British Journal of Pharmacology

August 1993

Volume 109 Number 4 pages 893–1290

Br. J. Pharmacol. (1993), **109**, 895–897

SPECIAL REPORT

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Caffeine-induced decreases in the inward rectifier potassium and the inward calcium currents in rat ventricular myocytes

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The effects of high (20 mM) concentrations of caffeine were studied on the transmembrane voltage and currents in rat single ventricular myocytes by the whole cell configuration of the patch clamp technique. Rapid application of caffeine released Ca^{2+} from the sarcoplasmic reticulum and induced a Ni^{2+}-sensitive transient inward current with concomitant change of the transmembrane voltage from -72.6 ± 0.4 to -68.0 ± 0.6 mV ($n = 4$). Maintained application of caffeine lengthened the action potential duration (APD_{90}) from 66.7 ± 16.9 to 135.1 ± 34.1 ms ($n = 4$) and depressed the amplitude of both the inward rectifier potassium and the inward calcium currents. It is concluded that these effects of caffeine should be recognized when it is used as a tool to study electromechnical coupling.

Keywords: Caffeine; heart; voltage clamp; calcium current; potassium current

Introduction Caffeine in high concentrations (5–20 mM) is widely used as a pharmacological tool in muscular tissues to study excitation-contraction coupling. Caffeine enhances Ca^{2+} permeability of the sarcoplasmic reticulum (SR) membrane by increasing the frequency and the duration of open events of the SR Ca-release channels (Rousseau & Meissner, 1989). The change of the intracellular Ca^{2+} concentration may affect the properties of various transmembrane ionic currents in the heart. Also, there are reports that caffeine may directly influence ionic currents (Goto *et al.*, 1979; Eisner *et al.*, 1979; Yatani *et al.*, 1984; Habuchi *et al.*, 1991). The effect of caffeine on the L-type Ca current is controversial. Some studies suggest an increase of I_{Ca} (Goto *et al.*, 1979; Yatani *et al.*, 1984) probably due to the suppression of phosphodiesterase activity (Beavo *et al.*, 1970). Other studies indicate an inhibitory action of caffeine on the I_{Ca} (Eisner *et al.*, 1979; Hughes *et al.*, 1990). Recently, it has also been reported that caffeine decreased the fast inward sodium current in guinea-pig ventricular myocytes (Habuchi *et al.*, 1991). However, there is still little information available about the effect of caffeine on the ionic currents in the heart. Therefore, in this study we investigated the effects of high (20 mM) concentrations of caffeine on the action potential and ionic currents in rat ventricular myocytes.

Methods Single myocytes isolated from the rat ventricle by a technique modified from that of Powell *et al.* (1980) were voltage clamped using the whole cell configuration of the patch clamp technique. Cell shortening was measured with a video edge-detector. Electrodes of 3–6 MΩ resistance were filled with the following solution (mM): potassium glutamate 140, KCl 10, NaCl 7, $MgCl_2$ 5, HEPES 10, K_2EGTA 0.1, K_2ATP 5, pH 7.2. The cells were bathed in the following solution (mM): NaCl 133.5, KCl 4, NaH_2PO_4 1.2, $MgSO_4$ 1.2, HEPES 10, glucose 11, $CaCl_2$ 1, pH = 7.4. All experiments were performed at room temperature (20–24°C).

Results Rapid application of 20 mM caffeine released Ca^{2+} from the SR, and induced an inward current (-45.4 ± 13.9 pA, $n = 4$, Figure 1b) at -80 mV. This current was completely abolished by 5 mM $NiCl_2$ (not shown) and therefore, was attributed to the Na-Ca exchange (Clusin, 1983). This transient caffeine-induced inward current changed the transmembrane potential from -72.6 ± 0.4 to -68.0 ± 0.6 mV ($n = 4$; Figure 1a). Following these transient effects, maintained application of caffeine lengthened the action potential duration at 50 and 90% repolarization from 13.9 ± 2.8 and 66.7 ± 16.9 ms to 51.5 ± 20.0 and 135.1 ± 34.1 ms, respectively ($n = 4$, $P < 0.05$, Figure 1c).

Maintained application of 20 mM caffeine decreased the amplitude of the steady state current voltage relation at the end of the 400 ms voltage pulse from -40 mV holding potential (Figure 2a). This effect of caffeine was completely reversed upon washout. A similar result was obtained in five other cells. When the inward rectifier potassium current was greatly reduced with 100 μM $BaCl_2$, caffeine did not change the steady-state current voltage relation (Figure 2a). The effects of caffeine on the inward calcium current were studied in the presence of 5 mM 4-aminopyridine to abolish the transient outward current which may interfere with I_{Ca} measurements. Cells were held at -40 mV and 400 ms voltage pulses were applied with a pulse frequency of 0.33 Hz. As Figure 2b shows, maintained application of 20 mM caffeine reversibly decreased the amplitude of the peak inward calcium current without shifting its voltage-dependence. Similar results were obtained in four other rat ventricular cells. Caffeine, however, not only decreased the amplitude of the inward calcium current but significantly slowed its inactivation kinetics (t_{inac} increased from 29.3 ± 5.2 to 42.7 ± 5.2 ms, $n = 5$, $P < 0.05$).

Discussion Caffeine has various effects on the myocardium. The nickel-sensitive transient effects of caffeine on both currents and transmembrane voltage can be satisfactorily explained by the Na-Ca exchanger (Clusin, 1983). The effect of caffeine on the action potential repolarization is not entirely clear. The lack of calcium release during the action potential greatly diminishes the depolarizing Na-Ca exchanger current, therefore, it should shorten the action potential duration in rat ventricular myocardium as it was reported with ryanodine (Mitchell *et al.*, 1984). However, the significant slowing of the inactivation kinetics of I_{Ca} and inhibition of I_{k1} have the opposite effect, i.e. may lengthen the repolarization. Also, an additional effect of caffeine on other currents such as the fast and slow component of the transient outward potassium current (Apkon & Nerbonne, 1991) cannot be ruled out. The depressant effect of caffeine on the steady state current-voltage relation is probably due to its inhibition of I_{k1}, because this effect was not seen in conditions where I_{k1} was greatly inhibited, i.e. in the presence of $BaCl_2$.

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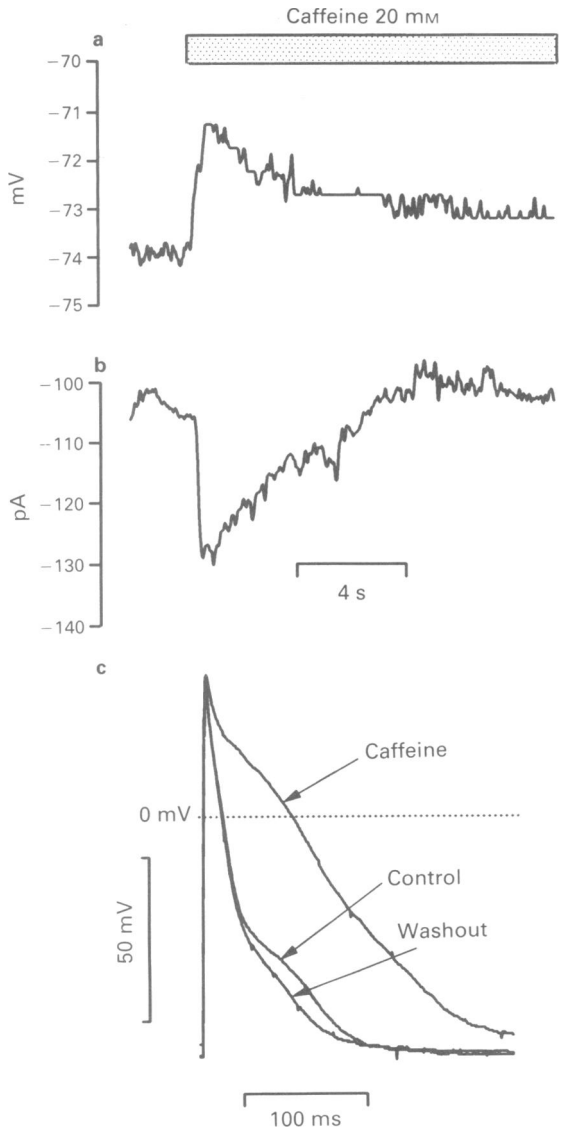


Figure 1 The effect of rapid application of 20 mM caffeine on transmembrane voltage (a) and transmembrane ionic currents (b). Cell was held at − 80 mV. (c) Shows the effect of maintained application of 20 mM caffeine on the action potential.

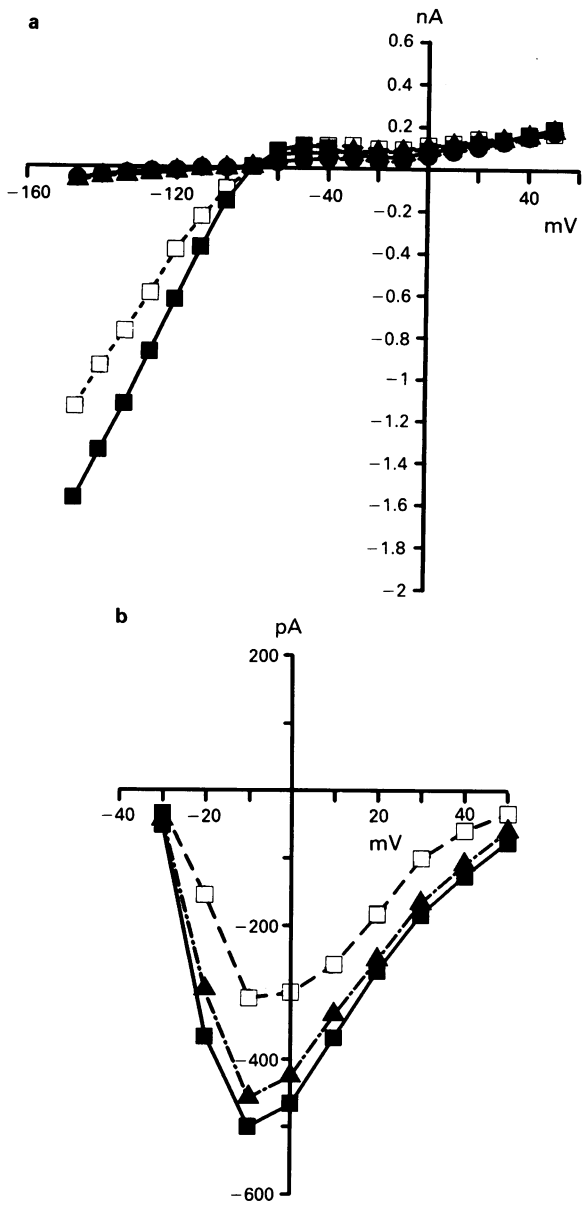


Figure 2 (a) The effect of maintained application of 20 mM caffeine on 'steady state' current voltage relation in the absence and presence of 100 μM $BaCl_2$: (■) control; (□) caffeine; (●) control + Ba; (▲) caffeine + Ba. (b) The effect of maintained application of 20 mM caffeine on peak inward calcium current: (■) control; (□) caffeine; (▲) washout. Calcium current was measured as the difference between the peak inward and the steady state current. Holding potential = − 40 mV, pulse duration = 400 ms, pulse frequency = 0.33 Hz. External solution contained 5 mM 4-aminopyridine to block the transient outward current.

The reported data with caffeine on the inward calcium current are conflicting. Yatani *et al.* (1984) found that caffeine increased the inward calcium current in rat single ventricular myocytes, but Eisner *et al.* (1979) observed the opposite effect with caffeine on the inward calcium current in sheep cardiac Purkinje fibres. Our data agree with the results of the latter study, but the differences between our present and previous studies is not understood. Although, the above effect of caffeine is not relevant therapeutically, we conclude that it should be recognized when the drug is used as a tool to study electromechanical coupling.

This work was supported by the Wellcome Trust and a grant from the Hungarian National Scientific Research Foundation (OTKA 676).

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(Received April 26, 1993
Accepted May 4, 1993)

Br. J. Pharmacol. (1993), **109**, 898–899

SPECIAL REPORT

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Antagonist effects of seglitide (MK 678) at somatostatin receptors in guinea-pig isolated right atria

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Somatostatin (SS) exerts a negative inotropic effect in isolated atria. Here we report that in guinea-pig isolated right atria, seglitide, a potent cyclic hexapeptide somatostatin agonist, behaves as a competitive somatostatin receptor antagonist with pA_2 values against SS_{14}, SS_{25} and SS_{28}, of 6.50 ± 0.40, 6.24 ± 0.08 and 6.09 ± 0.06, respectively. Seglitide had little or no effect on the negative inotropic action of carbachol or N^6-cyclohexyladenosine. Our findings indicate that the receptor-response coupling characteristics of guinea-pig atria are such that in this preparation seglitide has low intrinsic activity and behaves specifically as a somatostatin receptor antagonist.

Keywords: Seglitide; somatostatin; atria; antagonist

Introduction The tetradecapeptide somatostatin, first isolated from ovine hypothalamus (Brazeau *et al.*, 1973), exerts a negative inotropic effect in guinea-pig atria (Quirion *et al.*, 1979). However the pharmacology of somatostatin receptors is not well understood, due to a paucity of good drug tools.

The cyclic hexapeptide somatostatin analogue, seglitide (MK678), has been shown to be a potent somatostatin receptor agonist in various functional studies (Veber *et al.*, 1984) and selectively labels a somatostatin receptor subtype in radioligand binding studies (for review see Bell & Reisine, 1993). In the present study we have investigated the actions of some somatostatin analogues on spontaneously beating guinea-pig isolated right atria and shown that seglitide exhibits negligible agonist activity; rather, it acts as a somatostatin receptor antagonist in this preparation.

Methods Male Dunkin-Hartley guinea-pigs (220–450 g) were killed by cervical dislocation. Hearts were removed and transferred to a petri dish containing Krebs solution of the composition (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7, $MgSO_4.7H_2O$ 0.6, KH_2PO_4 1.2, D-glucose 11.1 and $CaCl_2.6H_2O$ 2.6.

Spontaneously beating right atria were prepared and mounted in 10 ml organ baths filled with Krebs solution, gassed with 95% O_2/5% CO_2 maintained at 32°C and connected to a Dynamometer UF1 isometric force transducer by a cotton thread. Isometric contractions were recorded on a Lectromed multitrace 8-channel chart recorder, through a Lectromed MT8P preamplifier.

A resting tension of 1 g was applied before leaving preparations to equilibrate for 1 h, during which time tension was readjusted to 1 g with washes every 15 min. After equilibration, cumulative concentration-effect curves to SS_{14} (1 nM–3 μM) were performed. Responses were expressed as percentage inhibition of developed tension. Following a 1 h interval with washes every 15 min, this was either repeated, or a cumulative concentration-effect curve to another agonist was constructed. When either antagonism or the influence of peptidase inhibitors was investigated, seglitide or peptidase inhibitors were added to the bath 30 min before the start of the repeat agonist concentration-effect curve. Following two further 15 min washout periods, preparations were exposed to 1 μM carbachol.

Equi-effective concentration-ratios for all experiments were calculated at the level of 20% inhibition. Antagonism was quantified according to the method of Arunlakshana & Schild (1959). Results are expressed as means ± s.e.mean of *n* observations. Statistical significance was ascertained by use of an unpaired *t* test, at a value of $P = 0.05$.

Drugs N^6-cyclohexyladenosine, carbamylcholine chloride (carbachol), somatostatin$_{14}$ (SS_{14}), somatostatin$_{28}$ (SS_{28}), somatostatin$_{25}$ (SS_{25}), phosphoramidon and amastatin were all obtained from Sigma. Seglitide (MK678) (cyclo(N-Me-Ala-Tyr-D-Trp-Lys-Val-Phe)) was synthesized by Dr J. Kitchen's Chemistry team at Glaxo Group Research, Greenford. All peptides used were acetate salts.

Results *Agonist studies* SS_{14} (1 nM–3 μM), SS_{25} (0.1 nM–0.3 μM) and SS_{28} (0.1 nM–0.3 μM) all decreased the force of developed tension in spontaneously beating guinea-pig right atria, (initial tension 476 ± 15 mg, $n = 92$) with maximum inhibitory effects of 46.6 ± 2.6%, 60.0 ± 2.0% and 55.8 ± 7.0% ($n = 4$), respectively at the highest concentration tested (Figure 1). Carbachol (1 μM) abolished spontaneously developed tension in all experiments. SS_{25} and SS_{28} were both more potent that SS_{14}, with equi-active molar ratios (EMR) of 0.08 ± 0.02 and 0.03 ± 0.01 ($SS_{14} = 1$), respectively ($n = 4$). Seglitide (1 nM–3 μM) had little agonist activity producing only 9.0 ± 1.1% ($n = 4$) inhibition of developed force in the absence and a 12.2 ± 4.8% ($n = 4$) in the presence of a combination of the peptidase inhibitors phosphoramidon (1 μM) and amastatin (10 μM). No significant chronotropic changes were seen with any of these peptides.

