar membranes than that of [^{3}H]-QMDP (Treherne *et al.*, 1991), 100 mM NaCl had a greater effect on the position of the curve of (+)-QMDP inhibition of [^{3}H]-mepyramine binding to cerebellar membranes, measured in 50 mM Tris HCl buffer, than on that of mepyramine (Figure 1). The very small effect of Na^+ on the position of the curve of mepyramine inhibition of [^{3}H]-mepyramine binding, indicates that there is only a small change in the K_d for mepyramine, since the concentration of the radioligand was the same throughout the experiment. The ratios of the K_ds in the presence and absence of Na^+ are set out in Table 1, together with the values for temelastine and histamine. The extent of the increase in the apparent K_d for the agonist histamine produced by 100 mM NaCl in 50 mM Tris HCl, 10.4 fold (Table 1), was similar to that, 9.8 fold, reported by Chang & Snyder (1980) using the same buffer.

Effect of Tris HCl buffer concentration on [^{3}H]-mepyramine binding and the modulatory action of Na$^+$

The initial choice of 50 mM Tris HCl buffer for our studies (Treherne *et al.*, 1991) was made for compatability with earlier measurements of the effects of cations on H_1-agonist inhibition of [^{3}H]-mepyramine binding (Chang & Snyder, 1980). However, Tris HCl itself proved to be an effective inhibitor of [^{3}H]-mepyramine binding to cerebellar membranes (Figure 2). There was little effect on the temelastine-insensitive binding of [^{3}H]-mepyramine, but the temelastine-sensitive binding (H_1-receptor binding) was markedly reduced as the buffer strength was increased, with an IC_{50} of approximately 75 mM Tris HCl, assuming that all of the temelastine-binding would have been inhibited (Figure 2). The pH was 7.5 at 30°C throughout.

The concentration of Tris HCl present had a marked effect on the modulation of [^{3}H]-mepyramine binding by Na^+. In 10 mM Tris HCl, [^{3}H]-mepyramine binding was inhibited more strongly by Na^+ than in 50 mM Tris HCl and in 200 mM Tris HCl the inhibitory action of Na^+ was abolished

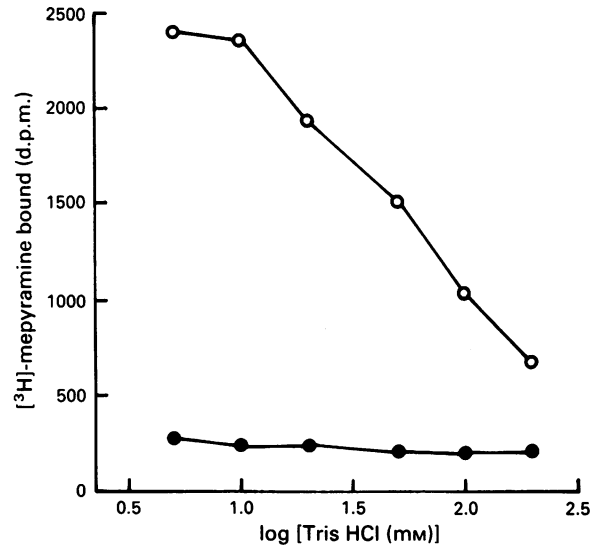


Figure 2 Inhibition of [^{3}H]-mepyramine binding by Tris HCl. Values are means ± s.e.mean of the binding of 0.35 nM [^{3}H]-mepyramine from quadruplicate determinations within a single experiment. Error bars were within the size of the symbols. The cerebellar homogenate used in these experiments was prepared in 10 mM Tris HCl. The whole experiment was repeated twice more with similar results. (○) Total binding; (●) binding in the presence of 1 μM temelastine.

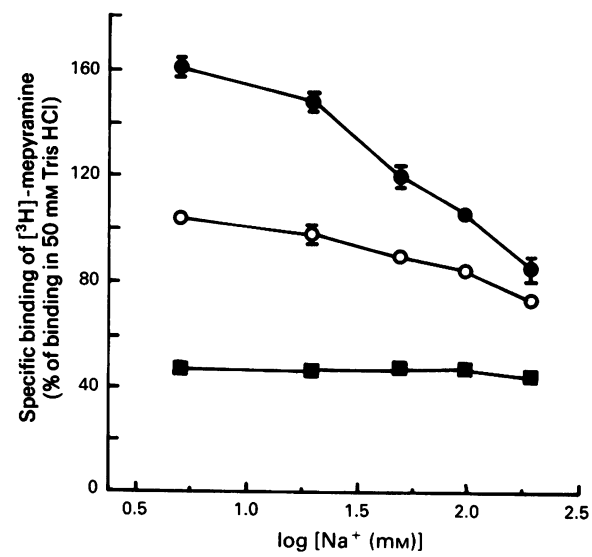


Figure 3 Effect of the concentration of Tris HCl buffer present on the inhibition of the H_1-receptor binding of [^{3}H]-mepyramine by NaCl. Values are means ± s.e.mean of quadruplicate measurements within a single experiment of the difference of the binding of 0.52 nM [^{3}H]-mepyramine in the absence and presence of 1 μM temelastine. Where no error bars are shown the error was within the size of the symbol. The whole experiment was repeated twice more with similar results. (●) 10 mM Tris; (○) 50 mM Tris; (■) 200 mM Tris.

(Figure 3). The implication is that Na^+ and Tris are both allosteric inhibitors of [^{3}H]-mepyramine binding. However, it is not clear that they are acting by a common mechanism, since the shift of the inhibition curve for histamine in 50 mM Tris HCl was as great as in 10 mM Tris HCl (Table 1).

The interaction of both Na^+ and Tris HCl with the H_1-receptor appeared to be reversible, since the effect of 100 mM NaCl on the saturation binding of [^{3}H]-mepyramine to the H_1-receptor measured in 10 mM Tris HCl was to cause a decrease in the affinity without any significant effect on the maximum binding (Figure 4a). The mean increase in the K_d

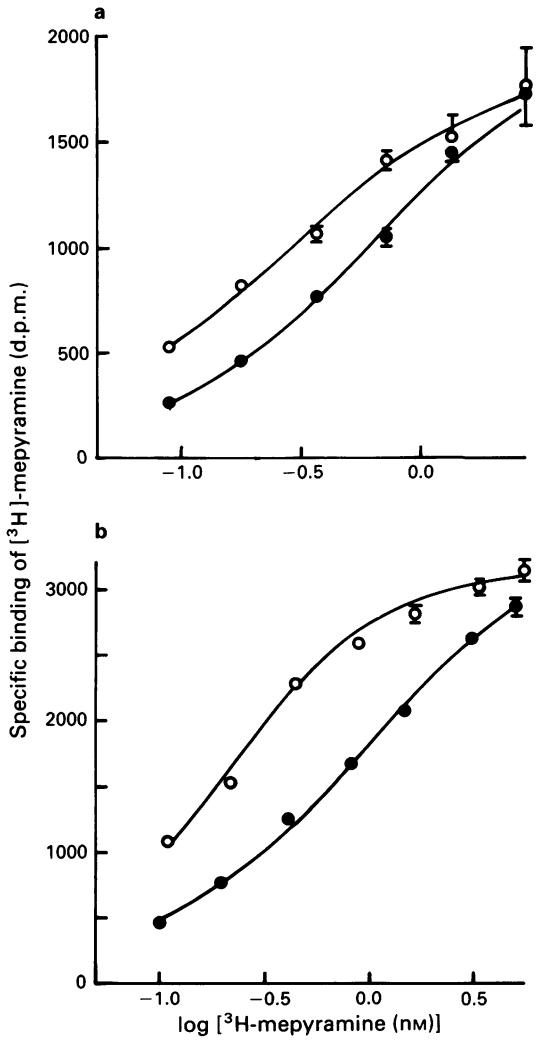


Figure 4 Binding of [^{3}H]-mepyramine to histamine H_1-receptors (a) in 10 mM Tris HCl in the presence and absence of 100 mM NaCl and (b) in 10 and 50 mM Tris HCl buffers. Values are the means ± s.e.mean from quadruplicate determinations within a single experiment of the difference in the binding of [^{3}H]-mepyramine to cerebellar membranes in the absence and presence of 1 μM temelastine. Where no error bars are shown the error was within the size of the symbol. The curves drawn are the weighted best-fit lines to a Hill equation (see Methods). Best-fit values: (a) Hill coefficient (n_H) 0.90 ± 0.10 and 0.99 ± 0.09; EC_{50} 0.26 ± 0.06 and 0.62 ± 0.15 nM, in the absence and presence of 100 nM NaCl, respectively; (b) n_H 1.14 ± 0.14 and 0.81 ± 0.09; EC_{50} 0.21 ± 0.03 and 0.99 ± 0.29 nM, in 10 and 50 mM Tris, respectively. Each experiment was repeated twice more with similar results. (a) (○) 10 mM Tris HCl; (●) 10 mM Tris HCl + 100 mM NaCl (b) (○) 10 mM Tris HCl; (●) 50 mM Tris HCl.

was by a factor of 2.2 ± 0.2 (three determinations). Similarly, the binding curve for [^{3}H]-mepyramine to 50 mM Tris HCl was shifted to lower affinity compared with that in 10 mM Tris HCl, but again without any significant effect on the maximum binding (Figure 4b). There were no consistent or significant differences in the best-fit Hill coefficients for the pairs of curves in either set of experiments. The mean increase in the K_d in 50 mM Tris was 2.5 ± 0.2 fold (3).

The overall mean K_d for [^{3}H]-mepyramine in 10 mM Tris HCl, taken from saturation binding and inhibition curves, was 0.24 ± 0.01 nM (eight determinations) and in 50 mM Tris HCl 0.77 ± 0.03 nM (7).

Effect of HEPES buffer on [^{3}H]-mepyramine binding

Increasing the concentration of HEPES KOH buffer from 25 to 50 mM caused an inhibition of [^{3}H]-mepyramine binding (Figure 5). K^+ is a relatively weak inhibitor of the binding of both [^{3}H]-mepyramine and [^{3}H]-QMDP (Treherne *et al.*, 1991), but the presence of an appreciable concentration of K^+, *circa* 36 mM in 50 mM HEPES KOH, potentially complicates the interpretation of the data and we have not examined higher HEPES KOH buffer concentrations. The amount of K^+ present was adjusted with KCl to be the same as in the 50 mM buffer throughout the experiment.

A possible interaction between HEPES and Na^+ was less clear (Figure 5) than in Tris HCl. The percentage inhibition of [^{3}H]-mepyramine binding by 100 mM NaCl was consistently greater in 50 mM than 10 mM HEPES (13 ± 1% and 23 ± 2% inhibition in 10 and 50 mM HEPES, respectively, 5 paired determinations), but the effect of NaCl appeared to be to reduce the temelastine-sensitive binding of [^{3}H]-mepyramine by a constant absolute amount (Figure 5).

The H_1-receptor binding of [^{3}H]-mepyramine in 50 mM HEPES KOH was shifted to lower affinity compared with the saturation binding in 10 mM HEPES KOH, in an analogous fashion to that observed in 10 and 50 mM Tris HCl (cf. Figure 4), without consistent effect on the maximum binding. The mean K_d for [^{3}H]-mepyramine in 50 mM HEPES was 1.0 ± 0.1 nM (3) and that in 10 mM HEPES, calculated from both saturation binding and from inhibition curves, was 0.25 ± 0.02 nM (6).

Dependence of the direction and magnitude of Na^+ shifts of inhibition curves on antagonist structure

The inhibition of [^{3}H]-mepyramine binding in 10 mM Tris HCl and 10 mM HEPES KOH appears to be minimal (Figures 2 and 5) and shifts in the curves of inhibition of [^{3}H]-mepyramine binding by mepyramine, (+)-QMDP, temelastine and histamine were similar in the two buffers. The ratios of the K_d values in the presence and absence of 100 mM NaCl, derived from the inhibition curves, are given in Table 1. The ratios for mepyramine and (+)-QMDP were greater in the 10 mM buffers than in 50 mM Tris HCl, but the difference was not statistically significant for mepyramine in 10 mM Tris HCl (as assessed by applying the Student-Newman-Keuls multiple range test to the weighted means derived

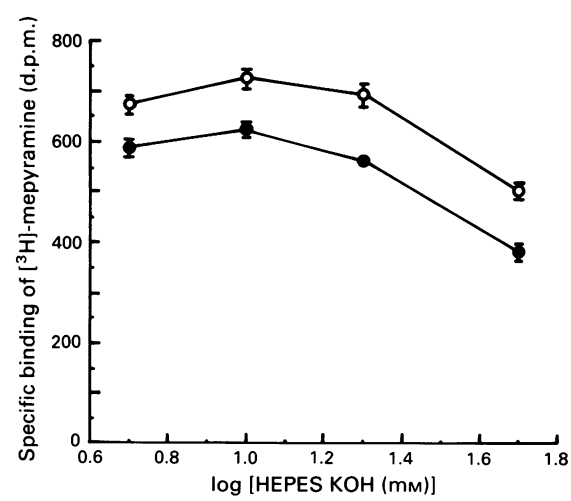


Figure 5 Inhibition of the histamine H_1-receptor binding of [^{3}H]-mepyramine by HEPES KOH in the presence and absence of 100 mM Na^+. The amount of K^+ present was adjusted to be the same throughout the experiment by addition of the appropriate amount of KCl to incubations in which [HEPES] was less than 50 mM. Values are means ± s.e.mean from quadruplicate determinations within a single experiment of the binding of 0.59 nM [^{3}H]-mepyramine sensitive to inhibition by 1 μM temelastine. Where no error bars are shown the error was within the size of the symbol. The whole experiment was repeated twice more with similar results. (○) HEPES alone; (●) HEPES + 100 mM NaCl.

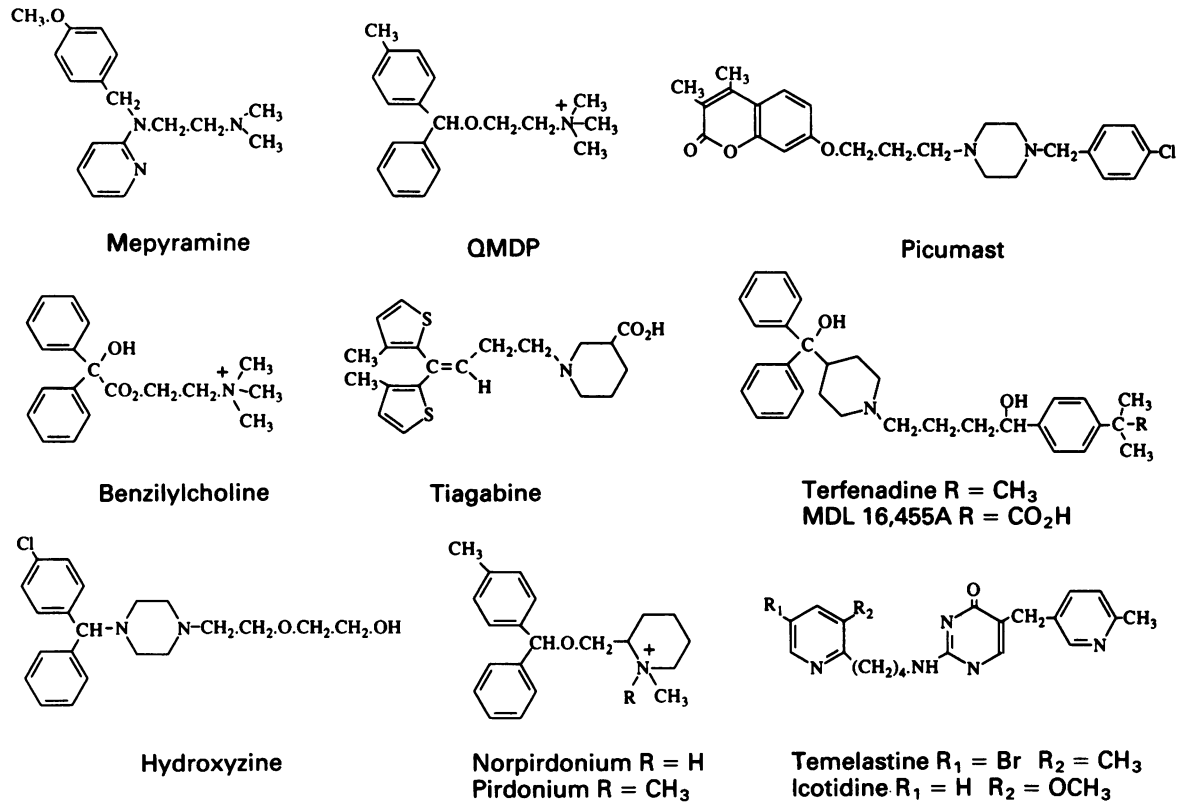


Figure 6 Structures of some of the antagonists examined.

from ratios ± approximate s.e.). In contrast, for histamine the ratio of apparent K_d values did not differ significantly in any of the buffers (Table 1). The Hill coefficients for the histamine inhibition curves were similar in the three buffers and in the presence and absence of 100 mM NaCl (values given in the legend to Tables 1 and 2). Temelastine was little affected by Na^+ in either 50 or 10 mM buffer (Table 1).

For the survey of the effect of Na^+ on a wider range of antagonists, measurements were made in 10 mM Tris HCl to avoid any possible complications introduced by the presence of K^+ in the buffer. The changes in affinity produced by 100 mM NaCl, expressed as the ratio of K_d values in the presence and absence of Na^+ are given in Table 2. The largest decreases in affinity, approximately 10 fold, were observed with benzilylcholine (primarily a muscarinic antagonist), (+)-chlorpheniramine and triprolidine. The K_d for temelastine was not significantly changed. However, for one of the antagonists, MDL 16,455A (Figure 6) the effect of Na^+ was to produce a significant increase in affinity, ratio of K_d values in the presence and absence of Na^+ 0.67 ± 0.04 ($P<0.05$, assuming the ratios to be normally distributed), but only by a factor of 1.5.

Table 1 Influence of buffer composition on the effect of 100 mM NaCl on K_d values of H_1-receptor ligands

Inhibitor	(K_d in presence of Na^+)/(K_d in absence of Na^+) measured in 50 mM Tris	10 mM Tris	10 mM HEPES
Mepyramine	1.1 ± 0.3 (4)	1.8 ± 0.2 (3)	2.5 ± 0.2 (3)
(+)-QMDP	4.0 ± 0.2 (3)	7.4 ± 0.5 (3)	9.4 ± 0.5 (3)
Temelastine	1.0 ± 0.1 (3)	0.9 ± 0.1 (3)	1.0 ± 0.1 (3)
Histamine	11.0 ± 0.5 (4)	10.0 ± 0.4 (3)	12.4 ± 0.8 (3)

Values of the ratios of K_d values are weighed means ± s.e.mean calculated from the ratios ± approximate s.e. of the best-fit values of the IC_{50} for the inhibition of the inhibitor-sensitive binding of 0.4–0.9 nM [^{3}H]-mepyramine in the presence and absence of 100 mM NaCl. The number of paired determinations of IC_{50} ± Na^+ is given in parentheses. Weighted mean K_d values in 50 mM Tris HCl, calculated from the best-fit IC_{50} values in the absence of Na^+: mepyramine 0.92 ± 0.02 nM, (+)-N-methyl-4-methyldiphenhydramine ((+)-QMDP) 0.76 ± 0.03 nM, temelastine 0.28 ± 0.01 nM and histamine (apparent K_d mean Hill coefficient 0.77 ± 0.02) 1,970 ± 50 nM. The mean Hill coefficient for histamine in 50 mM Tris HCl in the presence of Na^+ was 0.78 ± 0.02. K_d values in 10 mM HEPES KOH: mepyramine 0.27 ± 0.04 nM, (+)-QMDP 0.20 ± 0.01 nM, temelastine 0.26 ± 0.02 nM, histamine (apparent K_d), 1,550 ± 40 nM. The Hill coefficients for histamine inhibition curves in 10 mM HEPES KOH were 0.74 ± 0.02 and 0.77 ± 0.03 in the absence and presence of Na^+, respectively. K_d values in 10 mM Tris HCl are given in Table 2.

Discussion

It is clear from the data presented above that the modulatory action of Na^+ ions on ligand binding to the histamine H_1-receptor is not confined to agonists, as originally proposed (Chang & Snyder, 1980), but also affects the binding of antagonists in a direction and to an extent dependent on their structure. This is consistent with Na^+ acting directly at the receptor as an allosteric regulator of ligand binding in a way well established for a number of G-protein-coupled receptors (Hibert *et al.*, 1993). The enhancement of the binding of at least one of the antagonists studied is strong evidence that Na^+ does induce a conformation change. It is also clear, at least as far as antagonist binding is concerned, that the action of Na^+ can be obscured in the presence of a sufficient concentration of Tris HCl buffer. The simplest explanation is that Tris HCl is also an allosteric effector at the H_1-receptor, although inhibition of [^{3}H]-mepyramine binding is the only effect that we have demonstrated for Tris HCl acting alone. However, the antagonism between Na^+

Table 2 Effect of 100 mM NaCl on the affinity of antagonists for the histamine H_1-receptor in cerebellar membranes

Antagonist	K_d *(nM)*	$(K_d + Na^+)/(K_d$ *no* $Na^+)$
Benzilylcholine	830 ± 10	10.3 ± 0.3 (3)
(+)-Chlorpheniramine	0.12 ± 0.01	10.2 ± 0.6 (3)
Triprolidine	0.05 ± 0.01	9.6 ± 0.4 (3)
(−)-Chlorpheniramine	34 ± 1	8.7 ± 0.3 (3)
(+)-QMDP	0.24 ± 0.01	7.4 ± 0.5 (3)
Norpirdonium	0.43 ± 0.02	5.6 ± 0.2 (3)
Tiagabine	3,260 ± 170	3.3 ± 0.3 (2)
Pirdonium	0.36 ± 0.01	2.9 ± 0.1 (4)
Picumast	0.25 ± 0.30	2.5 ± 0.2 (3)
Hydroxyzine	1.0 ± 0.1	2.0 ± 0.3 (3)
Promethazine	0.36 ± 0.01	1.9 ± 0.1 (3)
Mepyramine	0.36 ± 0.03	1.8 ± 0.2 (3)
Temelastine	0.36 ± 0.02	0.9 ± 0.1 (3)
Icotidine	7,140 ± 200	0.9 ± 0.1 (3)
Terfenadine	4.3 ± 0.2	0.8 ± 0.1 (3)
MDL 16,455A	33 ± 2	0.7 ± 0.1 (3)
Agonist		
Histamine	(1,520 ± 50)*	10.0 ± 0.4 (3)

Measurements of the inhibition of the binding of 0.42–0.92 nM [^{3}H]-mepyramine to cerebellar membranes in the presence and absence of 100 mM NaCl were made in 10 mM Tris HCl buffer, pH 7.5. K_d values are weighted means ± s.e.mean calculated from the best-fit values of IC_{50} ± estimated error (see Methods) for inhibition curves in the absence of NaCl, taking the value of K_d for [^{3}H]-mepyramine to be 0.36 nM, as determined from mepyramine inhibition curves. The values of the ratio of K_d values in the presence and absence of Na^+ are the weighted means of the ratios from individual experiments, in which paired measurements were made of inhibition curves in the presence and absence of 100 mM NaCl (see legend to Table 1). There were no consistent changes in the Hill coefficient in the presence of Na^+ and no consistent divergence of Hill coefficients from unity.
*Apparent K_d. The weighted mean values of the Hill coefficients for histamine were 0.74 ± 0.02 and 0.74 ± 0.03 in the presence and absence of 100 mM NaCl, respectively.

and Tris HCl is clear, since in 10 mM Tris HCl, Na^+ inhibits [^{3}H]-mepyramine binding at concentrations above 20 mM, whereas in 200 mM Tris HCl there is no effect of Na^+ up to 100 mM (Figure 3). Were Tris HCl acting as a simple competitive antagonist at the H_1-receptor, then it would not be expected that the inhibitory action of Na^+ would be abolished at a concentration of Tris HCl that does not cause complete inhibition of [^{3}H]-mepyramine binding. The same antagonism between Tris HCl and Na^+ is apparent in the increased effect of Na^+ on the K_d for (+)-QMDP as the concentration of Tris HCl buffer is reduced from 50 mM to 10 mM (Table 1).

Both Na^+ and Tris HCl appear to be reversible allosteric inhibitors of [^{3}H]-mepyramine binding, since they both displace the K_d for [^{3}H]-mepyramine binding curves to higher concentrations, without any significant change in B_{max} (Figure 4). However, there is some indication that the mechanisms of interaction of Na^+ and Tris HCl with the H_1-receptor are not identical, since the extent of the decrease in the apparent K_d for histamine produced by 100 mM NaCl is not significantly different in 10 and 50 mM Tris HCl buffers (Table 1). Complexity of the conformational equilibria involved is further suggested by the observation that Li^+, which is also reported to inhibit the binding of histamine (Chang & Snyder, 1980), inhibits equally the binding of [^{3}H]-mepyramine and [^{3}H]-QMDP (Treherne *et al.*, 1991). Which Tris species, protonated or uncharged, is involved in this interaction is unknown, although it would seem most likely to be the Tris cation.

The action of Tris HCl in obscuring the inhibitory effect of Na^+ is one reason why the effect on antagonist binding was not more apparent in the original study of the effect of cations on [^{3}H]-mepyramine binding in which measurements were made in 50 mM Tris HCl (Chang & Snyder, 1980), although the K_d for triprolidine was in fact decreased by 100 mM NaCl by a factor of nearly four. This underlines the dependence of dissociation constants determined from inhibition of radioligand binding to membrane preparations on the assay conditions employed. The same point has been made with some force for the determination of the relative affinities of antagonists for muscarinic receptor subtypes (Pedder *et al.*, 1991). It also emphasises the fundamental problem inherent in the use of membrane preparations in binding assays, in that no medium can mimic the physiological situation with intact cells, in which the cytoplasmic and extracellular faces of the receptor are exposed to solutions of very different ionic composition.

Na^+ and Tris HCl are not the only agents which act as modulators of ligand binding to histamine H_1-receptors. We have recently demonstrated that Ni^{2+} is an allosteric inhibitor of [^{3}H]-mepyramine binding to a membrane preparation from guinea-pig cerebral cortex (Arias-Montaño & Young, 1993b). The approach we adopted there to demonstrate the allosteric mechanism of action, namely the simultaneous analysis of a family of Ni^{2+} inhibition curves measured in the presence of a range of concentrations of [^{3}H]-mepyramine, is not so easily applicable to Na^+, since the inhibition curves with Na^+ give no indication of having reached a level of maximum inhibition even at 200 mM. Whether Ni^{2+} and Na^+ act at a common site (or whether either ion acts at more than one site) is not known, although Ni^{2+} did inhibit histamine-induced inositol phosphate formation in intact HeLa cells, implying an extracellular site of action (Arias-Montaño & Young, 1993b). The evidence from studies with Na^+ and α_2-receptor-mediated responses in platelets is that it is intracellular Na^+ which is important (Motulsky & Insel, 1983). There is some evidence that Na^+ slows the inactivation of dopamine D_2-receptors by N-ethylmaleimide (Neve, 1991) but the sites of action of Na^+ and N-ethylmaleimide on histamine inhibition of [^{3}H]-mepyramine binding to guinea-pig cerebellar membranes are clearly distinct (Yeramian *et al.*, 1985). Dithiothreitol, which increased the efficacy of H_1-agonist-mediated biochemical responses (Donaldson & Hill, 1986a) and potentiates H_1-agonist inhibition of [^{3}H]-mepyramine binding (Donaldson & Hill, 1986b) in guinea-pig brain preparations, also remains effective in the presence of Na^+ (K.W. Young & J.M.Y., unpublished observations). There is no indication that either N-ethylmaleimide or dithiothreitol affect H_1-antagonist binding, but the mechanism of action of both agents could be to promote a conformational change induced by agonist binding, without themselves causing such a change. Just how Na^+ + Tris HCl might distinguish between agonist and antagonist binding, if this is established to be the case, remains to be determined.

For the majority of the antagonists surveyed, the effect of Na^+ was to decrease their affinity for the histamine H_1-receptor. This is not surprising, given that most of the compounds we examined were developed as antagonists at the unperturbed receptor. It is fortunate for the accuracy of the assay that the affinity of [^{3}H]-mepyramine itself is only modestly reduced by 100 mM NaCl, i.e., that there is not a large inhibition of [^{3}H]-mepyramine binding by Na^+ and that the affinity of temelastine, used to define H_1-receptor binding in saturation binding experiments, is effectively unaltered. There is no very clear relationship between antagonist structure and the affinity change induced by Na^+. The structures of a number of the antagonists examined are shown in Figure 6. On the basis of the greater effect of Na^+ on the binding of [^{3}H]-QMDP, a quaternary amine, than on the binding of [^{3}H]-mepyramine, a tertiary amine, we suggested that the presence of the permanent positive charge might be an important factor (Treherne *et al.*, 1991). However, the effect of Na^+ on the binding of norpirdonium and its quaternary derivative, pirdonium, is the reverse of what would have been predicted, since with this pair the shift of the inhibition curve for the tertiary amine is greater than with the permanently charged derivative. For only one of the compounds that we examined, the terfenadine metabolite, MDL 16,455A, was the affinity significantly increased in the presence of Na^+. The effect is much smaller than the order of magnitude increase in the binding affinity of benzamide antagonists at the dopamine D_2-receptor (Jarvie *et al.*, 1987; Neve, 1991), but it does suggest that it would be possible, at least in theory, to develop compounds selective for the conformation of the H_1-receptor stabilized by Na^+. Such a compound would be a useful pharmacological tool for an investigation of the functional significance of what appears to be a highly conserved mechanism for the regulation of agonist binding by intracellular Na^+ and H^+.

References

ARIAS-MONTAÑO, J.A. & YOUNG, J.M. (1993a). Characteristics of histamine H_1 receptors on HeLa cells. *Eur. J. Pharmacol.*, **245**, 291–295.

ARIAS-MONTAÑO, J.A. & YOUNG, J.M. (1993b). Locus of action of Ni^{2+} on histamine-induced inositol phosphate formation in brain slices and in HeLa cells. *Eur. J. Pharmacol.*, **245**, 221–228.

BOLGER, G.T. & SKOLNICK, P. (1986). Novel interactions of cations with dihydropyridine calcium antagonist binding sites in brain. *Br. J. Pharmacol.*, **88**, 857–866.

CHANG, R.S.L. & SNYDER, S.H. (1980). Histamine H_1-receptor binding sites in guinea-pig brain membranes: regulation of agonist interactions by guanine nucleotides and cations. *J. Neurochem.*, **34**, 916–922.

DONALDSON, J. & HILL, S.J. (1986a). Enhancement of histamine H_1-receptor agonist activity by 1,4-dithiothreitol in guinea-pig cerebellum and cerebral cortex. *J. Neurochem.*, **47**, 1476–1482.

DONALDSON, J. & HILL, S.J. (1986b). 1,4-Dithiothreitol-induced alteration in histamine H_1-agonist binding in guinea-pig cerebellum and cerebral cortex. *Eur. J. Pharmacol.*, **129**, 25–31.

FUJIMOTO, K., YOSHIYUKI, H., KAZUSHIGE, S., ITO, S., YE, Q.L. & FUKUI, H. (1993). Genomic cloning of the rat histamine H_1 receptor. *Biochem. Biophys. Res. Commun.*, **190**, 294–301.

HIBERT, M.F., TRUMPP-KALLMEYER, S., HOFLACK, J. & BRUINVELS, A. (1993). This is not a G protein-coupled receptor. *Trends Pharmacol. Sci.*, **14**, 7–12.

HORSTMAN, D.A., BRANDON, S., WILSON, A.L., GUYER, C.A., CRAGOE, E.J.Jr. & LIMBERD, L.E. (1990). An aspartate conserved among G-protein receptors confers allosteric regulation of α_2-adrenergic receptors by sodium. *J. Biol. Chem.*, **265**, 21590–21595.

JARVIE, K.R., NIZNIK, H.B. & SEEMAN, P. (1987). Dopamine D-2 receptors in canine brain: ionic effects on [^{3}H]neuroleptic binding. *Eur. J. Pharmacol.*, **144**, 163–171.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurements with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

MOTULSKY, H.J. & INSEL, P.A. (1983). Influence of sodium on the α_2-adrenergic receptor system of human platelets. *J. Biol. Chem.*, **258**, 3913–3919.

NEVE, K.A. (1991). Regulation of dopamine D2 receptors by sodium and pH. *Mol. Pharmacol.*, **39**, 570–578.

NEVE, K.A., COX, B.A., HENNINGSEN, R.A., SPANOYANNIS, A. & NEVE, R.L. (1991). Pivotal role for aspartate-80 in the regulation of dopamine D2 receptor affinity for drugs and inhibition of adenylyl cyclase. *Mol. Pharmacol.*, **39**, 733–739.

PATERSON, S.J., ROBSON, L.E. & KOSTERLITZ, H.W. (1986). Control by cations of opioid binding in guinea-pig brain membranes. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 6216–6220.

PEDDER, E.K., EVELEIGH, P., POYNER, D., HULME, E.C. & BIRDSALL, N.J.M. (1991). Modulation of the structure-binding relationships of antagonists for muscarinic acetylcholine receptor subtypes. *Br. J. Pharmacol.*, **103**, 1561–1567.

PERT, C.B. & SNYDER, S.H. (1974). Opiate receptor binding of agonists and antagonists affected differentially by Na^+. *Mol. Pharmacol.*, **10**, 868–879.

RODBARD, D. (1974). Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays. *Clin. Chem.*, **20**, 1255–1270.

TREHERNE, J.M., STERN, J.S., FLACK, W.J. & YOUNG, J.M. (1991). Inhibition by cations of antagonist binding to histamine H_1-receptors: differential effect of sodium ions on the binding of two radioligands. *Br. J. Pharmacol.*, **103**, 1745–1751.

YAMASHITA, M., FUKUI, H., SUGAMA, K., YOSHIYUKI, H., ITO, S., MIZUGUCHI, H. & WADA, H. (1991). Expression cloning of a cDNA encoding the bovine histamine H_1 receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 11515–11519.

YERAMIAN, E., GARBARG, M. & SCHWARTZ, J.-C. (1985). N-Ethylmaleimide-induced changes in agonist affinity for histamine H_1-receptors in the guinea-pig brain. *Mol. Pharmacol.*, **28**, 155–162.

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Role of histamine in rodent antinociception

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1 Effects of substances which are able to alter brain histamine levels on the nociceptive threshold were investigated in mice and rats by means of tests inducing three different kinds of noxious stimuli: mechanical (paw pressure), chemical (abdominal constriction) and thermal (hot plate).

2 A wide range of i.c.v. doses of histamine 2HCl was studied. Relatively high doses were dose-dependently antinociceptive in all three tests: 5–100 μg per rat in the paw pressure test, 5–50 μg per mouse in the abdominal constriction test and 50–100 μg per mouse in the hot plate test. Conversely, very low doses were hyperalgesic: 0.5 μg per rat in the paw pressure test and 0.1–1 μg per mouse in the hot plate test. In the abdominal constriction test no hyperalgesic effect was observed.

3 The histamine H_3 antagonist, thioperamide maleate, elicited a weak but statistically significant dose-dependent antinociceptive effect by both parenteral (10–40 mg kg^{-1}) and i.c.v. (1.1–10 μg per rat and 3.4–10 μg per mouse) routes.

4 The histamine H_3 agonist, (R)-α-methylhistamine dihydrogenomaleate was hyperalgesic, with a rapid effect (15 min after treatment) following i.c.v. administration of 1 μg per rat and 3 μg per mouse, or i.p. administration of 100 mg kg^{-1} in mice. In rats 20 mg kg^{-1}, i.p., elicited hyperalgesia only 4 h after treatment.

5 Thioperamide-induced antinociception was completely prevented by pretreatment with a non-hyperalgesic i.p. dose of (R)-α-methylhistamine in the mouse hot plate and abdominal constriction tests. Antagonism was also observed when both substances were administered i.c.v. in rats.

6 L-Histidine HCl dose-dependently induced a slowly occurring antinociception in all three tests. The doses of 250 and 500 mg kg^{-1}, i.p. were effective in the rat paw pressure test, and those of 500 and 1500 mg kg^{-1}, i.p. in the mouse hot plate test. In the mouse abdominal constriction test 500 and 1000 mg kg^{-1}, i.p. showed their maximum effect 2 h after treatment.

7 The histamine N-methyltransferase inhibitor, metoprine, elicited a long-lasting, dose-dependent antinociception in all three tests by both i.p. (10–30 mg kg^{-1}) and i.c.v. (50–100 μg per rat) routes.

8 To ascertain the mechanism of action of the antinociceptive effect of L-histidine and metoprine, the two substances were also studied in combination with the histamine synthesis inhibitor (S)-α-fluoromethylhistidine and with (R)-α-methylhistamine, respectively. L-Histidine antinociception was completely antagonized in all three tests by pretreatment with (S)-α-fluoromethylhistidine HCl (50 mg kg^{-1}, i.p.) administered 2 h before L-histidine treatment. Similarly, metoprine antinociception was prevented by (R)-α-methylhistamine dihydrogenomaleate 20 mg kg^{-1}, i.p. administered 15 min before metoprine. Both (S)-α-fluoromethylhistidine and (R)-α-methylhistamine were used at doses which did not modify the nociceptive threshold when given alone.

9 The catabolism product, 1-methylhistamine, administered i.c.v. had no effect in either rat paw pressure or mouse abdominal constriction tests.

10 These results indicate that the antinociceptive action of histamine may take place on the postsynaptic site, and that its hyperalgesic effect occurs with low doses acting on the presynaptic receptor. This hypothesis is supported by the fact that the H_3 antagonist, thioperamide is antinociceptive and the H_3 agonist, (R)-α-methylhistamine is hyperalgesic, probably modulating endogenous histamine release. L-Histidine and metoprine, which are both able to increase brain histamine levels, are also able to induce antinociception in mice and rats. Involvement of the histaminergic system in the modulation of nociceptive stimuli is thus proposed.

Keywords: Analgesia; antinociception; pain; histamine; L-histidine; (R)-α-methylhistamine; thioperamide; metoprine; (S)-α-fluoromethylhistidine; methylhistamine

Introduction

Modulation of nociception can occur via different neuronal systems. Many neuromediators besides enkephalins, such as acetylcholine (ACh) (Metys *et al.*, 1969; Bartolini *et al.*, 1992), γ-aminobutyric acid (GABA) (Liebman & Pastor, 1980; Malcangio *et al.*, 1992), catecholamines (Jones & Gebhart, 1986) and 5-hydroxytryptamine (5-HT) (Samanin & Valzelli, 1971) have been reported to be involved in nociception control. Recently histamine, which is regarded as an autacoid associated with cutaneous pain (Crossland, 1980), has also been shown to take part in antinociception. Intracerebroventricular (i.c.v.) administration elicits antinociception in both rats (Glick & Crane, 1978; Bhattacharya & Parmar, 1985; Parolaro *et al.*, 1989) and mice (Chung *et al.*, 1984; Oluyomi & Hart, 1991) at relatively high doses. Glick & Crane (1978) also reported that injection of histamine into the rat dorsal raphe nucleus and periaqueductal grey region caused antinociception, while its injection into the median raphe nucleus caused hyperalgesia.

Conversely, the role of endogenous histamine in antinociception has not yet been investigated. Its importance has grown in the last few years with the discovery and definition of a histaminergic neuronal system in mammalian brain. According to many research groups (Steinbusch & Mulder,

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1985; Panula et al., 1989; Wada et al., 1991), in addition to two ascending histaminergic pathways, there is a minor descending pathway which arises from hypothalamic neurones. Its fibres can be found in the dorsal raphe nucleus and periaqueductal grey region, areas which are considered to be important for pain modulation (Basbaum & Fields, 1984).

Furthermore, the existence of presynaptic histamine receptors, called H_3, was reported by Arrang et al. (1983). According to the authors, their stimulation inhibits histamine release (Arrang et al., 1983; Van der Werf et al., 1987) and synthesis (Arrang et al., 1987b). Lately, this same group described the effects of a potent and selective H_3 receptor agonist, (R)-α-methylhistamine (RAMH), and an antagonist, thioperamide (Arrang et al., 1987a; Garbarg et al., 1989). The two molecules were seen to be good tools for studying the role of endogenous histamine.

Besides investigating the effects of a wide range of histamine doses, or acting on the histamine H_3 receptor with RAMH and thioperamide, a further way to study the role of endogenous histamine in antinociception might be to alter histamine brain levels. Different substances such as the histamine precursor, L-histidine, the histidine decarboxylase [EC 4.1.1.22] (HDC), (S)-α-fluoromethylhistidine (FMH) (Kollonitsch et al., 1978) and the histamine-*N*-methyltransferase [EC 2.1.1.8] (HMT) inhibitor, metoprine (Duch et al., 1978), have in fact been described as able to alter selectively histamine brain levels. Oluyomi & Hart (1991) recently reported an antinociceptive effect for histidine, as well as for thioperamide, in the mouse hot plate test, while there are no reports on the effects of histamine synthesis or catabolism inhibitors.

We therefore considered it worthwhile investigating the role of the histaminergic system in antinociception by using all three of the aforementioned strategies in both mice and rats, with three different kinds of antinociceptive tests.

Preliminary data were presented at the XXIth and XXIIth Annual Meetings of the European Histamine Research Society, (Lamberti et al., 1992b; Malmberg-Aiello et al., 1992; 1993) and at the IXth Meeting of the European Society for Neurochemistry (Lamberti et al., 1992a).

Methods

Male Swiss-Webster mice (22–28 g) and Wistar rats (120–180 g) were used. Fifteen mice or four rats were housed per cage. The cages were taken to the experimental room 24 h before the experiment, for acclimatization. The animals were fed *ad libitum* a standard laboratory diet and tap water.

Hot plate test

The method described by O'Callaghan & Holtzman (1976) was adopted, using a stainless steel container (36 × 28 × 30 cm), thermostatically set at 52.5 ± 0.1°C, in a precision water-bath. Mice with a licking latency below 12 and over 18 s in the test before drug administration (30%) were rejected. An arbitrary cut-off time of 45 s was adopted.

Abdominal constriction test

The test was performed in mice according to Koster et al. (1959). The number of stretching movements was counted for 10 min, starting 5 min after 0.6% acetic acid injection.

Paw pressure test

The nociceptive threshold in rats was determined with an analgesymeter (Ugo Basile, Varese, Italy) according to the method described by Leighton et al. (1988). Rats scoring below 50 g or over 80 g during the test before drug administration (25%) were rejected. An arbitrary cut-off value of 250 g was adopted.

Rota-rod test

The integrity of motor coordination was assessed on the basis of the endurance time of the animals on the rotating rod according to Kuribara et al. (1977). On the day of the test, the performance time was measured before and 15 min after treatment.

Drugs

The following drugs were used: histamine dihydrochloride, L-histidine monohydrochloride monohydrate and 1-methylhistamine dihydrochloride (Sigma); (R)-α-methylhistamine dihydrogenomaleate (Bioprojet), (S)-α-fluoromethylhistidine monohydrochloride (Merck Sharp & Dohme Research Lab.), morphine hydrochloride (USL 10/D), metoprine (Burroughs Wellcome) and thioperamide maleate (RBI). The doses given in the text are expressed as salts. All drugs except metoprine were dissolved in isotonic (NaCl 0.9%) saline solution immediately before use. Metoprine was dissolved in 10% aqueous lactic acid and then diluted with saline (1:30). Drug concentrations were prepared in such a way that the necessary dose could be injected in a volume of 10 ml kg^{-1} by both s.c. and i.p. route.

I.c.v. administration was performed in two different ways. For the first method a short ether anaesthesia was adopted. Substances were injected in the necessary dose dissolved in 5 μl for mice and in 10 μl for rats, according to the method described by Haley & McCormick (1957) for mice and extended to rats by us. The second approach consisted of injecting the substances in conscious rats with permanent i.c.v. polyethylene cannulae (5 μl of drug solution + 2 μl air + 5 μl saline) implanted according to the method described by Altaffer et al. (1970), in order to avoid false responses due to the effect of ether. To ascertain the exact site of i.c.v. injection, some mice or rats were injected i.c.v. with 5 μl or 10 μl of 1:10 diluted Indian ink and their brains were examined macroscopically after sectioning.

Statistical analysis

Results are given as the mean ± s.e. Student's two-tailed *t* test was used to verify significance between two means. *P* values of less than 0.05 were considered significant. Multiple comparisons with appropriate controls were made with ANOVA, followed by the multiple range test for least significant differences (LSD). Means with 95% confidence intervals that did not overlap were considered significantly different. ED_{50} and ED_{30} values are the doses which produced respectively the 50% and 30% of the maximum possible effect with 95% confidence limits. Data were analyzed with computer programmes (Tallarida & Murray, 1984, and STATGRAPHICS, 1986, STSC Inc. U.S.A.).

Results

Histamine antinociceptive and hyperalgesic effects

Histamine was administered i.c.v. in doses ranging from 0.05 to 100 μg per mouse and from 0.1 to 100 μg per rat. Very low doses (0.1, 0.5 and 1 μg per mouse) induced significant hyperalgesia 15 min after treatment, while high doses (50 and 100 μg per mouse) elicited antinociception which was still evident 45 min after treatment in the hot plate test (Figure 1a). The ED_{30} for the antinociceptive effect (15 min after treatment) was 96.0 (71.9–144.7) μg per mouse i.c.v.

The same biphasic effect was observed in the paw pressure test on rats. Histamine caused statistically-significant hyperalgesia 30 min after treatment at 0.5 μg per rat administered i.c.v. during ether anaesthesia and at both 0.5 and 1 μg per rat i.c.v., 45 min after treatment; antinociception was detectable at doses of 5–50 μg per rat (Figure 2a). In order to

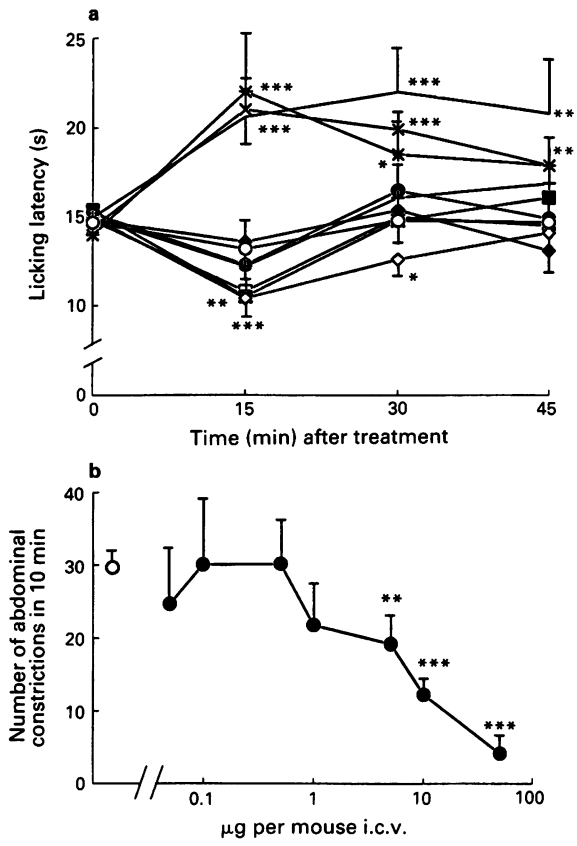


Figure 1 Effect of i.c.v. histamine 2HCl on the nociceptive threshold in mice. (a) Biphasic effect in the hot plate test. Groups were treated (μg per mouse i.c.v.) as follows: saline 5 μl (○); histamine 0.05 (●); 0.1 (□); 0.5 (■); 1 (◇); 5 (◆); 10 (+); 50 (×) and 100 (*). In comparison, the effect of morphine HCl 5 mg kg^{-1}, s.c. (no symbol) is shown. (b) Antinociceptive effect in the abdominal constriction test. Saline (○) and histamine (●) were injected 15 min before the test. $*P<0.05$; $**P<0.01$; $***P<0.001$ versus saline controls. Each point represents the mean (with s.e.mean) of 11–79 mice.

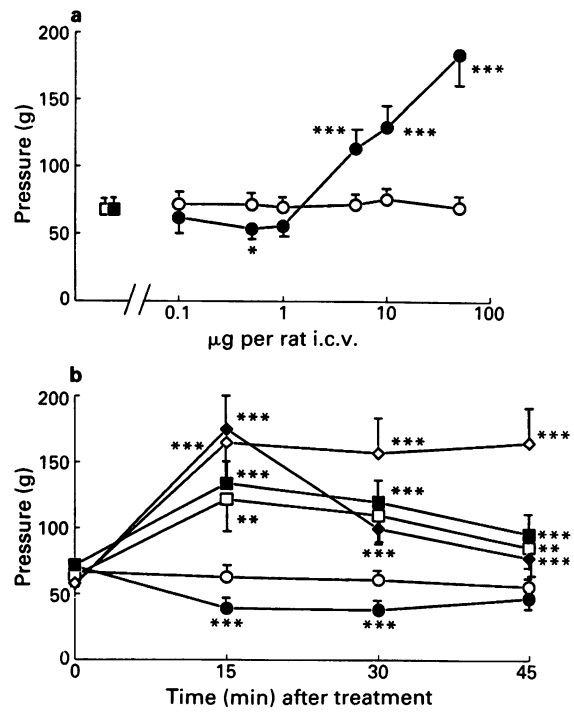


Figure 2 Biphasic effect of histamine on the nociceptive threshold in the rat paw pressure test. (a) Dose-response curve of histamine effect. Saline 10 μl per rat (○) and histamine 2HCl (●) were administered by i.c.v. injection during short ether anaesthesia, 30 min before test. Symbols (□) and (■) indicate pretest values of saline and histamine-treated groups respectively. (b) Effects on rats with permanent i.c.v. cannulae. Groups were (dose per rat i.c.v.): saline 5 μl (○), histamine 2HCl 0.5 μg (●), 10 μg (□), 50 μg (■), 100 μg (◆). In comparison the effect of morphine 5 mg kg^{-1}, s.c. (◇) is shown. $*P<0.05$; $**P<0.01$; $***P<0.001$ versus saline controls. Each point represents the mean (with s.e.mean) of 7–23 rats.

make sure that ether anaesthesia did not affect the results hyperalgesic and antinociceptive doses of histamine were also administered to conscious rats via permanent i.c.v. cannulae. The results obtained confirmed our previous observations and revealed a maximum effect at 15 min (Figure 2b), with an ED_{50} of 69.0 (13.3–356.9) μg per rat i.c.v.

In the mouse abdominal constriction test only the antinociceptive effect was detectable. Histamine induced a statistically-significant antinociception at 5, 10 and 50 μg per mouse with an ED_{50} of 6.7 (3.8–11.7) μg per mouse i.c.v. (Figure 1b). The antinociception induced by 50 μg per mouse is comparable to that induced by 2 mg kg^{-1} of morphine HCl (5.4 ± 2.0 constrictions 15 min after s.c. administration), and a comparison with the effects of morphine in the hot plate and paw pressure tests is given in Figures 1a and 2b, respectively.

The histamine dose of 50 μg per mouse was also used to study mouse rota-rod performance 15 min after treatment. No effect was seen on endurance time on the rod in histamine- and saline-treated mice (186 ± 40 s versus 229 ± 23 s of controls).

Thioperamide antinociceptive effect

In all three tests thioperamide was able to induce a statistically-significant antinociception. Both i.c.v. and parenteral routes of administration were used.

In the mouse hot plate test, 20 mg kg^{-1}, i.p. appeared to be the optimum dose for eliciting antinociception (Table 1), which persisted up to 30 min with a maximum response at 15 min. A lower (10 mg kg^{-1}, i.p.) or higher (40 mg kg^{-1}, i.p.) dose was less effective in raising the nociceptive threshold. When given i.c.v. in doses ranging from 0.5 to 10 μg per mouse, no statistically-significant effect was observed for thioperamide (data not shown).

Conversely, in the mouse abdominal constriction test, i.c.v. administration of thioperamide showed an initial dose-dependent action with a maximum effect at 10 μg per mouse, while a higher dose (30 μg per mouse) was not effective in significantly decreasing the number of abdominal constrictions (Figure 3a). Similarly, subcutaneous administration revealed a statistically-significant antinociception only for the same dose which elicited the maximum effect in the hot plate test (Figure 3b).

Thioperamide antinociception was confirmed in the rat paw pressure test, where the time-course for the dose of 20 mg kg^{-1}, i.p. reflected the one observed with the hot plate test (Figure 4a). Both 1.1 and 10 μg per rat administered via permanent i.c.v. cannulae were antinociceptive; the effect of the latter was still significant 2 h after treatment (Table 2).

Effect of (R)-α-methylhistamine (RAMH) on nociceptive threshold

When given at sufficiently high doses, RAMH was able to induce hyperalgesia in the hot plate and paw pressure tests.

In the mouse hot plate test, three different i.p. doses were used. The doses of 5 and 20 mg kg^{-1} were ineffective in modifying the nociceptive threshold, while 100 mg kg^{-1} elicited a highly significant hyperalgesia up to 60 min after treatment (Table 1). The dose 20 mg kg^{-1}, i.p. also had no effect in the abdominal constriction test (Figure 3b).

Table 1 Effects of thioperamide and (R)-α-methylhistamine (RAMH) i.p. alone and combined in the mouse hot plate test

Pretreatment (mg kg^{-1}, i.p.)	*Treatment* (mg kg^{-1}, i.p.)	n	*Licking latency* (s) *Pretest*	*15 min*	*30 min*	*45 min*	*60 min*
					after treatment		
Saline 10 ml	Saline 10 ml	47	14.6 ± 0.3	13.5 ± 0.4	12.9 ± 0.4	13.6 ± 0.4	13.9 ± 0.4
	Thioper 10	14	14.4 ± 0.4	16.0 ± 1.4*	15.3 ± 1.0*	14.0 ± 0.9	13.1 ± 0.7
Saline	Thioper 20	12	14.6 ± 0.3	19.6 ± 0.9***	18.3 ± 1.5***	15.5 ± 1.5	14.3 ± 0.7
	Thioper 40	14	14.5 ± 0.3	16.0 ± 1.0*	15.0 ± 1.1*	13.7 ± 1.1	14.2 ± 0.8
	RAMH 5	5	14.6 ± 1.1	12.4 ± 1.4	12.4 ± 1.1	12.2 ± 0.9	14.8 ± 0.7
RAMH 20	Saline	30	14.3 ± 0.4	12.2 ± 0.9	13.1 ± 0.7	13.5 ± 0.6	14.6 ± 1.1
	RAMH 100	11	14.5 ± 0.6	9.6 ± 0.8***	9.7 ± 1.0**	9.4 ± 1.0***	11.6 ± 0.9*
RAMH 20	Thioper 20	10	14.5 ± 0.6	12.7 ± 0.8^^	13.4 ± 0.9^	14.4 ± 1.2	13.0 ± 0.8

*$P<0.05$; **$P<0.01$; ***$P<0.001$ versus saline controls.
^P0.02; ^^$P<0.001$ versus thioperamide (20 mg kg^{-1})-treated mice.
Pretreatment was performed 15 min before treatment.

A wide range of doses, from 1 to 100 μg per mouse, was tested by i.c.v. route in the hot plate test, but only 3 μg per mouse significantly lowered the latency from 13.0 ± 0.5 s of controls to 9.7 ± 0.6 s in 17 mice 15 min after treatment. The highest doses tested, 50 and 100 μg per mouse, caused immobility and convulsions.

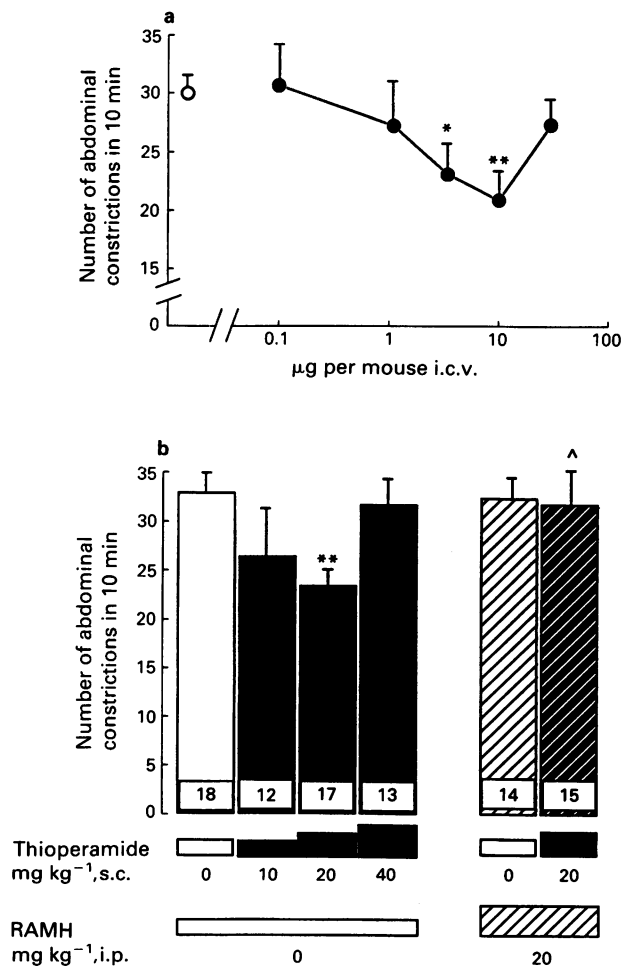


Figure 3 Antinociceptive effect of i.c.v. and s.c. thioperamide in the mouse abdominal constriction test. (a) Thioperamide maleate was administered i.c.v. 15 min before test. Each point represents the mean of 12–19 mice. (b) Antinociceptive effect of thioperamide and its antagonism by (R)-α-methylhistamine (RAMH). RAMH dihydrogenomaleate was administered 15 min before thioperamide and thioperamide s.c. 15 min before test. Inside the columns is shown the number of mice. Vertical lines give s.e.mean. *$P<0.02$; **$P<0.01$ versus saline controls. ^$P<0.05$ versus thioperamide (20 mg kg^{-1})-treated mice.

Similarly, in the rat paw pressure test 1 μg per rat administered i.c.v. via permanent cannulae was significantly hyperalgesic (Table 2). The dose of 20 mg kg^{-1} was tested by parenteral administration in rats. The threshold was gradually lowered, and a statistically-significant hyperalgesia was observed from 4 to 7 h after treatment (Figure 4b).

Antagonism by (R)-α-methylhistamine of thioperamide antinociception

In all three tests RAMH administered 15 min before thioperamide completely prevented the antinociception induced by the latter. When tested in mice, both RAMH and thioperamide were administered parenterally at 20 mg kg^{-1} (Table 1 and Figure 3b), while in the rat paw pressure test i.c.v. administration via permanent cannulae was adopted (Table 2).

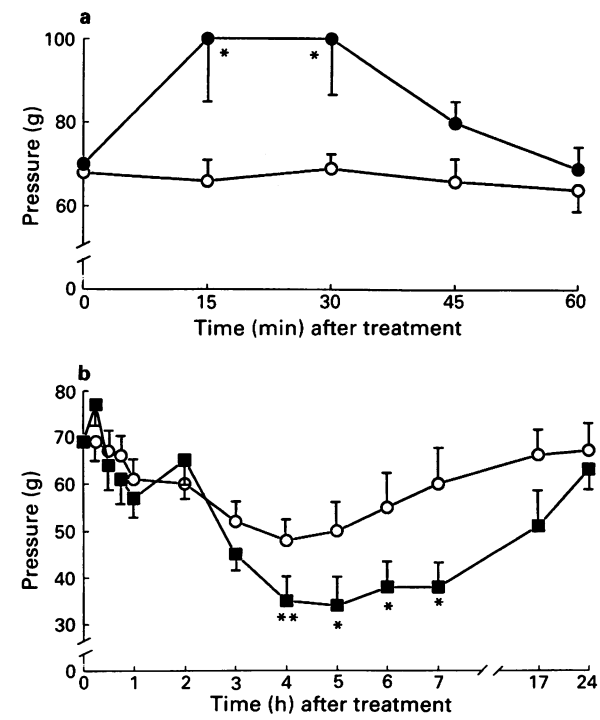


Figure 4 Effects of thioperamide and (R)-α-methylhistamine (RAMH) on nociceptive threshold in the rat paw pressure test. (a) Antinociceptive effect of thioperamide maleate 20 mg kg^{-1}, i.p. (●) and (b) hyperalgesic effect of RAMH dihydrogenomaleate 20 mg kg^{-1}, i.p. (■) in comparison with saline-treated (○) rats. Each point represents the mean with s.e.mean of 7–11 rats. *$P<0.05$; **$P<0.01$ versus saline controls.

Table 2 Effects of thioperamide and (R)-α-methylhistamine (RAMH) i.c.v. alone and combined in the rat paw pressure test

Pretreatment (μg per rat)	*Treatment* (μg per rat)	n	*Pressure* (g) *Pretest*	*15 min*	*30 min*	*45 min after treatment*	*60 min*	*120 min*
Saline 10 μl	Saline 10 μl	19	60 ± 2	60 ± 2	58 ± 1	57 ± 2	57 ± 2	57 ± 3
	Thioper 1.1	9	60 ± 2	87 ± 6***	77 ± 6**	63 ± 4	56 ± 2	–
Saline	Thioper 10	10	60 ± 2	102 ± 10***	84 ± 6***	68 ± 4*	66 ± 3*	71 ± 3**
RAMH 1	Saline	14	63 ± 1	49 ± 3**	54 ± 2	46 ± 2*	47 ± 2**	45 ± 2**
RAMH 1	Thioper 10	12	58 ± 2	64 ± 2^^	58 ± 2^^	56 ± 2^	58 ± 2^	–

*$P<0.05$; **$P<0.01$; ***$P<0.001$ versus saline-treated rats.
^P0.05; ^^$P<0.001$ versus thioperamide (10 μg)-treated rats.
Pretreatment was performed 15 min before treatment.

L-Histidine antinociceptive effect

L-Histidine was able to raise significantly the nociceptive threshold in both rats and mice in all three tests.

In the rat paw pressure test the antinociceptive effect was dose-dependent and for both doses used (250 and 500 mg kg^{-1}, i.p.), it lasted until 3 h after treatment, the highest one producing a peak effect 2 h after administration (Figure 5).

In the mouse abdominal constriction test also, histidine antinociception followed a pattern which was both time- and dose-dependent. Both doses used in this test, 500 and 1000 mg kg^{-1} i.p., decreased the number of abdominal constrictions with a maximum effect 2 h after treatment. At this same time the percentage of inhibition was 34% and 69% respectively (Figure 6a).

In the mouse hot plate test the dose-dependent rise in the nociceptive threshold was very small but significant, and for the highest dose (1500 mg kg^{-1}, i.p.) was detectable from 30 to 180 min after treatment (Figure 6b). Despite this high dose, the antinociception obtained produced no visible change in the animals' normal behaviour.

Antagonism of histidine-induced antinociception by (S)-α-fluoromethylhistidine (FMH)

In all three tests FMH, administered 2 h before histidine treatment, was able to prevent completely the antinociception induced by the latter (Figures 5 and 6). FMH was given at a dose (50 mg kg^{-1}, i.p.) which neither modified the nociceptive threshold when given alone, nor altered normal animal behaviour.

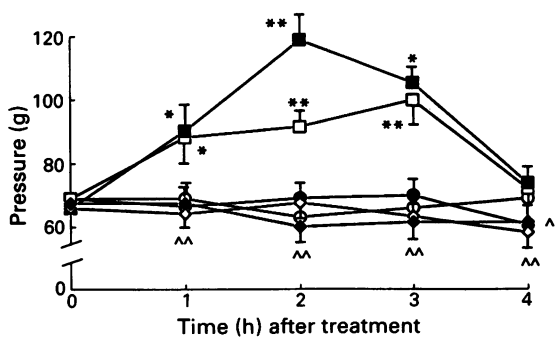


Figure 5 Antinociceptive effect of L-histidine and its antagonism by (S)-α-fluoromethylhistidine (FMH) in the rat paw pressure test. Groups (pretreatment i.p. + treatment i.p.) were as follows: saline 0.1 ml 10 g^{-1} + saline (○); FMH HCl 50 mg kg^{-1} + saline (●); saline + L-histidine HCl 250 mg kg^{-1} (□); saline + L-histidine 500 mg kg^{-1} (■); FMH 50 mg kg^{-1} + L-histidine 250 mg kg^{-1} (◇); FMH 50 mg kg^{-1} + L-histidine 500 mg kg^{-1} (◆). Pretreatment was performed 2 h before treatment. *$P<0.01$; **$P<0.001$ versus saline controls. ^$P<0.05$; ^^$P<0.01$ versus L-histidine-treated mice. Each point represents the mean (with s.e.mean) of 9–20 rats.

Metoprine antinociceptive effect

In the mouse hot plate test, doses of 5, 20 and 30 mg kg^{-1}, i.p. of metoprine were studied. The antinociception observed was dose-dependent: 5 mg kg^{-1} was ineffective, while at 20 mg kg^{-1} the antinociceptive effect was significant for 1 h and at 30 mg kg^{-1} a very strong effect lasted up to 24 h (Figure 7a). Mice treated with the highest dose showed slight excitation inside their cages; this became more noticeable when they were placed on the plate. Calculations revealed an ED_{50} of 19.5 (13.5–28.3) mg kg^{-1}, i.p. 15 min after treatment.

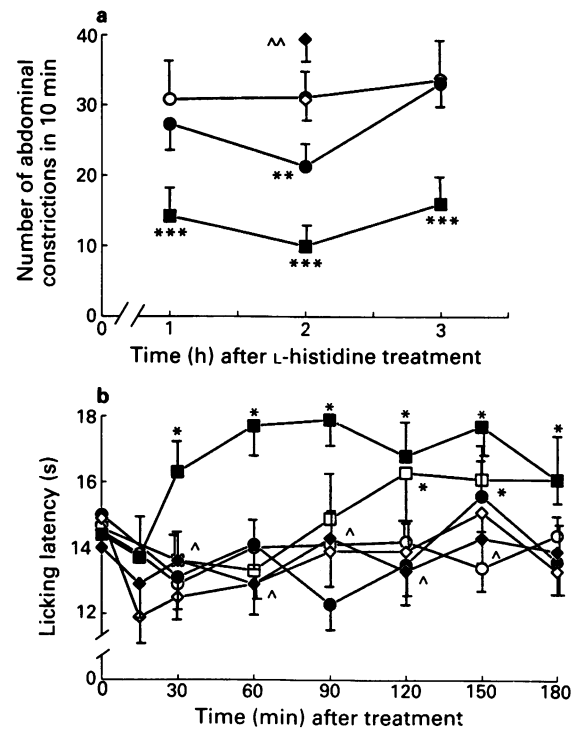


Figure 6 Time course of L-histidine antinociception and its antagonism by (S)-α-fluoromethylhistidine (FMH) in mice. (a) Abdominal constriction test. FMH was administered 2 h before L-histidine and L-histidine 1, 2 or 3 h before test. Groups (pretreatment i.p. + treatment i.p.) were: saline 0.1 ml 10 g^{-1} + saline (○); saline + L-histidine HCl 500 mg kg^{-1} (●); L-histidine 1000 mg kg^{-1} (■); FMH HCl 50 mg kg^{-1} + saline (◇); FMH + L-histidine 500 mg kg^{-1} (◆). Each point represents the mean of 9–16 mice. **$P<0.01$; ***$P<0.001$ versus saline controls. ^^$P<0.001$ versus L-histidine 500 mg kg^{-1} (2 h before test)-treated mice. (b) Hot plate test. Groups (pretreatment i.p. + treatment i.p.) were as follows: saline 0.1 ml 10 g^{-1} + saline (○); L-histidine 250 mg kg^{-1} (●); L-histidine 500 mg kg^{-1} (□); saline + L-histidine 1500 mg kg^{-1} (■); FMH 50 mg kg^{-1} + saline (◇); FMH 50 mg kg^{-1} + L-histidine 1500 mg kg^{-1} (◆). Pretreatment was performed 2 h before treatment. Each point represents the mean of 12–26 mice. *$P<0.05$ versus saline controls. ^$P<0.05$ versus L-histidine (1500 mg kg^{-1})-treated mice.

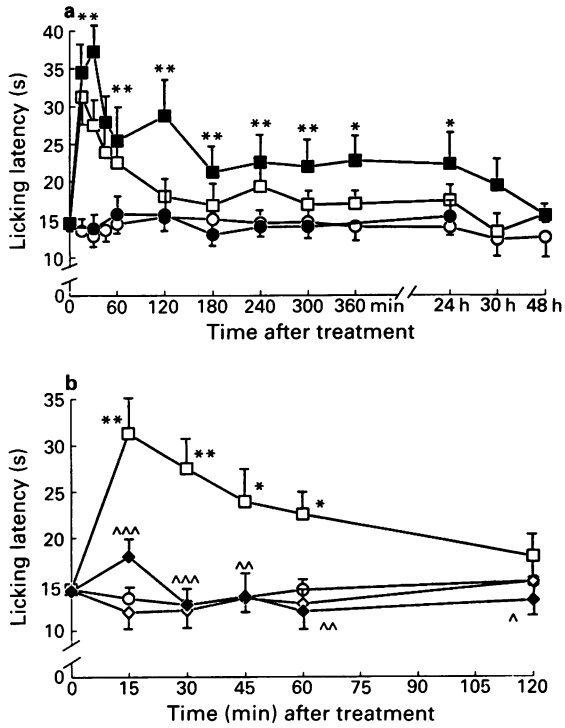


Figure 7 Antinociceptive effect of metoprine (a) and its antagonism by (R)-α-methylhistamine (RAMH) (b) in the mouse hot plate test. Groups (pretreatment i.p. + treatment i.p.) were as follows: saline 0.1 ml 10 g^{-1} + lactic acid 0.3% in saline (○); metoprine 5 mg kg^{-1} (●); saline + metoprine 20 mg kg^{-1} (□); metoprine 30 mg kg^{-1} (■); RAMH dihydrogenomaleate 20 mg kg^{-1} + lactic acid 0.3% (◇); RAMH + metoprine 20 mg kg^{-1} (◆). Pretreatment was performed 15 min before treatment. $^{*}P<0.01$; $^{**}P<0.001$ versus saline controls. $^{\wedge}P<0.05$; $^{\wedge\wedge}P<0.01$; $^{\wedge\wedge\wedge}P<0.001$ versus metoprine (20 mg kg^{-1})-treated mice. Each point represents the mean (with s.e.mean) of 11–24 mice.

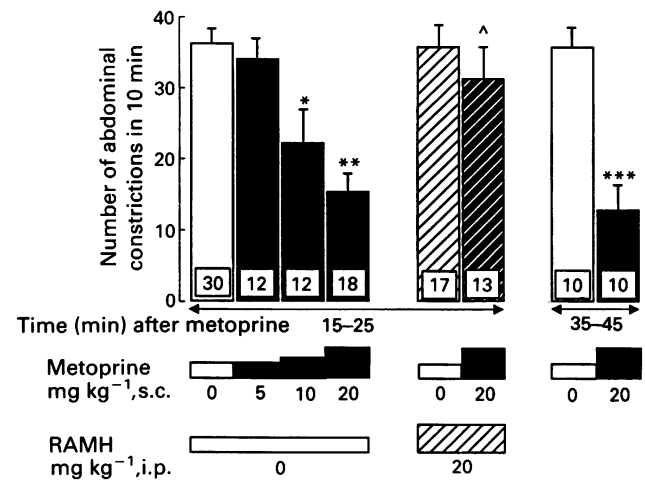


Figure 8 Metoprine antinociception and its antagonism by (R)-α-methylhistamine (RAMH) in the mouse abdominal constriction test. RAMH dihydrogenomaleate was administered 15 min before metoprine. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ versus controls. $^{\wedge}P<0.01$ versus metoprine (20 mg kg^{-1})-treated mice. Inside the columns is shown the number of mice.

The same dose-dependent antinociceptive effect was observed in the mouse abdominal constriction test. The number of stretching movements was significantly reduced with 10 and 20 mg kg^{-1}, i.p. 15–25 min after treatment with an ED_{50} of 15.3 (10.3–22.8) mg kg^{-1}, i.p. and 20 min later the effect for the dose 20 mg kg^{-1} was even more significant (Figure 8). In the rat paw pressure test, metoprine was studied after administration by both i.p. and i.c.v. route (Figure 9). Systemically the doses of 10 and 20 mg kg^{-1} were used; both were significantly antinociceptive and at the highest dose the effect lasted up to 6 h. For i.c.v. administration in rats with permanent cannulae, the doses of 50 and 100 μg per rat were also observed to produce a dose-dependent, long-lasting (6 h) antinociception.

Metoprine effect in the rota-rod test

Metoprine at 20 mg kg^{-1} did not cause any impairment to performance 15 min after i.p. administration. The endurance time on the rotating rod was 282 ± 17 s before and 285 ± 14 s after treatment for control group, and 291 ± 9 s and 297 ± 3 s respectively for metoprine-treated mice (10 animals per group).

Antagonism by (R)-α-methylhistamine (RAMH) of metoprine-induced antinociception

RAMH (20 mg kg^{-1}, i.p.) was able to prevent the antinociception induced by 20 mg kg^{-1}, i.p. of metoprine administered 15 min later in all three tests (Figures 7b, 8, 9a).

In both the rat paw pressure and mouse hot plate tests antinociception was significantly reduced as early as 15 min

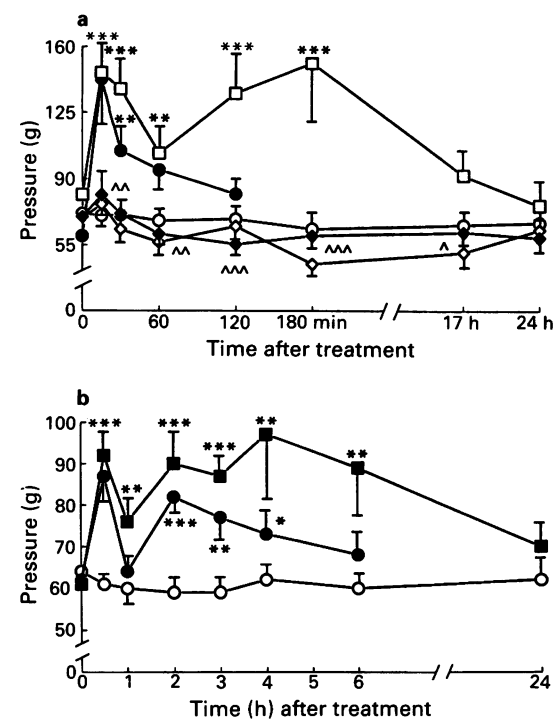


Figure 9 Antinociceptive effect of metoprine in the rat paw pressure test. (a) Effect of i.p. metoprine and its antagonism by (R)-α-methylhistamine (RAMH). Groups (pretreatment i.p. + treatment i.p.) were as follows: saline 0.1 ml 10 g^{-1} + lactic acid 0.3% in saline (○); metoprine 10 mg kg^{-1} (●); saline + metoprine 20 mg kg^{-1} (□); RAMH dihydrogenomaleate 20 mg kg^{-1} + lactic acid 0.3% (◇); RAMH + metoprine 20 mg kg^{-1} (◆). Pretreatment was performed 15 min before treatment. Each point represents the mean of 11–19 rats. (b) Effect of i.c.v. metoprine on rats with permanent cannulae. Groups were (dose per rat i.c.v.): lactic acid 1% in saline 5 μl (○); metoprine 50 μg (●); metoprine 100 μg (■). Each point represents the mean of 7–11 rats. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ versus control group. $^{\wedge}P<0.05$; $^{\wedge\wedge}P<0.01$; $^{\wedge\wedge\wedge}P<0.001$ versus metoprine (20 mg kg^{-1})-treated rats. Vertical lines give s.e.mean.

Table 3 Effect of i.c.v. 1-methylhistamine (MH) on pain threshold in the rat paw-pressure and mouse abdominal constriction tests

Treatment (μg per animal)	*Rat paw pressure test* *Pressure* (g) *Pretest*	*30 min* after treatment	*45 min* after treatment	*60 min* after treatment	*Abdom. constr. test* *Constrictions* *in 10 min*
Saline	59.1 ± 2.0 (6)	58.3 ± 3.0	56.6 ± 3.3	58.3 ± 3.0	28.6 ± 3.3 (19)
MH 0.5	61.6 ± 3.5 (9)	61.1 ± 3.0	62.2 ± 2.2	52.2 ± 2.7	–
MH 1	–	–	–	–	35.5 ± 5.5 (6)
MH 5	–	–	–	–	34.8 ± 4.8 (6)
MH 10	62.2 ± 3.6 (9)	63.3 ± 3.3	58.8 ± 3.8	58.8 ± 2.0	32.7 ± 1.5 (7)
MH 50	–	–	–	–	27.0 ± 3.5 (7)

In parentheses is shown the number of animals

after metoprine treatment, and from 30 min on the antagonism was complete.

The dose of RAMH used did not modify the nociceptive threshold in any of the three tests.

Lack of 1-methylhistamine effect in antinociception

1-Methylhistamine (MH) did not modify the pain threshold in either the mouse abdominal constriction test or the rat paw pressure test (Table 3).

In the abdominal constriction test, MH was studied at a wide range of doses (1–50 μg per mouse, i.c.v.). None of them reduced the number of constrictions induced by acetic acid.

In the paw pressure test also the two doses used (0.5 and 10 μg per rat i.c.v.) were ineffective in modifying the threshold pressure.

Discussion

The present results clearly show that histamine can be either antinociceptive or hyperalgesic depending on the dose. The apparently contradictory effects of this drug might be due to its dose-related action either on the presynaptic receptor or on a postsynaptic receptor, since the selective H_3 receptor antagonist thioperamide caused antinociception, and the selective H_3 receptor agonist, RAMH caused hyperalgesia.

Owing to the reportedly poor ability of histamine to cross the blood-brain barrier (Snyder *et al.*, 1964; Schwartz *et al.*, 1971a), the i.c.v. route of administration was always used. Histamine-induced antinociception, probably due to an action on postsynaptic H_1 or H_2 receptors, was observed in all three tests, showing a dose-dependent relationship. So far, our results confirm previous observations by Glick & Crane (1978), Bhattacharya & Parmar (1985) and Parolaro *et al.* (1989) on rats and Chung *et al.* (1984) and Oluyomi & Hart (1991) on mice. The paw pressure and abdominal constriction seem to be more sensitive tests than the hot plate for detecting histamine antinociceptive effects, as can be noticed from ED_X values. In fact, for the hot plate test, the same cut-off time of 45 s was maintained in order to allow comparisons with the other substances used, but an ED_{30} rather than ED_{50} value needed to be calculated, due to the low antinociceptive effect elicited by histamine. Such discrepancies might be due to the different kinds of noxious stimulus used, i.e. mechanical, chemical and thermal. However, a similar differential sensitivity to analgesic tests is also shared by morphine, some κ-agonists and eseroline (Tyers, 1980; Bartolini *et al.*, 1981b; Yaksh & Noueihed, 1985).

The high reaction scores measured in the three analgesic tests are likely to be due to a real antinociception and not to the loss of ability to react to noxious stimuli, since motor coordination assessed with the rota-rod test was unaffected after the administration of 50 μg per mouse of histamine. The latter dose was preferred to the highest one tested for the rota-rod experiment, since during the hot plate experiments some mice treated with 100 μg i.c.v. developed facial convulsions when held by the tail. Moreover, also Glick & Crane (1978) described catalepsy, besides antinociception, following i.c.v. administration of 200–400 μg per rat of histamine. Further support to histamine acting as a modulator of nociception is given by the experiments of Braga *et al.* (1992). These authors demonstrated that i.c.v. injection of histamine in arthritic rats at the same doses which were antinociceptive in our experiments in the paw pressure test, inhibited the firing of nociceptive thalamic neurones after a noxious stimulus.

Conversely, low doses of histamine were hyperalgesic in the mouse hot plate and rat paw pressure tests. Doses ranging from 50 to 500 fold lower than the analgesic dose in the hot plate and 10 fold lower in the paw pressure test induced a significant decrease in nociceptive threshold (Figures 1a and 2). The dose of 0.5 μg per rat was significantly effective both 30 and 45 min after treatment in the paw pressure test on rats into which histamine was injected during short ether anaesthesia, and the same was adopted for experiments on rats with permanent i.c.v. cannulae, to confirm the data in the absence of ether. This hyperalgesic effect might not seem contradictory when one takes into account the fact that H_3 receptors are 100 fold more sensitive to histamine than the postsynaptic receptors in rat brain slice preparations (Arrang *et al.*, 1983). Thus, hyperalgesia induced by low doses of histamine might be due to a preferential action on H_3 receptors, and, consequently, to inhibition of its own release.

On the other hand, in the mouse abdominal constriction test, histamine-induced hyperalgesia was not detected, as is the case with many other drugs such as arecaidine propargyl ester and CGP 35348, which are hyperalgesic in many tests but inactive in the mouse abdominal constriction test (Bartolini *et al.*, 1992; Malcangio *et al.*, 1991). One reason for this lack might be the abdominal constriction test itself. In fact, as reported by Itoh *et al.* (1989), histamine turnover is enhanced after i.p. injection of 0.7% acetic acid. Thus, if histamine release is enhanced during the abdominal constriction test, the putative hyperalgesic effect of low doses of exogenous histamine is overcome by the augmented amount of endogenous histamine in the brain.

Subsequently, in order to verify our hypothesis that low doses of histamine act at the presynaptic receptor, we studied the effects of both an antagonist and an agonist of the H_3 receptor. Thioperamide, the selective histamine H_3 receptor

antagonist, elicited antinociception in all three tests. It has been reported that parenteral administrations of thioperamide are able to increase histamine turnover (Arrang *et al.*, 1987a; Garbarg *et al.*, 1989; Oishi *et al.*, 1989; Taylor *et al.*, 1992) or release (Itoh *et al.*, 1991) in brain tissues, indicating that it crosses the blood-brain barrier. Thus, our first approach was to study thioperamide effects following parenteral administration. Subsequently, in order to verify whether the site of action of the antinociceptive effect observed with parenteral administration was in the CNS we used the i.c.v. route. Furthermore, for this route we used thioperamide doses (1.1 μg per mouse or rat) which corresponded on a molar basis to a hyperalgesic dose of histamine (0.5 μg per mouse or rat). Despite the lack of any statistically-significant effects of i.c.v.-administered thioperamide in the hot plate test, antinociception was evident in both abdominal constriction and paw pressure tests for both administration routes. This is in agreement with the reported action of thioperamide as a histamine H_3 receptor antagonist, and the antinociception observed might well be due to blockade of the H_3 receptor and, consequently, to increased endogenous histamine release. This mechanism of action is not surprising if one bears in mind that two postsynaptic antagonists of the opioid and cholinergic systems naloxone and atropine, have also been demonstrated to induce antinociception when administered at low doses which are ineffective on the postsynaptic site, but which are capable of blocking the respective presynaptic receptors (Levine *et al.*, 1979; Ghelardini *et al.*, 1990).

In the abdominal constriction and hot plate tests it was also observed that the antinociceptive effect diminished with increasing doses of thioperamide. Since thioperamide has been reported to have a K_i of 4 nM on the H_3 receptor and $>10{,}000$ nM on the H_1 or H_2 receptors (Schwartz *et al.*, 1990), the hypothesis of a postsynaptic antagonism can be ruled out. Instead, competition on the H_3 receptor between thioperamide and the endogenous histamine released might be postulated. When the dose of thioperamide is sufficiently strong, the amount of histamine released is high enough to compete with thioperamide, thus activating the mechanism of negative feedback on the release and, consequently, antagonizing thioperamide antinociceptive effect. This hypothesis might also explain the low maximum effect of thioperamide observed by us.

As for the histamine H_3 agonist, RAMH, hyperalgesia was observed at 3 μg per mouse or 1 μg per rat i.c.v. as soon as 15 min after treatment. In order to assess whether a quick hyperalgesia is also obtainable following systemic administration, a dose as high as 100 mg kg^{-1} was used in the hot plate test, since 20 mg kg^{-1}, i.p. had no effect within 1 h of treatment. Such a rapid effect is probably due to inhibition of histamine release. Conversely, the delayed hyperalgesia observed in the rat paw pressure test following the injection of 20 mg kg^{-1} i.p. (from 4 to 7 h after treatment) might be due to histamine synthesis inhibition. Garbarg *et al.* (1989) in fact reported an increase in synaptosomal histamine and a decrease in N^{τ}-methylhistamine levels which reached statistical significance 3 h after oral administration of 10 mg kg^{-1} of RAMH (expressed as the base), and a reduction in cortical [^{3}H]-histamine synthesis which was already significant 30 min after treatment. All these effects lasted up to 6 h. Thus, it might be postulated that the hyperalgesia observed in our conditions is due to a dose-dependent effect of RAMH on endogenous histamine release. Moreover, hyperalgesia induced by stimulation of autoreceptors is not unusual: another example is in the cholinergic system, where the presynaptic agonist, arecaidine is able to lower the nociceptive threshold in both hot plate and paw pressure tests (Bartolini *et al.*, 1992).

Pretreatment with an i.p. dose of RAMH which did not modify the pain threshold in mice completely prevented the antinociception induced by 20 mg kg^{-1} of thioperamide in both abdominal constriction and hot plate tests. This antagonism might reflect the action of both substances on the same receptor, since for both of them a high selectivity on the histamine H_3 receptor has been reported (Arrang *et al.*, 1987b; Garbarg *et al.*, 1989). In the rat paw pressure test a similar antagonism was also observed following i.c.v. administration. Although 1 μg per rat of RAMH had an hyperalgesic effect, the antagonism is probably not due to a simple summing of the effects of the two substances, since the values obtained with double treatment are much lower than those resulting from the subtraction of the hyperalgesic effect of RAMH from the analgesic effect of thioperamide. On the other hand, the dose of 10 μg per rat of thioperamide was preferred as eliciting a clearer and longer-lasting antinociception, while for RAMH the dose of 1 μg per rat was chosen as about a ten fold lower dose (on a molar basis) than the thioperamide one. The choice was made taking into account that for RAMH and thioperamide a 1:8 affinity ratio has been reported for the H_3 receptor (Taylor *et al.*, 1992). Conversely, when i.p. or s.c. administration was performed (i.e. in experiments on mice), two equal doses were adopted for the two substances, since, despite its higher affinity for the H_3 receptor, RAMH seems to have even more difficulty in crossing the blood-brain barrier than thioperamide (Taylor *et al.*, 1992).

The histaminergic system in antinociception was further studied by altering histamine brain levels. The choice of histidine as a tool for studying the role of endogenous histamine in antinociception was made on the basis that, as Schwartz *et al.* (1972) and Abou *et al.* (1973) demonstrated, its decarboxylating enzyme is not saturated in normal conditions. Systemic administrations of L-histidine are therefore able to enhance brain histamine levels.

In our experiments on rats, L-histidine induced antinociception with both doses used, with a maximum effect at 2–3 h after treatment. This time-course reflects that of endogenous brain histamine levels observed in rats by Schwartz *et al.* (1972) and Sheiner *et al.* (1985) after i.p. administration of L-histidine at the same doses: brain histidine levels were maximal within 1 h of a loading of 500 mg kg^{-1}, i.p., halved at 1.5 h and constantly decreased thereafter, while histamine levels reached peak values within 2–3 h of injection. This seems to support the hypothesis that antinociception is due to histamine deriving from the conversion of L-histidine, and not to histidine itself.

In mice, too, antinociception takes place slowly after L-histidine administration. In the abdominal constriction test the maximal inhibition of stretching movements was observed 2 h after treatment, while in the hot plate test antinociception reached a plateau from 1 to 2.5 h for the highest dose; in both tests the effect diminished 3 h after histidine injection. As seems also to be the case with rats, the antinociception in mice occurs parallel to, though later than, the alterations in brain histamine levels observed after a single (850 and 1000 mg kg^{-1}) or multiple (3 × 1000 mg kg^{-1}) i.p. L-histidine injection (Taylor & Sneider, 1972; Cosentin *et al.*, 1974).

With L-histidine as with histamine, the mouse hot plate is less sensitive than the mouse abdominal constriction and rat paw pressure tests. In fact, in the hot plate test, statistically-significant antinociception was found at the doses of 500 and 1500 mg kg^{-1}, i.p., although the effect was very small. The latter dose might seem to be high, but it should be considered that another amino acid, L-DOPA, also needs to be administered at a dose of 1000 mg kg^{-1} in order to show any effect (Blaschko & Chrusciel, 1960). Nevertheless, even at the highest dose of L-histidine, the gross behaviour of animals was not modified during our experiments, as formerly reported by Schwartz *et al.* (1972) for rats and by Abou *et al.* (1973) for rabbits.

Further evidence of the fact that L-histidine-induced antinociception is due to its conversion into histamine is given by our results obtained with FMH. This substance, developed by Kollonitsch *et al.* (1978) as a highly selective irreversible

HDC inhibitor, has been demonstraed to halve brain histamine levels in mice within 1 h, up to 24 h (Garbarg *et al.*, 1980; Maeyama *et al.*, 1982; 1983), and in rats (Oishi *et al.*, 1984). The brain histamine levels which are reduced by FMH are likely to be those of neural cells, since in mutant mice devoid of mast cells brain histamine is almost totally abolished by FMH (Maeyama *et al.*, 1983). Thus, total prevention by FMH of L-histidine-induced antinociception at all the times considered was probably due to blockade of neuronal HDC activity.

The present findings partly contradict those of Oluyomi & Hart (1991), who observed histidine-induced antinociception only in the mouse hot plate test. Moreover, this effect was already present 15 min after histidine administration (400 mg kg^{-1}, i.p.). The authors attributed the lack of effect in the mouse abdominal constriction test to a lack of action on peripheral histamine receptors.

Our further approach to the study of endogenous histamine effects in antinociception was to raise brain histamine levels by inhibiting its catabolism. Since brain histamine catabolism occurs almost exclusively via ring methylation (Schwartz *et al.*, 1971b; Schayer & Reilly, 1973), we used for this purpose metoprine, an antifolate which has been described as a highly potent, competitive HMT inhibitor devoid of any action on HDC (Duch *et al.*, 1978) and which is able to enhance histamine release in *in vivo* microdialysis studies following i.p. administration (Itoh *et al.*, 1991).

Due to its high liposolubility (Duch *et al.*, 1978), in the present experiments metoprine was usually administered i.p. in mice and rats to obtain a more general distribution of the substance within the central nervous system. However, in some experiments with the paw pressure test, metoprine was also given i.c.v. through permanent cannulae, in order to make sure that its site of action was in fact the CNS. In both cases and species the dose-dependence of the antinociceptive action of metoprine seems to reflect the degree of inhibition of brain HMT activity. Hough *et al.* (1986) reported that 5 mg kg^{-1}, i.p. of metoprine caused a reduction of 70% in whole brain HMT activity, while a 90% reduction can be obtained with 20–30 mg kg^{-1}. The present data seem to suggest that a 70% reduction is not sufficient to induce antinociception, since at least 10 mg kg^{-1} were found to be necessary to obtain a statistically significant rise in the nociceptive threshold (abdominal constriction test). A single dose (20 mg kg^{-1}, i.p.) was found to induce an antinociception whose potency was comparable to that induced by morphine (9 mg kg^{-1}, s.c. in the mouse hot plate test and 5 mg kg^{-1}, s.c. in the rat paw pressure test 15 and 30 min after treatment respectively). At the same dose metoprine-treated mice were able to stay balanced on the rotating rod, while morphine given at doses higher than 8.2 mg kg^{-1}, s.c. significantly impaired animal performance (data not shown).

Furthermore, metoprine antinociception was also longer-lasting than that of morphine; this is consistent with the reported metoprine half-life of 17 h (Duch *et al.*, 1978). In fact, a long-lasting antinociceptive effect is also shared by other neurotransmitter catabolism inhibitors, such as the enkephalinase inhibitors, thiorphan (Greenberg & O'Keefe, 1982), D-phenylalanine (Ehrenpreis *et al.*, 1983), azidothiorphan (Beaumont *et al.*, 1987) and SCH 34826 (Chipkin *et al.*, 1988), the GABA transaminase inhibitors amino-oxyacetic acid (Bartolini *et al.*, 1981a; Sawynok & LaBella, 1982) γ-acetylenic GABA and γ-vinyl GABA (Buckett, 1980; Sawynok & LaBella, 1982), and the cholinesterase inhibitor diisopropylfluorophosphate (DFP) (Costa & Murphy, 1986; Green & Kitchen, 1986).

To assess whether metoprine-induced antinociception was due to the blockade of HMT, animals were pretreated with RAMH. The complete antagonism observed is consistent with the results of Oishi *et al.* (1989), who reported a decrease in the levels of the catabolism product 1 h after RAMH treatment. Thus, since inhibition of histamine release by RAMH prevents metoprine-induced antinociception, it can be argued that the metoprine antinociceptive effect might be due to endogenous histamine which has not been catabolized.

The effect of MH was studied in the two tests most sensitive to histamine, the rat paw pressure and mouse abdominal constriction tests, at the same doses at which histamine was antinociceptive. As expected, the catabolism product was ineffective. Schwartz *et al.* (1971b) reported that 500 μg per rat intracisternally is able to inhibit HMT, but in their experiments the doses used were at least 10 fold higher than in ours.

In conclusion, our data taken together indicate that endogenous histamine is an antinociceptive neurotransmitter. All the substances used in the present work had an effect which was consistent with their reported ability to modulate endogenous histamine release or alter brain histamine levels. Thus when histamine brain levels or release are increased, antinociception can be observed, conversely, when the amount of histamine released is decreased, hyperalgesia occurs. It thus seems likely that the histaminergic system, like many other neuronal systems, plays an important role in the modulation of central perception of nociceptive stimuli.

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References

ABOU, Y.Z., ADAM, H.M. & STEPHEN, W.R.G. (1973). Concentration of histamine in different parts of the brain and hypophysis of rabbit: effect of treatment with histidine, certain other amino acids and histamine. *Br. J. Pharmacol.*, **48**, 577–589.

ALTAFFER, F.B., DE BALBIAN VERSTER, F., HALL, S., LONG, C.J. & D'ENCARNACAO, P. (1970). A simple and inexpensive cannula technique for chemical stimulation of the brain. *Physiol. Behav.*, **5**, 119–121.

ARRANG, J.-M., GARBARG, M., LANCELOT, J.C., LECOMTE, J.M., POLLARD, H., ROBBA, M., SCHUNACK, W. & SCHWARTZ, J.-C. (1987a). Highly potent and selective ligands for histamine H_3-receptors. *Nature*, **327**, 117–123.

ARRANG, J.-M., GARBARG, M. & SCHWARTZ, J.C. (1983). Autoinhibition of brain histamine release mediated by a novel class (H_3) of histamine receptor. *Nature*, **302**, 832–837.

ARRANG, J.-M., GARBARG, M. & SCHWARTZ, J.-C. (1987b). Autoinhibition of histamine synthesis mediated by H_3-receptors. *Neuroscience*, **23**, 149–157.

BARTOLINI, A., BARTOLINI, R., BISCINI, A., GIOTTI, A. & MALMBERG, P. (1981a). Investigations into baclofen analgesia: effect of naloxone, bicuculline, atropine and ergotamine. *Br. J. Pharmacol.*, **72**, 156P–157P.

BARTOLINI, A., GHELARDINI, C., FANTETTI, L., MALCANGIO, M., MALMBERG-AIELLO, P. & GIOTTI, A. (1992). Role of muscarinic receptor subtypes in central antinociception. *Br. J. Pharmacol.*, **105**, 77–82.

BARTOLINI, A., RENZI, R., GALLI, A., MALMBERG-AIELLO, P. & BARTOLINI, R. (1981b). Eseroline: a new antinociceptive agent derived from physostigmine with opiate receptor agonist properties. Experimental in vivo and in vitro studies on cats and rodents. *Neurosci. Lett.*, **25**, 179–183.

BASBAUM, A.I. & FIELD, H.L. (1984). Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. *Annu. Rev. Neurosci.*, **7**, 309–338.

BEAUMONT, A., HERNANDEZ, J.-F., CHAILLET, P., CRINE, P. & ROQUES, B.P. (1987). Irreversible photolabeling of active site of neutral endopeptidase-24.11 'enkephalinase' by azidothiorphan and [^{14}C]-azidothiorphan. *Mol. Pharmacol.*, **32**, 594–599.

BHATTACHARYA, S.K. & PARMAR, S.S. (1985). Antinociceptive effect of intracerebroventricularly administered histamine in rats. *Res. Commun. Chem. Pathol. Pharmacol.*, **49**, 125–136.

BLASCHKO, H. & CHRUSCIEL, T.L. (1960). The carboxylation of amino acids related to tyrosine and their awakening action in reserpine-treated mice. *J. Physiol.*, **151**, 272–284.

BRAGA, P.C., SIBILIA, V., GUIDOBONO, E., PECILE, A. & NETTI, C. (1992). Electrophysiological correlates for antinociceptive effects of histamine after intracerebral administration to the rat. *Neuropharmacology*, **31**, 937–941.

BUCKETT, W.R. (1980). Irreversible inhibitors of GABA transaminase induced antinociceptive effects and potentiate morphine. *Neuropharmacology*, **19**, 715–722.

CHIPKIN, R.E., BERGER, J.G., BILLARD, W., IORIO, L.C., CHAPMAN, R. & BARNETT, A. (1988). Pharmacology of SCH 34826, an orally active enkephalinase inhibitor analgesic. *J. Pharmacol. Exp. Ther.*, **245**, 829–838.

CHUNG, T.H., MIYAKE, H., KAMEI, C. & TASAKA, K. (1984). Analgesic effect of histamine induced by intracerebral injection into mice. *Agents Actions*, **15**, 137–142.

COSENTIN, J., SCHWARTZ, J.-C. & BOULU, R. (1974). Histamine et comportements. Effets de surcharges en L-histidine. *J. Pharmacol.*, **5**, 195–208.

COSTA, L.G. & MURPHY, S.D. (1986). Cholinergic and opiate involvement in the antinociceptive effect of diisopropylfluorophosphate. *Pharmacol. Biochem. Behav.*, **24**, 733–736.

CROSSLAND, J. (1980). Pain and itch. In *Lewis's Pharmacology*, Fifth edition, pp. 415–425. Edinburgh: Churchill Livingstone.

DUCH, D.S., BOWERS, S.W. & NICHOL, C.A. (1978). Elevation of brain histamine levels by diaminopyrimidine inhibitors of histamine N-methyltransferase. *Biochem. Pharmacol.*, **27**, 1507–1509.

EHRENPREIS, S., BALAGOT, R.C., GREENBERG, J., MYLES, S. & ELLYIN, F. (1983). Analgesic and other pharmacological properties of D-phenylalanine. In *Degradation of Endogenous Opioids. Its Relevance in Human Pathology and Therapy*. ed. Ehrenpreis, S. & Sicuteri, F. pp. 171–187. New York: Raven Press.

GARBARG, M., BARBIN, G., RODERGAS, E. & SCHWARTZ, J.-C. (1980). Inhibition of histamine synthesis in brain by α-fluoromethylhistidine, a new irreversible inhibitor: in vitro and in vivo studies. *J. Neurochem.*, **35**, 1045–1052.

GARBARG, M., TRUNG TUONG, M.D., GROS, C. & SCHWARTZ, J.C. (1989). Effects of histamine H_2-receptor ligands on various biochemical indices of histaminergic neuron activity in the rat brain. *Eur. J. Pharmacol.*, **164**, 1–11.

GHELARDINI, C., MALMBERG-AIELLO, P., GIOTTI, A. & BARTOLINI, A. (1990). Investigation into atropine-induced antinociception. *Br. J. Pharmacol.*, **101**, 49–54.

GLICK, S.D. & CRANE, L.A. (1978). Opiate-like and abstinence-like effects of intracerebral histamine administration in rats. *Nature*, **273**, 547–549.

GREEN, P.G. & KITCHEN, I. (1986). Di-isopropylfluorophosphate induced antinociception and its interactions with opioid drugs in the rat. *Toxicology*, **42**, 275–280.

GREENBERG, R. & O'KEEFE, E.H. (1982). Thiorphan potentiation of stress-induced analgesia in the mouse. *Life Sci.*, **31**, 1185–1188.

HALEY, T.J. & MCCORMICK, W.G. (1957). Pharmacological effects produced by intracerebral injections of drugs in the conscious mouse. *Br. J. Pharmacol. Chemother.*, **12**, 12–15.

HOUGH, L.B., KHANDELWAL, J.K. & GREEN, J.P. (1986). Inhibition of brain histamine metabolism by metoprine. *Biochem. Pharmacol.*, **35**, 307–310.

ITOH, Y., OISHI, R., NISHIBORI, M. & SAEKI, K. (1989). Effects of nociceptive stimuli on brain histamine dynamics. *Jpn. J. Pharmacol.*, **49**, 449–454.

ITOH, Y., OISHI, R., NISHIBORI, M. & SAEKI, K. (1991). Characterization of histamine release from the rat hypothalamus as measured by in vivo microdialysis. *J. Neurochem.*, **56**, 769–774.

JONES, S.L. & GEBHART, G.F. (1986). Characterization of coeruleospinal inhibition of the nociceptive tail-flick reflex in the rat: mediation by spinal α_2-adrenoreceptors. *Brain Res.*, **364**, 315–330.

KOLLONITSCH, J., PERKINS, L.M., PATCHETT, A.A. DOLDOURAS, G.A., MARBURG, S., DUGGAN, D.E., MAYCOCK, A.L. & ASTER, S.D. (1978). Selective inhibitors of biosynthesis of aminergic neurotransmitters. *Nature*, **274**, 906–908.

KOSTER, R., ANDERSON, M. & DE BEER, E.J. (1959). Acetic acid for analgesic screening. *Fed. Proc.*, **18**, 412.

KURIBARA, H., HIGUCHI, Y. & TAKADORO, S. (1977). Effects of central depressants on rota-rod and traction performances in mice. *Jpn. J. Pharmacol.*, **27**, 117–126.

LAMBERTI, C., GHELARDINI, C., BARTOLINI, A. & MALMBERG-AIELLO, P. (1992a). Effects of thioperamide and (R)-α-methylhistamine in antinociception. *Neurochem. Internat.*, **21**, Suppl. C4, 16.

LAMBERTI, C., MALMBERG-AIELLO, P., GHELARDINI, C., GIOTTI, A. & BARTOLINI, A. (1992b). Biphasic effect of i.c.v. histamine on pain threshold in rodents. *Eur. Histamine Res. Soc. XXI Annual Meeting*, Torremolinos-Malaga, Spain, May 13–17, Abstr. p. 109.

LEIGHTON, G.E., RODRIGUEZ, R.E., HILL, R.G. & HUGHES, J. (1988). κ-Opioid agonist produce antinociception after i.v. and i.c.v. but not intrathecal administration in the rat. *Br. J. Pharmacol.*, **93**, 553–560.

LEVINE, J.D., GORDON, N.C. & FIELDS, H.J. (1979). Naloxone dose dependently produces analgesia and hyperalgesia in postoperative pain. *Nature*, **278**, 740–741.

LIEBMAN, J.M. & PASTOR, G. (1980). Antinociceptive effects of baclofen and muscimol upon intraventricular administration. *Eur. J. Pharmacol.*, **61**, 225–230.

MAEYAMA, K., WATANABE, T., TAGUCHI, Y., YAMATODANI, A. & WADA, H. (1982). Effect of α-fluoromethylhistidine, a suicide inhibitor of histidine decarboxylase, on histamine levels in mouse tissues. *Biochem. Pharmacol.*, **31**, 2367–2370.

MAEYAMA, K., WATANABE, T., YAMATODANI, A., TAGUCHI, Y., KAMBE, H. & WADA, H. (1983). Effect of α-fluoromethylhistidine on the histamine content of the brain of W/Wv mice devoid of mast cells: turnover of brain histamine. *J. Neurochem.*, **41**, 128–134.

MALCANGIO, M., GHELARDINI, C., GIOTTI, A., MALMBERG-AIELLO, P. & BARTOLINI, A. (1991). CGP 35348, a new $GABA_B$-antagonist, prevents antinociception and muscle-relaxant effect induced by baclofen. *Br. J. Pharmacol.*, **103**, 1303–1308.

MALCANGIO, M., MALMBERG-AIELLO, P., GIOTTI, A., GHELARDINI, C. & BARTOLINI, A. (1992). Desensitization of $GABA_B$ receptor and antagonism by CGP 35348 prevent bicuculline and picrotoxin antinociception. *Neuropharmacology*, **31**, 783–791.

MALMBERG-AIELLO, P., LAMBERTI, C., GHELARDINI, C. & BARTOLINI, A. (1993). Metoprine antinociception prevented by the histamine H_3-receptor agonist (R)-α-methylhistamine. *Eur. Histamine Res. Soc. XXII Annual Meeting*, Cologne, Germany, May 19–22, Abstr. p. 114.

MALMBERG-AIELLO, P., LAMBERTI, C., GHELARDINI, C., GIOTTI, A. & BARTOLINI, A. (1992). Antinociception caused by L-histidine and metoprine in rodents. *Eur. Histamine Res. Soc. XXI Annual Meeting*, Torremolinos-Malaga, Spain, May 13–17, Abstr. p. 99.

METYS, J., WAGNER, N., METYSOVA, J. & HERTS, A. (1969). Studies on central antinoceptive actions of cholinergic agents. *Int. J. Neuropharmacol.*, **8**, 413–425.

O'CALLAGHAN, J.P. & HOLTZMAN, S.G. (1976). Prenatal administration of morphine to the rat: tolerance to the analgesic effect of morphine in the offspring. *J. Pharmacol. Exp. Ther.*, **197**, 533–544.

OISHI, R., ITOH, Y., NISHIBORI, M. & SAEKI, K. (1989). Effects of the histamine H_3-agonist (R)-α-methylhistamine and the antagonist thioperamide on histamine metabolism in the mouse and rat brain. *J. Neurochem.*, **52**, 1388–1392.

OISHI, R., NISHIBORI, M. & SAEKI, K. (1984). Regional differences in the turnover of neuronal histamine in the rat brain. *Life Sci.*, **34**, 691–699.

OLUYOMI, A.O. & HART, S.L. (1991). Involvement of histamine in naloxone-resistant and naloxone-sensitive models of swim stress-induced antinociception in the mouse. *Neuropharmacology*, **30**, 1021–1027.

PANULA, P., PIRVOLA, U., AUVINEN, S. & AIRAKSINEN, M.S. (1989). Histamine-immunoreactive nerve fibres in the rat brain. *Neuroscience*, **28**, 585–610.

PAROLARO, D., PATRINI, G., MASSI, P., MAINARDI, P., GIAGNONI, G., SALA, M. & GORI, E. (1989). Histamine as a central modulator of rat intestinal transit. *J. Pharmacol. Exp. Ther.*, **249,** 324–328.

SAMANIN, R. & VALZELLI, L. (1971). Increase of morphine-induced analgesia by stimulation of the nucleus raphe dorsalis. *Eur. J. Pharmacol.*, **16,** 298–302.

SAWYNOK, J. & LABELLA, F.S. (1982). On the involvement of GABA in the analgesia produced by baclofen, muscimol and morphine. *Neuropharmacology*, **21,** 397–403.

SCHAYER, R.W. & REILLY, M.A. (1973). Formation and fate of histamine in rat and mouse brain. *J. Pharmacol. Exp. Ther.*, **184,** 33–40.

SCHWARTZ, J.-C., ARRANG, J.M., GARBARG, M. & POLLARD, H. (1990). A third histamine receptor subtype: characterization, localization and function of the H_3-receptor. *Agents Action*, **30,** 13–23.

SCHWARTZ, J.-C., LAMPART, C. & ROSE, C. (1972). Histamine formation in rat brain in vivo: effects of histidine loads. *J. Neurochem.*, **19,** 801–810.

SCHWARTZ, J.-C., LAMPART, C., ROSE, C., REHAULT, M.C., BISCHOFF, S. & POLLARD, H. (1971a). Histamine formation in the rat brain during development. *J. Neurochem.*, **18,** 1787–1789.

SCHWARTZ, J.-C., POLLARD, H., BISCHOFF, S., REHAULT, M.C. & VERDIERE-SAHUQUE, M. (1971b). Catabolism of ^{3}H-histamine in the rat brain after intracisternal administration. *Eur. J. Pharmacol.*, **16,** 326–335.

SHEINER, J.B., MORRIS, P. & ANDERSON, G.H. (1985). Food intake suppression by histidine. *Pharmacol. Biochem. Behav.*, **23,** 721–726.

SNYDER, S.H., AXELROD, J. & BAUER, H. (1964). The fate of C^{14}-histamine in animal tissues. *J. Pharmacol. Exp. Ther.*, **144,** 373–379.

STEINBUSCH, H.W.M. & MULDER, A.H. (1985). Localization and projections of histamine-immunoreactive neurons in the central nervous system of the rat. In *Frontiers in Histamine Research, Advances in the Biosciences*, Vol. 51. ed. Ganellin, C.R. & Schwartz, J.-C. pp. 119–130. London: Pergamon Press.

TALLARIDA, R.J. & MURRAY, R.B. (1984). *Manual of Pharmacological Calculations with Computer Programs.* Second edition. New York: Springer-Verlag.

TAYLOR, K.M. & SNYDER, S.H. (1972). Dynamics of the regulation of histamine levels in mouse brain. *J. Neurochem.*, **19,** 341–354.

TAYLOR, S.J., MICHEL, A.D. & KILPATRICK, G.J. (1992). In vivo occupancy of histamine H_3 receptors by thioperamide and (R)-α-methylhistamine measured using histamine turnover and an ex vivo labelling technique. *Biochem. Pharmacol.*, **44,** 1261–1267.

TYERS, M.B. (1980). Classification of opiate receptors that mediate antinociception in animals. *Br. J. Pharmacol.*, **69,** 503–512.

VAN DER WERF, J.F., BAST, A., BIJLOO, G.J., VAN DER VLIET, A. & TIMMERMAN, H. (1987). HA autoreceptor assay with superfused slices of rat brain cortex and electrical stimulation. *Eur. J. Pharmacol.*, **138,** 199–206.

WADA, H., INAGAKI, N., ITOWI, N. & YAMATODANI, A. (1991). Histaminergic neuron system in the brain: distribution and possible functions. *Brain Res. Bull.*, **27,** 367–370.

YAKSH, T.L. & NOUEIHED, R. (1985). The physiology and pharmacology of spinal opiates. *Annu. Rev. Pharmacol. Toxicol.*, **25,** 433–462.

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Influence of S-nitrosothiols and nitrate tolerance in the rat gastric fundus

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1 The relaxant responses of S-nitroso-L-cysteine (CysNO), S-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), S-nitroso-*N*-acetyl-L-cysteine (SNAC) and S-nitrosoglutathione (GSNO) in the rat gastric fundus (forestomach) were studied and compared to the relaxant responses obtained in response to nitric oxide (NO) and electrical field stimulation (EFS, 10 s trains) of non-adrenergic non-cholinergic (NANC) nerves.

2 CysNO (10^{-7}–3×10^{-4} M) caused transient relaxation of the precontracted rat gastric fundus, comparable to the response to NO (10^{-6}–10^{-4} M) and EFS. SNAP, SNAC and GSNO elicited more sustained relaxations.

3 The cyclic GMP-specific phosphodiesterase inhibitor, zaprinast (3×10^{-5} M), increased the relaxant effect of CysNO, SNAP and GSNO while the NO-synthase inhibitor, N^G-nitro-L-arginine (L-NOARG, 3×10^{-4} M) had no influence.

4 In the presence of LY 83583 (10^{-5} M), which releases superoxide anions, the relaxant response to NO and CysNO was decreased, whereas that to all other stimuli was unaltered. The inhibitory effect of LY 83583 on CysNO-induced relaxations was prevented by superoxide dismutase (SOD, 1000 u ml^{-1}).

5 Tissues incubated for 1 h with 5.5×10^{-4} M nitroglycerin (GTN) became tolerant to GTN. In this condition, the relaxant response to 10^{-5} M NO was maintained, while the relaxations by EFS (8 Hz) and 3×10^{-5} M SNAP were significantly decreased. The reduction of the response to the other S-nitrosothiols was not significant.

6 The combination of nitrate tolerance and 10^{-5} M LY 83583 caused a significantly larger inhibition of the relaxant response to EFS (8 Hz) than nitrate tolerance alone. The combination of LY 83583 and GTN tolerance reduced the relaxant effect of 10^{-5} M NO to a similar extent to LY 83583 alone, while the relaxant response to 10^{-4} M GTN was reduced to the same extent as after 1 h exposure to 5.5×10^{-4} M GTN alone.

7 It is concluded that S-nitrosothiols potently relax the rat gastric fundus, possibly by a cyclic GMP-dependent mechanism and S-nitrosothiols such as SNAC and GSNO may be involved in NANC neurotransmission.

Keywords: S-nitrosothiols; rat gastric fundus; non-adrenergic non-cholinergic (NANC) nerves; nitrate tolerance

Introduction

In recent years, nitric oxide (NO) has emerged as a major mediator of a number of biological effects. Initially discovered as the endothelium-derived relaxing factor (EDRF, Palmer *et al.*, 1987; Ignarro *et al.*, 1987), NO has been implicated in neurotransmission in the central nervous system (Garthwaite, 1991) and inhibition of platelet aggregation (Radomski *et al.*, 1987). It was also identified as a neurotransmitter from non-adrenergic non-cholinergic (NANC) nerves in the respiratory (Belvisi *et al.*, 1992; Kannan & Johnson, 1992), urogenital (Gillespie *et al.*, 1989; Ignarro *et al.*, 1990) and gastrointestinal (Bult *et al.*, 1990) tract. Some observations led to the suggestion that NO is not transported freely from the synthesizing cell to the target cell. Myers *et al.* (1990) reported that the vasorelaxant properties of EDRF more closely resemble those of S-nitroso-L-cysteine than of NO. Hobbs *et al.* (1991) demonstrated in the mouse anococcygeus muscle, the guinea-pig trachea and the rat gastric fundus that the free radical scavenger hydroquinone was effective in inhibiting the relaxant effect of exogenous NO, but not that of NANC stimulation, suggesting that the endogenous substance released by NANC nerves may not be NO but an NO-containing or NO-generating substance.

In the rat gastric fundus, we observed that the relaxant response to exogenous NO is almost completely abolished by 6-anilino-5,8-quinolinedione (LY 83583), probably due to LY 85383-induced superoxide anion generation, while the response to shortlasting electrical field stimulation (EFS) of the NANC neurones was not influenced, suggesting the release of a stable NO-carrier complex from the NANC neurones (Barbier & Lefebvre, 1992a). In the bovine retractor penis muscle, Kerr *et al.* (1992) reported that the presence of a thiol is required for acid activation of nitrite to produce a vasodilator with the relaxant properties of the Inhibitory Factor, a NANC relaxant extract from this tissue; they concluded that not NO but an S-nitrosothiol is responsible for the activity of this Inhibitory Factor.

As S-nitrosothiols were proposed as nitrergic carriers in vascular (Myers *et al.*, 1990) and non-vascular smooth muscle (Kerr *et al.*, 1992), we have investigated the relaxant properties of several S-nitrosothiols in the rat gastric fundus and compared them to the effect of exogenously added NO and EFS of the NANC nerves. The influence of LY 83583, zaprinast (a guanosine 3′:5′-cyclic monophosphate (cyclic GMP)-specific phosphodiesterase inhibitor), N^G-nitro-L-arginine (a NO synthase inhibitor) and nitroglycerin-induced nitrate tolerance on the relaxant responses was investigated. A preliminary account of part of the results has been given (Barbier & Lefebvre, 1993).

Methods

Rat gastric fundus

Wistar rats of either sex (180–320 g) were fasted for 24 h but had free access to water. They were killed by a blow on the

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head and bleeding via the carotid arteries. Longitudinal gastric fundus strips (15 × 3 mm) were prepared as described by Vane (1957) and suspended (load 1 g) between platinum electrodes in 18 ml organ baths, containing Krebs solution (composition, mM: NaCl 118.5, KCl 4.8, $CaCl_2$ 1.9, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 10.1). It was kept at 37°C and bubbled with 95% O_2 and 5% CO_2. Atropine (10^{-6} M) and guanethidine (4×10^{-6} M) were present throughout the experiment to obtain NANC conditions. Tissues were equilibrated for 1 h with rinsing at 15 min intervals. Changes in length were recorded isotonically (Bioscience T3 Isotonic transducer) on a Graphtec Linearcorder F WR3701. Transmural electrical stimulation (40 V, 1 ms, 2–4–8 Hz, 10 s trains) was performed with a Grass S88 stimulator with a power magnification unit.

Synthesis of S-nitrosothiols

S-nitrosothiols were synthesized according to the method of Field *et al.* (1978) with a slight modification. This involved the addition of an excess of acidified sodium nitrite (0.5 N HCl, pH <2) to a solution of 0.1 M thiol. This nitrosylation reaction is essentially irreversible and complete (Aldred & Williams, 1982; Byler *et al.*, 1983). The solutions turned bright orange as the S-nitrosothiol was formed, except for S-nitroso-D,L-penicillamine (SNAP), which was green. S-nitroso-L-cysteine (CysNO), SNAP, S-nitroso-acetyl-L-cysteine (SNAC) and S-nitrosoglutathione (GSNO) were prepared from the corresponding thiols. The S-nitrosothiol solutions were prepared immediately before use and kept on ice.

Relaxant responses

After the 1 h equilibration period, contraction was induced by 10^{-6} M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to test the sensitivity of the tissues. After rinsing every 15 min for 1 h, a second $PGF_{2\alpha}$-contraction was induced and once the contraction had reached a plateau, the relaxant stimuli (S-nitrosothiols, 10^{-7} to 3×10^{-4} M, NO, 10^{-6} to 10^{-4} M or EFS, 2 to 8 Hz) were studied. Only one type of relaxant stimulus was investigated per tissue. Zaprinast (3×10^{-5} M) and LY 83583 (10^{-5} M) were applied for 30 min before the second plateau contraction with $PGF_{2\alpha}$ was induced, L-NOARG (3×10^{-4} M) for 10 min. The solvents of these substances were administered to parallel control strips. In some experiments superoxide dismutase (SOD, 1000 u ml^{-1}) was administered when the $PGF_{2\alpha}$-induced contraction had become stable and was left in contact with the tissue for 5 min before administration of CysNO.

Nitrate tolerance

Rat gastric fundus strips were made tolerant to nitroglycerin (GTN) according to a modification of a protocol described by Keith *et al.* (1983) for the guinea-pig ileum. In the first series of these experiments, a first contraction plateau was induced by 10^{-6} M $PGF_{2\alpha}$, and relaxation was consecutively induced by 10^{-5} M NO, EFS (8 Hz, 40 V, 1 ms, 10 s) and 10^{-4} M GTN. After rinsing, the strips were incubated for 1 h with 5.5×10^{-4} M GTN or solvent. Subsequently, the strips were rinsed 10 times over a 30 min period. In some groups of tissues, 10^{-5} M LY 83583 was left in contact with the tissues during this 30 min period after desensitization, to study the combined effect of nitrate tolerance and LY 85383. A second $PGF_{2\alpha}$-plateau was then induced and the relaxant stimuli were repeated. To study the influence of nitrate tolerance on the relaxant effects of the S-nitrosothiols (1 concentration), the GTN incubation for 1 h was begun immediately after the equilibration period. After rinsing 10 times over a 30 min period, a plateau contraction was induced with $PGF_{2\alpha}$ and

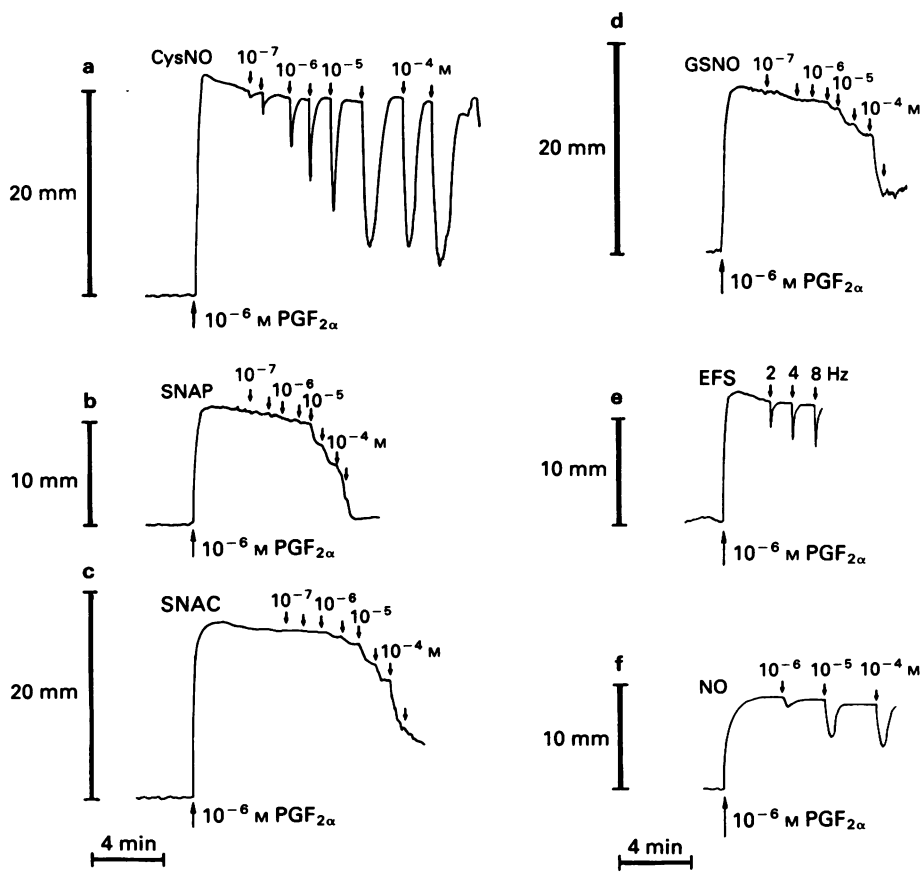


Figure 1 Representative traces of the relaxant responses of the rat gastric fundus to the stimuli studied, after induction of tone with 10^{-6} M $PGF_{2\alpha}$. (a) CysNO; (b) SNAP; (c) SNAC; (d) GSNO; (e) EFS (10 s trains); (f) NO. Unmarked arrows in (a) to (d) indicate 3×10^{-7} M, 3×10^{-6} M, 3×10^{-5} M, 3×10^{-4} M. For abbreviations, see text.

the relaxant response to an S-nitrosothiol was then studied and compared with that in a parallel control strip, incubated with the solvent of GTN. In the series investigating the effect of the combination of 10^{-5} M LY 83583 and nitrate tolerance, LY 83583 was present in the organ bath during the 30 min rinsing period after desensitization.

Materials

N-acetyl-L-cysteine, N-acetyl-D,L-penicillamine, atropine sulphate, glutathione, guanethidine sulphate, N^G-nitro-L-arginine, prostaglandin $F_{2\alpha}$ and superoxide dismutase from bovine erythrocytes were obtained from Sigma (St. Louis, U.S.A.), L-cysteine from Merck (Darmstadt, Germany), LY 83583 from Calbiochem (La Jolla, U.S.A.), zaprinast from Rhône-Poulenc (Dagenham, England) and nitroglycerin (glyceryl trinitrate, 1 mg ml^{-1} solution in dextrose 5%, PEG 400 0.5% and water) from Pohl Boskamp (Hohenlockstedt, Germany). A saturated NO solution was prepared from NO gas (l'Air Liquide, Belgium) as described by Kelm & Schrader (1990). Zaprinast was solved in 20% triethanolamine, N-acetyl-D,L-penicillamine was solved in ethanol. All other drugs were solved in water.

Statistics

Relaxant responses were expressed as percentage reduction of $PGF_{2\alpha}$-induced tone. Results are given as mean ± s.e.mean. Statistical analysis was by the Wilcoxon signed-ranks test for paired and the Mann-Whitney two-sample rank test for unpaired observations. Differences were considered significant when $P<0.05$. EC_{50} values were determined by linear interpolation from individual concentration-response curves.

Results

Relaxant effects of S-nitrosothiols, NO and EFS in the rat gastric fundus

Figure 1 shows representative examples of the relaxant stimuli and Figure 2 shows the mean concentration-response curves. CysNO, SNAP, SNAC and GSNO (all at 10^{-7}–3×10^{-4} M) all relaxed the rat gastric fundus in a concentration-dependent manner. The relaxations induced by CysNO, EFS and NO were transient and were usually followed by complete or nearly complete tone recovery. The relaxant effects of SNAP, SNAC and GSNO were more sustained, although at the higher concentrations, tone sometimes tended to recover after the peak relaxation had been reached. The potency order of the S-nitrosothiols was CysNO (EC_{50}: $5.4 \pm 1.7 \times 10^{-6}$ M) > SNAP ($1.5 \pm 0.4 \times 10^{-5}$ M) > GSNO ($2.4 \pm 0.4 \times 10^{-5}$ M) > SNAC ($4.7 \pm 0.9 \times 10^{-5}$ M, $n = 6$–8, Figure 2). The unnitrosylated thiols did not have any relaxant effect ($n = 3$–4 from 2 rats).

Influence of zaprinast, LY 83583 and L-NOARG on the relaxant effects of S-nitrosothiols, NO and EFS

Zaprinast (3×10^{-5} M) did not affect the relaxant responses to NO or EFS (Figure 3). By contrast, it caused a potentiation of the relaxant responses to higher concentrations of CysNO, SNAP and GSNO. For SNAC, the trend towards potentiation was present but it did not reach statistical significance. LY 83583 (10^{-5} M) caused a clear inhibition of the relaxant responses to NO (Figure 4a), while not influencing that to EFS (2–8 Hz, $n = 6$). Of the S-nitrosothiols (n at least 6 for each agent), only the relaxations caused by CysNO were significantly decreased in the presence of 10^{-5} M LY 83583 (Figure 4b). SOD (1000 u ml^{-1}) added 5 min before the start of the concentration-response curve to CysNO, abolished the inhibitory effect of LY 83583 (Figure 4c).

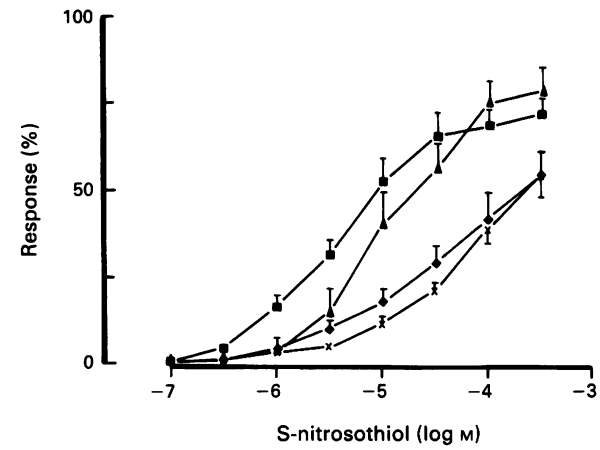


Figure 2 Mean concentration-response curves for the relaxant effect of the S-nitrosothiols in the rat gastric fundus: (■) CysNO; (▲) SNAP; (×) SNAC; (◆) GSNO. Mean ± s.e.mean of 6–8 experiments. For abbreviations, see text.

In the presence of L-NOARG (3×10^{-4} M), the relaxant response to EFS was significantly smaller than in control tissues. The relaxant responses to EFS at 2, 4 and 8 Hz were 21.8 ± 3.0, 29.7 ± 11.1 and 29.2 ± 4.9% in control tissues ($n = 7$), and 8.8 ± 3.3 ($P<0.05$), 8.2 ± 2.4 ($P<0.01$) and 13.1 ± 3.8% ($P<0.05$) in the presence of L-NOARG ($n = 8$). L-NOARG did not affect responses to NO or any of the S-nitrosothiols (n at least 6 for each stimulus).

Influence of nitrate tolerance, and the combination of nitrate tolerance and 10^{-5} M LY 83583, on the relaxant effects of NO, EFS, GTN and S-nitrosothiols

The relaxant responses to 10^{-5} M NO, EFS (8 Hz) and 10^{-4} M GTN were reproducible (Table 1). A representative example of the influence of 1 h incubation with 5.5×10^{-4} M GTN on these responses is shown in Figure 5. After incubation of the rat gastric fundus strips with 5.5×10^{-4} M GTN for 1 h, the relaxant response to 10^{-4} M GTN was significantly reduced from 58.5 ± 8.8% to 11.9 ± 1.0% (Table 1), indicating marked tolerance to GTN. The relaxation induced by 10^{-5} M NO, however, was not influenced by this nitrate tolerance, while the relaxant response obtained by EFS with a 10 s train at 8 Hz was slightly but significantly decreased. When 10^{-5} M LY 83583 was added to the desensitization regimen (Table 1), the relaxant response to EFS (8 Hz) was reduced from 33.6 ± 4.3% to 16.9 ± 3.8% ($n = 8$, $P<0.01$). This decrease is significantly more pronounced than that observed after induction of GTN tolerance alone ($P<0.01$), indicating that LY 83583, which does not influence EFS-induced relaxation *per se* (Table 1), has an inhibitory effect when added to the nitrate desensitization procedure. The combination of LY 83583 and GTN tolerance reduced the relaxant effect of 10^{-5} M NO to a similar extent to LY 83583 alone, while the relaxant response to 10^{-4} M GTN was reduced to the same extent as after 1 h exposure to 5.5×10^{-4} M GTN alone.

The relaxant response to 3×10^{-6} M CysNO, 3×10^{-5} M SNAP and SNAC and 10^{-4} M GSNO was 41.5 ± 6.0, 47.1 ± 8.8, 22.8 ± 1.0 and 35.0 ± 8.0% after incubation with the GTN solvent, and 21.5 ± 5.3, 20.1 ± 3.5, 13.4 ± 3.6 and 16.7 ± 6.7% after incubation with 5.5×10^{-4} M GTN ($n = 6$–7). Only for SNAP was the decrease statistically significant ($P<0.05$). The relaxant response to 10^{-4} M GSNO after induction of GTN tolerance and addition of 10^{-5} M LY 83583 (23.4 ± 2.7%, $n = 6$) was not different from that after induction of GTN tolerance alone.

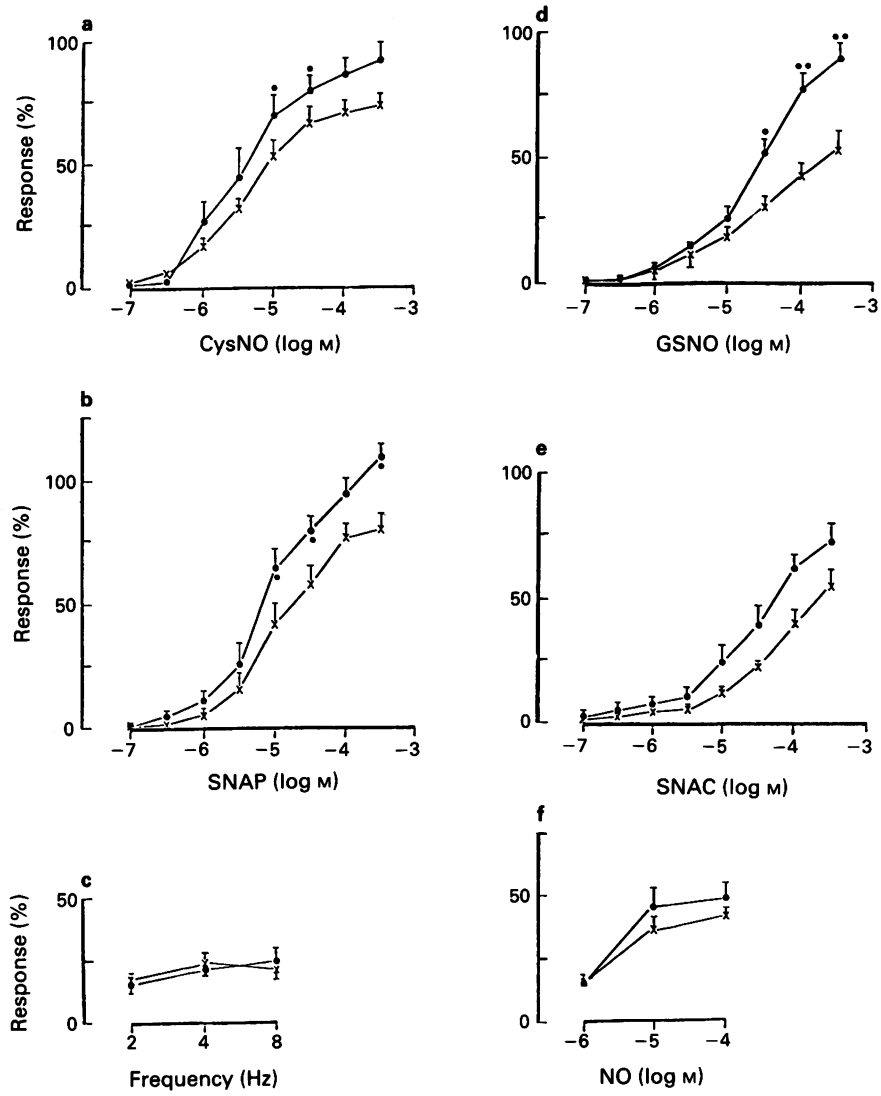


Figure 3 Influence of 3×10^{-5} M zaprinast on the relaxant response to: CysNO (a); SNAP (b); SNAC (e); GSNO (d); EFS (c) and NO (f) in the rat gastric fundus. Concentration- or frequency-response curves after incubation with 3×10^{-5} M zaprinast (●); concentration- or frequency-response curves in parallel control strips, incubated with the solvent of zaprinast (×). All data are the mean ± s.e.mean of 6–8 experiments. $^{*}P<0.05$, $^{**}P<0.01$: significantly different from control. For abbreviations, see text.

Table 1 Relaxant responses (%) to nitric oxide (NO), electrical field stimulation (EFS) and nitroglycerin (GTN) before and after incubation with GTN solvent, GTN, GTN + LY 83583 and LY 83583 ($n = 6$–8)

NO (10^{-5} M)	35.8 ± 6.7	33.2 ± 4.4	35.7 ± 2.8	38.3 ± 6.6
EFS (8 Hz)	30.5 ± 4.5	35.5 ± 3.3	33.6 ± 4.3	29.7 ± 4.9
GTN (10^{-4} M)	61.7 ± 7.3	58.5 ± 8.8	46.9 ± 5.8	41.0 ± 6.3
Incubation with	*GTN solvent*	*GTN* (5.5×10^{-4} M)	*GTN* (5.5×10^{-4} M) + *LY 83583* (10^{-5} M)	*LY 83583* (10^{-5} M)
NO (10^{-5} M)	35.4 ± 5.8	29.6 ± 5.2	5.9 ± 1.5**	8.3 ± 1.4*
EFS (8 Hz)	28.2 ± 3.1	27.7 ± 2.9*	16.9 ± 3.8**	23.9 ± 2.3
GTN (10^{-4} M)	58.7 ± 6.8	11.9 ± 1.0*	7.7 ± 1.8**	38.0 ± 6.6

$^{*}P<0.05$, $^{**}P<0.01$: significantly different from the response before incubation.

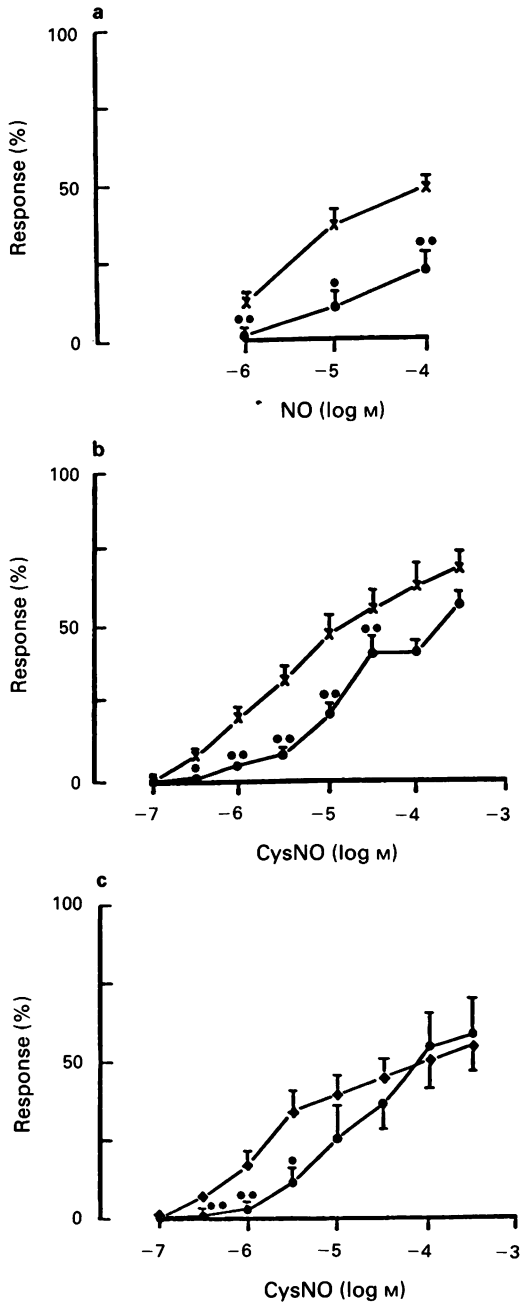


Figure 4 Influence of 10^{-5} M LY 83583 on the relaxant response to NO (a) and CysNO (b,c) in the rat gastric fundus: (×) concentration-response curves in control tissues; (●) concentration-response curves after incubation with 10^{-5} M LY 83583; (◆) concentration-response curves after incubation with 10^{-5} M LY 83583 + 1000 u ml^{-1} SOD. All data are mean ± s.e.mean of 6–7 experiments. $*P<0.05$, $**P<0.01$: significantly different from corresponding control. For abbreviations, see text.

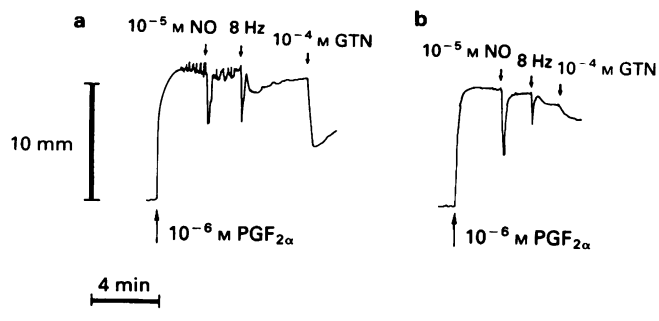


Figure 5 Trace showing the relaxant effect of 10^{-5} M NO, electrical field stimulation (8 Hz) and 10^{-4} M nitroglycerin (GTN) before (a) and after (b) 1 h exposure to 5.5×10^{-4} M GTN.

Discussion

In the rat gastric fundus, NO and vasoactive intestinal polypeptide (VIP) are probably inhibitory NANC cotransmitters, NO being preferentially released during short train (10–20 s) stimulation of the NANC neurones (Li & Rand, 1990; Boeckxstaens *et al.*, 1992; D'Amato *et al.*, 1992). This was again confirmed by the pronounced inhibition of the short train EFS-induced relaxations by the NO synthase inhibitor, L-NOARG, in this study. We previously showed that the relaxant response to exogenous NO in the rat gastric fundus is inhibited by LY 83583, probably via superoxide anion generation, as the effect is reversed by SOD (Barbier & Lefebvre, 1992a). However, the NANC relaxation to short train stimulation was not influenced by LY 83583, suggesting that NO synthesized in the NANC neurones is not transported to the smooth muscle cells as a free molecule. Similar observations have been made in other non-vascular smooth muscle preparations (Gillespie & Sheng, 1990; Hobbs *et al.*, 1991; Rajanayagam *et al.*, 1993). As S-nitrosothiols have been proposed to account for at least part of EDRF activity in vascular smooth muscle (Ignarro, 1990; Myers *et al.*, 1990; Rubanyi *et al.*, 1990; Bates *et al.*, 1991), the present study was conducted to investigate the effect of S-nitrosothiols in the rat gastric fundus.

Cysteine, acetylcysteine and glutathione are present in biological tissue (Meister & Anderson, 1983; Hecker *et al.*, 1992; Jansen *et al.*, 1992) while acetyl-D,L-penicillamine was selected as a representative non-biological thiol. The smooth muscle relaxing properties of the corresponding S-nitrosothiols have been shown in bovine coronary arteries (Henry *et al.*, 1989a), guinea-pig airways (Jansen *et al.*, 1992), the opossum lower oesophageal sphincter (Knudsen *et al.*, 1992) and the mouse anococcygeus muscle (Gibson *et al.*, 1992). The S-nitrosothiols also produced concentration-related relaxations in the rat gastric fundus. These relaxations were not due to activation of the endogenous NO synthase, as they were not influenced by L-NOARG in a concentration greatly reducing the electrically induced NANC responses. Although the short lasting responses to CysNO displayed the best resemblance to those induced by EFS with short trains, LY 83583 clearly reduced responses to CysNO and this inhibition was reversed by SOD. This corresponds to the effect of LY 83583 versus exogenous NO but contrasts with its non-effect on EFS-induced relaxations (Barbier & Lefebvre, 1992a). The nature and the sensitivity to LY 83583 of the CysNO-induced relaxation might be related to its rapid decomposition to NO and native thiol in solution (Ignarro *et al.*, 1981). Also in the mouse anococcygeus muscle, CysNO was the only S-nitrosothiol sensitive to the free radical scavenger hydroquinone (Gibson *et al.*, 1992). In the canine proximal colon (Thornbury *et al.*, 1991) and in circular muscle of the rat gastric fundus (Kitamura *et al.*, 1993), CysNO mimicked NANC hyperpolarization, but in the latter tissue, hydroquinone completely inhibited the hyperpolarization induced by CysNO, while not influencing the EFS-evoked inhibitory junction potentials. These and our observations make it unlikely that CysNO is released from the NANC neurones during EFS of the rat gastric fundus. The relaxant effect of SNAC and GSNO was not influenced by LY 83583, leaving open the possibility that they are involved in NANC neurotransmission. The fact that SNAC and GSNO relax the rat gastric fundus in a more sustained way than short train EFS might be related to the difference in local concentrations between administering these compounds exogenously to the organ bath and their possible release in the synaptic cleft during EFS.

NO induces smooth muscle relaxation by stimulation of cytosolic guanylate cyclase and increase of the intracellular cyclic GMP levels (Waldman & Murad, 1987). In the rat gastric fundus, we previously showed that zaprinast, an inhibitor of the cyclic GMP-specific phosphodiesterase (Thompson, 1991) potentiated the relaxant effect of the NO-

donor, sodium nitroprusside but not the response to NO itself or to short train stimulation of the NANC neurones (Barbier & Lefebvre, 1992b). It was suggested that zaprinast might successfully interfere with the degradation of cyclic GMP only when the cyclic GMP-pathway is activated in a more sustained way than with NO or short train EFS. The rather sustained relaxation induced by SNAC, SNAP and GSNO in the rat gastric fundus was potentiated by zaprinast, suggesting guanylate cyclase activation by these S-nitrosothiols. The shortlasting relaxations induced by CysNO were also potentiated by zaprinast; this shows that CysNO also acts by guanylate cyclase activation, but contrasts with the lack of effect of zaprinast on the NO response.

Prolonged exposure to GTN results in tolerance to the relaxant effect of organic nitrates in vascular smooth muscle, while the response to the direct NO donor, sodium nitroprusside, and NO is not or less markedly affected (Kowaluk *et al.*, 1987; Henry *et al.*, 1989b; Kowaluk & Fung, 1990b). Similar results were obtained in the guinea-pig ileum (Keith *et al.*, 1983). Although not all experimental findings support it (Bassenge & Zanzinger, 1992; Fung *et al.*, 1992), the most widespread concept to explain the selective organic nitrate tolerance is that intracellular thiols are required to liberate NO from organic nitrates; prolonged exposure to an organic nitrate will lead to depletion of the intracellular thiol pool and tolerance (Needleman & Johnson, 1973; Needleman *et al.*, 1973). Decreased bioconversion of GTN to NO in tolerant vascular smooth muscle cells has been described (Salvemini *et al.*, 1993). Although the mechanism of NO release from S-nitrosothiols is not completely clear, intracellular penetration of extracellular S-nitrosothiols does not seem required to induce relaxation. As the compounds are too polarized for easy transmembranar diffusion (Kowaluk & Fung, 1990a) and as the involvement of transporter mechanisms seems unlikely (Bates *et al.*, 1991), the S-nitrosothiols most probably decompose at the surface of the target cell, liberating NO, which will easily penetrate into the cell (Bates *et al.*, 1991). The denitrosation process seems to involve a metabolic activation step at external membranes (Kowaluk & Fung, 1990a), which could then explain why the S-nitrosothiols were insensitive to the superoxide anion generator, LY 83583 in the rat gastric fundus, except for the very unstable CysNO. In vascular tissue, tolerant to GTN, the relaxant effect of S-nitrosothiols is thus not or only marginally reduced (Henry *et al.*, 1989a; Kowaluk & Fung, 1990b).

In the rat gastric fundus, made tolerant to GTN, the relaxant effect of the S-nitrosothiols was however clearly decreased, although statistical significance was reached only with SNAP. The mechanism underlying this observation is unclear. Desensitization of guanylate cyclase in tissues, tolerant to organic nitrates, has been implicated as a secondary mechanism to explain the sometimes observed moderate degree of cross-tolerance between organic and non-organic nitrates (Van de Voorde, 1991). This seems unlikely in the rat gastric fundus as the response to NO was well maintained in tolerant strips. By contrast, the NANC relaxation induced by electrical stimulation was significantly reduced, suggesting that the NANC neurones cannot only release free NO. If an S-nitrosothiol were released, one might expect a more pronounced influence of nitrate tolerance on EFS-induced NANC relaxation, as the response to S-nitrosothiols was on average reduced by 50%. The observation that LY 83583, which *per se* does not influence the EFS- and GSNO-induced relaxation, further inhibited the EFS-evoked response in tolerant tissues but not that of GSNO, may clarify this. In circumstances of nitrate tolerance, thiol depletion may occur in NANC nerves and may impair the incorporation of NO into a thiol. This might result in a proportionally larger share of the NANC output being released as free NO, sensitive to LY 83583.

In conclusion, the S-nitrosothiols CysNO, SNAP, SNAC and GSNO potently relax the rat gastric fundus. S-nitrosothiols such as SNAC and GSNO may be involved in NANC neurotransmission.

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References

ALDRED, S.E. & WILLIAMS, D.L.H. (1982). Kinetics and mechanism of the nitrosation of alcohols, carbohydrates, and a thiol. *J. Chem. Soc. Perkin Trans.*, **II**, 777–782.

BARBIER, A.J.M. & LEFEBVRE, R.A. (1992a). Effect of LY 83583 on relaxation induced by non-adrenergic non-cholinergic nerve stimulation and exogenous nitric oxide in the rat gastric fundus. *Eur. J. Pharmacol.*, **219**, 331–334.

BARBIER, A.J. & LEFEBVRE, R.A. (1992b). Effect of 3-isobutyl-1-methylxanthine and zaprinast on non-adrenergic non-cholinergic relaxation in the rat gastric fundus. *Eur. J. Pharmacol.*, **210**, 315–323.

BARBIER, A.J. & LEFEBVRE, R.A. (1993). Influence of nitrosothiols on the rat gastric fundus. *Arch. Int. Pharmacodyn.*, **322**, 126.

BASSENGE, E. & ZANZINGER, J. (1992). Nitrates in different vascular beds, nitrate tolerance, and interactions with endothelial function. *Am. J. Cardiol.*, **70**, 23B–29B.

BATES, N., HARRISON, D.G., MYERS, P.R. & MINOR, R.L. (1991). EDRF: nitrosylated compound or authentic nitric oxide. *Basic Res. Cardiol.*, **86**, Suppl. 2, 17–26.

BELVISI, M.G., STRETTON, C.D., YACOUB, M. & BARNES, P.J. (1992). Nitric oxide is the endogenous neurotransmitter of bronchodilator nerves in humans. *Eur. J. Pharmacol.*, **210**, 221–222.

BOECKXSTAENS, G.E., PELCKMANS, P.A., DE MAN, J.G., BULT, H., HERMAN, A.G. & VAN MAERCKE, Y.M. (1992). Evidence for a differential release of nitric oxide and vasoactive intestinal polypeptide by nonadrenergic noncholinergic nerves in the rat gastric fundus. *Arch. Int. Pharmacodyn.*, **318**, 107–115.

BULT, H., BOECKXSTAENS, G.E., PELCKMANS, P.A., JORDAENS, F.H., VAN MAERCKE, Y.M. & HERMAN, A.G. (1990). Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. *Nature*, **345**, 346–347.

BYLER, D.M., GOSSER, D.K. & SUSI, H. (1983). Spectroscopic estimation of the extent of S-nitrosothiol formation by nitrite action on sulfhydryl groups. *J. Agric. Food Chem.*, **31**, 523–527.

D'AMATO, M., CURRO, D., MONTUSCHI, P., CIABATTONI, G., RAGAZZONI, E. & LEFEBVRE, R.A. (1992). Release of vasoactive intestinal polypeptide from the rat gastric fundus. *Br. J. Pharmacol.*, **105**, 691–695.

FIELD, L., DILTS, R.V., RAVICHANDRAN, R., LENHERT, P.G. & CARNAHAN, G.E. (1978). An unusually stable thionitrite from N-acetyl-D,L-penicillamine; X-ray crystal and molecular structure of 2-(acetylamino)-2-carboxy-1,1-dimethylethyl thionitrite. *J.C.S. Chem. Commun.*, 249–250.

FUNG, H.-L., CHUNG, S.-J., BAUER, J.A., CHONG, S. & KOWALUK, E.A. (1992). Biochemical mechanism of organic nitrate action. *Am. J. Cardiol.*, **70**, 4B–10B.

GARTHWAITE, J. (1991). Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends Neurosci.*, **14**, 60–66.

GIBSON, A., BABBEDGE, R., BRAVE, S.R., HART, S.L., HOBBS, A.J., TUCKER, J.F., WALLACE, P. & MOORE, P.K. (1992). An investigation of some S-nitrosothiols, and of hydroxy-arginine, on the mouse anococcygeus. *Br. J. Pharmacol.*, **107**, 715–721.

GILLESPIE, J.S., LIU, X. & MARTIN, W. (1989). The effects of L-arginine and N^G-monomethyl L-arginine on the response of the rat anococcygeus muscle to NANC nerve stimulation. *Br. J. Pharmacol.*, **98**, 1080–1082.

GILLESPIE, J.S. & SHENG, H. (1990). The effects of pyrogallol and hydroquinone on the response to NANC nerve stimulation in the rat anococcygeus and the bovine retractor penis muscles. *Br. J. Pharmacol.*, **99**, 194–196.

HECKER, M., SIEGLE, I., MACARTHUR, H., SESSA, W.C. & VANE, J.R. (1992). Role of intracellular thiols in release of EDRF from cultured endothelial cells. *Am. J. Physiol.*, **262**, H888–H896.

HENRY, P.J., DRUMMER, O.H. & HOROWITZ, J.D. (1989a). S-nitrosothiols as vasodilators: implications regarding tolerance to nitric oxide-containing vasodilators. *Br. J. Pharmacol.*, **98**, 757–766.

HENRY, P.J., HOROWITZ, J.D. & LOUIS, W.J. (1989b). Nitroglycerin-induced tolerance affects multiple sites in the organic nitrate bioconversion cascade. *J. Pharmacol. Exp. Ther.*, **248**, 762–768.

HOBBS, A.J., TUCKER, J.F. & GIBSON, A. (1991). Differentiation by hydroquinone of relaxations induced by exogenous and endogenous nitrates in non-vascular smooth muscle: role of superoxide anions. *Br. J. Pharmacol.*, **104**, 645–650.

IGNARRO, L.J. (1990). Biosynthesis and metabolism of endothelium-derived nitric oxide. *Annu. Rev. Pharmacol. Toxicol.*, **30**, 535–560.

IGNARRO, L.J., BUGA, G.M., WOOD, K.S., BYRNS, R.E. & CHAUDHURI, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 9265–9269.

IGNARRO, L.J., BUSH, P.A., BUGA, G.M., WOOD, K.S., FUKUTO, J.M. & RAJFER, J. (1990). Nitric oxide and cyclic GMP formation upon electrical field stimulation cause relaxation of corpus cavernosum smooth muscle. *Biochem. Biophys. Res. Commun.*, **170**, 843–850.

IGNARRO, L.J., LIPPTON, H., EDWARDS, J.C., BARICOS, W.H., HYMAN, A.L., KADOWITZ, P.J. & GRUETTER, C.A. (1981). Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates. *J. Pharmacol. Exp. Ther.*, **218**, 739–749.

JANSEN, A., DRAZEN, J., OSBORNE, J.A., BROWN, R., LOSCALZO, J. & STAMLER, J.S. (1992). The relaxant properties in guinea pig airways of S-nitrosothiols. *J. Pharmacol. Exp. Ther.*, **261**, 154–160.

KANNAN, M.S. & JOHNSON, D.E. (1992). Nitric oxide mediates the neural nonadrenergic, noncholinergic relaxation of pig tracheal smooth muscle. *Am. J. Physiol.*, **262**, L511–L514.

KEITH, R.A., BURKMAN, A.M., SOKOLOSKI, T.D. & FERTEL, R.H. (1983). Nitroglycerin tolerance and cyclic GMP generation in the longitudinal smooth muscle of the guinea-pig ileum. *J. Pharmacol. Exp. Ther.*, **225**, 29–34.

KELM, M. & SCHRADER, J. (1990). Control of coronary vascular tone by nitric oxide. *Circ. Res.*, **66**, 1561–1575.

KERR, S.W., BUCHANAN, L.V., BUNTING, S. & MATHEWS, W.R. (1992). Evidence that S-nitrosothiols are responsible for the smooth muscle relaxing activity of the bovine retractor penis inhibitory factor. *J. Pharmacol. Exp. Ther.*, **263**, 285–292.

KITAMURA, K., LIAN, Q., CARL, A. & KURIYAMA, H. (1993). S-nitrosocysteine, but not sodium nitroprusside, produces apamin-sensitive hyperpolarization in rat gastric fundus. *Br. J. Pharmacol.*, **109**, 415–423.

KNUDSEN, M.A., SVANE, D. & TØTTRUP, A. (1992). Action profiles of nitric oxide, S-nitroso-L-cysteine, SNP and NANC responses in opossum lower esophageal sphincter. *Am. J. Physiol.*, **262**, G840–G846.

KOWALUK, E. & FUNG, H.-L. (1990a). Spontaneous liberation of nitric oxide cannot account for in vitro vascular relaxation by S-nitrosothiols. *J. Pharmacol. Exp. Ther.*, **255**, 1256–1264.

KOWALUK, E.A. & FUNG, H.-L. (1990b). Dissociation of nitrovasodilator-induced relaxation from cyclic GMP levels during in vitro nitrate tolerance. *Eur. J. Pharmacol.*, **176**, 91–95.

KOWALUK, E.A., POLISZCZUK, R. & FUNG, H.-L. (1987). Tolerance to relaxation in rat aorta: comparison of an S-nitrosothiol with nitroglycerin. *Eur. J. Pharmacol.*, **144**, 379–383.

LI, C.G. & RAND, M.J. (1990). Nitric oxide and vasoactive intestinal polypeptide mediate non-adrenergic non-cholinergic inhibitory transmission to smooth muscle of the rat gastric fundus. *Eur. J. Pharmacol.*, **191**, 303–309.

MEISTER, A. & ANDERSON, M.E. (1983). Glutathione. *Annu. Rev. Biochem.*, **52**, 711–760.

MYERS, P.R., MINOR, R.L. Jr., GUERRA, R. Jr., BATES, J.N. & HARRISON, D.G. (1990). Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble S-nitrosocysteine than nitric oxide. *Nature*, **345**, 161–163.

NEEDLEMAN, P., JAKSCHIK, B. & JOHNSON, E.M. Jr. (1973). Sulfhydryl requirement for relaxation of vascular smooth muscle. *J. Pharmacol. Exp. Ther.*, **187**, 324–331.

NEEDLEMAN, P. & JOHNSON, E.M. Jr. (1973). Mechanism of tolerance development to organic nitrates. *J. Pharmacol. Exp. Ther.*, **184**, 709–715.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1987). The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. *Br. J. Pharmacol.*, **92**, 639–646.

RAJANAYAGAM, M.A.S., LI, C.G. & RAND, M.J. (1993). Differential effects of hydroxocobalamin on NO-mediated relaxations in rat aorta and anococcygeus muscle. *Br. J. Pharmacol.*, **108**, 3–5.

RUBANYI, G.M., JOHNS, A., HARRISON, D. & WILCOX, D. (1990). S-nitroso-L-cysteine, but not nitric oxide, behaves like EDRF in EPR analysis of nitrosylhemoglobin. *Arch. Int. Pharmacodyn.*, **305**, 298.

SALVEMINI, D., PISTELLI, A. & VANE, J. (1993). Conversion of glyceryl trinitrate to nitric oxide in tolerant and non-tolerant smooth muscle and endothelial cells. *Br. J. Pharmacol.*, **108**, 162–169.

THOMPSON, W.J. (1991). Cyclic nucleotide phosphodiesterases: pharmacology, biochemistry and function. *Pharmacol. Ther.*, **51**, 13–33.

THORNBURY, K.D., WARD, S.M., DALZIEL, H.H., CARL, A., WESTFALL, D.P. & SANDERS, K.M. (1991). Nitric oxide and nitrosocysteine mimic nonadrenergic noncholinergic hyperpolarization in canine proximal colon. *Am. J. Physiol.*, **261**, G553–G557.

VAN DE VOORDE, J. (1991). Mechanisms involved in the development of tolerance to nitrovasodilators. *J. Cardiovasc. Pharmacol.*, **17**, Suppl. 3, S304–S308.

VANE, J. (1957). A sensitive method for the assay of 5-hydroxytryptamine. *Br. J. Pharmacol. Chemother.*, **12**, 344–349.

WALDMAN, S.A. & MURAD, F. (1987). Cyclic GMP synthesis and function. *Pharmacol. Rev.*, **39**, 163–196.

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Non-adrenergic, non-cholinergic relaxation of the bovine retractor penis muscle: role of S-nitrosothiols

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1 This study examined the possibility that an S-nitrosothiol, rather than nitric oxide, functions as the non-adrenergic, non-cholinergic (NANC) inhibitory neurotransmitter in the bovine retractor penis (BRP) muscle.

2 Treatment of BRP muscle with either of two sulphydryl inactivating agents, diamide (1 mM) and N-ethylmaleimide (0.3 mM), inhibited NANC relaxation and this was prevented by pretreating tissues with L-cysteine (3 mM), L-glutathione (3 mM) or dithiothreitol (3 mM). Inhibition was not specific, however, since the inactivating agents also inhibited the relaxant actions of authentic nitric oxide (0.3 μM), glyceryl trinitrate (0.001–1 μM) and isoprenaline (0.01–1 μM).

3 Reacting nitric oxide with L-cysteine in nominally oxygen-free solution at pH 3, followed by purging to remove free nitric oxide and neutralisation, produced greater and more prolonged relaxant activity when assayed on rabbit aortic rings than could be attributed to nitric oxide alone. H.p.l.c. analysis of the mixture identified a new peak distinct from either L-cysteine or nitric oxide which was responsible for the relaxant activity. The spectral absorption of this new compound had two bands with peaks at 218 and 335 nm.

4 Using a series of structural analogues of L-cysteine (all at 15 mM) it was found that removal of the carboxyl group (L-cysteamine), replacement of the carboxyl with an ester function (L-cysteine methyl ester) or substitution at the amino group (N-acetyl-L-cysteine) had no effect on the ability to generate relaxant activity upon reaction with nitric oxide (0.1 mM). In contrast, substitution at the sulphydryl group (S-methyl-L-cysteine, L-cysteinesulfinic acid and L-cysteic acid), or formation of disulphides (L-cystine and L-cystamine) led to a complete loss of ability to generate relaxant activity. L-Glutathione was also able to react with nitric oxide to produce relaxant activity, and this too was blocked upon substitution of the free sulphydryl group (S-methyl-L-glutathione). A free sulphydryl group was therefore required to generate relaxant activity following reaction with nitric oxide.

5 Reacting L-cysteine (10 mM) with nitric oxide (~3 mM) under more stringent oxygen-free conditions followed by purging to remove free nitric oxide resulted in the generation of low relaxant activity and small absorption peaks at 218 and 335 nm and these were unaffected upon exposure to the air. In contrast, admitting air to the reaction chamber before purging enhanced both relaxant activity and the absorption peaks at 218 and 335 nm by some 40 fold and the solution turned pink due to the appearance of another absorption peak at 543 nm. This enhanced relaxant activity was not due to nitrogen dioxide being the reactive species, since at 0.1 mM this gas failed to react with L-cysteine to generate relaxant activity, and at 1 mM generated less activity than the equivalent concentration of nitric oxide.

6 The relaxant activity generated by reacting nitric oxide with L-cysteine or L-glutathione was abolished following treatment with haemoglobin (3 μM), methylene blue (10 μM) or N-methylhydroxylamine (100 μM), but was unaffected by N^G-nitro-L-arginine (30 μM). Furthermore, two agents that generate superoxide anion, pyrogallol (0.1 mM) and hydroquinone (0.1 mM), also inhibited this relaxant activity as well as that induced by authentic nitric oxide (0.3 μM) but as previously reported, had no effect on relaxation induced by NANC nerve stimulation. Superoxide dismutase (100 u ml^{-1}) reversed the actions of pyrogallol and hydroquinone but had no effect on NANC relaxation.