Antagonist studies Seglitide (1–10 μM) produced a concentration-dependent rightward shift of the concentration-effect curves to SS_{14}. Pre-incubation of 3 μM seglitide for periods of 15, 30 and 60 min gave SS_{14} concentration-ratios of 6.4 ± 0.8, 11.1 ± 6.4 and 7.2 ± 1.8 respectively ($n = 4$). Using a 30 min pre-incubation period, pA_2 values for seglitide against SS_{14}, SS_{25} and SS_{28} were 6.50 ± 0.40, 6.24 ± 0.08 and 6.09 ± 0.06, with slopes of the Schild plots of 0.86 ± 0.19, 0.97 ± 0.12 and 0.89 ± 0.05, respectively (see Figure 1). The pA_2 values were not statistically different from each other and the slopes of the Schild plots for all three agonists did not differ significantly from unity. In these experiments seglitide itself (10 μM), produced only a small decrease in force of contraction 4.8 ± 1.6% ($n = 12$) which was transient and completely reversed within 5 min.

While seglitide (10 μM), produced a marked displacement of the concentration-effect curves to SS_{14}, it had little or no effect on the negative inotropic action of either carbachol or cyclohexyladenosine, agonist concentration-ratios were 13.2 ± 2.1, 2.4 ± 0.4 and 0.8 ± 0.2, respectively. Corresponding control agonist concentration ratios were 1.2 ± 0.3, 0.9 ± 0.2 and 1.2 ± 0.4 ($n = 4$–6).

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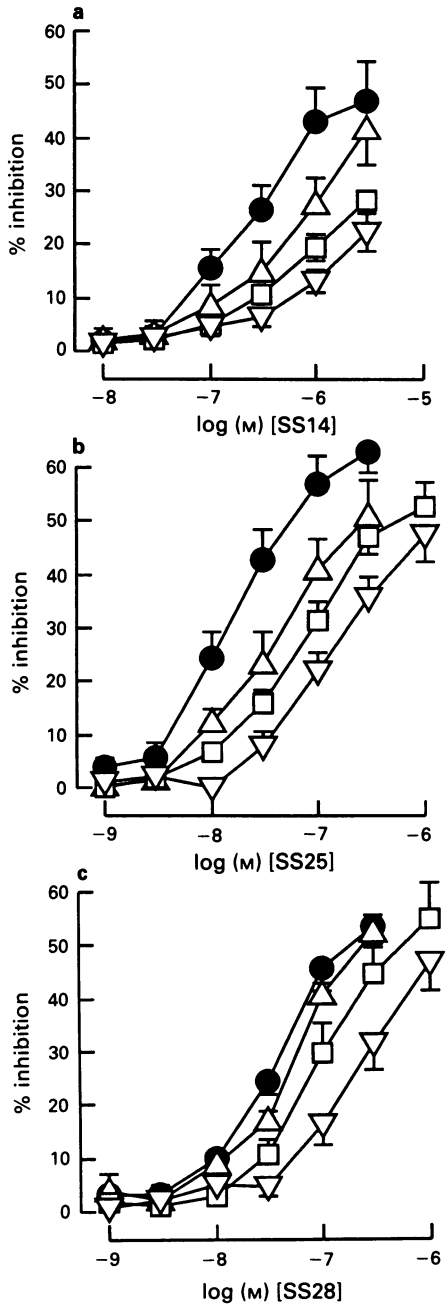


Figure 1 Inhibition of developed tension by (a) SS_{14}, (b) SS_{25} and (c) SS_{28} in the absence (●) and presence of seglitide 1 (△), 3 (□) and 10 μM (▽). The pre-incubation time for seglitide was 30 min. Values are means ± s.e.mean from 4 experiments.

Discussion Somatostatin produced a negative inotropic effect in atria, with SS_{25} and SS_{28} being approximately 13 and 30 times more potent than SS_{14}, respectively. However, seglitide which has been used to characterize somatostatin receptors in both functional and radioligand binding studies, behaved primarily as a somatostatin receptor antagonist.

Veber *et al.* (1984) reported that seglitide is a potent inhibitor of insulin, glucagon and growth hormone release in rats, while it has also been shown to inhibit forskolin stimulated adenylate cyclase, voltage activated Ca^{2+} currents and potentiate a delayed rectifier K^+ current in rat cultured neocortical neurones (Raynor *et al.*, 1991). Radioligand binding studies have demonstrated that seglitide shows high affinity for certain somatostatin receptor subtypes (Raynor & Reisine, 1993).

In the present study seglitide antagonized the negative inotropic response to SS_{14}, SS_{25} and SS_{28} but had little or no effect on the negative inotropic action of either carbachol or cyclohexyladenosine at 10 μM, indicating that seglitide acts specifically at somatostatin receptors. The antagonism displayed by seglitide was optimal with a 30 min incubation time, since extending this to 60 min incubation did not alter the degree of shift of the SS_{14} concentration-effect curve produced by 3 μM seglitide.

The Schild plots had slopes that were not significantly different from unity, indicating that the antagonism displayed by seglitide was competitive, such that the pA_2 values provide an estimate of the dissociation constant of seglitide for the somatostatin receptor subtype present in guinea-pig atria. Since the pA_2 values for the three agonists are not significantly different from each other, it seems likely that all three agonists are acting at a common receptor.

The low intrinsic activity of seglitide is unlikely to be explained by the hydrolysis of the peptide by endogenous peptidases, since a mixture of peptidase inhibitors, previously shown to potentiate the effects of SS_{14} in guinea-pig vas deferens (Feniuk *et al.*, 1993), had no significant effect on the response to seglitide in this preparation. Our findings suggest that seglitide is a low efficacy agonist and that either the receptor density and/or the receptor-response coupling efficiency in guinea-pig atria is low.

In conclusion, while seglitide has previously been shown to be a potent somatostatin receptor agonist, we describe here results which demonstrate that seglitide can behave as a specific antagonist, inhibiting the negative inotropic effect of somatostatin in guinea-pig isolated right atria, with little or no effect on the negative inotropic effects of either carbachol or cyclohexyladenosine.

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(Received April 30, 1993
Accepted May 5, 1993)

Br. J. Pharmacol. (1993), **109**, 900–901

SPECIAL REPORT

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Different effects of aspirin on blood pressure of spontaneously hypertensive rats (SHR) with high and spontaneously low levels of blood pressure

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Spontaneously hypertensive rats (SHR) of the Okamoto strain with blood pressure above 161 mmHg and SHR with blood pressure levels of less than 160 mmHg were treated with oral doses of aspirin (100 mg kg^{-1}) for three days. Whereas the blood pressure of SHR with blood pressure above 161 mmHg was decreased by aspirin, the blood pressure of SHR below 160 mmHg was increased by aspirin. The extent and direction of blood pressure change by aspirin was strongly correlated with the blood pressure of SHR before treatment ($r = -0.88$). The effect of aspirin supports an important role for endogenous prostanoids in the regulation of blood pressure of SHR.

Keywords: SHR; blood pressure; aspirin; prostanoids

Introduction Essential hypertension involves a number of cardiac and peripheral alterations (Folkow *et al.*, 1973), although its clear aetiology is still unknown. Despite the blood pressure effects of prostanoids and their analogues administered to animals with experimental hypertension, the potential role of endogenous protanoids formed by platelets, blood vessels or other tissues in the establishment or maintenance of hypertension has often been questioned (Stier & Itskovitz, 1988). It has been demonstrated that in spontaneously hypertensive rats (SHR) the availability of the prostanoid precursor arachidonic acid is increased by an enhanced phospholipase A_2 activity as compared to normotensive rats (Kawaguchi *et al.*, 1987). As the capacity of cyclo-oxygenase to generate the unstable prostaglandin H_2 (PGH_2)/PGG_2 seems not to be a limiting factor, the critical step might therefore be the formation of vasoactive prostanoids such as PGI_2, PGE, $PGF_{2\alpha}$ and thromboxane A_2 (TxA_2) or the sensitivity of the blood pressure regulatory system to these prostanoids (Taube & Schirner, 1992). Up to now, there has been no convincing experimental evidence that endogenous vasodilator prostanoids may decrease the hypertensive stage. Most recently, the failure to develop hypertension in adult offspring of SHR with hypertensive levels was observed (Sim & Singh, 1987). Using the cyclo-oxygenase inhibitor, aspirin, which may reduce the biosynthesis of prostanoids, the present study focused on the potential involvement of prostanoids in the normotension of adult offspring of SHR.

Methods Male, 4–6 month old SHR of the Okamoto strain (Institute of Pharmacology and Toxicology, University of Halle, Germany) were allocated to one of the three groups according to their blood pressure before treatment: (i) above 161 mmHg ($n = 13$); (ii) 141–160 mmHg ($n = 5$); (iii) below 140 mmHg ($n = 7$). As in other reports, we observed in 10–15% of the offspring of SHR, blood pressure levels of less than 160 mmHg. The blood pressure was determined by the tail cuff methods for three days running before the start of treatment. Blood pressure was recorded while the rats were resting quietly in a chamber at 35°C. Aspirin (acetylsalicyclic acid; Berlin-Chemie AG, Berlin, Germany) was

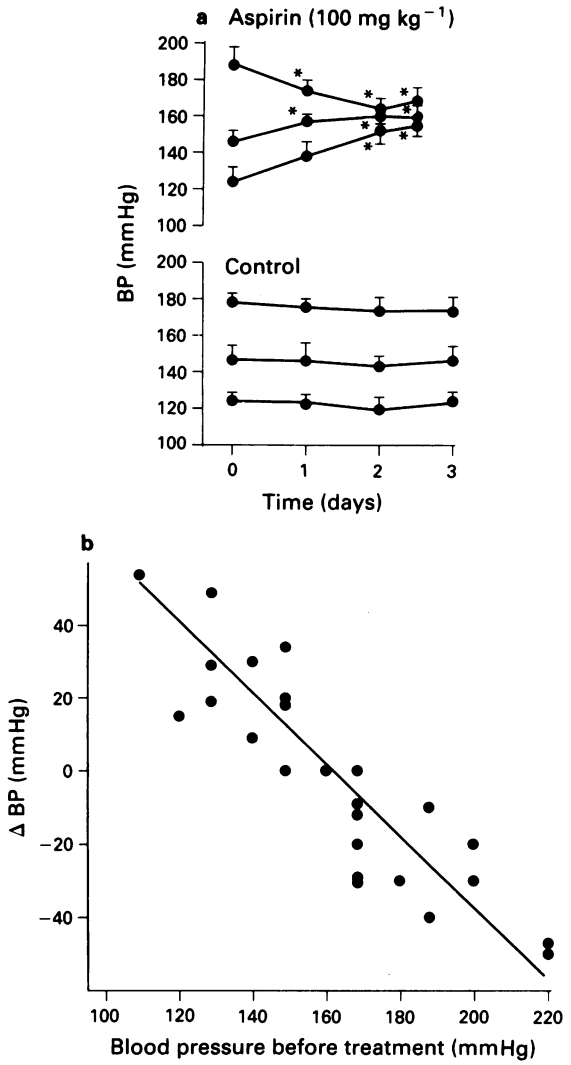


Figure 1 (a) Effect of aspirin (100 mg kg^{-1}) on the mean systolic blood pressure of SHR with different levels of blood pressure before start to treatment. Each point represents the mean ± s.e.mean. *Indicates significant difference ($P < 0.05$). (b) Regression analysis between the change of blood pressure by aspirin (day 3 – day 0) in individual SHR and their blood pressure before treatment (day 0); $n = 25$, $r = -0.88$, $P < 0.01$.

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given in single oral doses of 100 mg kg^{-1} for three days. Control animals received saline. The blood pressure was measured 1 h before treatment.

Statistical analysis was performed by the Friedman test.

Results Figure 1a demonstrates the antihypertensive effect of aspirin in SHR in which blood pressure was above 161 mmHg. The maximal blood pressure decrease was observed at day 2 (from 187 mmHg to 165 mmHg). At day 3, a slight return to higher blood pressure was found. In contrast, aspirin led to an increase in blood pressure in SHR with control blood pressure below 160 mmHg. SHR in which blood pressure was 147 mmHg before the start of treatment developed a significantly higher blood pressure following aspirin treatment (165 mmHg). The increase in blood pressure was more pronounced in SHR with a blood pressure of 125 mmHg before start of treatment. Aspirin treatment for three days led to an increase of 31 mmHg. No alteration in blood pressure was observed in the controls (Figure 1a).

Figure 1b shows the result of a regression analysis between the blood pressure before the start of treatment with aspirin (day 0) and the extent of blood pressure change induced by aspirin (day 3 − day 0) for each individual SHR. The analysis demonstrates a strong correlation ($r = -0.88$) between these two parameters.

There was no difference in formation of 6-keto-$PGF_{1\alpha}$ or TxB_2 in pieces of the aortae from the three blood pressure groups with or without aspirin treatment (data not shown).

Discussion The decrease in blood pressure in experimental models of hypertension following administration of vasodilator prostanoids and their analogues suggests a role for vasodilator prostanoids in the regulation of hypertension (Moncada, 1982). Despite this experimental evidence, there are some doubts about the importance of endogenous prostanoids in the maintenance and regulation of hypertension. In order to clarify this, cyclo-oxygenase inhibitors have been widely investigated in models of hypertension. Aspirin has been shown to decrease the blood pressure of SHR, suggesting a role for vasoconstrictor prostanoids (TxA_2; $PGF_{2\alpha}$) in the maintenance of hypertension (Tuttle *et al.*, 1988). The present investigation also demonstrates an antihypertensive effect of aspirin, which further supports a critical role of vasoconstrictor prostanoids in the hypertensive stage. In contrast, aspirin treatment has no significant effect on the blood pressure of normotensive Wistar-Kyoto rats (Tuttle *et al.*, 1988). More interestingly, 10–15% of offspring of SHR do not develop hypertension. Apart from an increased sensitivity of arterial vessels to vasoconstrictor stimuli, such as histamine and noradrenaline (Sim & Singh, 1987), there is no further explanation for this normotension. The present investigation demonstrates a marked increase in blood pressure induced by aspirin in SHR which were approximately normotensive before the start of treatment. Furthermore, the extent of the increase in blood pressure depended upon the blood pressure before administration of aspirin. As cyclo-oxygenase inhibition by aspirin in SHR with approximately normotensive blood pressure induced an increase to a hypertension, a contribution of vasodilator prostanoids to the failure to develop spontaneous hypertension might be expected. Since no differences in the formation of prostacyclin or TxA_2 in pieces of the aortae from the three blood pressure groups were seen, further investigations have to determine additional prostanoids as well as prostanoid biosynthesis in other organs. In conclusion, the results of the present investigation provide strong evidence for a critial role of vasodilator as well as vasoconstrictor prostanoids in experimental hypertension.

The authors thank Dr Fiona McDonald and Dr David Henderson for helpful advice and critical reading of the manuscript.

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(Received April 7, 1993
Accepted May 6, 1993)

Br. J. Pharmacol. (1993), **109**, 902–904

SPECIAL REPORT

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Antagonism of the inhibitory action of neuropeptide Y (NPY) in guinea-pig trachea by the C-terminal fragment NPY (2-36)

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The C-terminal fragment of neuropeptide Y (NPY), NPY(2-36) was used as a means of discriminating between two differently located NPY receptor sites in guinea-pig trachea. Both NPY and NPY(2-36) reduced the maximal relaxation elicited by vasoactive intestinal peptide (VIP). In contrast, the C-terminal fragment did not mimic the inhibitory action of NPY on the noradrenaline-(NA) evoked response. However, pretreatment of the trachea with 30 nM NPY(2-36), 5 min before generating NA and VIP concentration-response curves in the presence of NPY, abolished the inhibitory effect of NPY on NA-elicited response but did not affect the modulatory action of NPY on VIP-induced relaxation. These results suggest that the two differently located NPY receptor sites in guinea-pig trachea are of two distinct subpopulations.

Keywords: Neuropeptide Y (NPY); airways; neuropeptide Y(2-36); antagonism

Introduction Neuropeptide Y (NPY) has been shown to exert a broad variety of pharmacological actions including central, hormonal and peripheral effects (Dumont *et al.*, 1992). Agreement exists between authors that in the lung, NPY influences airway calibre by interfering with the release of transmitters both from non-adrenergic, non-cholinergic and sympathetic nerves (Grundemar *et al.*, 1990; Stretton *et al.*, 1990). Recently, we have shown that NPY is also capable of regulating airway smooth muscle tone by modulating the effector response of vasoactive intestinal peptide (VIP) and noradrenaline (NA) via two different mechanisms of action (T.-Benchekroun *et al.*, 1992). Whether this non-specific inhibitory action of NPY results from interaction with two different NPY receptor subtypes is unknown.

Results from structure activity studies have revealed that the full expression of the effects of NPY in most systems depends upon both intact C- and N-terminals (Dumont *et al.*, 1992). An interesting exception to this rule was the observation that NPY(2-36), injected intracerebroventricularly was more potent than NPY in increasing food intake whereas it was less potent than the native peptide in decreasing body temperature (Jolicoeur *et al.*, 1991). Since prominent differences were found between these two effects of NPY(2-36), these findings were used to address the possible existence of different NPY receptor subtypes in the central nervous system (Stanley *et al.*, 1992). In the present study, NPY(2-36) was used to explore further potential differences in NPY receptors mediating the modulatory influence of NPY on responses to NA and VIP in guinea-pig trachea.

Methods Tracheae from Hartley albino guinea-pigs were set up for isometric recording (Grass Instruments) in 5 ml organ baths (Krebs solution) under a resting tension of 1 g. Cumulative concentration-response curves for VIP and NA were generated either in the absence or in the presence of 30 nM (5 min) NPY or NPY (2-36). To evaluate the antagonistic properties of NPY(2-36), 30 nM of the C-terminal fragment was added to the organ bath 5 min before generating VIP or NA concentration-response curves in the presence of NPY. All data are given as means ± s.e.mean.