7 In conclusion, the reaction of nitric oxide with L-cysteine or L-glutathione generates relaxant activity which exceeds that of nitric oxide alone and probably results from formation of S-nitrosocysteine and S-nitrosoglutathione, respectively. The effects of pyrogallol and hydroquinone suggest that the NANC neurotransmitter is a superoxide anion-resistant, nitric oxide-releasing molecule and that neither S-nitrocysteine nor S-nitrosoglutathione is a suitable candidate for this.

Keywords: Nitric oxide; NANC nerves; S-nitrosothiols; S-nitrosocysteine; S-nitrosoglutathione; L-cysteine, bovine retractor penis; pyrogallol; hydroquinone; superoxide anion

Introduction

It is now well established that the L-arginine nitric oxide system (Moncada, 1992) mediates the phenomenon of endothelium-dependent relaxation of blood vessels first reported by Furchgott & Zawadzki (1980). This system is also responsible for the cytostatic and cytotoxic actions of macrophages (Hibbs *et al.*, 1988; Granger *et al.*, 1990), and for the hypotension associated with septic shock (Gray *et al.*, 1991; Kilbourn & Griffith, 1992). Largely on the basis of the actions of analogues of L-arginine that block nitric oxide synthesis (Bowman *et al.*, 1986; Moore *et al.*, 1990; Gibson *et al.*, 1990), we and others have recently proposed that this system can also account for the inhibitory non-adrenergic, non-cholinergic (NANC) transmission process in anococcygeus muscles of the rat and mouse and the retractor penis muscle of the bull (Gillespie *et al.*, 1989; Gibson *et al.*, 1990;

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Hobbs & Gibson, 1990; Martin *et al.*, 1993). Subsequently, this system was shown in a variety of species to mediate NANC relaxant responses in the gastrointestinal tract (Boeckxstaens *et al.*, 1991), the lung (Tucker *et al.*, 1990; Li & Rand, 1991), corpus cavernosus (Ignarro *et al.*, 1990; Pickard *et al.*, 1991), and penile and cerebral arteries (Toda & Okamura, 1990; Liu *et al.*, 1991). Furthermore, the same pathway is known to be present in certain neurones of the central nervous system (Garthwaite *et al.*, 1988; Bredt *et al.*, 1990; 1992), and may play a role in nociception (Moore *et al.*, 1993) as well as in long-term potentiation and inhibition (Schuman & Madison, 1991; Bredt & Snyder, 1992).

Controversy still exists, however, concerning the precise nature of the mediator produced by the L-arginine: nitric oxide system in the vascular endothelium. For example, although some authors have reported that the level of nitric oxide, measured either by chemiluminescence (Palmer *et al.*, 1987) or by oxidation of haemoglobin (Kelm & Schrader, 1988), can account for the activity of endothelium-derived relaxing factor (EDRF), others have challenged this view (Myers *et al.*, 1989; 1990). Indeed, Myers *et al.* (1990) have proposed that the stability and potency of EDRF more closely resembles that of S-nitrosocysteine than of nitric oxide. Doubt has also been cast on the assumption that the NANC transmitter in the anococcygeus and BRP is simply nitric oxide (Gillespie & Sheng, 1990; Gibson *et al.*, 1992). Specifically, generation of the superoxide anion by the xanthine: xanthine oxidase system or by drugs such as pyrogallol or hydroquinone destroys the activity of authentic nitric oxide and of EDRF either in cascade bioassay systems or in arterial rings (Gryglewski *et al.*, 1986; Moncada *et al.*, 1986; Rubanyi & Vanhoutte, 1986), but does not inhibit NANC relaxation (Gillespie & Sheng, 1990; Gibson *et al.*, 1992). It is possible therefore that the NANC neurotransmitter in these tissues may be a superoxide anion-resistant, nitric oxide-releasing molecule rather than free nitric oxide.

The aim of this study was to investigate the possibility that S-nitrosocysteine or a related S-nitrosothiol acts as the NANC neurotransmitter in the BRP muscle. This was attempted first, by examining the actions of two sulphydryl inactivating agents, diamide and N-ethylmaleimide (Minor *et al.*, 1989; Siegle *et al.*, 1993), on NANC relaxation, and secondly, by comparing the properties of the NANC transmitter with those of authentic nitric oxide and those of the relaxants produced following the reaction of nitric oxide with L-cysteine and certain structural analogues.

Methods

Preparation of tissues and tension recording

The bioassay of relaxant activity was assessed either on strips of bovine retractor penis (BRP) muscle or rings of rabbit aorta. BRP muscles were obtained from a local abattoir; some tissues were used that day but others were stored at 4°C in Krebs solution for use the following day. Strips of muscle (2 × 10 mm) were cut and suspended for tension recording either on glass hooks, or within Ag-AgCl ring electrodes if the NANC nerves were to be stimulated. The preparation of rabbit aortic rings was essentially as described previously (Martin *et al.*, 1985). Briefly, male New Zealand rabbits were killed by a lethal injection of pentobarbitone and exsanguination. The aorta was removed, cleared of adhering fat and connective tissue and cut into 2.5 mm wide transverse rings using a razor blade slicing device. Endothelial cells were removed from all rings by gently rubbing the intimal surface with moist filter paper. Rings were suspended for tension recording on stainless steel hooks. Both the aortic rings and BRP muscle strips were suspended under 2 g resting tension in 20 ml organ chambers and bathed at 37°C in Krebs solution comprising (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24, glucose 11 and gassed with 95% O_2 and 5% CO_2. Tension was measured with Grass FTO3C transducers and displayed on a Grass polygraph. Tissues were allowed to equilibrate for 90 min before experiments were begun. Relaxant studies were conducted following submaximal contraction of aortic rings and BRP strips with phenylephrine (0.3 μM). In experiments on the BRP in which NANC nerves were stimulated, adrenergic motor responses were blocked and the tone raised with guanethidine (30 μM). Electrical field stimulation was delivered from a Grass S88 stimulator at 2 Hz, 20 pulses, pulse width 0.5 ms and supramaximal voltage at 3 min intervals.

Nitric oxide solutions and reaction of nitric oxide with L-cysteine and analogues

Solutions of nitric oxide gas were prepared by submerging a brown bottle (volume 18 ml) in a beaker containing 200 ml of distilled, deionised water and gassing with oxygen-free nitrogen (British Oxygen Company) for 1 h. While still submerged, a rubber septum was used to seal the bottle and 4 μl of nitric oxide gas (99.9%, Air Products, U.K.) was injected into the bottle to give a concentration of 20 μM. These solutions of nitric oxide retained their activity for several hours while remaining sealed.

The reaction between nitric oxide and L-cysteine or its analogues (all at 15 mM), was performed under anaerobic conditions but experience showed us how difficult it was to achieve true oxygen-free conditions. Initially, similar precautions to those described in the preceding paragraph for nitric oxide solutions were used. When later experiments showed some oxygen remained and played an important part in the reaction, more elaborate precautions were taken. Specifically, a sealed cuvette containing an aqueous solution of L-cysteine or one of its analogues at pH 3 was submerged in distilled water within a sealed Amicon chamber. Both the cuvette contents and the surrounding distilled water were purged with oxygen-free nitrogen for 1 h. The nitrogen was purified by passing through a series of 300 ml wash bottles containing successively, Fieser's solution to remove any traces of oxygen, saturated lead acetate to remove H_2S from the Fieser's solution and 10% NaOH to remove any nitrogen

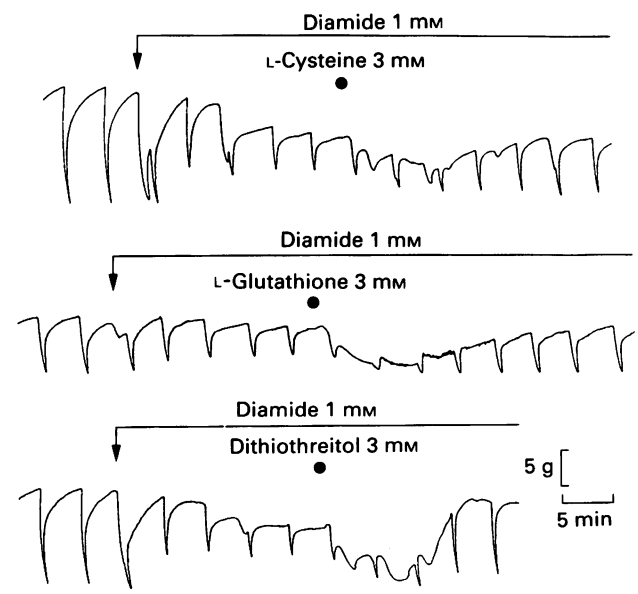


Figure 1 Following induction of tone and blockade of adrenergic motor responses, electrical field stimulation (2 Hz, 20 pulses) of strips of bovine retractor penis muscle elicited NANC relaxant responses. These relaxant responses were inhibited by treatment with diamide (1 mM) and this inhibition was partially reversed upon addition of L-cysteine, L-glutathione or dithiothreitol, all at 3 mM.

dioxide. Nitric oxide gas was passed through the same solutions directly into the cuvette containing the L-cysteine for saturated solutions (~3 mM) or measured volumes of nitric oxide gas were removed with a gas-tight syringe and injected into the L-cysteine solution to give concentrations of 0.1 or 1 mM. The tubing connecting the wash bottles was stainless steel since rubber or plastic tubing absorbed nitrogen dioxide and was permeable to oxygen and the gas-tight syringe was flushed several times with purified oxygen-free nitrogen. Fieser's solution was prepared by adding 60 g of potassium hydroxide, 6 g of sodium anthraquinone-β-sulphonate and 45 g of sodium dithionite to 300 ml of distilled water (Fieser & Fieser, 1967).

In some experiments, nitrogen dioxide (1 mM) was reacted with L-cysteine (10 mM) by the same procedure detailed above for nitric oxide. Nitrogen dioxide was prepared either by mixing purified nitric oxide gas with an equal volume of oxygen (British Oxygen Company) in a gas-tight syringe or liquid nitrogen dioxide was made by heating anhydrous lead nitrate and condensing the evolved nitrogen dioxide gas in a glass coil cooled with dry ice (Moody, 1991).

These solutions were taken for bioassay or spectrophotometric analysis on a Shimadzu UV 240 dual beam spectrophotometer. High performance liquid chromatography (h.p.l.c.) separation was performed using a μBondpac Radial-Pac C18 reverse-phase column (8 × 100 mm) with distilled, deionised water at pH 3 at a flow rate of 1.5 ml min^{-1} and detection was by absorbance at 254 nm.

Drugs

N-acetyl-L-cysteine, L-cystamine, L-cysteamine, L-cysteic acid, L-cysteine, L-cysteine methyl ester, L-cysteinesulphinic acid, L-cystine, L-glutathione, guanethidine sulphate, haemoglobin (bovine), hydroquinone, isoprenaline hydrochloride, L-methionine, S-methyl-L-cysteine, methylene blue, S-methyl-L-glutathione, N-methylhydroxylamine hydrochloride, N^G-nitro-L-arginine, phenylephrine hydrochloride and superoxide dismutase (bovine erythrocyte) were obtained from Sigma (U.K.); diamide, dithiothreitol and N-ethylmaleimide were obtained from Aldrich (U.K.); pyrogallol was obtained from BDH (U.K.) and glyceryl trinitrate (10% w/w in lactose) was obtained from Napp Laboratories (U.K.). All drugs were dissolved and dilutions made in distilled, deionised water.

Solutions of oxyhaemoglobin were prepared as previously described (Martin *et al.*, 1985). Briefly, a 10 fold molar excess of the reducing agent, sodium dithionite, was added to aqueous solutions of commercial haemoglobin (1 mM) and this was followed by dialysis against 100 volumes of distilled water for 2 h at 4°C. The resulting solutions were stored in aliquots at −20°C for up to 14 days.

Results

Effects of diamide and N-ethylmaleimide

Treatment with either of two sulphydryl inactivating agents, diamide (1 mM, Figure 1) and N-ethylmaleimide (0.3 mM), inhibited NANC relaxation of strips of BRP muscle. The blockade induced by diamide was partially reversed by treatment with the sulphydryl agents, L-cysteine (3 mM), L-glutathione (3 mM) or dithiothreitol (3 mM), but that induced by N-ethylmaleimide was completely unaffected. Pretreatment with L-cysteine (3 mM), L-glutathione (3 mM) or dithiothreitol (3 mM) completely prevented inhibition of NANC relaxation by diamide or N-ethylmaleimide. The relaxant actions of authentic nitric oxide (0.3 μM), glyceryl trinitrate (0.001–1 μM), and isoprenaline (0.01–1 μM) were also inhibited by treatment with diamide (1 mM) or N-ethylmaleimide (0.3 mM). In these experiments also, the inhibitory actions of diamide and N-ethylmaleimide were completely prevented by pretreatment with the three sulphydryl compounds. Once established, however, the inhibitory actions of diamide and N-ethylmaleimide were not reversed by the three sulphydryl compounds.

Effects of nitric oxide and L-cysteine

Nitric oxide (1–100 nM) in oxygen-free solution induced concentration-dependent, transient relaxation of phenylephrine-contracted rings of rabbit aorta and strips of BRP muscle (Figures 2 and 8). L-Cysteine (1.5 mM) had no effect by itself, but mixing nitric oxide with L-cysteine in nominally oxygen-free solution at pH 3 followed by purging with oxygen-free nitrogen to remove any free nitric oxide and neutralisation, giving final bath concentrations equivalent to 0.5 μM nitric oxide and 1.5 mM L-cysteine, produced more powerful and sustained relaxation than nitric oxide alone (Figure 2). Clearly, either L-cysteine protected nitric oxide or the two substances combined to form a new compound and these possibilities were tested by h.p.l.c. analysis. L-Cysteine itself produced two peaks in the h.p.l.c., representing its oxidised and reduced forms, respectively (Figure 3). After mixing L-cysteine (10 mM) with nitric oxide (50 μM) a third peak appeared indicating formation of a new compound and bioassay revealed that this peak contained all of the relaxant activity.

Functional groups of L-cysteine reacting with nitric oxide

L-Cysteine has three functional groups which could potentially react with nitric oxide namely, the sulphydryl, carboxyl and amino groups. A series of analogues (all at 15 mM) were used in which each of these groups was modified and the effect on the ability to form the new relaxant activity following reaction with nitric oxide (0.1 mM) examined. The results were clear cut: modification of the sulphydryl always abolished the ability to form the new relaxant activity whereas modification of either of the other two groups had no effect. For example, substitution of the sulphydryl group (S-methyl-L-cysteine, L-cysteinesulphinic acid and L-cysteic acid) or formation of disulphides (L-cystine and L-cystamine)

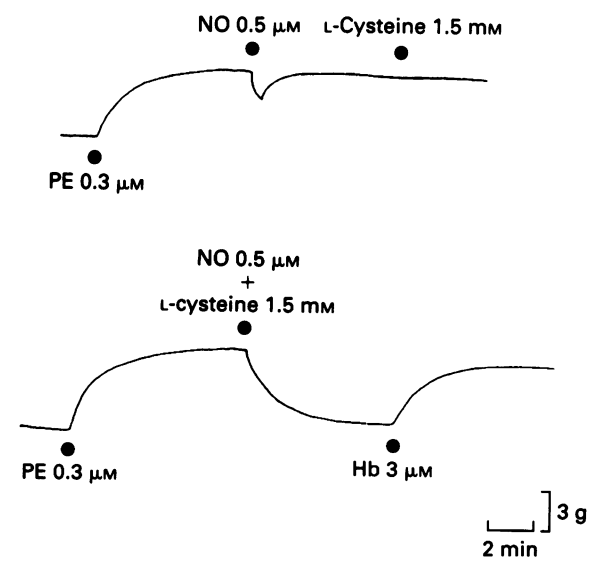


Figure 2 Aqueous solutions of nitric oxide (NO, 0.5 μM) produced transient relaxation of phenylephrine (PE, 0.3 μM)-contracted, endothelium-denuded rings of rabbit aorta, and L-cysteine (1.5 mM) by itself had no effect. Reacting nitric oxide with L-cysteine in nominally oxygen-free solution at pH 3, followed by purging with oxygen-free nitrogen to remove any free nitric oxide and neutralisation, giving final bath concentrations of 0.5 μM nitric oxide and 1.5 mM L-cysteine, produced more powerful and sustained relaxation than could be attributed to nitric oxide alone. This powerful relaxant activity was rapidly abolished upon addition of haemoglobin (Hb 3 μM).

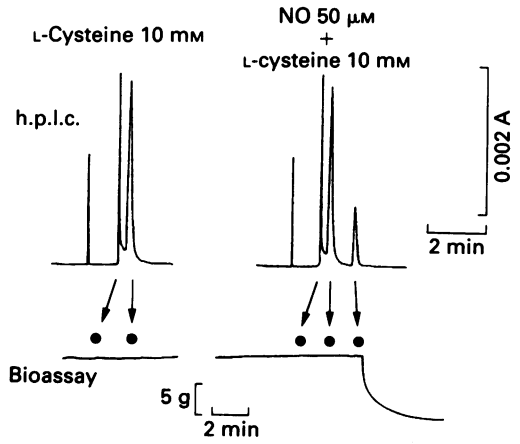


Figure 3 H.p.l.c. analysis split L-cysteine into two peaks representing its oxidised and reduced forms, respectively. Reacting nitric oxide (50 μM) with L-cysteine (10 mM) in nominally oxygen-free solution followed by purging with oxygen-free nitrogen to remove any free nitric oxide and neutralisation resulted in the formation of a new peak which was entirely responsible for relaxant activity as assessed on phenylephrine-contracted endothelium-denuded rings of rabbit aorta.

led to complete loss of the ability to generate relaxant activity, whereas removal of the carboxyl group (L-cysteamine), replacement of the carboxyl with an ester function (L-cysteine methyl ester) or substitution of the amino group (N-acetyl-L-cysteine) had no effect (Figure 4). L-Glutathione was also able to react with nitric oxide to produce relaxant activity, and this too was blocked upon substitution of the free sulphydryl group (S-methyl-glutathione). L-Methionine, which contains a methyl group on the sulphur atom was also unable to generate relaxant activity following reaction with nitric oxide.

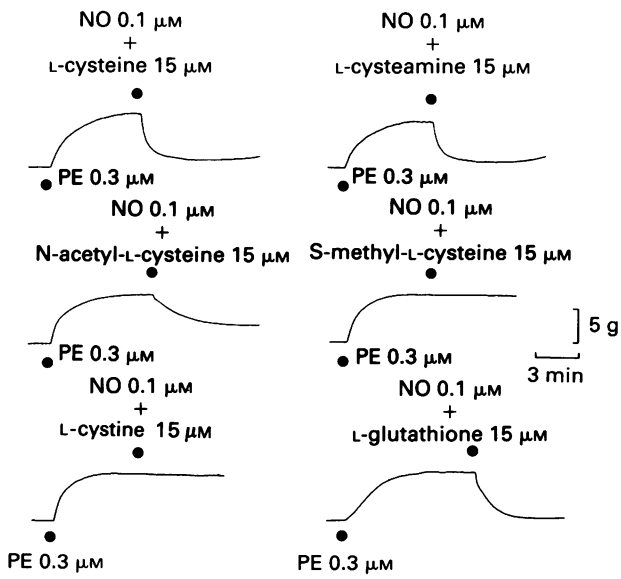


Figure 4 A series of structural analogues (all at 15 mM) was used to determine the functional group of the L-cysteine molecule with which nitric oxide (NO) reacts. Reacting nitric oxide (0.1 mM) with L-cysteine (15 mM) under nominally oxygen-free conditions at pH 3 produced powerful relaxant activity as assessed on phenylephrine (PE, 0.3 mM)-contracted, endothelium-denuded rings of rabbit aorta. L-cysteamine, which lacks the carboxyl group, and N-acetyl-L-cysteine, which has a substituent at the amino group, were able to react with nitric oxide to produce relaxant activity. In contrast, S-methyl-L-cysteine, which has a substituent at the sulphydryl, and L-cystine, the disulphide, failed to react with nitric oxide to produce relaxant activity. L-Glutathione, which also contains a free sulphydryl group reacted with nitric oxide to produce relaxant activity. Final bath equivalents of the reactants are given.

pH dependence

The reaction between L-cysteine and nitric oxide was examined over the range pH 1–8. Generation of powerful relaxant activity was strictly pH-dependent (Figure 5); following purging, neutralisation and assay, little activity had been generated at neutral pH or higher and maximal activity had been obtained at pH 1.

Spectral characteristics and the role of oxygen

As part of the characterization of the new relaxant formed in reacting nitric oxide with L-cysteine, we examined the full spectrum between 190 and 700 nm of each of the reactants and the product formed after mixing. In the course of these experiments it became clear that a low concentration of oxygen was necessary for full activity and that the usual method of producing an oxygen-free environment was inadequate. In our early experiments nitric oxide was reacted with a solution of L-cysteine at pH 3 sealed in a brown bottle immersed in a beaker of distilled water and both the distilled water and the L-cysteine purged with nitrogen before injecting a measured amount of nitric oxide from a gas-tight syringe into the L-cysteine. After allowing 10 min for reaction, free nitric oxide was removed by purging with oxygen-free nitrogen and the mixture was neutralised and assayed. The result was the development of powerful relaxant activity and the appearance of the third peak in the h.p.l.c. shown on Figure 3.

Subsequently, more stringent precautions were taken to exclude oxygen by immersing a sealed cuvette containing

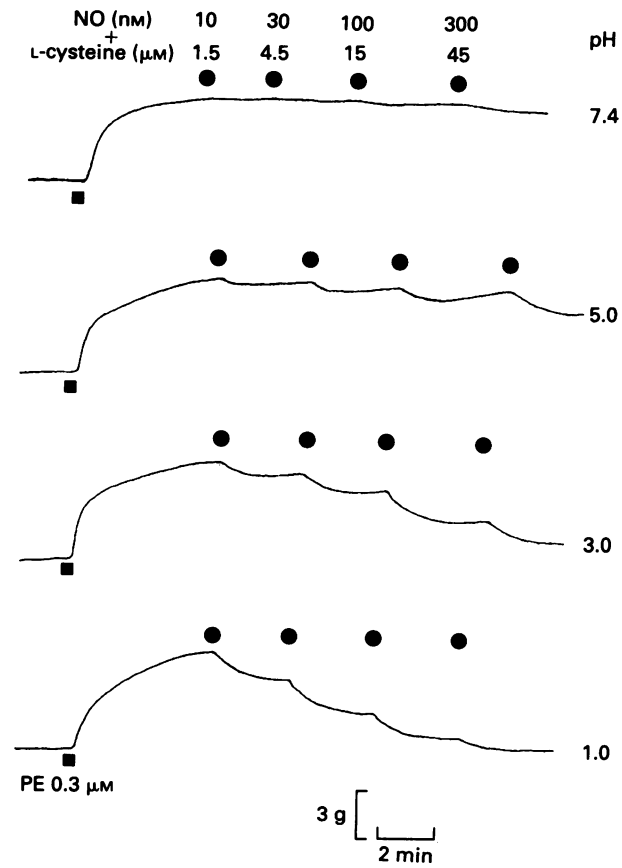


Figure 5 Reacting nitric oxide (NO, 0.1 mM) and L-cysteine (15 mM) in nominally oxygen free solution produced a pH-dependent generation of relaxant activity as assessed on phenylephrine (PE, 0.3 μM)-contracted endothelium-denuded rings of rabbit aorta. Little activity was generated at pH 7.4 and maximal activity was generated at pH 1. Concentrations shown give the final bath equivalent of the two reactants.

L-cysteine (10 mM) at pH 3 in distilled water in an Amicon chamber and purging with nitrogen which had been passed through Fieser's solution followed by bubbling with nitric oxide (~3 mM) for 10 min then purging to remove free nitric oxide. These solutions were clear and their relaxant activity was less (Figure 6) than in the earlier experiments (Figure 2). Exposure of these solutions to air did not affect their relaxant activity. If, however, these solutions were not purged to remove free nitric oxide, opening the cuvette to the air instantly resulted in the development of a pink colour and a ~40 fold increase in relaxant activity (Figure 6).

As would be expected, spectral changes accompanied this change in colour and activity. Where strict precautions were taken to exclude air and free nitric oxide was removed by purging, the clear solution had two new absorption peaks at 218 and 335 nm and these did not change upon exposing the cuvette contents to the air (Figure 6). If, however, the nitric oxide was not removed by purging, opening the cuvette to the air increased these two peaks some 40 fold, the same increase as was seen in relaxant activity. At the same time a third, new absorption peak appeared at 543 nm corresponding to the pink colour of the solution. Other experiments showed that if nitric oxide was not purged but left in contact with L-cysteine in the sealed cuvette than absorbance at 218, 335 and 543 nm increased slowly over a period of hours suggesting a slow entry of oxygen. Furthermore, if the cuvette was opened to the air at any time, the absorbance at these wavelengths increased within seconds to values similar to those shown on Figure 6.

Reactivity of nitric oxide compared with nitrogen dioxide

Since the presence of oxygen greatly increased the generation of relaxant activity, we examined whether or not nitrogen dioxide was the species which reacted with L-cysteine. When either nitric oxide or nitrogen dioxide at 0.1 mM was reacted with L-cysteine (10 mM) at pH 3, relaxant activity and the associated third peak in the h.p.l.c. was only generated with the former (Figure 7). If, however, the concentration of the two gases was increased to 1 mM, relaxant activity and the associated third peak in the h.p.l.c. were also generated with nitrogen dioxide, but the magnitude of these was lower than for nitric oxide. The effectiveness of this higher concentration probably results from the production of some nitrous acid and therefore nitric oxide from the solution of nitrogen dioxide.

Effects of haemoglobin, methylene blue, N-methylhydroxylamine and N^G-nitro-L-arginine

While the relaxant effect of the new compound was presumably due to nitric oxide acting on soluble guanylate cyclase it was possible that this did not need to be released

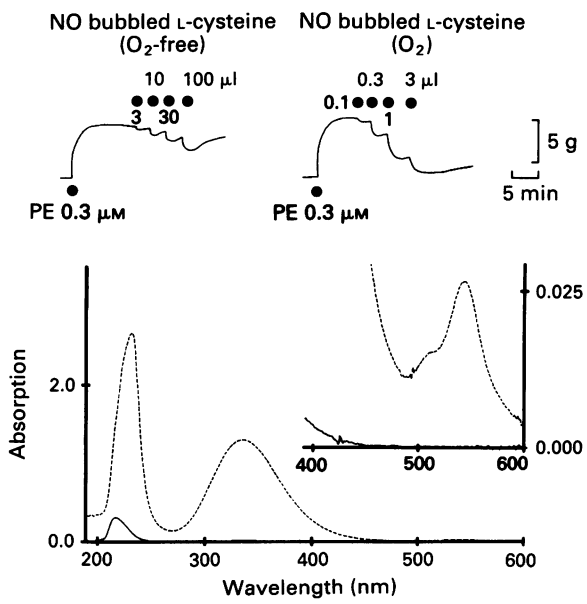


Figure 6 Bubbling nitric oxide gas to saturation (~3 mM) in solutions of L-cysteine (10 mM) in stringent oxygen-free conditions (O_2-free) followed by purging with oxygen-free nitrogen to remove free nitric oxide and neutralisation produced low relaxant activity as assessed on phenylephrine (PE, 0.3 µM)-contracted, endothelium-denuded rings of rabbit aorta. These solutions were colourless but contained two small absorption peaks at 218 and 335 nm (continuous line). If, however, nitric oxide was bubbled to saturation in solutions of L-cysteine (10 mM) and the reaction chamber was opened to the air (O_2) before purging and neutralisation, the relaxant activity generated was greatly (~40 fold) increased. The absorption peaks at 218 and 335 nm (discontinuous line) increased by a proportionate degree and a new peak became visible at 543 nm which was responsible for the development of a pink colour.

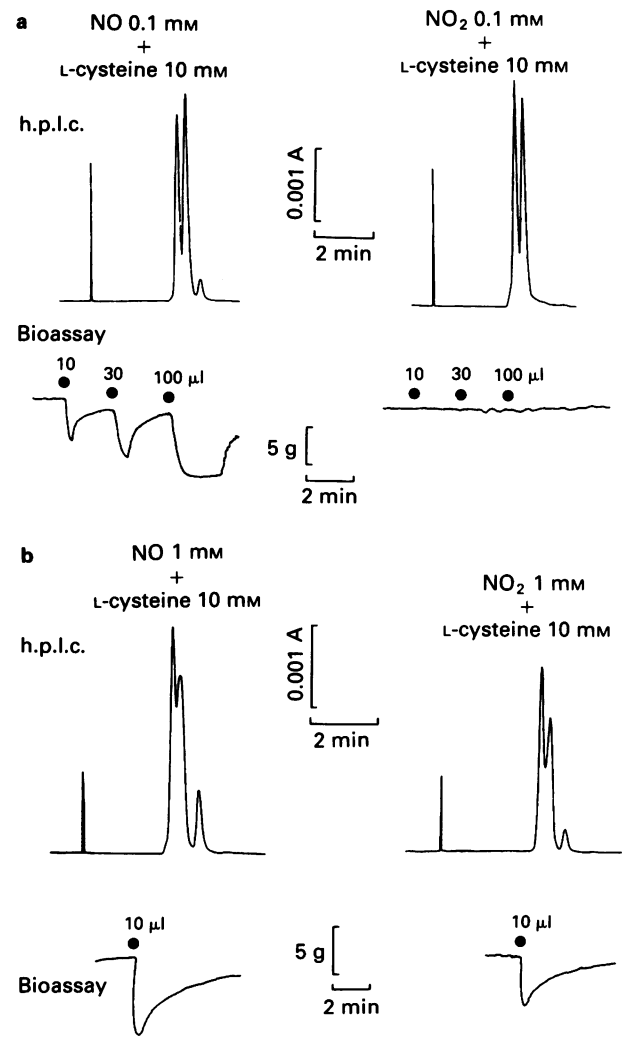


Figure 7 (a) Reacting nitric oxide (NO, 0.1 mM) with L-cysteine (10 mM) under stringent oxygen-free conditions at pH 3 led to the development of relaxant activity as assessed on phenylephrine (0.3 µM)-contracted strips of bovine retractor penis muscle, and h.p.l.c. analysis revealed the presence of three peaks, representing L-cystine, L-cysteine and a new substance that was entirely responsible for relaxant activity. Reacting nitrogen dioxide (NO_2, 0.1 mM) under identical conditions failed to generate relaxant activity or the associated third peak in the h.p.l.c. (b) If the concentration of nitrogen dioxide (NO_2) was increased to 1 mM, relaxant activity and the associated third peak in the h.p.l.c. were generated but the magnitude of these was lower than those generated by nitric oxide (NO) at 1 mM.

from the compound. A more unlikely hypothesis is that the compound could itself stimulate the synthesis of nitric oxide by the NANC nerves. If it releases nitric oxide then haemoglobin should block its action, if it acts via guanylate cyclase then methylene blue and N-methylhydroxylamine should also block, and finally if it activates nitric oxide synthase, N^G-nitro-L-arginine should block. Experimentally the results were clear. Haemoglobin (3 μM, Figure 2), methylene blue (10 μM) and N-methylhydroxylamine (100 μM) abolished the activity of the new relaxant formed from reacting nitric oxide with L-cysteine or L-glutathione but N^G-nitro-L-arginine (30 μM) had not effect.

Effects of pyrogallol and hydroquinone

As previously reported (Gillespie & Sheng, 1990), we found that treatment of strips of BRP with the superoxide anion-generating drugs, pyrogallol (0.1 mM, Figure 8) or hydroquinone (0.1 mM, data not shown), abolished the relaxant actions of authentic nitric oxide (0.3 μM) but had no effect on that to NANC nerve stimulation (2 Hz, 20 pulses). The effect on nitric oxide was blocked by superoxide dismutase (100 u ml^{-1}). Treatment with pyrogallol (0.1 mM) or hydroquinone (0.1 mM, data not shown) also abolished the activity of the new relaxant generated following reaction of nitric oxide (1 mM) with L-cysteine (10 mM) or L-glutathione (10 mM, data not shown), and this inhibition too was reversed by superoxide dismutase (100 u ml^{-1}). Furthermore, superoxide dismutase (100 u ml^{-1}) potentiated the actions of the new relaxant but had no effect on relaxation to NANC nerve stimulation. Other experiments showed that if pyrogallol (0.1 mM) or hydroquinone (0.1 mM) was added to the reaction chamber containing nitric oxide and L-cysteine or L-glutathione rather than to the BRP, then relaxant activity was also destroyed.

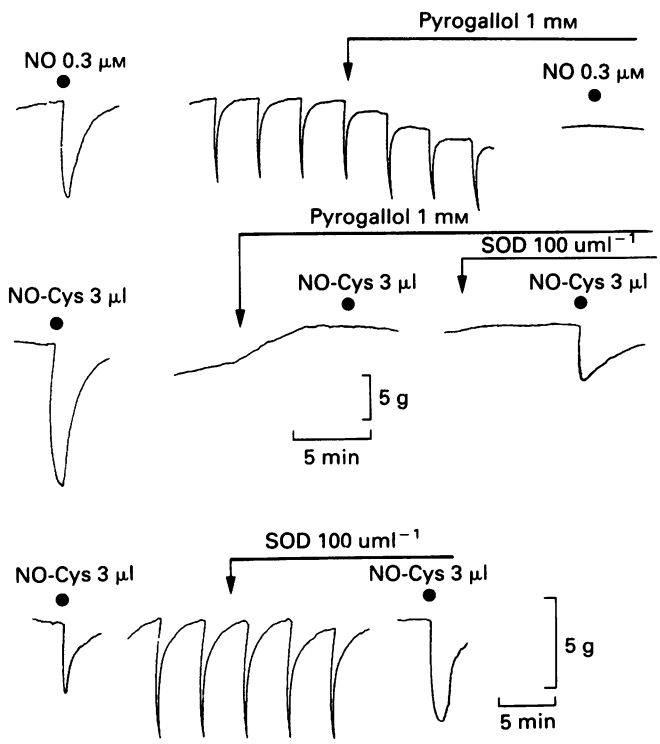


Figure 8 Authentic nitric oxide (NO, 0.3 μM), electrical field stimulation (2 Hz, 20 pulses) of the NANC nerves and the product (NO-Cys) of reacting nitric oxide (1 mM) with L-cysteine (10 mM) all relaxed strips of bovine retractor penis muscle. Treatment with pyrogallol (0.1 mM) abolished the relaxant actions of nitric oxide and of NO-Cys but had no effect on those to NANC nerve stimulation. The inhibitory effects of pyrogallol were reversed following treatment with superoxide dismutase (SOD, 100 u ml^{-1}). Superoxide dismutase also potentiated the relaxant actions of NO-Cys but had no effect on those to NANC nerve stimulation.

Discussion

It is now widely accepted that the L-arginine: nitric oxide system mediates inhibitory NANC transmission in the BRP and the related anococcygeus muscles of the rat and mouse (Gillespie *et al.*, 1989; Gibson *et al.*, 1990; Hobbs & Gibson, 1990; Martin *et al.*, 1993). The finding that superoxide anion-generating drugs such as pyrogallol and hydroquinone inhibit the relaxant actions of authentic nitric oxide but not of NANC nerve stimulation in the BRP and mouse anococcygeus (Gillespie & Sheng, 1990; Gibson *et al.*, 1992), suggests that the neurotransmitter in these tissues may be a superoxide anion-resistant, nitric oxide-releasing molecule rather than free nitric oxide. Indeed, an analogous debate continues concerning the precise nature of EDRF which Myers *et al.* (1990) suggest most closely resembles S-nitrosocysteine than nitric oxide. If a compound does exist which binds nitric oxide and acts as a delivery system between synthesis in the nerve endings or endothelium and action on guanylate cyclase in the effector cells it would ideally meet certain conditions. Specifically, it would bind nitric oxide securely enough to preclude non-specific interactions with proteins and other substances and prevent destruction by superoxide anion. Furthermore, the binding would be reversible so as to deliver nitric oxide to guanylate cyclase.

Sulphydryl inactivating agents have previously been employed to investigate the involvement of S-nitrosothiols in the activity of EDRF, but with equivocal results (Minor *et al.*, 1989; Siegle *et al.*, 1993). We examined the actions of two agents, diamide and N-ethylmaleimide, which respectively oxidise and alkylate sulphydryl groups (Kosower *et al.*, 1969; Siegle *et al.*, 1993). Consistent with involvement of an S-nitrosothiol, we found that both blocked NANC relaxation and this was prevented by pretreating the tissues with excess sulphydryl groups in the form of L-cysteine, L-glutathione or dithiothreitol. These three sulphydryl compounds were also effective in reversing the actions of diamide, presumably through reduction of the -S-S- bonds formed following its action. The actions of N-ethylmaleimide were not reversed, suggesting that blockade had not resulted from covalent inactivation of the sulphydryl groups of endogenous L-cysteine or L-glutathione. The most compelling evidence against the blockade having resulted from loss of the ability to form S-nitrosothiols was obtained when we found that relaxation to nitric oxide, glyceryl trinitrate and isoprenaline was also blocked. It was clear, therefore, that the sulphydryl inactivating agents were blocking the effector pathways for these diverse relaxants and that any additional action in preventing formation of S-nitrosothiols by the NANC nerves would be impossible to discern.

Our next approach was to examine the properties of S-nitrosothiols. A number of methods are available for the synthesis of these compounds, including the reaction of L-cysteine and nitrogen dioxide in cold methanol (Myers *et al.*, 1990), acidification of sodium nitrite in the presence of L-cysteine and other sulphydryl compounds (Kowaluk & Fung, 1990; Gibson *et al.*, 1992; Kerr *et al.*, 1993), and the reaction of nitric oxide with L-cysteine and other sulphydryl compounds under anaerobic conditions (Ignarro & Gruetter, 1980; Ignarro *et al.*, 1981). The S-nitrosothiols produced by each of these routes are powerful smooth muscle relaxants, normally pink in colour, and have a characteristic absorption peak at around 335 nm. There is, however, enormous variability in the reported half-lives of the nitrosothiols produced by these methods ranging from ~30 s (Myers *et al.*, 1990) to 7–10 days (Ignarro & Gruetter, 1980). We initially adopted the method of Ignarro & Gruetter (1980) since we wished to determine if nitric oxide itself rather than higher

oxides of nitrogen could react with the -S-H moiety of L-cysteine. This method has, however, been criticised by Feelisch (1991) who reported that the presence of oxygen was a prerequisite for nitrosation to proceed, and our observations supported this. We found that if nitric oxide was bubbled to saturation in solutions of L-cysteine under rigorous oxygen-free conditions that small absorption peaks were present at 218 and 335 nm, but the solution remained colourless. While still sealed, the absorption peaks at 218 and 335 nm grew slowly with time, but if the solution was opened to the air, they rose roughly 40 fold within a few seconds to stable new values and the solution became pink in colour owing to a new absorbance peak developing at 543 nm. It was likely, therefore, that the presence of oxygen was vital for nitric oxide and L-cysteine to react generating absorption peaks at 218, 335 and 543 nm, and that the small slowly growing peaks at 218 and 335 nm even under our most rigorous oxygen-free conditions were due to the slow entry of trace amounts of oxygen. The development of relaxant activity, as assessed on the BRP and rabbit aorta, was also critically dependent upon the availability of oxygen. Under our rigorous oxygen-free conditions when nitric oxide was allowed to react with L-cysteine, and then unreacted nitric oxide was removed by purging, some relaxant activity was generated, but this increased in direct proportion ($\sim$40 fold) to the increases in absorption at 218 and 335 nm if the solution was exposed to the air before purging.

The requirement for oxygen in the development of relaxant activity and associated absorption peaks showed that nitric oxide itself was unable to react with L-cysteine and that a higher oxide of nitrogen was the reactive species. There is debate over the precise nature of this nitrosating species; some have suggested that it is N_2O_3 or N_2O_4 (Leak *et al.*, 1990), but others dispute this suggesting that it is an as yet uncharacterized oxide of nitrogen (Wink *et al.*, 1993). We also dismissed the possibility that it was nitrogen dioxide, since this gas generated less relaxant activity than nitric oxide following reaction with L-cysteine.

H.p.l.c. analysis confirmed that nitric oxide had indeed reacted with L-cysteine to form a novel compound: the C18 column used fractionated the mixture into three peaks, representing L-cystine (the disulphide), L-cysteine and a third, new peak that was entirely responsible for relaxant activity. Our experiments with a series of structural analogues provided strong evidence that the reaction of nitric oxide or higher oxide was with the sulphydryl group. All derivatives of L-cysteine or L-glutathione in which this group was intact, irrespective of what changes were made in the amino or carboxyl groups, produced powerful relaxants whereas all the compounds in which the sulphydryl was modified were ineffective. It is likely therefore that the relaxants produced were the respective S-nitrosothiols. We confirmed, however, that the smooth muscle relaxant action of the nitrosothiols was derived from the nitric oxide they released (Ignarro *et al.*, 1981; Gibson *et al.*, 1992), by finding that haemoglobin, which binds and inactivates nitric oxide, and methylene blue and N-methylhydroxylamine, which inhibit soluble guanylate cyclase, blocked their activity.

Another striking finding was that the reaction of nitric oxide with L-cysteine was pH-dependent: little relaxant activity was generated at neutral pH, but as the pH was lowered, relaxant activity was increased until an optimum was reached at pH 1. This process is reminiscent of the 'acid-activation' of inhibitory factor extracted from BRP (Gillespie & Martin, 1980). The inactive form of inhibitory factor contains inorganic nitrite and upon acidification generates nitric oxide which binds to a substance which stabilizes its activity (Martin *et al.*, 1988). It has recently been proposed that the acid-activated inhibitory factor is S-nitrosocysteine or S-nitrosoglutathione (Yui *et al.*, 1989; Kerr *et al.*, 1993). Although it is well established that acidification of nitrite leads to liberation of nitric oxide (Furchgott, 1988), it is unclear why acidification should have the additional effect of catalysing the reaction of nitric oxide with L-cysteine. What is clear, however, is that as the chemical reaction between nitric oxide and L-cysteine proceeds so inefficiently at neutral pH, it is unlikely that sufficient S-nitrosocysteine is produced under normal physiological conditions to account for the activity of the NANC neurotransmitter.

Further evidence against S-nitrosocysteine or S-nitrosoglutathione acting as the NANC transmitter in the BRP was obtained using the superoxide anion-generating agents, pyrogallol and hydroquinone. These agents have previously been reported to destroy the activity of authentic nitric oxide but do not affect neurogenic relaxation in the BRP or mouse anococcygeus (Gillespie & Sheng, 1990; Gibson *et al.*, 1992), suggesting that the NANC transmitter involved is a superoxide anion-insensitive, nitric oxide-releasing molecule. We found that pyrogallol and hydroquinone both destroyed the relaxant activity of S-nitrosocysteine, S-nitrosoglutathione and nitric oxide when added to their respective solutions or to the BRP assay tissue, but as before, neither affected relaxation to NANC nerve stimulation. Furthermore, superoxide dismutase potentiated the relaxant actions of nitric oxide and the S-nitrosothiols but had no effect on those to NANC nerve stimulation. Our findings confirm those of Gibson *et al.* (1992) who also found that hydroquinone inhibits the relaxant activity of S-nitrosocysteine, but not of NANC nerve stimulation in the mouse anococcygeus. In contrast to our findings, however, they reported that hydroquinone did not block the actions of S-nitrosoglutathione. Whether this disparity is due to differences in concentrations of drugs used or methods in preparing the nitrosothiol is unclear at present. We are in agreement, however, that the ability of free radical-generating drugs to destroy the activity of S-nitrosocysteine precludes the involvement of this substance in NANC transmission in the BRP and anococcygeus muscles.

In conclusion, although the sulphydryl inactivating agents, diamide and N-ethylmaleimide, inhibit NANC relaxation in the BRP, this occurs through a non-selective action rather than from an inability to form an S-nitrosothiol. Nitric oxide can react in the presence of oxygen and at acid pH with sulphydryl compounds to produce S-nitrosothiols which are potent, long-lasting smooth muscle relaxants. The ability of superoxide anion-generating drugs to inhibit the relaxant actions of these S-nitrosothiols as well as of nitric oxide but not of the NANC nerves, suggests that these substances are not candidates for the inhibitory transmitter. The data suggest that the NANC neurotransmitter is more likely to be a superoxide anion-resistant, nitric oxide-releasing molecule.

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References

BOECKXSTAENS, G.E., PELCKMANS, P.A., RUYTJENS, I.F., BULT, H., DE MAN, J.G., HERMAN, A.G. & VAN MAERCKE, Y.M. (1991). Bioassay of nitric oxide released upon non-adrenergic, non-cholinergic nerves in the canine ileocolonic junction. *Br. J. Pharmacol.*, **103**, 1085–1091.

BOWMAN, A., GILLESPIE, J.S. & SOARES-DE-SILVA, P. (1986). A comparison of the actions of endothelium-derived relaxant factor and the inhibitory factor from the bovine retractor penis on rabbit aortic smooth muscle. *Br. J. Pharmacol.*, **87**, 175–181.

BREDT, D.S., FERRIS, C.D. & SNYDER, S.H. (1992). Nitric oxide synthase regulatory sites. *J. Biol. Chem.*, **267**, 10976–10981.

BREDT, D.S., HWANG, P.M. & SNYDER, S.H. (1990). Localisation of nitric oxide synthase indicating a neural role for nitric oxide. *Nature*, **347**, 768–770.

BREDT, D.S. & SNYDER, S.H. (1992). Nitric oxide, a novel neuronal messenger. *Neuron.*, **8**, 3–11.

FEELISCH, M. (1991). The biochemical pathways of nitric oxide formation from nitrovasodilators: appropriate choice of exogenous NO donors and aspects of preparation and handling of aqueous NO solutions. *J. Cardiovasc. Pharmacol.*, **17**, S25–S33.

FIESER, L.F. & FIESER, M. (1967). *Reagents for Organic Synthesis.* pp. 393. New York: John Wiley & Sons.

FURCHGOTT, R.F. (1988). Studies on relaxation of rabbit aorta by sodium nitrite. The basis for the proposal that the acid activatable inhibitory factor from bovine retractor penis is inorganic nitrite and the endothelium-derived relaxing factor is nitric oxide. In *Mechanisms of Vasodilatation.* 4th Ed., ed. Vanhoutte, P.M. pp. 401–414. New York: Raven Press.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GARTHWAITE, J., CHARLES, S.L. & CHESS-WILLIAMS, R. (1988). Endothelium-derived relaxing factor release on activation of NMDA receptors suggests a role as intercellular messenger in the brain. *Nature*, **366**, 385–388.

GIBSON, A., BABBEDGE, R.C., BRAVE, S.R., HART, S.L., HOBBS, A.J., TUCKER, J.F., WALLACE, P. & MOORE, P.K. (1992). An investigation of some S-nitrosothiols, and of hydroxy-arginine, on the mouse anococcygeus. *Br. J. Pharmacol.*, **107**, 715–721.

GIBSON, A., MIRAZAZADEH, S., HOBBS, A.J. & MOORE, P.K. (1990). L-N^G-monomethyl arginine and L-N^G-nitro arginine inhibit non-adrenergic, non-cholinergic relaxation of the mouse anococcygeus muscle. *Br. J. Pharmacol.*, **99**, 602–606.

GILLESPIE, J.S., LIU, X. & MARTIN, W. (1989). The effects of L-arginine and N^G-monomethyl L-arginine on the response of the rat anococcygeus to NANC nerve stimulation. *Br. J. Pharmacol.*, **98**, 1080–1082.

GILLESPIE, J.S. & MARTIN, W. (1980). A smooth muscle inhibitory material from the bovine retractor penis and rat anococcygeus muscles. *J. Physiol.*, **309**, 55–64.

GILLESPIE, J.S. & SHENG, H. (1990). The effects of pyrogallol and hydroquinone on the response to NANC nerve stimulation in the rat anococcygeus and bovine retractor penis muscles. *Br. J. Pharmacol.*, **99**, 194–196.

GRANGER, D.L., HIBBS, J.B. Jr., PERFECT, J.R. & DURACK, D.T. (1990). Metabolic fate of L-arginine in relation to microbiostatic capability of murine macrophages. *J. Clin. Invest.*, **85**, 264–273.

GRAY, G.A., SCHOTT, C., JULOU-SCHAEFFER, G., FLEMING, I., PARRATT, J.R. & STOCLET, J. (1991). The effect of inhibitors of the L-arginine/nitric oxide pathway on endotoxin-induced loss of vascular responsiveness in anaesthetized rats. *Br. J. Pharmacol.*, **103**, 1218–1224.

GRYGLEWSKI, R.J., PALMER, R.M.J. & MONCADA, S. (1986). Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature*, **320**, 454–456.

HIBBS, J.B. Jr., TAINTOR, R.R., VAVRIN, Z. & RACHLIN, E.M. (1988). Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.*, **157**, 87–94.

HOBBS, A.J. & GIBSON, A. (1990). N^G-nitro-L-arginine and its methyl ester are potent inhibitors of non-adrenergic, non-cholinergic transmission in the rat anococcygeus. *Br. J. Pharmacol.*, **100**, 749–752.

IGNARRO, L.J., BUSH, P.A., BUGA, G.M., WOOD, K.S., FUKUTO, J.M. & RAFER, J. (1990). Nitric oxide and cyclic GMP formation upon electrical field stimulation cause relaxation of corpus cavernosum smooth muscle. *Biochem. Biophys. Res. Commun.*, **170**, 843–850.

IGNARRO, L.J. & GRUETTER, C.A. (1980). Requirement of thiols for activation of coronary arterial guanylate cyclase by glyceryl trinitrate and sodium nitrite. *Biochim. Biophys. Acta.*, **631**, 221–231.

IGNARRO, L.J., LIPPTON, H., EDWARDS, J.C., BARICOS, W.H., HYMAN, A.L., KADOWITZ, P.J. & GRUETTER, C.A. (1981). The mechanism of smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates. *J. Pharmacol. Exp. Ther.*, **218**, 739–749.

KELM, M. & SCHRADER, J. (1988). Nitric oxide release from the isolated guinea pig heart. *Eur. J. Pharmacol.*, **155**, 317–321.

KERR, S.W., BUCHANAN, L.V., BUNTING, S. & MATHEWS, W.R. (1993). Evidence that S-nitrosothiols are responsible for the smooth muscle relaxant activity of the bovine rectractor penis inhibitory factor. *J. Pharmacol. Exp. Ther.*, **263**, 285–292.

KILBOURN, R.G. & GRIFFITH, O.W. (1992). Overproduction of nitric oxide in cytokine-mediated and septic shock. *J. Natl. Cancer Inst.*, **84**, 827–831.

KOSOWER, N.S., KOSOWER, E.M. & WERTHEIM, B. (1969). Diamide, a new reagent for the intracellular oxidation of glutathione to the disulfide. *Biochem. Biophys. Res. Commun.*, **37**, 593–596.

KOWALUK, E.A. & FUNG, H.-L. (1990). Spontaneous liberation of nitric oxide cannot account for *in vitro* vascular relaxation by S-nitrosothiols. *J. Pharmacol. Exp. Ther.*, **255**, 1256–1264.

LEAF, C.D., WISHNOK, J.S. & TANNENBAUM, S.R. (1990). Nitric oxide: the dark side. In *Nitric Oxide from L-Arginine: a Bioregulatory System.* ed. Moncada, S. & Higgs, E.A. pp. 291–299. Amsterdam: Excerpta Medica.

LI, C.G. & RAND, M.J. (1991). Evidence that part of the NANC relaxant response of guinea-pig trachea to electrical field stimulation is mediated by nitric oxide. *Br. J. Pharmacol.*, **102**, 91–94.

LIU, X., GILLESPIE, J.S., GIBSON, I.F. & MARTIN, W. (1991). Effects of N^G-substituted analogues of L-arginine on NANC relaxation of the rat anococcygeus and bovine retractor penis muscles and the bovine penile artery. *Br. J. Pharmacol.*, **104**, 53–58.

MARTIN, W., SMITH, J.A., LEWIS, M.J. & HENDERSON, A.H. (1988). Evidence that inhibitory factor extracted from bovine retractor penis is nitrite, whose acid-activated derivative is stabilized nitric oxide. *Br. J. Pharmacol.*, **93**, 579–586.

MARTIN, W., VILLANI, G.M., JOTHIANANDAN, D. & FURCHGOTT, R.F. (1985). Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. *J. Pharmacol. Exp. Ther.*, **232**, 708–716.

MARTIN, W., GILLESPIE, J.S. & GIBSON, I.F. (1993). Actions and interactions of N^G-substituted analogues of L-arginine on NANC neurotransmission in the bovine retractor penis and rat anococcygeus muscles. *Br. J. Pharmacol.*, **108**, 242–247.

MINOR, R.L.J., MYERS, P.R., BATES, J.N. & HARRISON, D.G. (1989). Basal EDRF release is reduced by depletion of cysteine from endothelial cells. *Circulation*, **80**, 281.

MONCADA, S. (1992). The L-arginine: nitric oxide pathway. *Acta Physiol. Scand.*, **145**, 201–227.

MONCADA, S., PALMER, R.M.J. & GRYGLEWSKI, R.J. (1986). Mechanism of action of some inhibitors of endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 9164–9168.

MOODY, B.J. (1991). *Comparative Inorganic Chemistry.* 3rd Ed., pp. 347–387. London: Edward Arnold.

MOORE, P.K., AL-SWAYEH, O.A., CHONG, N.W.S., EVANS, R.A. & GIBSON, A. (1990). L-N^G-nitro-arginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium-dependent relaxation *in vitro*. *Br. J. Pharmacol.*, **99**, 408–412.

MOORE, P.K., BABBEDGE, R.C., WALLACE, P., GAFFEN, Z.A. & HART, S.L. (1993). 7-Nitroindazole, an inhibitor of nitric oxide synthase, exhibits anti-nociceptive activity in the mouse without increasing blood pressure. *Br. J. Pharmacol.*, **108**, 296–297.

MYERS, P.R., GUERRA, R.J. & HARRISON, D.G. (1989). Release of NO and EDRF from cultured bovine aortic cells. *Am. J. Physiol.*, **256**, 1030–1037.

MYERS, P.R., MINOR, R.L.J., GUERRA, R.J., BATES, J.N. & HARRISON, D.G. (1990). Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble S-nitrocysteine than nitric oxide. *Nature*, **345**, 161–163.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

PICKARD, R.S., POWELL, P.H. & ZAR, M.A. (1991). The effect of inhibitors of nitric oxide biosynthesis and cyclic GMP formation on nerve evoked relaxation of human cavernosal smooth muscle. *Br. J. Pharmacol.*, **104**, 755–759.

RUBANYI, G.M. & VANHOUTTE, P.M. (1986). Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor. *Am. J. Physiol.*, **250**, H822–H827.

SCHUMAN, E.M. & MADISON, D.V. (1991). A requirement for the intercellular messenger nitric oxide in long-term potentiation. *Science*, **254**, 1503–1506.

SIEGLE, I., HECKER, M., SESSA, W.C. & VANE, J.R. (1993). Sulfhydryl reagents inhibit stimulated but not basal EDRF release from cultured endothelial cells. *Br. J. Pharmacol.*, **102**, 119P.

TODA, N. & OKAMURA, T. (1990). Modification by L-N^G-monomethyl arginine of the response to nerve stimulation in isolated dog mesenteric and cerebral arteries. *Jpn. J. Pharmacol.*, **52,** 170–173.

TUCKER, J.F., BRAVE, S.R., CHARALAMBOUS, L., HOBBS, A.J. & GIBSON, A. (1990). L-N^G-nitro arginine inhibits non-adrenergic, non-cholinergic relaxations of guinea-pig isolated tracheal smooth muscle. *Br. J. Pharmacol.*, **100,** 663–664.

WINK, D.A., DARBYSHIRE, J.F., NIMS, R.W., SAAVEDRA, J.E. & FORD, P.C. (1993). Reactions of the bioregulatory agent nitric oxide in oxygenated aqueous media: determination of the kinetics for oxidation and nitrosation by intermediates generated in the NO/O_2 reaction. *Chem. Res. Toxicol.*, **6,** 23–27.

YUI, Y., OHKAWA, S., OHNISHI, K., HATTORI, R., AOYAMA, T., TAKAHASHI, M., MORISHITA, H., TERAO, Y. & KAWAI, C. (1989). Mechanism for the generation of an active smooth muscle inhibitory factor (IF) from bovine retractor penis musle (BRP). *Biochem. Biophys. Res. Commun.*, **164,** 544–594.

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Pharmacological characterization and anatomical localization of prejunctional β-adrenoceptors in the rat kidney

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1 The subtype and anatomical localization of β-adrenoceptors mediating facilitation of stimulus-induced overflow of noradrenaline ('prejunctional β-adrenoceptors') are not conclusively known to date. The present study was undertaken to characterize these receptors by use of pharmacological methods as well as to define their localization (prejunctional or postjunctional) with radio-ligand binding and autoradiography techniques combined with surgical denervation of the sympathetic innervation to the rat kidney.

2 Exposure of the kidney to (−)-isoprenaline, the nonselective β-adrenoceptor agonist, resulted in a dose-dependent facilitation of stimulus-induced neurotransmitter overflow. This response was inhibited by propranolol, the β_1- and β_2-adrenoceptor antagonist, with a pA_2 of 9.20 suggesting that the prejunctional β-adrenoceptors are not of the β_3-subtype.

3 The rank order of potency and potency ratios of β-adrenoceptor agonists at renal prejunctional β-adrenoceptors (EC_{50} for agonist/EC_{50} for (−)-isoprenaline) were: (−)-isoprenaline (1) > procaterol (2) > salbutamol (3) > adrenaline (10) > (+)-isoprenaline (25). However, dobutamine, the β_1-adrenoceptor agonist, failed to enhance stimulus-induced overflow of noradrenaline. These results are indicative of the presence of β_2-adrenoceptors as prejunctional β-adrenoceptors.

4 Facilitation elicited by (−)-isoprenaline and procaterol, the selective β_2-adrenoceptor agonist, was inhibited by ICI 118,551, the selective β_2-adrenoceptor antagonist, with pK_b values of 9.20 and 9.35, respectively at renal prejunctional β-adrenoceptors. Similarly, the pK_b values of metoprolol, the selective β_1-adrenoceptor antagonist, at renal prejunctional β-adrenoceptors were determined to be 6.25 and 6.18 against (−)-isoprenaline and procaterol, respectively. These results suggest the presence of a homogeneous population of β_2-adrenoceptors as prejunctional β-adrenoceptors.

5 Radio-ligand binding analysis of renal β-adrenoceptors revealed the prevalence of the β_1-subtype as compared to the β_2-subtype (63% vs 37%). However, surgical denervation of the rat kidney, resulting in more than 90% reduction in renal noradrenaline content, selectively reduced the β_2-adrenoceptor population by 80%, implying the presence of β_2-adrenoceptors on renal sympathetic nerve terminals.

6 Autoradiographic analysis demonstrated the presence of β_1-adrenoceptors on cortical structures such as glomeruli and tubules. β_2-Adrenoceptors were found to be present on tubules (minor population), collecting tubules in outer medulla and the adventitia and adventitial-medial border of intraparenchymal branches of the renal artery. Surgical denervation of the rat kidney resulted in the disappearance of β_2-adrenoceptors associated with the intraparenchymal branches, without affecting the β-adrenoceptor populations at other sites. These results support the notion that the β_2-subtype is present on renal sympathetic nerve terminals and demonstrate that these prejunctional β_2-adrenoceptors are associated with the renal vasculature and not with renal tubules.

7 The results of the present investigation demonstrate that renal prejunctional β-adrenoceptors are of the β_2-subtype in nature. These receptors are present on sympathetic nerve terminals which are associated with the renal vasculature.

Keywords: Autoradiography; β-adrenoceptor; characterization; facilitation; prejunctional; rat kidney; sympathetic neurotransmission

Introduction

Regulation of sympathetic neurotransmission is achieved at various levels in the body. An important regulatory site is the sympathetic nerve terminal which is presumably endowed with various 'prejunctional' receptors. Activation of these receptors results in either inhibition or facilitation of exocytotic noradrenaline (NA) release from the nerve terminals. One of the facilitatory receptor systems is the prejunctional β-adrenoceptor system.

Prejunctional β-adrenoceptors enhance stimulus-induced (S-I) overflow of NA from sympathetic varicosities. The presence of these receptors has been demonstrated in various tissues and species (cf. Misu & Kubo, 1986). It has been suggested that physiologically, prejunctional β-adrenoceptors may be activated by neuronally released NA (Adler-Graschinsky & Langer, 1975). Alternatively, adrenaline may assume the role of physiological activator for these receptors (Stjarne & Brundin, 1976). However, the question of physiological agonist for prejunctional β-adrenoceptors is intimately related to the β-adrenoceptor subtype mediating the facilitatory response because, NA is equipotent with or more potent than adrenaline at the β_1- and β_3-subtypes while adrenaline is more potent than NA at the β_2-subtype (Lands *et al.*, 1967; Bond & Clarke, 1988).

Attempts have been made to characterize prejunctional β-adrenoceptors, but have yielded contradictory results. For example, the involvement of β_1-adrenoceptors in the facilitatory response was implied in the cat hindlimb (Dahlof *et*

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al., 1975), rabbit ear artery (Majewski & Rand, 1981) and rat portal vein (Ortiz de Urbina *et al.*, 1992). Conversely, β_2-adrenoceptors were suggested to mediate the facilitatory response in the human omental artery and vein (Stjarne & Brundin, 1976), rat mesentery (Kawasaki *et al.*, 1982) and guinea-pig papillary muscle (Valenta & Singer, 1991). Biological variation between different tissues and species cannot account for these controversial results, as different groups have reported different prejunctional β-adrenoceptor subtypes in the same tissue (Dahlof *et al.*, 1978; Westfall *et al.*, 1979; Remie *et al.*, 1988; Ortiz de Urbina *et al.*, 1992). Instead, the primary reason for these conflicting results seems to be the use of inadequate methods (such as, using only one or two concentrations of the β-adrenoceptor agents or failing to achieve equilibrium conditions) instead of appropriate pharmacological techniques, to characterize the receptors. Thus, a proper characterization of prejunctional β-adrenoceptors with the aid of suitable pharmacological methods is warranted.

Another controversial issue surrounding peripheral prejunctional β-adrenoceptors is their anatomical localization. Although these receptors are believed to be present on sympathetic nerve terminals (hence 'prejunctional'), anatomical evidence supporting this concept is sparse to date. The prejunctional localization of these receptors has been reported in a few tissues, such as the rat and cat spleen (Sharma & Banerjee, 1978; Dahlof *et al.*, 1986), while supportive evidence has been difficult to obtain in other tissues, such as the rat kidney cortex (Fortin & Sundaresan, 1989). In addition, it has been proposed recently that β-adrenoceptors mediating facilitation of S-I overflow of NA in the rat vena cava may be postjunctionally located and may elicit facilitation by releasing a transjunctional mediator (Gothert & Kollecker, 1986), thus raising a question regarding the concept of prejunctional localization of 'prejunctional' β-adrenoceptors. Also, even in tissues where the prejunctional localization of these receptors has been reported (Sharma & Banerjee, 1978; Dahlof *et al.*, 1986), the precise anatomical location of the sympathetic varicosities containing these receptors within the tissue has not been examined.

Therefore, the present investigation was undertaken to characterize prejunctional β-adrenoceptors in the rat kidney by use of pharmacological techniques such as pK_b determination for antagonists and determination of potency ratio of agonists. The putative prejunctional localization of these receptors also was studied by examining β-adrenoceptor populations in the innervated and surgically denervated preparations by radio-ligand binding and autoradiography.

Methods

Isolated perfused rat kidney preparation

Surgical procedure The rat kidney was isolated as described previously (Schwartz & Eikenburg, 1988). Male Wistar rats (Charles-River, PA, U.S.A.) weighing 250–300 g were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.). The abdominal cavity was opened by a midline incision and the right kidney, right renal artery, superior mesenteric artery and aorta were located and cleaned of surrounding tissues. Fifteen minutes following heparinization (1500 i.u., i.v.), the superior mesenteric artery was cannulated and then the cannula was advanced further into the renal artery. The right kidney along with the renal artery was isolated from the rat, placed in the perfusion apparatus and perfused (6 ml min^{-1}) with carbogenated Krebs bicarbonate solution (pH: 7.4) maintained at 37°C. The composition of the Krebs bicarbonate solution in mM was: KH_2PO_4 1.2, NaCl 118.4, KCl 4.8, $NaHCO_3$ 25.0, glucose 5.0, $MgSO_4\,7H_2O$ 1.2, $Na_2S_2O_5$ 0.1, EDTA 0.03 and $CaCl_2{\cdot}2H_2O$ 2.5. Cocaine (10 μM) and corticosterone (40 μM) were added to the solution in order to inhibit neuronal and extraneuronal uptake, respectively. A bipolar platinum electrode was placed around the renal artery for periarterial nerve stimulation. Perfusion pressure was monitored by a pressure transducer connected to the perfusion system and recorded on a Grass polygraph. Any preparation in which perfusion pressure changed by more than 5 mmHg in the absence of any stimulation was discarded, though this happened very rarely.

Experimental protocol Following a 30 min stabilization period, a priming stimulus was delivered (480 pulses, 4 Hz, 1 ms, supramaximal V) after which the α-adrenoceptor antagonist, phentolamine (10 μM) was added to the perfusate to minimize the influences of prejunctional α-adrenoceptors and S-I perfusion pressure changes on S-I NA overflow. Captopril (0.1 μM) and indomethacin (3 μM) also were added to the perfusate to inibit renal synthesis of angiotensin II and prostaglandins, respectively. The kidney was allowed to stabilize for an additional 45 min after which 9 consecutive stimulations (S1 to S9) were delivered at 20 min intervals using a Grass stimulator (10 pulses, 1 Hz, 1 ms, supramaximal V). During each stimulation and 1 min following that, the perfusate was collected and analyzed for NA content. At the end of the experiment, the kidney was weighed and homogenized with 5 ml of 1.0 N $HClO_4$ and an aliquot of the homogenate supernatant was analyzed for determination of the total renal NA content. The weight of kidney ranged from 1.5 to 2 g.

Estimation of NA NA was extracted from the perfusate and homogenate samples by an alumina extraction method (Schwartz & Eikenburg, 1988) and the content in the final eluate was analyzed by reverse phase high pressure liquid chromatography coupled with electrochemical detection (h.p.l.c.-e.c.). The overflow of NA in the absence of stimulations was non-detectable.

Calculation and expression of NA release S-I release of NA has been expressed as absolute and fractional overflows. These terms were calculated using the following formulae:

$$\text{Absolute overflow (pmol g}^{-1}) = \frac{\text{Stimulus-induced overflow (pmol)}}{\text{Kidney weight (g)}}$$

$$\text{Fractional overflow (\%)} = \frac{\text{Absolute overflow (pmol g}^{-1})}{\text{Renal NA content at the time of stimulation (pmol g}^{-1})} \times 100$$

When a stimulation was performed in the presence of a β-adrenoceptor agonist, facilitation of S-I overflow of NA was observed which was calculated as:

$$\text{Facilitation (\%)} = \frac{(\text{Fractional overflow}_{\text{Agonist}}) - (\text{Fractional overflow}_{\text{Control}})}{\text{Fractional overflow}_{\text{Control}}} \times 100$$

Here, Fractional $\text{overflow}_{\text{Agonist}}$ = Fractional overflow in the presence of the agonist

Fractional $\text{overflow}_{\text{Control}}$ = Fractional overflow in the absence of the agonist

Fractional overflow of NA in the absence of agonist was calculated by taking an average of the fractional overflow at S1 and S2.

Construction of agonist concentration-response curve On the basis of preliminary experiments, an appropriate agonist concentration-range (over which the responses are concentration-dependent) was chosen and 6 to 7 concentrations of the agonist, half a log unit apart, were selected from this range to generate a concentration-response curve. It was ensured that the maximum effective concentration of the agonist was used in each experiment. The first 2 stimulations (S1 and S2) were carried out in the absence of an agonist. The agonist was added to the perfusion fluid at a fixed time (2 min or 10 min) prior to each stimulation beginning with S3 and remained until the end of the stimulation. Agonist was

added in the order of increasing concentrations from S3 to S9 and the facilitatory response at each concentration was measured to generate a concentration-facilitatory response curve. A single concentration-response curve was generated in each experiment.

When agonist concentration-facilitatory response curves were generated in the presence of various concentrations of an antagonist, an appropriate concentration of the antagonist was added to the perfusion fluid 45 min prior to S1 and was perfused throughout the experiment. The agonist concentration-response curve was generated in the presence of the antagonist as described above.

Fitting of agonist concentration-response curve The concentration-response curve to an agonist was fitted to a logistic equation using a computer programme (Kaleidagraph, Abelbeck Software). The concentrations producing 50% of the maximum response (EC_{50} values) were obtained directly from the equation. The logistic equation which was used to best fit the curves is as follows (Waud & Parker, 1971):

$$\text{Response} = \frac{M \times [A]^n}{[A]^n + (EC_{50})^n}$$

Here, M = maximum response; [A] = molar agonist concentration; n = slope of the curve; EC_{50} = agonist concentration producing half the maximum response.

Determination of rank order of potency and potency ratio The rank order of potency and the potency ratios for β-adrenoceptor agonists relative to (−)-isoprenaline were determined. For the potency ratio, the EC_{50} value for the agonist was divided by the EC_{50} value for (−)-isoprenaline. Thus, any potency ratio greater than 1 indicates that the agonist is that many fold less potent than (−)-isoprenaline.

Determination of pA_2 and pK_b values In some cases when only one concentration of the antagonist was used, the pA_2 value was calculated from the Furchgott equation (Furchgott, 1972) which is as follows:

$$pA_2 = \log ([A']/[A] - 1) - \log [B]$$

Here, [A'] = EC_{50} in the presence of the antagonist; [A] = EC_{50} in the absence of the antagonist; [B] = molar antagonist concentration.

In other cases, Schild regression analysis was performed to determine the pK_b values of the antagonists. This analysis is based upon following equation (Arunlakshana & Schild, 1959):

$$\log (\text{concentration ratio} - 1) = \log [B] - \log K_B$$

Here, [B] = molar antagonist concentration, K_B = equilibrium dissociation constant of the antagonist.

Once the criteria of similar maximum responses and parallelism of the agonist concentration-response curves in the absence and presence of the antagonist was satisfied (Kenakin, 1984), Schild regression analysis was carried out. The concentration-ratio (shift) of the agonist concentration-response curve in the presence of a particular concentration of the antagonist as compared to that in the absence of the antagonist was calculated by dividing the EC_{50} value obtained in the presence of the antagonist by the EC_{50} value obtained in the absence of the antagonist. Then a Schild plot was obtained by plotting log molar concentrations of the antagonist on the abscissa scale and log (concentration ratio − 1) on the ordinate scale. The data points were fitted by simple regression and the slope of the regression line was determined. If the slope was not significantly different from 1 then a line with the slope of 1 was best fitted to the points (MacKay, 1978) and was extrapolated to determine the x-intercept at y = 0. This x-intercept was accepted as the pK_b value for the antagonist at the receptors.

Radio-ligand binding and autoradiography studies

Animal and tissue treatment Twenty male Wistar rats of 235–265 g were anaesthetized with pentobarbitone (60 mg kg^{-1}, i.p.) and the right renal artery was exposed. In 10 rats, the right kidney was denervated by stripping the right renal artery surgically and swabbing it with a 10% phenol solution dissolved in ethanol. Ten other rats were sham-operated. Animals were allowed to survive for one week after denervation and then were killed by pentobarbitone overdose. The right kidney was dissected out and washed with an ice-cold 0.9% NaCl solution to remove blood and cell debris. The upper half of the right kidney was weighed and used for NA assay. The lower half of the kidney was cut into 0.3 cm slices which were frozen in isopentane cooled with liquid nitrogen and used for β-adrenoceptor assay and autoradiography. Sections, 8 μm thick, were obtained with a −20°C microtome cryostat and mounted on pre-weighed gelatin-coated microscope slides.

NA assay The upper half of the right kidney was homogenized in ice-cold 0.1 M $HClO_4$ (5 ml g^{-1} tissue). The homogenate was centrifuged at 2000 *g* for 20 min in a refrigerated centrifuge and the resulting supernatant was used for NA assay. NA was assayed by h.p.l.c.-e.c. according to the procedure detailed elsewhere (Amenta *et al.*, 1987).

Radio-ligand binding Sections from surgically denervated or sham-operated kidneys were preincubated for 30 min at room temperature in Krebs bicarbonate solution. Then they were incubated in the same solution for 120 min at room temperature with increasing concentrations of radiolabelled iodocyanopindolol ([^{125}I]-CYP) (0.05 to 1 nM). Selective binding to the different β-adrenoceptor subtypes was evaluated by incubating parallel sections in the presence of different concentrations of ICI 89,406 (selective β_1-adrenoceptor antagonist) or ICI 118,551 (selective β_2-adrenoceptor antagonist) (Bilski *et al.*, 1983; Engel *et al.*, 1985). The optimal binding time and temperature were determined in a series of preliminary experiments. Nonspecific binding was evaluated by incubating some sections in the presence of 1 μM (−)-propranolol. Furthermore, to assess the specificity of [^{125}I]-CYP binding to β-adrenoceptors, other sections were incubated in the presence of different concentrations of (−)- and (+)-propranolol, isoprenaline, NA and phentolamine.

After incubation, the sections were washed in cold buffer (2 times, 5 min each), rapidly rinsed in distilled water, wiped onto Whatman glass filters and placed in scintillation vials. Vials were then placed in a gamma counter (LKB, Bromma, Sweden) for radioactivity determination. Further details of the radio-ligand binding procedures are reported elsewhere (Amenta *et al.*, 1991).

Autoradiography For autoradiographic experiments, the sections were incubated according to the optimal binding parameters determined in preliminary experiments using a radio-ligand concentration of 0.4 nM. Such a concentration produced the highest specific/nonspecific binding ratio, as determined in radio-ligand binding experiments (see results). The selective antagonists ICI 89,406 and ICI 118,551 were used at concentrations of 100 nM and 70 nM, respectively, on the basis of radio-ligand binding data. At the end of the incubation, the sections were washed as above, rinsed quickly in distilled water and air dried. Then they were post-fixed by exposure to formaldehyde vapours at 80°C for 30 min and processed for autoradiography according to the method of Young & Kuhar (1979). Briefly, coverslips coated with Ilford L4 nuclear emulsion (diluted 1:1 with distilled water) were attached to the slides; then they were exposed for 3–6 days in light-proof boxes, developed with Kodak D19, fixed with Agefix-Agfa, stained with toluidine blue and observed under a light microscope equipped with both bright- and dark-field condensers.

The density of silver grains developed by autoradiography and representing [^{125}I]-CYP binding sites was assessed according to the procedure detailed in an earlier paper (Amenta *et al.*, 1991). Briefly, silver grains were counted in an area of 500 μm^2 as determined by an image analyzer connected via a TV camera to the microscope, using a final magnification at the camera of × 400. Counts were made by three investigators independently on at least 5 consecutive sections per kidney ($n = 10$, denervated or sham-operated).

Statistical evaluation

Fractional overflows of NA in different groups in the absence and presence of various antagonist concentrations but in the absence of agonist (at S1 and S2) were compared by unpaired *t*-test or one factor analysis of variance. Maximum facilitation in different groups was compared by an unpaired *t*-test or one factor analysis of variance. To test parallelism of concentration-response curves, each average concentration-response curve was converted to a straight line by transforming the values of mean facilitatory response at each concentration into probits which were plotted on the ordinate scale against log molar concentration of the agonist on the abscissa scale. The slopes of the straight lines were then compared by analysis of covariance. The slope of the Schild regression plot was tested by *t*-test to determine whether it was significantly different from unity. In the radio-ligand binding and autoradiography studies, the densities of [^{125}I]-CYP binding among the various groups were compared by one factor analysis of variance (ANOVA). A significance level of $P < 0.05$ was accepted.

Drugs and chemicals

Chemicals used in preparation of the Krebs bicarbonate solution and elsewhere were reagent or h.p.l.c. grade and obtained from standard sources. The following stock solutions were divided into aliquots and stored at − 80°C. Solutions of (−)-isoprenaline (Sigma), (+)-isoprenaline (> 99%, Sigma) and (−)-adrenaline (Sigma) were prepared in 0.01 N hydrochloric acid containing 1 mM sodium metabisulphite.

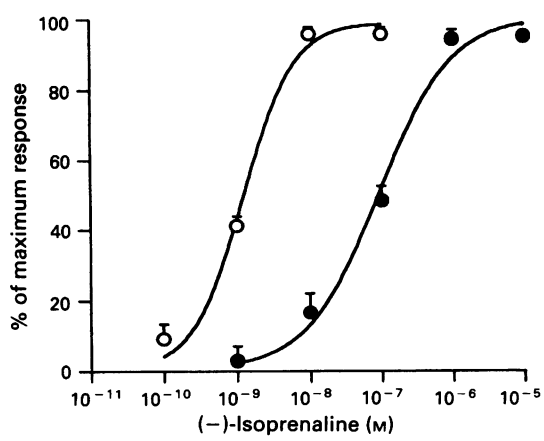


Figure 1 Concentration-facilitatory response curves to (−)-isoprenaline in the absence (○) and presence of 30 nM propranolol (●), $n = 5$. Data are presented as mean ± s.e.mean. The maximum facilitatory responses were 93 ± 6% and 107 ± 14% in the control and propranolol groups, respectively. The fractional overflows of noradrenaline (NA) in the absence of (−)-isoprenaline were 0.58 ± 0.03% and 0.57 ± 0.06% while the renal NA contents were 495 ± 83 and 435 ± 23 pmol g^{-1} in control and propranolol groups, respectively. These experiments were performed in the presence of cocaine (10 μM) and corticosterone (40 μM) to inhibit neuronal and extraneuronal uptake, phentolamine (10 μM) to block prejunctional α-adrenoceptors, indomethacin (3 μM) to inhibit renal prostaglandin synthesis and captopril (0.1 μM) to inhibit renal angiotensin II synthesis.

Solutions of dobutamine (Eli Lilly), procaterol (Sigma), metoprolol (Sigma) and ICI 118,551 (Imperial Chemical Industries) were prepared in distilled water. The following stock solutions were stored at 4°C. A solution of indomethacin (Sigma) was prepared in ethanol. Solutions of cocaine (Sigma) and corticosterone (Sigma) were prepared in 0.01 N hydrochloric acid and 90% dimethylsulfoxide, respectively. All stock solutions were diluted with Krebs bicarbonate solution to the appropriate concentrations on the day of the experiment. A fresh stock solution of salbutamol (Sigma) was prepared daily and was diluted with Krebs bicarbonate solution to the appropriate concentrations. Captopril (Sigma) and phentolamine mesylate (Ciba-Geigy) were weighed daily, dissolved in distilled water and added to Krebs bicarbonate solution. [^{125}I]-cyanopindolol (specific activity 2,000 Ci $mmol^{-1}$) was purchased from the Radiochemical Centre of Amersham (Buckinghamshire, UK).

Results

Control experiments performed in the absence of an agonist demonstrated that S-I fractional overflow of NA was reproducible during the course of the experiment (data not shown). The β-adrenoceptor antagonists, when given alone, did not affect S-I fractional overflow of NA (data shown in legends to figures representing agonist concentration-response curves in the absence and presence of the antagonist).

Effects of the non-selective β-adrenoceptor agonist, isoprenaline, on neurotransmitter overflow

Exposure of the kidney to the non-selective β-adrenoceptor agonist, (−)-isoprenaline, results in dose-dependent facilitation of S-I neurotransmitter overflow (Figure 1). The tissue was exposed to each agonist concentration for 2 min to avoid desensitization of prejunctional β-adrenoceptors (Lakhlani & Eikenburg, 1993). The maximum facilitatory response was 93 ± 6% while the EC_{50} concentration was 1.2 nM (Figure 1). The concentration-effect curve to (−)-isoprenaline was shifted 52 fold to the right in a parallel fashion by propranolol, the β_1- and β_2-adrenoceptor antagonist, without significantly altering the maximum facilitatory response (107 ± 14%) (Figure 1). The pA_2 for propranolol at prejunctional β-adrenoceptors, determined from the Furchgott equation (Furchgott, 1972), was estimated to be 9.2.

The rank order of potency and the potency ratios of β-adrenoceptor agonists

As a first step in identifying the β-adrenoceptor subtype responsible for the facilitatory effect on neurotransmitter overflow, the rank order of potency and the potency ratios of various β-adrenoceptor agonists were determined. An agonist exposure time of 2 min was used. The observed rank order of potency was (−)-isoprenaline > procaterol > salbutamol > adrenaline > (+)-isoprenaline (Figure 2a). In addition, procaterol, salbutamol, adrenaline and (+)-isoprenaline were observed to be 2-fold, 3-fold, 10-fold and 25-fold less potent than (−)-isoprenaline, respectively (Table 1). It should be noted here that adrenaline, a mixed β- and α-adrenoceptor agonist, did not affect the perfusion pressure (data not shown), suggesting that α-adrenoceptor blockade by phentolamine remained effective in the presence of adrenaline.