The composition of the Krebs solution was as follows (mM): NaCl 118.1, KCl 4.7, $MgSO_4$ $7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5 and glucose 11.1. Porcine (p)-NPY(1-36), pNPY(2-36) and VIP were synthesized in our laboratories. Noradrenaline ((−)-arterenol hydrochloride) (NA) was purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Other experimental conditions and data analysis methods were the same as those described previously by T.-Benchekroun *et al.* (1992).

Results Mean maximal amplitudes of responses induced by VIP (10^{-10} to 10^{-6} M) and NA (10^{-8} to 6×10^{-5} M) reached 0.59 ± 0.05 and 0.59 ± 0.06 g, respectively. As demonstrated earlier (T.-Benchekroun *et al.*, 1992), preincubation of tracheae with 30 nM NPY (5 min) resulted in significant reductions of the maximal response to VIP and NA (by 37.2 and 40.6%, respectively) without affecting their pD_2 values (Figure 1a,b; Table 1).

C-terminal fragment NPY(2-36) had no effect on its own on the resting tone of the trachea. When added 5 min before generating VIP or NA concentration-response curves, NPY(2-36) reduced by 35.5% ($P < 0.05$) the maximal response induced by VIP whereas it did not cause any change in the magnitude of the relaxation elicited by NA. Furthermore, in neither case, were the pD_2 values of both agonists altered in the presence of NPY(2-36) (Table 1). However, as shown in Figure 1a and b, following a 5 min pretreatment of the tracheae with 30 nM NPY(2-36), NPY failed to reduce the NA-evoked maximal response but maintained its inhibitory influence on VIP-induced relaxation. However, the magnitude of the inhibitory effect of NPY on the response to VIP in the presence of NPY(2-36) was similar to that obtained with NPY alone, and averaged 45.7%. Maximal responses and pD_2 values for both agonists in the presence of NPY plus NPY(2-36) are summarized in Table 1.

Discussion The present results confirm previous observations that NPY exerts a potent inhibitory action on VIP- and NA-induced relaxation in guinea-pig trachea (T.-Benchekroun *et al.*, 1992). Moreover, our results provide further evidence of the importance of the N-terminal portion of the NPY molecule for adequate interaction with its receptors and for the maintenance of its full biological activity (Dumont *et al.*, 1992). Deletion of the Tyr^1 of NPY resulted in the formation of a peptide that behaved differently from NPY as a modulatory agent on the relaxation induced by the two agonists. NPY(2-36) mimicked to a similar degree the effect of NPY on VIP-evoked responses whereas it was totally inactive on NA-elicited relaxation. This finding that NPY(2-36) may be as active as NPY in the trachea is similar to what has been observed in behavioural studies (Jolicoeur *et al.*, 1992; Stanley *et al.*, 1992) and is in line with previous reports

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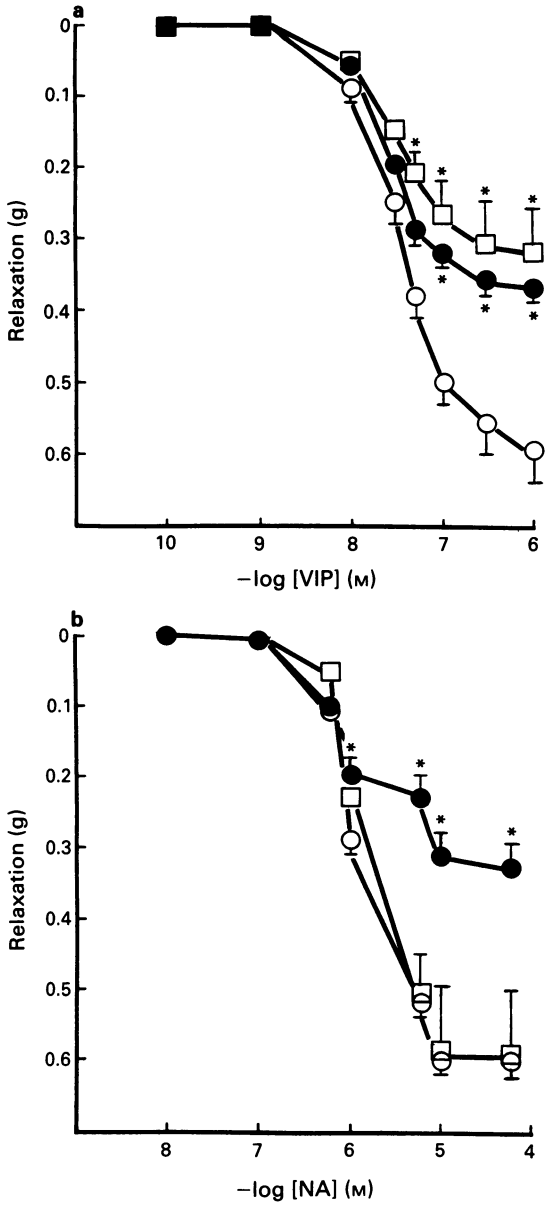


Figure 1 Effects of neuropeptide Y(2-36) (NPY(2-36), □) on the inhibitory action of NPY (●) on (a) vasoactive intestinal peptide (VIP)- and (b) noradrenaline (NA)-induced cumulative concentration-response curves in guinea pig trachea; (○) VIP (a) or NA (b) alone. NPY(2-36) (30 nM) was added to the organ bath 5 min before the administration of NPY (30 nM) which was also added 5 min prior to (a) VIP or (b) NA. Responses are expressed as absolute grams of relaxation. Means are shown with s.e.mean. $*P<0.05$ as compared with control values (VIP or NA alone) by use of Student's *t* test for unpaired data.

Table 1 Maximal responses (m.r.) and pD_2 values for vasoactive intestinal peptide (VIP) and noradrenaline (NA) in guinea-pig trachea in the absence or presence of neuropeptide Y (NPY, 30 nM) and/or C-terminal fragment NPY(2-36) (30 nM)

Pretreatment	*m.r.* (g)	*pD_2*
Control VIP	0.59 ± 0.05	7.55 ± 0.09
NPY + VIP	0.37 ± 0.02[a]	7.54 ± 0.06
NPY(2-36) + VIP	0.38 ± 0.04[a]	7.50 ± 0.08
NPY(2-36) + NPY + VIP	0.32 ± 0.06[a]	7.50 ± 0.1
Control NA	0.59 ± 0.06	5.92 ± 0.05
NPY + NA	0.35 ± 0.06[a]	6.04 ± 0.09
NPY(2-36) + NA	0.57 ± 0.06	6.16 ± 0.02
NPY(2-36) + NPY + NA	0.59 ± 0.09	5.85 ± 0.06

The m.r. and pD_2 values are expressed as means ± s.e.mean of data obtained from 4 to 14 tissues.
[a] $P<0.05$ as compared with control responses (VIP or NA alone) by means of Student's *t* test for unpaired data.

in which various *in vitro* pharmacological effects of NPY and its related fragments have been measured (Rioux *et al.*, 1986; Donoso *et al.*, 1988). However, the interesting observation of the present study is that the C-terminal fragment NPY(2-36) acted as a full antagonist of NPY on NA-induced relaxation. To our knowledge, it is the first time that such antagonistic properties of NPY(2-36) have been observed.

We have previously reported that the inhibitory action of NPY in guinea-pig trachea is expressed following interaction with both pre- (NA) and post-junctional (VIP) receptors (T.-Benchekroun *et al.*, 1992). These receptors were initially detected as a single population with specific affinity for NPY. However, the present data strongly suggest that these differently located NPY receptor sites are of two distinct subpopulations. The receptors involved in the modulatory action of NPY on VIP-induced relaxation would be similar to those encountered in brain and other peripheral tissues where deletion of Tyr^1 also resulted in the formation of a peptide with an intrinsic activity similar to that of NPY (Rioux *et al.*, 1986; Martel *et al.*, 1986; Donoso *et al.*, 1988; Dumont *et al.*, 1992). In contrast, the receptors mediating the inhibitory action of NPY on NA-evoked responses would belong to a possible new subpopulation.

In conclusion, this study shows that NPY receptors in guinea-pig trachea are far from being homogeneous. That NPY(2-36) expresses antagonistic property specifically on one NPY receptor subpopulation exemplifies the diverse roles of NPY fragments as biologically active molecules.

This study was supported by the Medical Research Council of Canada (Program Grant PG 11125) and the 'Association Pulmonaire du Québec'. The authors thank Mrs. Lucie Dionne for excellent secretarial assistance. A.F. and A.C. are scholars of the 'Fonds de la Recherche en Santé du Québec' (F.R.S.Q.).

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(Received May 4, 1993
Accepted May 17, 1993)

Br. J. Pharmacol. (1993), **109**, 905–912 © Macmillan Press Ltd, 1993

Inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate binding sites in smooth muscle

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1 We have previously demonstrated that activation of M_3 muscarinic receptors increases inositol 1,4,5-trisphosphate ($InsP_3$) and inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) accumulation in colonic smooth muscle.

2 In the present study, we demonstrate the existence of $InsP_3$ and $InsP_4$ binding sites in colonic circular smooth muscle by use of radioligand binding methods. Both [^{3}H]-$InsP_3$ and [^{3}H]-$InsP_4$ bound rapidly and reversibly to a single class of saturable sites in detergent-solubilized colonic membranes with affinities of 5.04 ± 1.03 nM and 3.41 ± 0.78 nM, respectively. The density of [^{3}H]-$InsP_3$ binding sites was 335.3 ± 19.3 fmol mg^{-1} protein which was approximately 2.5 fold greater than that of [^{3}H]-$InsP_4$ sites (127.3 ± 9.1 fmol mg^{-1} protein).

3 The two high affinity inositol phosphate binding sites exhibited markedly different pH optima for binding of each radioligand. At pH 9.0, specific [^{3}H]-$InsP_3$ binding was maximal, whereas [^{3}H]-$InsP_4$ binding was only 10% that of [^{3}H]-$InsP_3$. Conversely, at pH 5.0, [^{3}H]-$InsP_4$ binding was maximal, while [^{3}H]-$InsP_3$ binding was reduced to 15% of its binding at pH 9.0.

4 $InsP_3$ was about 20 fold less potent (K_I = 50.7 ± 8.3 nM) than $InsP_4$ in competing for [^{3}H]-$InsP_4$ binding sites and could compete for only 60% of [^{3}H]-$InsP_4$ specific binding. $InsP_4$ was also capable of high affinity competition with [^{3}H]-$InsP_3$ binding (K_I = 103.5 ± 1.5 nM), and could compete for 100% of [^{3}H]-$InsP_3$ specific binding.

5 [^{3}H]-$InsP_3$ binding in subcellular fractions separated by discontinuous sucrose density gradients followed NADPH-cytochrome c reductase activity, suggesting an intracellular localization for the majority of $InsP_3$ receptors in this tissue, whereas [^{3}H]-$InsP_4$ binding appeared to be equally distributed between plasma membrane and intracellular membrane populations.

6 These results suggest the existence of distinct and specific $InsP_3$ and $InsP_4$ binding sites which may represent the physiological receptors for these second messengers; differences in the subcellular distribution of these receptors may contribute to differences in their putative physiological roles.

Keywords: $InsP_3$ receptor; $InsP_4$ receptor; radioligand binding; smooth muscle; colon (canine)

Introduction

It has been well established that the phosphoinositide cascade plays a central role in the transduction of many extracellular stimuli. Activation of phosphoinositide-specific phospholipase C generates inositol 1,4,5,-trisphosphate ($InsP_3$), which in turn releases Ca^{2+} from an intracellular store (Berridge & Irvine, 1989). In most tissues, $InsP_3$ is metabolized by either 5-dephosphorylation to inositol 1,4-bisphosphate ($InsP_2$) or 3-phosphorylation to inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) (Irvine *et al.*, 1986; Shears, 1989). $InsP_4$ has also been implicated in Ca^{2+} homeostasis. Proposals for the action of $InsP_4$ have included activation of extracellular Ca^{2+} entry (Irvine & Moor, 1986; Changya *et al.*, 1989), modulation of the $InsP_3$-sensitive Ca^{2+} pool (Gawler *et al.*, 1991), and a direct effect on intracellular Ca^{2+} mobilization (Hill *et al.*, 1988).

$InsP_3$ binding sites have been identified in a number of tissues (Worley *et al.*, 1987; Enyedi *et al.*, 1989; Varney *et al.*, 1990; Van Delden *et al.*, 1992), including smooth muscle (Chadwick *et al.*, 1990). Comparison of the smooth muscle $InsP_3$ receptor with that from cerebellum indicates that the two receptors are similar both structurally and functionally (Marks *et al.*, 1990). The $InsP_3$ receptor has been purified from cerebellum and its primary structure has been determined (Furuichi *et al.*, 1989; Maeda *et al.*, 1990). Functional identity of the $InsP_3$ receptor as an intracellular Ca^{2+} release channel has been indicated by incorporation of the cerebellar and smooth muscle $InsP_3$ receptors into phospholipid vesicles and observing an $InsP_3$-activated Ca^{2+} conductance (Ferris *et al.*, 1989; Mayrleitner *et al.*, 1991). Compared to the $InsP_3$ receptor, however, our understanding of the $InsP_4$ receptor is poor. $InsP_4$ receptors have been described by radioligand binding methods in cerebellum, HL-60 cells, and parathyroid glands (Bradford & Irvine, 1987; Enyedi *et al.*, 1989; Donie *et al.*, 1990). Recently, a putative high-affinity $InsP_4$ receptor was purified from pig cerebellar membranes (Donie & Reiser, 1991). While a role for $InsP_4$ action in smooth muscle has not been established, generation of $InsP_4$ in smooth muscle has been described by ourselves (Zhang & Buxton, 1991; Schiemann *et al.*, 1991) and others (Bielkiewicz-Vollrath *et al.*, 1987; Pijuan & Litosch, 1988; Salmon & Bolton, 1988) although the location and nature of smooth muscle $InsP_4$ receptors have not been described. It is still unknown whether $InsP_4$ acts at the plasma membrane, on internal Ca^{2+} stores, or on a structural element which connects the plasma membrane and the Ca^{2+} store. Subcellular localization along with functional studies and reconstitution experiments of $InsP_4$ receptors should shed light on these questions.

We have recently demonstrated the coexistence of muscarinic receptors of the M_2 and M_3 subtypes in canine colonic circular smooth muscle (Zhang *et al.*, 1991). Activation of M_3 muscarinic receptors leads to a rapid accumulation of $InsP_3$ while $InsP_4$ accumulation is delayed. The rapid accumulation of $InsP_3$ is likely to play a direct role in initiation of the early tonic phase of contraction of the muscle following activation of muscarinic receptors since removal of extracellular Ca^{2+} does not abolish this phase of contraction or the M_3-mediated Ca^{2+}-transient seen in single colonic myocytes loaded with fura-2, while the phasic contractions that follow are entirely dependent on extracellular Ca^{2+} (unpublished observation). The role of $InsP_4$ in regulating intracellular

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Ca^{2+} oscillations, which correlate well with phasic contractions in colon smooth muscle is not known.

To clarify the role of $InsP_3$ and $InsP_4$ as second messengers in colonic smooth muscle, we have examined the binding of these two inositol polyphosphates to detergent-solubilized colonic smooth muscle membranes. We have resolved distinct, high affinity binding sites for $InsP_3$ and $InsP_4$ and have determined the intracellular distribution of these sites by means of subcellular fractionation techniques.

Methods

Solubilized membrane preparations

Canine colonic circular smooth muscle was prepared as described previously (Zhang *et al.*, 1991). The smooth muscle was minced and suspended in 10 volumes of Buffer A (composition, mM: Tris/HCl 20, NaCl 20, KCl 100, EDTA 1, BSA 1 mg ml^{-1}, NaN_3 0.02%, pH 7.7). Tissue was then homogenized in a Polytron tissue homogenizer at setting 3.5 in two bursts of 15 s each. The homogenate was centrifuged at 100,000 *g* for 30 min. Pellets were re-homogenized in half the original volume of Buffer A at setting 3.5 for 15 s, and re-centrifuged using the same conditions. The resulting supernatant was discarded and the pellet was solubilized as described by Hingorani & Agnew (1991). Briefly, the pellet was resuspended in Buffer B (composition mM: Tris/HCl 20, KCl 100, NaCl 20, EDTA 1, NaN_3 0.02%, pH 8.5) containing 2% (w/v) Lubrol-PX. The suspension was gently homogenized with 20 strokes in a glass-glass homogenizer on ice, followed by incubation on ice with stirring for 20 min. The detergent extract was then centrifuged at 80,000 *g* for 40 min. For [^{3}H]-$InsP_4$ binding, the supernatant pH was adjusted to 5.0 with 1 mM HCl.

Radioligand binding studies

Binding of the [^{3}H]-$InsP_3$ and [^{3}H]-$InsP_4$ to solubilized colonic smooth muscle membranes was measured by the syringe assay as previously described (Hingorani & Agnew, 1991). The syringe ion-exchange column was prepared with 0.5–0.6 ml of Dowex AG 1-X2 resin equilibrated in 2 volumes of Buffer C (composition mM: Tris/HCl 50, EDTA 1, NaN_3 0.02%, pH 8.5) containing BSA 10 mg ml^{-1} for 2 h. A small Spectra/Mesh nylon disk (10 μm pore size) was inserted into the barrel of a 3 ml plastic syringe, followed by a disk of Whatman No. 1 paper filter and then Dowex AG 1-X2 resin8. Radioligand binding assays were carried out in a final volume of 200 μl consisting of 175 μl solubilized membrane supernatant, 20 μl radioligand, and 5 μl buffer or unlabelled inositol phosphates. Saturation binding experiments employed concentrations of [^{3}H]-$InsP_3$ or [^{3}H]-$InsP_4$ from 0.15 nM to 80 nM and nonspecific binding was determined by the addition of the appropriate unlabelled ligand (12 μM). Equilibrium binding was carried out at 4°C for 15 min. Bound and free radioligand were separated by rapidly pushing equilibrated samples through the syringe columns. The separation could be completed reliably within approximately 1 s. The labelled receptor-ligand complex was collected directly into a vial for counting at 45% efficiency in a Beckman LS 6000IC liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA, U.S.A.). All determinations were made in triplicate. Specific binding of [^{3}H]-$InsP_3$ or [^{3}H]-$InsP_4$ at 10 nM was examined in competition studies employing increasing concentrations of unlabelled inositol phosphates. Protein was determined by the method of Bradford (1976).

Preparation of subcellular fractions

Freshly isolated colonic circular smooth muscle was minced and homogenized on ice in Buffer D (sucrose 300 mM, Tris/base 20 mM, DTT 5 mM, pH 7.2) by 20 strokes in a Duall No. 20 tissue grinder. All samples were kept at 4°C throughout purification. The homogenate was centrifuged at 2,500 *g* for 20 min, after which the pellet was again homogenized, diluted, and centrifuged. A postnuclear supernatant was obtained by centrifuging the combined supernatants at 15,000 *g* for 20 min. The resulting supernatant was then centrifuged at 40,000 *g* for 90 min, and the pellet was resuspended in 4 ml Buffer E (NaCl 80 mM, Na-oxalate 5 mM). This suspension was separated on a discontinuous sucrose density gradient (24%, 28%, 33%, 45%, w/v) by centrifuging at 64,000 *g* for 3 h in a swinging bucket rotor as described by Carsten & Miller (1980). PMSF (0.2 mM final concentration) was added to gradient media to inhibit proteinase. Three bands of decreasing densities were identified: a heavy-density band (interface 45% and 33% sucrose), an intermediate-density band (interface 33% and 28% sucrose) and a low-density band (interface 28% and 24% sucrose). The gradient was separated into 8 fractions including the pellet, which was discarded. Collected fractions were diluted 7 fold in Buffer E and centrifuged at 70,000 *g* for 2 h. The resulting pellets were resuspended in 1 ml of Buffer D and 0.1 ml of the suspensions was reserved for marker enzyme and protein assay. The remaining samples were diluted in binding buffer A and centrifuged at 70,000 *g* for 2 h, after which pellets were solubilized as described above.

Biochemical marker determinations

K^+-dependent *p*-nitrophenyl phosphatase (*p*-NPPase) was assayed by the method of Murer *et al.* (1976) and NADPH-cytochrome c reductase by the method of Mircheff *et al.* (1983). Protein was determined by the method of Bradford (1976) with BSA as the standard.

ATP-dependent calcium accumulation

Pellets following fractionation were resuspended in 350 μl of a solution of the following composition (mM): KCl 150, HEPES 20 (pH 7.0, adjusted with KOH). Calcium uptake was measured according to the method of Moore *et al.* (1975) in the following medium (mM): KCl 130, HEPES 20, $MgCl_2$ 5, ammonium oxalate 5 (to precipitate intravesicular Ca^{2+}), $CaCl_2$ 0.02 and $^{45}CaCl_2$ 0.6 μCi ml^{-1}, with or without ATP 5 (pH adjusted to 7.0 with KOH). The incubation was carried out at 37°C for 10 min and the vesicle accumulated ^{45}Ca was separated from medium by rapid filtration through 0.45 μm millipore membrane filters. The filter was washed twice with 4 ml ice cold solution of KCl (150 mM) and HEPES (20 mM, pH 7.0) and left in a scintillation vial overnight at room temperature to dry. ^{45}Ca was determined by liquid scintillation spectrometry. The ratio of ^{45}Ca uptake in the presence of ATP to that in the absence of ATP was determined.

Data analysis

Saturation binding data were analyzed by both Scatchard plot and computer assisted non-linear least-squares regression to fit binding data (GraphPad Inplot v. 4.0, Graphpad Software, San Diego, CA, U.S.A.). Both methods yielded similar K_D and B_{max} values (Schiemann *et al.*, 1990). For competition binding data, the non-linear least-squares approach fits the data to either one or two classes of binding sites and assists in determining if the two site model is significantly better than the one-site fit of the data (*F* test). Data are expressed as the mean ± s.e.

Materials

The materials and their sources were as follows: [^{3}H]-inositol 1,4,5-trisphosphate ([^{3}H]-$InsP_3$, 17 Ci $mmol^{-1}$), [^{3}H]-inositol 1,3,4,5-tetrakisphosphate ([^{3}H]-$InsP_4$, 17 Ci $mmol^{-1}$) and $^{45}CaCl_2$ (18 Ci g^{-1}) were from Dupont-NEN (Boston, MA,

U.S.A.); inositol 1,4,5-trisphosphate ($InsP_3$), inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) and bovine serum albumin (BSA) were from Calbiochem (La Jolla, CA, U.S.A.); Lubrol-PX, Dowex AG 1-X2 resin (chloride form, 200–400 mesh), sucrose, reduced β-nicotinamide adenine dinucleotide phosphate (NADPH), *p*-nitrophenyl phosphate, adenosine triphosphate (ATP) Sigma 104, EDTA, Tris/HCl, NaN_3, phenylmethylsulphonyl fluoride (PMSF) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); Spectra/Mesh nylon filters (10 μm pore size) were from Spectrum (Los Angeles, CA, U.S.A.); Whatman No. 1 paper filters were from Whatman (Hillsboro, OR, U.S.A.).

Results

Radioligand binding characteristics

Studies of the binding of [^{3}H]-$InsP_3$ and [^{3}H]-$InsP_4$ to detergent-solubilized membranes prepared from colon circular smooth muscle indicated that the binding of these two inositol polyphosphates was specific and exhibited markedly different pH optima (Figure 1). By increasing the pH from 4 to 9, specific binding of [^{3}H]-$InsP_3$ increased 8 fold. At pH 5.0, little specific binding of [^{3}H]-$InsP_3$ to solubilized membranes could be detected. In contrast, specific binding of [^{3}H]-$InsP_4$ was maximal at pH 5.0, but decreased dramatically as the assay pH was increased, and was minimal at pH 9.0. This finding is in agreement with previous studies which demonstrated that optimal binding of [^{3}H]-$InsP_3$ and [^{3}H]-$InsP_4$ in cerebellum occurred at pH 8.0 and pH 5.0, respectively (Donie & Reiser, 1989; Challiss *et al.*, 1991).

At pH 8.5, binding of [^{3}H]-$InsP_3$ to detergent-solubilized membranes of colon circular smooth muscle was specific and saturable (Figure 2). Non-specific binding represented approximately 10% of the total binding at the concentration of [^{3}H]-$InsP_3$ near the K_D (5 nM). Saturation isotherms employing [^{3}H]-$InsP_3$ consistently yielded linear transforms (bound/free *vs* bound) and were best described by an interaction of the radioligand with a single class of binding sites with an apparent dissociation constant (K_D) of 5.04 ± 1.03 nM and

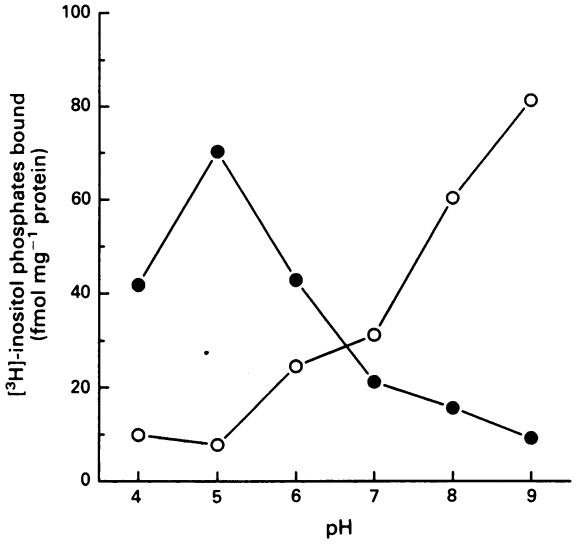


Figure 1 Effect of pH on [^{3}H]-$InsP_3$ and [^{3}H]-$InsP_4$ specific binding to detergent-solubilized membranes prepared from canine colonic circular smooth muscle. Binding of [^{3}H]-$InsP_3$ (15 nM; ○) and [^{3}H]-$InsP_4$ (15 nM; ●) at different pH values was determined as described in Methods. Non-specific binding was determined in the presence of 12 μM unlabelled ligand in each case. Values represent means of triplicate determinations from a single representative experiment. Standard deviations were less than 20%.

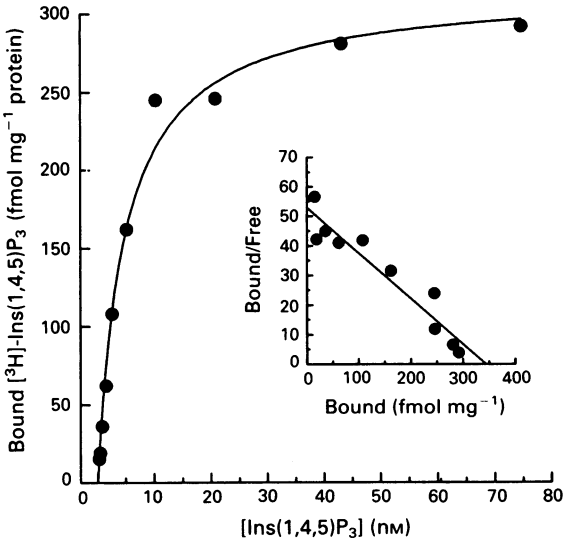


Figure 2 Saturation binidng of [^{3}H]-$InsP_3$ to detergent-solubilized membranes prepared from canine colonic circular smooth muscle. Increasing concentrations of [^{3}H]-$InsP_3$ (0.15 to 80 nM) were incubated with the solubilized sarcolemmal membrane at pH 8.5 as described in Methods. Specific [^{3}H]-$InsP_3$ binding, defined as the difference between total binding and nonspecific binding observed in the presence of 12 μM unlabelled $InsP_3$, is presented as fmol of receptor bound per mg of solubilized protein. Nonspecific binding represented about 10% of total binding at 5 nM [^{3}H]-$InsP_3$. Analysis of the specific binding data by Scatchard plot (inset) confirmed that [^{3}H]-$InsP_3$ bound to a single class of binding sites in the solubilized membranes. The receptor affinity (K_D) and density (B_{max}) are 4.8 nM and 345 fmol mg^{-1} protein respectively for the experiment shown. Data points represent the means of triplicate determinations in a single experiment. Mean data obtained from several such experiments ($n = 6$) are presented in the text.

B_{max} of 335.3 ± 19.3 fmol mg^{-1} protein ($n = 6$). Saturation binding of [^{3}H]-$InsP_4$ to solubilized membranes was performed at pH 5.0 (Figure 3). Consistent with the data reported above for [^{3}H]-$InsP_3$, analysis of [^{3}H]-$InsP_4$ binding data demonstrated that [^{3}H]-$InsP_4$ bound to an apparently homogeneous receptor population with a K_D of 3.41 ± 0.78 nM and a B_{max} of 127.3 ± 9.1 fmol mg^{-1} protein ($n = 7$). Non-specific binding of [^{3}H]-$InsP_4$ was slightly higher than that of [^{3}H]-$InsP_3$ and was 40% of total binding at 4 mM.

In order to investigate the specificity of [^{3}H]-$InsP_3$ and [^{3}H]-$InsP_4$ binding to solubilized membranes, unlabelled $InsP_3$ and $InsP_4$ were employed in radioligand binding competition studies. In a manner consistent with the saturation binding data, $InsP_3$ and $InsP_4$ competed with their radioligand form with high affinity (Figure 4). $InsP_3$ *vs* [^{3}H]-$InsP_3$ competition gave a K_I of 4.4 ± 1.2 nM and $InsP_4$ *vs* [^{3}H]-$InsP_4$ of 2.8 ± 0.9 nM ($n = 3$). Computer assisted analysis of the competition curves indicated that both inositol polyphosphates bound to a single class of sites. Despite marked differences in pH optima between [^{3}H]-$InsP_3$ and [^{3}H]-$InsP_4$ binding, there is significant cross-specificity of each ligand. As shown in Figure 4, the specific binding of [^{3}H]-$InsP_3$ was not only competed for by $InsP_3$ but also by $InsP_4$. $InsP_4$ was about 20 fold less potent than $InsP_3$ in competition for specific [^{3}H]-$InsP_3$ binding with K_I being 103.5 ± 1.5 nM ($n = 3$). Similar results were obtained with $InsP_3$ competitions for [^{3}H]-$InsP_4$ specific binding ($K_I = 50.7 \pm 8.3$ nM, $n = 3$). Unlike $InsP_4$ competition for [^{3}H]-$InsP_3$ binding however, $InsP_3$ was not able to compete for some 40% of [^{3}H]-$InsP_4$ binding sites. While the reason for this discrepancy is not clear, it is possible that some $InsP_4$ binding sites are not capable of binding $InsP_3$. Such a possibility suggests the presence of two disparate $InsP_4$ receptors.

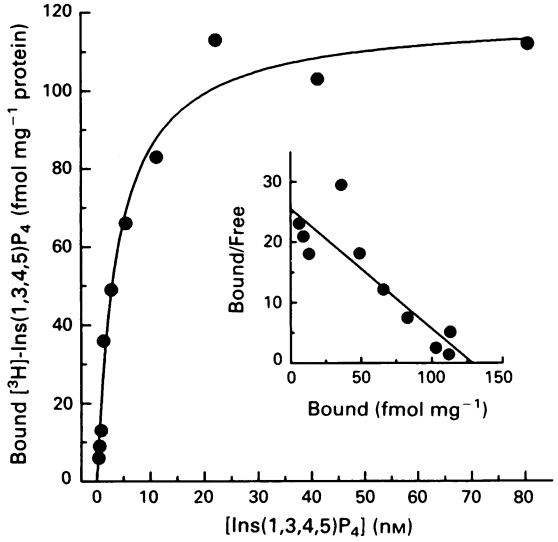


Figure 3 Saturation binding of [^{3}H]-InsP$_4$ to detergent-solubilized membranes prepared from canine colonic circular smooth muscle. Increasing concentrations of [^{3}H]-InsP$_4$ (0.15 to 80 nM) were incubated with the solubilized sarcolemmal membrane at pH 5.0 as described in Methods. Specific [^{3}H]-InsP$_4$ binding, defined as the difference between total binding and nonspecific binding observed in the presence of 12 μM unlabelled InsP$_4$, is presented as fmol of receptor bound per mg of solubilized protein. Nonspecific binding represented about 40% of total binding at 4 nM [^{3}H]-InsP$_4$. Analysis of the specific binding data by Scatchard plot (inset) confirmed that [^{3}H]-InsP$_4$ bound to a single class of binding sites in the solubilized membranes. The receptor affinity (K_D) and density (B_{max}) are 3.9 nM and 129 fmol mg^{-1} protein respectively for the experiment shown. Data points represent the means of triplicate determinations in a single experiment. Mean data obtained from several such experiments ($n = 7$) are presented in the text.

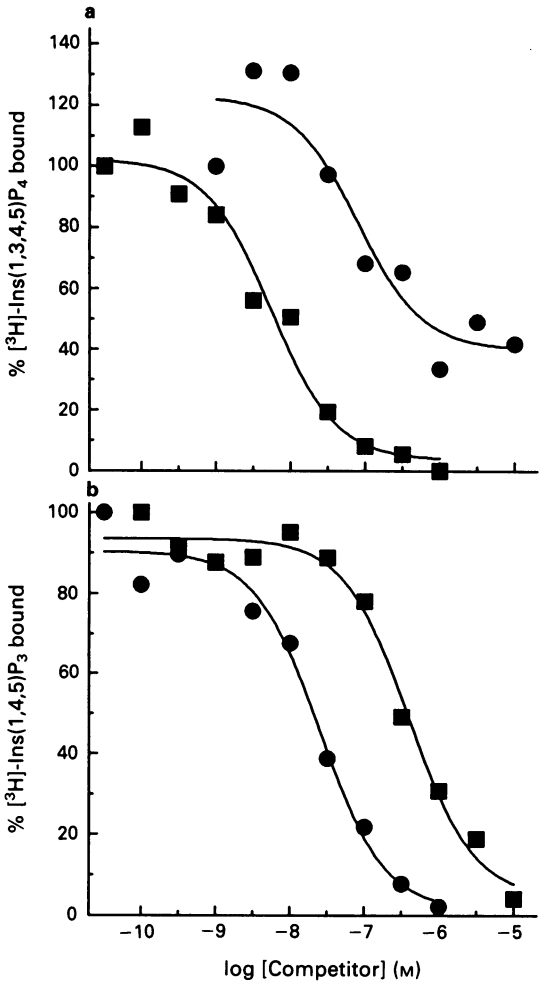


Figure 4 Competition of [^{3}H]-InsP$_3$ and [^{3}H]-InsP$_4$ by unlabelled inositol phosphates in detergent-solubilized membranes prepared from canine colonic circular smooth muscle. The solubilized membranes were incubated with 10 nM [^{3}H]-InsP$_3$ (b) or 10 nM [^{3}H]-InsP$_4$ (a) in the presence and absence of increasing concentrations of unlabelled InsP$_3$ (●) and InsP$_4$ (■). Data, normalized to binding in the absence of competitor (100%) and that occurring in the presence of 12 μM unlabelled InsP$_3$ or InsP$_4$, are expressed as the percentage of maximal [^{3}H]-InsP$_3$ or [^{3}H]-InsP$_4$ binding. Data points represent the means of triplicate determinations in a single representative experiment. Binding parameters determined in several such experiments ($n = 3$) are presented in the text.