In contrast to the agonists described above, dobutamine, the β_1-adrenoceptor agonist, did not produce any effect on sympathetic neurotransmission in the rat kidney (Figure 2b) within the concentration range of 1 nM to 10 μM. Dobutamine, also an α-adrenoceptor agonist at higher concentrations, did not influence perfusion pressure within this concentration-range, suggesting that α-adrenoceptor blockade by phentolamine remained effective. At the concentrations of 100 μM and 1 mM, dobutamine produced marked vasoconstriction,

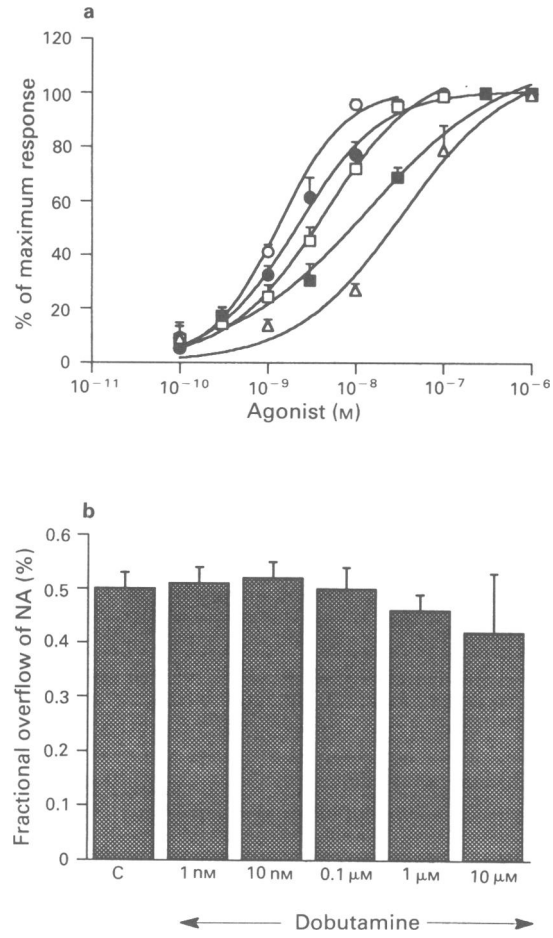


Figure 2 (a) The concentration-facilitatory response curves to (−)-isoprenaline (○), procaterol (●), salbutamol (□), adrenaline (■) and (+)-isoprenaline (△) at prejunctional β-adrenoceptors, $n = 3-5$. Data are presented as mean ± s.e.mean. The maximum facilitatory responses for (−)-isoprenaline, procaterol, salbutamol, epinephrine and (+)-isoprenaline were 93 ± 6%, 96 ± 6%, 99 ± 3%, 115 ± 11% and 104 ± 27%, respectively. The fractional overflows of noradrenaline (NA) in the absence of agonist were 0.58 ± 0.03%, 0.40 ± 0.10%, 0.36 ± 0.05%, 0.48 ± 0.05 and 0.53 ± 0.06% while renal NA contents were 495 ± 80, 601 ± 54, 511 ± 4, 484 ± 46 and 389 ± 51 pmol g^{-1} in (−)-isoprenaline, procaterol, salbutamol, adrenaline and (+)-isoprenaline groups, respectively. For other experimental conditions, refer to Figure 1. (b) Stimulus-induced fractional overflow of NA in the absence (C) and presence of the β_1-adrenoceptor agonist, dobutamine, $n = 4$. The data are presented as mean ± s.e.mean. The renal NA content was 442 ± 21 pmol g^{-1}. For other experimental conditions, refer to Figure 1.

suggesting its action at postjunctional (and probably at prejunctional) α-adrenoceptors (data not shown). Hence, its effect on renal sympathetic neurotransmission at these concentrations was not evaluated.

Determination of the pK_b for antagonists at prejunctional β-adrenoceptors

In the first series of experiments, ICI 118,551 was used as a representative of selective β_2-adrenoceptor antagonists and its pK_b was determined with (−)-isoprenaline and procaterol, the selective β_2-adrenoceptor agonist, to activate the facilitatory β-adrenoceptors. A β_1-adrenoceptor agonist could not be used for reasons explained above. When an agonist exposure time of 2 min or 5 min was used in these experiments, an apparent noncompetitive behaviour by ICI 118,551 was observed (data not shown). When agonist exposure time was increased to 10 min in these experiments, parallel shifts indicative of competitive antagonism were observed. Therefore, an agonist exposure time of 10 min was used in all experiments. The appropriate antagonist concentration was added to the perfusate 45 min prior to the first stimulation and was present for the remainder of the experiment.

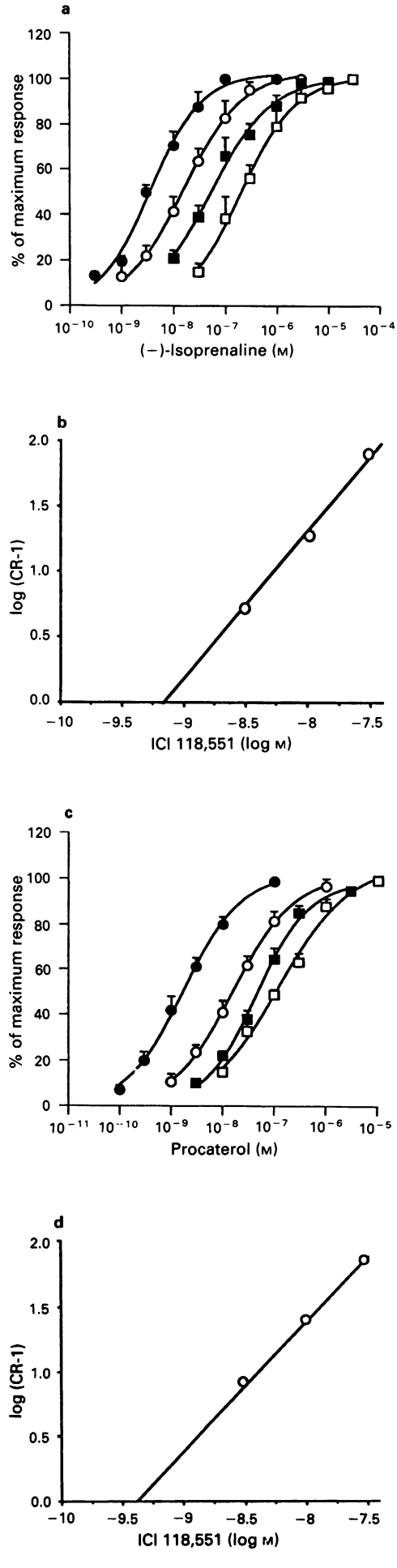
When concentration-facilitatory response curves to (−)-isoprenaline were generated in the absence and presence of ICI 118,551 (3–30 nM), the maximum facilitatory responses observed in the different groups were not significantly different (Figure 3a and Table 2). The curves were parallel and suggested a competitive antagonism by ICI 118,551 (Figure 3a). The slope of the regression line from the Schild plot was not significantly different from unity and when constrained to a slope of 1 the pK_b value was determined to be 9.20 (Figure 3b and Table 2). Similarly, ICI 118,551 (3–30 nM) produced parallel dextral shifts of the procaterol concentration-facilitatory response curves. The maximum response to procaterol was not significantly affected by any concentration of ICI 118,551 (Figure 3c and Table 2). When the Schild analysis was performed, the slope of the regression line was not significantly different from unity and when constrained to a slope of one the pK_b value for ICI 118,551 against procaterol was determined to be 9.35 (Figure 3d).

In a second series of experiments, the pK_b of the selective β_1-adrenoceptor antagonist, metoprolol, was determined with (−)-isoprenaline and procaterol. Metoprolol produced parallel dextral shifts of the isoprenaline concentration-facilitatory response curves without significantly affecting the maximum responses (Figure 4a and Table 2). Schild analysis revealed that slope of the regression line was not significantly different from unity and when the plot was constrained to a slope of unity, the pK_b value of metoprolol against (−)-isoprenaline was 6.25 (Figure 4b and Table 2). Similarly, when the effects of ICI 118,551 on the procaterol concentration-facilitatory

Table 1 Potency ratios of β-adrenoceptor agonists relative to (−)-isoprenaline at prejunctional β-adrenoceptors, β_1-adrenoceptors and β_2-adrenoceptors

Agonist	*Potency ratio*[a]		
	Prejunctional β-adrenoceptors[b]	*β_2-adrenoceptors*	*β_1-adrenoceptors*
(−)-Isoprenaline	1	1	1
Procaterol	2	0.5 to 0.14[c]	127 to 1000[c]
Salbutamol	3	2 to 6[d]	170 to 2500[d]
Adrenaline	10	20 to 33[e]	4 to 8[e]
(+)-Isoprenaline	25	46[f]	35[f]

[a]Potency ratio was calculated by dividing EC_{50} value for the agonist by EC_{50} value for (−)-isoprenaline. Thus, any potency ratio greater than 1 indicates that the agonist is that many fold less potent than (−)-isoprenaline while any potency ratio less than 1 indicates that the agent is more potent than (−)-isoprenaline.
[b]Potency ratios of the agonists at prejunctional β-adrenoceptors were derived from the data presented in Figure 4a.
[c]Yabuuchi (1977); O'Donnell & Wanstall (1985).
[d]Farmer *et al.* (1970); O'Donnell (1972).
[e]Lands *et al.* (1967); Furchgott (1972).
[f]Bowman & Rand (1982).



response curves were examined, ICI 118,551 produced parallel dextral shifts of the procaterol curves without significantly altering the maximum response (Figure 4c and Table 2). The Schild analysis of the metoprolol-procaterol data resulted in a regression line with a slope that was not significantly different from unity and when a line with the slope of 1 was best fitted to the points, the pK_b value of metoprolol at prejunctional β-adrenoceptors was determined to be 6.18 (Figure 4d).

NA levels in sham-operated and denervated rat kidneys

The results of the NA assay in control (sham-operated) or surgically denervated kidneys are summarized in Table 3. Surgical kidney denervation reduced the renal NA content by more than 90%. This indicated the effectiveness of the denervation procedures.

Radio-ligand binding

[^{125}I]-CYP binding to sections of the rat kidney was time-, temperature- (data not shown) and concentration-dependent (Figure 5a). The plateau in specific binding was reached at a [^{125}I]-CYP concentration of about 0.4 nM; K_d value was 0.20 nM and B_{max} was 108 fmol mg^{-1} tissue (Figure 5a). About 81% of [^{125}I]-CYP binding to sections of rat kidney was specific (Figure 5a). Experiments performed using the selective β_1- and β_2-adrenoceptor antagonists, ICI 89,406 and ICI 118,551, respectively, revealed that ICI 89,406 was effective in inhibiting [^{125}I]-CYP binding at lower concentrations than ICI 118,551 (Figure 5b). The analysis of binding to each β-adrenoceptor subtype demonstrated a prevalence of the β_1-subtype as compared to the β_2-subtype (63% vs 37%) (Table 3).

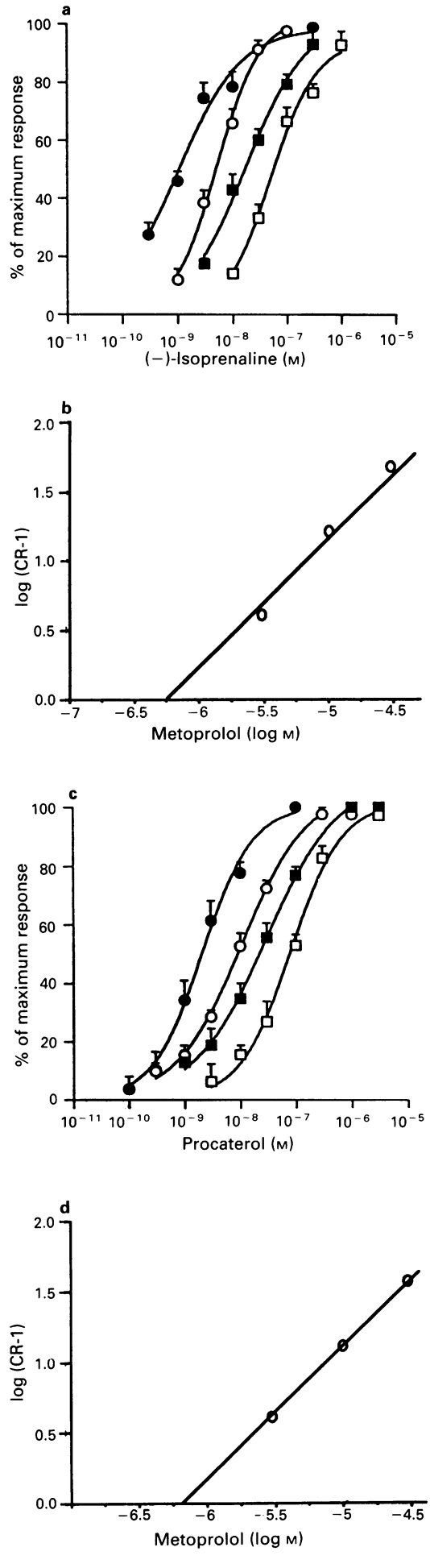
Surgical denervation of the rat kidney resulted in a 44% reduction in the total β-adrenoceptor density and an 80% reduction in the β_2-adrenoceptor sites (Table 3). In contrast, β_1-adrenoceptors were not significantly affected.

Autoradiography

Silver grains developed by autoradiography and corresponding to [^{125}I]-CYP binding sites could be detected both in kidney cortex and outer medulla, as well as intraparenchymal renal blood vessels. The incubation in the presence of the two specific antagonists allowed for discrimination between the two β-adrenoceptor subtypes.

In cortical structures (glomeruli and renal tubules), β_1-adrenoceptors were dominant (Figure 6). Incubation in the presence of ICI 89,406, however, demonstrated the presence

Figure 3 (a) and (c) Concentration-facilitatory response curves to (−)-isoprenaline (a) and procaterol (c) in the absence (●) and presence of 3 nM (○), 10 nM (■) and 30 nM (□) ICI 118,551, $n = 4-6$. Data are presented as mean ± s.e.mean. The following values are for control, ICI 118,551 (3 nM), ICI 118,551 (10 nM) and ICI 118,551 (30 nM) groups, respectively: Fractional overflow of noradrenaline (NA) in the absence of (−)-isoprenaline – 0.33 ± 0.05%, 0.32 ± 0.03%, 0.29 ± 0.06% and 0.34 ± 0.05%; renal NA content in (−)-iosprenaline group – 388 ± 31, 424 ± 74, 419 ± 38 and 485 ± 63 pmol g^{-1}; fractional overflow of NA in the absence of procaterol – 0.43 ± 0.04%, 0.37 ± 0.05%, 0.42 ± 0.05% and 0.39 ± 0.04%; renal NA content in procaterol group – 468 ± 37, 481 ± 12, 476 ± 18 and 423 ± 22 pmol g^{-1}. For other experimental conditions, refer to Figure 1. (b) and (d) Schild plots of ICI 118,551 at prejunctional β-adrenoceptors using (−)-isoprenaline (b) and procaterol (d) as agonists. The slopes of the regression lines in (−)-isoprenaline experiments (1.19) and procaterol experiments (0.95) were not significantly different from unity. Therefore, presented above are the lines constrained to a slope of 1, which were best fitted to the points. From these lines, the pK_b values were determined to be 9.20 and 9.35.



of a small tubular population of the β_2-subtype (Figure 6). Surgical denervation had little effect on this pattern (data not shown). β_2-Adrenoceptors also were present in the outer medulla, where they are most probably associated with collecting tubules (Figure 7). Intraparenchymal branches of the renal artery displayed silver grains in the adventitia and adventitial-medial border; these binding sites were displaced by ICI 118,551 and thus represent β_2-adrenoceptors (Figure 8). No silver grain was detectable in the arterial muscle layer (Figure 8). Importantly, surgical denervation resulted in a complete disappearance of this population, suggesting a prejunctional localization of these binding sites (Figure 8). No specific β_1-adrenoceptor binding was noticeable within sections of intraparenchymal branches of the renal artery (data not shown).

Discussion

β-Adrenoceptor activation results in facilitation of S-I NA overflow from sympathetic varicosities. However, some basic aspects of these receptors have not been delineated properly. It is not known exactly which subtype of β-adrenoceptors mediates the facilitatory response. Similarly, the anatomical location of these receptors within peripheral organs has not been examined in detail. The present investigation was undertaken to define these aspects by use of two different approaches: pharmacological and anatomical. Pharmacologically, the receptors were characterized using classical pharmacological methods; examination of agonist potency ratios and pA_2 or pK_b determinations. Anatomically, the receptors were characterized and their anatomical location was defined using radio-ligand binding and autoradiography techniques.

Although the classical pharmacological methods (potency ratio determination, pA_2 or pK_b determination) have been used extensively to characterize postjunctional β-adrenoceptors, these methods have not been applied to date to characterize prejunctional β-adrenoceptors because of various complications in achieving proper experimental conditions. One prerequisite for the correct characterization of any receptor is that the concentration of an agonist in the vicinity of the receptors should be estimated as accurately as possible (Furchgott, 1972). Also, the receptor system should be functionally isolated and the receptor-mediated response should be minimally influenced by other endogenous or exogenous factors (Furchgott, 1972). Hence, with regard to prejunctional β-adrenoceptors, the final determination of the potency ratio or pK_b value may be influenced by (1) a co-transmitter released with NA (Bartfai *et al.*, 1988) which may influence the facilitatory response; (2) simultaneous activation of other

Figure 4 (a) and (c) Concentration-facilitatory response curves to (−)-isoprenaline (a) and procaterol (c) in the absence (●) and presence of 3 μM (○), 10 μM (■) and 30 μM (□) metoprolol, $n = 4-6$. Data are presented as mean ± s.e.mean. The following values are for control, metoprolol (3 μM), metoprolol (10 μM) and metoprolol (30 μM) groups, respectively: fractional overflow of noradrenaline (NA) in the absence of (−)-isoprenaline – 0.57 ± 0.09%, 0.52 ± 0.07%, 0.58 ± 0.07% and 0.56 ± 0.10%; renal NA content in (−)-isoprenaline group – 349 ± 49, 327 ± 21, 332 ± 29 and 326 ± 34 pmol g^{-1}; fractional overflow of NA in the absence of procaterol – 0.46 ± 0.04%, 0.42 ± 0.04%, 0.43 ± 0.03% and 0.51 ± 0.11%; renal NA content in procaterol group – 346 ± 18, 333 ± 31, 382 ± 37 and 437 ± 88 pmol g^{-1}. For other experimental conditions, refer to Figure 1. (b) and (d) Schild plots of metoprolol at prejunctional β-adrenoceptors using (−)-isoprenaline (b) and procaterol (d) as agonists. The slopes of the regression lines in (−)-isoprenaline experiments (1.08) and procaterol experiments (0.97) were not significantly different from unity. Therefore, presented above are the lines constrained to a slope of 1, which were best fitted to the points. From these lines, the pK_b values were determined to be 6.25 and 6.18.

Table 2 Effects of β-adrenoceptor antagonists on agonist-concentration-facilitatory response curves

Agonist	*Antagonist*	*Schild plot slope*[a] (95% confid. limits)	pK_b[b]	*Antagonist conc.*	*Shift of agonist curve*[c]	*Agonist max. response*[d]
(−)-Isoprenaline	ICI 118,551	1.19 (0.3–2.0)	9.2	0		114 ± 33%
				3 nM	6	109 ± 18%
				10 nM	20	123 ± 13%
				30 nM	83	117 ± 17%
Procaterol	ICI 118,551	0.95 (0.89–1.00)	9.35	0		101 ± 8%
				3 nM	8	128 ± 15%
				10 nM	25	103 ± 19%
				30 nM	72	108 ± 8%
(−)-Isoprenaline	Metoprolol	1.08 (0.48–1.67)	6.25	0		87 ± 11%
				3 μM	5	76 ± 9%
				10 μM	17	101 ± 9%
				30 μM	50	86 ± 9%
Procaterol	Metoprolol	0.97 (0.93–1.00)	6.18	0		97 ± 21%
				3 μM	5	108 ± 5%
				10 μM	13	89 ± 15%
				30 μM	38	79 ± 17%

[a]Schild plot slope of the line obtained by best fit of the data points in Figures 3b, 3d, 4b or 4d along with the 95% confidence limits for that line.
[b]X-intercept of Schild Plots shown in Figures 3b, 3d, 4b or 4d where a line constrained to a slope of 1 was best-fitted to the data points.
[c]Dextral parallel shifts (EC_{50} of the agonist in presence of [antagonist]/EC_{50} of the agonist in absence of antagonist). Data are shown in Figures 3a, 3c, 4a and 4c.
[d]Facilitation of stimulus-induced fractional neurotransmitter overflow expressed as a percentage of the overflow in the absence of agonist. The antagonists alone had no effect on overflow at the concentrations studied.

Table 3 Effect of surgical sympathetic denervation on noradrenaline (NA) content, β_1-adrenoceptors and β_2-adrenoceptors in the rat kidney

	NA content (ng g^{-1} tissue)	*β_1-Adrenoceptors* (fmol mg^{-1} tissue)	*β_2-Adrenoceptors* (fmol mg^{-1} tissue)
Control (sham-operated)	34 ± 5	68 ± 10	40 ± 11
Denervation	4 ± 2*	53 ± 14	8 ± 7*

*Significantly different as compared to the control condition, $P<0.01$.

prejunctional receptors on sympathetic nerve terminals (Rump, 1987); (3) active removal of NA or β-adrenoceptor agents, and (4) endogenous factors generated in the organ, especially angiotensin II and prostaglandins in the present case (Boke & Malik, 1983; Malik, 1988). Therefore, care was taken in the present study to minimize the influence of these factors. First, it has been suggested that ATP plays the role of a sympathetic co-transmitter in the rat kidney which, however, has no effect on S-I overflow of NA (Schwartz & Malik, 1989). Second, only prejunctional α-adrenoceptors are tonically active under the experimental conditions of the present study and influence of prejunctional α-adrenoceptors on sympathetic neurotransmission was minimized in the present study by using phentolamine. Third, active uptake of NA or β-adrenoceptor agents was prevented by inhibiting neuronal and extraneuronal uptake. Finally, synthesis of renal angiotensin II and prostaglandins was inhibited with captopril and indomethacin to eliminate their potential influence on β-adrenoceptor-mediated facilitation.

Another important point in the use of pharmacological methods for characterizing prejunctional β-adrenoceptors is the choice of β-adrenoceptor agents. O'Donnell & Wanstall (1979) pointed out that the pA_2 values obtained with selective receptor agonists and antagonists provide the best basis for receptor differentiation. They demonstrated that the pA_2 values for a selective antagonist may differ against agonists of different selectivities in tissues containing more than one receptor subtype. Hence, β-adrenoceptor agonists and antagonists with different selectivities for β-adrenoceptor subtypes were used in the present investigation to differentiate between the β_1-, β_2- and β_3-subtypes as prejunctional β-adrenoceptors.

One strategy which can provide evidence for the involvement of a particular receptor subtype in a functional response is examination of the rank order of potency and potency ratios of various β-adrenoceptor agonists (Kenakin, 1984). In the present investigation, all the agonists, except dobutamine, acted as full agonists at prejunctional β-adrenoceptors in the rat kidney. The rank order of potency of β-adrenoceptor agonists was, (−)-isoprenaline > procaterol > salbutamol > adrenaline > (+)-isoprenaline, which is indicative of the β_2-subtype since at the β_1-subtype, adrenaline is more potent than salbutamol and procaterol. This rank order of potency also confirmed the stereoselectivity of prejunctional β-adrenoceptors as (−)-isoprenaline was observed to be more potent at prejunctional β-adrenoceptors than (+)-isoprenaline. Another important observation in the present investigation was that the potency ratios of the β-adrenoceptor agonists are similar to their reported ratios at β_2-adrenoceptors, and differ significantly from their reported ratios at β_1-adrenoceptors (see Table 1). Thus, these results suggest that prejunctional β-adrenoceptors are of the β_2-subtype.

In contrast to the other β-adrenoceptor agonists used, the preferential β_1-adrenoceptor agonist, dobutamine, did not produce any facilitation of S-I overflow of NA. This may be due to the presence of the β_2-subtype as prejunctional β-adrenoceptors. However, two alternative explanations should be considered. One possibility is that the concentrations of dobutamine used in the present study may not be sufficient to activate prejunctional β-adrenoceptors. Dobutamine is 200 to 1000 fold less potent than isoprenaline at the β-adrenoceptor subtypes (Wagner & Schumann, 1979; Kenakin, 1981). Since

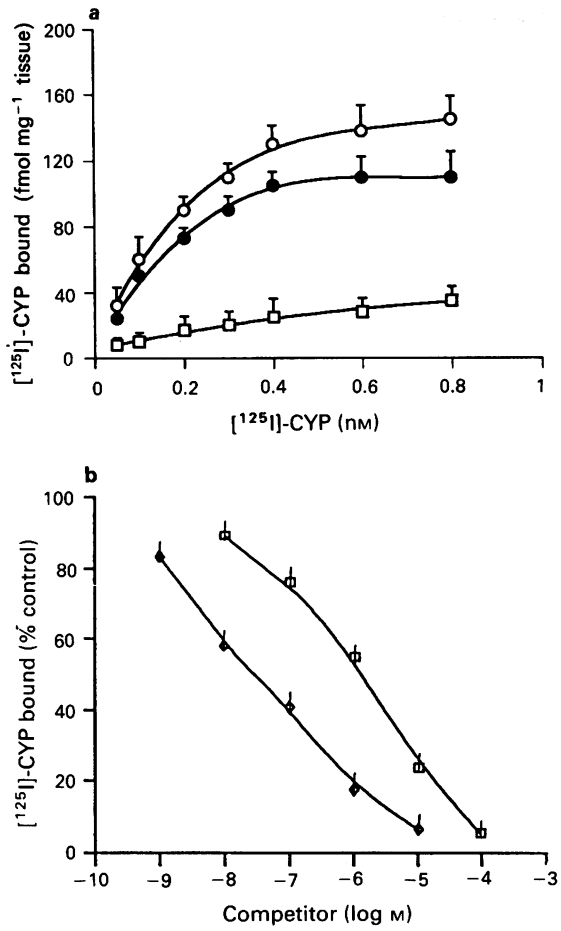


Figure 5 (a) Total (○), specific (●) and nonspecific (□) [^{125}I]-cyanopindolol ([^{125}I]-CYP) binding to sections of the rat kidney, $n = 10$. Data are presented as mean ± s.e.mean. The [^{125}I]-CYP binding was concentration-dependent. (b) Displacement curves of [^{125}I]-CYP binding by ICI 89,406 (◇) and ICI 118,551 (□), $n = 10$. The data are presented as mean ± s.e.mean.

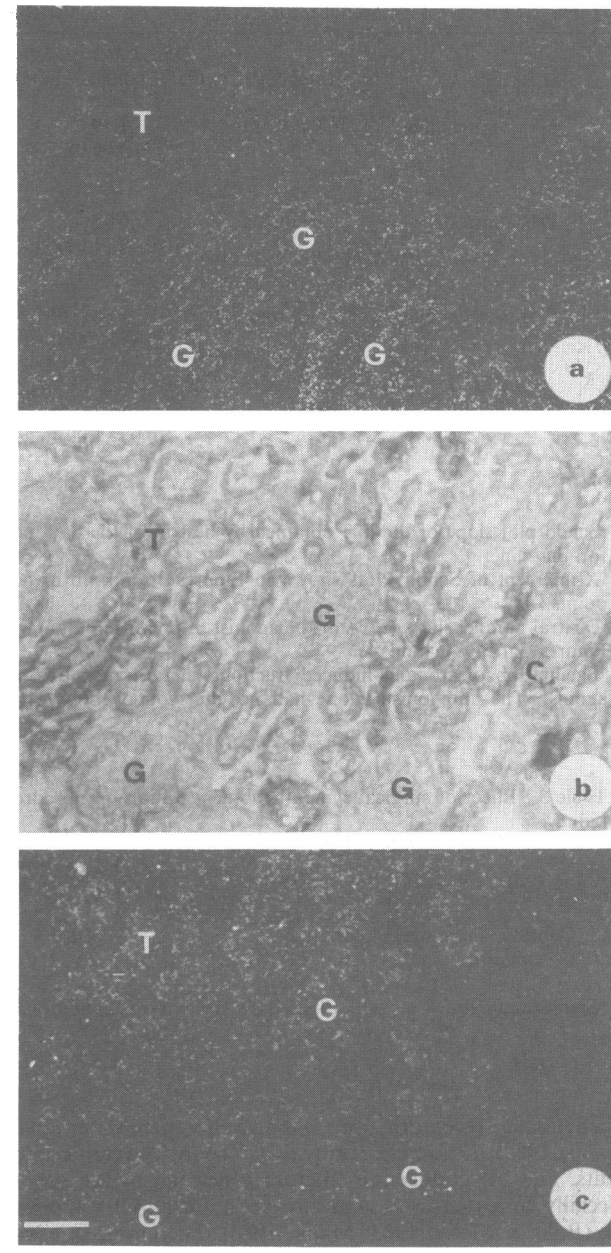


Figure 6 Autoradiography of [^{125}I]-cyanopindolol ([^{125}I]-CYP) binding in the rat renal cortex. (a) Dark-field picture of a section incubated in the presence of ICI 118,551 to block binding to β_2-adrenoceptors. β_1-Adrenoceptors are present mainly within glomeruli. (b) Bright-field picture of the same field as shown in (a) to verify anatomical details. (c) Dark-field picture of a section incubated in the presence of ICI 89,406 to block binding to β_1-adrenoceptors. β_2-Adrenoceptors are associated mainly with renal tubules. G = glomeruli, T = tubules. (Magnification: × 125, calibration bar = 50 μm).

the EC_{50} value for (−)-isoprenaline at prejunctional β-adrenoceptors was observed to be 1–2 nM, dobutamine should produce facilitation in the concentration range of 1 to 10 μM which was examined in the present study. Thus, the concentrations of dobutamine studied were appropriate. Second, dobutamine can activate α-adrenoceptors in addition to its action at β-adrenoceptors (Ruffolo *et al.*, 1981). Although experiments in the present study were performed in the presence of 10 μM phentolamine, it is possible that dobutamine may have overcome the α-adrenoceptor blockade, especially at higher concentrations, and may have produced inhibition of sympathetic neurotransmission by activating prejunctional α-adrenoceptors. This inhibition may have counterbalanced the facilitatory influence of dobutamine by β-adrenoceptor stimulation. This possibility is unlikely since no vasoconstriction (indicative of α-adrenoceptor activation) was observed in the presence of dobutamine. Thus, the results with dobutamine support the presence of the β_2-adrenoceptors as prejunctional β-adrenoceptors.

Another strategy which was used in the present study to characterize the prejunctional β-adrenoceptor was the determination of antagonist pA_2 or pK_b values. Propranolol, the β-adrenoceptor antagonist, has high affinity (in nM range) for the β_1- and β_2-subtypes, but low affinity (in μM range) for β_3-adrenoceptors (Bond & Clark, 1988). Hence, this property of propranolol was taken advantage of to examine whether β_3-adrenoceptors are responsible for the facilitatory response. Theoretically, if β_3-adrenoceptors contributed significantly to the facilitation of sympathetic neurotransmission in the rat kidney, 30 nM propranolol should produce a shift of the concentration-response curve to (−)-isoprenaline, the nonselective β-adrenoceptor agonist, which is less than the shift expected for antagonism at β_1- or β_2-adrenoceptors. However, a parallel, dextral shift of the (−)-isoprenaline curve was observed in the presence of 30 nM propranolol and the pA_2 of propranolol at prejunctional β-adrenoceptors was determined to be 9.20. This pA_2 value is similar to the pA_2 values of propranolol in guinea-pig atria (β_1-subtype) and guinea-pig trachea (β_2-subtype) which are 8.6 and 8.4, respectively, (Bertholet *et al.*, 1981), but significantly different from its pA_2 value at β_3-adrenoceptors which is 6.6 (Wilson *et al.*, 1984).

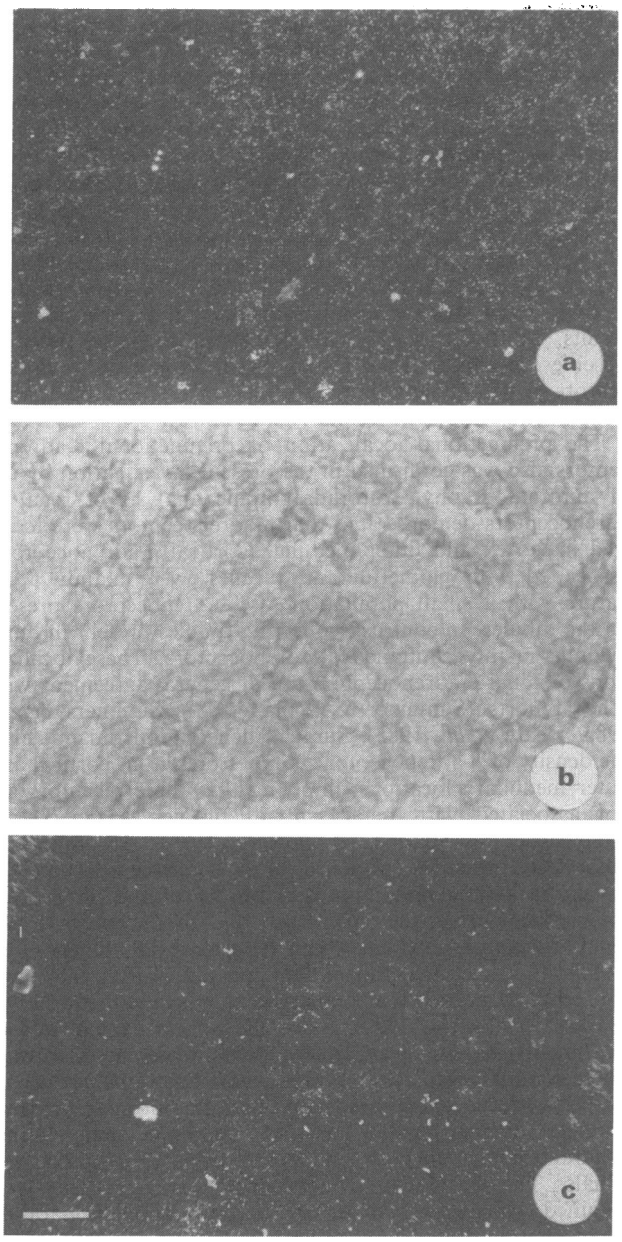


Figure 7 Autoradiography of [^{125}I]-cyanopindolol ([^{125}I]-CYP) binding in the rat renal outer medulla. (a) Dark-field picture of a section incubated without displacing agents. (b) Bright-field picture of the same field as shown in (a) to verify microanatomical details. (c) Dark-field picture of a section incubated in the presence of ICI 118,551 to block binding to β_2-adrenoceptors. Silver grains are remarkably reduced (magnification: × 125, calibration bar = 50 μm).

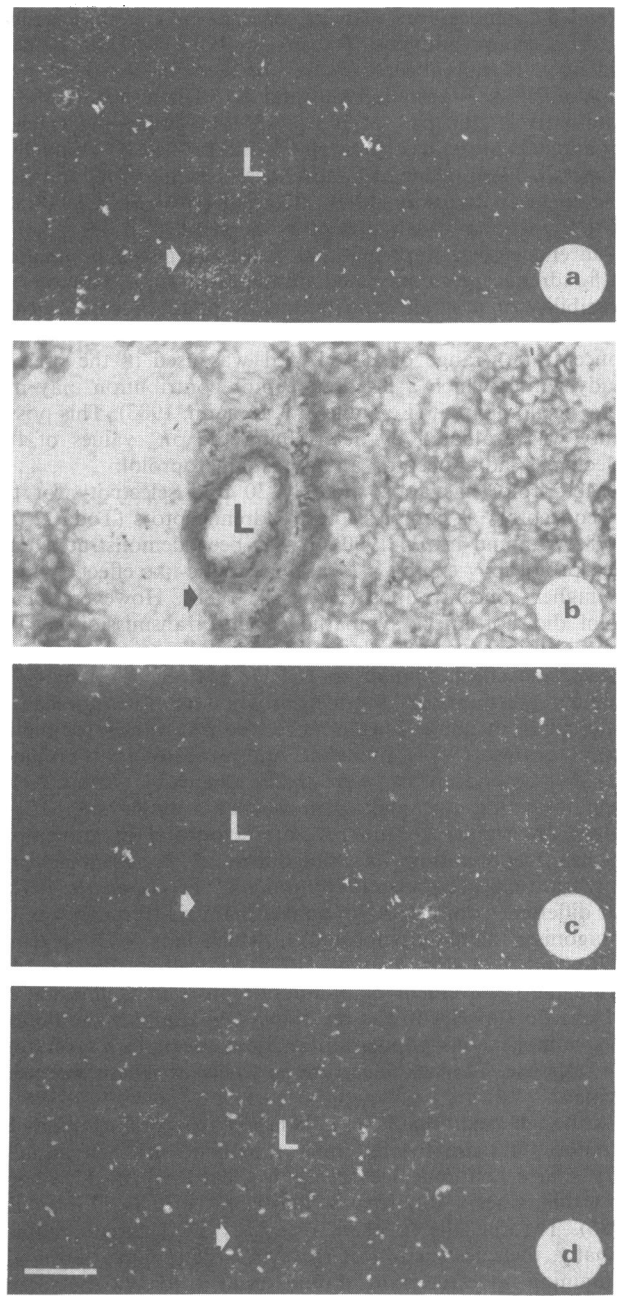


Figure 8 Autoradiography of [^{125}I]-cyanopindolol ([^{125}I]-CYP) binding in the intraparenchymal branch of the renal artery. (a) Dark-field picture of a section incubated in the presence of ICI 89,406 to block binding to β_1-adrenoceptors. Silver grains representing β_2-adrenoceptors are located in the adventitia and adventitial-medial border. (b) Bright-field picture of the same field as shown in (a) to verify microanatomical details. (c) Dark-field picture of a section incubated in the presence of (−)-propranolol to display nonspecific binding. (d) Dark-field picture of a section from a surgically denervated animal. The density of silver grains is remarkably reduced. L = lumen; Arrows indicate the adventitial-medial border. (Magnification: × 160, calibration bar = 50 μm).

These results imply that the β_3-adrenoceptor subtype does not significantly contribute to the facilitatory response in the rat kidney.

To differentiate between the β_1- and β_2-subtypes as prejunctional β-adrenoceptors, the pK_b values for ICI 118,551 and metoprolol at prejunctional β-adrenoceptors also were determined. ICI 118,551 exhibits 100 to 300 fold selectivity for β_2-adrenoceptors over β_1-adrenoceptors (Bilski *et al.*, 1983; Lemoine *et al.*, 1985). It has been suggested that ICI 118,551 may produce nonspecific inhibitory effects at 1 μM or higher concentrations (Bilski *et al.*, 1983). However, no nonspecific effect of ICI 118,551 was observed at the concentrations used. Importantly, the slopes of the Schild regression lines for ICI 118,551 obtained using (−)-isoprenaline and procaterol, the selective β_2-adrenoceptor agonist, were observed not to be different from unity. Furthermore, the pK_b values for ICI 118,551 against (−)-isoprenaline and procaterol (9.20 and 9.35) were only 0.15 log unit apart. This demonstration of the same pK_b values and Schild regressions of unity for ICI 118,551 against agonists of differential selectivity is indicative that a homogeneous population of β-adrenoceptors is mediating the facilitation (O'Donnell & Wanstall, 1979). To determine exactly which subtype was involved in the facilitation, we compared the pK_b of ICI 118,551 at prejunc-

tional β-adrenoceptors with its reported pA_2 at the classical β-adrenoceptor subtypes. The pA_2 of ICI 118,551 in guinea-pig atria (β_1-adrenoceptors) has been reported to be 6.96 (O'Donnell & Wanstall, 1980) and 7.17 (Bilski et al., 1983). Alternatively, the pA_2 of ICI 118,551 in guinea-pig trachea (β_2-adrenoceptors) has been reported to be 8.69 (O'Donnell & Wanstall, 1980), while at β_3-adrenoceptors, it is 5.49 and 5.33 (Hollenga & Zaagsma, 1989). Thus, the pA_2 of ICI 118,551 at prejunctional β-adrenoceptors is similar to its pA_2 at β_2-adrenoceptors, implying that a homogeneous population of β_2-adrenoceptors may mediate the facilitation. However, a possibility of a small contribution by β_1-adrenoceptors cannot be eliminated on the basis of these results, as a narrow concentration-range of ICI 118,551 was used in the present study within which a β_1-adrenoceptor contribution may not have been apparent (Lemoine & Kaumann, 1983). This possibility was evaluated by determining the pK_b values of the selective β_1-adrenoceptor antagonist, metoprolol.

Metoprolol has approximately 30 fold selectivity for β_1-adrenoceptors as compared to β_2-adrenoceptors (Toda et al., 1978; Wahlund et al., 1990). It has been demonstrated that metoprolol may exhibit a local anaesthetic-like effect at 1 μM or higher concentrations (Doggrell, 1988). However, metopolol did not affect sympathetic neurotransmission at the concentrations used implying that it did not produce any nonspecific effect. Similar to ICI 118,551, the slopes of the Schild regression lines for metoprolol were not significantly different from unity. Furthermore, the pK_b values for metoprolol against (−)-isoprenaline and procaterol at prejunctional β-adrenoceptors were 6.25 and 6.18, respectively, suggesting that its antagonism was at a single site. These values are similar to the pA_2 of metoprolol in guinea-pig trachea (representing its antagonism at β_2-adrenoceptors) which are 6.06 (Harms, 1976) and 6.25 (Toda et al., 1978), but different from its pA_2 in guinea-pig atria, representing its antagonism at β_1-adrenoceptors, which are 7.43 (Harms, 1976) and 7.93 (Toda et al., 1978). Together these results refute the involvement of β_1-adrenoceptors in facilitation of S-I overflow of NA in the rat kidney and support the notion of a homogeneous population of β_2-adrenoceptors mediating the response. The next question is: where are these receptors located?

Although traditionally it is believed that prejunctional β-adrenoceptors are located on sympathetic nerve terminals, very sparse anatomical evidence has been obtained to support this concept (Sharma & Banerjee, 1978; Dahlof et al., 1986). Theoretically, if these receptors are located prejunctionally, then destruction of the sympathetic nerve terminals containing these receptors should result in loss of the receptors. We utilized this theoretical concept to examine the location (prejunctional or postjunctional) of 'prejunctional' β-adrenoceptors in the rat kidney using radio-ligand binding and autoradiography combined with surgical denervation.

The binding of [^{125}I]-CYP in the rat kidney slices was saturable and of high affinity. Furthermore, the affinity of [^{125}I]-CYP for renal β-adrenoceptors observed in the present investigation is similar to that reported in previous studies with the same radio-ligand in the rat kidney (Munzel et al., 1984; Summers et al., 1985). The binding studies revealed that of the total β-adrenoceptor population in the rat kidney, β_1-adrenoceptors were 63% while β_2-adrenoceptors were 37%. Other investigators also have reported similar values for renal β-adrenoceptor subtypes (Snavely et al., 1982; Summers et al., 1985). However, a more important observation was that surgical denervation, accompanied by more than 90% reduction in renal NA content, resulted in 80% reduction in the β_2-adrenoceptors. Disappearance of the β_2-sites upon surgical denervation suggests a prejunctional location for these receptors. This result is in strong agreement with the results obtained by pharmacological methods which also demonstrated that prejunctional β-adrenoceptors are of the β_2-subtype. In contrast to β_2-adrenoceptors, the β_1-adrenoceptor population was not significantly affected by surgical denervation. This suggests that β_1-adrenoceptors are not present on sympathetic nerve terminals. This suggestion is supported by the pharmacological demonstration that β_1-adrenoceptors are not involved in facilitation of S-I overflow of NA in the rat kidney. The results obtained in the present study are at variance with those by Fortin & Sundaresan (1987) and Woodcock et al. (1985) who failed to observe any effect of sympathectomy on β-adrenoceptor population in the rat kidney. However, Fortin & Sundaresan (1989) used only the renal cortex to examine the β-adrenoceptor population in that study, in contrast to the whole kidney preparation used in the present study, and this may account for the different results. On the other hand, Woodcock et al. (1985) failed to observe the effect of chemical sympathectomy on β-adrenoceptors in the whole kidney. The reason for the discrepancy between this and our results is not known.

The prejunctional localization of prejunctional β-adrenoceptors also was confirmed in the present investigation by use of autoradiography. Autoradiographic analysis of renal β-adrenoceptors demonstrated that β-adrenoceptors were localized in cortex and outer medulla. Cortical β-adrenoceptors, associated with glomeruli and tubules, were mainly β_1 in nature with a small population of β_2-adrenoceptors present on the tubules. In contrast, collecting tubules present in outer medulla mainly contained β_2-adrenoceptors. These localizations of renal β-adrenoceptors are in general agreement with other reports (Summers & Kuhar, 1983; Summers et al., 1985; Healy et al., 1985). Surgical denervation did not alter the density of β-adrenoceptor subtypes present at cortical and outer medullary locations, implying that these receptors are not associated with renal sympathetic nerves innervating these structures (Barajas & Powers, 1990). An important observation was the presence of β_2-adrenoceptors on the adventitia and adventitial-medial border of the intraparenchymal branches of the renal artery. β_2-Adrenoceptors on the renal vasculature also have been reported by Summers et al. (1985). It has been demonstrated previously that the intraparenchymal branches are innervated by sympathetic nerves (Barajas & Powers, 1990). Since the adventitia and adventitial-medial border of an artery represent the vascular zones where sympathetic nerve plexus ends (cf. Amenta et al., 1991), β_2-adrenoceptors associated with the intraparenchymal branches of the renal artery may represent prejunctional β_2-adrenoceptors located on renal sympathetic nerve terminals. The conclusive evidence for this hypothesis was obtained when it was observed that this population of β_2-adrenoceptors disappeared following destruction of the nerve terminals by surgical denervation. Thus, these results provide the direct anatomical evidence for the presence of β_2-adrenoceptors on the renal sympathetic nerve terminals associated with renal vasculature. Furthermore, these results suggest that prejunctional β-adrenoceptors in the rat kidney may not affect tubular function directly. Instead, they may influence renal function by altering renal hemodynamics.

In summary, the present study demonstrates that renal prejunctional β-adrenoceptors are of the β_2-subtype in nature. Anatomical evidence was obtained suggesting that these receptors are located on sympathetic nerve terminals associated with the renal vasculature rather than renal tubules. Thus, prejunctional β_2-adrenoceptors in the rat kidney may influence renal function by altering sympathetic neurotransmission at the renal vasculature level.

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References

ADLER-GRASCHINSKY, E. & LANGER, S.Z. (1975). Possible role of a β-adrenoceptor in the regulation of noradrenaline release by nerve stimulation through a positive feed-back mechanism. *Br. J. Pharmacol.*, **53**, 43–50.

AMENTA, D., CAVALLOTTI, C., COLLIER, W.L., DE ROSSI, M., RICCI, A. & AMENTA, F. (1987). Noradrenergic innervation of the superior mesenteric artery in streptozotocin-diabetic rats. *Acta Histochem. Cytochem.*, **20**, 177–184.

AMENTA, F., COPPOLA, L., GALLO, P., FERRANTE, F., FORLANI, A., MONOPOLI, A. & NAPOLEONE, P. (1991). Autoradiographic localization of β-adrenergic receptors in human large coronary arteries. *Circ. Res.*, **68**, 1591–1599.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BARAJAS, L. & POWERS, K. (1990). Monaminergic innervation of the rat kidney: A quantitative study. *Am. J. Physiol.*, **259**, F503–F511.

BARTFAI, T., IVERFELDT, K., FISONE, G. & SERFOZO, P. (1988). Regulation of the release of coexisting neurotransmitters. *Annu. Rev. Pharmacol. Toxicol.*, **28**, 285–310.

BERTHOLET, A., MILAVEC, M., WAITE, R. & SCHILD, H.O. (1981). A study of β-adrenoceptors in the guinea-pig. *Postgrad. Med. J.*, **57** (Suppl. 1), 9–17.

BILSKI, A.J., HALLIDAY, S.E., FITZGERALD, J.D. & WALE, J.L. (1983). The pharmacology of a β_2-selective adrenoceptor antagonist (ICI 118,551). *J. Cardiovasc. Pharmacol.*, **5**, 430–437.

BOKE, T. & MALIK, K.U. (1983). Enhancement by locally generated angiotensin II of release of the adrenergic transmitter in the isolated rat kidney. *J. Pharmacol. Exp. Ther.*, **226**, 900–907.

BOND, R.A. & CLARKE, D.E. (1988). Agonist and antagonist characterization of a putative adrenoceptor with distinct pharmacological properties from the α- and β-subtypes. *Br. J. Pharmacol.*, **95**, 723–734.

BOWMAN, W.C. & RAND, M.J. (1982). Principles of drug action. In *Textbook of Pharmacology*, pp. 39.1–39.69. St. Louis: Blackwell Scientific Publications.

DAHLOF, C., ABLAD, B., BORG, K.O., EK, L. & WALDECK, B. (1975). Prejunctional inhibition of adrenergic nervous vasomotor control due to β-receptor blockade. In *Chemical Tools in Catecholamine Research*, Vol. 2. ed. Almgren, O., Carlsson, A. & Engel, J. pp. 201–210. Amsterdam: North-Holland Publishing Co.

DAHLOF, C., HEDBERG, A. & NERME, V. (1986). Evidence for prejunctionally located β_2-adrenoceptors in the cat spleen. *Naunyn-Schmied. Arch. Pharmacol.*, **333**, 362–367.

DAHLOF, C., LJUNG, B. & ABLAD, B. (1978). Increased noradrenaline release in rat portal vein during sympathetic nerve stimulation due to activation of presynaptic β-adrenoceptors by noradrenaline and adrenaline. *Eur. J. Pharmacol.*, **50**, 75–78.

DOGGRELL, S.A. (1988). Simultaneous assessment of membrane-stabilizing and β-adrenoceptor blocking activity of drugs with the rat isolated left atria. *J. Pharmacol. Meth.*, **19**, 93–107.

ENGEL, G., MAURER, R., PERROT, K. & RICHARDSON, B.P. (1985). β-Adrenoceptor subtypes in sections of rat and guinea-pig kidney. *Naunyn-Schmied. Arch. Pharmacol.*, **328**, 354–357.

FARMER, J.B., KENNEDY, I., LEVY, G.P. & MARSHALL, R.J. (1970). A comparison of the β-adrenoceptor stimulant properties of isoprenaline, with those of orciprenaline, salbutamol, soterenol and trimetoquinol on isolated atria and trachea of the guinea-pig. *J. Pharm. Pharmacol.*, **22**, 61–63.

FORTIN, T.L. & SUNDARESAN, P.R. (1989). Reserpine but not surgical denervation regulates rat renal β-adrenergic receptors. *Am. J. Physiol.*, **256**, F532–F539.

FURCHGOTT, R.F. (1972). The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. In *Handbook of Experimental Pharmacology*, Vol. 33. ed. Blaschko, H. & Muscholl, E. pp. 283–335. New York: Springer-Verlag.

GOTHERT, M. & KOLLECKER, P. (1986). Subendothelial β_2-adrenoceptors in the rat vena cava: Facilitation of noradrenaline release via local stimulation of angiotensin II synthesis. *Naunyn-Schmied. Arch. Pharmacol.*, **334**, 156–165.

HARMS, H.H. (1976). Isoprenaline antagonism of cardioselective β-adrenergic receptor blocking agents: A comparative study of human and guinea-pig cardiac and bronchial β-adrenergic receptors. *J. Pharmacol. Exp. Ther.*, **199**, 329–335.

HEALY, D.P., MUNZEL, P.A. & INSEL, P.A. (1985). Localization of β_1- and β_2-adrenergic receptors in rat kidney by autoradiography. *Circ Res.*, **57**, 278–284.

HOLLENGA, C. & ZAAGSMA, J. (1989). Direct evidence for the atypical nature of functional β-adrenoceptors in rat adipocytes. *Br. J. Pharmacol.*, **98**, 1420–1424.

KAWASAKI, H., CLINE, W.H. Jr. & SU, C. (1982). Enhanced presynaptic β-adrenoceptor-mediated modulation of vascular adrenergic neurotransmission in spontaneously hypertensive rats. *J. Pharmacol. Exp. Ther.*, **223**, 721–728.

KENAKIN, T.P. (1981). An *in vitro* quantitative analysis of the α-adrenoceptor partial agonist activity of dobutamine and its relevance to inotropic selectivity. *J. Pharmacol. Exp. Ther.*, **216**, 210–219.

KENAKIN, T.P. (1984). The classification of drugs and drug receptors in isolated tissues. *Pharmacol. Rev.*, **36**, 165–222.

LAKHLANI, P.P. & EIKENBURG, D.C. (1993). Dependence of prejunctional β-adrenoceptor facilitation of sympathetic neurotransmission on stimulus train length and agonist exposure time. *Eur. J. Pharmacol.*, **230**, 107–110.

LANDS, A.M., ARNOLD, A., MCAULIFF, J.P., LUDUENA, F.P. & BRONW, T.G. Jr. (1967). Differentiation of receptor systems activated by sympathomimetic amines. *Nature*, **214**, 597–598.

LEMOINE, H., BERNHARD, B. & KAUMANN, A.J. (1985). Direct labelling of β_2-adrenoceptors. Comparison of binding potency of ^{3}H-ICI 118,551 and blocking potency of ICI 118,551. *Naunyn-Schmied. Arch. Pharmacol.*, **331**, 40–51.

LEMOINE, H. & KAUMANN, A.J. (1983). A model for the interaction of competitive antagonists with two receptor subtypes characterized by a Schild plot with apparent slope unity. *Naunyn-Schmied. Arch. Pharmacol.*, **322**, 111–120.

MACKAY, D. (1978). How should values of pA_2 and affinity constants for pharmacological competitive antagonists be estimated? *J. Pharm. Pharmacol.*, **30**, 312–313.

MAJEWSKI, H. & RAND, M.J. (1981). An interaction between prejunctional α-adrenoceptors and prejunctional β-adrenoceptors. *Eur. J. Pharmacol.*, **69**, 493–498.

MALIK, K.U. (1988). Interaction of arachidonic acid metabolites and adrenergic nervous system. *Am. J. Med. Sci.*, **295**, 280–286.

MISU, Y. & KUBO, T. (1986). Presynaptic β-adrenoceptors. *Med. Res. Rev.*, **6**, 197–225.

MUNZEL, P.A., HEALEY, D.P. & INSEL, P.A. (1984). Autoradiographic localization of β-adrenergic receptors in rat kidney slices using (^{125}I)iodocyanopindolol. *Am. J. Physiol.*, **246**, F240–F245.

O'DONNELL, S.R. (1972). An examination of some β-adrenoceptor stimulants for selectivity using the isolated trachea and atria of the guinea-pig. *Eur. J. Pharmacol.*, **19**, 371–379.

O'DONNELL, S.R. & WANSTALL, J.C. (1979). The importance of choice of agonist in studies designed to predict β_2:β_1 adrenoceptor selectivity of antagonists from pA_2 values on guinea-pig trachea and atria. *Naunyn-Schmied. Arch. Pharmacol.*, **308**, 183–190.

O'DONNELL, S.R. & WANSTALL, J.C. (1980). Evidence that ICI 118,551 is a potent, highly β_2-selective adrenoceptor antagonist and can be used to characterize β-adrenoceptor populations in tissues. *Life Sci.*, **27**, 671–677.

O'DONELL, S.R. & WANSTALL, J.C. (1985). Responses to the β_2-selective agonist procaterol of vascular and atrial preparations with different functional β-adrenoceptor populations. *Br. J. Pharmacol.*, **84**, 227–235.

ORTIZ DE URBINA, A.V., DAVY, M., MIDOL-MONNET, M., HEIMBURGER, M., BESLOT, F. & COHEN, Y. (1992). Modulation of noradrenergic transmission in the rat isolated portal vein: Role of prejunctional α_2-adrenoceptors and β-adrenoceptors. *Gen. Pharmacol.*, **23**, 763–767.

REMIE, R., KNOT, H.J., BOS, E.A. & ZAAGSMA, J. (1988). Characterization of presynaptic β-adrenoceptors facilitating endogenous noradrenaline release in the portal vein of permanently cannulated, freely moving rats. *Eur. J. Pharmacol.*, **157**, 37–43.

RUFFOLO, R.R.Jr., SPRADLIN, T.A., POLLOCK, G.D., WADDELL, J.E. & MURPHY, P.J. (1981). α- and β-adrenergic effects of the stereoisomers of dobutamine. *J. Pharmacol. Exp. Ther.*, **219**, 447–452.

RUMP, L.C. (1987). Modulation of renal noradrenaline release by prejunctional system *in vitro*. *Clin. Exp. Physiol. Pharmacol.*, **14**, 423–428.

SCHWARTZ, D.D. & EIKENBURG, D.C. (1988). Enhanced endogenous neurotransmitter overflow in the isolated perfused rat kidney after chronic epinephrine administration: Lack of a prejunctional β-adrenoceptor influence. *J. Pharmacol. Exp. Ther.*, **244**, 11–18.

SCHWARTZ, D.D. & MALIK, K.U. (1989). Renal periarterial nerve stimulation-induced vasoconstriction at low frequencies is primarily due to release of a purinergic transmitter in the rat. *J. Pharmacol. Exp. Ther.*, **250**, 764–771.

SHARMA, V.K. & BANERJEE, S.P. (1978). Prejunctional adrenoceptors in rat peripheral tisues. In *Catecholamines. Basic and Clinical Frontiers*. ed. Usdin, E., Kopin, E.J. & Barchas, J. pp. 945–947. New York: Pergamon Press.

SNAVELY, M.D., MOTULSKY, H.J., MOUSTAFA, E., MAHAN, L.C. & INSEL, P.A. (1982). β-Adrenergic receptor subtypes in the rat renal cortex. Selective regulation of β_1-adrenergic receptors by pheochromocytoma. *Circ. Res.*, **51**, 504–513.

STJARNE, L. & BRUNDIN, J. (1976). β_2-Adrenoceptors facilitating noradrenaline secretion from human vasoconstrictor nerves. *Acta Physiol. Scand.*, **97**, 88–93.

SUMMERS, R.J. & KUHAR, M.J. (1983). Autoradiographic localization of β-adrenoceptors in rat kidney. *Eur. J. Pharmacol.*, **91**, 305–310.

SUMMERS, R.J., STEPHENSON, J.A. & KUHAR, M.J. (1985). Localization of β-adrenoceptor subtypes in rat kidney by light microscopic autoradiography. *J. Pharmacol. Exp. Ther.*, **232**, 561–569.

TODA, N., HAYASHI, S., HATANO, Y., OKUNISHI, H. & MIYAZAKI, M. (1978). Selectivity and steric effects of metoprolol isomers on isolated rabbit atria, arteries and tracheal muscles. *J. Pharmacol. Exp. Ther.*, **207**, 311–319.

VALENTA, B. & SINGER, E.A. (1991). Presynaptic β-adrenoceptors in guinea-pig papillary muscle: Evidence for adrenaline-mediated positive feedback on noradrenergic transmission. *J. Cardiovasc. Pharmacol.*, **17**, 256–260.

WAGNER, J. & SCHUMANN, H.J. (1979). Stimulation of β-adrenoceptors by dobutamine in the guinea-pig atrium and tracheal chain. *Naunyn-Schmied. Arch. Pharmacol.*, **308**, 19–23.

WAHLUND, G., NERME, V., ABRAHAMSSON, T. & SJOQUIST, P.-O. (1990). The β_1- and β_2-adrenoceptor affinity and β_1-blocking potency of S- and R-metoprolol. *Br. J. Pharmacol.*, **99**, 592–596.

WAUD, D.R. & PARKER, R.B. (1971). Pharmacological estimation of drug-receptor dissociation constants. Statistical Evaluation. II. Competitive antagonists. *J. Pharmacol. Exp. Ther.*, **177**, 13–24.

WESTFALL, T.C., PEACH, M.J. & TITTERMARY, V. (1979). Enhancement of the electrically induced release of norepinephrine from the rat portal vein: mediation by β_2-adrenoceptors. *Eur. J. Pharmacol.*, **58**, 67–74.

WILSON, C., WILSON, S., PIERCY, V., SENNIT, M.V. & ARCH, J.R.S. (1984). The rat lipolytic β-adrenoceptor: Studies using novel β-adrenoceptor agonists. *Eur. J. Pharmacol,*. **100**, 309–319.

WOODCOCK, E.A., MORRIS, M.J., MCLEOD, J.K. & JOHNSTON, C.I. (1985). Specific increase in renal α_1-adrenergic receptors following unilateral renal denervation. *J. Recept. Res.*, **5**, 133–146.

YABUUCHI, Y. (1977). The β-adrenoceptor stimulant properties of OPC-2009 on guinea-pig isolated tracheal, right atrial and left atrial preparations. *Br. J. Pharmacol.*, **61**, 513–521.

YOUNG, W.S.III & KUHAR, M.J. (1979). A new method for receptor autoradiography: (^{3}H)opioid receptors in rat brain. *Brain Res.*, **179**, 255–270.

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Time-dependent enhancement or inhibition of endotoxin-induced vascular injury in rat intestine by nitric oxide synthase inhibitors

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1 The effects of the nitric oxide (NO) synthase inhibitors, N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA), on the vascular damage induced by the endotoxin, *E. coli* lipopolysaccharide (LPS), in the ileum and colon were investigated in the conscious rat over a 5 h period.

2 Administration of LPS (3 mg kg^{-1}, i.v.) increased ileal and colonic vascular injury after a lag period of 2 h, as determined by the leakage of radiolabelled albumin.

3 Administration of L-NAME (1–5 mg kg^{-1}, s.c.) concurrently with LPS, produced a dose-dependent increase in vascular albumin leakage in the intestinal tissues, when determined over a 5 h period. Vascular albumin leakage with LPS and L-NAME (5 mg kg^{-1}) was substantially increased after 1 h, reached maximal levels 3 h after administration, and then slowly declined.

4 L-NMMA (50 mg kg^{-1}, s.c.), likewise elevated intestinal albumin leakage when administered concurrently with LPS, but this reached maximal levels after 1 h and rapidly declined over the subsequent 2 h.

5 In control rats, in the absence of LPS challenge, neither L-NAME (5 mg kg^{-1}, s.c.) nor L-NMMA (50 mg kg^{-1}, s.c.) increased intestinal vascular leakage of albumin over a 5 h period.

6 By contrast, when L-NAME (1–5 mg kg^{-1}, s.c.) or L-NMMA (12.5–50 mg kg^{-1}, s.c.) was injected 3 h after LPS, a dose-dependent reduction in the LPS-provoked vascular albumin leakage was observed.

7 Pretreatment with L-arginine (300 mg kg^{-1}, s.c.) 15 min prior to the NO synthase inhibitors, reversed either the potentiation or the inhibition by L-NAME (5 mg kg^{-1}, s.c.) or L-NMMA (50 mg kg^{-1}, s.c.) of the LPS-induced intestinal vascular damage.

8 These findings indicate that initial suppression of the constitutive NO synthase by L-NAME or L-NMMA following challenge with LPS aggravates the acute vascular injury in the ileum and colon, suggesting a defensive role of NO. By contrast, the delayed administration of NO synthase inhibitors, at a time of known expression of the inducible NO synthase, provides protection against the subsequent damage to the intestinal vasculature.

Keywords: Nitric oxide; endotoxin; NO synthase inhibitors; N^G-monomethyl-L-arginine; N^G-nitro-L-arginine methyl ester; rat intestinal vasculature; intestinal inflammation; endothelial injury

Introduction

Nitric oxide (NO), formed from L-arginine by constitutive NO synthase (Palmer *et al.*, 1988; Moncada *et al.*, 1991), plays an important role in the maintenance of gastrointestinal vascular integrity under physiological and pathophysiological circumstances (Whittle *et al.*, 1990; Hutcheson *et al.*, 1990; Boughton-Smith *et al.*, 1990; Kubes & Granger, 1992). Concurrent intravenous administration of the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) with high doses of endotoxin augments the vascular injury in rat gastrointestinal tissue observed after 15 min; this effect can be reversed by concurrent administration of the NO donor, S-nitroso-*N*-acetyl-penicillamine (Hutcheson *et al.*, 1990; Boughton-Smith *et al.*, 1990). Furthermore, administration of the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) increases microvascular fluid and protein flux in the cat small intestine, effects attenuated by nitroprusside (Kubes & Granger, 1992), again suggesting an important protective action of endogenous NO in the vasculature.

NO can also be formed by an inducible isoenzyme in activated inflammatory cells, particularly macrophages (Marletta *et al.*, 1988; Hibbs *et al.*, 1988; Stuehr *et al.*, 1989) and neutrophils (Rimele *et al.*, 1988; Salvemini *et al.*, 1989; Schmidt *et al.*, 1989; Wright *et al.*, 1989; McCall *et al.*, 1989). Moreover, vascular smooth muscle cells can also express an inducible isoform of NO synthase following prolonged exposure to cytokines or endotoxin (Radomski *et al.*, 1990; Busse & Mulsch, 1990). In endothelial cells in culture, the excessive local generation of NO or its metabolites following NO synthase induction can lead to cellular damage (Palmer *et al.*, 1992). Administration of low doses of endotoxin *in vivo* can induce a calcium-dependent NO synthase in the rat intestine 3 h after administration (Salter *et al.*, 1991), which is associated with microvascular injury in the jejunum and colon (Boughton-Smith *et al.*, 1993). Pretreatment with the anti-inflammatory corticosteroid, dexamethasone, which can inhibit the induction of NO synthase (Radomski *et al.*, 1990; Knowles *et al.*, 1990; Rees *et al.*, 1990), reduces the vascular damage induced by low doses of endotoxin in these gastrointestinal tissues (Boughton-Smith *et al.*, 1993).

In the present study, the potential for endogenous NO to produce both protective or damaging actions in the intestinal microcirculation have been investigated. Thus, changes in vascular leakage of radiolabelled albumin in the ileum and colon, were measured at various times after endotoxin challenge in the conscious rats. Previous studies have demonstrated that increases in plasma albumin leakage in the rat intestine, provoked by endotoxin, are associated with histological evidence of intestinal vascular injury (Hutcheson *et al.*, 1990). For evaluation of the role of the constitutive NO synthase in the maintenance of the vascular integrity in the acute phase following endotoxin challenge, the NO synthase inhibitors L-NAME or L-NMMA were injected concurrently with lipopolysaccharide (LPS) from *E. coli*. Changes in

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albumin extravasation were then determined over a 5 h period. To evaluate the role of the inducible NO synthase in the later phase of vascular injury following endotoxin challenge, these inhibitors were administered 3 h after LPS, at a time of known expression of this isoenzyme (Salter *et al.*, 1991; Boughton-Smith *et al.*, 1993), and the extent of albumin extravasation was subsequently determined.

A preliminary account of some of this work has been presented to the British Pharmacological Society (Laszlo *et al.*, 1993).

Methods

Albumin leakage

Male Wistar rats (225–275 *g*) were fasted overnight, but received water *ad libitum*. For the determination of the vascular albumin leakage as an index of endothelial injury, rats were anaesthetized with halothane and ^{125}I-labelled human serum albumin ([^{125}I]-HSA) was administered (2 μCi kg^{-1}) via the tail vein. In all experiments, the radiolabelled albumin was administered immediately (time zero) after the injection of either *E. coli* lipopolysaccharide (LPS; 3 mg kg^{-1}, i.v.) or isotonic saline (the control vehicle) and the animals allowed to recover from the anaesthetic. At various times (1 to 5 h) after LPS or saline injection, the rats were re-anaesthetized with halothane and blood (1 ml) from the abdominal aorta was collected in tubes containing trisodium citrate (final concentration 0.318%) and centrifuged (10,000 *g*, 10 min, 4°C).

Following cervical dislocation, segments of the ileum and colon were ligated, removed and blotted dry from the outside surface of the tissue. The [^{125}I]-HSA contents in segments of whole tissue and in aliquots of plasma (100 μl) were determined in a gamma-spectrometer (Nuclear Enterprises NE1600). The organs were then opened, blotted dry from the inside surface and weighed, and the total radiolabelled albumin content in the intestinal tissue was calculated. Control values were subtracted from the treated values and the data were expressed as the content of plasma in albumin equivalents (μl g^{-1} of tissue).

Changes in intravascular volume in the intestinal tissue were determined in an additional group of rats by administering [^{125}I]-HSA via the tail vein at each time point and to each group, 2 min before tissue removal. The tissue and plasma radiolabel content were determined and the intravascular volume was calculated. This value was subtracted from the appropriate value from the plasma leakage studies to obtain a measure of the intestinal plasma albumin leakage, and expressed as Δ μl g^{-1} tissue.

Effects of NO synthase inhibitors

L-NAME (1–5 mg kg^{-1}, s.c.) or L-NMMA (12.5–50 mg kg^{-1}, s.c.) was administered either concurrently with LPS treatment (time 0), or 3 h after LPS administration. The ileum and the colon were removed 5 h after LPS challenge for determination of the vascular leakage of albumin.

In a separate time-course study, L-NAME (5 mg kg^{-1}) or L-NMMA (50 mg kg^{-1}) was injected s.c., concurrently with LPS. Likewise, in a further series of studies, L-NAME or L-NMMA in these doses was administered 3 h after the LPS challenge. In both these studies, vascular leakage of albumin in the ileum and colon was determined hourly over the 5 h study period.

In order to have an indication of the duration of action of these NO synthase inhibitors, rats were anaesthetized with pentobarbitone (60 mg kg^{-1}, i.p.) and the right carotid artery cannulated for the determination of systemic arterial blood pressure (BP), utilizing a pressure transducer (Elcometic) and chart recorder (Grass system). L-NMMA (50 mg kg^{-1}) or L-NAME (5 mg kg^{-1}) were injected subcutaneously and BP was determined continuously over the subsequent 2 h period.

L-Arginine reversal of the effects of the NO synthase inhibitors

In separate groups of rats from both series of studies, where the NO synthase inhibitor was administered either concurrently with LPS, or 3 h after LPS challenge, L-arginine (300 mg kg^{-1}, s.c.) was injected 15 min before L-NAME (5 mg kg^{-1}, s.c.) or L-NMMA (50 mg kg^{-1}, s.c.) administration. Vascular albumin leakage in the ileum and the colon was measured 1 h after administration of these inhibitors; thus, measurements were performed on tissue obtained either 1 or 4 h after the LPS challenge.

Materials

L-NMMA was synthesized in the Department of Medicinal Chemistry, Wellcome Research Laboratories. [^{125}I]-HSA (3.7 kBq mg^{-1}) was obtained from Amersham International (U.K.). All other reagents (LPS 0.111:B4, L-arginine hydrochloride and L-NAME) were obtained from the Sigma Chemical Company (Poole, Dorset).

Statistical analysis

The data are expressed as mean ± s.e.mean from (*n*) rats per experimental group. For statistical comparisons, analysis of variance with the Bonferroni test for multiple comparisons was used; $P<0.05$ was taken as statistically significant.

Results

Effects of concurrent administration of NO synthase inhibitors on vascular leakage

Administration of LPS (3 mg kg^{-1}) caused a significant increase in albumin leakage into the ileal and colonic tissue when determined 5 h after challenge. Concurrent administration of L-NAME (1–5 mg kg^{-1}, s.c.) provoked a dose-dependent aggravation of the LPS-induced albumin leakage, in both ileal and colonic tissue, determined after 5 h (Figure 1). However, plasma leakage in the ileum 5 h following LPS challenge, was not significantly increased by concurrent administration of L-NMMA (12.5, 25 and 50 mg kg^{-1}, s.c.)

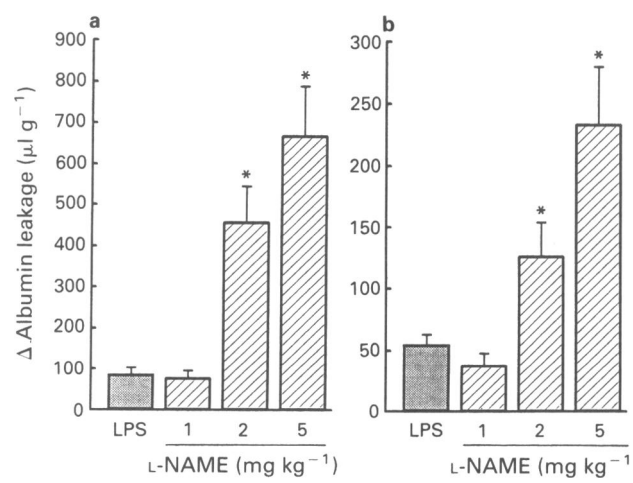


Figure 1 Potentiation of lipopolysaccharide (LPS, 3 mg kg^{-1}, i.v.)-induced vascular leakage of radiolabelled albumin, in (a) rat ileum and (b) colon by concurrent administration of N^{G}-nitro-L-arginine methyl ester (L-NAME, 1–5 mg kg^{-1}, s.c.). The columns show the leakage of plasma (Δ μl g^{-1} tissue) 5 h after challenge with LPS alone (stippled column) or LPS with L-NAME (hatched columns). Data are given as the mean ± s.e.mean, where (*n*) is at least 8; statistical significance is shown as $*P<0.05$ compared to the control LPS-alone group.

being Δ94 ± 16 compared with Δ86 ± 9, 84 ± 9 and 111 ± 33 μl plasma g^{-1} tissue, respectively. Similarly, plasma leakage in the colon following LPS challenge alone after 5 h was not significantly increased by concurrent administration of these doses of L-NMMA, being Δ70 ± 18 compared with Δ66 ± 20, 82 ± 20 and 95 ± 27 μl plasma g^{-1} tissue, respectively.

In the study on the time-course of vascular injury following LPS administration, albumin leakage was not changed significantly as compared with the control groups, when determined 1 or 2 h after LPS administration. However, 3 h after LPS administration, the albumin leakage into the ileal and colonic tissue had begun to rise, and further increases were observed 4 and 5 h following LPS injection (Figure 2). L-NAME (5 mg kg^{-1}, s.c.), administered concurrently with LPS challenge, induced a significant plasma leakage into the ileum and the colon at each time period measured from 1 to 5 h. As compared with the LPS-treated control groups, the LPS-induced albumin leakage was significantly elevated by L-NAME during the whole 5 h period, with the maximal effect being observed at 3 h (Figure 2), which only slowly declined.

Concurrent administration of L-NMMA (50 mg kg^{-1}, s.c.) with LPS also provoked significant increases in albumin leakage into the intestinal tissues 1 and 2 h after its administration. This response reached maximal levels after 1 h, and then declined 2 and 3 h, and was absent at 4 and 5 h after administration of L-NMMA (Figure 2).

Neither L-NAME (5 mg kg^{-1}, s.c.) nor L-NMMA (50 mg kg^{-1}, s.c.) significantly affected albumin leakage under control conditions (in the absence of LPS challenge) in the colon during the 5 h period, and small, inconsistent, non-significant changes were observed in the ileum (Table 1).

The intravascular volumes were not significantly different, when comparisons were made with any group at any time point in the ileum. Thus, the value for the control intravascular volume in the ileum (30 ± 4 μl g^{-1} tissue; $n = 4$) was not significantly different 5 h after saline or LPS administration (34 ± 4 and 28 ± 6 μl g^{-1} tissue, respectively), nor 5 h after administration of L-NAME or L-NMMA in control (35 ± 4 and 26 ± 5 μl g^{-1} tissue, respectively) or LPS-treated rats (36 ± 5 and 27 ± 4 μl g^{-1} tissue, respectively). Similarly, intravascular volume in the colon was not significantly altered by any of the treatment or time-points studied ($n = 4$ for each; data not shown).

Table 1 Radiolabelled albumin leakage following administration of N^G-nitro-L-arginine methyl ester (L-NAME, 5 mg kg^{-1}, s.c.) or N^G-monomethyl-L-arginine (L-NMMA, 50 mg kg^{-1}, s.c.) alone over a 5 h period in the ileum and colon of the conscious rat

Treatment	1 h	2 h	3 h	4 h	5 h
			Ileum		
L-NAME	24 ± 9	11 ± 16	2 ± 14	7 ± 10	11 ± 13
L-NMMA	17 ± 12	21 ± 10	2 ± 10	2 ± 10	0 ± 10
			Colon		
L-NAME	1 ± 6	4 ± 3	10 ± 9	23 ± 16	10 ± 7
L-NMMA	3 ± 7	10 ± 8	13 ± 9	15 ± 10	15 ± 9

Data are expressed as plasma leakage, Δ μl g^{-1} tissue, compared with the control values determined at each time point in groups of rats receiving saline (1.0 ml kg^{-1}, s.c.) alone, and shown as mean ± s.e.mean ($n = 7$–9). No significant difference was found between control values and any of the groups.

In studies on the duration of action of these NO synthase inhibitors on systemic arterial BP in anaesthetized rats, the hypertensive action of L-NMMA (50 mg kg^{-1}, s.c.) reached its peak effect (Δ49 ± 3 mmHg, $n = 7$; $P<0.001$) 15 min after administration and then declined over the ensuing 60 min to Δ13 ± 3 mmHg. The hypertensive action of L-NAME (5 mg kg^{-1}, s.c.) reached its peak value (Δ57 ± 5 mmHg, $n = 7$; $P<0.001$) 30 min after administration, which only declined to Δ40 ± 5 mmHg ($P<0.01$) after 120 min.

Effects of delayed administration of NO synthase inhibitors

In a time course study, when L-NAME (5 mg kg^{-1}, s.c.) or L-NMMA (50 mg kg^{-1}, s.c.) was administered 3 h after LPS

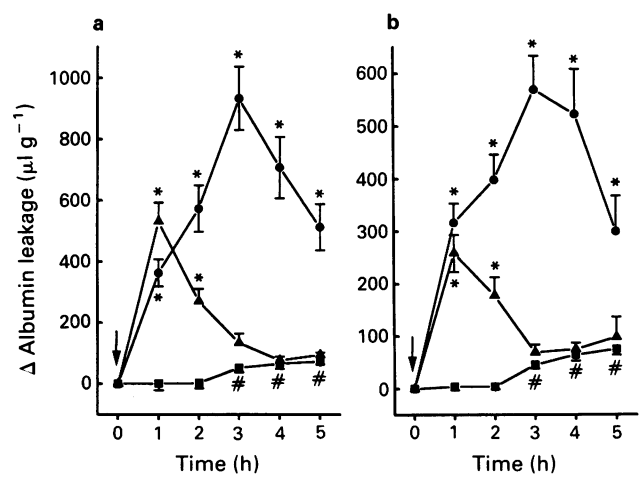


Figure 2 Potentiation of lipopolysaccharide (LPS, 3 mg kg^{-1}, i.v.)-induced albumin leakage in (a) rat ileum and (b) colon by N^G-nitro-L-arginine methyl ester (L-NAME, 5 mg kg^{-1}, s.c.) or N^G-monomethyl-L-arginine (L-NMMA, 50 mg kg^{-1}, s.c.) when administered concurrently with LPS, over a 5 h period. LPS (■), LPS with L-NAME (○) or LPS with L-NMMA (▲) were administered at time zero (shown by arrow); albumin leakage (expressed as plasma leakage Δ μl g^{-1} tissue) was determined at each time point in separate groups of rats. Data are shown as means ± s.e.mean of at least 8 experiments for each group, where statistical significant increase from the control time zero value or the control LPS alone group is shown as $\#P<0.05$ or $*P<0.05$, respectively.

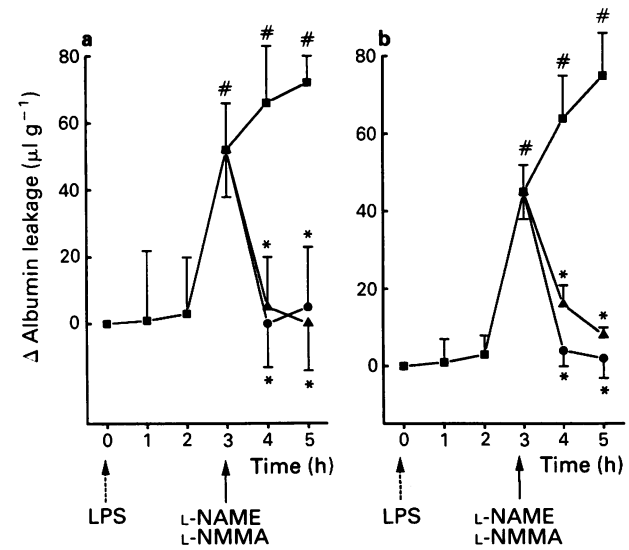


Figure 3 Inhibition of lipopolysaccharide (LPS, 3 mg kg^{-1}, i.v.)-induced albumin leakage in (a) rat ileum and (b) colon by N^G-nitro-L-arginine methyl ester (L-NAME, 5 mg kg^{-1}, s.c.) or N^G-monomethyl-L-arginine (L-NMMA, 50 mg kg^{-1}, s.c.) when administered 3 h after challenge with LPS. LPS (■) was administered at time zero (shown by dotted arrow), whereas L-NAME (●) or L-NMMA (▲) was administered at 3 h (shown by arrow); albumin leakage (expressed as plasma leakage, Δ μl g^{-1} tissue) was determined at each time point in separate groups of rats. Data are shown as the mean ± s.e.mean, where (n) is at least 8 for each group, and where statistical significance is given as $*P<0.05$ compared to LPS groups; $\#P<0.05$ compared to control groups.

treatment, a significant reduction in LPS-induced albumin leakage into the ileum and the colon was found 1 h later (Figure 3).