Subcellular fractionation

In order to delineate the intracellular distribution of inositol phosphate binding in colonic circular smooth muscle, tissue homogenates were subjected to a combination of differential sedimentation and equilibrium density gradient centrifugations, followed by assays for membrane marker enzymes and total protein. Figure 5 depicts the results of a typical density gradient distribution of marker activities including *p*-NPPase (known to be equivalent to the activity of ouabain-sensitive Na,K-ATPase). *p*-NPPase activity has traditionally been used as a marker for plasma membranes, but is also known to be present in significant quantities within intracellular membranes such as the Golgi complex (Yiu *et al.*, 1991). Also depicted in Figure 5 are the distributions of membrane-associated protein and the endoplasmic reticulum marker NADPH-cytochrome c reductase; while the latter activity is also present within the membranes of the mitochondria, the vast majority of this potentially contaminating activity was removed from the preparation along with intact mitochondria by means of a differential sedimentation step, prior to density gradient separation (not shown). The activities present within the density gradients suggest the presence of at least 3 major membrane populations with distribution modes in density fractions II, IV, and V (Figure 5). The distribution of *p*-NPPase activity is distinctly bimodal, with peaks of activity present within fractions II and V; this observation is consistent with the localization of sodium pump proteins on intracellular as well as plasma membrane populations (Yiu *et al.*, 1991). In contrast, NADPH-cytochrome c reductase exhibits a major mode only within density fraction II, and is only nominally present within fractions IV–VII; the latter activities could be due to a skewed distribution of a single population, or might represent minor activities present on the *p*-NPPase-containing membranes in the population localized to fraction V. Correlating well with the peaks of *p*-NPPase and NADPH-cytochrome c reductase activities in fraction II is a large peak of membrane-bound protein, a result which has been found to be consistent with the presence of intracellular (eg. Golgi complex and endoplasmic reticulum) membranes (Bradley & Mircheff, unpublished observation). These data, taken together, suggest an intracellular origin for the membranes equilibrating within density fraction II.

When ratios of percentage recovered marker enzyme activity to recovered protein are calculated, an indication of the enrichments of the enzyme activities as a result of density gradient separations is obtained. As can be seen in Table 1, small amounts of protein recovered in density fraction V provide for large enrichments for all activities present in this fraction, and the large peak of *p*-NPPase activity in fraction V, when normalized to protein, results in the highest observed enrichment factor (6.8) obtained from any of the markers. The enrichment for *p*-NPPase, coupled with the large (at least 25%) amount of this activity recovered in fraction V, as well as the relatively low amounts of intracel-

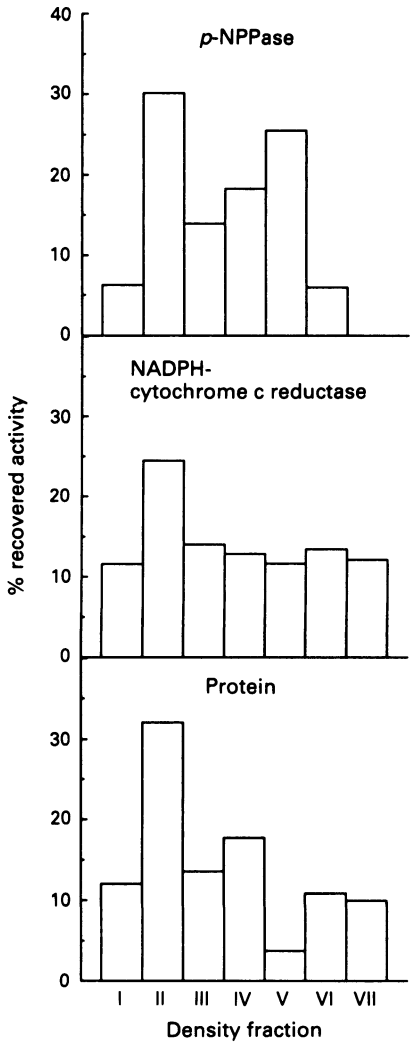


Figure 5 Density distribution of membrane markers in circular smooth muscle membrane. Colonic circular smooth muscle homogenates were first subjected to low-speed and then high-speed differential centrifugation procedures, and then loaded onto discontinuous sucrose density gradients for equilibrium density centrifugation as described in Methods. Values given are representative of results from 3 different preparations, and are presented as percentages of the total activity recovered from the gradient.

Table 1 Marker enrichment factors

Marker	I	II	III	IV	V	VI	VII
p-NPPase	0.5	0.9	1.0	1.0	6.8	0.6	0.0
NADPH-cytochrome c reductase	1.0	0.8	1.0	0.7	3.1	1.2	1.2
$InsP_3$	0.2	1.2	0.8	0.7	4.4	1.4	0.6
$InsP_4$	0.1	0.7	1.5	1.2	6.4	0.1	1.0

Activity enrichments resulting from equilibrium density gradient separation and high-speed differential sedimentation of membrane vesicles from colonic smooth muscle. Enrichments were calculated as the ratio of percentage recovered enzyme or receptor activity to that of protein contained within the same density fraction, and reflect the concentrations of specific markers within membrane populations.

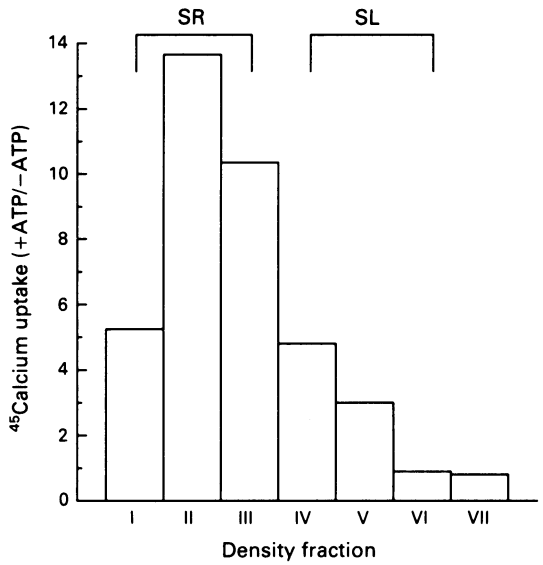


Figure 6 Distribution of ATP-dependent ^{45}Ca uptake in circular smooth muscle membrane density fractions. ^{45}Ca uptake was measured as described in Methods. The ratio of ^{45}Ca uptake in the presence of ATP to that in the absence of ATP was calculated for each fraction. SR: sarcoplasmic reticulum; SL: sarcolemmal. Values represent means of four experiments. Standard deviations were less than 15%.

lular markers present, provide strong evidence for the plasmalemmal origin of the membranes in this region of the gradient.

In accordance with the enzyme markers, ATP-dependent calcium accumulation was found highest within density fraction II and very weak in density fraction V (Figure 6). There was four times as much ATP-dependent calcium accumulation in density fraction II as in the fraction V. Although thapsigargin inhibited 50% of calcium uptake into the microvesicles before sucrose density gradient separation (data not shown), the inhibition was not seen in the fractions from the density gradient.

Subcellular distribution of inositol phosphate binding

Figure 7 presents the results of inositol phosphate binding assays performed on vesicles separated by the procedures summarized above and subsequently solubilized as described in Methods. The largest peak of recovered $InsP_3$ activity was found in fraction II of the density gradient, and therefore appears to co-equilibrate with intracellular membrane marker activities present in this region (Figure 5). Significant $InsP_3$ binding activity was also found in the region of density fractions IV–VI, albeit much less localized than the $InsP_3$ binding activity seen in fraction II (Figure 7a). $InsP_4$ binding displayed a well-defined bimodal distribution (Figure 7b) which resembled closely that of *p*-NPPase, with distribution modes centered in density fractions II and V. The highest enrichments for both $InsP_3$ and $InsP_4$ binding were found in fraction V; enrichment for $InsP_4$ binding in this fraction was 1.5 × that of $InsP_3$ binding (Table 1), and reflects the relatively large peak of $InsP_4$ binding found in this region of the gradient. Indeed, assuming a bimodal distribution in fractions II and V, one can see that the overlap in these two patterns sums in fractions III and IV. $InsP_4$ binding is therefore equally distributed between regions of the density gradient which appear to contain intracellular membranes (e.g. fraction II) and regions containing membranes of plasma membrane origin (e.g. fraction V).

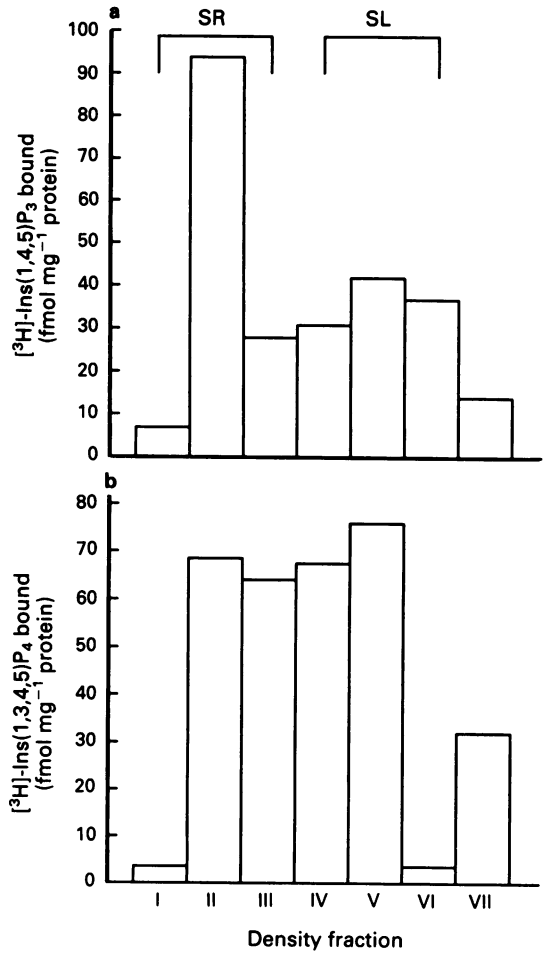


Figure 7 Distribution of [^{3}H]-InsP$_3$ and [^{3}H]-InsP$_4$ specific binding in subcellular fractions prepared from canine colonic circular smooth muscle. The subcellular fractions of colon smooth muscle were prepared from discontinuous sucrose density gradients as described in Methods. The binding of [^{3}H]-InsP$_3$ (15 nM; a) and [^{3}H]-InsP$_4$ (15 nM; b) to the fractions was performed at pH 8.5 ([^{3}H]-InsP$_3$) and pH 5.0 ([^{3}H]-InsP$_4$). Nonspecific binding was obtained in the presence of 12 μM unlabelled InsP$_3$ or InsP$_4$. The highest of [^{3}H]-InsP$_3$ specific binding was in the light-density fraction II, whereas [^{3}H]-InsP$_4$ specific binding was equally distributed between II and V fractions. SR: sarcoplasmic reticulum; SL: sarcolemmal. Values are means of two separate experiments performed in triplicate. Standard deviations were less than 15%.

Discussion

This study demonstrates for the first time the presence of high affinity InsP$_4$ binding sites in smooth muscle and their localization to plasma as well as intracellular membranes. Both InsP$_3$ and InsP$_4$ binding sites have been demonstrated in gradient-fractionated native membranes and in solubilized membranes. Detergent solubilization of InsP$_3$ and InsP$_4$ binding sites in smooth muscle increased radioligand binding with affinities comparable to those of the receptors in native membranes measured by others (Donie *et al.*, 1990; Hingorani & Agnew, 1991). The distinct pH-dependencies of the InsP$_3$ and InsP$_4$ receptor binding are in broad agreement with previous findings by other groups studying brain (Challiss *et al.*, 1991; Donie & Reiser, 1991; Theibert *et al.*, 1990) and is likely to relate to the required ionization state of the phosphate substituents of the ligand. By using ^{1}H- and ^{31}P-n.m.r. spectroscopy, White *et al.* (1991) could demonstrate that InsP$_3$ had five equatorial substituents and one axial substituent. No detectable change in conformation was observed over the pH range 4–11. The increase in affinity of InsP$_3$ for its receptor with increasing pH was explained by ionization of the 4- and 5-phosphate groups. On the other hand, the high affinity conformation of the InsP$_4$ binding sites was stabilized at acidic pH, facilitating their detection by radioligand binding studies. Such cellular pH changes occurring during Ca^{2+} regulation following addition of agonists (Danthuluri *et al.*, 1990; Kohmoto *et al.*, 1990) are likely to play an important role in modifying the binding properties of the inositol polyphosphates and may be the critical factor in understanding the action of InsP$_4$ in excitable cells.

InsP$_3$ receptors have been identified in a number of tissues, including smooth muscle (Chadwick *et al.*, 1990). The cerebellar membrane contains a high density of apparently homogeneous InsP$_3$ receptors which have been cloned and shown to have a molecular weight of 313,000 (Furuichi *et al.*, 1989; Maeda *et al.*, 1990). InsP$_3$ receptors isolated from smooth muscle are structurally and functionally similar to those from cerebellum (Marks *et al.*, 1990). Our data demonstrate a relatively high density of InsP$_3$ receptors in detergent-solubilized membranes of canine colon smooth muscle. The affinity of InsP$_3$ receptors defined in the present study is similar to that found in other smooth muscle preparations (Chadwick *et al.*, 1990; Varney *et al.*, 1990). In accordance with previous reports by a number of groups (Worley *et al.*, 1987; Chadwick *et al.*, 1990; Varney *et al.*, 1990; Challiss *et al.*, 1991), InsP$_3$ receptors characterized in colon smooth muscle are apparently homogeneous. Although there are slight differences in the affinities of the InsP$_3$ receptor for InsP$_3$ among tissues, there is no consistent pattern to suggest distinct subtypes of the InsP$_3$ receptor (Varney *et al.*, 1990).

The initial phase of receptor-mediated Ca^{2+} signalling, involving Ca^{2+} release from intracellular stores by InsP$_3$ is well characterized. It has been demonstrated that InsP$_3$ induces Ca^{2+} release from reconstituted vesicles containing the brain InsP$_3$ receptor (Ferris *et al.*, 1989). More recently, the purified InsP$_3$ receptor from smooth muscle has been reconstituted into planar lipid bilayers and demonstrated to be an InsP$_3$ activated Ca^{2+} channel (Mayrleitner *et al.*, 1991). The intracellular localization of the InsP$_3$-sensitive Ca^{2+} pool is thought to be in endoplasmic reticulum (so called sarcoplasmic reticulum in smooth muscle). Subcellular fractionation studies did in fact localize the InsP$_3$-sensitive Ca^{2+} pool to the microsomal fraction most enriched in endoplasmic reticulum (Joseph *et al.*, 1984; Prenki *et al.*, 1984; Ross *et al.*, 1989). In the present study, the subcellular fraction with the largest peak of InsP$_3$ binding was found to contain membrane vesicles of predominantly intracellular origin on the basis of its content of the endoplasmic reticulum marker NADPH-cytochrome c reductase as well as large amounts of membrane-associated protein. In addition to enzyme markers, ATP-dependent calcium accumulation was also found to be highest in these membrane vesicles. The same result has been demonstrated in uterine smooth muscle where ATP-dependent calcium accumulation is a property of sarcoplasmic reticulum (Carsten & Miller, 1980). Although it is possible for the cell membrane to have intrinsic ATP-dependent calcium accumulation, this activity is at best weak, both in colon and myometrium. The loss of inhibition of calcium uptake by thapsigargin in density fractions may be due to the loss of its binding sites during sucrose density gradient separation. Interestingly, InsP$_3$ binding was also present in detectable quantities in regions of the density gradient thought to contain vesicles derived from the plasma membrane (Figure 5). Similar results were obtained in subcellular fractions prepared from rat hepatocytes (Guillemette *et al.*, 1988; Rossier *et al.*, 1991). There is considerable evidence that at least a portion of the InsP$_3$-sensitive pool, so called subplasmalemmal cisternae, might be localized near the plasma membrane. Using polyclonal antibodies, Ross *et al.* (1989) localized the InsP$_3$ receptor not only to the endoplasmic reticulum, but also to the subplasmalemmal cisternae. It is possible that the enrichment of InsP$_3$ binding activity in plasma membrane fractions in the present study is due to

co-equilibration of subplasmalemmal cisternae with the plasma membranes. On the other hand, the $InsP_3$ receptor may also localize directly to the plasma membrane. In fact, electron microscopy studies using three different monoclonal antibodies indicated the presence of $InsP_3$ receptors on the plasma membranes in cerebellar Purkinje cells (Furuichi *et al.*, 1989). The localization of $InsP_3$ receptor on the plasma membrane or on the cell organelle near plasma membrane may suggest an important role for the $InsP_3$ receptor in regulation of Ca^{2+} homeostasis. It has been demonstrated that muscarinic stimulation of parotid cells induces the opening of Ca^{2+}-sensitive ion channels in the plasma membrane prior to increases in cytosolic Ca^{2+} concentrations (Foskett *et al.*, 1989). More directly, $InsP_3$ has been found to activate Ca^{2+} channels in the plasma membrane of T lymphocytes (Kuno & Gardner, 1987).