Likewise, administration of L-NAME (1–5 mg kg^{-1}, s.c.) or L-NMMA (12.5–50 mg kg^{-1}, s.c.) 3 h after LPS challenge produced a dose-dependent reduction in this vascular albumin leakage in both the ileum and the colon, when determined 2 h later (i.e. 5 h after the LPS challenge), the albumin leakage being abolished at the higher dose of either agent (Figures 4 and 5).

Effects of L-arginine on the actions of NO synthase inhibitors

Pretreatment (15 min) with L-arginine (300 mg kg^{-1}, s.c.) abolished the increase in albumin leakage, observed after 1 h, into the ileum and the colon induced by L-NAME (5 mg kg^{-1}, s.c.) or L-NMMA (50 mg kg^{-1}, s.c.), when administered concurrently with LPS challenge, as shown in Figure 6.

The inhibitory effect of L-NAME (5 mg kg^{-1}, s.c.) or L-NMMA (50 mg kg^{-1}, s.c.) on LPS-induced albumin leakage, injected 3 h after the LPS challenge and determined 1 h later, was also abolished by pretreatment with L-arginine (300 mg kg^{-1}, s.c.), as shown in Figure 7. L-Arginine (300 mg kg^{-1}, s.c.) did not itself affect albumin leakage, when administered concurrently with, or 3 h following, LPS treatment (Figures 6 and 7).

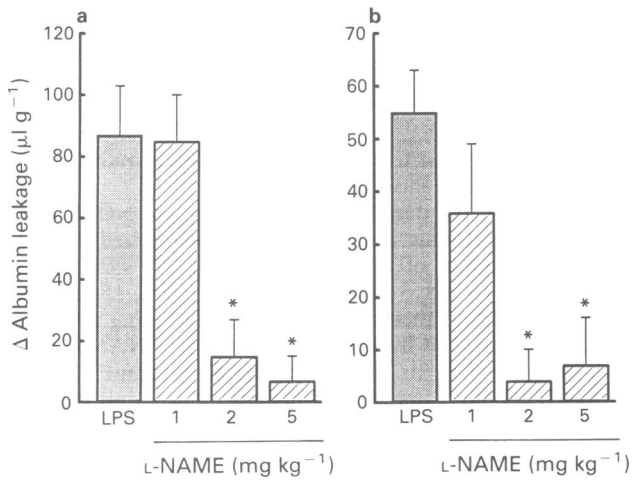


Figure 4 Inhibition of lipopolysaccharide (LPS, 3 mg kg^{-1}, i.v.)-induced albumin leakage in (a) rat ileum and (b) colon by N^G-nitro-L-arginine methyl ester (L-NAME, 5 mg kg^{-1}, s.c.) when administered 3 h after challenge with LPS and determined 2 h later. The columns show leakage of plasma albumin (Δ μl g^{-1} tissue) 5 h after challenge with LPS alone (stippled column) and LPS with L-NAME (hatched columns). Data are shown as the mean ± s.e.mean, where (*n*) is at least 8; statistical significance is shown as $*P<0.05$ compared to the control LPS-alone group.

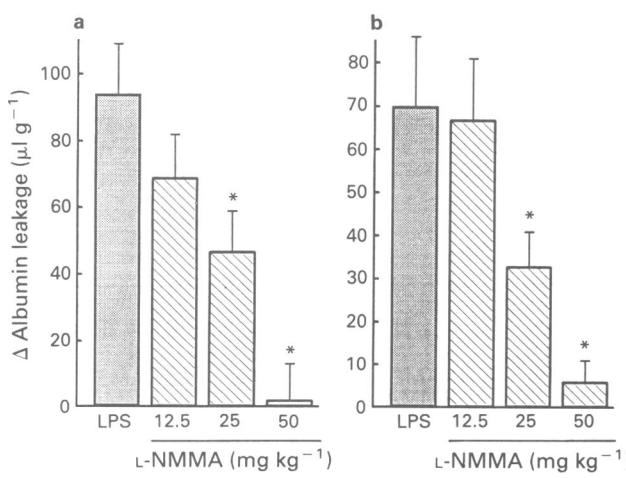


Figure 5 Inhibition of lipopolysaccharide (LPS, 3 mg kg^{-1}, i.v.)-induced albumin leakage in (a) rat ileum and (b) colon by N^G-monomethyl-L-arginine (L-NMMA, 50 mg kg^{-1}, s.c.) when administered 3 h after challenge with LPS and determined 2 h later. The columns show leakage of plasma albumin (Δ μl g^{-1} tissue) 5 h after challenge with LPS alone (stippled column) and LPS with L-NMMA (hatched columns). Data are the mean ± s.e.mean, where (*n*) is at least 8; statistical significance is shown as $*P<0.05$ compared to the control LPS-alone group.

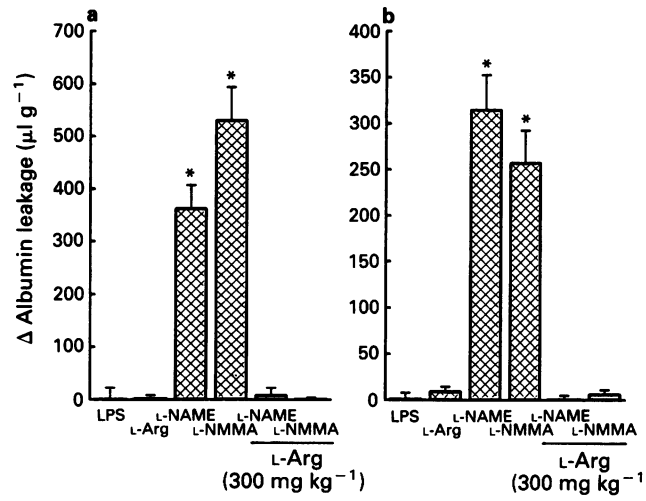


Figure 6 Inhibitory effects of L-arginine (300 mg kg^{-1}, s.c.) on the potentiation of lipopolysaccharide (LPS)-induced (3 mg kg^{-1}, i.v.) albumin leakage (expressed as plasma leakage, Δ μl g^{-1} tissue) in (a) rat ileum and (b) colon by N^G-nitro-L-arginine methyl ester (L-NAME, 5 mg kg^{-1}, s.c.) or N^G-monomethyl-L-arginine (L-NMMA, 50 mg kg^{-1}, s.c.). The NO synthase inhibitors were administered concurrently with LPS challenge, with L-arginine being injected 15 min before challenge. The tissues were removed 1 h after LPS administration. Data are shown as the mean ± s.e.mean, where (*n*) is at least 6, and where statistical significance is given as $*P<0.05$ compared with LPS group.

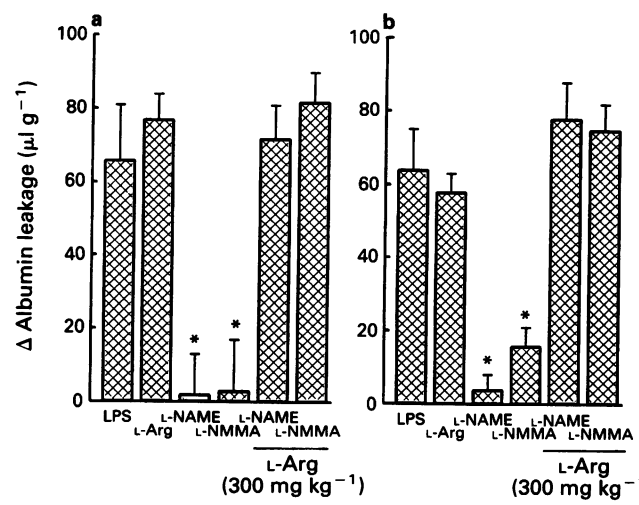


Figure 7 Effects of L-arginine (300 mg kg^{-1}, s.c.) on the inhibition of lipopolysaccharide (LPS)-induced (3 mg kg^{-1}, i.v.) albumin leakage (expressed as plasma leakage Δ μl g^{-1} tissues) in (a) rat ileum and (b) colon by N^G-nitro-L-arginine methyl ester (L-NAME, 5 mg kg^{-1}, s.c.) or N^G-monomethyl-L-arginine (L-NMMA, 50 mg kg^{-1}, s.c.). The NO synthase inhibitors were administered 3 h after LPS challenge, with L-arginine being injected 15 min before the NO synthase inhibitors. The tissues were removed 1 h later (i.e. 4 h after LPS treatment). Data are shown as the mean ± s.e.mean, where (*n*) is at least 6, and where statistical significance is given as $*P<0.05$ compared with LPS group.

Discussion

The findings of this study clearly demonstrate that the time of administration of NO synthase inhibitors has a critical bearing on their actions on microvascular injury in the rat intestine following intravenous challenge with low doses of endotoxin. Thus, L-NMMA and L-NAME may either increase or decrease the vascular injury following endotoxin administration. These divergent actions may reflect the detrimental effects of inhibiting constitutive NO synthase during the early phases of the syndrome following endotoxin challenge, leading to acute vascular injury in the ileum and colon. These effects contrast with the beneficial actions of delayed administration of the NO synthase inhibitors, which would reduce the activity of the inducible NO synthase, during the later phase of endotoxin-induced vascular injury in this model.

In earlier studies in the rat, L-NMMA was shown to exacerbate the acute gastrointestinal vascular injury, as determined by leakage of radiolabelled albumin and histological evaluation, 15 min following challenge with a high dose (50 mg kg^{-1}, i.v.) of endotoxin (Hutcheson *et al.*, 1990). In the current study, a much lower dose of lipopolysaccharide from *E. coli* was used, that itself did not provoke any detectable vascular injury until 2 h after the time of administration. Under these conditions, concurrent administration of L-NMMA produced substantial acute vascular injury, determined as the extravasation of radiolabelled albumin, 1 h after endotoxin challenge, which decreased towards control levels over the ensuing 2–3 h. The subsequent decrease in tissue albumin levels may reflect the recovery of vascular integrity following acute injury, with interstitial albumin being removed by lymphatic drainage and other processes. By contrast to the findings with L-NMMA, the more-potent NO synthase inhibitor L-NAME (Rees *et al.*, 1990) progressively augmented vascular injury over the 3 h period following endotoxin challenge, that subsequently only slowly declined. This may reflect the differences between the distribution, pharmacokinetics and duration of action of the two NO synthase inhibitors, as indeed suggested by the evaluation of their hypertensive actions following subcutaneous administration. Furthermore, this could also reflect the degree of the changes in vascular integrity, which could extend from disruption of continuity of the endothelial barrier for protein flux to extensive endothelial cell injury. In previous studies in the rabbit, the detrimental cardiovascular effects that follow intravenous administration of L-NMMA during the initial 30 min following endotoxin challenge have also been demonstrated (Wright *et al.*, 1992).

Despite the substantial augmentation of the acute vascular injury by L-NMMA and L-NAME when administered at the time of endotoxin challenge, subcutaneous administration of these agents did not themselves affect the intestinal vascular permeability to albumin in the conscious rat over the 5 h period. These present findings thus contrast with the observations in the exposed mesenteric vascular bed of the anaesthetized cat, where local intra-arterial infusion of L-NAME was found to enhance resting vascular permeability (Kubes & Granger, 1992). Apart from a species distinction or route of administration, other differences, such as the use of anaesthesia and surgical trauma following mesenteric cannulation in the cat, may contribute to this apparent discrepancy.

Neutrophil adherence to endothelial cells is now considered as an important component of vascular damage within the gastrointestinal microcirculation, including the mucosal injury induced by haemorrhagic shock or ischaemia-reperfusion, as well as that following the administration of PAF, ethanol or non-steroid anti-inflammatory agents (Smith *et al.*, 1987; Hernandez *et al.*, 1987; Granger *et al.*, 1989; Kubes *et al.*, 1990a,b; Kvietys *et al.*, 1990; Wallace *et al.*, 1990; 1991). Adherence of such inflammatory cells to the endothelium, which is promoted by the expression of adhesion molecules on both neutrophils and endothelial cells (see Springer, 1990; Zimmerman *et al.*, 1992 for review), can lead to the local release of cytotoxic mediators, including oxygen radicals (Grisham & Granger, 1988), known to provoke microvascular injury (Parks & Granger, 1983; Parks *et al.*, 1984). Since NO can attenuate neutrophil adherence to the endothelium (Kubes *et al.*, 1991), inhibition of endogenous NO formation may be anticipated to enhance the adherence of such cells, especially when activated, and indeed L-NAME does promote leukocyte adherence in the cat mesenteric vasculature (Kubes *et al.*, 1991). Endotoxin challenge can lead to the activation of both leukocytes and endothelium and the expression of adhesion receptors (see Springer, 1990; Zimmerman *et al.*, 1992). Such actions may therefore account for the apparent priming of the vasculature by low doses of LPS in the present study, leading to the observed acute detrimental effects of NO synthase inhibitors in the intestinal tissue under these conditions.

Delay of the administration of L-NMMA or L-NAME following challenge with endotoxin, until the time of significant expression of the inducible NO synthase in the intestine (Salter *et al.*, 1991; Boughton-Smith *et al.*, 1993), produced a dose-dependent reduction in the subsequent appearance of vascular damage in the ileum and colon. These findings thus extend and confirm previous preliminary studies in this model, that administration of L-NMMA 3 h after endotoxin challenge could reduce vascular injury in the jejunum and colon (Boughton-Smith *et al.*, 1993). NO synthase inhibitors can also reverse the hypotension and cardiovascular collapse associated with endotoxin shock in the dog and rat (Kilbourn *et al.*, 1990; Thiemermann & Vane, 1990; Nava *et al.*, 1991), as well as in patients with septic shock (Petros *et al.*, 1991). The processes by which excessive NO could produce microvascular injury are not clear, and although the cytotoxic hydroxyl radical, derived from decomposition of the peroxynitrite moiety produced by NO and superoxide, or peroxynitrite itself (Beckman *et al.*, 1990; Lipton *et al.*, 1993), may be involved, evidence for a role of such a reactive species *in vivo* has yet to be demonstrated.

Extravasation of plasma in rat skin induced by pro-inflammatory mediators can be attenuated by NO synthase inhibitors (Hughes *et al.*, 1990; Warren *et al.*, 1992; Ialenti *et al.*, 1992). As in the skin, the reduction in intestinal extravasation of albumin by NO synthase inhibitors could, in part, reflect a local reduction in blood flow within the small and large intestine. Although L-NMMA exerts little action on resting blood flow in the rat jejunum or colon under control conditions (Pique *et al.*, 1992), a reduction in blood flow by L-NMMA is feasible following endotoxin challenge, where excessive vasodilatation may be associated with induction of NO synthase. Comparative studies on local intestinal blood flow and permeability changes with NO synthase inhibitors and directly acting vasoconstrictor agents following endotoxin challenge may shed some light on this possibility, but interpretation is likely to be confounded by the influences of the concurrent various systemic haemodynamic changes produced by these different agents.

Endogenous NO, formed by the constitutive NO synthase, thus appears to play a key role as a protective mediator in the intestinal microvasculature. Such defensive effects may reflect modulation of local blood flow (Whittle *et al.*, 1990; Boughton-Smith *et al.*, 1990) and, possibly, the attenuation of platelet aggregation or adhesion (Radomski *et al.*, 1987a,b) and leukocyte adhesion to the endothelium (Kubes *et al.*, 1991). Clarification of such a physiological role, as well as the involvement of excessive NO formed by the inducible enzyme in the intestinal vascular injury associated with endotoxaemia, will be made easier by the availability of NO synthase inhibitors that demonstrate selectivity between the different isoforms *in vivo*. Such inhibitors, directed towards the inducible NO synthase, would offer obvious potential therapeutic benefits in the management of the adverse vascular events contributing to the pathogenesis of septic shock.

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References

BECKMAN, J.S., BECKMAN, T.W., CHEN, J., MARSHALL, P.A. & FREEMAN, B.A. (1990). Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 1620–1624.

BOUGHTON-SMITH, N.K., EVANS, S.M., LASZLO, F., WHITTLE, B.J.R. & MONCADA, S. (1993). The induction of nitric oxide synthase and intestinal vascular permeability by endotoxin in the rat. *Br. J. Pharmacol.*, **110**, 1189–1195.

BOUGHTON-SMITH, N.K., HUTCHESON, I.R., DEAKIN, A.M., WHITTLE, B.J.R. & MONCADA, S. (1990). Protective effect of S-nitroso-N-acetyl-penicillamine in endotoxin-induced acute intestinal damage in the rat. *Eur. J. Pharmacol.*, **191**, 485–488.

BUSSE, R. & MULSCH, A. (1990). Induction of nitric oxide by cytokines in vascular smooth muscle cells. *FEBS Lett.*, **275**, 87–90.

GRANGER, D.N., BENOIT, J.N., SUZUKI, M. & GRISHAM, M.B. (1989). Leukocyte adherence to venular endothelium during ischemia-reperfusion. *Am. J. Physiol.*, **257**, G683–G688.

GRISHAM, M.B. & GRANGER, D.N. (1988). Neutrophil-mediated mucosal injury: role of reactive oxygen metabolites. *Dig. Dis. Sci.*, **33**, 6S–15S.

HERNANDEZ, L.A., GRISHAM, M.B., TWOHIG, B., ARFORS, K.E., HARLAN, J.M. & GRANGER, D.N. (1987). Role of neutrophils in ischemia/reperfusion-induced microvascular injury. *Am. J. Physiol.*, **253**, H699–H703.

HIBBS, J.B. Jr., TAINTOR, R.R., VAVRIN, Z. & RACHLIN, E.M. (1988). Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.*, **157**, 87–94.

HUGHES, S.R., WILLIAMS, T.J. & BRAIN, S.D. (1990). Evidence that endogenous nitric oxide modulates oedema formation induced by substance P. *Eur. J. Pharmacol.*, **191**, 481–484.

HUTCHESON, I.R., WHITTLE, B.J.R. & BOUGHTON-SMITH, N.K. (1990). Role of nitric oxide in maintaining vascular integrity in endotoxin-induced acute intestinal damage in the rat. *Br. J. Pharmacol.*, **101**, 815–820.

IALENTI, A., IANARO, A., MONCADA, S. & DI ROSA, M. (1992). Modulation of acute inflammation by endogenous nitric oxide. *Eur. J. Pharmacol.*, **211**, 177–182.

KILBOURN, R.G., JUBRAN, A.N., GROSS, S.S., GRIFFITH, O.W., LEVI, R., ADAMS, J. & LODATO, R.F. (1990). Reversal of endotoxin-mediated shock by N^G-methyl-L-arginine, an inhibitor of nitric oxide synthesis. *Biochem. Biophys. Res. Commun.*, **172**, 1132–1138.

KNOWLES, R.G., SALTER, M., BROOKS, S.L. & MONCADA, S. (1990). Anti-inflammatory glucocorticoids inhibit the induction by endotoxin of nitric oxide synthase in the lung, liver and aorta of the rat. *Biochem. Biophys. Res. Commun.*, **172**, 1042–1048.

KUBES, P. & GRANGER, D.N. (1992). Nitric oxide modulates microvascular permeability. *Am. J. Physiol.*, **262**, H611–H615.

KUBES, P., IBBOTSON, G., RUSSELL, J., WALLACE, J.L. & GRANGER, D.N. (1990a). Role of platelet-activating factor in ischemia/reperfusion-induced leukocyte adherence. *Am. J. Physiol.*, **259**, G300–G305.

KUBES, P., SUZUKI, D. & GRANGER, D.N. (1990b). Platelet-activating factor-induced microvascular dysfunction: role of adherent leukocytes. *Am. J. Physiol.*, **258**, G158–G163.

KUBES, P., SUZUKI, M. & GRANGER, D.N. (1991). Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 4651–4655.

KVIETYS, P.R., TWOHIG, B., DANZELL, J. & SPECIAN, R.D. (1990). Ethanol-induced injury to the rat gastric mucosa. Role of neutrophils and xanthine oxidase-derived radicals. *Gastroenterology*, **98**, 909–920.

LASZLO, F., BOUGHTON-SMITH, N.K., WHITTLE, B.J.R. & MONCADA, S. (1993). Potentiation and inhibition of endotoxin-induced vascular injury in rat intestine by nitric oxide synthase inhibitors. *Br. J. Pharmacol.*, **110**, 84P.

LIPTON, S.A., CHOI, J.B., PAN, Z.H., LEI, S.Z., CHEN, J.S.V., SUCHER, N.J., LOSCALZO, J., SINGEL, D.J. & STAMLER, J.S. (1993). A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature*, **364**, 626–632.

MCCALL, T.B., BOUGHTON-SMITH, N.K., PALMER, R.M.J., WHITTLE, B.J.R. & MONCADA, S. (1989). Synthesis of nitric oxide from L-arginine by neutrophils. Release and interaction with superoxide anion. *Biochem. J.*, **261**, 293–296.

MARLETTA, M.A., YOON, P.S., IYENGAR, R., LEAF, C.D. & WISHNOK, J.S. (1988). Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry*, **27**, 8706–8711.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol Rev.*, **43**, 109–141.

NAVA, E., PALMER, R.M.J. & MONCADA, S. (1991). Inhibition of nitric oxide synthesis in septic shock: how much is beneficial? *Lancet*, **338**, 1555–1557.

PALMER, R.M.J., ASHTON, D.S. & MONCADA, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333**, 664–666.

PALMER, R.M.J., BRIDGE, L., FOXWELL, N.A. & MONCADA, S. (1992). The role of nitric oxide in endothelial cell damage and its inhibition by glucocorticoids. *Br. J. Pharmacol.*, **105**, 11–12.

PARKS, D.A. & GRANGER, D.N. (1983). Ischemia-induced vascular changes: role of xanthine oxidase and hydroxyl radicals. *Am. J. Physiol.*, **245**, G285–G289.

PARKS, D.A., SHAH, A.K. & GRANGER, D.N. (1984). Oxygen radicals: effects on intestinal vascular permeability. *Am. J. Physiol.*, **247**, G167–G170.

PETROS, A., BENNETT, D. & VALLANCE, P. (1991). Effect of nitric oxide on hypotension in patients with septic shock. *Lancet*, **338**, 1557–1558.

PIQUE, J.M., PIZCUETA, M.P., BOSCH, J., FERNANDEZ, M., WHITTLE, B.J.R. & MONCADA, S. (1992). Role of nitric oxide in the hyperdynamic splanchnic circulation of portal hypertensive rats. In *Biology of Nitric Oxide. 1. Physiological and Clinical Aspects.* ed. Moncada, S., Marletta, M.A., Hibbs, J.B.Jr. & Higgs, E.A. pp. 60–64. London and Chapel Hill: Portland Press.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1987a). Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet*, **ii**, 1057–1058.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1987b). The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. *Br. J. Pharmacol.*, **92**, 639–646.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1990). Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 10043–10047.

REES, D.D., PALMER, R.M.J., SHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **101**, 746–752.

RIMELE, T.J., STURM, R.J., ADAMS, L.M., HENRY, D.E., HEASLIP, R.J., WEICHMAN, B.M. & GRIMES, D. (1988). Interaction of neutrophils with vascular smooth muscle: identification of a neutrophil-derived relaxing factor. *J. Pharmacol. Exp. Ther.*, **245**, 102–111.

SALTER, M., KNOWLES, R.G. & MONCADA, S. (1991). Widespread tissue distribution, species distribution and changes in activity of Ca^{2+}-dependent and Ca^{2+}-independent nitric oxide synthases. *FEBS Lett.*, **291**, 145–149.

SALVEMINI, D., DE NUCCI, G., GRYGLEWSKI, R.J. & VANE, J.R. (1989). Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 6328–6332.

SCHMIDT, H.H.H.W., SEIFERT, R. & BOHME, E. (1989). Formation and release of nitric oxide from human neutrophil and HL-60 cells induced by chemotactic peptide, platelet activating factor and leukotriene B_4. *FEBS Lett.*, **244**, 357–360.

SMITH, S.M., HOLM-RUTILI, L., PERRY, M.A., GRISHAM, M.B., ARFORS, K.-E., GRANGER, D.N. & KVIETYS, P.R. (1987). Role of neutrophils in hemorrhagic shock-induced gastric mucosal injury in the rat. *Gastroenterology*, **93**, 466–471.

SPRINGER, T.A. (1990). Adhesion receptors of the immune system. *Nature*, **346**, 425–434.

STUEHR, D., GROSS, S., SAKUMA, I., LEVI, R. & NATHAN, C. (1989). Activated murine macrophages secrete a metabolite of arginine with the bioactivity of endothelium-derived relaxing factor and the chemical reactivity of nitric oxide. *J. Exp. Med.*, **169**, 1011–1020.

THIEMERMANN, C. & VANE, J. (1990). Inhibition of nitric oxide synthesis reduces the hypotension induced by bacterial lipopolysaccharides in the rat in vivo. *Eur. J. Pharmacol.*, **182,** 591–595.

WALLACE, J.L., ARFORS, K.-E. & MCKNIGHT, G. (1991). A monoclonal antibody against the CD_{18} leukocyte adhesion molecule prevents indomethacin-induced gastric damage in the rabbit. *Gastroenterology*, **100,** 878–883.

WALLACE, J.L., KEENAN, C.M. & GRANGER, D.N. (1990). Gastric ulceration induced by non-steroidal anti-inflammatory drugs is a neutrophil-dependent process. *Am. J. Physiol.*, **259,** G462–G467.

WARREN, J.B., COUGHLAN, M.L. & WILLIAMS, T.J. (1992). Endotoxin-induced vasodilatation in anaesthetized rat skin involves nitric oxide and prostaglandin synthesis. *Br. J. Pharmacol.*, **106,** 953–957.

WHITTLE, B.J.R., LOPEZ-BELMONTE, J. & MONCADA, S. (1990). Regulation of gastric mucosal integrity by endogenous nitric oxide: interactions with prostanoids and sensory neuropeptides in the rat. *Br. J. Pharmacol.*, **99,** 607–611.

WRIGHT, C.E., REES, D.D. & MONCADA, S. (1992). Protective and pathological roles of nitric oxide in endotoxin shock. *Cardiovasc. Res.*, **26,** 48–57.

WRIGHT, D.E., MULSCH, A., BUSSE, R. & OSSWALD, H. (1989). Generation of nitric oxide by human neutrophils. *Biochem. Biophys. Res. Commun.*, **160,** 813–819.

ZIMMERMAN, G.A., PRESCOTT, S.M. & MCINTYRE, T.M. (1992). Endothelial cell interactions with granulocytes: tethering and signalling molecules. *Immunol. Today*, **13,** 93–100.

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Evidence for a cooperation between adenosine A_2 receptors and β_1-adrenoceptors on cardiac automaticity in the isolated right ventricle of the rat

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1 The effects of the adenosine receptor agonists, 5'-N-ethyl-carboxamidoadenosine (NECA) and 2-[4-(2-carboxyethyl)phenethylamino]-5'-N-ethylcarboxamidoadenosine (CGS-21680) on ventricular automaticity induced by a local injury in the isolated right ventricle of the rat were studied.

2 In concentrations ranging from 0.1 to 100 nM, NECA significantly increased ventricular automaticity. This effect was more reproducible when the adenosine receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) was present at 5 nM, a concentration that blocks A_1 adenosine receptors.

3 The excitatory effect of NECA was not observed when DPCPX was present at a concentration of 10 μM, which antagonizes both A_1 and A_2 adenosine receptors, as well as when rats were reserpinized.

4 In reserpinized rats, NECA increased ventricular automaticity in the presence of isoprenaline and the β_2-adrenoceptor antagonist, ICI-118,551, but not in the presence of the β_1-adrenoceptor antagonists, bisoprolol or atenolol.

5 The A_{2a}-selective adenosine receptor agonist, CGS-21680 (0.1 nM–10 μM) was devoid of excitatory effect on ventricular automaticity. Binding studies of this compound to the rat ventricular membranes revealed that in the preparation there was no specific binding.

6 These results suggest that the excitatory effect of NECA on ectopic ventricular automaticity is dependent on endogenous catecholamines and is mediated by an A_2 adenosine receptor which is in some way 'linked' to the β_1-adrenoceptor. These A_2 receptors do not appear to be of the A_{2a} adenosine receptor subtype.

Keywords: Cardiac automaticity; adenosine receptors; β-adrenoceptors; isolated right ventricle of rat

Introduction

In previous work Hernandez *et al.* (1989) showed that the non selective adenosine receptor agonist, 5'-N-ethyl-carboxamidoadenosine (NECA), increases ventricular automaticity induced by a local injury in the isolated right ventricle of the rat. This effect of NECA was obtained with low concentrations (0.1–10 nM). Adenosine (ADO), in similar concentrations, also causes, as NECA does, an increase in ventricular automaticity. This effect seems to be dependent on the integrity of the β-adrenoceptors because it is prevented by the non selective β-blocker, propranolol (Hernandez *et al.*, 1989).

In the present work we investigated further the nature of the adenosine receptor involved in the excitatory effect of NECA on ectopic ventricular automaticity, and its relation to β-adrenoceptors. To this purpose we studied the effect of NECA, a compound that in rat ventricular myocyte membranes exhibits a typical A_2 agonist effect by stimulating adenylate cyclase activity in the presence of the adenosine receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (see Romano *et al.*, 1989), which, at low concentrations (5 nM) selectively antagonizes A_1 adenosine receptors and at high concentrations (10 μM) antagonizes both A_1 and A_2 adenosine receptors (Bruns *et al.*, 1987; Correia-de-Sá *et al.*, 1991). The subtype of β-adrenoceptor involved in the excitatory effect of NECA was explored by studying the relation between the β agonist isoprenaline and NECA, in the presence of either the β_1 antagonists atenolol (Hoffman & Lefkowitz, 1990) or bisoprolol (Wang *et al.*, 1985) and the highly selective β_2 antagonist ICI-118,551 (Bilski *et al.*, 1983).

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Methods

After Sprague-Dawley rats of either sex had been killed by a blow to the head, the heart was removed and placed in warm Tyrode solution containing (mM): NaCl 136.9, KCl 5, $MgCl_2$ 1.05, $CaCl_2$ 1.8, NaH_2PO_4 0.4, $NaHCO_3$ 11.9 and dextrose 5. The right ventricle was freed as previously described by Hernandez & Serrano (1982); then the atrial end was damaged by crushing it with a pair of Starling forceps, and the ventricle fixed to a metallic support. The apical end was attached to a Grass FT-03 force-displacement transducer by a nylon thread. A 30 ml organ bath was used and the bathing Tyrode solution was maintained at 37°C, pH 7.4 and bubbled with a mixture of 95% O_2 and 5% CO_2.

Contractions were displayed on the screen of a computer by using a Stemtech amplifier (Stemtech Inc., Houston, U.S.A.) and the AT-CODAS computer programme (Dataq Instruments, Ohio, U.S.A.). The resting tension was set at 1 *g*. The preparation was then allowed to stabilize for at least 15 min. Only ventricles having a stable basal rate, with a variation of less than 10 beats min^{-1} and contractile activity at the end of the stabilization period, were used.

After the stabilization period, the drugs were applied and each concentration left 5–10 min, after which a higher concentration of the test drug was applied until a cumulative concentration-response curve had been constructed. Following the application of the last concentration, the preparation was washed until recovering the control (pre-drug) frequency. In the experiments where interactions between drugs were studied, the concentration-response curves were obtained in the presence of a fixed concentration of the other drug, which was applied at least 15 min before starting the cumulative concentration-response curve. In each experiment only one preparation taken from one rat was used.

Recorded data were then processed and the frequency (contraction rate per min) was calculated as the mean of the last

5 min incubation period for each drug concentration, using a BASIC programme developed by us.

Reserpine

In some experiments, rats were reserpinized with 5 mg kg^{-1} intraperitoneal reserpine, given at least 24 h before each experiment.

Binding studies

Preparation of right heart ventricle membranes The membranes were prepared according to Leung *et al.* (1988). Male rats between 200–250 *g* weight were killed by a blow on the head and exsanguinated. The right ventricles were dissected, freed of blood vessels and suspended in 10% (w/v) sucrose containing 0.1 mM phenylmethylsulphonyl fluoride and buffer A (20 mM Tris HCl, pH 7.5, at room temperature (22–25°C), 2 mM EDTA, 0.1 mM dithiothreitol, 0.1 mM benzamidine, 0.1 mg ml^{-1} of bacitracin and 0.01 mg ml^{-1} of soybean trypsin inhibitor). The tissue was homogenized (1 to 4 w/v) using two 5 s bursts with a Ultra-Torrax T25 homogenizer at setting 20500. The homogenate was centrifuged for 40 min at 100,000 *g* at 40°C. The pellet was resuspended in 10% sucrose in buffer A using a 2 s burst in the Ultra-Torrax T25 at setting 20500, layered over a discontinuous sucrose gradient of 15% and 36% sucrose solutions in buffer A and centrifuged at 178,000 *g* for 90 min at a SW 40. The protein based at the 15%–36% sucrose interface was collected with a Pasteur pipette, diluted 5 fold with the buffer A and recentrifuged at 100,000 *g* for 40 min. The pellet was diluted to a protein concentration of 10 mg ml^{-1} with buffer A and stored in liquid nitrogen.

Binding assay Membranes were incubated with adenosine deaminase (ADA) (5 units ml^{-1}) at 22°C. [^{3}H]-CGS-21680 was used as the radioligand. Membranes and [^{3}H]-CGS-21680 with or without the unlabelled competing ligand were incubated in a total volume of 250 µl. Incubations were carried out at 22°C for 90 min on a shaker. Radioligand concentration was 1–300 nM for [^{3}H]-CGS-21680. Incubations were ended by rapid collection of membranes on GF/C glass fibre filters (Whatman GF/C) presoaked with 0.01% CHAPS to reduce nonspecific binding. The filters were washed three times in 10 s with 4 ml aliquots of ice-cold 50 mM Tris HCl buffer, pH 7.5, containing 8 mM $MgCl_2$ and counted in a scintillation cocktail (Cocktail T from BDH). Nonspecific binding of [^{3}H]-CGS-21680 was defined as the amount of radioligand bound in the presence of 2-chloroadenosine (CADO) (20 µM).

Protein assay

Protein contents of membrane preparations were determined according to the method of Peterson (1977) with bovine serum albumin used as standard.

Drugs

The following drugs were used: DPCPX and NECA were purchased from RBI (U.S.A.) ICI-118,551 (erythro-DL-1-(7-methylindan-4-yloxy)-3-isopropyl-aminobutane-2-ol) was generously supplied by ICI Laboratory (U.K.), reserpine atenolol and isoprenaline from Sigma (Spain), CGS-21680 (2-[4-(2-carboxyethyl)phenethyl amino]-5'-N-ethyl-carboxamido adenosine) from Ciba-Geigy Corporation (N.J., U.S.A.), [^{3}H]CGS-21680 with a specific activity of 47.2 Ci $mmol^{-1}$, from Amersham (U.K.) and bisoprolol was kindly supplied by Merck Laboratory (Spain).

Statistics

The significance between means was calculated by Student's paired *t* test (Tallarida & Murray, 1987). *P* values of 0.05 or less were considered to represent significant differences.

Results

We performed a total of 57 experiments in which a spontaneous automaticity appeared, using the technique described above. The mean control ventricular rate before addition of any drug was 30.0 ± 4.3 beats min^{-1}.

NECA

Figure 1 shows an example of the response to addition of NECA (1 nM) to a bathing solution containing a right ventricle with ectopic automaticity. As can be seen, NECA increased the automatic activity of the preparation during the time it was present in the medium. Similar experiments were carried out on eight preparations and the results for the different concentrations of NECA used (0.1–100 nM) are summarized in Figure 2. The effect of NECA was variable with only 8 out of 12 preparations responding. In the reserpinized rats, NECA (0.1–100 nM) did not induce any excitation on ventricular automaticity but instead, it reduced ventricular rate at all concentrations used (Figure 2).

NECA + DPCPX

To investigate the nature of the adenosine receptor involved in the excitatory effect of NECA, concentration-response

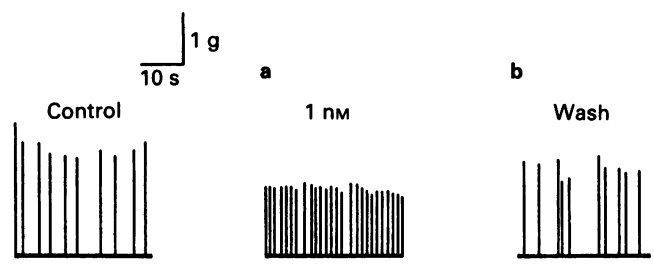


Figure 1 Recorded data from a representative experiment showing the effect of the addition of 1 nM of 5'-N-ethyl-carboxamidoadenosine (NECA) (a) to the organ bath containing an isolated right ventricle of rat with ectopic automaticity. (b) After washing, recovery of the control rate.

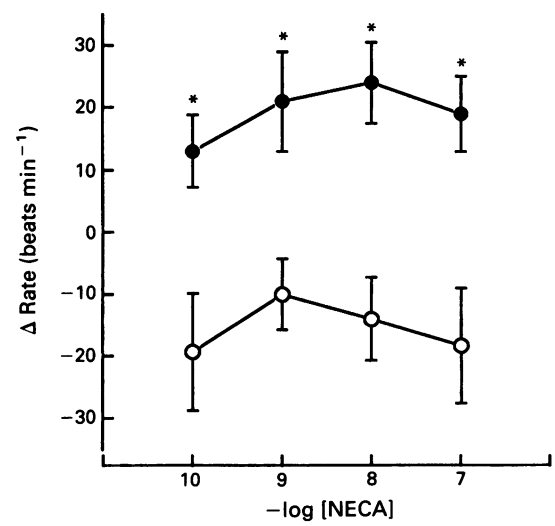


Figure 2 Concentration-response curves for the effect of 5'-N-ethyl-carboxamidoadenosine (NECA) on ventricular automaticity in normal (●) and in reserpinized rats (○; 5 mg kg^{-1}, i.p. reserpine). In each experiment frequency was calculated as the mean of the last 5 min incubation period for each concentration. *Differences from control considered significant for *P* values less than 0.05 (Student's paired *t* test). The average ventricular rate ± s.e.mean during the pre-drug (control) periods was 32.4 ± 6.9 beats min^{-1}. Values are the means ± s.e.mean of the differences from control ($n = 8$).

curves for NECA were obtained in the presence of two different concentrations of the A_1-selective adenosine receptor antagonist, DPCPX. When 5 nM DPCPX was present in the medium, NECA (0.1–100 nM) still caused a consistent excitatory effect on ventricular automaticity in all the preparations tested ($n = 5$). However, a higher concentration of DPCPX (10 μM) blocked this excitatory effect of NECA (Figure 3). DPCPX alone, did not have any effect on ventricular automaticity, at either of the concentrations used.

Reserpinized rats

The experiments aimed to study the interaction between NECA and β-adrenoceptors were performed in reserpinized rats to prevent the effect of endogenous catecholamines. The control (pre-drug) frequency was slightly decreased in reserpinized rats, but not statistically significantly different from non reserpinized rats ($P > 0.05$; normal = 31.3 ± 6.1, reserpinized = 27.2 ± 5.7). To obtain consistent excitatory effects with NECA, this compound was used in the presence of DPCPX (5 nM) to block the influence of A_1 adenosine receptor activation (see above, results from NECA + DPCPX). NECA, either alone or in the presence of DPCPX (5 nM), was devoid of any excitatory effect on ventricular automaticity in reserpinized rats (see Figure 4).

In order to determine the involvement of β_1-adrenoceptor activation in the effect of NECA, concentration-response curves to this ADO analogue were performed in the presence of the non selective β-adrenoceptor agonist, isoprenaline. Isoprenaline (0.1 nM), slightly increased ventricular frequency by 7.7 ± 2.7 beats min^{-1}, compared with the control value ($n = 15$). The selective β_2-adrenoceptor antagonist, ICI-118,551 (0.1 μM) was present in the medium to block β_2-adrenoceptor activation. In these conditions, NECA (0.1–100 μM) produced a marked, concentration-dependent, increase in ventricular frequency. Similar results were obtained for NECA in the presence of isoprenaline without β-blockade. However, the excitatory effect of NECA in the presence of isoprenaline was prevented by the β_1-adrenoceptor antagonists, either the classical atenolol (0.1 μM) or the highly selective bisoprolol (10 nM), Figure 4.

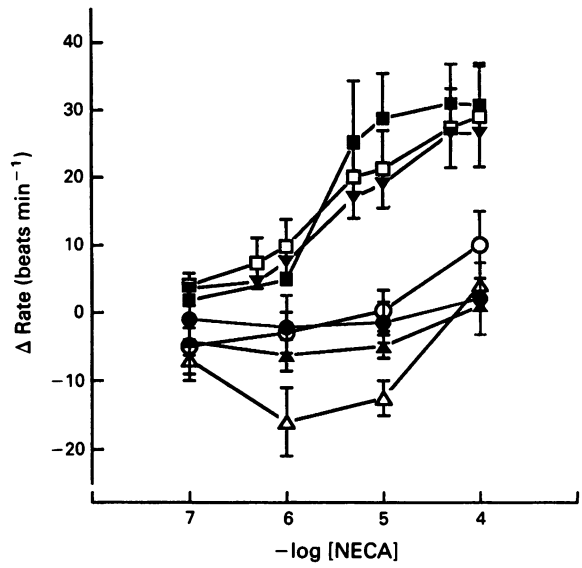


Figure 4 Interactions between adenosine receptors and β-adrenoceptors on ventricular automaticity in reserpinized rats (5 mg kg^{-1}, i.p. reserpine). Isoprenaline (0.1 nM) was added to the organ bath and in the experiments with the β-adrenoceptor antagonists, ICI-118,551 (0.1 μM, ■), atenolol (0.1 μM, ●), or bisoprolol (10 nM, ○), were added after a 5–10 min stabilization period. After a stable rate was reached in the presence of the β-adrenoceptor antagonists (10–15 min), concentration-response curves for 5'-N-ethylcarboxamidoadenosine (NECA) were performed as described in Figure 2 and in the presence of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 5 nM) to obtain consistent excitatory effects. In the absence of these selective β-adrenoceptor blockers, NECA still produced excitatory effect in the presence of isoprenaline both in the absence (▼) or the in the presence (□) of DPCPX (5 nM), (△) NECA alone, and (▲) NECA + DPCPX (5 nM). The average ventricular rate ± s.e.mean during the pre-drug period was 27.1 ± 6.7. Isoprenaline, slightly increased ventricular frequency by 7.7 ± 2.7 beats min^{-1}. Values are the means ± s.e.mean ($n = 4–7$). The effects of NECA in the presence of isoprenaline, in the absence or in the presence of DPCPX and ICI-118,551 were statistically significantly different ($P < 0.05$) from 0 for concentrations higher than 1 μM.

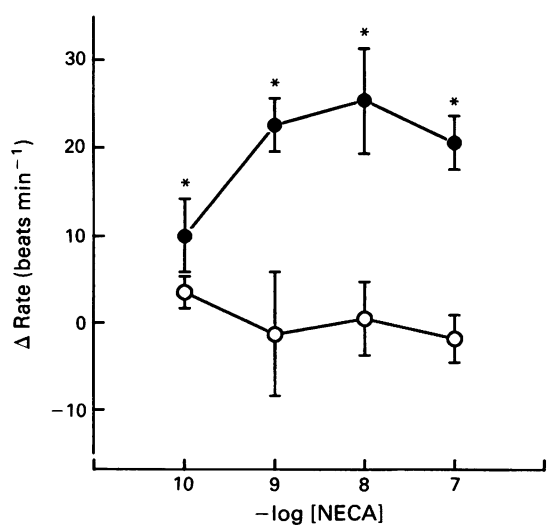


Figure 3 Effects of the adenosine receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) on ventricular frequency responses to 5'-N-ethyl-carboxamidoadenosine (NECA) (see legend to Figure 2 for details). DPCPX, in concentrations of 5 nM (●) and 10 μM (○) was added to the organ bath 5–10 min before starting the addition of NECA and was present throughout the experiments. The average ventricular rate ± s.e.mean during the pre-drug periods was 27.1 ± 6.7 beats min^{-1}. Values are the means ± s.e.mean ($n = 5$). *$P < 0.05$.

Effects of isoprenaline

The excitatory effect of isoprenaline was studied in five experiments in reserpinized rats. Isoprenaline (0.1–10 nM) increased ventricular automaticity in a concentration-dependent manner. Applied in the presence of the adenosine receptor antagonist, DPCPX (10 μM), isoprenaline (0.1–10 nM) consistently increased ventricular automaticity in approximately the same proportion as that observed when used alone. These results suggest that DPCPX does not block β-adrenoceptors.

CGS-21680

The effect of the A_{2a}-selective adenosine receptor agonist, CGS-21680 (0.1 nM–10 μM) was studied in four experiments, in normal rats and no detectable excitatory effect was observed. This suggests that in this preparation there are no functional A_{2a} adenosine receptors. However, as NECA was active in high concentrations in reserpinized rats, it is possible that the A_2 adenosine receptor in the right ventricle is an A_{2b} subtype.

CGS-21680 binding

[^{3}H]-CGS-21680 was used in concentrations between 1–300 nM. No saturation was achieved for this range of concentrations and no marked differences were obtained between total and specific binding for CGS-21680. CADO (20 μM) was used as the displacer.

These results suggest that CGS-21680 does not specifically bind to the rat heart ventricle membranes. As this compound binds with high affinity to A_{2a} adenosine receptors, the results indicate absence of these receptors in the rat ventricle.

Discussion

The present results show that NECA, in concentrations ranging from 0.1 to 100 nM, increased ventricular automaticity in the rat heart. This effect was blocked by the adenosine receptor antagonist, DPCPX in a concentration (10 μM) that blocks both A_1 and A_2 adenosine receptors (see Bruns *et al.*, 1987).

The A_{2a}-selective agonist, CGS-21680, was virtually devoid of excitatory effect and in binding studies no specific binding to right ventricle membranes was detected. These results preclude the existence of A_{2a} adenosine receptors in this tissue in the rat.

Adenosine and adenosine analogues have been considered cardiodepressant agents because of their negative chronotropic and inotropic effects (Drury & Szent-Gyorgy, 1929). However, some cardioexcitatory effects such as positive chronotropic effects as well as positive inotropic effects have been described (Burnstock & Meghji, 1983; Watt & Routledge, 1986). Recently it has been shown that both adenosine (La Orden *et al.*, 1986) and its stable analogue, NECA (Hernandez *et al.*, 1989), increased cardiac automaticity in the isolated right ventricle of the rat. This effect was further confirmed in the present study and agrees with the fact that intracoronary administration of adenosine increases the number of single extrasystoles and salvos, resulting in an augmented ventricular ectopic activity following coronary artery occlusion in dogs (Wainwright & Parratt, 1988). Adenosine also seems to increase cardiac automaticity when used for therapeutic purposes. In fact, it has been reported that there is an incidence of about 40% of adenosine-induced ventricular premature complexes or non-sustained ventricular tachycardia in patients immediately upon termination of paroxysmal supraventricular tachycardia (Garratt *et al.*, 1989; Rankin *et al.*, 1990).

The excitatory effect of NECA in the present work was blocked by high (10 μM) but not by low (5 nM) concentrations of the A_1-selective adenosine receptor antagonist, DPCPX. It is known that the K_i of this antagonist for the A_1 adenosine binding site is about 1 nM, and for the A_2 adenosine binding site is about 340 nM (Bruns *et al.*, 1987). So, the excitatory effect of NECA was prevented when both A_1 and A_2 receptors were blocked, but not when only A_1 adenosine receptors were blocked by 5 nM DPCPX. Thus, it appears that the excitatory effect of NECA on ventricular automaticity is mainly mediated by A_2 adenosine receptor activation. This agrees with previous results on ventricular automaticity in which NECA was shown to be more potent than CADO, with **R**-PIA being ineffective, and in relation to the inhibitory effect, **R**-PIA was about equipotent with CADO, both being more potent than NECA (Hernandez *et al.*, 1989). It has been suggested (Xu *et al.*, 1992) that the cardioinhibitory effect of the A_1 adenosine receptor is counteracted by the cardioexcitatory effect mediated by A_2 adenosine receptors. Thus, when A_1 receptors are blocked, the excitatory effect of adenosine and adenosine analogues become more evident. This could explain why NECA did not increase cardiac automaticity in some preparations, but caused consistent excitatory effects in the presence of 5 nM DPCPX. Romano *et al.* (1989), found that NECA in the same concentrations (0.1–10 μM) as those used in the present work, stimulates adenylate cyclase activity in the rat ventricular myocyte membranes (a typical A_2 adenosine effect), only when the A_1 receptor is blocked with DPCPX.

A point of interest in the present results is that the excitatory effect of NECA on ventricular automaticity was not apparent when rats were reserpinized. This agrees with previous observations in which the excitatory effect of adenosine on ventricular automaticity was absent in reserpinized rats, suggesting the involvement of catecholamines (Hernandez *et al.*, 1989).

In reserpinized rats, the excitatory effect of NECA on ventricular automaticity could be shown in the presence of the β-agonist, isoprenaline along with the β_2 antagonist, ICI-118,551, but not when β_1-adrenoceptors were blocked by either bisoprolol or atenolol. These results suggest that the effect of NECA depends on the functional integrity of the β_1 subtype adrenoceptors. This was to be expected since the actions of catecholamines on the heart seem to be mainly mediated by the β_1-adrenoceptors. In fact, by using radioligand binding techniques, Hancock *et al.* (1979) found that the rat ventricle contains exclusively β_1-adrenoceptors, whereas Minneman *et al.* (1979) suggested that the rat heart contains 83% β_1 and 17% $\beta_2$receptors. An approximate 2:1 ratio was found by Juberg *et al.* (1985). These authors suggested that β_1- and β_2-adrenoceptors coexist in both left and right rat atria, but only β_1-adrenoceptors mediate the chronotropic and inotropic effects of β-adrenoceptor agonists. However, the presence of β_2-receptors in the rat heart has also been established (Freyss-Beguin *et al.*, 1983) and it has been suggested that they might play a more important role in regulating heart frequency than force of contraction. In fact, it has been demonstrated that ICI-118,551 antagonized the isoprenaline-induced tachycardia much more efficiently than the β_1-selective antagonist, atenolol (Arnold *et al.*, 1985; Zerkowski *et al.*, 1986). However, this kind of receptor does not seem to play any significant role in the excitatory effect of NECA observed in the present work.

The results in our study indicate that NECA could activate a given system (receptor) that sensitizes the heart for the action of β_1-agonists. It is interesting to note that the excitatory effect of NECA, in reserpinized rats, in the presence of isoprenaline and ICI-118, 551, was obtained with concentrations higher than 1 μM. However, in normal rats or in reserpinized rats in the presence of noradrenaline, this effect was produced by concentrations around 1 nM (Hernandez *et al.*, 1989). This suggests that, in addition to the β_1-adrenoceptor activation, other mechanisms could also be involved in the effect of NECA.

The interaction between β_1 and A_2 receptors could occur at the level of the cascade process which leads to the formation of the second messenger, adenosine 3′:5′-cyclic monophosphate (cyclic AMP). In fact, activation of both A_2 (Van Calker *et al.*, 1979) and β_1 (Brodde *et al.*, 1984) receptors results in an increase of the intracellular levels of this nucleotide. This kind of interaction has been demonstrated in the central nervous system, where adenosine applied together with noradrenaline, produces a potentiating action of cyclic AMP accumulation in brain slices (Sattin & Rall, 1970). This is also in agreement with the ability of the non metabolizable cyclic AMP analogue, dibutyryl cyclic AMP to restore the excitatory effect of adenosine on ventricular automaticity in reserpinized rats (Hernandez *et al.*, 1989).

In summary, the adenosine receptor agonist NECA, increases ventricular frequency and this effect is more reproducible when A_1 adenosine receptors are blocked. This effect seems to be dependent on the functional integrity of the β_1-adrenoceptors because it was observed only when these receptors were activated. Therefore, the excitatory effect of NECA on ectopic ventricular automaticity may be mediated by an A_2 adenosine receptor, and seems to be dependent on the activation of an adrenoceptor of the β_1 subtype.

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References

ARNOLD, J.M.O., O'CONNOR, P.C., RIDDELL, J.G., HARRON, D.W.G., SHANKS, R.G. & McDEVITT, D.G. (1985). Effects of the beta-2 adrenoceptor antagonist ICI-118,551 on exercise tachycardia and isoprenaline-induced α-adrenoceptor responses in man. *Br. J. Clin. Pharmacol.*, **19**, 619–630.

BILSKI, A.J., HALLIDAY, S.E., FITZGERALD, J.D. & KALE, J.L. (1983). The pharmacology of a beta-2 selective adrenoceptor antagonist (ICI-118,551). *J. Cardiovasc. Pharmacol.*, **5**, 430–437.

BRODDE, O.E., O'HARA, N., ZERKOWSKY, H.R. & ROHM, N. (1984). Human cardiac β-adrenoceptors: both β_1- and β_2-adrenoceptors are functionally coupled to adenylate cyclase in right atrium. *J. Cardiovasc. Pharmacol.*, **6**, 1184–1191.

BRUNS, R.F., FERGUS, J.H., BADGER, E.W., BRISTOL, J.A., SANTAY, L.A., HARTMANN, J.D., HAYS, S.J. & HUANG, C.C. (1987). Binding of the A_1-selective adenosine antagonist β-cyclo-1,3-dipropylxanthine to rat brain membranes. *Naunyn Schmied. Arch. Pharmacol.*, **335**, 59–63.

BURNSTOCK, G. & MEGHJI, P. (1983). The effect of adenyl compounds on the rat heart. *Br. J. Pharmacol.*, **79**, 211–218.

CORREIA-DE-SÁ, P., SEBASTIÃO, A.M. & RIBEIRO, J.A. (1991). Inhibitory and excitatory effects of adenosine receptor agonists on evoked transmitter release from phrenic nerve endings of the rat. *Br. J. Pharmacol.*, **103**, 1614–1620.

DRURY, A.M. & SZENT-GYORGY, A. (1929). The physiological activity of adenine compounds with special references to their action on the mammalian heart. *J. Physiol.*, **68**, 213–237.

FREYSS-BEGUIN, M., GRIFFATON, G., LECHAT, P., PICKEN, D., QUENNEDY, M.C., ROUOT, B. & SCHWARTZ, J. (1983). Comparison of the chronotropic effect and the cyclic-AMP accumulation induced by β_2-agonists in rat heart cell culture. *Br. J. Pharmacol.*, **78**, 717–723.

GARRATT, C., LINKEN, N., GRIFFITH, M., WARD, D. & CAMM, J. (1989). Comparison of adenosine and verapamil for termination of paroxysmal ventricular tachycardia. *Am. J. Cardiol.*, **64**, 1310–1316.

HANCOCK, A.A., DE LEAN, A.L. & LEFTKOWITZ, R.J. (1979). Quantitative resolution of beta-adrenergic receptor subtypes by selective ligand binding: application of a computerized model fitting technique. *Mol. Pharmacol.*, **16**, 1–9.

HERNANDEZ, J. & SERRANO, J.S. (1982). Experimental automaticity induced by mechanical lesion in the isolated rat ventricle: the effects of quinidine, phenytoin and propranolol. *J. Pharmacol. Meth.*, **7**, 255–261.

HERNANDEZ, J., LA ORDEN, M.L., RUIZ, F. & RIBEIRO, J.A. (1989). Effects of adenosine and its analogues on ventricular automaticity induced by a local injury: role of catecholamines and of cyclic-AMP. *Arch. Int. Pharmacodyn.*, **297**, 49–59.

HOFFMAN, B.B. & LEFKOWITZ, R.J. (1990). Adrenergic receptor subtypes. In *The Pharmacological Basis of Therapeutics* ed. Gilman, A.G., Rall, T.W., Nies, A.S. & Taylor, P. 8th ed. pp. 221–243. New York: Pergamon Press Inc.

JUBERG, E.N., MINNEMAN, K.P. & ABEL, P.W. (1985). β_1- and β_2-adrenoceptor binding and functional response in right and left atria of rat heart. *Naunyn-Schmied. Arch. Pharmacol.*, **330**, 193–202.

LA ORDEN, M.L., HERNANDEZ, J. & RIBEIRO, J.A. (1986). The effects of adenosine, ATP and ADP on ventricular automaticity induced by a local injury in the isolated right ventricle of the rat. *Arch. Int. Pharmacodyn.*, **279**, 258–267.

LEUNG, E., KWATRA, M.M., HOSEY, M.M. & GREEN, R.D. (1988). Characterization of cardiac A-1 adenosine receptors by ligand binding and photoaffinity labeling. *J. Pharmacol. Exp. Ther.*, **244**, 1150–1156.

MINNEMAN, K.P., HEGSTRAND, L.R. & MOLINOFF, P.B. (1979). Simultaneous determination of beta-1 and beta-2 adrenergic receptors in tissues containing both receptor subtypes. *Mol. Pharmacol.*, **16**, 34–46.

PETERSON, G.L. (1977). A simplification of the protein assay method of Lowry *et al.*, which is more generally applicable. *Anal. Biochem.*, **83**, 346–356.

RANKIN, A.C., OLDROYD, K.G., CHONG, E., DOW, J.W., RAE, A.P. & COBBE, S.M. (1990). Adenosine or adenosine triphosphate for supraventricular tachycardias? Comparative double-blind randomized study in patients with spontaneous or induced arrhythmias. *Am. Heart J.*, **119**, 316–323.

ROMANO, F.D., MACDONALD, S.G. & DOBSON, J.G. Jr. (1989). Adenosine receptor coupling to adenylate cyclase of rat ventricular myocyte membranes. *Am. J. Physiol.*, **257**, H1088–H1095.

SATTIN, A. & RALL, T.W. (1970). The effect of adenosine and adenine nucleotides on the cyclic adenosine 3',5'-phosphate content of guinea-pig cerebral cortical slices. *Mol. Pharmacol.*, **6**, 13–23.

TALLARIDA, R.J. & MURRAY, R.B. (1987). *Manual of pharmacologic calculations with computer program.* New York: Springer-Verlag.

VAN CALKER, D., MULLER, M. & HAMPRECHT, B. (1979). Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J. Neurochem.*, **33**, 999–1005.

WAINWRIGHT, C.L. & PARRATT, J.R. (1988). An antiarrhythmic effect of adenosine during myocardial ischaemia and reperfusion. *Eur. J. Pharmacol.*, **145**, 183–194.

WANG, X.L., BRINKMANN, M. & BRODE, O.E. (1985). Selective labeling of β_1-adrenoceptors in rabbit lung membranes by (−)-^{3}H-bisoprolol. *Eur. J. Pharmacol.*, **114**, 157–165.

WATT, A.H. & ROUTLEDGE, P.A. (1986). Transient bradycardia and subsequent sinus tachycardia produced by intravenous adenosine in healthy adult subjects. *Br. J. Clin. Pharmacol.*, **21**, 533–536.

XU, D., KONG, H. & LIANG, B.T. (1992). Expression and pharmacological characterization of a stimulant subtype of adenosine receptor in fetal chick ventricular myocytes. *Circ. Res.*, **70**, 56–65.

ZERKOWSKI, H.R., IKEZONO, K., ROHM, N., REIDEMEISTER, J. CHR. & BRODDE, O.E. (1986). Human myocardial β-adrenoceptors: demonstration of both β_1- and β_2-adrenoceptors mediating contractile reponses to β-agonists on the isolated right atrium. *Naunyn-Schmied. Arch. Pharmacol.*, **332**, 142–147.

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Differential effects of ω-conotoxin GVIA and tetrodotoxin on vasoconstrictions evoked by electrical stimulation and nicotinic receptor stimulation in canine isolated, perfused splenic arteries

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1 The effects of ω-conotoxin GVIA (ω-CgTX) and tetrodotoxin (TTX) on vasoconstrictions induced by acetylcholine (ACh) and nicotine were investigated and compared with those induced by periarterial electrical stimulation in the isolated and perfused canine splenic arteries.

2 ACh and nicotine at doses of 0.01 to 1 μmol constricted the splenic artery, dose-dependently. ACh induced consistent responses, but the vasoconstrictor responses to nicotine became significantly smaller with repeated administration of nicotine.

3 Periarterial electrical stimulation produced a vasoconstriction that was abolished by either TTX (30 nmol) or ω-CgTX (3 nmol), but the vasoconstrictor response to nicotine was not significantly affected by the same doses of TTX and ω-CgTX. Inhibitions by TTX and ω-CgTX of ACh-induced vasoconstrictions were small but statistically significant, showing that the percentage inhibition was less than 15%. TTX and ω-CgTX did not affect the vasoconstrictor responses to exogenous noradrenaline (NA).

4 ACh did not produce any vasoconstriction in the preparations treated either with α-adrenoceptor antagonists (10 μM bunazosin and 10 μM midaglizole) or with 30 μM guanethidine. NA-induced responses were abolished by α-adrenoceptor antagonists, but not affected by guanethidine treatment.

5 Vascular responses to ACh were completely inhibited by 1 μmol hexamethonium. In the preparations treated with 100 nmol nicotine, ACh did not produce any vasoconstriction. However, the NA-induced vasoconstriction was affected by neither hexamethonium nor nicotine treatment.

6 Atropine (1 μM) significantly inhibited but did not abolish the vasoconstrictor responses to ACh. The vascular responses to nicotine and NA were also significantly inhibited by atropine treatment.

7 These results indicate that (1) ACh constricts the splenic artery through the activation of presynaptic nicotinic receptors present on the sympathetic nerves; (2) differential effects of TTX and ω-CgTX on the vascular responses to ACh and nicotine, and to electrical stimulation suggest that the receptor-operated ion channels are mainly responsible for NA release induced by nicotinic receptor stimulation, but N-type VOCCs are responsible for that by electrical stimulation; (3) atropine may have an inhibitory action on nicotine-related responses, in addition to its inhibitory action on NA.

Keywords: Sympathetic nervous system; presynaptic nicotinic receptor; electrical stimulation; acetylcholine; ω-conotoxin GVIA; receptor-operated ion channels; canine splenic artery; vasoconstriction

Introduction

Voltage-operated calcium channels (VOCCs) have been subdivided into L-, N- and T-types on the basis of differences in unitary barium conductance and gating properties (Nowycky *et al.*, 1985; Miller, 1987). ω-Conotoxin GVIA (ω *Conus geographus* toxin, ω-CgTX), a 27-amino acid peptide purified from a fish-hunting snail *Conus geographus* (Kerr & Yoshikami, 1984) blocks neuronal N- and L-type VOCCs, but not non-neuronal L-type VOCCs in patch-clamp electrophysiological and biochemical studies (McCleskey *et al.*, 1986; 1987; Cruz *et al.*, 1987). ω-CgTX inhibits physiological responses and the release of noradrenaline (NA) in response to electrical stimulation in the tissues innervated by adrenergic nerves (Mohy El-Din & Malik, 1988; Clasbrummel *et al.*, 1989; McKnight *et al.*, 1989; De Luca *et al.*, 1990), indicating that neuronal N-type VOCCs participate in NA release induced by electrical stimulation in the peripheral adrenergic nervous system. Recently, P-type VOCCs have been proposed, but they have been found to be present only in the brain areas to date (Llinas *et al.*, 1989; Hillman *et al.*, 1991).

On the other hand, it is well-known that nicotinic receptors are present on adrenergic nerve terminals in sympathetic effector organs, and activation of these receptors elicits a release of NA (Muscholl, 1970). There may be two modes of nicotine-induced NA release, sodium action potential-dependent and potential-independent modes, in many tissues including the heart and arteries. The former but not latter mode is sensitive to tetrodotoxin (TTX), a neuronal sodium channel inhibitor, which is effective against electrical stimulation-induced functional responses and transmitter release (Bell, 1968; Westfall & Brasted, 1972; Jayasundar & Vohra, 1978; Ren *et al.*, 1991). Action potentials induce nerve terminal membrane depolarization after which the calcium ion enters from the extracellular space through the opening calcium channels (Illes, 1986). The requirement of calcium ions for a nicotine-induced NA release is generally accepted (Jayasundar & Vohra, 1977; Starke & Weitzell, 1978; Kirpekar *et al.*, 1980; Abajo *et al.*, 1989). However, the information about the characterization of calcium channels in nicotine-induced functional responses and NA release is not known with any degree of certainty. Haass *et al.* (1990; 1991) reported that the release of NA evoked by nicotine or electrical stimulation in the guinea-pig isolated heart was dependent on calcium influx through N-type VOCCs. In contrast, Lou *et al.* (1992) reported that TTX and ω-CgTX did not affect nicotine-induced bronchoconstriction and nerve stimulation but inhibited electrical stimulation-induced bronchoconstriction, in the guinea-pig lung preparation.

Even though the sympathetic innervation in arteries is important to maintain vascular resistance and nicotine can evoke a neurogenic vasoconstriction (Su & Bevan, 1970; Ikushima *et al.*, 1982; Muramatsu *et al.*, 1984), there is no

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available information on the modification by ω-CgTX of nicotine-induced vasoconstriction in vasculature. In the present study, therefore, we tried to investigate whether ω-CgTX and TTX inhibited differently the vasoconstrictor responses to periarterial sympathetic nerve stimulation and to nicotinic receptor stimulation, using the cannula insertion method (Hongo & Chiba, 1983; Tsuji & Chiba, 1984) in the canine isolated splenic artery.

Methods

Arterial preparations

After treatment with an overdose of barbiturate (sodium pentobarbitone 65 mg kg^{-1}, i.v.) and sodium heparin (200 units kg^{-1}, i.v.), the spleen with the splenic artery was isolated from mongrel dogs (8–15 kg) of either sex, and immersed in cold Krebs-Henseleit solution (4°C). Many branches of the splenic artery supply blood to the spleen, pancreas and stomach but only the arterial branches running into the spleen were used in the present experiments. The arteries were carefully isolated and cleaned of loose adipose and connective tissue. All side branches of the artery were tied with silk threads. The arteries (0.8–1.6 mm in outer diameter) were cut into segments 15–20 mm in length, and each segment was cannulated and set up for perfusion as described previously (Hongo & Chiba, 1983; Tsuji & Chiba, 1984). Briefly, a 3–4 cm long stainless steel cannula (0.6–1.4 mm in outer diameter) with small holes 5 mm from the distal sealed end was inserted into the arterial segment from the distal to the proximal end, and a proximal portion of the segment was fixed at the distal portion of the needle with silk threads. The isolated, cannulated artery was placed in a cup-shaped glass bath, and was perfused by a peristaltic pump (Tokyo Rikakikai) with Krebs-Henseleit solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 10, gassed with 95% O_2 and 5% CO_2. The flow rate was kept at about 1 ml min^{-1}. Perfusion pressure was measured with an electric manometer (Nihon Kohden, MPU-0.5A) and recorded by a rectigraph (Nihon Kohden, WT-685G). In order to observe nicotinic receptor stimulation-induced vascular responses we modified the method as follows. After an initial increase at the beginning of perfusion, the perfusion pressure gradually decreased and reached a stable baseline (20–50 mmHg); then the preparation was moved from the bath-solution and fixed in a vertical position to keep its adventitial surface superperfused with the outflow of Krebs-Henseleit solution at the same time. The organ bath was sealed with plastic film to maintain the preparation at 37°C by a thermostat pump (Haake FJ). A stabilization period of 60 min was allowed before any experimental procedures were carried out.

In the experiments on periarterial nerve stimulation, electrical stimulation was applied to periarterial nerves with two platinum wires, 0.5 mm in diameter. The platinum wires, which were coated with enamel, except for the cut point which was gently placed on the extraluminal side of the artery wall, served as a pair of stimulating electrodes (Chiba & Tsukada, 1985). Electrical stimuli of 1 ms pulse duration and an amplitude of 10 V was used.

Agonists were administered by a microsyringe (Ito Co.) into the rubber tube connecting the cannula in a volume of 0.01–0.03 ml for 4 s. Antagonists were dissolved in the physiological perfusion solution except where mentioned. If the preparation was perfused with one or more antagonists, a perfusion period of 60 min was needed before administration of the agonist.

Drugs

Drugs used were: noradrenaline hydrochloride (NA, Sankyo, Tokyo, Japan); acetylcholine chloride (ACh, Daiichi Pharmaceutical Co., Tokyo, Japan); disodium adenosine triphosphate (ATP, Sigma, St. Louis, U.S.A.); nicotine bitartrate (Tokyo Kasei Kogyo, Tokyo, Japan); tetrodotoxin (TTX, Sigma); guanethidine sulphate (Sigma); ω-conotoxin GVIA (ω-CgTX, Sigma); atropine sulphate (Wako Pure Chemical Ind., Osaka, Japan); bunazosin hydrochloride (Eisai, Tokyo, Japan); midaglizole (DG-5128, 2-[2-(4,5-dihydro-1H-imidazol-2-yl)-1-phenylethyl] pyridine dihydrochloride sesquihydrate, Daiichi Pharmaceutical Co.) and hexamethonium bromide (Yamanouchi, Tokyo, Japan). ω-CgTX was dissolved in 0.5% (w/v) bovine serum albumin in distilled water. Other drugs were dissolved in physiological saline before the start of the experiment.

Statistical analysis

Vascular responses to drugs and electrical stimulation were expressed as changes in perfusion pressure (mmHg) from their control levels. Values presented here are the mean ± s.e.mean. An analysis of variance was used to evaluate the data. If the F statistic was significant, we compared the individual datum with its respective control value by simultaneous multiple comparisons, Scheffé's method (Wallenstein et al., 1980). P values less than 0.05 were considered statistically significant.

Results

Vascular responses to nicotine and ACh

Nicotine and ACh were administered by bolus injections at intervals of more than 20 min to avoid tachyphylaxis. Nicotine (0.01–1 μmol) when applied to isolated arterial preparations produced concentration-dependent vasoconstrictions, but the vasoconstrictor response to increased concentrations (3 μmol) was significantly smaller than that elicited by 1 μmol nicotine (Figure 1). ACh (0.01–3 μmol) induced concentration-related vasoconstrictions, and the maximal response to ACh occurred at 1 μmol (Figure 1).

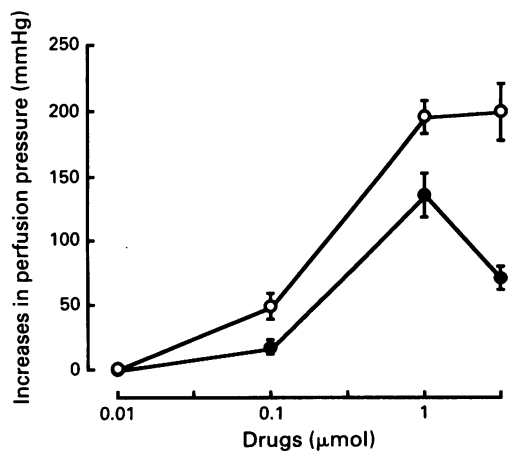


Figure 1 Dose-response curves for acetylcholine (○) and nicotine (●) in the isolated and perfused splenic arteries of the dog. Points represent the mean values with s.e.mean, $n = 6–8$.

When four bolus injections of 1 μmol of nicotine or ACh were given at intervals of more than 20 min in 10 preparations, vasoconstrictor responses to repeated administration of ACh were not significantly different but responses to repeated administration of nicotine decreased significantly by 167 ± 38 (mmHg, the first time), 134 ± 37 (the second time), 115 ± 33 (the third time) and 104 ± 28 (the fourth time). Because the statistical analysis did not show significant differences between the responses to the second and the third administration of 1 μmol nicotine, we observed the effects of

antagonists between the second and third administration of nicotine in the following experiments.

Effects of TTX and ω-CgTX on the vasoconstrictor responses to electrical stimulation, nicotine and ACh

Vasoconstrictor responses to periarterial electrical stimulation with 1 ms pulse duration, 10 V and 10 Hz for 100 pulses were abolished by a bolus injection of TTX at a concentration of 10 or 30 nmol, but the response to 0.3 nmol NA was not affected by the same treatment with TTX (Figure 2a). ω-CgTX (0.3–3 nmol) inhibited the vasoconstriction induced by electrical stimulation in a concentration-related manner, but it did not affect the response to exogenous NA (Figure 2b).

Figure 3 shows the effects of 30 nmol TTX and 3 nmol ω-CgTX on nicotine-induced vasoconstrictions. Vasoconstrictor responses to 1 μmol nicotine were not significantly changed by treatment with TTX or ω-CgTX. The vasoconstriction induced by 1 μmol ACh was unaffected by 10 nmol TTX, but was significantly inhibited (14.1%) by 30 nmol TTX (Figure 4a). Similarly, 3 nmol ω-CgTX slightly but significantly inhibited the vascular response to 1 μmol ACh by 13.7% (Figure 4b).

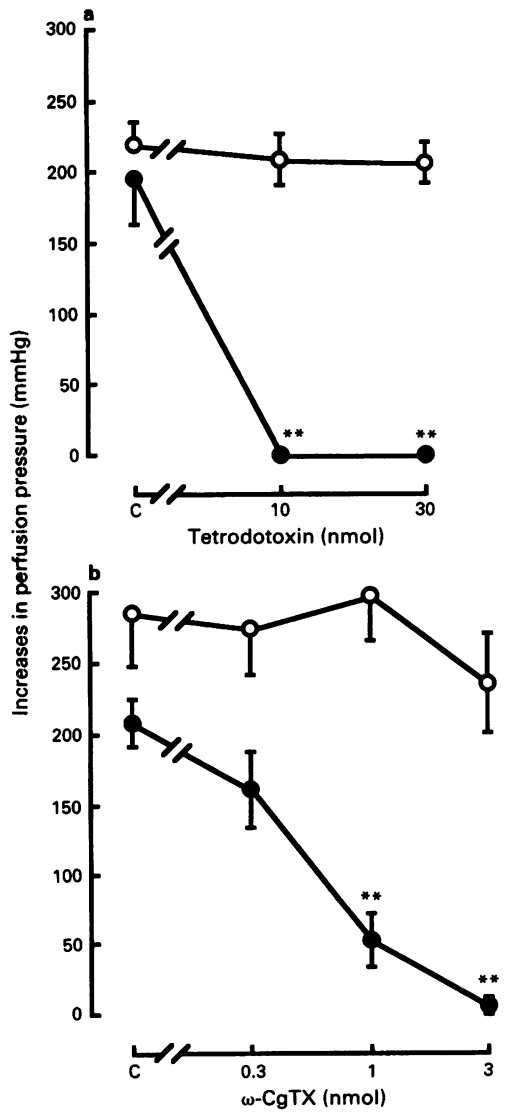


Figure 2 Effects of tetrodotoxin (a) and ω-CgTX (b) on the vascular responses to noradrenaline (○) and to periarterial nerve stimulation with 10 V, 1 ms pulse duration, 10 Hz and 100 pulses (●) in the canine splenic arteries. Points represent the mean values with s.e.mean. Asterisks represent statistical significance *vs* the respective control value: $^{**}P<0.01$, $n=5-7$.

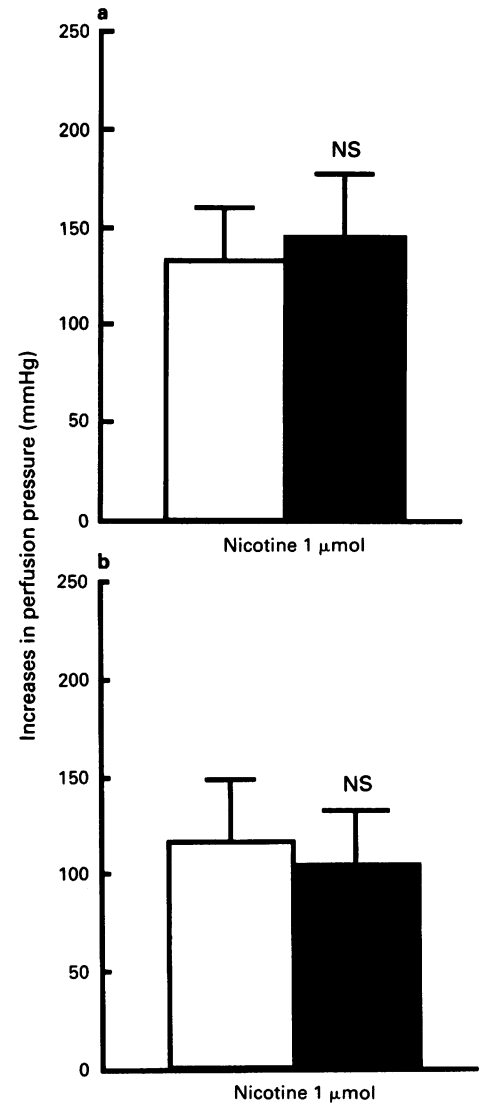


Figure 3 Vascular responses to 1 μmol nicotine before (open columns) and after (solid columns) treatment with 30 nmol tetrodotoxin (a) and 3 nmol ω-CgTX (b). Columns represent the mean values with s.e.mean. NS: not significantly different from control values, $n=6-8$.

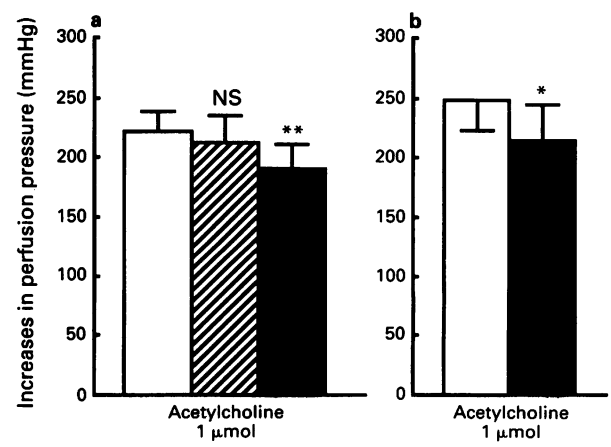


Figure 4 Vascular responses to 1 μmol acetylcholine before (open columns, a and b) and after treatment with 10 nmol (hatched column, a) and 30 nmol (solid column, a) tetrodotoxin and 3 nmol ω-CgTX (solid column, b). Columns represent the mean values with s.e.mean. Asterisks represent statistical significance *vs* the respective control value: $^{*}P<0.05$; $^{**}P<0.01$, $n=5-7$.

Effects of α-adrenoceptor antagonists or guanethidine on the vasoconstriction induced by ACh

ACh, NA and ATP constricted the splenic arterial segments in a concentration-dependent manner. Perfusion with 3 μM guanethidine, an adrenergic neurone blocker, significantly inhibited the vascular responses to ACh, and 30 μM guanethidine completely suppressed the ACh-induced vasoconstriction. Guanethidine perfusion (3 or 30 μM), however, did not affect the responses to NA and ATP (Figure 5).

Figure 6 shows the effect of bunazosin (a selective α_1-adrenoceptor antagonist) and midaglizole (a selective α_2-adrenoceptor antagonist) on ACh-induced vasoconstrictions. Perfusion with 10 μM bunazosin and 10 μM midaglizole abolished the vasoconstrictor responses to ACh (10–1000 nmol) or NA (0.01–0.3 nmol), but did not change the responses to ATP (0.1–100 nmol).

Effects of hexamethonium and nicotine on ACh-induced vasoconstriction

A bolus injection of hexamethonium at 1 μmol inhibited the ACh-induced vasoconstrictor response and the response to ACh recovered 60 min after the injection of hexamethonium. The vasoconstriction elicited by NA was not significantly affected by hexamethonium (Figure 7).

Figure 8 shows the interaction between nicotine and ACh. Twenty min after the first administration of 1 μmol ACh, a bolus injection of 100 nmol nicotine was given to the preparation. When a small increase in perfusion pressure elicited by 100 nmol nicotine returned to a base line, the second administration of 1 μmol ACh was injected immediately. The treatment with nicotine almost completely inhibited the vasoconstrictor response to ACh, but did not affect the response to NA. The response to ACh recovered 60 min after the treatment with nicotine (Figure 8).