Although the $InsP_4$ receptor has been described in cerebellum, parathyroid glands, adrenal cortex, and HL-60 cells (Bradford & Irvine, 1987; Enyedi & Williams, 1988; Enyedi *et al.*, 1989; Donie *et al.*, 1990), it has not yet been characterized in smooth muscle. In the present study, we have identified binding sites for $InsP_4$ with an affinity of approximately 3 nM at a concentration of 127 fmol mg^{-1} protein in detergent-solubilized membranes prepared from canine colonic circular smooth muscle. Similar affinities for $InsP_4$ were reported in cerebellum (Doine *et al.*, 1990; Theibert *et al.*, 1991). The finding that competition for [^{3}H]-$InsP_4$ by unlabelled $InsP_3$ is incomplete raises the possibility of heterogeneity of [^{3}H]-$InsP_4$ binding sites. In fact, heterogeneous [^{3}H]-$InsP_4$ binding sites have been reported in cerebellum, adrenal cortex, and parathyroid glands (Enyedi & Williams, 1988; Enyedi *et al.*, 1989; Challiss *et al.*, 1991). However, both saturation and competition analysis of $InsP_4$ receptor binding in the present study reveal a single class of binding sites for $InsP_4$ with an affinity similar to that of the high affinity binding sites described in other tissues. The low affinity binding sites may not have been observed in the present study simply because the concentrations of [^{3}H]-$InsP_4$ used in the present study were not high enough. Alternatively, it is possible that the heterogeneity of $InsP_4$ binding sites only exists with respect to an action of $InsP_3$. That is, perhaps $InsP_3$ acts as an endogenous competitor at some but not all $InsP_4$ receptors. This possibility is intriguing in light of the distribution of smooth muscle $InsP_4$ binding sites to two distinct regions of the muscle cell.

Our data provide evidence that the $InsP_4$ receptor is present in significant quantities on the plasma membrane, as well as on membranes of intracellular origin. High enrichment factors for $InsP_4$ binding in density fractions thought to contain vesicles of plasma membrane origin may suggest a significant function for $InsP_4$ in the plasma membrane (Table 1). It has been proposed that $InsP_4$ acts with $InsP_3$ as a second messenger by promoting extracellular Ca^{2+} entry (Gawler *et al.*, 1991). Using single cell voltage clamping methods, Morris *et al.* (1987) have demonstrated that $InsP_4$, in the presence of $InsP_3$, evokes a sustained increase in Ca^{2+}-activated K^+ current which is dependent on external Ca^{2+}. While it is intriguing to consider a potential role for $InsP_3$/$InsP_4$ action in regulation of colonic smooth muscle Ca^{2+}-activated K^+ channels in light of the presence of this channel in circular muscle cells (Carl *et al.*, 1991), it is unlikely that this is the site of action of $InsP_4$ in smooth muscle since our repeated attempts to regulate this channel with $InsP_4$ in the presence and absence of $InsP_3$ failed (data not shown). The localization of $InsP_4$ binding to the sarcoplasmic reticulum in the present study lends support to the proposed role of $InsP_4$ in the modulation of an $InsP_3$-sensitive Ca^{2+} pool as well as possible direct effect upon intracellular Ca^{2+} mobilization. The presence of an additional [^{3}H]-$InsP_4$ binding to fraction VII is intriguing but not readily explained. What is clear, however, is that [^{3}H]-$InsP_4$ binds to both plasma and intracellular membranes and may enjoy a physiological function in the regulation of intracellular calcium in smooth muscle.

The demonstration of high affinity $InsP_3$ and $InsP_4$ receptors in canine colon circular smooth muscle provides evidence for the roles of $InsP_3$ and $InsP_4$ as second messengers in this tissue. In our recent study, we have demonstrated that activation of muscarinic receptors in colon smooth muscle produces a rapid accumulation of $InsP_3$ (Zhang & Buxton, 1991). $InsP_4$ accumulation was relatively delayed, which might be due to the similar activities of $InsP_3$ 3-kinase and the 5-phosphatase enzymes responsible for the formation and removal of $InsP_4$, respectively. Alternatively, it is possible that the radioactive method we have employed does not permit detection of labelled $InsP_4$ until late times due to the low abundance of this inositol isomer expected in the smooth muscle cells.

The rapid accumulation of $InsP_3$ is likely to play a direct role in initiation of the early tonic contraction of colon smooth muscle following activation of muscarinic receptors since removal of extracellular Ca^{2+} does not abolish the contraction, while subsequent phasic contractions are dependent on extracellular Ca^{2+}. Recently, Murthy *et al.* (1991) have demonstrated the existence of high affinity $InsP_3$ receptors in circular smooth muscle of guinea-pig intestine, in which $InsP_3$ elicited concentration-dependent contractions and increases in intracellular Ca^{2+}. The role of $InsP_4$ in regulating intracellular Ca^{2+} oscillations, which correlate well with phasic contractions in colon smooth muscle, is not entirely clear. Given the fact that $InsP_4$ is generated in response to M_3 muscarinic receptor stimulation and $InsP_4$ receptors are present on plasma membranes, $InsP_4$ may play an important role in regulating Ca^{2+} homeostasis based on the model proposed by Irvine (1990; 1992).

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(Received November 3, 1992
Revised March 4, 1993
Accepted March 12, 1993)

Br. J. Pharmacol. (1993), **109**, 913–917 © Macmillan Press Ltd, 1993

Characterization of CCK receptors in a novel smooth muscle preparation from the guinea-pig stomach by use of the selective antagonists CI-988, L-365,260 and devazepide

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1 The cholecystokinin receptors mediating motor responses in a novel smooth muscle preparation from the corpus region of the guinea-pig stomach have been characterized by use of five agonist peptides and the antagonists CI-988, L-365,260 and devazepide.

2 Mucosa-denuded strips of circular muscle were contracted in a concentration-dependent manner by the five cholecystokinin (CCK)-related peptides CCK-8S, pentagastrin, gastrin-I, CCK-8US and CCK-4.

3 CI-988 was a powerful antagonist of the response to pentagastrin with an affinity ($pK_B = 9.49$) similar to that obtained in CCK_B receptor binding assays. With CCK-8S as the agonist, CI-988 was approximately 1000 fold less powerful as an antagonist.

4 Devazepide powerfully blocked responses to CCK-8S with an affinity ($pK_B = 9.54$) that was in agreement with reported functional data obtained in pancreatic amylase secretion studies, a system exhibiting CCK_A receptor activity. Devazepide displayed lower affinity against pentagastrin than against CCK-8S.

5 CI-988 blocked responses to pentagastrin in an insurmountable manner in the presence of 3 nM devazepide; a concentration previously shown to block the CCK_A receptor. The nature of the antagonism observed with L-365,260 was unaltered by the presence of devazepide.

6 The guinea-pig stomach corpus smooth muscle preparation contains both subtypes of CCK receptor and will be useful as a pharmacological tool for investigating the functional effects of novel CCK ligands.

Keywords: Cholecystokinin (CCK) receptors; CCK_B antagonists; CI-988; smooth muscle; guinea-pig stomach

Introduction

Cholecystokinin (CCK) and gastrin which share a common COOH-terminal pentapeptide amide sequence (Rehfeld, 1981) interact with at least two receptors that can be distinguished by the relative potencies of sulphated CCK8 (CCK-8S), pentagastrin, gastrin I, unsulphated CCK8 (CCK-8US) and CCK-4 (Jensen, 1989; Knapp *et al.*, 1990). The CCK_A receptor has a high affinity for CCK-8S and a 7000 fold lower affinity for gastrin I (Hughes *et al.*, 1990). This receptor subtype is present on a number of peripheral tissues and cells particularly in the alimentary tract (Zetler, 1984; Innis & Snyder, 1980) but also occurs in some highly localized regions of the brain (Hill *et al.*, 1987) for example in dorsal raphe neurones (Pinnock *et al.*, 1990).

The CCK_B/gastrin receptor is recognised by gastrin, CCK-8S, CCK-8US and CCK-4 with approximately equal affinity (Hughes *et al.*, 1990). Currently the CCK_B and gastrin receptor are pharmacologically indistinguishable (Freidinger, 1989). The CCK_B receptor exists predominantly in the central nervous system (Hughes *et al.*, 1990) while the gastrin receptor is found primarily in the alimentary canal, for example on gastric parietal cells, pancreatic acinar cells and smooth muscle cells (Grider & Makhlouf, 1987; Cherner *et al.*, 1988).

CCK receptor heterogeneity has been confirmed following the development of high affinity and selective antagonists. The benzodiazepine ligand devazepide (formerly L-364,718, MK-329) has high affinity for the CCK_A receptor with 150 fold selectivity relative to the CCK_B receptor (Chang & Lotti, 1986). L-365,260, also a benzodiazepine has conversely high affinity for the CCK_B receptor although it does not possess the same degree of selectivity (Lotti & Chang, 1989). Recently developed in our laboratories is a series of dipeptoid ligands with high affinity and high selectivity for the CCK_B/gastrin receptor, the prototype of which is C1-988 (Hughes *et al.*, 1990).

The development of the benzodiazepine antagonists has allowed the characterization of CCK receptors that exist on a variety of smooth muscle preparations with standard organ bath techniques. With few exceptions it has become apparent that the majority of peripheral tissues contain only the CCK_A receptor subtype (Meldrum *et al.*, 1986; Woodruff & Hughes, 1991). Until recently the dearth of a simple and robust smooth muscle functional assay for the CCK_B/gastrin receptor has led a number of groups to examine the ability of CCK analogues to contract isolated, individual smooth muscle cells obtained from the fundic region of the guinea-pig stomach (Bitar & Makhlouf, 1982; Collins & Gardner, 1982) or the guinea-pig gallbladder and stomach (Grider & Makhlouf, 1990). It has been established that CCK-8S and gastrin I both cause contraction of isolated smooth muscle cells from these regions (Huang *et al.*, 1989). More recently it has been suggested that CCK_B/gastrin receptors mediate the transient contractile response to pentagastrin and CCK-4 in guinea-pig ileum longitudinal muscle myenteric plexus (Lucaites *et al.*, 1991; Patel & Spraggs, 1992).

In studies on isolated cells, conflicting results have been obtained with the antagonists L-365,260 and devazepide with the contractile effect of gastrin in isolated dispersed gastric smooth muscle cells being blocked more effectively by L-365,260 in one study (Huang *et al.*, 1989) but by devazepide in another (Grider & Makhlouf, 1990).

In the present study we have employed a previously uncharacterized preparation: the isolated smooth muscle strip from the corpus region of the guinea-pig stomach, in an attempt to shed light on these recent anomalies. A preliminary account of this work has been presented to the British Pharmacological Society (Boyle *et al.*, 1992).

Methods

Tissue preparation

Adult male Dunkin-Hartley guinea-pigs (wt. 330–400 g) with free access to food and water were killed by cervical dislocation and the stomach rapidly removed. The fundus was discarded and the stomach was opened along the greater curvature, pinned out on a petri-dish with the mucosa uppermost. The mucosal and submucosal layers were removed by dissection to reveal the underlying smooth muscle layer. Strips of circular muscle (2 × 25 mm) from the corpus region of the stomach were obtained by cutting inwards, following the striations at the lesser curvature. Muscle strips were mounted in siliconised 3 ml organ baths containing Krebs-Henseleit 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 11.1. The solution was maintained at 37°C and was gassed continuously with a mixture of 95% O_2/5% CO_2. After preliminary experiments the buffer was modified to include 5 μM indomethacin. Isometric contractile responses were measured with Grass FT.03 force-displacement transducers and recorded on Graphtec Linearcorders (Mark VII).

Experimental procedure

Tissues were placed under 1 g tension and allowed to equilibrate for 30 min after which time they were contracted with a submaximal dose of carbamylcholine (10 nM). Using a 12 min dose cycle concentration-response curves were established for a range of agonists that included CCK-8S, CCK-8US, gastrin I, CCK-4 and pentagastrin. Drugs were added in volumes that did not exceed 10 μl. For studies with the antagonists, tissues were exposed to antagonists for 15 min before re-exposure to agonists.

Analysis of results

Contractile responses to exogenously applied agonist were expressed as absolute changes in tension and were transformed to a percentage of the maximal response achieved for that agonist in order to obtain potency values. EC_{50} values were obtained graphically for individual concentration-response curves using an iterative curve-fitting programme (InPlot) available from GraphPad Software Inc. and are expressed as geometric mean with range. Responses obtained to agonists in the presence of antagonists are expressed as a percentage of the control maximum response obtained in the same tissue preparation. Agonist concentration-response curves in the absence and presence of increasing concentrations of CCK receptor antagonist were obtained and the method of Arunlakshana & Schild (1959) was used to provide Schild plots of the data, obtain affinity constants for the antagonists, and where applicable confirm the competitive nature of the antagonism. Linear regression for Schild plot analysis was carried out with RS1 curve-fitting software and data are expressed as pA_2 and slope value, with 95% confidence limits in parentheses. Where the slope of the interaction was not significantly different from unity, pK_B values are also supplied.

Compounds

CCK-8S, pentagastrin, CCK-8US, gastrin I and CCK-4 were obtained from Bachem UK Ltd and were dissolved in dimethylsulphoxide (DMSO) at a concentration of 10 mM and subsequently diluted in Krebs-Henseleit solution. Indomethacin, atropine, methysergide, mepyramine and tetrodotoxin were obtained from Sigma Chemical Co. and dissolved in saline. Devazepide and L-365,260 were synthesized by the Medicinal Chemistry Department of Parke-Davis and were dissolved in DMSO at a concentration of 10 mM. CI-988 (4-{[2-[[3- (1H-indol-3-yl)-2- methyl-1-oxo-2-[[(tricyclo [3.3. 1.1.^{3,7}] dec-2-yloxy) carbonyl]amino] propyl]amino]-1-phenylethyl] amihno}-4-oxo-[**R**-(**R***,**R***)-butanoate *N*-Methyl-D-glucamine), was also synthesized by the Medicinal Chemistry Department at Parke-Davis and was dissolved in Krebs-Henseleit solution.

Results

Effects of agonists

Addition of 5 μM indomethacin to the bathing solution resulted in abolition of the spontaneous contractions of the corpus muscle strip without affecting the potency or efficacy of pentagastrin (Figure 1). Five CCK-related peptides induced concentration-dependent contractions of the stomach preparation. CCK-8S produced a contractile response that was qualitatively similar to that produced by the other agonists but had greater efficacy producing a maximum contraction twice that observed with gastrin, pentagastrin and CCK-4 (Table 1). CCK-8US was of intermediate efficacy producing a maximum contractile response midway between

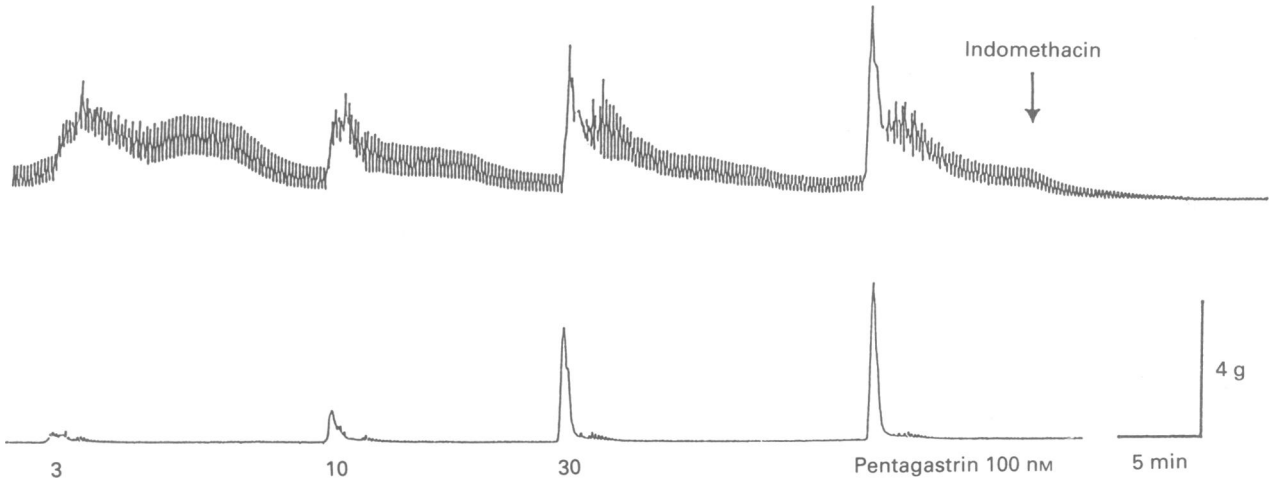


Figure 1 Representative continuous recording of the dose-dependent contractile action of pentagastrin in the guinea-pig stomach corpus smooth muscle preparation. The top panel of the recording demonstrates the inherent myogenic tone that exists in the tissue. Addition of indomethacin 5 μM removes the spontaneous activity but leaves the responses to pentagastrin unaffected (lower panel).

Table 1 Efficacy and potencies of cholecystokinin (CCK)-related peptides in isolated smooth muscle from the corpus region of the guinea-pig stomach

Agonist	*Maximum response* (g)	EC_{50} (nM)
CCK-8S	8.6 ± 0.54	13.1 (2.1–29.8)
Pentagastrin	4.1 ± 0.22	10.0 (3.2–15.4)
Gastrin 1	3.7 ± 0.35	33.1 (12.5–70.1)
CCK-8US	6.1 ± 0.58	102 (30.3–320)
CCK4	3.9 ± 0.29	214 (60.5–471)

Efficacy values are expressed as mean response in g tension ± s.e.mean for > 5 observations. Potency values are expressed as the geometric mean with range in parentheses for > 5 observations.

that observed with CCK-8S and the other agonists. In terms only of the respective EC_{50} values, of the five peptides employed pentagastrin and CCK-8S were the most potent while gastrin I was of intermediate potency relative to the other agonists (Table 1).

Effects of antagonists

The ability of a number of antagonists to influence the contractile responses of the preparation were examined. Atropine, mepyramine and methysergide each at 5 μM caused no significant alterations in the ability of the tissue to contract to either pentagastrin or CCK-8S. Similarly 1 μM tetrodotoxin also was without effect. (Data not shown).

The specific CCK_B receptor antagonist, CI-988, caused a concentration-dependent rightward shift of the concentration-response curve for pentagastrin (Figure 2). Schild plot analysis revealed a pA_2 of 9.49 (95% c.l. 9.29–9.79), a slope of − 1.14 (0.48–1.38) and a corresponding pK_B of 9.64 (9.38–9.91). CI-988 was also an antagonist of responses to CCK-8S (Figure 3). The pA_2 value for this interaction was 6.60 (6.13–7.07) with a slope = 0.79 (0.14–1.57). This represents a 1000 fold selectivity for CI-988 when pentagastrin is the contractile agent. L-365,260 also caused a concentration-dependent rightward shift in the concentration-response curves to pentagastrin and CCK-8S (data not shown) with respective pA_2 values of 8.79 (8.49–9.09, slope − 0.75 (0.39–1.10)) and 6.86 (6.56–7.16, slope − 1.11 (0.55–1.65)) representing a much lower level of specificity than CI-988.

The specific CCK_A receptor angatonist, devazepide, caused a concentration-dependent rightward shift of the concentration-response curve to CCK-8S (Figure 4) and Schild plot analysis by linear regression revealed a competitive interaction with a pA_2 value of 9.54 (9.95–9.13) a slope of − 0.98 (0.48–1.46) and a corresponding pK_B value of 9.51 (9.24–9.77). Devazepide was also a competitive antagonist of responses to pentagastrin with a pA_2 value of 7.55 (7.25–7.85) a slope of − 0.81 (0.30–1.26) and a corresponding pK_B value of 7.46 (7.27–7.65).

In order to preclude any interaction of pentagastrin at the CCK_A receptor, the ability of the two CCK_B receptor antagonists CI-988 and L-365,260 to antagonize responses to the pentapeptide were re-examined in the presence of 3 nM devazepide. Under these circumstances L-365,260 produced an almost identical competitive interaction at the CCK_B receptor in comparison to that observed in the absence of devazepide, with a pA_2 of 8.17 (8.00–8.34) a slope of − 0.87 (0.58–1.19) and a corresponding pK_B of 8.02 (7.89–8.15). Figure 5 demonstrates that the addition of devazepide to the bathing solution transformed the nature of the antagonism observed with CI-988 against the specific CCK_B receptor agonist pentagastrin. Under these conditions CI-988 produced an insurmountable blockade at the CCK_B receptor.

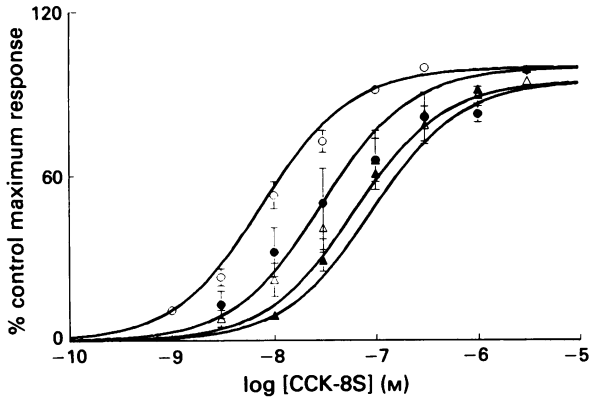


Figure 3 Inhibitory effect of the CCK_B receptor antagonist, CI-988, on control concentration-response curves to CCK-8S (○). Increasing concentrations of CI-988 300 nM (●), 1 μM (△) and 3 μM (▲) resulted in a concentration-dependent rightward shift of the CCK-8S concentration-response curve. Data are expressed as percentage of control maximum response to CCK-8S and each point represents the mean (± s.e.mean) of not less than 4 observations.

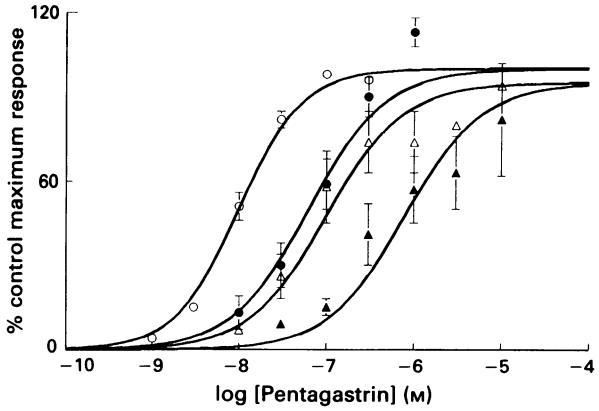


Figure 2 Inhibitory effect of the CCK_B receptor antagonist, CI-988, on control concentration-response curves to pentagastrin (○). Increasing concentrations of CI-988 1 nM (●), 3 nM (△) and 10 nM (▲) resulted in a concentration-dependent rightward shift of the pentagastrin concentration-response curve. Data are expressed as percentage of control maximum response to pentagastrin and each point represents the mean ± s.e.mean of not less than 4 observations.

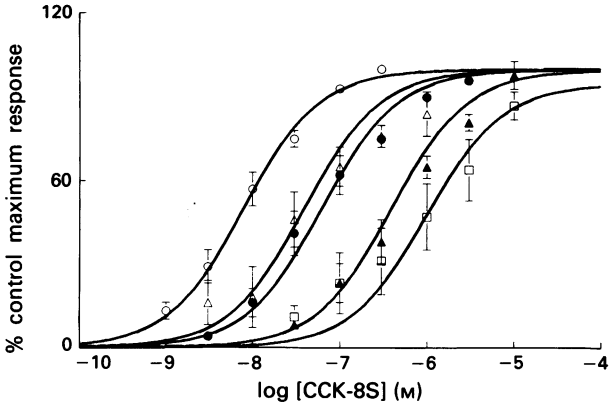


Figure 4 Inhibitory effect of the CCK_A receptor antagonist, devazepide, on control concentration-response curves to CCK-8S (○). Increasing concentrations of devazepide 1 nM (●), 3 nM (△) 10 nM (▲) and 30 nM (□) resulted in a concentration-dependent rightward shift of the CCK-8S concentration-response curve. Data are expressed as percentage of control maximum response to CCK-8S and each point represents the mean (± s.e.mean) of not less than 4 observations.

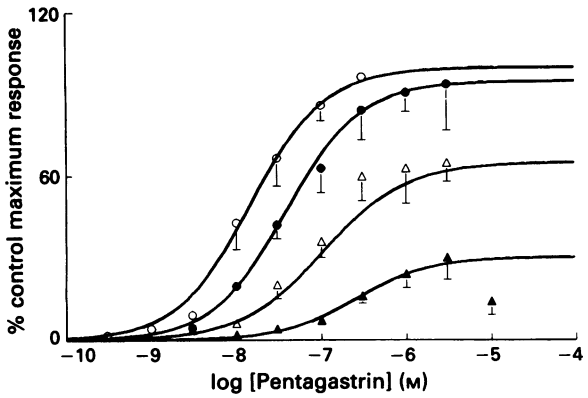


Figure 5 Insurmountable blockade of the pentagastrin concentration-response curve by increasing concentrations of CI-988 in the presence of 3 nM devazepide. The maximum response of the control (○) concentration-response curve was concentration-dependently: 1 nM (●), 3 nM (△), 10 nM (▲) reduced by CI-988. Data are expressed as percentage of control maximum response to pentagastrin and each point represents the mean (± s.e.mean) of not less than 4 observations.

It was apparent, however, that blockade of the CCK_B receptor with 10 nM CI-988 did not affect the nature of the antagonism observed with devazepide against CCK-8S. Under these circumstances devazepide blocked responses to CCK-8S with a pA_2 value of 9.84 (9.45–10.23) a slope of −0.94 (0.48–1.38) and a corresponding pK_B of 9.77 (9.48–10.07).

Discussion

The aim of the present study was to characterize the nature of the contractile effects of a number of CCK-related peptides in a novel smooth muscle preparation obtained from the corpus region of the guinea-pig stomach. CCK_A receptor-mediated contractile effects are readily accessible on a number of tissue preparations from several species, for example guinea-pig gall-bladder and ileum (Zetler, 1984), the pyloric and fundic regions of the rat stomach (Margolis *et al.*, 1989) and guinea-pig trachea (Stretton & Barnes, 1989). Previous attempts to investigate CCK_B receptor-mediated contractions have necessitated the use of isolated smooth muscle cell preparations from guinea-pig stomach (Bitar & Makhlouf, 1982; Grider & Makhlouf, 1990) or in guinea-pig ileum (Patel & Spraggs, 1992) where the response is indirect and apparently due to the release of acetylcholine. The novel preparation investigated in this study provides an accessible and robust means of investigating CCK_A and CCK_B receptor interactions.

Preliminary experiments revealed the ability of the cyclo-oxygenase inhibitor, indomethacin, to inhibit myogenic activity in the preparation without affecting the efficacy or potency of the CCK_B selective agonist pentagastrin or the non-selective agonist CCK-8S. The inability of micromolar concentrations of atropine, mepyramine and methysergide to affect contractions to either agonist suggested that the peptides were not causing release of acetylcholine, histamine or 5-hydroxytryptamine. Similarly the inability of the sodium channel blocker, tetrodotoxin, to influence the contractile response of the preparation suggested that the peptides were producing motor effects via receptors situated post-synaptically on the smooth muscle walls.

The non-selective CCK receptor agonist, CCK-8S, contracted the tissue with the greatest efficacy of the five peptides examined, presumably due to its ability to stimulate receptor-effector coupling at both receptor sites. The intermediate level of efficacy observed with CCK-8US is perhaps due to its ability at higher concentrations to interact with the CCK_A receptor in the preparation, while the other peptides pentagastrin, gastrin I and CCK-4 retain the ability to interact only with the CCK_B receptor.

The observed efficacy of these peptide agonists coupled with the high potency seen for pentagastrin and gastrin I and the comparatively high potencies observed for CCK-4 and CCK-8US suggest the presence of CCK_B receptors in this smooth muscle preparation that mediate contractile responses. This was confirmed by the observation that two highly selective CCK_B receptor antagonists, CI-988 and L-365,260, both antagonized responses to pentagastrin with affinities that suggested an interaction at the CCK_B receptor. The observed affinity of CI-988 is in close agreement with that observed in radio-ligand binding studies in mouse cortex and in functional electrophysiological studies in the ventromedial nucleus of the hypothalamus (Woodruff *et al.*, 1990) while the affinity observed with L-365,260 is in close agreement with that seen in the guinea-pig ileum where pentagastrin was also used as the agonist, albeit in the presence of devazepide (100 nM) (Patel & Spraggs, 1992).

While the functional affinity of L-365,260, for example in rat hypothalamic brain slices (Kemp *et al.*, 1989) or in guinea-pig dispersed smooth muscle cells (Huang *et al.*, 1989), is lower than its affinity in binding studies; using the guinea-pig isolated stomach preparation as a functional model, the affinity of L-365,260 was maintained in relation to its binding affinity. The 1000 fold lower affinity of CI-988 and the 30 fold lower affinity of L-365,260 obtained against CCK-8S as the agonist as opposed to pentagastrin, suggested that CCK-8S was mediating its contractile response via the CCK_A receptor. This was confirmed with devazepide, the highly selective CCK_A receptor antagonist. Devazepide antagonized responses to CCK-8S both in the presence and absence of CI-988 with equal and high affinity. The pK_B value of 9.5 that was obtained for devazepide is similar to that previously reported for guinea-pig ileum (Chang & Lotti, 1986; Patel & Spraggs, 1992). In the guinea-pig stomach preparation it would seem that the 'non-selective' agonist, CCK-8S, mediates its contractile effects largely through the CCK_A receptor. This is at odds with the higher level of efficacy observed with CCK-8S and suggests that in the presence of CCK_B receptor blockade, the CCK_A receptor assumes total responsibility for contraction of the tissue, possibly due to more efficient receptor effector coupling. The low affinity of devazepide observed against pentagastrin was indicative of an interaction at the CCK_B receptor and is similar to the binding affinity at the CCK_B binding site in guinea-pig cortical membranes (Knapp *et al.*, 1990).

CI-988 and L-365,260 are more powerful antagonists of pentagastrin-induced contractions than devazepide. The data obtained in this study disagree with the observations made in isolated cell preparations wherein the CCK_A selective antagonist, devazepide, was more powerful than L-365,260 at inhibiting the contractile responses to gastrin (Grider & Makhlouf, 1990). However, the data are in agreement with those obtained in a similar isolated cell preparation in which L-365,260 was more powerful than devazepide at blocking the contractile effects of CCK_B-selective agonists (Huang *et al.*, 1989).

In order to preclude an interaction of pentagastrin at the CCK_A receptor, concentration-response curves to this peptide were carried out in the presence of a concentration of devazepide (3 nM) that was ten times its affinity at the CCK_A receptor. Under these circumstances the interaction between L-365,260 and pentagastrin seemed unchanged with the estimated affinity for L-365,260 being similar to that obtained in the absence of devazepide. In contrast, with CI-988 in the presence of devazepide, an insurmountable block at the CCK_B receptor was observed. This effect suggests the possibility that with CI-988, the dipeptoid ligand, but not L-365,260, the benzodiazepine, a previously unrecognised interaction is revealed between the two subtypes of CCK receptor. The selectivity we observed for CI-988 (in the absence of

devazepide) in favour of the blockade of pentagastrin versus CCK-8S suggests that the concentrations of CI-988 that produce an apparently competitive blockade cannot be interacting at the CCK_A receptor to any significant degree. The circumstances of the insurmountable blockade are unique since in the converse experiment where CCK-8S is the agonist and the CCK_B receptor is blocked with CI-988, devazepide retains its competitive action. Indeed no evidence exists to suggest that either compound can influence the binding of the other at either CCK receptor subtype. Therefore the specificity of circumstances under which the effect is observed suggests something other than a simple ligand-receptor interaction and the possibility of an allosteric interaction between the CCK_A and CCK_B receptors that is revealed under this set of conditions must be considered.

It is possible that such experiments designed to investigate any potential interaction may have to be carried out in this novel preparation if the observed effects are indeed unique to guinea-pig stomach smooth muscle. If radio-ligand binding experiments cannot supply the answer to this interesting observation, then second messenger assays that permit study of receptor-effector coupling may shed light on the reasons for the transformation of the antagonism observed with CI-988 in the presence of devazepide.

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(Received November 11, 1992
Revised March 4, 1993
Accepted March 12, 1993)

Br. J. Pharmacol. (1993), **109**, 918–924 © Macmillan Press Ltd, 1993

Block of voltage-dependent sodium currents by the substance P receptor antagonist (±)-CP-96,345 in neurones cultured from rat cortex

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1 Whole cell patch clamp recordings of voltage- and tetrodotoxin-sensitive Na^+ currents were made from cultured rat neocortical neurones (E18). The effects of the non-peptide NK_1 receptor antagonist, (±)-CP-96,345 on Na^+ currents was examined, relative to the effect of the local anaesthetic lignocaine and tetrodotoxin.

2 Sodium currents were reversibly depressed by bath application of (±)-CP-96,345 with a half-maximally effective concentration of 18 ± 2 μM at a stimulation frequency of 0.1 Hz. Likewise the concentrations required to half-maximally inhibit sodium currents by tetrodotoxin and lignocaine were 10 ± 2 nM and 1.3 ± 0.2 mM respectively.

3 The depression of sodium currents by (±)-CP-96,345 (10 μM) was use-dependent in that raising the stimulus frequency from 0.1 Hz to 10 Hz further decreased the magnitude of sodium currents from 60 ± 5% to 37 ± 5% of control values respectively. Similarly, the depression of sodium currents by lignocaine (500 μM) and tetrodotoxin (30 nM) was also accentuated by raising the stimulus frequency from 0.1 Hz to 10 Hz.

4 The effect of (±)-CP-96,345 was not associated with a change in either the activation or steady-state inactivation characteristics of these currents, suggesting that its mechanism of action was via open channel blockade.

5 These data demonstrate that in addition to antagonizing NK_1 receptors, (±)-CP-96,345 also acts as a channel blocker on sodium channels at micromolar concentrations, an effect which should be taken into consideration when examining the antinociceptive or anti-inflammatory action of this compound.