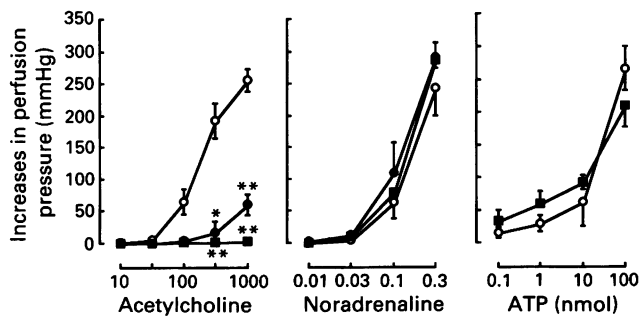


Figure 5 Vascular responses to acetylcholine ($n = 4$–8), noradrenaline ($n = 4$) and ATP ($n = 4$) before (○) and after perfusion with 3 μM guanethidine (●) and 30 μM guanethidine (■) in the canine splenic arteries. Points represent the mean values with s.e.mean. Asterisks represent statistical significance *vs* the respective control value: $*P < 0.05$; $**P < 0.01$.

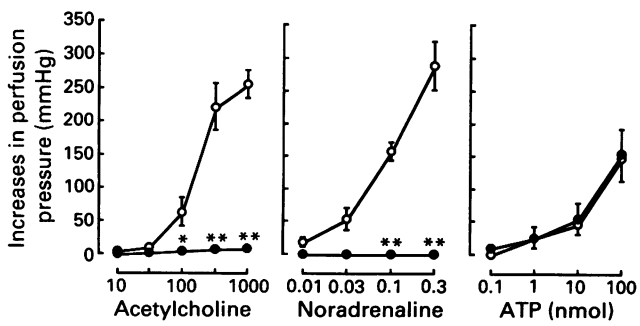


Figure 6 Vascular responses to acetylcholine ($n = 6$), noradrenaline ($n = 3$) and ATP ($n = 4$) before (○) and after (●) perfusion with 10 μM bunazosin and 10 μM midaglizole in the canine splenic arteries. Points represent the mean values with s.e.mean. Asterisks represent statistical significance *vs* the respective control value: $*P < 0.05$; $**P < 0.01$.

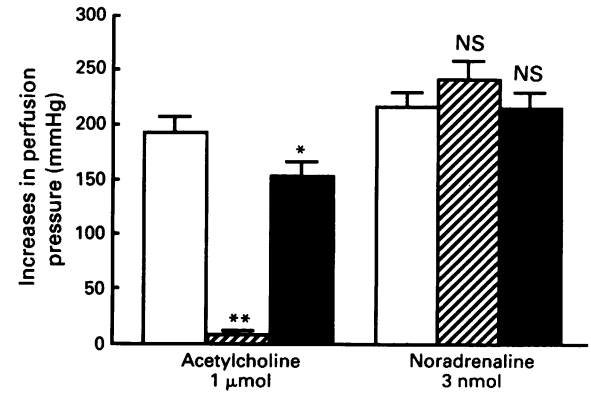


Figure 7 Vascular responses to 1 μmol acetylcholine and 3 nmol noradrenaline before (open columns) and after treatment with 1 μmol hexamethonium (hatched columns) and 60 min after hexamethonium treatment (solid columns). Columns represent the mean values with s.e.mean. Asterisks represent statistical significance *vs* the respective control value: $*P < 0.05$; $**P < 0.01$, NS: not significantly different from control values, $n = 7$.

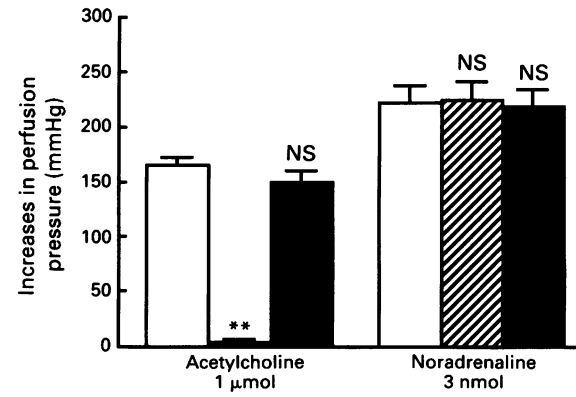


Figure 8 Vascular responses to 1 μmol acetylcholine and 3 nmol noradrenaline before (open columns) and after treatment with 0.1 μmol nicotine (hatched columns) and 60 min after nicotine treatment (solid columns). Columns represent the mean values with s.e.mean. Asterisks represent statistical significance *vs* the respective control value: $**P < 0.01$, NS: not significantly different from control values, $n = 7$.

Effects of perfusion with atropine on the vascular responses to ACh, NA and nicotine

Perfusion with 1 μM atropine did not change the resting perfusion pressure. However, the vasoconstrictor responses to ACh, NA and nicotine (1 μmol) were significantly inhibited (Figure 9). The percentage inhibitions of 0.3 nmol NA, 1000 nmol ACh and 1 μmol nicotine by atropine were 31.6%, 47.6% and 70.6%, respectively.

Discussion

In the present study, ACh induced vasoconstriction through activation of presynaptic nicotinic receptors present on the sympathetic nerves, and the nicotinic receptor stimulation-induced vasoconstriction was not sensitive to TTX and ω-CgTX. On the contrary, electrical stimulation-induced vasoconstriction was readily inhibited by TTX and ω-CgTX. These findings suggest that receptor-operated ion channels are mainly responsible for the nicotinic receptor stimulation-induced NA release, but N-type VOCCs are distinctly sen-

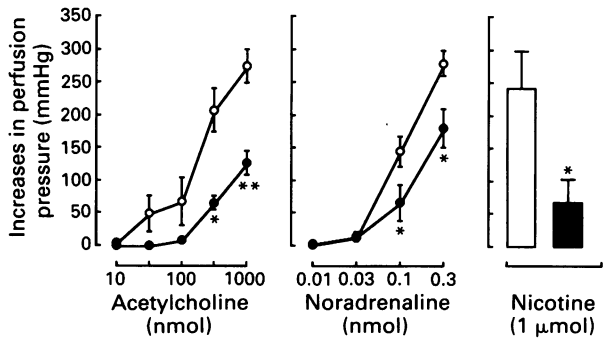


Figure 9 Vascular responses to acetylcholine, noradrenaline and nicotine before (○ and open column) and after (● and solid column) perfusion with 1 μM atropine in the canine splenic arteries. Points and columns represent the mean values with s.e.mean. Asterisks represent statistical significance *vs* the respective control value: $*P<0.05$; $**P<0.01$, $n=4$.

sitive to the electrical stimulation-induced one in the canine splenic artery.

Nicotinic receptor stimulation-induced and sympathetic nerve-related vasoconstrictions

Though ACh produces a vasoconstriction in the porcine and bovine isolated coronary arteries with endothelium, through muscarinic receptors (Gräser *et al.*, 1986; Brunner *et al.*, 1991), it often causes a concentration-dependent relaxation but not a vasoconstriction in many kinds of arteries, including the canine splenic artery with endothelium, when they are precontracted with a vasoconstrictive agent (De Mey & Vanhoutte, 1982; Jaiswal *et al.*, 1991). Even after removing the endothelium of the canine splenic artery, ACh does not contract the artery (De Mey & Vanhoutte, 1982). In the present studies, vasodilator effects of ACh were not observed, since the canine splenic artery was not precontracted by a vasoconstrictor agent. This result is consistent with a previously reported observation (De Mey & Vanhoutte, 1982). On the other hand, ACh-induced vasoconstriction may result from stimulation of muscarinic receptors and/or nicotinic receptors, as it is generally accepted that ACh is an agonist for both receptors.

For observing the nicotine-like action of ACh, we should have used a selective muscarinic receptor antagonist, atropine, at a sufficient concentration (1 μM) to antagonize muscarinic receptors completely. However, in the present experiments this concentration of atropine significantly inhibited the vasoconstrictor responses to NA and also to nicotine though it inhibited ACh-induced vasoconstrictions. Several reports have indicated that a high concentration of atropine (1 μM to 0.1 mM) antagonizes NA-induced constriction in the rabbit aorta and smooth muscle strips from the reticular groove of cattle (Denac *et al.*, 1991; Satake *et al.*, 1992). Our present results provide further evidence in support of their observations. Moreover, since the inhibition by atropine of 1 μmol nicotine-induced vasoconstriction (by 70.6%) was markedly greater than that of 0.3 nmol NA-induced vasoconstriction (by 31.6%), a high concentration of atropine might have an inhibitory action on the nicotine-related response, except for its action against NA. Therefore, perfusion with atropine could not be used in the present experiments for observing nicotine-like actions of ACh.

Though the preparations were not pretreated with atropine, the conclusion that ACh contracted the splenic artery through activation of the presynaptic nicotinic receptors of the sympathetic nerves, but not the muscarinic receptors of smooth muscle was strongly supported by the following evidence. First, ACh and nicotine induced a similar strong vasoconstriction in the splenic artery, and ACh contracted the artery at the same dose-range as nicotine. Furthermore, when the artery was pretreated with nicotine to cause desensitization of nicotinic receptors, ACh did not produce any vascular responses. Secondly, hexamethonium, a ganglionic nicotinic receptor antagonist, inhibited ACh-induced vasoconstrictions, but did not affect the vascular response to exogenous NA. Thirdly, in the preparation perfused with α-adrenoceptor antagonists, ACh and NA did not induce any vasoconstriction, but ATP still contracted the artery well. In addition, guanethidine, a sympathetic neurone blocking agent, prevented the vasoconstrictor responses to ACh, but not to NA and ATP. Finally, perfusion with atropine at a concentration high enough to block muscarinic receptors did not abolish the ACh-induced vasoconstriction. All the results show that ACh stimulates the presynaptic nicotinic receptors present on the sympathetic nerves, inducing NA release, which in turn contracts the splenic artery.

Actions of TTX and ω-CgTX on vasoconstrictor responses to nicotinic receptor-stimulation

TTX selectively blocks voltage-dependent sodium channels (Ceña *et al.*, 1983), and ω-CgTX selectively blocks neuronal N- and L-type VOCCs (McCleskey *et al.*, 1986; 1987; Cruz *et al.*, 1987). Vasoconstrictions and NA release in response to electrical stimulation in the rabbit and rat isolated arteries are sensitive to TTX and ω-CgTX, indicating that these channels are responsible for electrical stimulation-induced NA release from sympathetic nerve endings (Su & Bevan, 1970; Furchgott *et al.*, 1975; De Luca *et al.*, 1990; Pruneau & Angus, 1990; Russell *et al.*, 1990). In the canine splenic artery, TTX and ω-CgTX completely inhibited the vasoconstrictor responses to periarterial electrical stimulation without affecting NA-induced vascular responses (Figure 2), and electrical stimulation-induced vasoconstriction was also inhibited by guanethidine (unpublished data), suggesting that voltage-dependent sodium channels and neuronal type VOCCs were involved in the sympathetic transmission evoked by electrical stimulation.

In contrast with electrical stimulation, there is conflicting information about the mechanism of nicotine-induced NA release in tissues innervated by sympathetic nerves. TTX blocks the functional responses and NA release in response to low doses of nicotine but does not modify that to high doses in the rabbit ear artery and atrium (Furchgott *et al.*, 1975; Kirpekar *et al.*, 1980). On the other hand, it has been reported that TTX does not inhibit NA release by nicotine at any concentrations used in the guinea-pig atrium, rabbit pulmonary artery and adrenal chromaffin cells (Su & Bevan, 1970; Westfall & Brasted, 1972; Jayasundar & Vohra, 1978; Ceña *et al.*, 1983). In the canine atrium perfused by blood, we observed that the positive cardiac responses to nicotine were caused by both TTX-sensitive and TTX-insensitive NA release mechanisms (Ren *et al.*, 1991). Therefore, the mechanisms by which nicotine and ACh cause NA release are very complicated.

In recent years, ω-CgTX, a selective neuronal VOCCs blocker, has been widely used, especially in the study of neurotransmission induced by electrical stimulation. On the other hand, no direct information is available to date on the effect of ω-CgTX on sympathetic nerve-related responses to nicotinic receptor stimulation in vasculature. In the guinea-pig lung preparations, TTX and ω-CgTX inhibit the bronchoconstriction and peptide release evoked by vagal nerve stimulation, but they only marginally influence the bronchoconstriction and peptide release evoked by nicotine, suggesting that nicotine mainly acts via receptor-operated channels to induce a peptide release and functional response (Lou *et al.*, 1992). Haass *et al.* (1991) reported that an N-type VOCCs blocker, ω-CgTX, reduced the nicotine-induced NA release to 20% of the control value in the guinea-pig heart, suggesting a role of N-type VOCCs in mediating the nicotine-induced transmitter release, but they

did not observe the effect of TTX in their study (Haass et al., 1991). Westfall & Brasted (1972) and Jayasundar & Vohra (1978) found that TTX did not affect the nicotine-induced NA release and functional responses in the guinea-pig heart, but Bell (1968) reported that TTX abolished nicotine-induced functional responses in the preparation. Furchgott et al. (1975) found that the state of the preparation influences the effect of TTX. Therefore, the response to nicotine reported by Haass et al. (1991) might be sensitive to TTX, i.e. the administration of nicotine may cause an opening of sodium channels and trigger propagated action potentials along the adrenergic nerve fibres, and finally activate VOCCs.

In the present study, we demonstrated that more than 85% of the vasoconstrictor responses to nicotine and ACh were not affected by either TTX or ω-CgTX in doses which completely inhibited the vascular responses to electrical stimulation. The fact that TTX failed to block the vascular responses to nicotinic receptor stimulation indicates that the NA release induced by nicotine or ACh is independent of the propagated action potentials along the adrenergic nerve fibres and of nerve terminal membrane depolarization induced by the opening of voltage-dependent sodium channels. As VOCCs on the nerve terminals were not activated, the vasoconstrictor responses to nicotine and ACh were not affected by ω-CgTX. However, the requirement of calcium ions for the nicotine-induced NA release is generally accepted (Jayasundar & Vohra, 1977; Starke & Weitzell, 1978; Kirpekar et al., 1980; Abajo et al., 1989). Calcium ions might enter the nerve terminals through other kinds of calcium ion channels. Since it has been suggested that receptor-operated ion channels are responsible for the nicotinic receptor stimulation-induced NA release from the chromaffin cells (Ceña et al., 1983) and that calcium ions enter the human neuroblastoma cells through nicotinic receptor-operated calcium channels (Noronha-Blob et al., 1989), it may be reasonable to speculate that receptor-operated ion channels are responsible for the nicotinic receptor stimulation-induced NA release in the canine splenic artery. Since selective antagonists of receptor-operated ion channels for sodium or calcium ions have not yet been developed, it is difficult to characterize precisely the receptor-operated ion channels pharmacologically at present.

References

ABAJO, F.J., CASTRO, M.A.S., LOPO, C.R., GARIJO, B., SANCHEZ-GARCIA, P. & GARCIA, A.G. (1989). Sodium-dependent and sodium-independent nicotine-evoked catecholamine release from cat adrenals. *Neurosci. Lett.*, **101**, 101–106.

BELL, C. (1968). Differential effects of tetrodotoxin on sympathomimetic actions of nicotine and tyramine. *Br. J. Pharmacol. Chemother.*, **32**, 96–103.

BRUNNER, F., KÜHBERGER, E., SCHLOOS, J. & KUKOVETZ, W.R. (1991). Characterization of muscarinic receptors of bovine coronary artery by functional and radioligand binding studies. *Eur. J. Pharmacol.*, **196**, 247–255.

CEÑA, V., NICOLAS, G.P., SANCHEZ-GARCIA, P., KIRPEKAR, S.M. & GARCIA, A.G. (1983). Pharmacological dissection of receptor-associated and voltage-sensitive ionic channels involved in catecholamine release. *Neuroscience*, **10**, 1455–1462.

CHIBA, S. & TSUKADA, M. (1985). Different sensitivity of blocking effects of α-adrenoceptor blocking agents on vascular responses to intraluminal norepinephrine and periarterial stimulation in isolated dog arteries. *Jpn J. Pharmacol.*, **38**, 83–89.

CLASBRUMMEL, B., OSSWALD, H. & ILLES, P. (1989). Inhibition of noradrenaline release by ω-conotoxin GVIA in the rat tail artery. *Br. J. Pharmacol.*, **96**, 101–110.

CRUZ, L.J., JOHNSON, D.S. & OLIVERA, B.M. (1987). Characterization of the ω-conotoxin target. Evidence for tissue-specific heterogeneity in calcium channel types. *Biochemistry*, **26**, 820–824.

DE LUCA, A., LI, C.G., RAND, M.J., REID, J.J., THAINA, P. & WONG-DUSTING, H.K. (1990). Effects of ω-conotoxin GVIA on autonomic neuroeffector transmission in various tissues. *Br. J. Pharmacol.*, **101**, 437–447.

DE MEY, J.G. & VANHOUTTE, P.M. (1982). Heterogeneous behavior of the canine arterial and venous wall (importance of the endothelium). *Circ. Res.*, **51**, 439–447.

DENAC, M., KUMIN, G. & SCHARRER, E. (1991). Effect of noradrenaline on smooth muscle strips from the reticular groove of adult cattle. *J. Vet. Med.*, **38**, 383–388.

FURCHGOTT, R.F., STEINSLAND, O.S. & WAKADE, T.D. (1975). Studies on prejunctional muscarinic and nicotinic receptors. In *Chemical Tools in Catecholamine Research*, Vol. 2, ed. Almgren, O., Carlsson, A. & Engel, J., pp. 167–174, Amsterdam: North-Holland Publishing Company.

GRÄSER, T., LEISNER, H. & TIEDT, N. (1986). Absence of role of endothelium in the response of isolated porcine coronary arteries to acetylcholine. *Cardiovasc. Res.*, **20**, 299–302.

HAASS, M., FÖRSTER, C., KRANZHÖFER, R., RICHARDT, G. & SCHÖMIG, A. (1990). Role of calcium channels and protein kinase C for release of norepinephrine and neuropeptide Y. *Am. J. Physiol.*, **259**, R925–R930.

HAASS, M., RICHARDT, G., BRENN, T., SCHÖMIG, E. & SCHÖMIG, A. (1991). Nicotine-induced release of noradrenaline and neuropeptide Y in guinea-pig heart: role of calcium channels and protein kinase C. *Naunyn-Schmied. Arch. Pharmacol.*, **344**, 527–531.

HILLMAN, D., CHEN, S., AUNG, T.T., CHERKSEY, B., SUGIMORI, M. & LLINAS, R.R. (1991). Localisation of P-type calcium channels in the central nervous system. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 7076–7080.

HONGO, K. & CHIBA, S. (1983). A new method for measuring vascular responsiveness of relatively larger arteries of dogs. *J. Pharmacol. Methods*, **9**, 83–91.

IKUSHIMA, S., MURAMATSU, I. & FUJIWARA, M. (1982). Nicotine-induced response in guinea-pig aorta enhanced by goniopora toxin. *J. Pharmacol. Exp. Ther.*, **223**, 790–794.

ILLES, P. (1986). Mechanisms of receptor-mediated modulation of transmitter release in noradrenergic cholinergic and sensory neurons. *Neuroscience*, **14**, 909–928.

JAISWAL, N., LAMBRECHT, G., MUTSCHLER, E., TACKE, R. & MALIK, K.U. (1991). Pharmacological characterization of the vascular muscarinic receptors mediating relaxation and contraction in rabbit aorta. *J. Pharmacol. Exp. Ther.*, **258**, 842–850.

JAYASUNDAR, S. & VOHRA, M.M. (1977). Mechanism of nicotine-induced release of noradrenaline from adrenergic nerve endings. *Br. J. Pharmacol.*, **61**, 183–188.

JAYASUNDAR, S. & VOHRA, M.M. (1978). Mechanism of nicotine-induced release of norepinephrine from adrenergic nerve endings: is generation and propagation of impulses necessary? *Arch. Int. Pharmacodyn. Ther.*, **232**, 202–210.

KERR, L.M. & YOSHIKAMI, D. (1984). A venom peptide with a novel presynaptic blocking action. *Nature*, **308**, 282–284.

KIRPEKAR, S.M., GARCIA, A.G. & PRAT, J.C. (1980). Action of nicotine on sympathetic nerve terminals. *J. Pharmacol. Exp. Ther.*, **213**, 133–138.

LLINAS, R.R., SUGIMORI, M. & CHERKSEY, B. (1989). Voltage-dependent calcium conductances in mammalian neurons: the P channel. *Ann. N.Y. Acad. Sci.*, **560**, 103–111.

LOU, Y.P., FRANCO-CERECEDA, A. & LUNDBERG, J.M. (1992). Different ion channel mechanisms between low concentrations of capsaicin and high concentrations of capsaicin and nicotine regarding peptide release from pulmonary afferents. *Acta Physiol. Scand.*, **146**, 119–127.

MCCLESKEY, E.W., FOX, A.P., FELDMAN, D.H., CRUZ, L.J., OLIVERA, B.M., TSIEN, R.W. & YOSHIKAMI, D. (1987). ω-Conotoxin: direct and persistent blockade of specific types of calcium channels in neurons but not muscle. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 4327–4331.

McCLESKEY, E.W., FOX, A.P., FELDMAN, D. & TSIEN, R.W. (1986). Different types of calcium channels. *J. Exp. Biol.*, **124**, 177–190.

McKNIGHT, A.T., MAGUIRE, J.J. & WOODRUFF, G.N. (1989). Selective block by ω-conotoxin GVIA of motor adrenergic nerves in rat anococcygeus, *in vitro*. *J. Physiol.*, **409**, 50P.

MILLER, R.J. (1987). Multiple calcium channels and neuronal function. *Science*, **235**, 46–52.

MOHY EL-DIN, M.M. & MALIK, K.U. (1988). Differential effect of ω-conotoxin on release of the adrenergic transmitter and the vasoconstrictor response to noradrenaline in the rat isolated kidney. *Br. J. Pharmacol.*, **94**, 355–362.

MURAMATSU, I., KIGOSHI, S. & OSHITA, M. (1984). Nonadrenergic nature of prazosin-resistant, sympathetic contraction in the dog mesenteric artery. *J. Pharmacol. Exp. Ther.*, **229**, 532–538.

MUSCHOLL, E. (1970). Cholinomimetic drugs and release of the adrenergic transmitter. In *New Aspects of Storage and Release Mechanism of Catecholamines*. ed. Schümann, H.J. & Kroneberg, G., pp. 168–186. New York: Springer-Verlag.

NORONHA-BLOB, L., GOVER, R. & BAUMGOLD, J. (1989). Calcium influx mediated by nicotinic receptors and voltage sensitive calcium channels in SK-N-SH human neuroblastoma cells. *Biochem. Biophys. Res. Commun.*, **162**, 1230–1235.

NOWYCKY, M.C., FOX, A.P. & TSIEN, R.W. (1985). Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature*, **316**, 440–443.

PRUNEAU, D. & ANGUS, J.A. (1990). ω-Conotoxin GVIA is a potent inhibitor of sympathetic neurogenic responses in rat small mesenteric arteries. *Br. J. Pharmacol.*, **100**, 180–184.

REN, L.-M., FURUKAWA, Y., KARASAWA, Y., MURAKAMI, M., TAKEI, M., NARITA, M. & CHIBA, S. (1991). Effects of tetrodotoxin and imipramine on the cardiac responses to nicotine in isolated, blood-perfused canine heart preparations. *J. Cardiovasc. Pharmacol.*, **18**, 77–84.

RUSSELL, J.A., GIESE, E.C. & MOORE, T.L. (1990). Neuronal type N calcium channels mediate norepinephrine release in isolated rabbit pulmonary artery. *Pulmonary Pharmacol.*, **3**, 41–46.

SATAKE, N., KIYOTO, S., SHIBATA, S., GANDHI, V., JONES, D.J. & MORIKAWA, M. (1992). Possible mechanisms of inhibition with atropine against noradrenaline-induced contraction in the rabbit aorta. *Br. J. Pharmacol.*, **107**, 553–558.

STARKE, K. & WEITZELL, R. (1978). Is histamine involved in the sympathomimetic effect of nicotine? *Naunyn-Schmied. Arch. Pharmacol.*, **304**, 237–248.

SU, C. & BEVAN, J.A. (1970). Blockade of the nicotine-induced norepinephrine release by cocaine, phenoxybenzamine and desipramine. *J. Pharmacol. Exp. Ther.*, **175**, 533–540.

TSUJI, T. & CHIBA, S. (1984). Potentiating effect of methysergide on norepinephrine-induced constriction of the internal carotid artery of the dog. *Jpn. J. Pharmacol.*, **34**, 95–100.

WALLENSTEIN, S., ZUCKER, C.L. & FLEISS, J.L. (1980). Some statistical methods useful in circulation research. *Circ. Res.*, **47**, 1–9.

WESTFALL, T. & BRASTED, M. (1972). The mechanism of action of nicotine on adrenergic neurons in the perfused guinea-pig heart. *J. Pharmacol. Exp. Ther.*, **182**, 409–418.

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Mediation by nitric oxide formation in the preoptic area of endotoxin and tumour necrosis factor-induced inhibition of water intake in the rat

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1 Drinking was induced in rats by 24 h of water deprivation. Water intake (ml) was evaluated for a 1 h period.

2 N^G-nitro-L-arginine methyl ester (L-NAME, 5–10 μg, i.c.v., 50–100 ng into the preoptic area (POA)), an inhibitor of nitric oxide (NO) synthase, and methylene blue (50–100 ng into POA), an inhibitor of guanylate cyclase activation, antagonized the inhibition of drinking induced by *E. coli* endotoxin (LPS, 640 μg kg^{-1}, i.v.) and tumour necrosis factor (TNFα, 40 ng, i.c.v.) in 24 h water-deprived rats.

3 L-Arginine (25, 50 and 100 ng), the precursor aminoacid of NO, but not the stereoisomer D-arginine (100 ng), inhibited drinking induced by water deprivation when injected into the POA 30 min before water presentation (74.4% of inhibition with the highest dose). A dose of 12.5 ng L-arginine into the POA did not exhibit antidipsogenic effects.

4 TNFα (20 and 40 ng, i.c.v.; 1.25, 2.5 and 5 ng into the POA) showed a dose-dependent and powerful inhibition of drinking behaviour in water-deprived rats (70.4% and 80.8%, i.c.v. and into POA, with the highest doses, respectively). A dose of 10 ng of TNFα given i.c.v. had no effect on the intake of water.

5 LPS and TNFα, given at doses (160 μg kg^{-1}, i.v. and 10 ng, i.c.v., respectively) that did not influence drinking in water-deprived rats, exhibited a strong antidipsogenic effect in water-deprived rats treated with a dose of L-arginine (12.5 ng, into the POA) which did not modify drinking by itself. (LPS + L-arginine: 53.6% of inhibition; TNFα + L-arginine: 52.0% of inhibition).

6 These results suggest that NO into the POA: (1) acts as an inhibitory mechanism on thirst and (2) plays a role in the antidipsogenic effect of LPS and TNFα.

Keywords: Nitric oxide; drinking; LPS; TNFα; preoptic area; N^G-nitro-L-arginine methyl ester (L-NAME); methylene blue

Introduction

Intravenous (i.v.) injection of *Escherichia coli* endotoxin (lipopolysaccharide, LPS) causes a potent, dose-dependent and long-lasting inhibition of the rat's drinking behaviour induced by water deprivation, as well as by intracerebroventricular (i.c.v.) injection of angiotensin II, carbachol or hypertonic NaCl solution. The inhibitory effect of LPS on water intake is not a mere consequence of behavioural alteration, including a depressed motor activity, since this point has been investigated in previous work (Foca' *et al.*, 1983) and we concluded that this hypothesis can be excluded.

This antidipsogenic effect becomes evident 30 min after the injection, reaches a maximum after 3 h and is prevented by acetylsalicylic acid injected into the hypothalamic preoptic area (POA; Foca' *et al.*, 1985; Calapai *et al.*, 1990). Furthermore, LPS injected directly into the POA inhibits drinking induced by water deprivation (Foca' *et al.*, 1985).

LPS has been shown to induce a release of tumour necrosis factor (TNFα) from neuronal and glial cells (Koenig, 1991) and, in water-deprived rats, a serum, containing antibodies raised against TNFα, antagonizes LPS induced inhibition of thirst (Calapai *et al.*, 1991). Moreover, i.c.v. injection of TNFα in water-deprived rats mimics the antidipsogenic action of LPS (Calapai *et al.*, 1992b).

More recently, we found that nitric oxide (NO) acts as an inhibitory mechanism when thirst is stimulated by water deprivation or by angiotensin II, and we suggested that the POA could be one of the central sites of its antidipsogenic activity (Calapai *et al.*, 1992a).

Nitric oxide synthase in the brain has been shown, characterized, purified, and cloned as a cytosolic and Ca^{2+}-dependent enzyme (Knowles *et al.*, 1989; Forstermann *et al.*, 1990; Bredt & Synder, 1990; Bredt *et al.*, 1990). It is present in the largest concentration in the cerebellum, followed by the hyopthalamus, midbrain, striatum, hippocampus, and medulla oblongata (Bredt & Snyder, 1992). This enzyme is competitively inhibited by analogues of L-arginine (Schmidt *et al.*, 1989; Knowles *et al.*, 1990a; Forstermann *et al.*, 1990). Nitric oxide activates the soluble guanylate cyclase in the brain and L-arginine can be considered the endogenous stimulator of the soluble guanylate cyclase in the CNS (Knowles *et al.*, 1989).

Recently it has been demonstrated that NO could also play a role in food intake and in the regulation of body weight (Morley & Flood, 1991; 1992; Squadrito *et al.*, 1993), as well as in the regulation of drinking behaviour (Calapai *et al.*, 1992a).

Several effects, induced by both LPS and TNFα, are mediated, at least in part, by an overproduction of NO, which is caused by the induction of the Ca^{2+}-independent NO synthase in endothelial and smooth muscle cells, in macrophages and in other cells and tissues (Moncada *et al.*, 1992). Moreover, recent data indicate that a NO synthase-like activity can be induced in microglia and astrocytes by LPS (Simmons & Murphy, 1992).

In the light of the above findings we have investigated whether NO in the POA could be involved in drinking behaviour and water intake inhibition induced by LPS and TNFα in water-deprived rats.

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Methods

Animals

Adult male Sprague-Dawley rats weighing 280–320 g were used. The animals were housed at a constant temperature of 22 ± 2°C under a 12/12 h light-dark cycle (lights on at 6 h 00 min), with free access to Purina rat chow pellets and tap water, unless otherwise stated.

Water intake evaluations

Drinking was elicited by 24 h water deprivation. The rats had free access to food during this period. Water intake following deprivation was monitored for a 1 h period and expressed as ml/rat. Water was provided in graduated burettes, with drinking spouts, allowing direct volumetric measurement of intake to the nearest 0.1 ml. In order to avoid appearance of tolerance to LPS, each animal was treated only once with this substance and not used for other experiments.

Intracerebral and intravenous injections

Stainless steel guide cannulae (o.d. = 0.66 mm) were inserted 7 days before the experiments. The rats were anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.) and placed in a stereotaxic instrument. For i.c.v. injections, cannulae were implanted in the left lateral ventricle (stereotaxic coordinates: AP = 1 mm behind the bregma, L = 2.5 mm from the midsaggital suture and V = 2 mm from the dura). For injections into the POA, the cannulae were implanted, aimed 2 mm above the left medial preoptic area, at the following stereotaxic coordinates: AP = 7.9 mm anterior to the interaural line, L = 0.7 mm from the midsagittal suture, V = 3.4 mm up from the interaural line. The incisor bar of the stereotaxic was elevated 5 mm above the interaural line. In a group of animals cannulae were implanted both in the lateral ventricle and into the POA. Injections into the lateral ventricle (5 µl) or into the POA (1 µl) were made by a 30 gauge injector temporarily inserted into the guide-cannula and protruding 2 mm beyond the cannula tip. Injections were carried out over a period of 1–2 min. I.v. injections were made through the tail vein under light ether anaesthesia.

Treatments

LPS was injected i.v. in 24 h water-deprived rats 3 h before water presentation, in order to obtain its maximum inhibitory antidipsogenic effect (Foca' *et al.*, 1983), while naive water deprived animals received saline solution (0.1 ml 100 g^{-1}, wt.).

TNFα was injected i.c.v. or into the POA in 24 h water-deprived rats 20 min before water presentation. Animals treated i.c.v. (5 µl) or into the POA (1 µl) with bovine serum albumin (BSA; 125 µg rat), the solvent of TNFα, served as controls.

L-Arginine, D-arginine, N^G-nitro-L-arginine methyl ester (L-NAME) and methylene blue were injected into the POA (1 µl) of water-deprived rats, 30 min before water presentation. Control animals received saline solution.

In order to study the effects on water intake for 24 h, four groups of animals were treated at 10 h 00 min with a single injection into the preoptic area (1 µl) of only one of the following drugs: L-NAME (100 ng), L-arginine (100 ng), D-arginine (100 ng), methylene blue (100 ng). The intake of water was evaluated 24 h after drug administration.

Drugs

Escherichia coli lipopolysaccharide (LPS) (055: B5 phenol extract) was purchased from the Sigma Chemical Company, U.S.A.; recombinant human tumour necrosis factor-α (TNF-α) from Nuclear Laser Medicine (Milan, Italy); L-arginine and D-arginine and N^G-nitro-L-arginine methyl ester (L-NAME) from the Sigma Chemical Company; methylene blue from Hoechst, Italy. RhTNFα was dissolved in bovine serum albumin (BSA). Lipopolysaccharide (LPS), L-arginine, D-arginine, L-NAME and methylene blue were dissolved in 0.9% NaCl solution warmed to 37°C before the injection.

Statistical analysis

Data are expressed as the means ± s.d. Statistical analysis of data was performed by ANOVA (one way analysis of variance). Statistical significance was set at $P<0.05$.

Table 1 Effect of isotonic saline (Sal) or N^G-nitro-L-arginine methyl ester (L-NAME) given i.c.v. (5 µl) or into the preoptic area (POA, 1 µl) on 1 h water intake (ml/rat) stimulated by 24 h water deprivation in rats pretreated with *E. coli* LPS (160, 320 and 640 µg kg^{-1}, i.v.)

No. of animals	*Treatment* (i.v.)	*Pretreatment*	*Water intake* (ml/rat)	*% inhibition water intake* vs Sal + Sal
		(i.c.v.; µg)		
5	Sal	Sal	12.5 ± 1.8	–
8	LPS 160	Sal	10.4 ± 0.9	16.8
5	LSP 320	Sal	7.9 ± 2.0*	36.8
7	LPS 640	Sal	3.9 ± 1.0*	68.8
8	LPS 640	L-NAME 2.5	4.0 ± 0.6	68.0
6	LPS 640	L-NAME 5	8.2 ± 1.4**	34.4
6	LPS 640	L-NAME 10	11.3 ± 2.0**	9.6
6	Sal	L-NAME 10	12.0 ± 2.0	4.0
		(into POA; ng)		
6	Sal	Sal	12.0 ± 1.3	–
7	LPS 640	Sal	4.1 ± 0.7***	65.8
6	LPS 640	L-NAME 10	4.5 ± 1.0	62.5
5	LPS 640	L-NAME 50	7.9 ± 2.0****	34.2
7	LPS 640	L-NAME 100	11.3 ± 1.7****	8.3
5	Sal	L-NAME 100	10.6 ± 2.0	16.6

LPS was injected 3 h before water presentation.
*$P<0.05$ vs Sal + Sal i.c.v.;
**$P<0.05$ vs LPS 640 + Sal i.c.v.;
***$P<0.05$ vs Sal + Sal into POA;
****$P<0.05$ vs LPS 640 + Sal into POA.

Results

Effect of L-NAME on LPS inhibition of thirst (Table 1)

L-NAME, given i.c.v. (5 and 10 μg) or into the POA (50 and 100 ng), 30 min before water presentation in water-deprived rats, significantly inhibited the antidipsogenic effect induced by i.v. injections of LPS (640 μg kg^{-1}). Lower doses (2.5 μg, i.c.v.; 10 ng into the POA) were without effect. L-NAME, either i.c.v. (10 μg) or into the POA (100 ng), by itself, did not modify drinking behaviour induced by 24 h water-deprivation.

Effect of L-arginine and D-arginine on water intake induced by water deprivation and on its inhibition induced by LPS

L-arginine (25, 50 and 100 ng), given into the POA 30 min before water presentation in 24 h water-deprived rats, inhibited drinking in a dose-dependent way (Table 2). A dose of 12.5 ng of L-arginine, as well as a dose of 100 ng of D-arginine did not modify water deprivation-induced drinking (Table 2).

A significant inhibition of water intake (Figure 1a) was observed in water-deprived rats when animals were treated with LPS (160 μg kg^{-1}, i.v.) together with L-arginine (12.5 ng, into the POA). Both doses were without inhibitory effects, by themselves. L-Arginine (12.5 ng) was injected 30 min before water presentation and 150 min after LPS. When D-arginine (100 ng) was injected into the POA, the LPS dose of 160 μg kg^{-1} did not inhibit drinking (Figure 1a).

Effect of TNFα on drinking behaviour induced by water-deprivation

TNFα given i.c.v. (20 and 40 ng) or into the POA (1.25, 2.5 and 5 ng) inhibited the intake of water deprived rats. Lower doses were without effect (Figure 2).

Effect of the injection of L-NAME and L-arginine into the preoptic area on the antidipsogenic effect induced by TNFα injected i.c.v.

For these experiments rats were prepared with two cerebral cannulae. Injections into the POA were made 10 min before TNF (40 ng) i.c.v. injection.

In water-deprived rats, L-NAME (100 ng) given into the POA inhibited (by 70%) the antidipsogenic effect induced by the i.c.v. injection of TNFα (Figure 1b). In water-deprived rats, injection into the POA of L-arginine, 12.5 ng (not antidipsogenic, by itself) given 10 min before the i.c.v. injection of TNF, 10 ng, (not antidipsogenic, by itself), showed a powerful antidipsogenic effect in 24 h water-deprived rats. D-Arginine (100 ng into the POA) did not inhibit drinking when associated with TNFα (10 ng, i.c.v.).

Table 2 Effect of injections of L-arginine and D-arginine into the preoptic area (POA) on 1 h water intake (ml/rat) of rats stimulated by 24 h water deprivation

No. of animals	*Treatment (i.c.v.; μg)*	*Water intake*	*% inhibition water intake vs Sal + Sal*
4	Sal	12.5 ± 2.0	–
5	L-Arg 12.5	10.4 ± 2.0	16.8
5	L-Arg 25	7.8 ± 1.5*	37.6
5	L-Arg 50	5.7 ± 1.0*	54.4
8	L-Arg 100	3.2 ± 0.6*	74.4
4	D-Arg 100	11.5 ± 2.3	8.0

*$P<0.05$ vs Sal

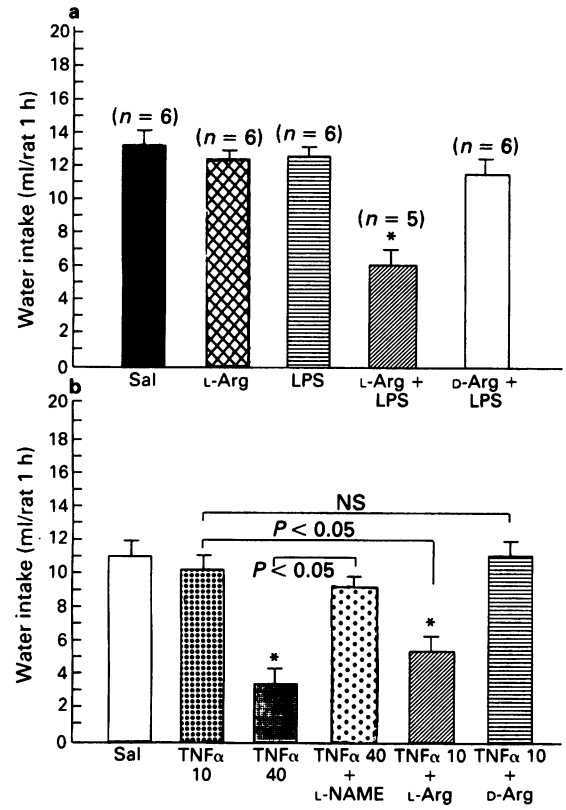


Figure 1 (a) Effect of injection of saline (Sal, into the preoptic area), L-arginine (L-Arg, 12.5 ng i.c.v.), LPS (160 μg kg^{-1}, i.v.), L-Arg + LPS (12.5 ng, POA + 160 μg kg^{-1}, i.v.,) and D-arginine (100 ng, POA) on 1 h water intake in animals deprived of water for 24 h. *$P<0.01$ vs Sal. Number of rats used in parentheses. (b) Water intake in 24 h water-deprived rats treated as following: BSA i.c.v. + Sal into POA ($n=4$); tumour necrosis factor (TNFα, 10 ng, i.c.v.) + Sal into POA ($n=6$); TNFα (40 ng, i.c.v.) + Sal into POA ($n=6$); TNFα (40 ng, i.c.v.) + L-NAME into POA (100 ng; $n=6$); TNFα (10 ng, i.c.v) + L-Arg into POA (12.5 ng); TNFα (10 ng, i.c.v.) + D-Arg (12.5 ng) into POA. *$P<0.01$ vs saline.

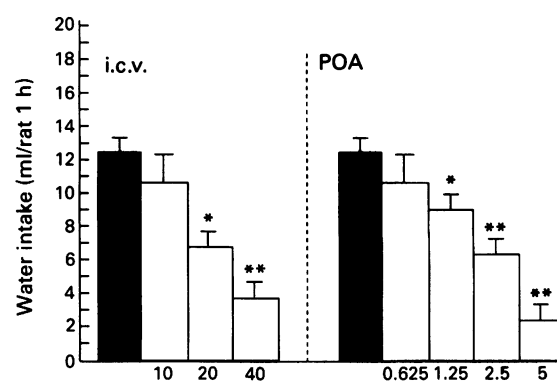


Figure 2 Effect of injection i.c.v. or into the preoptic area (POA) of bovine serum albumin (BSA) (solid columns) or tumour necrosis factor (TNFα; ng) on 1 h water intake in animals deprived (open columns) of water for 24 h. Each column represents the average of 5–6 animals. *$P<0.01$ and **$P<0.01$ vs BSA.

Effects of methylene blue on LPS and TNF-α inhibition of drinking behaviour induced by water-deprivation

Methylene blue (50 and 100 ng) injected into the POA produced a reduction of the inhibition of drinking induced by LP (640 μg kg^{-1}, i.v.) of TNFα (40 ng, i.c.v.) in water-deprived rats. (Figure 3). Methylene blue (100 ng) into the POA did not modify, by itself, drinking stimulated by water-deprivation (data not shown).

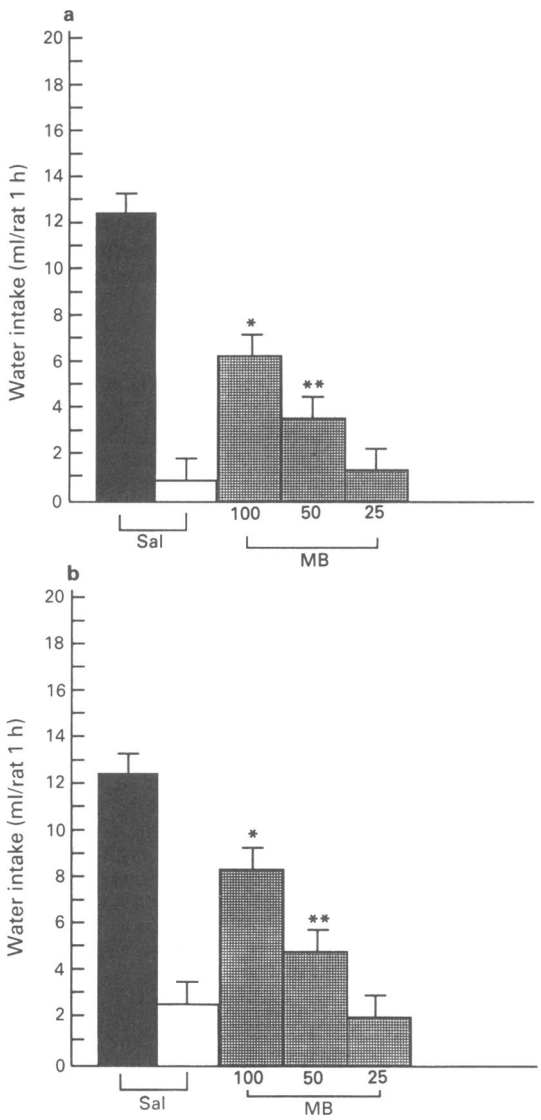


Figure 3 Effect of injection into the preoptic area (POA) of methylene blue (MB, ng) or saline (Sal) on 1 h water intake in animals deprived of water for 24 h and i.v. injected 3 h before testing with (a) saline (■) or LPS (640 μg kg^{-1}; □, ▨), $*P<0.01$ vs □; $**P<0.05$ vs ▨; or (b) i.c.v. with saline (■), or tumour necrosis factor (TNFα, 40 ng; □, ▨), $*P<0.01$ vs □. Each column represents the average ± s.d. of five animals.

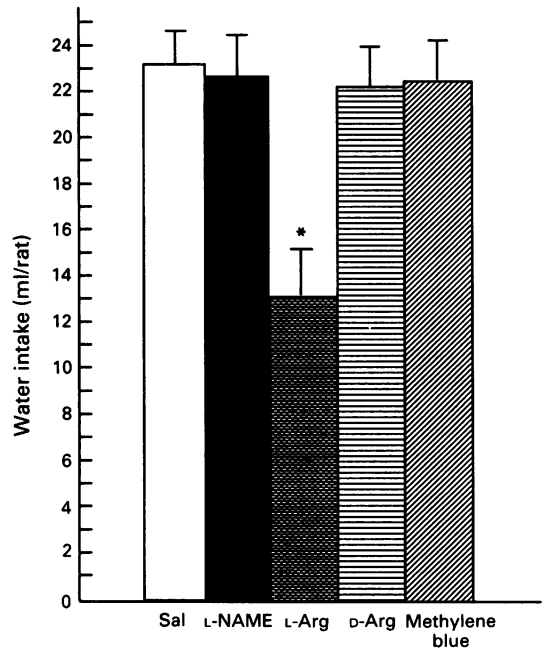


Figure 4 Effect of a single injection of saline (Sal, 1 μl into the preoptic area (POA)), N^G-nitro-L-arginine methyl ester (L-NAME, 100 ng, POA), L-arginine (L-Arg 100 ng, POA), D-arginine (D-Arg, 100 ng, POA) and methylene blue (100 ng, POA) on 24 h water intake of non-deprived animals. Each column represents the mean of six animals. $*P<0.01$ vs saline.

Effect of injection into the preoptic area of L-arginine, D-arginine, L-NAME and methylene blue of 24 h water intake

A single administration of L-arginine when injected into the preoptic area of non-deprived rats produced a significant decrease (43,53%) of the intake of water compared to the controls (saline). The intake of water during 24 h was unaffected by the other drugs (Figure 4).

Discussion

The results of the present experiments strongly support the role of nitric oxide in the POA in the antidipsogenic effect of LPS and TNFα. In fact, the peripheral administration of LPS and the intracerebroventricular injection of TNFα produced inhibition of drinking behaviour, which was partially abolished by the injection into the POA of L-NAME, a competitive inhibitor of NO synthase. Antagonism of L-NAME of inhibition of drinking behaviour was stronger when inhibition was induced by i.c.v. TNFα than i.v. LPS.

The experiments carried out with the injection of methylene blue (an inhibitor of guanylate cyclase activation by NO, Murad *et al.*, 1978) into the POA indicate that the enzyme in this area is involved in LPS and TNFα inhibition of drinking behaviour. Since biological effects of NO are accompanied by enhanced cyclic GMP levels, these data further support the idea that LPS and TNFα inhibit water intake through formation of NO in the preoptic area.

These results indicate that the inhibition of the enzymes (NO synthase and guanylate cyclase) antagonizes the effects of LPS and TNFα. Even when this inhibition was produced in the absence of LPS or TNFα or in non-deprived rats, there were no changes in the intake of water. Therefore, when rats were water-deprived, the stimulation of NO production by LPS or TNFα inhibited drinking, while the inhibition of the enzymes NO synthase or guanylate cyclase was not effective. We have already suggested that water deprivation, for 24 h, which produces intra- and extracellular dehydration, could be a stimulus able, by itself, to modify the activity of the enzymes (Calapai *et al.*, 1992a).

Experiments carried out with L-arginine and TNFα injected into the preoptic area confirm the role of this area in the regulation of water intake. Finally, L-arginine, but not D-arginine, when injected into the POA at doses not antidipsogenic by themselves, made doses of LPS and TNFα antidipsogenic which did not otherwise impair water intake in thirsty rats. Therefore, both LPS and TNF require NO formation in the preoptic area to elicit their inhibitory effect.

In the brain, astrocytes or microglia synthesize or secrete cytokines, such as interleukin-1 (IL-1), IL-6 and TNF, following infection or injury (Hauser *et al.*, 1990; Morganti-Kossmann *et al.*, 1992). In culture, glial cells and microglia release TNF following LPS stimulation (Morganti-Kossmann *et al.*, 1992). The injection of LPS into experimental animals has been demonstrated to produce TNFα (Carswell *et al.*, 1975). A trace amount of TNF can be detected in serum 15 to 20 min after injection of endotoxin and the concentration of TNF peaks after 1 or 2 h (Beutler *et al.*, 1985; Waage, 1987).

The fact that LPS is a very large molecule, which does not cross the blood brain barrier (Calapai *et al.*, 1990), raises the question of how peripherally administered LPS might exert its effect at the level of NO-synthase in the POA. Several hypotheses may be proposed: (1) TNFα, synthesized in peripheral cells following LPS, could enter the brain at specific sites and reach the POA, where it stimulates NO-synthase; (2) the synthesis of TNFα may be induced directly into the brain by LPS acting on astrocytes lying on the blood brain barrier; (3) LPS could stimulate some neural afferents, outside the brain, which in turn trigger the release of TNF in the preoptic area.

LPS and TNFα stimulate NO production, through the induction of a calcium-independent NO synthase, in endothelial and smooth muscle cells, in macrophages and in other cells and tissues (Knowles *et al.*, 1990b; Moncada *et al.*, 1991). Furthermore, treatment of glial cells for 16–18 h with LPS induces a NO synthase-like activity that is L-arginine dependent and Ca^{2+}-independent. The induction is evident after 4 h and depends on the dose of LPS and requires protein synthesis (Simmons & Murphy, 1992). It might be hypothesized that TNFα could induce this NO synthase in glial cells or, alternatively, it could increase L-arginine uptake into neuronal cells, thus increasing NO production through a calcium-dependent and constitutive NO synthase.

In conclusion, the results of the present study indicate that nitric oxide in the preoptic area is an inhibitory mechanism of drinking behaviour stimulated by water-deprivation and is one of the mediators of the antidipsogenic effects of TNFα and LPS.

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References

BEUTLER, B.A., MILSARK, I.W. & CERANI, A. (1985). Cachetin/tumor necrosis factor: production, distribution, and metabolic fate in vivo. *J. Immunol.*, **135**, 3972–3977.

BREDT, D.S., HWANG, P.M. & SNYDER, S.H. (1990). Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature*, **347**, 768–770.

BREDT, D.S. & SNYDER, S.H. (1990). Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 682–685.

BREDT, D.S. & SNYDER, S.H. (1992). Nitric oxide, a novel neuronal messenger. *Neuron*, **8**, 3–11.

CALAPAI, G., SQUADRITO, F., ALTAVILLA, D., ZINGARELLI, B., CAMPO, G.M., CILIA, M. & CAPUTI, A.P. (1992a). Evidence that nitric oxide modulates drinking behaviour. *Neuropharmacol.*, **31**, 761–764.

CALAPAI, G., SQUADRITO, F., ALTAVILLA, D., ZINGARELLI, B., CAMPO, G.M., SAITTA, A., IOCULANO, M., URNA, G., SARDELLA, A. & CAPUTI, A.P. (1991). Tumor necrosis factor involvement in lipopolysaccharide-induced inhibition of drinking in the rat. *Neurosci. Res. Comm.*, **9**, 53–61.

CALAPAI, G., MAZZAGLIA, G., MARCIANO, M.C., SQUADRITO, F., ALTAVILLA, D., ZINGARELLI, B., PROSDOCIMI, M. & CAPUTI, A.P. (1992b). Cloricromene antagonizes antidipsogenic effects induced by LPS, but not by TNF-α in the rat. *Life Sci.*, **51**, 2041–2048.

CALAPAI, G., SQUADRITO, F., MASSI, M., CAPUTI, A.P. & DE CARO, G. (1990). Endotoxin inhibition of drinking in the rat. *Pharmacol. Res.*, **22**, 161–170.

CARSWELL, E.A., OLD, L.J., KASSEL, R.L., GREEN, S., FIORE, N. & WILLIAMSON, B. (1975). An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. Natl. Acad. Sci. USA*, **72**, 3666–3670.

FOCA', A., MATERA, G., MASTROENI, P. & CAPUTI, A.P. (1985). Evidence that prostaglandins within preoptic area (POA) may mediate the antidipsogenic effect of Escherichia coli endotoxin in the rat. *Circ. Shock*, **17**, 137–145.

FOCA', A., NICOLETTA, P., MATERA, G., MASTROENI, P. & CAPUTI, A.P. (1983). Antidipsogenic effect of endotoxin in the rat. *Circ. Shock*, **11**, 341–350.

FORSTERMANN, U., GORSKY, L.D., POLLOCK, J.S., SCHMIDT, H.H.-H.W., HELLER, M. & MURAD, F. (1990). Regional distribution of EDRF/NO synthesizing enzyme(s) in rat brain. *Biochem. Biophys. Res. Commun.*, **168**, 727–732.

HAUSER, S.L., DOOLITTLE, T.H., LINCOLN, R., BROWN, R.H. & DINARELLO, C.A. (1990). Cytokine accumulation in CSF of multiple sclerosis patients: frequent detection of interleukin-1 and tumor necrosis factor, but not interleukin-6. *Neurology*, **40**, 1735–1739.

KNOWLES, R.G., PALACIOS, M., PALMER, R.M.J. & MONCADA, S. (1989). Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 5159–5162.

KNOWLES, R.G., PALACIOS, M., PALMER, R.M.J. & MONCADA, S. (1990a). Kinetic characteristics of nitric oxide synthase from rat brain. *Biochem. J.*, **269**, 207–210.

KNOWLES, R.L., SALTER, M., BROOKS, S.L. & MONCADA, S. (1990b). Anti-inflammatory glucocortoids inhibit the induction by endotoxin of nitric oxide synthase in the lung, liver and aorta of the rat. *Biochem. Biophys. Res. Commun.*, **172**, 1042–1048.

KOENIG, J.I. (1991). Presence of cytokines in the hypothalamic-pituitary axis. *Prog. NeuroEndocrinImmunol.*, **4**, 143–153.

MONCADA, S., MARLETTA, M.A., HIBBS, Jr. J.A. & HIGGS, E.A. (1992). *The Biology of Nitric Oxide. 1. Physiological and Clinical Aspects.* London and Chapel Hill: Portland Press.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.

MORGANTI-KOSSMAN, M.C., KOSSMAN, T. & WAHL, S.M. (1992). Cytokines and neuropathology. *Trends Pharmacol. Sci.*, **13**, 286–291.

MORLEY, J.E. & FLOOD, J.F. (1991). Evidence that nitric oxide modulates food intake in mice. *Life Sci.*, **49**, 707–711.

MORLEY, J.E. & FLOOD, J.F. (1992). Competitive antagonism of nitric oxide synthetase causes weight loss in mice. *Life Sci.*, **51**, 1285–1289.

MURAD, F., MITTAL, C.K., ARNOLD, W.P., KATSUKI, S. & KINURA, H. (1978). Guanylate cyclase activation by azide, nitro compounds, nitric oxide, and hydroxyl radical and inhibition by hemoglobin and myoglobin. *Adv. Cyclic Nucleotide Res.*, **9**, 145–158.

SCHMIDT, H.H.H.W., WILKE, P., EVERS, B. & BOHME, E. (1989). Enzymatic formation of nitrogen oxides from L-arginine in bovine brain cytosol. *Biochem. Biophys. Res. Commun.*, **165**, 284–291.

SIMMONS, M.L. & MURPHY, S. (1992). Induction of nitric oxide synthase in glial cells. *J. Neurochem.*, **59**, 897–905.

SQUADRITO, F., CALAPAI, G., CUCINOTTA, D., ALTAVILLA, D., ZINGARELLI, B., IOCULANO, M.P., URNA, G., SARDELLA, A., CAMPO, G.M. & CAPUTI, A.P. (1993). Anorectic activity of L-NO ARG, an inhibitor of brain NO synthase, in obese zucker rats. *Eur. J. Pharmacol.*, **230**, 125–128.

WAAGE, A. (1987). Production and clearance of tumor necrosis factor in rats exposed to endotoxin and dexamethasone. *Clin. Immunol. Immunopathol.*, **45**, 348–355.

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Effects of Cl channel blockers on Ca-activated chloride and potassium currents in smooth muscle cells from rabbit portal vein

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1 The effects of some chloride channel antagonists were studied on the calcium-activated chloride current ($I_{Cl(Ca)}$) in smooth muscle cells from the rabbit portal vein with the perforated patch technique.

2 4-Acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid (SITS) and 4,4'-diisothiocyanato-stilbene-2,2'-disulphonic acid (DIDS) reduced the amplitude of spontaneous transient inward currents (STICs, calcium-activated chloride currents) in a concentration-dependent manner. The concentrations required to reduce the amplitude by 50% (IC_{50}) of STICs were 2.1×10^{-4} M and 6.4×10^{-4} M for DIDS and SITS, respectively. This effect was not voltage-dependent.

3 The time constant of decay of STICs (τ), which is voltage-dependent, was increased by about 30% by SITS and decreased by about 20% by DIDS. The effect of DIDS and SITS on τ was similar at holding potentials of − 50 and + 50 mV.

4 These compounds did not modify the characteristics of spontaneous transient outward currents (STOCs, calcium-activated potassium currents).

5 DIDS and SITS decreased the amplitude of $I_{Cl(Ca)}$ evoked by noradrenaline and caffeine less potently than STICs with IC_{50} values of 7.5×10^{-4} M and 1.8×10^{-3} M, respectively.

6 DIDS and SITS increased the calcium-activated potassium current ($I_{K(Ca)}$) evoked by noradrenaline and caffeine by 3–6 fold.

7 Anthracene-9-carboxylic acid (A-9-C) inhibited STICs in a voltage-dependent fashion and was about 3 fold more active at + 50 mV than at − 50 mV. A-9-C increased STIC τ and this effect was enhanced by depolarization.

8 A-9-C also inhibited caffeine-evoked $I_{Cl(Ca)}$ but less potently than STICs and also increased the evoked $I_{K(Ca)}$ without altering spontaneous $I_{K(Ca)}$.

9 The results from the present work are compared with the pharmacology of other chloride conductances and the mechanism of action of the chloride channel antagonists in vascular smooth muscle is discussed.

Keywords: Pharmacological blockade; calcium-activated chloride current; vascular smooth muscle; rabbit portal vein; Cl channel blockers

Introduction

A calcium-activated chloride current ($I_{Cl(Ca)}$) was originally identified in rod segments from the salamander retina (Bader *et al.*, 1982) and in *Xenopus laevis* oocytes (Miledi, 1982). Subsequently this membrane mechanism has been observed in many types of tissue including exocrine gland cells, neurones and both skeletal and cardiac muscle. $I_{Cl(Ca)}$ was first shown in smooth muscle in the rat anococcygeus muscle where it was demonstrated that both α-adrenoceptor and muscarinic receptor activation evokes this membrane conductance (Byrne & Large, 1987a,b), Since then $I_{Cl(Ca)}$ has been observed in venous (Byrne *et al.*, 1988), and arterial smooth muscle (Amédée *et al.*, 1990a). More recently, $I_{Cl(Ca)}$ has been recorded in canine and guinea-pig trachea (Janssen & Sims, 1992), rat intestine (Ohta *et al.*, 1993) and rabbit oesophageal smooth muscle (Akbarali & Giles, 1993). Thus it is evident that $I_{Cl(Ca)}$ is found in many different types of smooth muscle and since it is now well established that chloride is concentrated inside the smooth muscle cell (the equilibrium potential E_{Cl} is between − 20 and − 30 mV, see Aickin, 1990) the calcium-activated chloride conductance represents a potentially important depolarizing mechanism in smooth muscle.

In the present study we have investigated the characteristics of the inhibition of $I_{Cl(Ca)}$ by two stilbene derivatives, 4-acetamido-4'-isothiocyanotostilbene-2,2'-disulphonic acid (SITS) and 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) and anthracene-9-carboxylic acid (A-9-C) on spontaneous and evoked $I_{Cl(Ca)}$ in rabbit portal vein. These agents have a varying degree of selectivity and potency but they have been widely used in the analysis of chloride currents and quantitative data would allow pharmacological comparison of $I_{Cl(Ca)}$ in smooth muscle with other types of chloride conductances. The present work represents the start of a programme to investigate systematically the pharmacology of $I_{Cl(Ca)}$ in smooth muscle and in this first series of experiments we have used the most commonly used blockers of different types of chloride conductances in a wide range of tissues. It has been shown that some of these compounds do inhibit $I_{Cl(Ca)}$ in smooth muscle (Baron *et al.*, 1991; Amédée *et al.*, 1990b) and in the present work we extend those observations and provide some indication of the mechanism of action of these chloride channel blocking compounds. Our approach to the latter problem was to investigate the action of blocking agents on the amplitude and decay time course of spontaneous transient inward currents (STICs) in rabbit portal vein cells. These random events are chloride currents, with no cation contribution, activated by calcium released from caffeine- and noradrenaline-sensitive intracellular stores (Wang *et al.*, 1992). An interesting characteristic of STICs is that the decay of these currents is exponential and the time

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constant of decay (τ) is voltage-dependent (Hogg *et al.*, 1993). At -50 mV, τ was 80–90 ms and was increased 3 fold for a depolarization of about 120 mV. Evidence was put forward to suggest that the decay of STICs was determined by closure of calcium-activated chloride channels and that τ approximated to the channel mean open time (Hogg *et al.*, 1993). Consequently it seemed that STICs may represent a good model for studying chloride channel antagonists in vascular smooth muscle. The results from the present work show that DIDS, SITS and A-9-C inhibit spontaneous chloride currents more readily than $I_{Cl(Ca)}$ activated by noradrenaline and caffeine. Moreover these agents differ in their effects on the STIC τ value and their voltage-dependence.

Methods

Experiments were carried out on freshly dispersed smooth muscle cells from the rabbit portal vein. Rabbits (2–2.5 kg) of either sex were killed by an overdose of i.v. sodium pentobarbitone and single cells were obtained by enzymatic dissociation with collagenase and papain as described previously (Hogg *et al.*, 1993). The cells were stored in a physiological salt solution with 0.75 mM Ca^{2+} at 4°C and were used on the same day. Whole-cell currents were measured with the perforated patch method with a patch clamp amplifier (List EPC7; List-Electronic; Darmstadt, Germany) at room temperature. In order to obtain a perforated patch, nystatin (75–200 μg ml^{-1}) was contained in the patch pipette solution. The external salt solution contained (mM): NaCl 126, KCl 6, $MgCl_2$ 1.2, $CaCl_2$ 1.5, HEPES 10 and glucose 11 and the pH was adjusted to 7.2 with NaOH. In potassium-free conditions, 6 mM KCl was omitted and in some experiments 5 mM tetraethylammonium chloride (TEA Cl) was added to the bathing solution. The pipette solution contained (mM): KCl 126, $MgCl_2$ 1.2, HEPES 10, glucose 11 and EGTA 0.2. In potassium-free conditions, 126 mM KCl was replaced by an equimolar amount of NaCl. In experiments where noradrenaline was used, 10^{-6} M propranolol was included in the bathing solution to remove any β-adrenoceptor-mediated response. The recordings were usually made in static bathing solution (i.e. not continuously perfused) and the external solution was changed with a push-pull arrangement with syringes.

Some records were illustrated by playback from videotape onto a Gould brush recorder. Analysis of the time course of spontaneous transient currents was carried out on a personal computer. Signals were filted at 400 Hz prior to digitisation and currents were sampled at 800 Hz using a CED 1401 laboratory interface and captured on the hard disk of the computer. Capture and averaging of signals were performed with SIGAVG signal-averaging programme and curve fitting was done with a voltage clamp analysis programme (both Cambridge Electronic Design, Science Park, Cambridge). Exponential fit was obtained with a sum-of-squares routine. From each cell 10–20 spontaneous chloride or potassium currents were averaged to obtain amplitude and time course values. In the text n values refer to the number of cells used to obtain the mean determinations. The values given in the text are means ± s.e.mean and statistical significance was estimated with either Student's t test or paired t test. Chemicals used were anthracene-9-carboxylic acid (A-9-C), 4,4'-diisothiocyanoto-stilbene-2,2'-disulphonic acid (DIDS), propranol and 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid (SITS) (Aldrich, Gillingham, Dorset), bovine albumin (fatty acid free), caffeine, dithiothreitol, noradrenaline bitartrate, nystatin, papain (type IV), TEA Cl

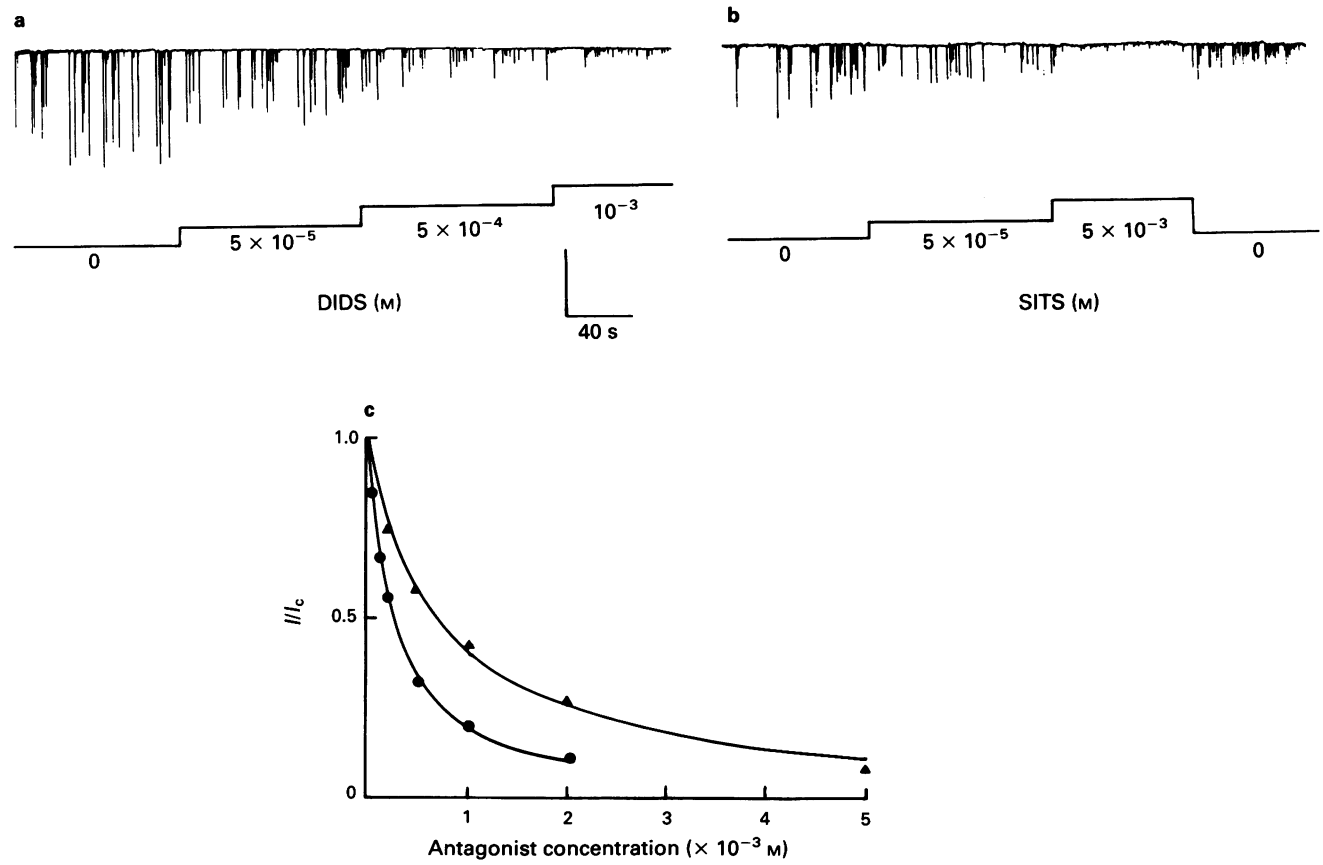


Figure 1 The effect of DIDS and SITS on STIC amplitude. DIDS (a) and SITS (b) were applied in the bathing solution at the concentrations indicated below the records. Holding potential of -77 mV with potassium-containing solution. The vertical calibration bar represents 50 pA for (a) and 10 pA for (b). In (c) the concentration-dependence of both DIDS (●) and SITS (▲) is illustrated. Each point is the mean of 4–8 cells and the s.e.means were less than 15% of the mean value but are not shown for clarity. I and I_c are the amplitude of the current in the presence and absence of the blocker, respectively.

(all Sigma, Poole Dorset); collagenase (CLS2 247 u ml^{-1}, Worthington, Reading, Berkshire).

Results

Effect of DIDS and SITS on STIC amplitude

In the first series of experiments we investigated the effects of DIDS and SITS on spontaneous chloride currents and Figure 1 illustrates the effect of these agents on STIC amplitude. Figure 1a and b show experimental records in which DIDS and SITS reduced the amplitude of STICs in a concentration-dependent manner. The effects of both agents were rapid in onset and seemed to be maximal in the time taken to change the bathing solution (5–10 s). The degree of reversibility was rather variable and depended to some extent on the concentration used and the time for which the agent was in contact with the tissue. In general SITS was washed out more readily than DIDS and a degree of reversibility to SITS is shown in Figure 1b where on removal of this compound the amplitude of the STICs recovered a little after several minutes contact time and with a high concentration (5 mM) of SITS. The concentration-dependence of the block by DIDS and SITS is demonstrated in Figure 1c. Hill plots of the blocking effects gave slopes of 0.9 and 0.7 for DIDS and SITS respectively and the continuous lines in Figure 1c have been drawn according to the conventional drug-receptor scheme using the Hill slope values. Presumably the non unity slopes indicate that 1 molecule binding to 1 receptor site is not a satisfactory explanation for the interaction of these blocking agents although the precise interpretation is unclear. The concentrations required to reduce the amplitude of the currents to 50% of the control value (IC_{50}) were 2.1×10^{-4} M and 6.4×10^{-4} M for respectively DIDS and SITS. The experiment shown in Figure 1 was carried out with potassium-containing solutions with the holding potential at − 77 mV, E_K, to remove calcium-activated potassium currents. In other experiments to study the voltage-dependence of the blockers it was necessary to remove potassium from both the pipette and external solutions (see Methods) but this experimental manipulation did not alter the potency of DIDS and SITS.

In a previous paper it was shown that the inhibition of STIC amplitude by A-9-C was voltage-dependent (Hogg *et al.*, 1993 and see later) and we carried out experiments to ascertain whether this property was shared by DIDS and SITS. Figure 2 shows the inhibitory effect of 10^{-4} M DIDS and 10^{-3} M SITS at holding potentials of − 50 and + 50 mV in potassium-free solutions. From the experimental records in Figure 2 it seems that the reduction of STIC amplitude by both DIDS and SITS is similar at both test potentials and this impression is confirmed by the pooled data given in Table 1. There was no significant difference in the reduction

Table 1 Lack of voltage-dependent inhibitory effect of DIDS and SITS on STICs

	$\frac{I}{I_c}$ *Membrane potential*	
Blocking agent	− 50 mV	+ 50 mV
DIDS 10^{-4} M	0.55 ± 0.07 (8)	0.54 ± 0.08
DIDS 5×10^{-4} M	0.30 ± 0.05 (4)	0.23 ± 0.04
SITS 10^{-3} M	0.43 ± 0.02 (6)	0.44 ± 0.05
SITS 2×10^{-3} M	0.29 ± 0.05 (4)	0.32 ± 0.06

I and I_c are the amplitude of STICs in the presence and absence of the blocking agent, respectively. Number of experiments given in parentheses. For abbreviations, see text.

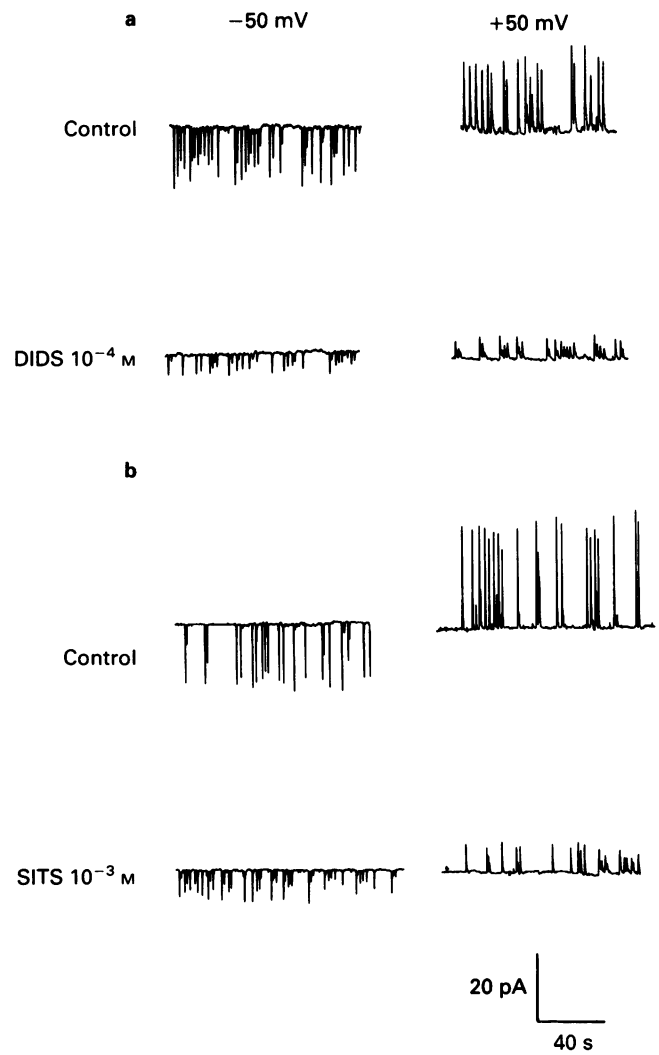


Figure 2 The inhibitory effect of DIDS and SITS on STIC amplitude at − 50 mV and + 50 mV holding potential. Potassium-free conditions. These are typical records in the absence and presence of the stated concentration of the blockers.

of STIC amplitude at − 50 and + 50 mV for two concentrations of both DIDS and SITS and it can be concluded that the reduction of the spontaneous chloride currents by these agents is not voltage-dependent between − 50 and + 50 mV, unlike the effect of A-9-C (Hogg *et al.*, 1993).

Effect of SITS and DIDS on STIC decay time constant

Previously it has been demonstrated that A-9-C prolonged the decay of STICs (Hogg *et al.*, 1993) and experiments were carried out to see if SITS and DIDS also altered the STIC time course. Figure 3a illustrates the effect of 10^{-3} M SITS on averaged STICs at holding potentials of − 50 mV and + 50 mV and the decays of these currents are plotted in Figure 3b. It can be seen that the reduction in STIC amplitude was accompanied by a small increase in the decay time (Figure 3b). In the presence of SITS the decay of the STICs conformed to a single exponential but at − 50 mV, τ increased from a control value of 110 ms to 136 ms and at + 50 mV, τ increased from 230 ms to 284 ms. Thus in the presence of SITS the decay of STICs was lengthened but was still similarly voltage-dependent. At − 50 mV the control τ (τ_c) was 103 ± 9 ms and in the presence of 10^{-3} M SITS the mean τ was 135 ± 13 ms ($n = 7$). At + 50 mV τ_c was 214 ± 28 ms and in SITS τ was 261 ± 29 ms. Normalized results from several experiments are given in Table 2 and it is apparent

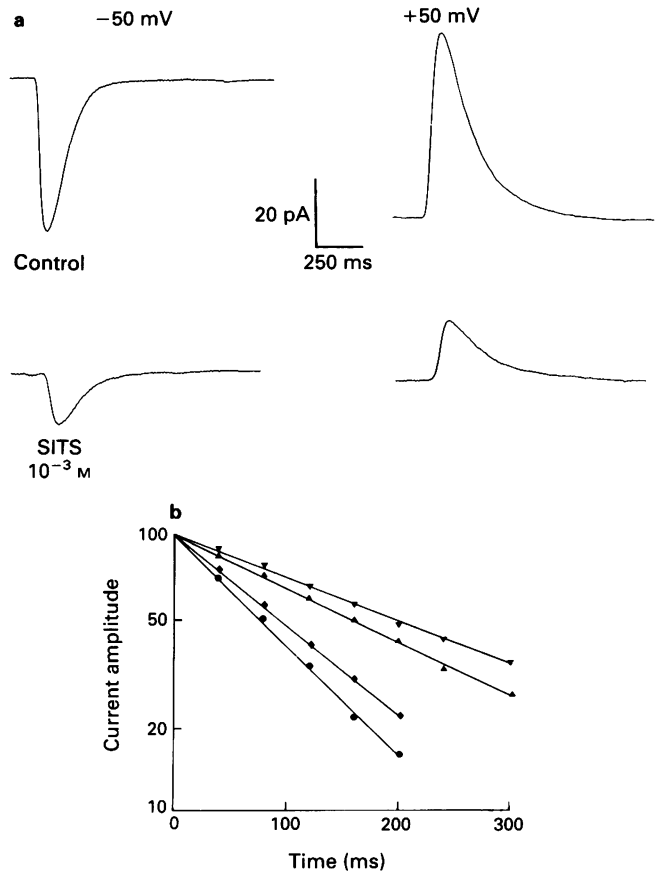


Figure 3 The effects of SITS on the STIC time course. (a) Averaged STICs at − 50 mV and + 50 mV in the absence and presence of 10^{-3} M SITS. (b) The decay of the currents in (a) are plotted against time. Note the logarithmic scale of normalized current amplitude. Controls, (●) − 50 mV, (▲) + 50 mV; SITS (◆) − 50 mV, (▼) + 50 mV. Potassium-free conditions.

Table 2 Effect of blocking agents on STIC decay time constant

	$\frac{\tau}{\tau_c}$ *Membrane potential*	
Blocking agent	− 50 mV	+ 50 mV
SITS 10^{-3} M	1.31 ± 0.04 (7)*	1.21 ± 0.06*
SITS 2×10^{-3} M	1.35 ± 0.07 (4)*	1.21 ± 0.11*
DIDS 10^{-4} M	0.75 ± 0.05 (8)**	0.73 ± 0.05**
DIDS 5×10^{-4} M	0.82 ± 0.05 (8)**	0.81 ± 0.04**
DIDS 10^{-3} M	0.78 ± 0.09 (4)**	0.81 ± 0.08**

*$P<0.01$, **$P<0.05$ represent statistically different values of the time constant of decay in the presence of the drug (τ) compared to the value in the absence of the drug (τ_C).

that SITS produced a similar increase in τ at − 50 and at + 50 mV. However, an interesting observation was that 2×10^{-3} M SITS produced no further increase in τ compared to 10^{-3} M SITS (Table 2) even though the higher concentration produced a greater reduction in the STIC amplitude than the lower concentration (see Figure 1c). Thus the maximal effect on STIC τ was achieved at lower concentrations of SITS than on STIC amplitude.

Figure 4a–c demonstrates the effect of 5×10^{-4} M DIDS on the time course of averaged STICS in one experiment at a holding potential of − 50 mV. In Figure 4c the trace in the presence of DIDS has been scaled up and the currents in Figure 4a and b have been superimposed. It can be seen that the rise times are identical but in the presence of DIDS the STIC decays more rapidly. In Figure 4d it is shown that in the presence of the blocking agent the STIC decays exponentially with a τ value of 81 ms compared to the control τ of 105 ms. In eight experiments, 5×10^{-4} M DIDS reduced τ from a control value of 94 ± 10 ms to 77 ± 11 ms at − 50 mV. At + 50 mV the corresponding values were 220 ± 26 ms (control) and 180 ± 19 ms (DIDS). The normalized data in Table 2 illustrate that the reduction in τ by DIDS was not voltage-dependent as the degree of shortening of the τ values was similar at − 50 mV and + 50 mV. Also it was found that low concentrations reduced both STIC τ and amplitude whereas with higher concentrations of DIDS the increased reduction of STIC amplitude was not accompanied by further shortening of the STIC decay time.

Effect of SITS and DIDS on spontaneous potassium currents

STICs are activated by calcium released from an intracellular caffeine-sensitive store (Wang *et al.*, 1992) and it is possible that blocking agents might affect STICs by an action on the intracellular calcium store. This possibility can be tested directly by studying the action of these compounds on spontaneous transient outward (potassium) currents (STOCs) which are also activated by calcium released from the caffeine-sensitive store (Benham & Bolton, 1986).

Figure 5a and b show the effect of high concentrations of SITS and DIDS on STOCs recorded with potassium-containing solutions at a holding potential of 0 mV (i.e. close to E_{Cl} so that these currents are pure calcium-activated potassium currents). Figure 5c and d are averaged STOCs from Figure 5a and b and it is apparent from this Figure and the pooled data from several experiments shown in Table 3 that the blocking agents had no effect on any of the measured parameters of STOCs. Therefore it can be concluded that it is unlikely that the blocking effect of DIDs and SITS on STICs is mediated by an effect on the intracellular calcium store.

Effect of SITS and DIDS on evoked $I_{Cl(Ca)}$

It was of interest to study the effect of the chloride channel antagonists on $I_{Cl(Ca)}$ evoked by either noradrenaline or caffeine for comparison with their effects on spontaneous currents. Typical records of these experiments are shown in Figure 6a and b and it can be seen that there is a concentration-dependent inhibition of both noradrenaline- and

Table 3 The effect of antagonists on the characteristics of STOCs

Blocking agent	*Amplitude*	*Rise time*	*Half-decay time*
SITS 10^{-3} M	1.0 ± 0.11 (4)	1.01 ± 0.02	0.99 ± 0.11
SITS 2×10^{-3} M	0.88 ± 0.09 (4)	0.99 ± 0.05	0.89 ± 0.09
DIDS 10^{-4} M	1.0 ± 0.01 (4)	1.12 ± 0.18	0.98 ± 0.09
DIDS 5×10^{-4} M	1.08 ± 0.08 (4)	1.06 ± 0.13	1.08 ± 0.08
DIDS 10^{-3} M	1.01 ± 0.09 (3)	1.11 ± 0.06	1.08 ± 0.08

The numbers given are the ratios of the values in the presence of the drug compared to the control values.

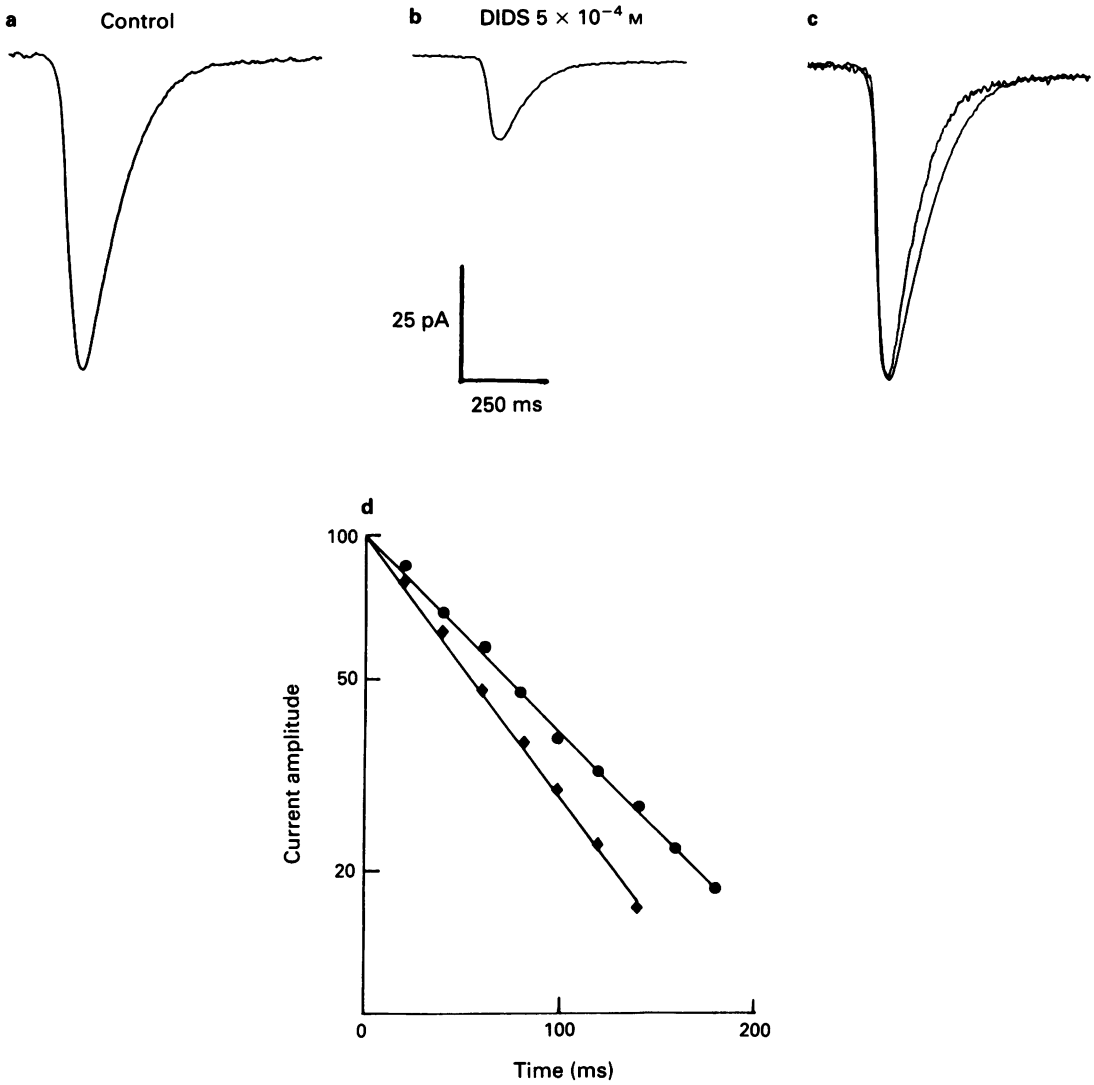


Figure 4 The effects of DIDS on the STIC time course. Records (a) and (b) are averaged STICs recorded at − 50 mV in potassium-free solutions in the absence and presence of 5×10^{-4} M DIDS, respectively. In (c) the current in (b) is scaled up and superimposed over the control STIC in (a). The current with the faster decay was recorded in 5×10^{-4} M DIDS. In (d) the decay of the currents in (a) and (b) are plotted semi-logarithmically and the τ values were 105 ms (control, ●) and 81 ms (DIDS, ◆).

caffeine-induced $I_{Cl(Ca)}$. There did not seem to be any difference in the effects of the antagonists on caffeine- or noradrenaline-evoked currents and therefore the results with caffeine and noradrenaline were pooled. As with STICs there was some reversibility of the inhibitory effect if the contact time was not too great (Figure 6b). The concentration-effect of the inhibitory effect against evoked $I_{Cl(Ca)}$ is shown graphically in Figure 6c and the IC_{50} values for DIDS and SITS were estimated to be 7.5×10^{-4} M and 1.8×10^{-3} M, respectively. A notable consequence of these results is that the IC_{50} values against evoked currents are about 2–3 times higher than the respective values against spontaneous currents (DIDS, 2×10^{-4} M and SITS, 6.4×10^{-4} M). This difference in effect can be seen quite clearly in many of the records shown. For example compare the effect of 10^{-3} M SITS on the evoked current in Figure 6a with the action on the spontaneous current in Figure 3a. An interesting observation is that 5×10^{-4} M SITS appeared to potentiate induced $I_{Cl(Ca)}$ although this effect was not statistically significant.

Effect of DIDS and SITS on evoked calcium-activated potassium currents

The difference in potency of the blocking agents against evoked and spontaneous $I_{Cl(Ca)}$ was a surprising and interesting result and therefore we decided to investigate the effect of SITS and DIDS on caffeine- and noradrenaline-evoked calcium-activated potassium currents ($I_{K(Ca)}$) which are mediated by the same intracellular calcium stores that are responsible for noradrenaline-induced $I_{Cl(Ca)}$ (Wang & Large, 1991b). An additional benefit of these experiments is that it has been shown that noradrenaline stimulates both $I_{Cl(Ca)}$ and $I_{K(Ca)}$ by an action of the α_1-adrenoceptor (Wang & Large, 1991a) and thus possible interference with the pharmacological recognition site can be appraised by studying the effect of the blocking agents on $I_{K(Ca)}$. Figure 7a shows that bath application of 10^{-3} M SITS produced a marked increase in the amplitude of the noradrenaline-evoked $I_{K(Ca)}$. This enhancement of the potassium current was sustained in the continued presence of SITS and was partially reversible after washout of the compound. The enhancement of $I_{K(Ca)}$ was concentration-dependent as the increase of the evoked $I_{K(Ca)}$ was 3.8 ± 1.7 fold and 5.7 ± 1.6 fold for respectively 10^{-3} M and 2×10^{-3} M SITS ($n = 5$ for both concentrations). The increase of $I_{K(Ca)}$ was observed whether noradrenaline or caffeine was used to evoke the current. Figure 7b illustrates that 10^{-4} M DIDS produced a large increase in the amplitude of the caffeine-evoked $I_{K(Ca)}$. DIDS 10^{-4} M and 10^{-3} M increased the amplitude of the evoked $I_{K(Ca)}$ by 3.0 ± 0.6 and 4.0 ± 0.5 times, respectively ($n = 6$ for each concentration).

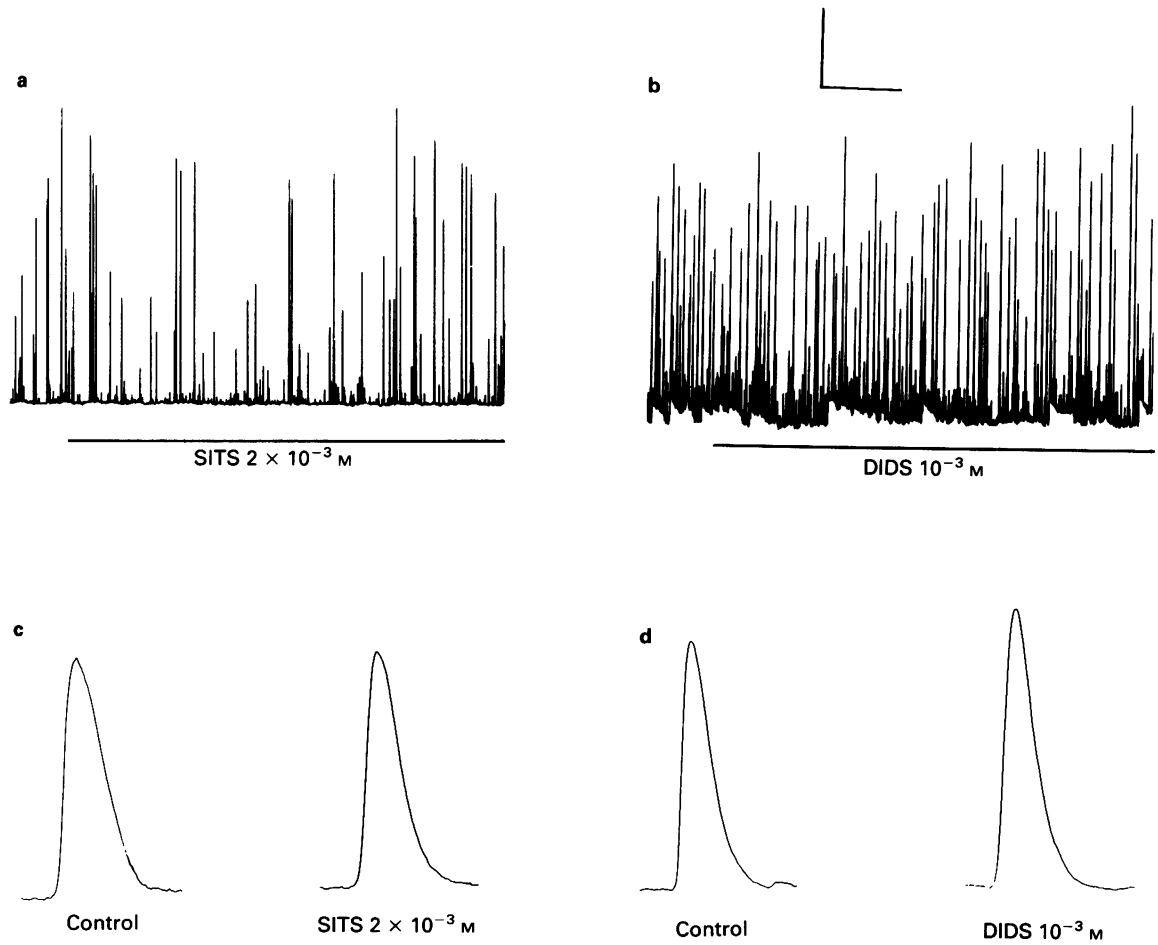


Figure 5 The effect of SITS and DIDS on STOCs. (a) and (b) are experimental records in which SITS and DIDS were applied as indicated by the horizontal bar; (c) and (d) show averaged STOCs taken from the traces in (a) and (b). Vertical calibration, 100 pA for (a) and (c) and 50 pA for (b) and (d). Horizontal calibration, 40 s for (a) and (b) and 100 ms for (c) and (d). Holding potential of 0 mV with potassium-containing solutions.

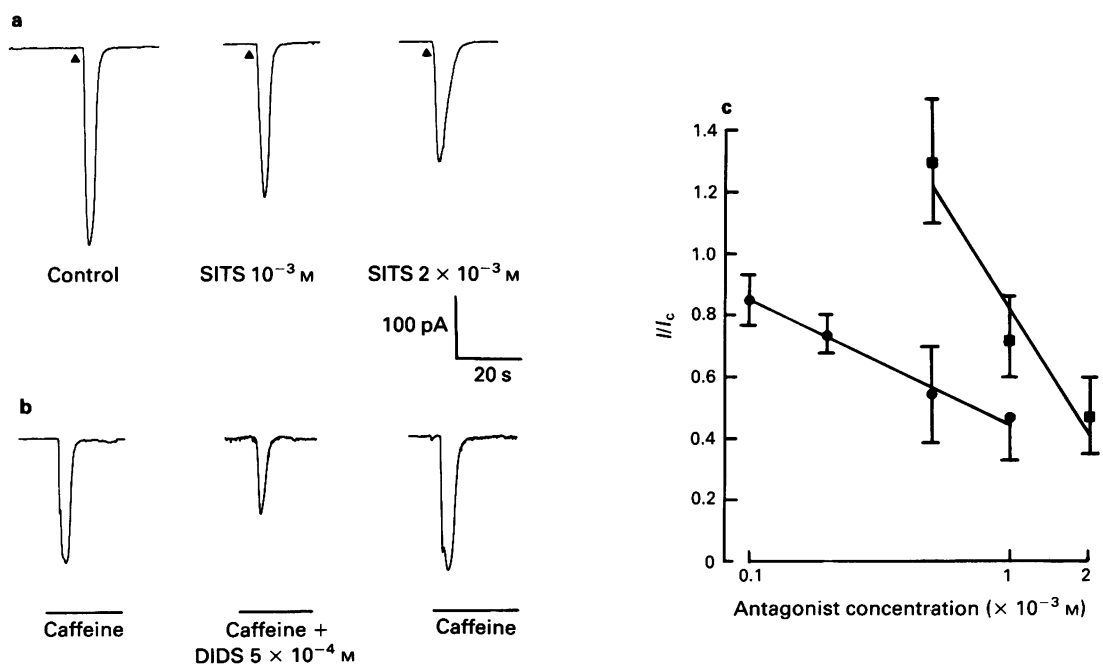


Figure 6 The effect of DIDS and SITS on $I_{Cl(Ca)}$ evoked by noradrenaline and caffeine. In (a), the currents were induced by ionophoretic application of noradrenaline (50 nA for 200 ms) indicated by the arrowheads. In (b) $I_{Cl(Ca)}$ was activated by bath-applied caffeine (5 mM). In these experiments the gap in the records represents about 5 min. Holding potential, − 50 mV in potassium-free solutions. In (c), the concentration-effect relationship is plotted. I and I_c are the current amplitudes in the presence and absence of the antagonists respectively and a ratio of I/I_c greater than 1.0 represents potentiation. Each point is the mean of 4–6 cells and the lines were drawn by eye: (■) SITS; (●) DIDS.

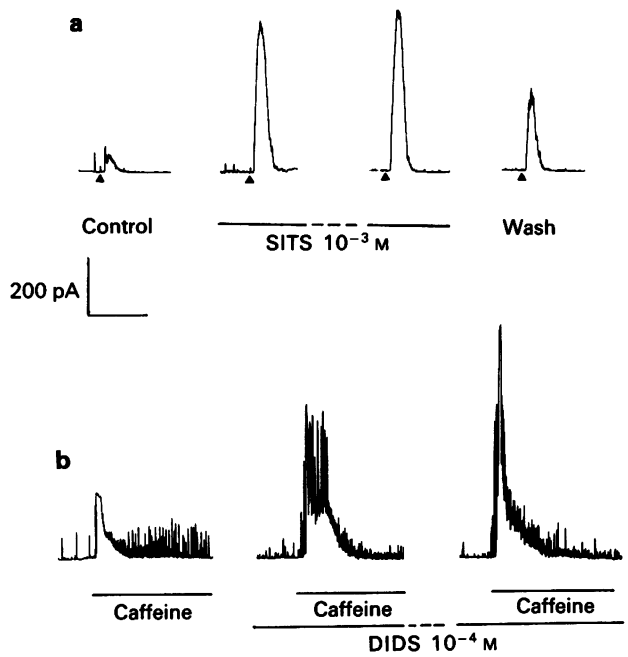


Figure 7 The action of SITS and DIDS on $I_{K(Ca)}$ evoked by noradrenaline and caffeine. In (a) noradrenaline was applied by ionophoresis at arrowheads (50 nA for 400 ms). The middle records were obtained 3 and 6 min after 10^{-3} M SITS had been added to the bathing solution and the wash was recorded 8 min after the antagonist had been removed. In (b) 10^{-3} M caffeine was bath-applied to activate the currents before, 1 and 4 min after 10^{-4} M DIDS had been included in the external solution. Horizontal calibration bar, 10 s for (a) and 20 s for (b). Holding potential of 0 mV in potassium-containing solutions.

The effect of A-9-C on spontaneous and evoked $I_{Cl(Ca)}$ *and* $I_{K(Ca)}$

Preliminary data from Hogg *et al.* (1993) showed that 2×10^{-4} M A-9-C produced a voltage-dependent decrease in STIC amplitude and increase in STIC decay time constant. We have extended that work to obtain IC_{50} values of A-9-C on spontaneous and evoked calcium-activated currents. The estimated IC_{50} values of A-9-C against STICs at + 50 and − 50 mV were 9×10^{-5} M and 3×10^{-4} M, respectively and therefore the potency of A-9-C on STIC amplitude increased 3.3 fold for a 100 mV change in membrane potential. The voltage-dependence of the effect of A-9-C on amplitude was

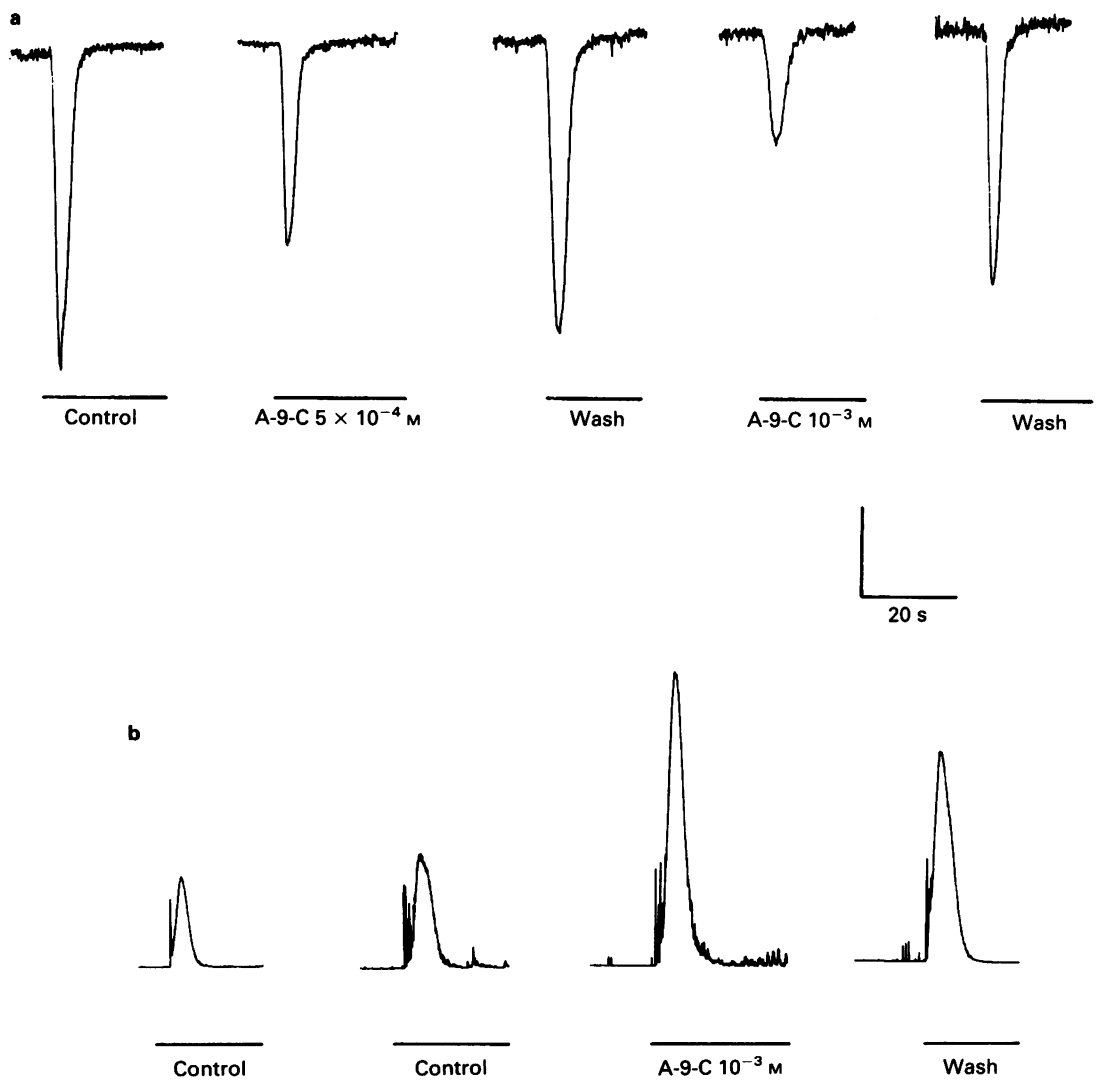


Figure 8 The action of A-9-C on caffeine-evoked $I_{Cl(Ca)}$ and $I_{K(Ca)}$. Bath-applied caffeine (5×10^{-3} M) was added (shown by horizontal bars) to evoke currents in both (a) and (b). In (a) $I_{Cl(Ca)}$ was induced in potassium-free conditions at − 50 mV and the gap between each record represents about 5 min. (b) $I_{K(Ca)}$ was recorded with potassium-containing solutions at 0 mV. Two control records are illustrated in this experiment to demonstrate the reproducibility of the responses. Vertical calibration bar, 50 pA in (a) and 100 pA in (b).

accompanied by a voltage-dependent increase in STIC decay time. With 10^{-4} M A-9-C τ was increased from a control value of 91 ± 9 ms to 101 ± 16 ms at − 50 mV ($n = 7$). τ_c was 201 ± 16 ms at + 50 mV and τ was 496 ± 52 ms in the presence of 10^{-4} M A-9-C. Thus the increase in τ produced by 10^{-4} M A-9-C was 1.09 ± 0.09 fold at − 50 mV and 2.47 ± 0.27 fold at + 50 mV. With 2×10^{-4} M A-9-C, τ increased by 1.20 ± 0.07 times at − 50 mV and 2.65 ± 0.13 times at + 50 mV ($n = 7$, data from Table 2, Hogg *et al.*, 1993). Therefore as with DIDS and SITS, reduction of amplitude by A-9-C was not associated with a change in τ throughout the full dose-response curve. It is possible that this characteristic might have been unmasked with higher concentrations of A-9-C but an unfavourable signal to noise ratio precluded the analysis. Other experiments with potassium-containing solutions at 0 mV demonstrated that 2×10^{-4} M, 5×10^{-4} M and 10^{-3} M A-9-C ($n = 3$ for each concentration) had no effect on STOCs.

In the light of the results with DIDS and SITS we carried out further experiments to ascertain the effect of A-9-C on evoked $I_{Cl(Ca)}$ and $I_{K(Ca)}$. The traces in Figure 8 show the results from 2 experiments. In potassium-free conditions at a holding potential of − 50 mV A-9-C produced a concentration-dependent decrease of the caffeine-evoked $I_{Cl(Ca)}$ (Figure 8a). In 7 experiments, 5×10^{-4} M and 10^{-3} M A-9-C decreased the evoked $I_{Cl(Ca)}$ to 0.55 ± 0.1 and 0.42 ± 0.07 of the control currents, respectively. Assuming a linear relationship between the degree of inhibition and concentration in this concentration-range, the estimated IC_{50} is about 6.5×10^{-4} M A-9-C. This value against evoked currents is about double the IC_{50} (3×10^{-4} M at − 50 mV) for A-9-C against STICs. In potassium-containing solutions at a holding potential of 0 mV, 10^{-3} M A-9-C increased the amplitude of the caffeine-evoked $I_{K(Ca)}$ which was only partially reversible after washout of A-9-C (Figure 8b). The increase in the amplitude of the evoked $I_{K(Ca)}$ by 5×10^{-4} M and 10^{-3} M A-9-C was 1.84 ± 0.07 times and 2.08 ± 0.34 times, respectively ($n = 4$ for each concentration). Therefore it can be concluded that A-9-C enhanced the amplitude of the evoked $I_{K(Ca)}$ in the same concentration-range which reduced $I_{Cl(Ca)}$.

Discussion

The two main aims of the present work were first to characterize pharmacologically $I_{Cl(Ca)}$ in the rabbit portal vein for comparison with other chloride conductances. Secondly, we attempted to gain an insight into the mechanism of action of some commonly-used chloride channel antagonists on $I_{Cl(Ca)}$ in vascular smooth muscle. It was hoped that such experiments might reveal important characteristics associated with blockade of $I_{Cl(Ca)}$ in smooth muscle. There have been only a few quantitative studies on the pharmacological blockade of $I_{Cl(Ca)}$ in smooth muscle and many reports are limited to a single concentration of an antagonist. Nevertheless, we will compare the IC_{50} values of the antagonists on STICs from the present work with previous published data. Baron *et al.* (1991) investigated the effects of DIDS and A-9-C on $I_{Cl(Ca)}$ evoked by either caffeine or the influx of calcium through voltage-dependent calcium channels in the rat portal vein. They reported IC_{50} values of 16.5 μM for DIDS and 117 μM for A-9-C. The values for A-9-C in the previous paper and the present investigation (3×10^{-4} M against STICs) are comparable. However, the earlier value for DIDS is an order less then the value against STICs from the present work (2.1×10^{-4} M). Against noradrenaline- or caffeine-evoked $I_{Cl(Ca)}$ the estimated IC_{50} for DIDS was even higher (7.5×10^{-4} M) in our study (see later). There is no obvious explanation for the increased potency of DIDS observed in the rat portal vein compared to the rabbit portal vein except for a difference in species and experimental conditions. In rat intestine it was shown that 0.1 mM DIDS and 0.5 mM SITS reduced $I_{Cl(Ca)}$ by 50% and 39%, respectively (Ohta *et al.*, 1993) and therefore these results are comparable to the present data. Akbarali & Giles (1993) also reported that 300 μM A-9-C reduced $I_{Cl(Ca)}$ in rabbit oesophageal smooth muscle but the data are far too limited at present to make comparisons with the present results. In other tissues it was claimed that 10^{-4} M DIDS blocked totally $I_{Cl(Ca)}$ in rabbit ventricular myocytes (Zygmunt & Gibbons, 1991). Since this concentration reduced the STIC amplitude to only 55% of the control value there may be a difference between the pharmacology of $I_{Cl(Ca)}$ in ventricle and venous muscle. In cystic fibrosis epithelial cells, 0.5 mM DIDS reduced $I_{Cl(Ca)}$ by 87% (Anderson & Welsh, 1991) and 1–2 mM SITS produced a significant but incomplete block of $I_{Cl(Ca)}$ in avian neurones (Bader *et al.*, 1987) and cultured pituitary cells (Korn & Weight, 1987). The degree of inhibition of $I_{Cl(Ca)}$ by the stilbene derivatives in these tissues are similar to the reduction of STICs in the rabbit portal vein produced by the same concentrations of the antagonists in the present study. These findings indicate a similar sensitivity of $I_{Cl(Ca)}$ to DIDS and SITS in epithelial, neuronal and smooth muscle cells.

Some interesting observations arise from comparison with other types of chloride conductances. For example it appears that DIDs does not inhibit the adenosine 3':5'-cyclic monophosphate (cyclic AMP)-mediated chloride current in cystic fibrosis airway epithelia (Anderson & Welsh, 1991). In cardiac myocytes, 100 μM SITS and DIDS actually enhanced the isoprenaline-induced chloride current which is also mediated by cyclic AMP (Harvey, 1993) although this conductance is sensitive to A-9-C (Harvey & Hume, 1989). In contrast, the voltage-gated chloride conductance in rat cultured astrocytes is significantly inhibited by low concentrations of DIDS (20 μM) but is insensitive to A-9-C (Gray & Ritchie, 1986). Consequently it is apparent that different chloride conductances have quite disparate sensitivities to the stilbene derivatives and A-9-C and although these agents are not potent, their use helps to establish the pharmacological profiles of different chloride conductances. It should be noted that many useful channel blocking chemicals possess low potency and have IC_{50} values in the range of 0.1–10 mM (e.g. tetraethylammonium on potassium channels).

The study of the blocking agents on the spontaneous chloride currents showed that the three compounds had quite distinctive effects on STICs. None of the inhibitors in the concentrations used altered the amplitude, rise time or half-decay of the spontaneous calcium-activated potassium currents which indicates that the blockers did not act on the intracellular calcium store (which is the primary source of calcium for triggering both STOCs and STICs) or on the potassium channels. It is possible that these agents inhibited the STICs by blocking the binding site with which calcium combines to open the chloride channel. Little is known about this site and we cannot rule out this possibility but there is some evidence that A-9-C, at least, inhibited the chloride currents by blocking the channel itself. A-9-C produced a voltage-dependent reduction in amplitude and lengthening of the decay phase of the spontaneous chloride currents which was readily reversible. Voltage-dependent reduction of a membrane current is normally interpreted to mean that molecules of the blocking agent bind to a receptor site within the membrane electrical field. The rapid onset of effect of A-9-C suggests that the most likely locus for its action is the channel pore itself and thus the data suggest that as the inside of the membrane is made more positive, A-9-C is driven into the chloride channel to produce a greater block. A corollary of this hypothesis is that it is the negatively charged ion of A-9-C that is responsible but further experiments with pH changes are necessary to confirm this conclusion.

There is less evidence to suggest that DIDS and SITS act as channel blockers. These agents produced only a small change in the STIC τ value and a clear concentration-dependence was not obtained. The fact that they did not block the currents completely, that the Hill plots are non-

linear and the poor reversibility indicate that these compounds have a more complicated action. It is intriguing that DIDS and SITS are inhibitors of both anion transporters and chloride channels. It is tempting to speculate that they bind to something in common in both these membrane mechanisms, perhaps the anion recognition site.

An unexpected finding was that all three blocking agents inhibited spontaneous chloride currents more readily than the response evoked by caffeine and noradrenaline. The IC_{50} values were 2–4 fold higher for the evoked compared to the spontaneous currents. A possible explanation for this discrepancy is that there are two classes of calcium-activated chloride channels in rabbit portal vein cells. However, since STICs and evoked $I_{Cl(Ca)}$ are triggered by calcium from the same intracellular store and the permeability ratios of various anions for spontaneous and evoked currents are similar (Wang & Large, 1991a; Wang *et al.*, 1992), we feel that this proposal is unlikely. A more attractive hypothesis is that in addition to blocking $I_{Cl(Ca)}$ in the sarcolemma, the agents may also increase the amount of calcium that is released from the sarcoplasmic reticulum in response to the agonists caffeine and noradrenaline which may tend to counteract the blockade of $I_{Cl(Ca)}$. All of the compounds produced a marked increase in the amplitude of $I_{K(Ca)}$ evoked by caffeine and noradrenaline. Since there was no effect on the calcium-activated potassium currents which were triggered by the spontaneous release of calcium, the increase of the evoked-$I_{K(Ca)}$ is presumably due to an increased release of calcium from the sarcoplasmic reticulum rather than a direct effect on the potassium channels. No direct measurement of calcium was made in the present work but recently it has been shown in T84 cells that stilbene derivatives elevate the intracellular calcium concentration which activates an anion conductance (Brayden *et al.*, 1993). Also it has been shown that DIDS and SITS open the Ca-release channel of the sarcoplasmic reticulum in skeletal muscle (Kawasaki & Kasai, 1989). If this action occurred in smooth muscle it is possible that chloride channel antagonists increased the amount of caffeine- and noradrenaline-evoked release of calcium from the sarcoplasmic reticulum which tended to overcome to some extent the blockade of $I_{Cl(Ca)}$ in the sarcolemma produced by the same compounds.

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References

AICKIN, C.C. (1990). Chloride transport across the sarcolemma of vertebrate smooth and skeletal muscle. In *Chloride Channels and Carriers in Nerve, Muscle and Glial cells*. ed. Alvarez-Leefmans, F.J. & Russell, J.M. New York: Plenum Press.

AKBARALI, H.I. & GILES, W.R. (1993). Ca^{2+} and Ca^{2+}-activated Cl^- currents in rabbit oesophageal smooth muscle. *J. Physiol.*, **460**, 117–133.

AMÉDÉE, T., BENHAM, C.D., BOLTON, T.B., BYRNE, N.G. & LARGE, W.A. (1990a). Potassium chloride and non-selective cation conductances opened by noradrenaline in rabbit ear artery cells. *J. Physiol.*, **423**, 551–568.

AMÉDÉE, T., LARGE, W.A. & WANG, Q. (1990b). Characteristics of chloride currents activated by noradrenaline in rabbit ear artery cells. *J. Physiol.*, **428**, 501–516.

ANDERSON, M.P. & WELSH, M.J. (1991). Calcium and cAMP activate different chloride channels in the apical membrane of normal and cystic fibrosis epithelia. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 6003–6007.

BADER, C.R., BERTRAND, D. & SCHLICHTER, R. (1987). Calcium-activated chloride current in cultured sensory and parasympathetic quail neurones. *J. Physiol.*, **394**, 125–148.

BADER, C.R., BERTRAND, D. & SCHWARTZ, E.A. (1982). Voltage-activated and calcium-activated currents studied in solitary rod inner segments from the salamander retina. *J. Physiol.*, **331**, 253–284.

BARON, A., PACAUD, P., LOIRAND, G., MIRONNEAU, C. & MIRONNEAU, J. (1991). Pharmacological block of Ca^{2+}-activated Cl^- current in rat vascular smooth muscle cells in short term primary culture. *Pflügers Archiv.*, **419**, 553–558.

BENHAM, C.D. & BOLTON, T.B. (1986). Spontaneous transient outward currents in single visceral and vascular smooth muscle cells of the rabbit. *J. Physiol.*, **381**, 385–406.

BRAYDEN, D.J., KROUSE, M.E., LAW, T. & WINE, J.J. (1993). Stilbenes stimulate T85 Cl^- secretion by elevating Ca^{2+}. *Am. J. Physiol.*, **264**, G325–G333.

BYRNE, N.G. & LARGE, W.A. (1987a). Action of noradrenaline on single smooth muscle cells freshly dispersed from the rat anococcygeus muscle. *J. Physiol.*, **389**, 513–525.

BYRNE, N.G. & LARGE, W.A. (1987b). Membrane mechanism associated with muscarinic receptor activation in single cells freshly dispersed from the rat anococcygeus muscle. *Br. J. Pharmacol.*, **92**, 371–379.

BYRNE, N.G. & LARGE, W.A. (1988). Membrane ionic mechanisms activated by noradrenaline in cells isolated from the rabbit portal vein. *J. Physiol.*, **404**, 557–573.

GRAY, P.T.A. & RITCHIE, J.M. (1986). A voltage-gated chloride conductance in rat cultured astrocytes. *Proc. R. Soc. B.*, **228**, 267–288.

HARVEY, R.D. (1993). Effects of stilbenedisulfonic acid derivatives on the cAMP-regulated chloride current in cardiac myocytes. *Pflügers Arch.*, **422**, 436–442.

HARVEY, R.D. & HUME, J.R. (1989). Autonomic regulation of a chloride current in heart. *Science*, **244**, 983–985.

HOGG, R.C., WANG, Q. & LARGE, W.A. (1993). Time course of spontaneous calcium-activated chloride currents in smooth muscle cells from the rabbit portal vein. *J. Physiol.*, **464**, 15–31.

JANSSEN, L.J. & SIMS, S.M. (1992). Acetylcholine activates non-selective cation and chloride conductances in canine and guinea-pig tracheal myocytes. *J. Physiol.*, **453**, 197–218.

KAWASAKI, T. & KASAI, M. (1989). Disulfonic stilbene derivatives open the Ca^{2+} release channel of sarcoplasmic reticulum. *J. Biochem.*, **106**, 401–405.

KORN, S.J. & WEIGHT, F.F. (1987). Patch-clamp study of the calcium-dependent chloride current in AtT-20 pituitary cells. *J. Neurophysiol.*, **58**, 1431–1451.

MILEDI, R. (1982). A calcium-dependent outward current in Xenopus laevis oocytes. *Proc. R. Soc. B.*, **215**, 491–497.

OHTA, T., ITO, S. & NAKAZATO, Y. (1993). Chloride currents activated by caffeine in rat intestinal smooth muscle cells. *J. Physiol.*, **465**, 149–162.

WANG, Q., HOGG, R.C. & LARGE, W.A. (1992). Properties of spontaneous inward currents in smooth muscle cells isolated from the rabbit portal vein. *J. Physiol.*, **451**, 535–537.

WANG, Q. & LARGE, W.A. (1991a). Noradrenaline-evoked cation conductance recorded with the nystatin whole-cell method in rabbit portal vein cells. *J. Physiol.*, **435**, 21–39.

WANG, Q. & LARGE, W.A. (1991b). Modulation of noradrenaline-induced membrane currents by papaverine in rabbit vascular smooth muscle cells. *J. Physiol.*, **439**, 501–512.

ZYGMUNT, A.C. & GIBBONS, W.R. (1991). Calcium-activated chloride current in rabbit ventricular myocytes. *Circ. Res.*, **68**, 424–437.

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Investigation of the specificity of FK 888 as a tachykinin NK_1 receptor antagonist

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1 A recently described peptide tachykinin (NK_1) receptor antagonist, FK 888, was found to inhibit the electrically-evoked, tachykinin-mediated contractile responses of the rabbit iris sphincter in a concentration-dependent manner; the pIC_{50} value was 6.6 ± 0.08.

2 Contractions induced by a selective NK_1 receptor agonist, [Sar^9,Met(O_2)^{11}]substance P, were inhibited competitively by FK 888; the pK_B value was 7.1.

3 FK 888 (1 nM–100 μM) was without effect on the electrically-evoked, cholinergic response of the rabbit iris sphincter and the electrically-evoked, sympathetic response of the guinea-pig vas deferens. The contractions of the rabbit iris sphincter, induced by either carbachol (10 nM–30 μM) or noradrenaline (0.1–100 μM), were not affected by 10 μM FK 888.

4 FK 888 (1–30 μM) did not induce histamine release from rat peritoneal mast cells.

5 FK 888 (33 and 333 μM) was without effect on the electrically-evoked action potentials of the frog sciatic nerve. Thus, FK 888 is a moderately high affinity and selective tachykinin (NK_1) receptor antagonist.

Keywords: FK 888; tachykinin (NK_1) receptor antagonist; rabbit iris sphincter; guinea-pig vas deferens; rat peritoneal mast cells; frog sciatic nerve

Introduction

The mammalian tachykinins, substance P (SP), neurokinin A (NKA) and neurokinin B (NKB), are the preferred endogenous agonists at receptors designated NK_1, NK_2 and NK_3, respectively (for reviews see Maggio, 1988; Guard & Watson, 1991; Maggi *et al.*, 1993; Otsuka & Yoshioka, 1993). During recent years, considerable progress has been made towards the development of tachykinin receptor antagonists. CP-96,345 (Snider *et al.*, 1991), RP 67580 (Garret *et al.*, 1991) (both selective for NK_1 receptors) and SR 48968 (selective for NK_2 receptors) (Emonds-Alt *et al.*, 1992) have attracted much interest, since they represent the first generation of potent, non-peptide, tachykinin receptor antagonists (Watling, 1992). Several high affinity peptide antagonists, such as spantide II (Folkers *et al.*, 1990; Håkanson *et al.*, 1990; 1991) and FK 888 (Fujii *et al.*, 1992; Hirayama *et al.*, 1993), have also recently been described.

However, non-peptide antagonists have been found to possess effects not related to tachykinin receptor antagonism (Lecci *et al.*, 1991; Nagahisa *et al.*, 1992; Schmidt *et al.*, 1992; Wang & Håkanson, 1992a; Guard *et al.*, 1993; Tamura *et al.*, 1993), including non-specific effects on neurotransmission (Wang & Håkanson, 1992a; Wang *et al.*, 1994) and local anaesthetic-like actions (Caeser *et al.*, 1993; Tamura *et al.*, 1993; Wang *et al.*, 1994). The concentrations needed to exert these non-specific actions are approximately 10 times higher than those needed to produce blockade of tachykinin receptors (Wang & Håkanson, 1992a; Wang *et al.*, 1994).

In the present study, we have investigated the effects of the recently described peptide tachykinin (NK_1) receptor antagonist, FK 888, on both endogenous and exogenous tachykinin-mediated contractile responses of the rabbit iris sphincter muscle. A series of experiments was also performed to examine the pharmacological selectivity of FK 888.

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Methods

General

Adult pigmented rabbits of either sex (1.5–3.0 kg) and male guinea-pigs (200–250 g) were killed by a blow on the neck and exsanguinated. The iris sphincter muscle of the rabbit and the vas deferens of the guinea-pig were dissected out and each was mounted vertically in a perspex holder in modified Krebs solution of the following composition (mM): NaCl 133, $NaHCO_3$ 16.3, KCl 4.7, $MgCl_2$ 1.0, NaH_2PO_4 1.4, $CaCl_2$ 2.5 and glucose 7.8 (Stjernquist *et al.*, 1983; Wahlestedt *et al.*, 1985). The preparations were stretched with a force of 1.5 mN (iris sphincter) or 5 mN (vas deferens). Before the start of each experiment, the preparations were allowed to equilibrate for 60–90 min.

Electrically-evoked contractile responses in the rabbit iris sphincter and the guinea-pig vas deferens

Electrical stimulation with square wave pulses (25 V, voltage drop 14–17 V over the electrodes, 0.3–1 ms duration) (Stjernquist *et al.*, 1983; Fujiwara *et al.*, 1984; Wahlestedt *et al.*, 1985) was applied to both tissues by means of platinum electrodes connected to a Grass S4C stimulator. All electrically-evoked responses were abolished by 1 μM tetrodotoxin (TTX) (Stjernquist *et al.*, 1983; Leander & Håkanson, 1985; Wahlestedt *et al.*, 1985). Drugs were given in a cumulative manner. Two pieces of iris sphincter from the same eye or two pieces of vas deferens from the same animal were mounted in separate baths, one segment being exposed to drugs and the other to the corresponding vehicle (control preparation). The preparations were exposed to each concentration of drug or vehicle for 15 min prior to electrical stimulation.

Contractile responses to [Sar^9,Met(O_2)^{11}] SP, carbachol or noradrenaline in the rabbit iris sphincter

Concentration-response curves were constructed by cumulative application of agonists. Ony one concentration-response curve for [Sar^9,Met(O_2)^{11}] SP (a selective NK_1 recep-

tor agonist) (Regoli *et al.*, 1988) was obtained from each individual preparation since, in preliminary experiments, this agonist was found to cause long-lasting (2–3 h) contraction even after extensive washing with fresh Krebs solution every 15 min. Thereafter, the response of the preparation to a second application of drugs was reduced dramatically. Parallel experiments were carried out on two preparations from the same eye mounted in separate baths, with one concentration of FK 888 (15 min preincubation time) or with the corresponding vehicle.

In another series of experiments, concentration-response curves for either carbachol (10 nM–30 μM) or noradrenaline (0.1–100 μM) were constructed in the presence of 10 μM FK 888 or vehicle as above.

Histamine release from rat peritoneal cells

Experiments were conducted as described previously (Håkanson *et al.*, 1983; 1990; Folkers *et al.*, 1984). Briefly, a male Sprague-Dawley rat (250–300 g) was killed by decapitation following light diethyl ether anaesthesia. Peritoneal saline washings were collected and the cells, consisting of 3–6% mast cells (Kurose & Saeki, 1981), were spun down at 350 *g* for 5 min in a centrifuge and washed twice in a buffer solution containing (mM) NaCl 145, KCl 2.7, Na_2HPO_4 6.7 and KH_2PO_4 6.7 (pH 6.8). The cells were suspended in 10 ml of the buffer to which was added 0.1% bovine serum albumin and 1 mM $CaCl_2$ at room temperature. Aliquots of 0.9 ml were transferred to polypropylene test tubes and preincubated for 5 min at 37°C before adding 0.1 ml of a solution containing FK 888 or vehicle (see Drugs). Experiments were performed in duplicate. The mixture was incubated for a further 5 min and the reaction was terminated by placing the samples on ice. Samples were then centrifuged at 600 *g* for 5 min at 4°C. Aliquots (0.1 ml) of the supernatant were taken for fluorometric assay of histamine (Håkanson & Rönnberg, 1974). The cell pellet was suspended in 1 ml redistilled water (causing lysis of the cells) and centrifuged at 600 *g* for 5 min. Aliquots of the supernatant were also taken for histamine determination. The histamine release is expressed as a percentage of the total amount of histamine present in the peritoneal cells.

Electrically-evoked action potentials in the frog sciatic nerves

Bullfrogs (*Rana catesbeiana*) were killed by decapitation following hypothermic general anaesthesia (30 min at − 20°C). The sciatic nerves were excised, desheathed and split longitudinally into two bundles. Each preparation was mounted in a sucrose-gap chamber as previously described (Strong *et al.*, 1978; Hahin & Strichartz, 1981). One end of the nerve was stimulated in drug-free Ringer solution with Ag/AgCl or platinum electrodes, using square-wave supermaximal current pulses of 0.05 ms duration (Grass Instrument Co., Braintree, MA, Model S44). Similar Ag/AgCl or platinum electrodes were used to record potential changes between two regions of the nerve separated by a sucrose gap. These changes in potential were amplified and recorded as compound action potentials on an a.c.-coupled, dual-beam storage oscilloscope (Tektronix 5113). All sucrose-gap experiments were conducted at 21–24°C.

The nerve was first stimulated to determine a supramaximal stimulus. Next, the flow of sucrose was started, rapidly at first (20–30 ml min^{-1} for 30 s) to flush out the system, and then maintained at 2–3 ml min^{-1} for the duration of the experiment. The test chamber held 350 μl solution. During the stabilization period, the solution in the test chamber was changed twice with normal frog Ringer solution to eliminate potential solution switching artifacts. This baseline period usually lasted 20–30 min. Afterwards, stimulation at 1 s^{-1} showed steady compound action potentials, changing by less than 5% over a 15 min period. Compound action potentials were measured in frog Ringer solution containing (mM): NaCl 110, KCl 2.5, $CaCl_2$ 2.0, morpholino-propanesulphonic acid 5.0, the pH was adjusted to 7.2 with NaOH. Inhibition in the presence of drug was expressed as a fraction of the initial control compound action potential amplitude.

Drugs

FK 888, a synthetic dipeptide with the structure N^2-[(4**R**)-4-hydroxy-1-(1-methyl-1H-indol-3-yl)carbonyl-L-prolyl]-*N*-methyl-*N*-phenylmethyl-3-(2-napthyl)-L-alaninamide, was a gift from Dr T. Fujii, Fujisawa Pharmaceutical, Osaka, Japan. Atropine was from Alcon, TX, U.S.A. and guanethidine from CIBA-Geigy, Basel, Switzerland. FK 888 was dissolved in dimethylsulphoxide (DMSO) at a concentration of 0.1 M and diluted to 0.01 M with a solvent consisting of 10% DMSO, 20% Tween 80 and 70% redistilled water (v/v). This solution could be further diluted with redistilled water as required. For the study of histamine release, FK 888 was dissolved in 50% DMSO in redistilled water at a concentration of 1 mM and then diluted with redistilled water, to avoid any possible influence of Tween 80. [Sar^9,$Met(O_2)^{11}$] SP (Peninsula, Merseyside, U.K.) was dissolved in pure DMSO at 0.01 M and diluted with redistilled water. NKA (Peninsula, Merseyside, U.K.) was dissolved in redistilled water.

Analysis of results

Drug-exposed and vehicle-exposed preparations were stimulated in parallel. Repeated electrical stimulations caused reproducible cholinergic-mediated contractile responses of the rabbit iris sphincter and sympathetic-mediated contractile responses of the guinea-pig vas deferens throughout the duration of the experiment. However, repeated electrical stimulations led to a gradual reduction (fatigue) in tachykinin-mediated contractile responses of the rabbit iris sphincter (Håkanson *et al.*, 1987). Tachykinins are synthesized in the nerve cell body and transported to the nerve endings (see Gamse *et al.*, 1982) and it is likely that tachykinin-mediated responses are more rapidly exhausted than responses that involve acetylcholine or sympathetic transmitters, which are being continuously resynthesized locally. Thus, in the present study, the contractile responses of drug-exposed preparations were expressed as a percentage of those of parallel control preparations. Concentration-response curves were constructed and the pIC_{50} values (the negative logarithm of the molar concentration of the antagonist producing 50% inhibition of the electrically-evoked contraction) were estimated by linear regression analysis of the results in the 10–90% response interval.

The contractions of the rabbit iris sphincter induced by the direct application of either [Sar^9,$Met(O_2)^{11}$] SP, carbachol or noradrenaline were expressed as a percentage of the response to 0.1 μM NKA (submaximal concentration). NKA was chosen instead of SP because both agonists are recognized by NK_1 receptors and because the response to the former was reversed rapidly while that to the latter was slow to decline. Data from each experiment were combined and calculated as mean values ± s.e.mean. The pD_2 value is the negative logarithm of the molar concentration of agonist producing 50% of the maximal response. The pK_B value, the negative logarithm of the affinity of antagonist, was determined by the Gaddum-Schild equation (Kenakin, 1984). Regression analysis was used to show whether or not the slope of the Schild plot was significantly different from unity.

Results

Effects of FK 888 on tachykinin-mediated responses of the electrically-stimulated rabbit iris sphincter

In the presence of 1 μM atropine and 5 μM guanethidine, the contractile response of the rabbit iris sphincter to electrical

stimulation (20 Hz, 25 V, 10 s) is mediated by tachykinins (Wahlestedt *et al.*, 1985; Wang & Håkanson, 1992b). FK 888 inhibited the tachykinin-mediated contraction in a concentration-dependent manner (1 nM–3 μM) (Figure 1), possessing a pIC_{50} value of 6.6 ± 0.08 ($n = 6$).

Effects of FK 888 on the contractile responses to [Sar⁹,Met(O₂)¹¹] SP in the rabbit iris sphincter

[Sar⁹,Met(O₂)¹¹] SP contracted the rabbit iris sphincter in a concentration-dependent manner (0.1–300 nM) displaying a pD_2 value of 8.2 ± 0.04 ($n = 6$). FK 888 (1, 3 and 10 μM) competitively inhibited the contractions with a pK_B value of 7.1; Schild plot analysis revealed a slope of 0.99, which was not significantly different from unity (Figure 2).

Effects of FK 888 on the contractile responses to carbachol and noradrenaline in the rabbit iris sphincter

Both carbachol (0.01–30 μM) and noradrenaline (0.1–100 μM) contracted the rabbit iris sphincter in a concentration-dependent manner. FK 888, at 10 μM, was without effect on these contractions (data not shown).

Effects of FK 888 on electrically-evoked, non-tachykinin-mediated contractile responses of the rabbit iris sphincter and the guinea-pig vas deferens

The rabbit iris sphincter muscle responds to single pulse stimulation (1 pulse/60 s, 25 V) with a twitch-like contraction. This contraction can be blocked by 1 μM atropine (Wahlestedt *et al.*, 1985). The guinea-pig vas deferens responds to low frequency stimulation (5 Hz, 25 V, 3 s) with a twitch-like contraction. This contraction is unaffected by 1 μM atropine, but abolished by 5 μM guanethidine (Stjernquist *et al.*, 1983). FK 888 (1 nM–100 μM) was without effect on either contractile response (Figure 1).

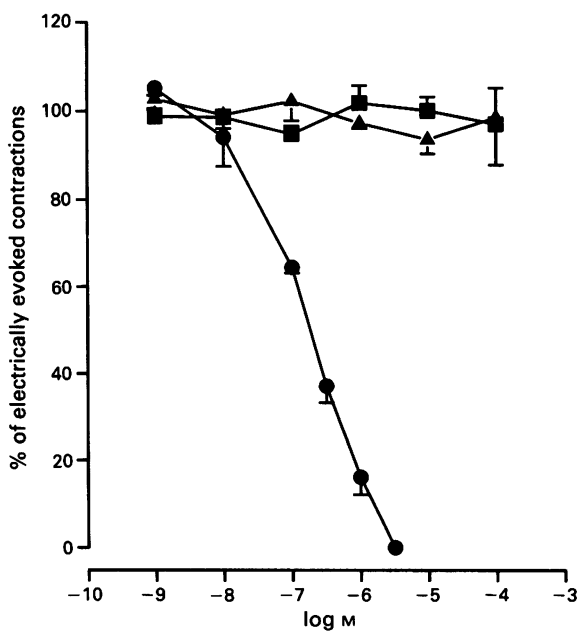


Figure 1 Inhibition of the electrically-evoked tachykinin-mediated contractile responses of the rabbit iris sphincter muscle by FK 888 (●). The electrically-evoked cholinergic response of the rabbit iris sphincter (▲) and the sympathetic response of the guinea-pig vas deferens (■) were not affected. $n = 4–8$ experiments. Values are mean with s.e.mean.

Effects of FK 888 on histamine release from rat peritoneal mast cells

FK 888 did not stimulate histamine release from rat peritoneal mast cells. In the presence of 1, 3, 10 and 30 μM FK 888, the histamine release was 9.7 ± 1%, 9.6 ± 1%, 9.7 ± 0.7% and 10.3 ± 0.7% respectively while the basal release (without FK 888) was 9.4 ± 0.7% ($n = 3$). In parallel experiments, the histamine release induced by 30 μM spantide, a widely used tachykinin receptor antagonist (Folkers *et al.*, 1984), was 61.9 ± 10.2% ($n = 3$); this result is consistent with a previous report (Schultheiss & Hörig, 1985).

Effects of FK 888 on electrically-evoked compound action potentials of the frog sciatic nerve

Depolarization of nerves opens sodium channels, thereby giving rise to transient action potentials. FK 888 at 33.3 μM ($n = 4$) and at 333 μM ($n = 2$) was without effect on the

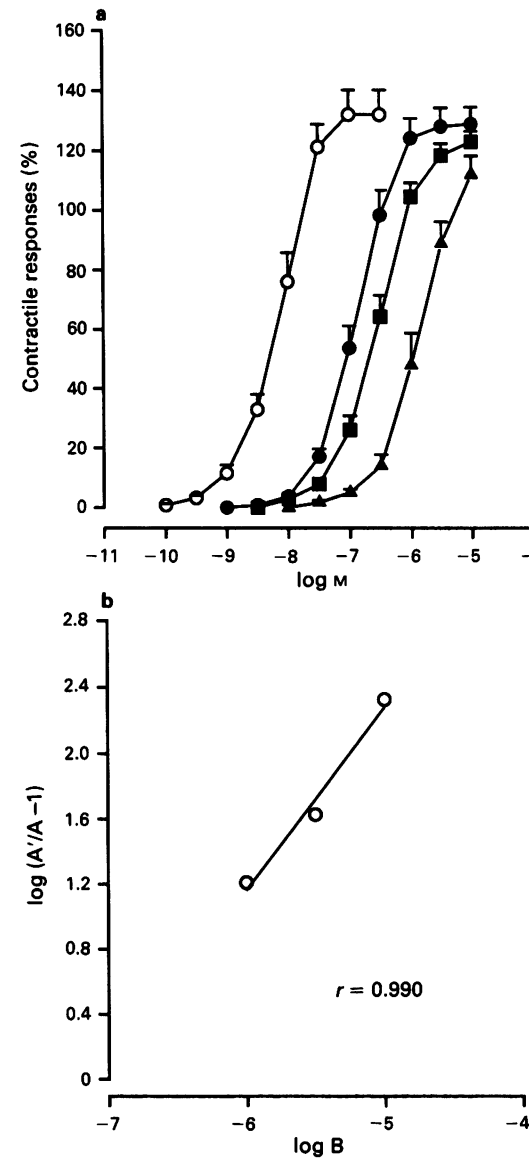


Figure 2 Rabbit iris sphincter: (a) cumulative concentration-response curves illustrating the contractions induced by [Sar⁹,Met(O₂)¹¹] SP in the absence (○) and presence of FK 888: 1 (●), 3 (■) and 10 μM (▲). Each value represents the mean with s.e.mean of 6 experiments. (b) Schild plot analysis reveals a slope of 0.99, which is not significantly different from unity, suggesting competitive antagonism.

electrically-evoked compound action potentials generated in the frog sciatic nerve, a preparation previously shown to be sensitive to local anaesthetics (Courtney & Strichartz, 1987) (data not shown).

Discussion

FK 888 has been reported to be a high affinity, peptide tachykinin receptor antagonist (Fujii *et al.*, 1992; Hirayama *et al.*, 1993). It is at least 10,000 times more potent in blocking NK_1 receptors than NK_2 and NK_3 receptors (Fujii *et al.*, 1992). Hence, the present finding that FK 888 inhibits both the electrically-evoked contractile response (mediated by endogenous tachykinins) and the exogenously applied NK_1 receptor agonist-induced contractions of the rabbit iris sphincter in a concentration-dependent manner support the view that NK_1 receptors predominate in this preparation (Hall *et al.*, 1991; 1993b,c; 1994; Wang & Håkanson, 1992b; 1993).

Since 1979, numerous peptide tachykinin receptor antagonists have been described (for reviews, see Håkanson & Sundler, 1985; Maggi *et al.*, 1993). However, these molecules generally possess low affinity and many have been claimed to be partial agonists. Moreover, they show poor selectivity for the different tachykinin receptor types and, in addition, possess other effects, such as mast cell degranulation, local anaesthetic properties or neurotoxicity (see Maggi *et al.*, 1993). Of the antagonists tested on the NK_1 receptor-mediated contractions of the rabbit iris sphincter preparation in our laboratory, FK 888 displays the highest pIC_{50} and pK_B values. The affinity of FK 888 is similar to that of another peptide NK_1 receptor antagonist GR 82334, which has also been examined in this preparation (Hall *et al.*, 1993b). Even so, the affinity of FK 888 was lower than expected from previously published reports. For instance, FK 888 exerts NK_1 receptor antagonism with a pA_2 value of 9.3 in the guinea-pig ileum (Fujii *et al.*, 1992). At present, we have no satisfactory explanation for the discrepancy between the rabbit iris sphincter and the guinea-pig ileum. Recently, species-related differences and even tissue-specific differences in the affinities of some selective NK_1 receptor antagonists have been reported (see Maggi *et al.*, 1993; Hall *et al.*, 1993a). It is possible that the rabbit iris sphincter expresses a subtype of the NK_1 receptor that is distinct from the subtype(s) found in other tissues (Hall *et al.*, 1994).

The recently described non-peptide NK_1 receptor antagonists, such as CP-96,345 and RP 67580, are known to inhibit several electrically-evoked cholinergic as well as sympathetic responses *in vitro*, suggesting that such drugs may exert non-specific actions on neurotransmission (Wang & Håkanson, 1992a; Wang *et al.*, 1994). Moreover, they have been found to inhibit the action potentials of the frog sciatic nerve and sodium currents of cultured neurones in a manner reminiscent of that of local anaesthetics (Wang *et al.*, 1994; Caeser *et al.*, 1993). However, the present study suggests that, unlike the non-peptide antagonists, and also unlike some peptidic antagonists (Post *et al.*, 1985; Maggi *et al.*, 1993), FK 888 does not possess these activities. In addition, FK 888 was not found to affect the contractions induced by either carbachol or noradrenaline of the rabbit iris sphincter. Finally, FK 888 does not behave as a partial agonist, nor does it induce histamine release from rat peritoneal mast cells. Thus, in conclusion, our results confirm that FK 888 is a moderately high affinity and apparently selective tachykinin (NK_1) receptor antagonist.

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References

CAESER, M., SEABROOK, G.R. & KEMP, J.A. (1993). Block of voltage-dependent sodium currents by the substance P receptor antagonist (±)-CP-96,345 in neurones cultured from rat cortex. *Br. J. Pharmacol.*, **109**, 918–924.

COURTNEY, K.R. & STRICHARTZ, G.R. (1987). Structural elements which determine local anesthetic activity. In *Handbook of Exp. Pharmacol.: Local Anesthetics*. ed. Strichartz, G.R. pp. 53–94. New York: Springer-Verlag.

EMONDS-ALT, X., VILAIN, P., GOULAOUIC, P., PROIETTO, V., BROECK, D.V., ADVENIER, C., NALINE, E., NELIAT, G., LE FUR, G. & BRELIÈRE, J.C. (1992). A potent and selective non-peptide antagonist of the neurokinin A (NK_2) receptor. *Life. Sci.*, **50**, PL 101–106.

FOLKERS, K., FENG, D.-M., ASANO, N., HÅKANSON, R., WEISENFELD-HALLIN, Z. & LEANDER, S. (1990). Spantide II, an effective tachykinin receptor antagonist having high potency and negligible neurotoxicity. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 4833–4836.

FOLKERS, K., HÅKANSON, R., HÖRIG, J., XU, J.-C. & LEANDER, S. (1984). Biological evaluation of substance P antagonists. *Br. J. Pharmacol.*, **83**, 449–456.

FUJII, T., MURAI, M., MORIMOTO, H., MAEDA, Y., YAMAOKA, M., HAGIWARA, D., MIYAKE, H., IKARI, N. & MATSUO, M. (1992). Pharmacological profile of a high affinity dipeptide NK_1 receptor antagonist, FK 888. *Br. J. Pharmacol.*, **107**, 785–789.

FUJIWARA, M., HAYASHI, H., MURAMATSU, I. & UEDA, N. (1984). Supersensitivity of the rabbit iris sphincter muscle induced by trigeminal denervation: the role of substance P. *J. Physiol.*, **350**, 583–597.

GAMSE, R., PETSCHE, U., LEMBECK, F. & JANCSO, G. (1982). Capsaicin applied to peripheral nerve inhibits axoplasmic transport of substance P and somatostatin. *Brain Res.*, **239**, 447–454.

GARRET, C., CARRUETTE, A., FARDIN, V., MOUSSAOUI, S., FEYRONEL, J.-F., BLANCHARD, J.-C. & LADURON, P.M. (1991). Pharmacological properties of a potent and selective nonpeptide substance P antagonist. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 10208–10212.

GUARD, S., BOYLE, S.J., TANG, K.-W., MCKNIGHT, A.T., WATLING, K.J. & WOODRUFF, G.N. (1993). The interaction of the NK_1 receptor antagonist CP-96,345 with L-type calcium channels and its functional consequences. *Br. J. Pharmacol.*, **110**, 385–391.

GUARD, S. & WATSON, S.P. (1991). Tachykinin receptor types: classification and membrane signalling mechanisms. *Neurochem. Int.*, **18**, 149–165.

HAHIN, R. & STRICHARTZ, G.R. (1981). Effects of deuterium oxide on the rate and dissociation constants for saxitoxin and tetrodotoxin action. *J. Gen. Physiol.*, **78**, 118.

HÅKANSON, R., BEDING, B., EKMAN, R., HEILIG, M., WAHLESTEDT, C. & SUNDLER, F. (1987). Multiple tachykinin pools in sensory nerve fibres in the rabbit iris. *Neurosci.*, **21**, 943–950.

HÅKANSON, R., LEANDER, S., ANDERSSON, R.G.G. & HÖRIG, J. (1983). Substance P antagonists release histamine from peritoneal mast cells. *Acta Physiol. Scand.*, **117**, 319–320.

HÅKANSON, R., LEANDER, S., ASANO, N., FENG, D.-M. & FOLKERS, K. (1990). Spantide II, a novel tachykinin receptor antagonist having high potency and low histamine-releasing effect. *Regul. Pept.*, **31**, 75–82.

HÅKANSON, R. & RÖNNBERG, A.-L. (1974). Improved fluorometric assay of histamine: condensation with *o*-phthalaldehyde at −20°. *Anal. Biochem.*, **60**, 560–567.

HÅKANSON, R. & SUNDLER, F. (1985). *Tachykinin Antagonists*. Amsterdam: Elsevier.

HÅKANSON, R., WANG, Z.-Y. & FOLKERS, K. (1991). Comparison of Spantide II and CP-96,345 for blockade of tachykinin-evoked contractions of smooth muscle. *Biochem. Biophys. Res. Commun.*, **178**, 297–301.

HALL, J.M., CAULFIELD, M.P., WATSON, S.P. & GUARD, S. (1993a). Receptor subtypes or species homologues: relevance to drug discovery. *Trends Pharmacol. Sci.*, **14**, 376–383.

HALL, J.M., MITCHELL, D. & MORTON, I.K.M. (1991). Neurokinin receptors in the rabbit iris sphincter characterised by novel agonist ligands. *Eur. J. Pharmacol.*, **199**, 9–14.

HALL, J.M., MITCHELL, D. & MORTON, I.K.M. (1993b). Tachykinin receptors mediating responses to sensory nerve stimulation and exogenous tachykinins and analogues in the rabbit isolated iris sphincter. *Br. J. Pharmacol.*, **109**, 1008–1013.

HALL, J.M., MITCHELL, D. & MORTON, I.K.M. (1993c). Does substance P release mediate NANC nerve-mediated responses to field stimulation and ligands, in the rabbit isolated sphincter pupillae? *Regul. Pept.*, **46**, 278–281.

HALL, J.M., MITCHELL, D. & MORTON, I.K.M. (1994). Tachykinin NK_1 receptor ligands show two distinct pharmacological profiles in rabbit iris sphincter. *Br. J. Pharmacol.* (in press).

HIRAYAMA, Y., LEI, Y.-H., BARNES, P.J. & ROGERS, D.F. (1993). Effects of two novel tachykinin receptor antagonists, FK 224 and FK 888, on neurogenic airway plasma exudation, bronchoconstriction and systemic hypotension in guinea-pigs *in vivo*. *Br. J. Pharmacol.*, **108**, 844–851.

KENAKIN, T.P. (1984). The classification of drugs and drug receptors in isolated tissues. *Pharmacol. Rev.*, **36**, 165–222.

KUROSE, M. & SAEKI, K. (1981). Histamine release induced by neurotensin from rat peritoneal mast cells. *Eur. J. Pharmacol.*, **76**, 129–136.

LEANDER, S. & HÅKANSON, R. (1985). Are substance P antagonists general neurosuppressive agents? In *Tachykinin Antagonsits*. ed. Håkanson, R. & Sundler, F. pp. 395–404. Amsterdam: Elsevier.

LECCI, A., GIULIANI, S., PATACCHINI, R., VITI, G. & MAGGI, C.A. (1991). Role of NK_1 tachykinin receptors in thermonociception: effect of (±) CP-96,345, a non-peptide substance P antagonist, on the hot plate test in mice. *Neurosci. Lett.*, **129**, 299–302.

MAGGI, C.A., PATACCHINI, R., ROVERO, P. & GIACHETTI, A. (1993). Tachykinin receptors and tachykinin receptor antagonists. *J. Auton. Pharmacol.*, **13**, 23–93.

MAGGIO, J.E. (1988). Tachykinins. *Annu. Rev. Neurosci.*, **11**, 13–28.

NAGAHISA, A., ASAI, R., KANAI, Y., MURASE, A., TSUCHIYA-NAKAGAKI, M., NAKAGAKI, T., SHIEH, T.-C. & TANIGUCHI, K. (1992). Non-specific activity of (±)-CP-96,345 in models of pain and inflammation. *Br. J. Pharmacol.*, **107**, 273–275.

OTSUKA, M. & YOSHIOKA, K. (1993). Neurotransmitter functions of mammalian tachykinins. *Physiol. Rev.*, **73**, 229–308.

REGOLI, D., DRAPEAU, G., DION, S. & COUTURE, R. (1988). New selective agonists for neurokinin receptors: pharmacological tools for receptor characterization. *Trends Pharmacol. Sci.*, **9**, 290–295.

POST, C., BUTTERWORTH, J.F., STRICHARTZ, G.R., KARLSSON, J.A. & PERSSON, C.G.A. (1985). Tachykinin antagonists have potent local anaesthetic actions. *Eur. J. Pharmacol.*, **117**, 347–354.

SCHMIDT, A.W., MCLEAN, S. & HEYM, J. (1992). The substance P receptor antagonist CP-96,345 interacts with Ca^{2+} channels. *Eur. J. Pharmacol.*, **19**, 351–352.

SCHULTHEISS, H. & HÖRIG, J. (1985). Histamine release from rat peritoneal cells by substance P antagonists and fragments – a study on structure activity relationship. In *Tachykinin Antagonists*. ed. Håkanson, R. & Sundler, F. pp. 413–420. Amsterdam: Elsevier.

SNIDER, R.M., CONSTANTINE, J.W., LOWE, III, J.A., LONGO, K.P., LEBEL, W.S., WOODY, H.A., DROZDA, S.E., DESAI, M.C., VINICK, F.J., SPENCER, R.W. & HESS, H.-J. (1991). A potent nonpeptide antagonist of the substance P (NK_1) receptor. *Science*, **251**, 435–437.

STJERNQUIST, M., HÅKANSON, R., LEANDER, C., OWMAN, C., SUNDLER, F. & UDDMAN, R. (1983). Immunohistochemical localization of substance P, vasoactive intestinal polypeptide and gastrin-releasing peptide in vas deferens and seminal vesicle, and the effect of these and eight other neuropeptides on resting tension and neurally evoked contractile activity. *Regul. Pept.*, **7**, 67–86.

STRONG, P.N., SMITH, J.T. & KEANA, J.T.-W. (1978). A convenient bioassay for detecting nanomolar concentrations of tetrodotoxin. *Toxicon*, **11**, 435.

TAMURA, K., MUTABAGANI, K. & WOOD, J.D. (1993). Analysis of a nonpeptide antagonist for substance P on myenteric neurons of guinea-pig small intestine. *Eur. J. Pharmacol.*, **232**, 235–239.

WAHLESTEDT, G., BYNKE, G., BEDING, B., VON LEITNER, P. & HÅKANSON, R. (1985). Neurogenic mechanisms in control of the rabbit iris sphincter muscle. *Eur. J. Pharmacol.*, **117**, 303–309.

WANG, Z.-Y. & HÅKANSON, R. (1992a). (±) CP-96,345, a selective tachykinin NK_1, receptor antagonist, has non-specific actions on neurotransmission. *Br. J. Pharmacol.*, **107**, 762–765.

WANG, Z.-Y. & HÅKANSON, R. (1992b). The electrically-evoked, tachykinin-mediated contractile response of the isolated rabbit iris sphincter muscle involves NK_1 receptors only. *Eur. J. Pharmacol.*, **216**, 327–329.

WANG, Z.-Y. & HÅKANSON, R. (1993). The rabbit iris sphincter contains NK_1 and NK_3 but not NK_2 receptors: a study with selective agonists and antagonists. *Regul. Pept.*, **44**, 269–275.

WANG, Z.-Y., TUNG, S.R., STRICHARTZ, G.R. & HÅKANSON, R. (1994). Non-specific actions of non-peptide tachykinin receptor antagonists CP-96,345, RP 67580 and SR 48968 on neurotransmission. *Br. J. Pharmacol.*, **111**, 179–184.

WATLING, K.J. (1992). Nonpeptide antagonists herald new era in tachykinin research. *Trends Pharmacol. Sci.*, **13**, 266–269.

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Prevention by NMDA receptor antagonists of the centrally-evoked increases of cardiac inotropic responses in rabbits

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1 The purpose of this study was to investigate further the role of the excitatory amino acid (EAA) system of neurotransmission, particularly of the NMDA receptor, in the central regulation of cardiac function.

2 Electrical stimulation of the paraventricular nucleus of the hypothalamus (PVN) in pentobarbitone anaesthetized rabbits induced a cardiovascular response mainly characterized by a positive inotropic effect, hypertension and a marked increase in the myocardial oxygen demand index.

3 The intracerebroventricular (i.c.v.) or intravenous (i.v.) injection of different EAA antagonists acting on different sites of the NMDA receptor/channel complex dose-dependently blunted the excitatory cardiovascular effects of PVN stimulation.

4 5,7 Dichlorokynurenic acid was used as a specific glycine site antagonist and 2-amino-5-phosphonovaleric acid was used to block the agonist recognition site; ketamine was used as a channel blocker site antagonist and ifenprodil as a blocker of the polyamine binding site.

5 5,7 Dichlorokynurenic acid (125 and 250 $\mu g\,kg^{-1}$, i.c.v.) virtually abolished the cardiovascular responses, inducing only haemodynamic depression at the highest dose used. 2-Amino-5-phosphonovaleric acid (0.1 to 1.0 $mg\,kg^{-1}$, i.c.v.) elicited a reduction of the peak values observed during PVN stimulation which was accompanied by a decrease of the basal cardiovascular parameters. Ketamine (2.5 and 10 $mg\,kg^{-1}$) and ifenprodil (1 $mg\,kg^{-1}$), injected intravenously, blocked the haemodynamic response induced by PVN stimulation without marked reduction of the basal haemodynamics.

6 It is concluded that glutamate neurotransmission is not only involved in vasomotor tone control but also in the central control of cardiac function and can therefore modulate the myocardial oxygen demand.

Keywords: Glutamate; NMDA antagonists; CNS; PVN stimulation; haemodynamic responses

Introduction

It is now generally accepted that excitatory amino acids (EAA), mainly glutamate, are important and ubiquitous excitatory neurotransmitters in the mammalian central nervous system and are involved in the mediation of physiological functions such as learning, memory, synaptic plasticity and cardiovascular regulation (Collingridge & Lester, 1989; Monaghan *et al.*, 1989; Young & Fagg, 1990). Moreover, EAA receptors are known to mediate synaptic excitations throughout the central nervous system (Headley & Grillner, 1990). The role of glutamatergic neurotransmission in the central regulation of cardiovascular function is emerging. There is increasing evidence for the involvement of EAA in the modulation of the baroreceptor reflex and of vasomotor tone (Cotman *et al.*, 1987; Guyenet *et al.*, 1987; Kubo & Kihara, 1988; Le Galloudec *et al.*, 1989). This system can modulate the activity of the vasomotor bulbospinal neurones of the ventrolateral medulla (Kubo *et al.*, 1986; Kihara *et al.*, 1989; Kao *et al.*, 1991; Chalmers & Pilowsky, 1991) which in turn modulates the activity of the sympathetic preganglionic neurones of the intermediolateral cell column in the spinal cord (Mills *et al.*, 1988; 1990). In this context, it has been recently reported that the intrathecal administration of glutamate antagonists in normotensive rats can attenuate the hypertensive response evoked by electrical stimulation of the rostral ventrolateral medulla, without affecting the resting levels of the cardiovascular parameters (Mills *et al.*, 1988). Moreover, the glutamatergic system of neurotransmission seems to be involved in the pathophysiology of arterial hypertension in genetically hypertensive rats (Mills *et al.*, 1990).

In the present work, we focused our attention on the involvement of glutamatergic neurotransmission in the modulation of cardiac inotropism which is one of the most important components of myocardial oxygen demand (MOD).

It had been shown previously that the activation of different brain regions, especially hypothalamic, could induce not only excitatory haemodynamic responses but also myocardial ischaemia and ventricular fibrillation (Lown *et al.*, 1977). For instance, Azevedo *et al.* (1980) reported that electrical stimulation of the paraventricular nucleus of the hypothalamus (PVN) in anaesthetized rabbits induced excitatory cardiovascular responses mainly characterized by important increases in cardiac output.

We used an experimental model of electrical stimulation of the PVN in the rabbit, which markedly increased arterial pressure and cardiac inotropic state, and studied the ability of NMDA receptor antagonists to modulate these responses.

Methods

Animals and haemodynamic measurements

Normotensive male rabbits (Zika strain) weighing between 2.5 and 3.5 kg were anaesthetized with 40 $mg\,kg^{-1}$ sodium

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pentobarbitone injected through the marginal vein of the ear; anaesthesia was complemented by another i.v. injection of pentobarbitone of 5 mg kg^{-1} immediately before the beginning of control electrical stimulations. Rectal temperature was maintained at 38 ± 0.5°C with the aid of a warming blanket as soon as anaesthesia was established (Harvard Apparatus LTD, Millis, MA, U.S.A.). The animals were tracheotomized, immobilized with pancuronium bromide (1 mg kg^{-1}, i.v.) and artificially ventilated with room air (Hugo Sachs Elektronic model 6025, March-Hugstetten, Germany). The ventilation parameters were adjusted to maintain PaO_2 at approximately 100 mgHg and $PaCO_2$ below 40 mmHg.

The right femoral vein was catheterized to permit i.v. injections and the instantaneous arterial pressure was measured through a catheter placed in the abdominal aorta via the right femoral artery connected to a Statham P23 Db transducer, which was in turn connected to a pressure processor and recorder (Gould Electronics model BS-272, Longjumeau, France). Mean arterial pressure (MAP) was calculated as diastolic pressure plus one third of the differential pressure (Ganong, 1971). Heart rate was also continuously monitored from the pressure signal with a Gould Biotach amplifier (model 13-4615-66). Left ventricular pressure and the maximum rate of rise of left ventricular pressure (dP/dt_{max}) were measured with a micromanometer tipped catheter (Gaeltec LTD, model ICT/B, Dunvegan, U.K.) placed in the left ventricle via the right carotid artery. The dP/dt_{max} was obtained with a Philips differentiator model 133-1-4331 (Bern, Switzerland). The myocardial oxygen demand was evaluated by the 'triple product' index (Baller *et al.*, 1979). This index was calculated by multiplying the heart rate by the systolic blood pressure and the maximum rate of rise in left ventricular pressure divided by 10^6 to reduce it to convenient units. This product will be referred to subsequently as the 'triple product'.

Electrical stimulation of the PVN

The head of the animal was placed in a stereotaxic apparatus (Unimécanique, Epinay/Seine, France). A craniotomy was performed and the dura mater drilled to permit stereotaxic placement of the concentric bipolar stainless steel electrode of 0.1 mm diameter (Rhodes Medical, model SNE-100). The stimulus consisted of a 5-s train of 1 ms rectangular pulses at a frequency of 100 Hz. In preliminary experiments, increasing current intensities ranging from 50 to 500 μA (Hugo Sachs T stimulator and a type 251 constant current unit, March-Hugstetten, Germany) were applied in order to establish intensity-effect relationships in our experimental model. We observed intensity-related increases in dP/dt_{max} until 500 μA, when a plateau was attained. Higher intensities induced cardiac rhythm disturbances without further increases in dP/dt_{max}. We then used current intensities ranging from 100 to 300 μA, inducing dP/dt_{max} increases of about 30%. The sites of stimulation were located according to the atlas of Sawyer *et al.* (1954) and the following coordinates were used: AP = from + 0.5 to 1.0 mm from bregma; L = from 0.5 to 1 mm and V = from − 12 to − 13 mm.

Intracerebral injections

A second craniotomy was performed to inject drugs into the left lateral ventricle. Normal saline solution (control group) or glutamate antagonists which do not cross the blood-brain barrier were injected in a constant volume of 100 μl of saline solution by use of a Hamilton micro-syringe (Hamilton Bonaduz AG, Switzerland) in the following stereotaxic coordinates: AP = − 4.5 mm from bregma; L = 8 mm and 6 mm down from the cranial surface (Sawyer *et al.*, 1954).

Experimental procedure

The electrodes were inserted stereotaxically in the PVN and at least three electrical stimulations at 5 min intervals inducing similar rises in dP/dt_{max} were performed and averaged before drug injections. When the haemodynamic responses obtained varied by less than 10% between stimulations, the response was considered to be stable and the drugs were injected either i.v. in 500 μl or i.c.v. in 100 μl normal saline solution followed by electrical stimulation 5 min later.

Location of sites of stimulation

At the end of the experiments to determine the location of the hypothalamic area where the electrical stimulation provoked reproducible significant increases of dP/dt_{max}, electrolytic lesions were made by a current of 1 mA for 10 s. After the animals were killed, their brains were fixed *in situ* by perfusion of a 10% formol saline solution through the carotid artery, then removed and conserved in 10% formol saline before embedding in paraffin Serial sections (10 μm) were cut and stained with haematoxyllin-eosin. The electrode position was located by use of the atlas of Sawyer *et al.* (1954).

Drugs

The following drugs were used: sodium pentobarbitone (Nembutal, Abbott Lab., North Chicago, IL, U.S.A.); pancuronium bromide (Pavulon, Organon Tecknica, Fresnes, France); 5-7 dichloro-kynurenic acid (Tocris Neuramin, England); ifenprodil (Vadilex, Synthélabo, France); ketamine (Ketalar, Parke Davis, France); 2-amino-5-phosphonovaleric acid (Tocris Neuramin, England).

Statistical analysis

All results are expressed as means ± s.e.mean. The effects of the treatments on baseline haemodynamics and on the responses to electrical stimulation were analysed by repeated ANOVA measures followed by Scheffe's test to determine statistically significant differences. Comparisons between the control group (saline-injected) and treated groups (animals injected with antagonists in saline solution) were made by one way ANOVA. All calculations were made by computer-assisted analyses with the Statview II programme (Abacus Concepts, Inc, Berkeley, U.S.A.).

Results

Control experiments

The repetitive intracerebroventricular injections of saline solution (100 μl) did not induce significant changes in the baseline haemodynamic parameters or alterations in the cardiovascular response to electrical stimulation of the PVN throughout the period of the experiment (Table 1).

Haemodynamic effects of electrical stimulation of the PVN region

Electrical stimulation of the PVN in the pentobarbitone-anaesthetized rabbit induced significant increases in dP/dt_{max} (Table 1) which began immediately after stimulation was started and ended when it ceased, reaching a maximum of + 35 ± 2% above the baseline value ($n = 6$, $P < 0.01$). Neither saline injected i.c.v. nor the time (up to 60 min) influenced this response. There were also increases in mean arterial pressure reaching a maximum of + 42 ± 5% ($n = 6$, $P < 0.01$) when compared to the initial values of 101 ± 5 mmHg. The positive inotropic and hypertensive responses were accompanied by mild bradycardia during the first 2 stimulation periods and, when repeated, increased to reach a maximum of − 19 ± 9%; $n = 6$, $P < 0.05$ (Table 1). These positive inotropic and vasopressive responses led to marked

increases in the triple product index of myocardial oxygen demand, which increased regularly by about 60%.

Effects of i.c.v. injection of NMDA antagonists on the haemodynamic responses to electrical stimulation of the PVN

5,7 Dichloro-kynurenic acid (5,7 diCl-KYNA) The marked increases in dP/dt_{max} induced by electrical stimulation of the PVN were inhibited by prior i.c.v. injections of 125 and 250 $\mu g\,kg^{-1}$ of 5,7 diCl-KYNA in a dose-related manner. The arterial pressure followed the same pattern of response (Table 2). In this series of experiments, 5,7 diCl-KYNA produced a dose-related inhibition of the increase in the triple product elicited by electrical stimulation of the PVN (Figure 1).

2-amino-5-phosphonovaleric acid (APV) The i.c.v. injection of increasing doses of APV (0.1, 0.3 and 1 $mg\,kg^{-1}$) reduced dose-dependently the peak values of the cardiovascular parameters observed during the electrical stimulation of the PVN. This effect was mainly obtained by decreasing the basal haemodynamics (Table 3, Figure 2).

Effects of the intravenous injection of NMDA antagonists on the haemodynamic responses to electrical stimulation of the PVN

Ifenprodil The i.v. injection of 1 $mg\,kg^{-1}$ of ifenprodil reduced the response observed during PVN stimulation for all cardiovascular parameters (Figure 3). The triple product (TP) response decreased from 178 ± 18 to 131 ± 9 ($P<0.05$). In this series of experiments, the basal level of the TP was slightly reduced by ifenprodil while the basal value of dP/dt_{max} was not significantly changed (Table 4).

Ketamine The i.v. administration of ketamine (2.5 and 10 $mg\,kg^{-1}$) dose-dependently reduced the peak values obtained during PVN stimulation of all cardiovascular parameters. All the stimulation-induced responses were abolished by the highest dose of 10 $mg\,kg^{-1}$. In these two groups of experiments, ketamine never significantly modified the basal values of the cardiovascular parameters (Table 5, Figure 4).

Table 1 Control and peak values of the cardiovascular parameters observed upon electrical stimulation of the PVN of the anaesthetized rabbit before and after repetitive i.c.v. injection of 100 µl of isotonic saline solution (control experiments)

	Saline (number of i.c.v. injection)							
	Control values		1		2		3	
	C	*S*	*C*	*S*	*C*	*S*	*C*	*S*
dP/dt_{max} ($mmHg\,s^{-1}$)	2960 ± 240	3975 ± 284	2920 ± 224	3970 ± 286	2980 ± 286	3830 ± 414	3150 ± 250	4070 ± 303
SBP (mmHg)	123 ± 6	162 ± 11	121 ± 6	166 ± 11	123 ± 6	164 ± 10	124 ± 6	165 ± 10
DBP (mmHg)	90 ± 5	132 ± 10	89 ± 5	131 ± 9	90 ± 5	133 ± 9	91 ± 6	135 ± 10
MBP (mmHg)	101 ± 5	142 ± 10	100 ± 6	142 ± 10	101 ± 6	145 ± 4	102 ± 6	145 ± 10
HR (beats min^{-1})	278 ± 10	251 ± 12	272 ± 12	254 ± 9	276 ± 9	230 ± 16	272 ± 12	216 ± 20
TP ($mmHg^2\,s^{-1}$ b.p.m. 10^{-6})	102 ± 12	162 ± 19	96 ± 12	163 ± 20	100 ± 13	150 ± 22	106 ± 12	144 ± 23

The excitatory cardiovascular responses to electrical stimulation were highly reproducible over time (60 min). Data are represented as means ± s.e.mean of 6 experiments. (ANOVA for repeated measures). SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; HR, heart rate; TP, triple product; C, baseline values, S, electrical stimulation

Table 2 Effects of the cumulative intracerebroventricular administrations of 5,7 di-chloro kynurenic acid on basal haemodynamics and on the cardiovascular response to electrical stimulation of the PVN of the pentobarbitone anaesthetized rabbit

	Before treatment		*5,7 di-chloro Kynurenic acid* 125 $\mu g\,kg^{-1}$, *i.c.v.*		250 $\mu g\,kg^{-1}$, *i.c.v.*	
	C	*S*	*C*	*S*	*C*	*S*
dP/dt_{max} ($mmHg\,s^{-1}$)	4673 ± 486	6260 ± 248	4060 ± 380	5300 ± 141*	3700 ± 591†	4120 ± 668**
SBP (mmHg)	108 ± 9	145 ± 6	96 ± 9	119 ± 6*	93 ± 14	100 ± 14**
DBP (mmHg)	83 ± 7	125 ± 5	72 ± 9	99 ± 5*	71 ± 12	82 ± 13*
MBP (mmHg)	91 ± 7	131 ± 5	80 ± 9	106 ± 5*	78 ± 13	88 ± 14*
HR (beats min^{-1})	275 ± 10	253 ± 9	253 ± 9	246 ± 12NS	244 ± 7	250 ± 9NS
TP ($mmHg^2\,s^{-1}$ b.p.m. 10^{-6})	143 ± 23	211 ± 9	104 ± 18	156 ± 14*	93 ± 26†	115 ± 29**

C = baseline values; S = electrical stimulation. Each value represents the mean ± s.e.mean of 5 experiments. *$P<0.05$; **$P<0.01$ statistically significant effect of electrical stimulation when compared to values during stimulation before treatment; †$P<0.05$; significant effect of the treatment on basal cardiovascular parameters when compared to control values before treatment. (ANOVA for repeated measures followed by Scheffé's test). Abbreviations as in Table 1.

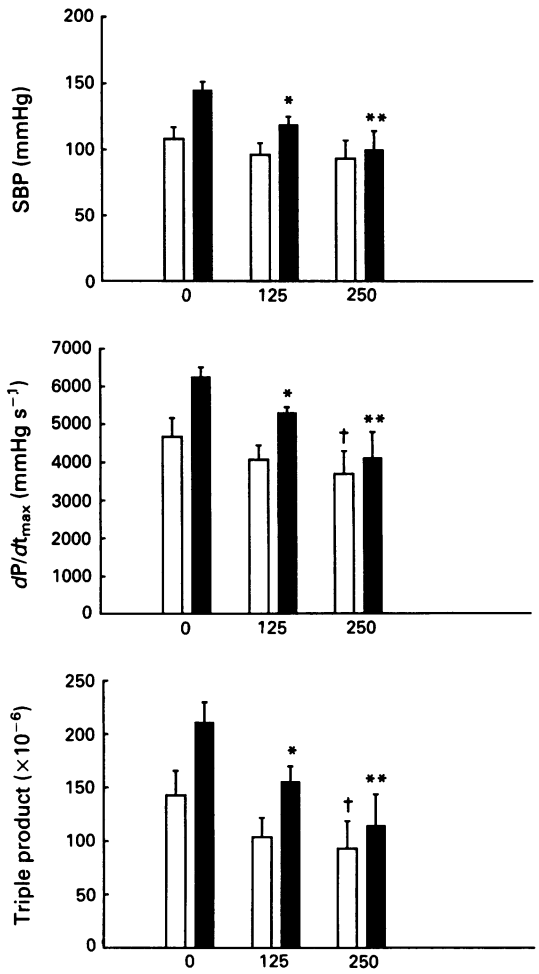


Figure 1 Effects of the intracerebroventricular injection of cumulative doses of 5,7 dichloro-kynurenic acid on cardiovascular responses elicited by electrical stimulation of the paraventricular nucleus of the hypothalamus (PVN) in pentobarbitone anaesthetized rabbits: open columns, control; solid columns, electrical stimulation. SBP: systolic blood pressure. Values are represented as means ± s.e.mean of 5 experiments. $*P<0.05$; $**P<0.01$: significant difference between values observed during stimulation before and after treatment; $\dagger P<0.05$: significant differences between basal values before and after treatment.

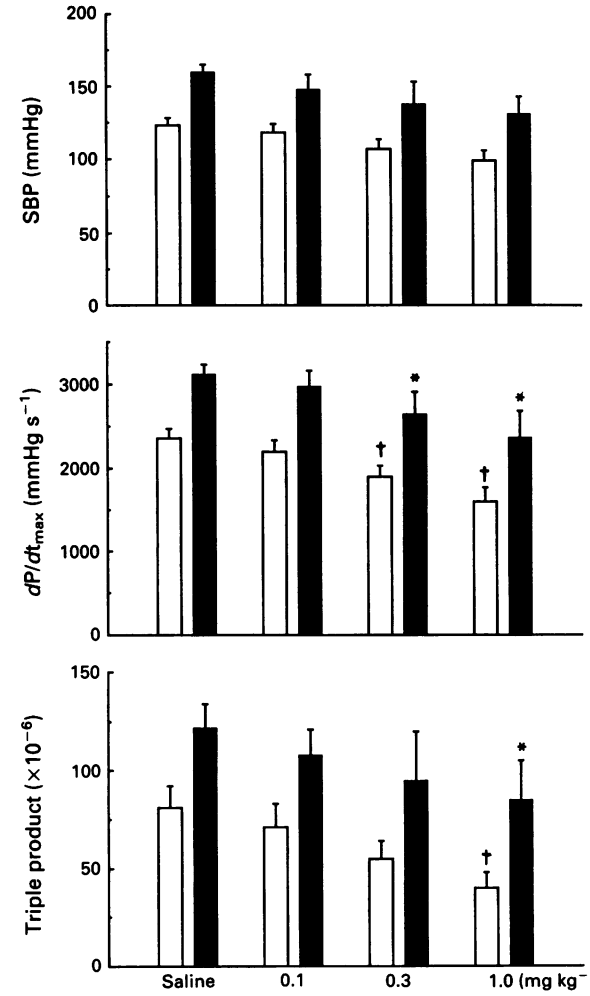


Figure 2 Effects of the intracerebroventricular administration of cumulative doses of 2-amino-5-phosphonovaleric acid (APV) on the cardiovascular responses elicited by electrical stimulation of the paraventricular nucleus of the hypothalamus (PVN) in pentobarbitone anaesthetized rabbits: open column, baseline; solid column, electrical stimulation. Data are represented as means ± s.e.mean of 6 experiments. $*P<0.05$: significant difference between values observed during stimulation before and after treatment; $\dagger P<0.05$: significant differences between basal values before and after treatment.

Table 3 Effects of increasing doses of intracerebroventricular 2-amino-5-phosphonovaleric acid on basal haemodynamics and on the cardiovascular responses to electrical stimulation of the PVN of the pentobarbitone anaesthetized rabbit

	2-Amino-5-phosphonovaleric acid (APV) (mg kg^{-1}, i.c.v.)							
	0		0.1		0.3		1.0	
	C	*S*	*C*	*S*	*C*	*S*	*C*	*S*
dP/dt_{max} (mmHg s^{-1})	2360 ± 111	3120 ± 110	2200 ± 136	2980 ± 179	1900 ± 134†	2650 ± 260*	1600 ± 171†	2370 ± 312*
SBP (mmHg)	123 ± 5	160 ± 5	118 ± 6	148 ± 10	107 ± 6.5	138 ± 11.5	99 ± 7	131 ± 12
DBP (mmHg)	90 ± 5	132 ± 3	87.5 ± 6	122 ± 7	74 ± 5	113 ± 9	73.5 ± 7	108 ± 9
MBP (mmHg)	101 ± 5	141 ± 4	97.5 ± 6	131 ± 8	85 ± 6	123 ± 10	79 ± 6	113 ± 12
HR (beats min^{-1})	266 ± 13	242 ± 13	258 ± 12	236 ± 12	243 ± 13	231 ± 19	230 ± 14	228 ± 15
TP (mmHg^2 s^{-1} b.p.m. 10^{-6})	81 ± 11	122 ± 12	71 ± 12	108 ± 13	55 ± 9	95 ± 25	40 ± 8†	85 ± 20*

C = baseline values; S = electrical stimulation. Each value represents the mean ± s.e.mean of 6 experiments. $*P<0.05$: statistically significant effect of electrical stimulation when compared to values during stimulation before treatment; $\dagger P<0.05$: significant effect of the treatment on basal cardiovascular parameters when compared to control values (ANOVA for repeated measures followed by Scheffé's test). Abbreviations as in Table 1.

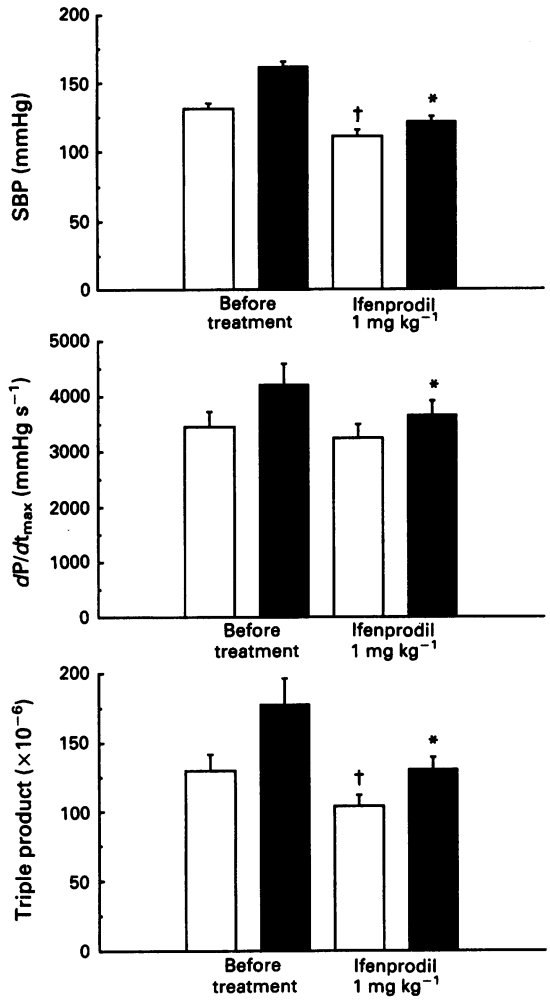


Figure 3 Effects of the intravenous administration of ifenprodil on the cardiovascular responses elicited by electrical stimulation of the paraventricular nucleus of the hypothalamus (PVN) in pentobarbitone anaesthetized rabbits: open columns, baseline; solid columns, stimulation. Data are represented as means ± s.e.mean of 5 experiments. $*P<0.05$: significant difference between values observed during stimulation before and after treatment; $\dagger P<0.05$: significant difference between basal values before and after treatment.

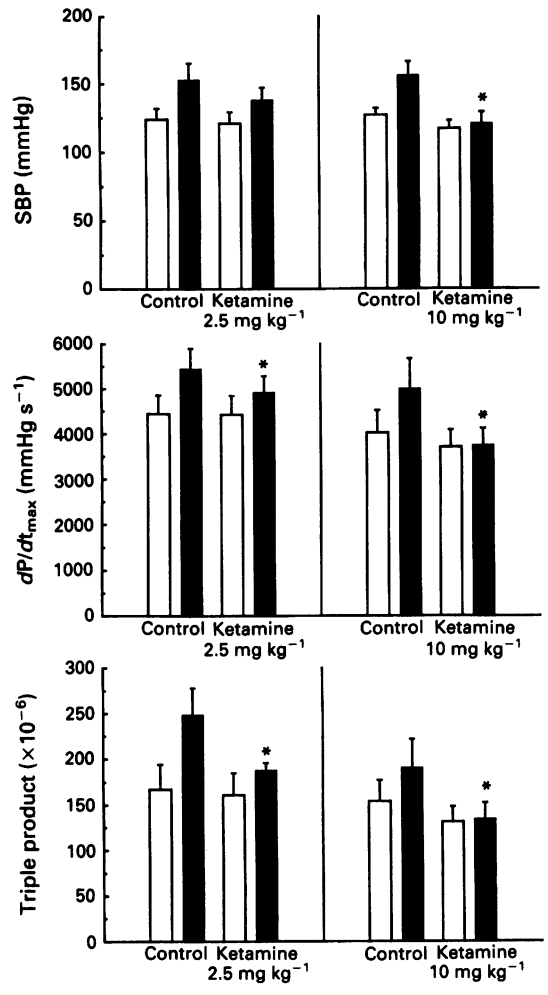


Figure 4 Effects of the intravenous administration of ketamine on the cardiovascular responses elicited by electrical stimulation of the paraventricular nucleus of the hypothalamus (PVN) in pentobarbitone anaesthetized rabbits: open columns, baseline; solid columns, stimulation. Data are represented as means ± s.e.mean of 5 experiments. $*P<0.05$: significant difference between values observed during stimulation before and after treatment.

Table 4 Effects of the intravenous administration of ifenprodil on the cardiovascular response elicited by electrical stimulation of the PVN of the pentobarbitone anaesthetized rabbit

	Before treatment		*Ifenprodil* (1 mg kg^{-1}, i.v.)	
	C	*S*	*C*	*S*
dP/dt_{max} (mmHg s^{-1})	3452 ± 274	4217 ± 373	3250 ± 248	3667 ± 250*
SBP (mmHg)	131 ± 4	162 ± 4	111 ± 5†	122 ± 3*
DBP (mmHg)	104 ± 3	132 ± 3	86 ± 4†	100 ± 3*
MBP (mmHg)	113 ± 3	141 ± 3	95 ± 4†	107 ± 3*
HR (beats min^{-1})	289 ± 16	258 ± 8	292 ± 13	297 ± 15*
TP (mmHg^2 s^{-1} b.p.m. 10^{-6})	130 ± 12	178 ± 18	104 ± 8†	131 ± 9*

C = baseline values; S = electrical stimulation. Each value represents the mean ± s.e.mean of 5 experiments. $*P<0.05$: statistically significant effect of electrical stimulation when compared to values during stimulation before treatment; $\dagger P<0.05$: significant effect of the treatment on basal cardiovascular parameters when compared to control values. (ANOVA for repeated measures followed by Scheffé's test). Abbreviations as in Table 1.

Table 5 Effects of the intravenous administration of ketamine on the cardiovascular response elicited by electrical stimulation of the PVN of the pentobarbitone anaesthetized rabbit

	Ketamine (2.5 mg^{-1}, i.v.)				*Ketamine* (10 mg kg^{-1}, i.v.)			
	Before treatment		*After treatment*		*Before treatment*		*After treatment*	
	C	*S*	*C*	*S*	*C*	*S*	*C*	*S*
dP/dt_{max} (mmHg s^{-1})	4440 ± 415	5447 ± 445	4420 ± 428	4920 ± 361*	4020 ± 486	5000 ± 660	3700 ± 380	3740 ± 370*
SBP (mmHg)	124 ± 8	153 ± 12	121 ± 4	138 ± 8	127 ± 5	156 ± 10	117 ± 6	121 ± 8*
DBP (mmHg)	91 ± 7	123 ± 7	88 ± 7	114 ± 9	91 ± 5	129 ± 8	85 ± 5	96 ± 7*
MBP (mmHg)	102 ± 7	133 ± 9	99 ± 7	122 ± 9	103 ± 4	138 ± 8	96 ± 5	104 ± 7*
HR (beats min^{-1})	281 ± 33	298 ± 12	296 ± 12	286 ± 21	299 ± 8	246 ± 10	298 ± 7	294 ± 7*
TP (mmHg^2 s^{-1} b.p.m. 10^{-6})	155 ± 27	249 ± 29	158 ± 24	188 ± 8*	154 ± 23	191 ± 31	131 ± 17	134 ± 18*

C = baseline values; S = electrical stimulation. Each value represents the mean ± s.e.mean of 5 experiments. *$P<0.05$: statistically significant effect of electrical stimulation when compared to values during stimulation before treatment; (ANOVA for repeated measures followed by Scheffé's test).

Discussion

In the present study, we investigated the possible involvement of a EAA neurotransmitter system in the pathways conducting the excitatory cardiovascular responses elicited by electrical stimulation of the PVN in anaesthetized rabbits.

In untreated animals, the electrical stimulation of the PVN always induced a classical cardiovascular response, i.e. hypertension, bradycardia and increase in dP/dt_{max}. The heart rate response was the most variable, bradycardia being probably due to the baroreflex activation. This excitatory haemodynamic reaction mimicked the one provoked by situations of stress or physical effort in conscious animals (Hilton & Redfern, 1986).

In this model we examined the involvement of NMDA receptors in these responses. Two main receptor subtypes for the EAA are involved in the glutamatergic neurotransmission in the mammalian central nervous system: the N-methyl-D-aspartate receptor/channel complex (NMDA) and the non NMDA receptor, AMPA/kainate (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) (Watkins & Evans, 1981; Collingridge & Lester, 1989; Honore, 1989; Lodge & Johnson, 1990; Watkins *et al.*, 1990). It is well known that the NMDA receptor is widely distributed throughout the brain (Cotman *et al.*, 1987; Sundaram *et al.*, 1989; Young & Fagg, 1990).

5,7 Dichlorokynurenic acid is a derivative of kynurenic acid (a tryptophan metabolite), an endogenous substance that has been shown to block selectively the glutamate-like neurotransmission. It has been demonstrated recently that the EAA antagonist effects of 5,7 diCl-KYNA are due to the selective blocking of the glycine allosteric modulatory unit of the NMDA receptor/channel complex (Johnson & Ascher, 1987; Birch *et al.*, 1988; Foster *et al.*, 1992).

The central administration of 5,7 diCl-KYNA clearly protected the animals against the cardiovascular responses provoked by PVN activation in a dose-related manner with a weak influence on the basal haemodynamic parameters (arterial pressure, heart rate and dP/dt_{max}). This protective effect is unlikely to be due to a diminished neuronal response to electrical stimulation over time (60 min); in control experiments there were no significant differences in the peak values of cardiovascular parameters observed during electrical stimulation of the PVN before and after repetitive i.c.v. administration of saline solution. This was also true with regard to the repetitive intravenous injection of 500 µl of saline solution (data not shown).

Results obtained with 5,7 diCl-KYNA strongly suggested that NMDA receptors were in fact involved in the central effect on the cardiovascular function, in particular the inotropic one.

We also used 2-amino-5-phosphonovaleric acid (APV). APV belongs to the series of the ω-phosphonic-α-carboxylic amino acids developed as antagonists acting on the agonist recognition site of the NMDA receptor (Evans *et al.*, 1982). This acid is a potent and highly selective NMDA antagonist both *in vitro* and in functional studies and is devoid of antagonist effects on AMPA/kainate-induced responses (Davies *et al.*, 1981; Evans *et al.*, 1982; Kehl & McLennan, 1983; Childs *et al.*, 1988). In addition APV exhibited no effects on responses induced by other neurotransmitters or neuromodulators of the central nervous system such as acetylcholine, 5-hydroxytryptamine, noradrenaline or substance P (Childs *et al.*, 1988).

In the present study, APV blunted the excitatory cardiovascular responses evoked by electrical stimulation of the PVN, but it also reduced the resting haemodynamic parameters *per se*, at least at the highest doses used in this study. Therefore, these results suggest that the NMDA recognition agonist site might be involved in the tonic regulation of the cardiovascular function.

In order to characterize this system further, we also used ifenprodil, a NMDA antagonist modulating the polyamine binding site (Carter *et al.*, 1989). This neuroprotective agent has been shown to have cerebral anti-ischaemic properties without producing any behavioural effect (Carter *et al.*, 1988; Gotti *et al.*, 1988). This latter feature of ifenprodil-like NMDA antagonists has made them potential candidates as novel therapeutic agents which could have an application in cardiovascular disorders.

Ifenprodil is a quite interesting drug for at least two reasons: (i) it readily crosses the blood-brain barrier when injected systemically and (ii) it is not a blocker but a modulator of the NMDA receptor/channel complex, and so was expected to block the cardiovascular responses induced by PVN stimulation without inducing any significant cardiodepression. In fact, our results showed that the i.v. injection of ifenprodil reduced the inotropic and vasopressor responses and consequently the increases in the MOD caused by PVN electrical stimulation. The basal arterial blood pressure was also reduced, probably because this drug is also an α-adrenoceptor blocking drug (Chenard *et al.*, 1991). Nevertheless, the classical α-blocker, prazosin, injected intravenously, reduced the vasopressor response but not the increases in dP/dt_{max} induced by electrical stimulation of the PVN (data not shown) while ifenprodil also affected the inotropic response.

Numerous findings suggest that the 'dissociative anaes-

thetics' like ketamine (which is a lipophilic compound and readily crosses the blood-brain barrier) are non-competitive antagonists of the EAA neurotransmission system (Martin & Lodge, 1985; Collingridge & Lester, 1989; Lodge & Johnson, 1990). It has been shown that ketamine binds to a specific site within the NMDA ion channel complex, called the phencyclidine site (PCP site) (Anis *et al.*, 1983; Honey *et al.*, 1985; Davies *et al.*, 1988; Halliwell *et al.*, 1989; MacDonald & Novak, 1990).

We wished to determine whether the influence of the blockade of glutamate effects induced by competitive and non-competitive NMDA antagonists on nervous regulation would be different. In fact, if the cardiovascular responses provoked by electrical stimulation of the PVN were due to the conversion of the cation channel coupled to the NMDA receptor from the closed to the open state, it is tempting to speculate that ketamine would inhibit the response without affecting the resting cardiovascular parameters. Indeed, in our experimental model, the i.v. injection of ketamine (2.5 and 10 mg kg^{-1}) prevented not only the hypertensive and positive inotropic responses to electrical stimulation of the PVN but also the increase of the triple product. These data showed that this drug is capable of inducing a central cardioprotective effect. It is noteworthy that these doses of ketamine are quite low, i.e. about 10 times lower than the anaesthetic ones in this species. Interestingly, the protective effects of ketamine, which were related to the dose, did not significantly reduce the resting haemodynamic parameters.

Thus, the results obtained with these two latter drugs injected via the i.v. route, are very similar to those observed with 5,7 diCl-KYNA and APV. The common effect of all these drugs was their blockade of the central NMDA receptors, suggesting that they blunted the cardiovascular responses to PVN stimulation probably by inhibiting the link(s) in the central nervous command to the cardiac function, including cardiac contractility, operating by EAA as neurotransmitters.

As a consequence of their inhibitory effect on the haemodynamic responses to hypothalamic stimulation, all the EAA inhibitors decreased oxygen consumption as measured by the triple product index. We recently described a similar action of baclofen (Tibiriça *et al.*, 1993), a $GABA_B$ agonist, known to inhibit the presynaptic release of glutamate (Bowery *et al.*, 1980).

Thus, modulation of the central regulation of cardiac function with drugs acting on glutamatergic structures, might open a new therapeutic perspective i.e. non-psychotropic centrally-acting cardioprotective drugs. Whether or not some specific antagonists of the NMDA receptor sites will be prototypes has to be investigated further. However, the data we obtained here, especially with ifenprodil, known to be devoid of any psychotropic toxicity, already look promising.

References

ANIS, N.A., BERRY, S.C., BURTON, N.R. & LODGE, D. (1983). The dissociative anaesthetics, ketamine and phencyclidine, selectively reduce excitation of central mammalian neurons by N-methyl-aspartate. *Br. J. Pharmacol.*, **79**, 565–575.

AZEVEDO, A.D., HILTON, S.M. & TIMMS, R.J. (1980). The defence reaction elicited by midbrain and hypothalamic stimulation in the rabbit. *J. Physiol.*, **301**, 56P–57P.

BALLER, D., BRETSCHNEIDER, H.J. & HELLIGE, G. (1979). Validity of myocardial oxygen consumption parameters. *Clin. Cardiol.*, **2**, 317–327.

BIRCH, P.J., GROSSMAN, C.J. & HAYES, A.G. (1988). Kynurenic acid antagonises responses to NMDA via an action at the strychnine-insensitive glycine receptor. *Eur. J. Pharmacol.*, **154**, 85–87.

BOWERY, N.G., HILL, D.R., HUDSON, A.L., DOBLE, A., MIDDLEMISS, D.N., SHAW, J. & TURNBULL, M.J. (1980). Baclofen decreases neurotransmitter release in the mammalian CNS by an action at a novel GABA receptor. *Nature*, **283**, 92–94.

CARTER, C., BENAVIDES, J., LEGENDRE, P., VINCENT, J.-D., NOEL, F., THURET, F., LLOYD, K.G., ARBILLA, S., ZIVKOVIC, B., MACKENZIE, E.T., SCATTON, B. & LANGER, S.Z. (1988). Ifenprodil and SL 820715 as cerebral anti-ischemic agents. II. Evidence for N-methyl-D-aspartate receptor antagonist properties. *J. Pharmacol. Exp. Ther.*, **247**, 1222–1232.

CARTER, C., RIVY, J.-P. & SCATTON, B. (1989). Ifenprodil and SL 82.0715 are antagonists at the polyamine site of the N-methyl-D-aspartate (NMDA) receptor. *Eur. J. Pharmacol.*, **164**, 611–612.

CHALMERS, J. & PILOWSKY, P. (1991). Brainstem and bulbospinal neurotransmitter systems in the control of blood pressure. *J. Hypertension*, **9**, 675–694.

CHENARD, B.L., SHALABY, I.A., KOE, B.K., RONAU, R.T., BUTLER, T.W., PROCHNIAK, M.A., SCHMIDT, A.W. & FOX, C.B. (1991). Separation of α_1 adrenergic and N-methyl-D-aspartate antagonist activity in a series of ifenprodil compounds. *J. Med. Chem.*, **34**, 3085–3090.

CHILDS, A.M., EVANS, R.H. & WATKINS, J.C. (1988). The pharmacological selectivity of three NMDA antagonists. *Eur. J. Pharmacol.*, **145**, 81–86.

COLLINGRIDGE, G.L. & LESTER, R.A. (1989). Excitatory amino acid receptors in the vertebrate central nervous system. *Pharmacol. Rev.*, **40**, 143–210.

COTMAN, C.W., MONAGHAN, D.T., OTTERSEN, O.P. & STORM-MATHISEN, J. (1987). Anatomical organisation of excitatory amino acid receptors and their pathways. *Trends Neurosci.*, **7**, 273–280.

DAVIES, J., FRANCIS, A.A., JONES, A.W. & WATKINS, J.C. (1981). 2-amino-5-phosphono-valerate (2APV), a potent and selective antagonist of amino acid induced synaptic excitation. *Neurosci. Lett.*, **21**, 77–81.

DAVIES, S.N., MARTIN, D., MILLAR, J.D., ARAM, J.A., CHURCH, J. & LODGE, D. (1988). Differences in results from in vivo and in vitro studies on the use-dependency of N-methylaspartate antagonism by MK-801 and other phencyclidine receptor ligands. *Eur. J. Pharmacol.*, **145**, 141–151.

EVANS, R.H., FRANCIS, A.A., JONES, A.W., SMITH, D.A.S. & WATKINS, J.C. (1982). The effects of a series of ψ-phosphonic α-carboxylic amino acids on electrically-evoked and excitant amino acid-induced responses in isolated spinal cord preparations. *Br. J. Pharmacol.*, **75**, 65–75.

FOSTER, A.C., KEMP, J.A., LEESON, P.D., GRIMWOOD, S., DONALD, A.E., MARSHALL, G.R., PRIESTLEY, T., SMITH, J.D. & CARLING, R.W. (1992). Kynurenic acid analogues with improved affinity and selectivity for the glycine site on the N-Methyl-D-aspartate receptor from rat brain. *Mol. Pharmacol.*, **41**, 914–922.

GANONG, W.F. (1971). *Review of Medical Physiology*. p. 421. Los Altos, CA, U.S.A.: Lange Medical Publication.

GOTTI, B., DUVERGER, D., BERTIN, J., CARTER, C., DUPONT, R., FROST, J., GAUDILLIERE, B., MACKENZIE, E.T., ROUSSEAU, J., SCATTON, B. & WICK, A. (1988). Ifenprodil and SL 8250715 as cerebral anti-ischemic agents. I. Evidence for efficacy in models of focal cerebral ischemia. *J. Pharmacol. Exp. Ther.*, **247**, 1211–1232.

GUYENET, P.G., FILTZ, T.M. & DONALDSON, S.R. (1987). Role of excitatory aminoacids in rat vagal and sympathetic baroreflexes. *Brain Res.*, **407**, 272–284.

HALLIWELL, R.F., PETERS, J.A. & LAMBERT, J.J. (1989). The mechanism of action and pharmacological specificity of the anticonvulsant NMDA antagonist MK-801: a voltage clamp study on neuronal cells in culture. *Br. J. Pharmacol.*, **96**, 480–494.

HEADLEY, P.M. & GRILLNER, S. (1990). Excitatory amino acids and synaptic transmission: the evidence for a physiological function. *Trends Pharmacol. Sci.*, **11**, 205–211.

HILTON, S.M. & REDFERN, W.S. (1986). A search for brain stem cell groups integrating the defence reaction in the rat. *J. Physiol.*, **378**, 213–228.

HONEY, C.R., MILJKOVIC, Z. & MACDONALD, J.F. (1985). Ketamine and phencyclidine cause a voltage-dependent block of responses to L-aspartic acid. *Neurosci. Lett.*, **61**, 135–139.

HONORE, T. (1989). Excitatory amino acid receptor subtypes and specific antagonists. *Med. Res. Rev.*, **9**, 1–23.

JOHNSON, J.W. & ASCHER, P. (1987). Glycine potentiates the NMDA response in cultured mouse brain neurones. *Nature*, **325**, 529–533.

KAO, M.C., LEE, H.K., CHAI, C.Y. & WANG, Y. (1991). NMDA antagonists attenuate hypertension induced by carotid clamping in the rostral ventrolateral medulla of rats. *Brain Res.*, **549**, 83–89.

KEHL, S.J. & MCLENNAN, M. (1983). The antagonism of amino acid-induced excitations of rat hippocampal CA1 neurones *in vitro*. *J. Physiol.*, **334**, 19–31.

KIHARA, M., MISU, Y. & KUBO, T. (1989). Release of electrical stimulation of endogenous glutamate, gamma-aminobutyric acid and other amino acids from slices of the rat medullar oblongata. *J. Neurochem.*, **52**, 261–267.

KUBO, T. & KIHARA, M. (1988). Evidence of N-methyl-D-aspartate receptor-mediated modulation of the aortic baroreceptor reflex in the rat nucleus tractus solitarii. *Neurosci. Lett.*, **87**, 69–74.

KUBO, T., NAGURA, J., KIHARA, M. & MISU, Y. (1986). Cardiovascular effects of L-glutamate and gamma-aminobutyric acid injected into the rostral ventrolateral medulla in normotensive rats and spontaneously hypertensive rats. *Arch. Int. Pharmacodyn. Ther.*, **279**, 150–161.

LE GALLOUDEC, E.,MERAHI, N. & LAGUZZI, R. (1989). Cardiovascular changes induced by the local application of glutamate-related drugs in the nucleus tractus solitarii. *Brain Res.*, **503**, 322–325.

LODGE, D. & JOHNSON, K.M. (1990). Noncompetitive excitatory amino acid receptor antagonists. *Trends Pharmacol. Sci.*, **11**, 81–86.

LOWN, B., VERRIER, R. & RABINOWITZ, S. (1977). Neural and psychologic mechanisms and the problem of cardiac sudden death. *Am. J. Cardiol.*, **39**, 890–902.

MACDONALD, J.F. & NOVAK, L.M. (1990). Mechanisms of blockade of excitatory amino acid receptor channels. *Trends Pharmacol. Sci.*, **11**, 167–172.

MARTIN, D. & LODGE, D. (1985). Ketamine acts as a non-competitive N-methyl-D-aspartate antagonist on frog spinal cord in vitro. *Neuropharmacology*, **24**, 993–1003.

MILLS, E., MINSON, J., DROLET, G. & CHALMERS, J.P. (1990). Effect of aminoacid receptor antagonists on basal blood pressure and pressor responses to brainstem stimulation in normotensive and hypertensive rats. *J. Cardiovasc. Pharmacol.*, **15**, 877–883.

MILLS, E., MINSON, J., PILOWSKY, P. & CHALMERS, J.P. (1988). N-methyl-D-aspartate receptors in the spinal cord mediate pressor responses to stimulation of the ventrolateral medulla in the rat. *Clin. Exp. Pharmacol. Physiol.*, **15**, 147–155.

MONAGHAN, D.T., BRIDGES, R.J. & COTMAN, C.W. (1989). The excitatory amino acid receptors: their classes, pharmacology and distinct properties in the function of the central nervous system. *Annu. Rev. Pharmacol. Toxicol.*, **29**, 365–402.

SAWYER, C.H., EVERETT, J.W. & GREEN, J.D. (1954). The rabbit diencephalon in stereotaxic coordinates. *J. Comp. Neurol.*, **101**, 801–824.

SUNDARAM, K., MURUGAIAN, J. & SAPRU, H. (1989). Cardiac responses to the microinjections of excitatory amino acids into the intermediolateral cell column of the rat spinal cord. *Brain Res.*, **402**, 12–22.

TIBIRICA, E., MONASSIER, L., FELDMAN, J., BRANDT, C., VERDUN, A. & BOUSQUET, P. (1993). Baclofen prevents the increase of myocardial oxygen consumption indexes evoked by hypothalamic stimulation in rabbits. *Naunyn-Schmied. Arch. Pharmacol.*, **348**, 164–171.

WATKINS, J.C. & EVANS, R.H. (1981). Excitatory amino acid transmitters. *Annu. Rev. Pharmacol. Toxicol.*, **21**, 165–204.

WATKINS, J.C., KROGSGAARD-LARSEN, P. & HONORE, T. (1990). Structure-activity relationships in the development of excitatory amino acid receptor antagonists. *Trends Pharmacol. Sci.*, **11**, 25–33.

YOUNG, A.B. & FAGG, G.E. (1990). Excitatory amino acid receptors in the brain: membrane binding and receptor autoradiographic approaches. *Trends Pharmacol. Sci.*, **11**, 126–133.

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Activation of chloride current by P_2-purinoceptors in rat ventricular myocytes

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1 Rat ventricular myocytes were dissociated and their responses to extracellularly applied ATP were recorded using patch pipettes under the whole cell configuration.

2 ATP initially induced an inward current followed by an outward current at − 50 mV. With a Cs-rich pipette solution the late outward current was blocked, leaving a sustained inward current (I_{ATPs}) suggesting that a K^+ conductance underlies the late response.

3 When the extracellular Cl^- concentration was changed, the reversal potential of I_{ATPs} corresponded well to the shift of the Cl^- equilibrium potential. I_{ATPs} was reversibly blocked by the chloride channel blocker, 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS).

4 The concentration-response curve of I_{ATPs} had a Hill coefficient of 0.98 and an EC_{50} value of 5.2×10^{-6} M.

5 ATP was more potent than ADP, while AMP and adenosine were ineffective, suggesting that P_2-purinoceptor activation induced I_{ATPs}.

6 The activation of I_{ATPs} was depressed by depleting the extracellular Mg^{2+} and increased by adding Mg^{2+}.

7 Our results strongly suggest that P_2-purinoceptor activation by ATP induces both a Cl^--conductance (I_{ATPs}) and a K^+-conductance in rat ventricular myocytes.

Keywords: Ventricular myocytes; ATP; chloride current; P_2-purinoceptor

Introduction

There are many reports that adenosine 5′-triphosphate (ATP) acts as a neurotransmitter through activation of P_2-purinoceptors in a variety of tissues (Hoyle & Burnstock, 1991). In the heart, after the establishment of the presence of P_{2x}-purinoceptors (frog: Hoyle & Burnstock, 1986; rat: Fleetwood & Gordon, 1987), later reports focused on the ionic mechanisms of purinoceptors in the heart. In guinea-pig atrium, it has been shown that ATP responses are mediated by an activation of Cl conductance (Matsuura & Ehara, 1992). However, the ionic mechanism in the rat ventricle is confusing. Christie *et al.* (1992) reported that the activation of P_2-purinoceptor phosphorylates extracellular membrane-bound proteins, leading to the activation of a channel which carries Na^+ and Ca^{2+}. On the other hand, Pùceat *et al.* (1991b) reported that ATP binding to a putative P_3-purinoceptor activates an electrically neutral HCO_3^--Cl^- exchanger causing intracellular acidification. The intracellular acidification was thought to induce an increase in $[Ca^{2+}]_i$ (Scamps & Vassort, 1990; Púceat *et al.*, 1991a) and finally the activation of a Ca-dependent cation conductance (Colquhoun *et al.*, 1981). Thus, the relationship between the ionic mechanisms of ATP-induced responses and the types of purinoceptors was unclear in the rat ventricle. In the present experiments, therefore, we have used the patch clamp recording method to analyse the ionic mechanisms of ATP-induced responses and the possible types of purinoceptors in rat ventricular myocytes. We find that activation of a P_2-purinoceptor induced two different conductances, a Cl^--dependent current and a K^+-dependent current, which have not been reported previously. In the discussion the possible relationship between Cl^- movement through an activation of P_2-purinoceptor and an ATP-induced intracellular acidification has been suggested.

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Methods

Preparation

Isolated myocytes were obtained from male Sprague-Dawley rats (300–400 g) by collagenase digestion (0.1 mg ml^{-1}, Yakult, Tokyo, Japan) (Powell & Twist, 1976). The isolated cells were collected by sedimentation. The pellet was diluted with control solution to give a final concentration of 10^5 cells ml^{-1}; 200 μl of cell suspension was transferred to concanavalin A (Sigma Chemical Co., St Louis, MO, USA)-coated dishes containing 5 ml of control solution (extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) = 0.1 mM). Within 1 h cells were anchored on the dish. Cells were kept at room temperature until use and $[Ca^{2+}]_o$ was gradually increased up to 1 mM just before use. Cells were viable for 6–8 h in the present experiments.

Solutions

External and internal solutions used in the present experiments are shown in Table 1. To isolate Cl^- conductance we used a N-methyl-D-glucamine (NMG)-rich solution intra- and extracellularly. In the NMG-rich solution replacement of the membrane-permeable cations (Na^+, K^+ and Ca^{2+}) by the membrane-impermeable cation, NMG, blocks cation conductances, background conductances (Hagiwara *et al.*, 1992), the Na-K pump and the Na-Ca exchanger (Kimura *et al.*, 1986; Mechmann & Pott, 1986). Second, the possibility of the passage of Mg^{2+} or contaminating Ca^{2+} through voltage-gated Ca channels was excluded with verapamil. Third, the movement of residual intracellular K^+ through voltage-gated K^+ channels was also inhibited by perfusing the cells intracellularly with tetraethyl ammonium (TEA). Fourth, the Na-K pump was directly inhibited by ouabain.

A rapid superfusion system was used for solution exchange (Suzuki *et al.*, 1990). In this system solution exchange around the cells was complete within 100 ms. All experiments were

Table 1 Composition of solutions

External solutions (in mM)

	NaCl	*NaH_2PO_4*	*KCl*	*$CaCl_2$*	*$MgCl_2$*	*HEPES*	*Glucose*	*NMG*
Control	144	0.33	5.4	1	2	5	10	
K-free	150	0.33		1	2	5	10	
NMG-rich					2	5	10	150

Solutions contained 0.1 mg ml^{-1} bovine serum albumin and 0.001% phenol red. pH was adjusted at 7.4 with NaOH (control and K-free) or HCl (NMG-rich). NMG-rich solutions contained 5 μM verapamil and 10 μM ouabain. NMG: N-methyl-D-glucamine.

Internal solutions (in mM)

	CsOH	*CsCl*	*EGTA*	*HEPES*	*AA*	*ATP*	*NaCr*	*$MgCl_2$*	*$CaCl_2$*	*NMG*	*TEACl*
Cs-rich	110	20	10	5	90	5	5	1	0.5		
NMG-rich			10	5		5		1		110	20

pH was adjusted at 7.2 with CsOH (Cs-rich) or HCl (NMG-rich). Final Cl^- concentration of NMG-rich solution was 101 mM. AA: aspartic acid. ATP salts used are as follows: Cs-rich, Na_2-ATP; NMG-rich, Tris ATP.

carried out at room temperature. Unless otherwise mentioned, experiments were repeated on at least 3 cells.

Current recording

Currents were recorded by use of a patch clamp recording system in the whole cell configuration (Hamill *et al.*, 1981). The resistance of fire polished patch pipettes, filled with internal solution was 5–10 MΩ. The outer wall of the pipette, except for the tip, was coated with apiezon wax dissolved in chloroform (Apiezon Products Ltd., London, U.K.) to reduce the capacitance. The reference electrode was an Ag-AgCl wire connected to the dish by a 140 mM NaCl agar bridge. Currents were recorded by a patch clamp amplifier (CEZ-2200, Nihon Kohden, Tokyo, Japan). The ramp pulse was generated by a function generator (FG-121B, NF Circuit Design Block Co. Ltd., Yokohama, Japan). Current and voltage signals were recorded on a video tape recorder (HV-S21, Mitsubishi, Tokyo, Japan) through a PCM processor (PCM-DP16, Shoshin EM Corp., Okazaki, Japan). Current and voltage signals were also monitored by a chartrecorder (WR3701, Graphtec, Yokohama, Japan). The recorded current and voltage signals were filtered at 1 kHz through a low pass filter (E3201-A, NF Circuit Design Block Co. Ltd., Yokohama, Japan) and analysed by an off-line computer (PC-9801RX, NEC, Tokyo, Japan). A hard copy of individual current-voltage (*I-V*) relationship was plotted by an X-Y plotter (MIPLOT III, Graphtec, Yokohama, Japan) through the off-line computer.

Correction of holding potential

In the present experiments the holding potential (V_H) was corrected for the effect of liquid junction potentials (Hagiwara & Ohmori, 1982).

Correction of chloride concentration

The equilibrium potential of Cl^- was calculated using the activity coefficient of Cl^-. The activity coefficient of Cl^- in NMG-Cl solution was aproximated by that in KCl solution. The activity coefficient of $[Cl^-]_o$ was estimated as 0.74 at 153 mM, 0.82 at 51 mM and 0.88 at 15 mM. The activity coefficient of the intracellular Cl^- concentration ($[Cl^-]_i$) was estimated as 0.77 when cells were fully perfused by NMG-rich intracellular solution ($[Cl^-]_i$ = 101 mM). The corrected $[Cl^-]_o$ and $[Cl^-]_i$ was expressed as the product of the activity coefficient and Cl^- concentration.

Results

ATP-induced response

As shown in Figure 1, the application of 10^{-5} M ATP induced a biphasic current response at a V_H of − 49 mV. The initial inward current was observed immediately after the start of ATP superfusion and was followed by a gradual outward current. Removal of ATP induced a further transient outward current. In the experiment shown in Figure 1, a Cs-rich pipette solution was used to suppress outward K^+ conductances. The gradual loss of the ATP-induced outward current (compare 3 min and 42 min) probably reflects equilibration of this Cs-containing pipette solution with the intracellular medium. Moreover, as the outward current was not observed when cells were held at − 89 mV (not shown), this current may be carried by K^+. Separation of the inward and outward responses was performed by subtraction of the response recorded at 97 min from that at 3 min (Figure 1, subtract). The outward response reached a peak at around 10 s and decayed gradually. The average time after patch rupture until the disappearance of the outward current was 21.5 ± 7.5 min (n = 23). Out of 57 cells tested at 4 min after the establishment of intracellular perfusion, 52 had both an inward and an outward current and 5 cells had only an inward current. In the present experiment we have analysed only the sustained inward current (I_{ATPs}) in detail.

I_{ATPs} was repeatedly evoked when 10^{-4} M ATP was applied every 4 min. Therefore, in the present experiments the interval of ATP application was set at 4–5 min.

Ionic selectivity of I_{ATPs}

In previous reports, two different cation conductances activated by ATP have been reported in rat ventricular myocytes; a cation conductance (Christie *et al.*, 1992), and a Ca-dependent cation conductance (Púceat *et al.*, 1991a). In our experiments, cells were intra- and extracellularly perfused with an NMG-rich solution designed to block cation conductances (see Methods). However, even under these ionic conditions, the application of ATP induced I_{ATPs}. In the NMG-rich solution the average time until the isolation of I_{ATPs} was 7.7 ± 2.9 min (n = 42). Therefore, we conclude that I_{ATPs} response is not mediated by an increase in cation conductance. In addition, I_{ATPs} could be observed when an NMG-rich solution containing 10 mM EGTA was used for the extracellular perfusate, suggesting that an influx of Ca^{2+} is not involved in the generation of I_{ATPs}.

The Cl^--sensitive nature of I_{ATPs} was indicated by measurement of the reversal potential. In the experiment shown in Figure 2a, ATP was applied at various holding potentials. The response was sustained during the application of ATP at every membrane potential and reversed between − 5 and − 15 mV. The peak amplitude voltage relation plotted in Figure 2b, was linear and indicated a reversal potential (E_{ATPs}) of − 12 mV. In 6 experiments E_{ATPs} was − 13.3 ± 1.7 mV. This value is quite close to the equilibrium potential for Cl^- (E_{Cl}) of − 9.5 mV.

To facilitate the measurement of E_{ATPs}, the *I-V* relation was determined by the use of a ramp pulse (Figure 3a). The background conductance obtained before the application of ATP showed an outward going rectification. When $[Cl^-]_o$ and $[Cl^-]_i$ were 153 and 101 mM, respectively, the *I-V* curve in the presence of ATP showed large slope conductance and crossed with the control *I-V* curve at 0 mV (Figure 3b, left). When $[Cl^-]_o$ was reduced to 15 mM, the *I-V* curve also showed rectification and two *I-V* curves determined before and after ATP application crossed at + 31 mV (Figure 3b, right). The difference obtained by subtracting the control *I-V* curve from those in the presence of ATP was almost linear in every case (Figure 3c). The reversal potentials thus determined were corrected for the liquid junction potential and plotted against the activity of $[Cl^-]_o$ (Figure 4). These values corresponded well with the shift of Cl^- equilibrium potential calculated from the Nernst equation. When $[Cl^-]_o$ was changed from 153 mM to 15 mM, for a theoretical shift of Cl^- equilibrium potential of 54.1 mV, the observed shift of E_{ATPs} was 38.2 mV.

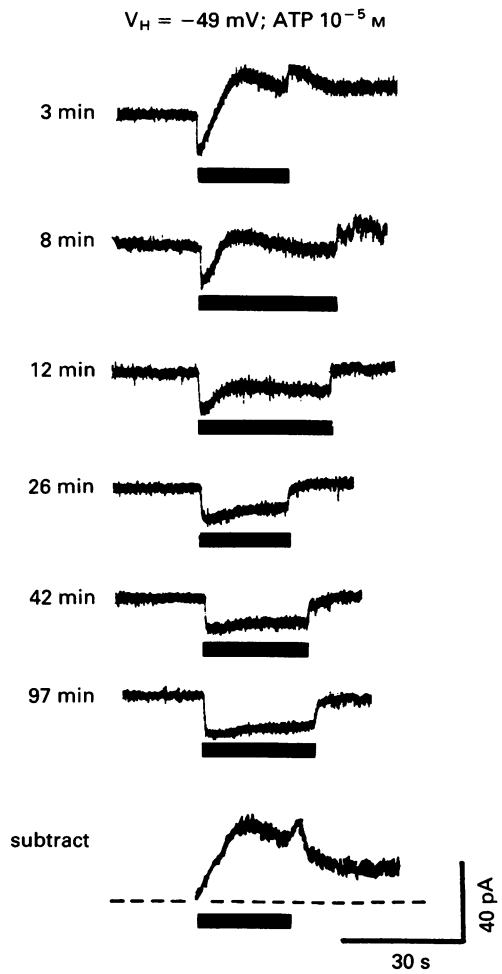


Figure 1 ATP-induced current recorded at different times. Times shown at the left, indicate the time after the establishment of the intracellular perfusion. Bars shown below each trace correspond to the duration of ATP application. V_H = − 49 mV. External solution: K-free, pipette solution: Cs-rich.

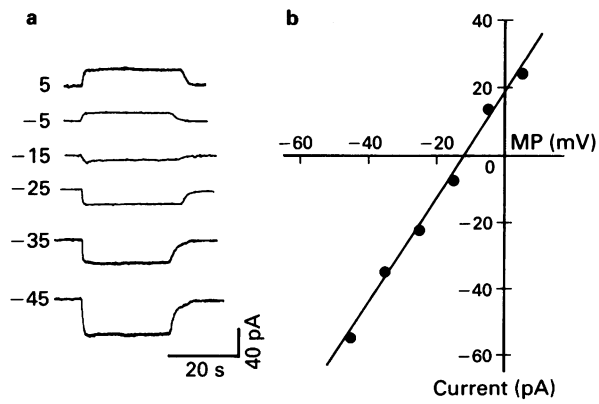


Figure 2 Current-voltage (*I-V*) relationship of I_{ATPs}. (a) I_{ATPs}s recorded at different holding potentials. (b) *I-V* relationship of I_{ATPs}. (a) and (b) were obtained from the same cell. Applied ATP concentration: 10^{-5} M, external solution: NMG-rich, pipette solution: NMG-rich.

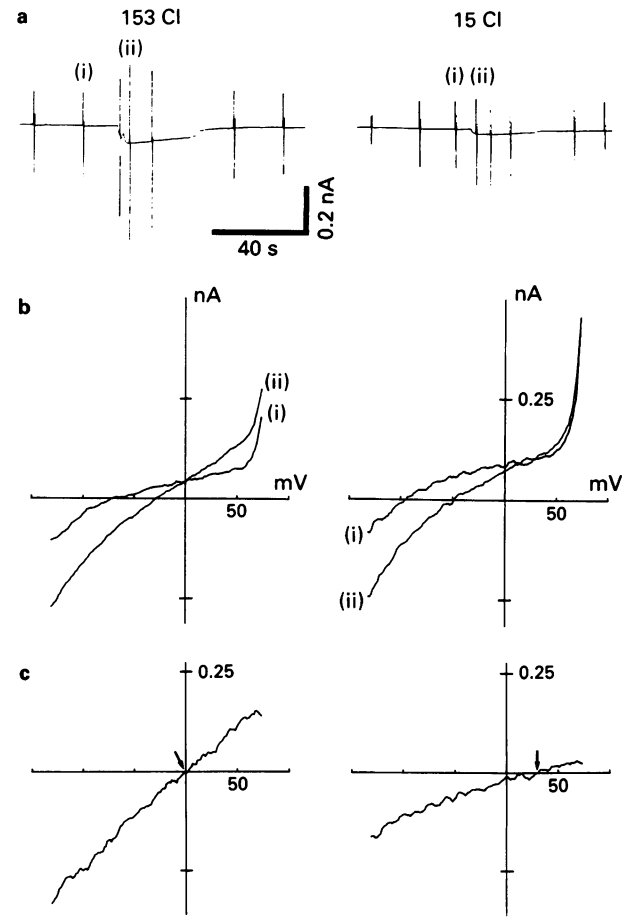


Figure 3 *I-V* relationship of I_{ATPs}. (a) Reponse to ramp pulse application. The sharp vertical lines on the current trace indicate the current evoked by the ramp pulse. Speed of ramp pulse was 420 mV s^{-1} (range − 140 mV to 70 mV). In the presence of ATP, ramp pulse was applied at around the peak of I_{ATPs}. (b) Estimation of E_{ATPs} by the ramp pulse. Individual *I-V* relationships correspond to (i) and (ii) shown in (a). The crossing points correspond to E_{ATPs}. (c) Subtraction of ramp pulse in the presence and absence of ATP. Arrows indicate E_{ATPs}. Applied ATP concentration: 10^{-5} M, external solution: NMG-rich, pipette solution: NMG-rich.

Effects of DIDS on I_{ATPs}

The Cl^--sensitive nature of I_{ATPs} was further confirmed by the use of Cl^- channel blocker, DIDS (de Lisle & Hopfer, 1986). In the presence of 0.2 mM DIDS, I_{ATPs} induced by 10^{-5} M ATP was suppressed by 70% (Figure 5). The inhibitory effects of DIDS on I_{ATPs} was reversible. The average of inhibition was 61 ± 8% ($n = 3$).

Response to various purinoceptor agonists

Purinoceptors can be pharmacologically classified into P_1 and P_2 types; P_1 was characterized by a high affinity for AMP and adenosine while P_2 is characterized by the high affinity for ATP and ADP (Burnstock, 1978). At a V_H of −45 mV, 10^{-5} M ATP induced I_{ATPs} (Figure 6). Similarly 10^{-4} M ADP also evoked I_{ATPs}, though the amplitude of I_{ATPs} was only 15% of that evoked by 10^{-5} M ATP. In contrast, AMP and adenosine did not evoke any response at 10^{-4} M (Figure 6). These results indicate that I_{ATPs} is mediated by a P_2-purinoceptor in the rat ventricular myocytes.

However, the P_{2X}-purinoceptor agonist, α, β-methyl-adenosine 5′-phosphate (AMP-CPP) and β, γ-methyl-adenosine 5′-phosphate (AMP-PCP) (Burnstock, 1990; Olsson & Pearson, 1990) did not evoke any response at 10^{-4} M (not shown). The subtype classification of P_2-purinoceptor therefore remains to be determined although our data might also indicate the presence of P_{2Y}-purinoceptors in rat ventricular myocytes (Björnsson *et al.*, 1989).

Concentration-response relationship of I_{ATPs}

At a V_H of −45 mV, application of 3×10^{-7} M ATP failed to evoke a detectable response (Figure 7a). I_{ATPs} was first observed at 6×10^{-7} M ATP and I_{ATPs} increased its amplitude with increasing ATP concentration. Figure 7b shows the concentration-response relationship of I_{ATPs}. In this figure the amplitude of individual responses was normalized to the amplitude of I_{ATPs} evoked by 10^{-5} M ATP. I_{ATPs} had a threshold at around 3×10^{-7} M and increased its amplitude in a sigmoidal fashion with increasing ATP concentration. The half maximal response was obtained at 5.2×10^{-6} M. At 10^{-4} M I_{ATPs} reached maximum. The concentration-response relationship of I_{ATPs} was fitted by the following equation:

$$I = I_{max}(C^n/(C^n + EC_{50}{}^n))$$

where I is the observed I_{ATPs}, I_{max} is the maximum current, C is the applied ATP concentration, EC_{50} is the ATP concen-

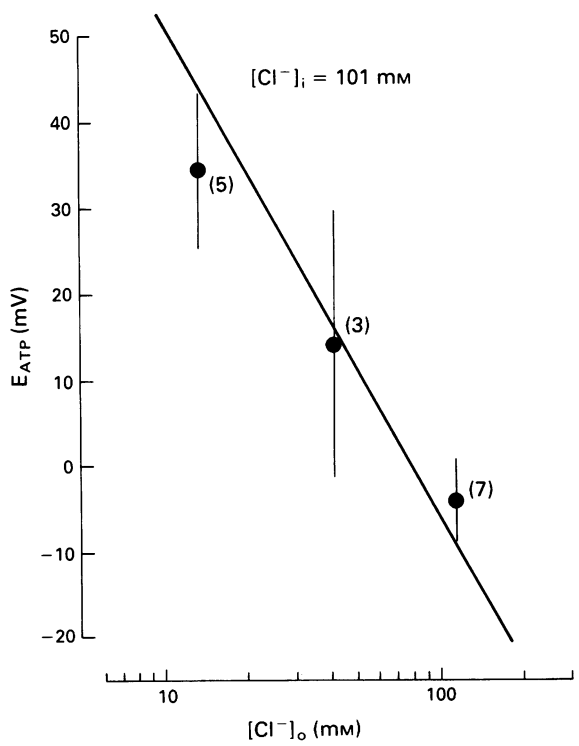


Figure 4 Relationship between $[Cl^-]_o$ and E_{ATPs}. $[Cl^-]_o$ was corrected by the activity coefficient of Cl^-. E_{ATPs} were corrected by the tip potential and the liquid junction potential. Symbols show the average with s.d. Solid line represents the equilibrium potential of Cl^- calculated from the Nernst equation. Number in parentheses indicates n values. Applied ATP concentration: 10^{-5} M, external solution: NMG-rich, pipette solution: NMG-rich.

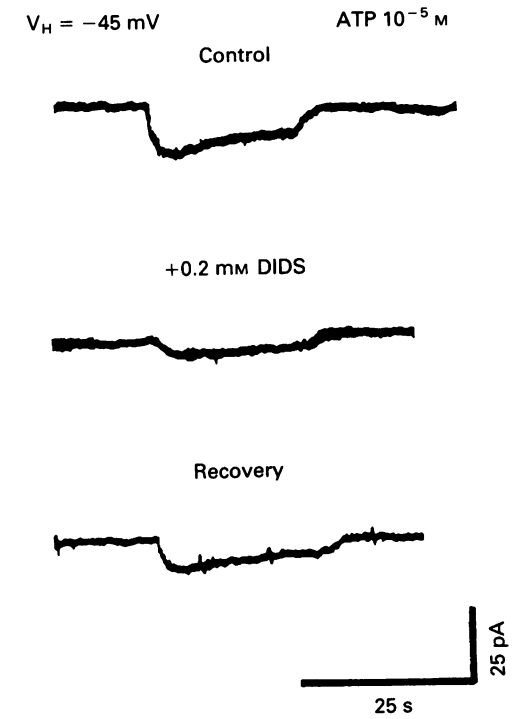


Figure 5 Effects of DIDS on I_{ATPs}. DIDS was perfused for 30 s prior to the ATP application. $V_H = -45$ mV. Applied ATP concentration: 10^{-5} M, external solution: NMG-rich, pipette solution: NMG-rich.

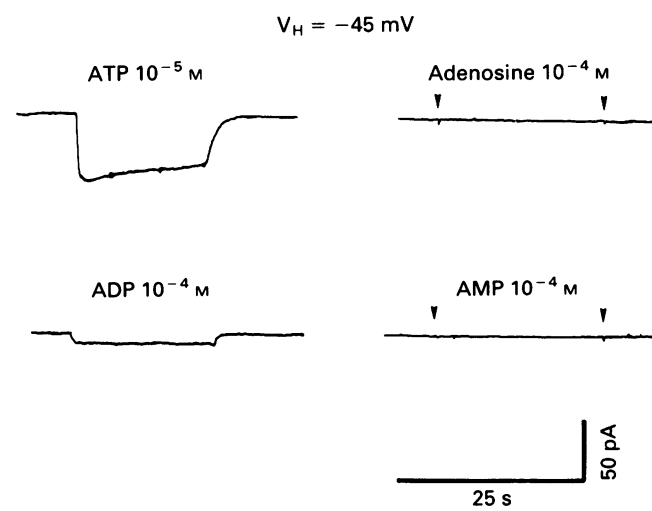


Figure 6 Responses to purinoceptor agonists. Arrows shown in the trace of AMP and adenosine indicate the start and the finish of the application. All responses are recorded from the same cell. $V_H = -45$ mV. External solution: NMG-rich, pipette solution: NMG-rich.

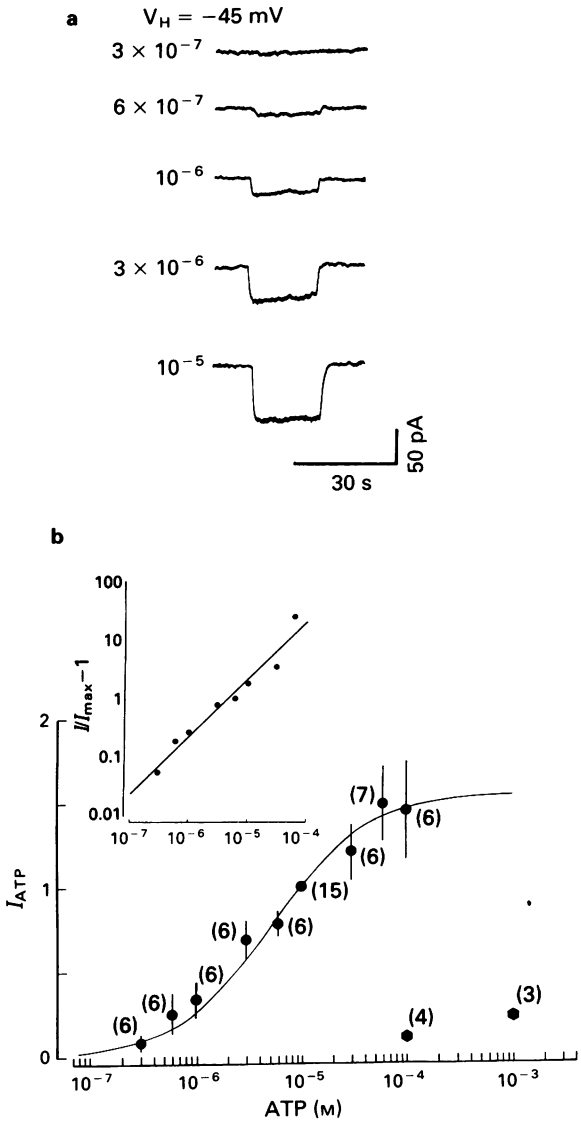


Figure 7 (a) I_{ATPs} evoked by different ATP concentrations (M) B. (b) Concentration-response relationship of I_{ATPs}. I_{ATPs} were normalized by I_{ATPs} evoked by 10^{-5} M ATP and plotted against ATP concentration. Symbols shown in the figure indicate the average with s.d. Numbers in parentheses indicate n values. Inset shows the Hill plot of I_{ATPs}. Solid line was drawn by the least squares method. V_H = − 45 mV. External solution: NMG-rich, pipette solution: NMG-rich. (●): ATP; (◆): ADP.

tration that evokes half maximal response, and n is the Hill coefficient. From the Hill plot of peak I_{ATPs} (inset), the Hill coefficient was determined as 0.98, indicating that the binding of one ATP molecule to P_2-purinoceptors activates I_{ATPs} in rat ventricular myocytes. Individual EC_{50} was also determined by the same equation in 7 cells having more than 5 points. An average of EC_{50} was $7.15 \times 10^{-6} \pm 6.20 \times 10^{-6}$ M (mean ± s.d.). In the Cs-rich solution the concentration-response relationship of I_{ATPs} was fitted by the similar parameters (EC_{50}, 2.2×10^{-6} M; Hill coefficient, 0.95).

Effects of extracellular Mg^{2+} ($[Mg^{2+}]_o$) on I_{ATPs}

It has been reported that ATP responses are sensitive to $[Mg^{2+}]_o$ (Scamps *et al.*, 1990). We tested the effect of $[Mg^{2+}]_o$ on I_{ATPs} evoked by 10^{-5} M ATP (Cs-rich pipette solution; Figure 8). The individual currents evoked at different $[Mg^{2+}]_o$ were normalized to the I_{ATPs} evoked in 2 mM Mg^{2+}. Then, normalized currents were averaged and plotted against

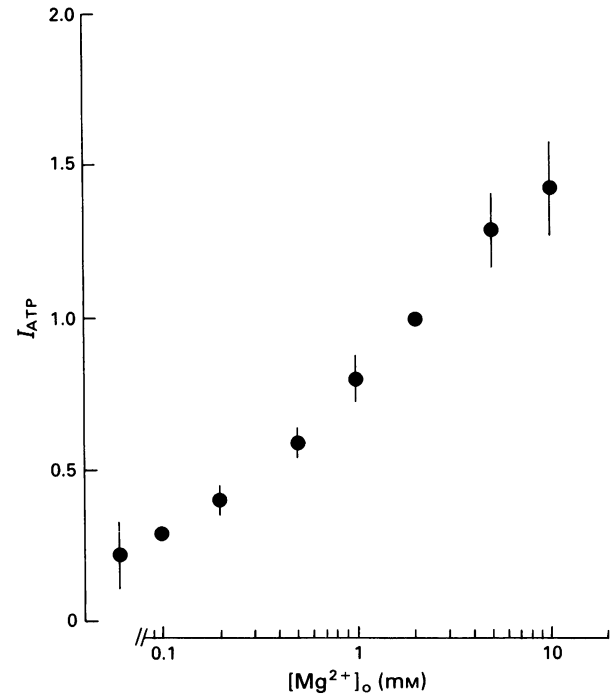


Figure 8 Effects of $[Mg^{2+}]_o$ on I_{ATPs}. I_{ATPs} were normalized by I_{ATPs} evoked in 2 mM $[Mg^{2+}]_o$. Concentration of applied ATP was 10^{-5} M. $MgCl_2$ was used for $[Mg^{2+}]_o$ change. Symbols show the average with s.d. V_H = − 49 mV. External solution: K-free, pipette solution: Cs-rich.

$[Mg^{2+}]_o$. When cells were perfused with Mg^{2+}-free solution, the observed amplitude of I_{ATPs} was 22 ± 11% of that obtained in 2 mM Mg^{2+} (n = 5). I_{ATPs} increased its amplitude with increasing $[Mg^{2+}]_o$. In 10 mM Mg^{2+} the amplitude of I_{ATPs} was 1.4 times larger than in 2 mM Mg^{2+} (n = 7). Thus in rat ventricular myocytes I_{ATPs} is affected by $[Mg^{2+}]_o$.

Discussion

We have identified an ATP-activated Cl^--current in rat ventricular myocytes by the intra- and extracellular perfusion of Na, K, Ca-free NMG solution. Under these conditions, the only permeant ions are anions, including Cl^-. In fact, the shift of E_{ATPs} fitted well to the theoretical value of E_{Cl} calculated from the Nernst equation, strongly suggesting that I_{ATPs} is carried by Cl^-. A similar ATP-induced Cl^- current was recently identified in guinea-pig atrial cells (Matsuura & Ehara, 1992).

In the present experiments we also observed ATP-induced K^+ currents. Thus, in ventricular myocytes, P_2-purinoceptors activation appears to induce both Cl^-- and K^+-conductances independently. In previous reports it has been shown that ATP activates two different types of conductances in guinea-pig (Hirano *et al.*, 1991) and bullfrog atria (Friel & Bean, 1988), one component of which was considered to be carried by K^+. In bullfrog atrium, ATP-induced K^+ conductances are thought to be identical to muscarinic ATP-induced K^+ conductances, which do not exist in ventricular cells (Friel & Bean, 1988). Thus, it would be interesting to see if ATP-induced K^+ conductances are sharing same K^+ channels with other K^+ conductances in rat ventricular myocytes.

In symmetrical Cl^- I_{ATPs} reversed its polarity at around 0 mV and had a linear I-V relationship. Previous studies of ATP-currents reported linear I-V relationships and reversal potentials around 0 mV in symmetrical Cl^- solution in the rat ventricular myocytes (Scamps & Vassort, 1990; Christie *et al.*, 1992). From the characteristics of I-V curve these authors

considered the existence of cation conductances. In the present experiments, however, the observed I_{ATPs} was mediated by Cl^-. Therefore, we conclude that the ATP-induced conductance having a linear *I-V* relationship is mediated by Cl^- and not by cations.

In the rat ventricular myocytes, previous reports show an increase in $[Ca^{2+}]_i$ by an application of ATP (Danziger *et al.*, 1988; Björnsson *et al.*, 1989; de Young & Scarpa, 1987, 1989). In ventricular myocytes, the equilibrium potential for Cl^- is thought to be more positive than the resting potential. Activation of I_{ATPs} would therefore induce depolarization within a few seconds. In fact, ATP-induced depolarization has been reported in rat ventricular myocytes, where superimposition of action potentials occurs within 1–2 s after the start of depolarization (Christie *et al.*, 1992). Therefore, $[Ca^{2+}]_i$ increase as a result of the activation of voltage-gated Ca^{2+} channels was considered. Actually this hypothesis corresponds well to the previous observation of simultaneous recording of membrane potentials and $[Ca^{2+}]_i$ in the rat (Björnsson *et al.*, 1989). As to the initiation of an increase in $[Ca^{2+}]_i$ the possibilities of the involvement of the intracellular acidification by a putative P_3-purinoceptor (Púceat *et al.*, 1991b) and extracellular membrane phosphorylation (Christie *et al.*, 1992) have been reported. However, these two possibilities are not likely to be the triggering mechanism since it seems difficult to explain the time course of $[Ca^{2+}]_i$ increase by these mechanisms. Using fluorescent dyes they reported that the increase in $[Ca^{2+}]_i$ reached a peak at around 15–20 s after the ATP application and then elevated $[Ca^{2+}]_i$ started to return to the control level. On the other hand, peak responses for both intracellular acidification (Púceat *et al.*, 1991b) and extracellular phosphorylation (Christie *et al.*, 1992) were observed at 60 s after the ATP application. Thus, $[Ca^{2+}]_i$ increase reached a peak before the other two responses reached that peak. Therefore, we concluded that the initiation of $[Ca^{2+}]_i$ increase was triggered by an activation of ATP-induced depolarization which is followed by voltage gated Ca^{2+} channel activation.

In the rat, it has been reported that ATP induces intracellular acidification by the activation of a putative P_3-purinoceptor which is coupled to a HCO_3^--Cl^- exchanger (Púceat *et al.*, 1991b). In the present experiments we found P_2-purinoceptor-activated Cl^- conductance. If the activation of I_{ATPs} occurs at the resting potential, Cl^- ions would move from the intracellular space to the extracellular space. This depletion of the intracellular Cl^- could activate the HCO_3^--Cl^- exchanger leading to intracellular acidification. Thus, it is also possible to explain the previously reported intracellular acidification by the activation of a P_2-purinoceptor. Actually, the extracellular ATP concentrations which gave the threshold, EC_{50} and the maximal response of I_{ATPs} in the present experiments were similar to those reported for the ATP-induced intracellular acidification (Púceat *et al.*, 1991b). Thus, it will be interesting to see if the present result could explain some of the previous observation that have been ascribed to the existence of a putative P_3-purinoceptors.

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References

BJÖRNSSON, O.G., MONCK, J.R. & WILLIAMSON, J.R. (1989). Identification of P_{2Y}-purinoceptors associated with voltage-activated cation channels in cardiac ventricular myocytes of the rat. *Eur. J. Biochem*, **186**, 395–404.

BURNSTOCK, G. (1978). A basis for distinguishing two types of purinergic receptor. In *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach*, ed. Bolis, L. & Straub, R.B., pp. 107–118, New York: Raven Press.

BURNSTOCK, G. (1990). Overview-Purinergic mechanisms. In *Biological Actions of Extracellular ATP*. Ann. N.Y. Acad. Sci. ed. G.R. Dubyak & J.A. Fedan, Vol. 603, pp. 1–18, New York: New York Academy of Sciences.

CHRISTIE, A., SHARMA, V.K. & SHEU, S.-S. (1992). Mechanism of extracellular ATP-induced increase of cytosolic Ca^{2+} concentration in isolated rat ventricular myocytes. *J. Physiol.*, **445**, 369–388.

COLQUHOUN, D., NEHER, E., REUTER, H. & STEVENS, C.F. (1981). Inward current channels activated by intracellular Ca in cultured cardiac cells. *Nature*, **294**, 752–754.

DANZIGER, R.S., RAFFAELI, S., MORENO-SANCHEZ, R., SAKAI, M., CAPOGROSSI, M.C., SPURGEON, H.A., HANSFORD, R.G. & LAKATTA, E.G. (1988). Extracellular ATP has a potent effect to enhance cystolic calcium and contractility in single ventricular myocytes. *Cell Calcium*, **9**, 193–199.

FLEETWOOD, G. & GORDON, J.L. (1987). Purinoceptors in the rat heart. *Br. J. Pharmacol.*, **90**, 219–227.

FRIEL, D.D. & BEAN, B.P. (1988). Two ATP-activated conductances in bullfrog atrial cells. *J. Gen. Physiol.*, **91**, 1–27.

HAGIWARA, N., IRISAWA, H., KASANUKI, H. & HOSODA, S. (1992). Background current in sino-atrial node of the rabbit heart. *J. Physiol.*, **448**, 53–72.

HAGIWARA, S. & OHMORI, H. (1982). Studies of calcium channels in rat clonal pituitary cells with patch electrode voltage clamp. *J. Physiol.*, **331**, 231–252.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.*, **391**, 85–100.

HIRANO, Y., ABE, S., SAWANOBORI, T. & HIRAOKA, M. (1991). External ATP-induced changes in $[Ca^{2+}]_i$ and membrane currents in mammalian atrial myocytes. *Am. J. Physiol.*, **260**, C673–C680.

HOYLE, C.H.V. & BURNSTOCK, G. (1986). Evidence that ATP is a neurotransmitter in the frog heart. *Eur. J. Pharmacol.*, **124**, 285–289.

HOYLE, C.H.V. & BURNSTOCK, G. (1991). ATP receptors and their physiological roles. In *Adenosine in the Nervous-System*. ed. Stone, T.W. pp. 43–76, London: Academic Press.

KIMURA, J., NOMA, A. & IRISAWA, H. (1986). Na-Ca exchange in mammalian heart cells. *Nature*, **319**, 596–597.

DE LISLE, R.C. & HOPFER, U. (1986). Electrolyte permeabilities of pancreatic zymogen granules: implications for pancreatic secretion. *Am. J. Physiol.*, **250**, G489–G496.

MATSUURA, H. & EHARA, T. (1992). Activation of chloride current by purinergic stimulation in guinea pig heart cells. *Circ. Res.*, **70**, 851–855.

MECHMANN, S. & POTT, L. (1986). Identification of Na-Ca exchange current in single cardiac myocytes. *Nature*, **319**, 597–599.

OLSSON, R.A. & PEARSON, J.D. (1990). Cardiovascular purinoceptors. *Physiol. Rev.*, **70**, 761–845.

POWELL, T. & TWIST, V.W. (1976). A rapid technique for the isolation and purification of adult cardiac muscle cells having respiratory control and a tolerance to calcium. *Biochem. Biophys. Res. Commun.*, **72**, 327–333.

PÚCEAT, M., CLÉMENT, O., SCAMPS, F. & VASSORT, G. (1991a). Extracellular ATP-induced acidification leads to cytosolic calcium transient rise in single rat cardiac myocytes. *Biochem. J.*, **274**, 55–62.

PÚCEAT, M., CLÉMENT, O. & VASSORT, G. (1991b). Extracellular MgATP activates the Cl^-/HCO_3^- exchanger in single rat cardiac cells. *J. Physiol.*, **444**, 241–256.

SCAMPS, F., LEGSSYER, E., MAYOUX, E. & VASSORT, G. (1990). The mechanism of positive intropy induced by adenosine triphosphate in rat heart. *Circ. Res.*, **7**, 1007–1016.

SCAMPS, F. & VASSORT, G. (1990). Mechanism of extracellular ATP-induced depolarization in rat isolated ventricular cardiomyocytes. *Pflügers Arch.*, **417**, 309–316.

SUZUKI, S., TACHIBANA, M. & KANEKO, A. (1990). Effects of glycine and GABA on isolated bipolar cells of the mouse retina. *J. Physiol.*, **421**, 645–662.

DE YOUNG, M.B. & SCARPA, A. (1987). Extracellular ATP induces Ca^{2+} transients in cardiac myocytes which are potentiated by norepinephrine. *FEBS Lett.*, **223**, 53–58.

DE YOUNG, M.B. & SCARPA, A. (1989). ATP receptor-induced Ca^{2+} transients in cardiac myocytes: sources of mobilized Ca^{2+}. *Am. J. Physiol.*, **257**, C750–C758.

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