Keywords: Lignocaine (lidocaine); substance P; neocortex; CP-96,345, tetrodotoxin

Introduction

Voltage-sensitive sodium channels are modulated by a number of organic toxins including the alkaloid veratridine, saxitoxin, and tetrodotoxin (Mosher *et al.*, 1964; Hille, 1968; Barnes & Hille, 1988). Similarly, several synthetic agents including pyrethroid insecticides, anticonvulsants and type 1 antiarrhythmic agents are known to exert their effects by modulating sodium channel function (Lund & Narahashi, 1981; Willow & Catterall, 1982; Narahashi, 1992; Grant & Wendt, 1992). In particular, amine local anaesthetics, like lignocaine, exert their action by blocking voltage-sensitive sodium channels in a use-dependent manner (Courtney, 1975; Hille, 1977).

Since the discovery of the neurokinin peptide substance P (Von Euler & Gaddum, 1931; Chang & Leeman, 1970), its role in the perception of pain has been the subject of much interest. Based upon both anatomical and physiological evidence, the NK_1 subtype of neurokinin receptor (reviewed in Iversen *et al.*, 1990), is thought to play an important role in nociception as well as inflammatory processes (Otsuka & Yanagisawa, 1987; Lotz *et al.*, 1988; Mantyh *et al.*, 1989). The development of potent non-peptide antagonists of receptors for substance P (reviewed in Watling, 1992) has enabled a more thorough examination of the role that these receptors have in nociception and inflammation. One such NK_1-antagonist, CP-96,345 (Snider *et al.*, 1991a,b) binds with a high affinity to NK_1 receptors in various regions of the brain (McLean *et al.*, 1991). This antagonist also inhibits SP-induced firing in locus coeruleus neurones (McLean *et al.*, 1991; Seabrook *et al.*, 1992) and reduces the magnitude of excitatory postsynaptic potentials in dorsal horn neurones *in vivo* produced by noxious stimulation (DeKoninck & Henry, 1991). Furthermore, this compound has antinociceptive effects, and reduces plasma extravasation in animal models of inflammation (Radhakrishnan & Henry, 1991; Birch *et al.*, 1992; Lembeck *et al.*, 1992; Xu *et al.*, 1992).

In order to attribute unequivocally the block of physiological processes by NK_1 receptor antagonists to an action upon substance P pathways, it is important to demonstrate that there are no other actions of these drugs. CP-96,345 has been shown to displace diltiazem binding as well as enhance nitrendipine binding to voltage-sensitive calcium channels (Schmidt *et al.*, 1992). It is known that calcium channel antagonists can have antinociceptive actions (Miranda *et al.*, 1992) and similarly, local anaesthesia may also be achieved via blockade of sodium channels. Consequently, to test the hypothesis that CP-96,345 may also block sodium channels, this study examined its effect on voltage-sensitive sodium currents in neurones cultured from rat cortex.

Methods

Preparation of cultures

Neocortical cells were maintained in culture as described previously (Priestley *et al.*, 1990). In brief, cortical hemispheres were taken from brains of 16–18 day gestation foetal rats, mechanically and enzymatically dissected, transferred into ice-cold Hanks balanced salt solution (without magnesium and calcium) and centrifuged at 2000 r.p.m. for 2 min. In order to prevent bacterial infections, all solutions contained 100 μg ml^{-1} streptomycin and 100 u ml^{-1} penicillin

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(Sigma). For enzymatic digestion the pellet was then transferred into a solution containing 0.25% trypsin (type IX, Sigma) and 0.05% deoxyribonuclease (DNAaseI, type II, Sigma), gently mechanically dissected by triturating through a 5 ml pipette and incubated in the same solution for 12 min at room temperature. Enzymatic digestion was terminated by addition of 0.5% soybean trypsin inhibitor (Sigma). The cell suspension was then centrifuged and the pellet suspended in final growth medium. Cells were then plated onto glass coverslips coated with poly-L-lysine (mol. wt. 50–150,000 Da, Sigma) at a density of approx. 10^6 cells per well and cultivated in an incubator with 95% O_2:5% CO_2 atmosphere. Cultures were incubated in Eagle's Minimum Essential Medium (Flow), containing 10% foetal bovine serum (Flow), 15 mM D-glucose and 2 mM L-glutamine (Flow). To reduce extensive glial cell proliferation, antimitotic substances (uridine and fluorodeoxyuridine, approx. 10^{-5} M, both Sigma) were added to the culture medium during the first 3 to 4 days *in vitro*.

Recording and analysis

Whole cell voltage-clamp recordings were made from neocortical neurones which had been grown in tissue culture for 7 to 14 days. To optimize the quality of space clamp, small cells with few unbranching dendrites were selected for recordings. Cultures were placed into a recording chamber (approx. 1.5 ml) and continuously superfused at a rate of (2 ml min^{-1}) with a solution containing (in mM): $MgCl_2$ 4, KCl 5, NaCl 135, HEPES 10, D-glucose 10, TEA 10 and EGTA 1, adjusted to pH 7.4, 22°C. High resistance giga-ohm seals were obtained with patch pipettes pulled from thin walled borosilicate glass (Clark Electromedical Glass) in two steps (BB-CH puller, Mecanex Geneva) and filled with an internal solution of (in mM): CsF 20, CsCl 10, HEPES 10, Cs-EGTA 10, $CaCl_2$ 0.5, adjusted to pH 7.4 with CsOH. The voltage-dependence, activation and inactivation characteristics of sodium currents were measured using a pipette solution containing 10 mM NaCl and 110 mM CsF. Electrode tip resistances measured in the bath were between 2 to 8 Mohms. Recordings were made with a List EPC-7 patch clamp amplifier (List Instruments) and CED electrophysiology software packages. Currents were filtered above 3 kHz, and digitized (12 to 25 kHz) using a CED 1401 laboratory interface (Cambridge Electronic Design). All currents were analyzed following leak and capacitance subtraction.

To examine the current-voltage relationship of Na^+ currents, cells were voltage-clamped at − 80 mV and currents were evoked by 20 ms depolarizing command pulses to membrane potentials ranging between − 60 and + 70 mV. Steady-state inactivation was determined by stepping from the initial holding potential of − 80 mV to a pre-pulse potential between − 80 and − 20 mV (5 mV step, 1.5 s duration) prior to a test pulse to − 20 mV. The maximum conductance was calculated at each membrane potential and activation and inactivation curves were fit to Boltzmann function $Y = 1/[1 + \exp((V_{\frac{1}{2}}-V)/k)]$, where $V_{\frac{1}{2}}$ is the potential for half maximal activation/inactivation, V the applied voltage and k the slope factor. The frequency-dependent depression of Na^+ currents was measured by raising the stimulation frequency from 0.1 Hz (control frequency) to 0.3, 1, 3 and 10 Hz. For each stimulation frequency, blocks of 35 successive voltage test pulses were applied.

Concentration-effect curves were fitted by least-squares analysis of variance to the equation $Y = Y_{max}-Y_{max}/(1 + (EC_{50}/\text{antagonist concentration})^{nH})$, with EC_{50} as the half maximal effective concentration, Y_{max} the maximal current response and nH the Hill coefficient, using an iterative procedure on a VAX computer with Research System 1 software (BBN Software Products Corporation). Data are expressed as the mean ± standard error (s.e.).

Drugs

(±)-CP-96,345 (*cis*-2-(diphenylmethyl)-N-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3 amine) and the 2S3S diastereomer were synthesized at Merck Sharp & Dohme

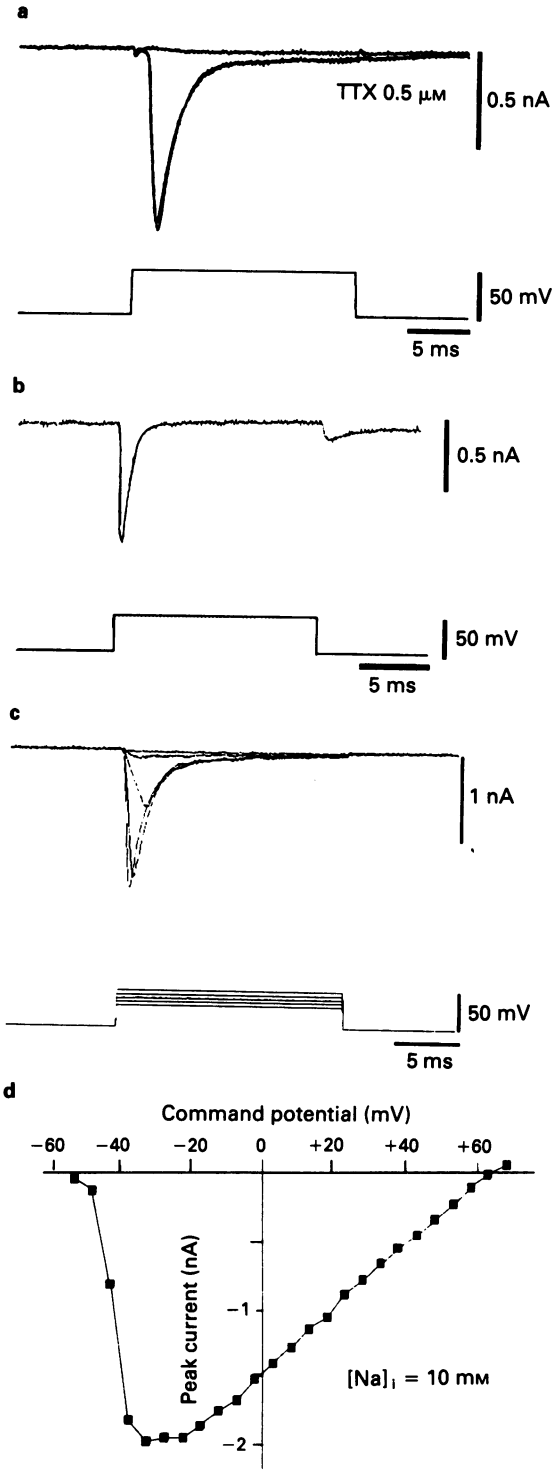


Figure 1 Whole cell sodium currents recorded from cultured neocortical neurones. Currents were activated by positive voltage command pulses from a holding potential of − 80 mV. (a) Reversible block of inward currents, evoked by a voltage step to − 20 mV, after exposure to tetrodotoxin (TTX, 0.5 μM). (b) Sodium currents in a different neurone showing a TTX-sensitive tail current following repolarization to − 80 mV. (c) Family of sodium currents activated at test potentials ranging from − 55 mV to − 35 mV. (d) Current-to-voltage relationship of peak sodium currents taken from the cell shown in (c).

Research Laboratories. (±)-CP-96,345 was dissolved in dimethylsulphoxide (DMSO), applied at a final bath concentration of <0.1% DMSO, which did not affect the magnitude or time course of sodium currents in these neurones ($n = 8$). Tetrodotoxin (TTX) and lignocaine were purchased from Calbiochem and Sigma, respectively.

Results

Biophysics of sodium currents in neocortical neurones

For whole cell patch clamp recordings neocortical cells were voltage clamped at − 80 mV and currents were activated by depolarizing voltage command pulses at 20 ms duration to potentials greater than − 60 mV (Figure 1). Evoked inward currents were completely and reversibly abolished by superfusion with TTX (500 nM; Figure 1a). Sodium currents were fully activated within 2 ms and in most cases were completely inactivated within 10 ms (Figure 1b). In some neurones a TTX-sensitive steady state current was present which was not totally inactivated within 20 ms (Figure 1a). TTX-sensitive tail currents were also observed in some, but not all, neurones upon membrane repolarization to − 80 mV (Figure 1b). The threshold for sodium current activation was > − 50 mV and reached a maximum at test depolarizations to about − 35 mV (Figure 1c,d). The reversal potential of sodium currents, with 10 mM $[Na]_i$, was + 65 ± 2 mV ($n = 7$).

The voltage-dependence of sodium current activation and inactivation were determined from the conductance-calculated from the peak current flowing at each test potential (Figure 2). The mean membrane potential for half-maximal activation was − 36 ± 2 mV with a steepness of 3 ± 1 mV per e-fold change in membrane potential ($n = 7$). Similarly, half-maximal inactivation occurred at a prepulse potential of − 53 ± 2 mV, with a steepness of 5 ± 1 mV per e-fold change in membrane potential ($n = 6$).

Depression of sodium currents by (±)-CP-96,345, lignocaine and TTX

Sodium currents were reversibly depressed, in a concentration-dependent manner by 1–100 μM (±)-CP-96,345 (Figure 3). At a concentration of 100 μM (±)-CP-96,345 decreased

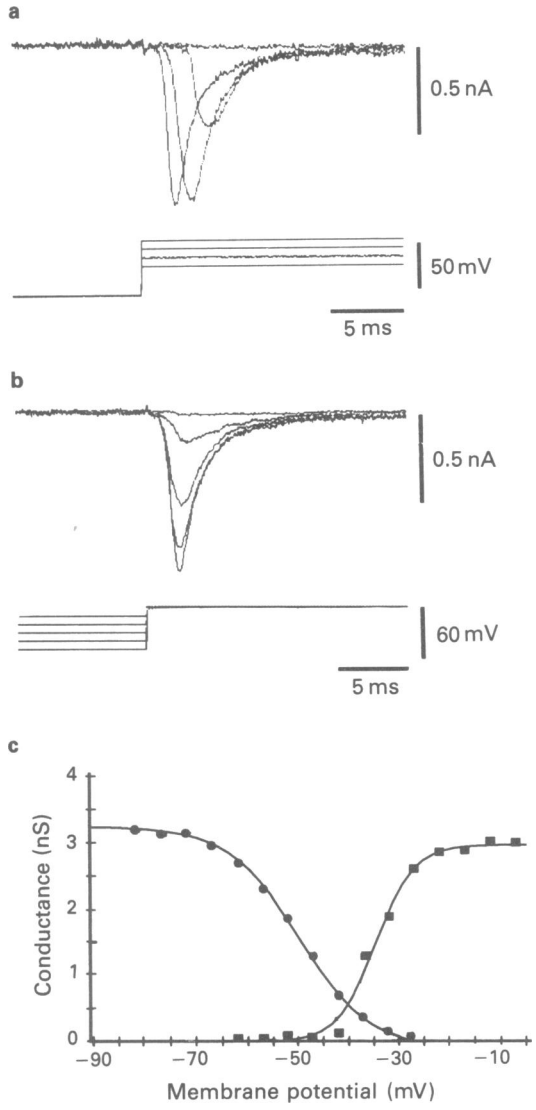


Figure 2 Voltage-dependence of activation and steady-state inactivation. (a) Activation protocol. Inward currents were evoked by voltage command pulses from a holding potential of − 80 mV to potentials exceeding − 60 mV. (b) Steady state inactivation protocol. Currents were evoked at − 20 mV membrane potential following stepwise depolarization of the holding potential from − 80 mV to − 20 mV for 1.5 s. (c) Data taken from (a) and (b) and plotted as an activation and steady state inactivation curve for peak conductances. Maximum conductance was calculated at each membrane potential and activation and inactivation curves were fit to a Boltzmann function. The potential for half-maximal activation and inactivation in this neurone were − 34 and − 48 mV respectively.

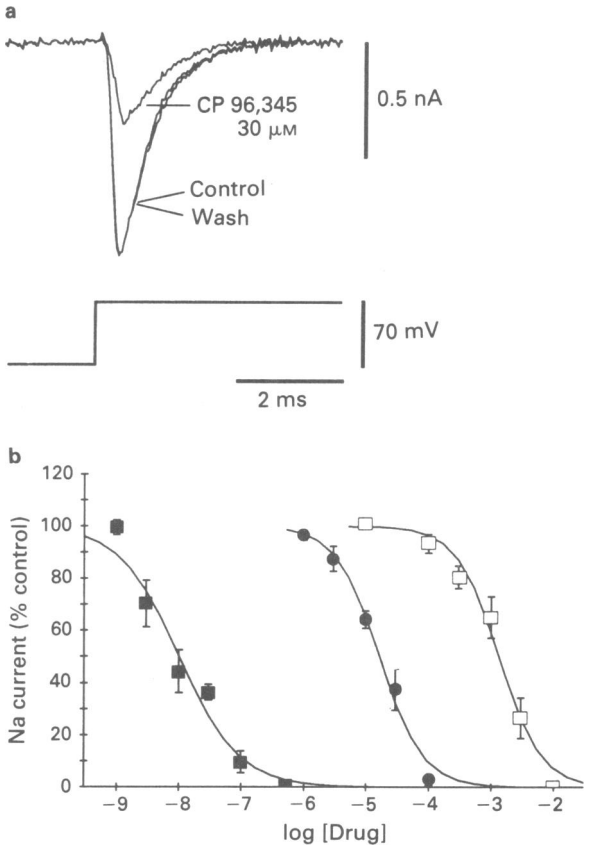


Figure 3 Concentration-dependent depression of Na^+ currents by tetrodotoxin (TTX), (±)-CP-96,345, and lignocaine. Currents were evoked by stepping the membrane potential from − 80 mV to − 20/ − 30 mV at a stimulus frequency of 0.1 Hz. Drugs were bath applied for 100 s. (a) Typical current traces illustrating the reversible depression of sodium currents by 30 μM (±)-CP-96,345 obtained by applying a depolarizing voltage command pulse to − 20 mV. (b) Concentration-effect relationships of TTX (■), (±)-CP-96,345 (●), and lignocaine (□). The half-maximal inhibitory concentrations of each drug were 10 ± 2 nM, 18 ± 2 μM and 1.3 ± 0.2 mM respectively. Each data point represents the mean ± s.e.mean of 4 to 14 cells.

sodium currents to 3 ± 2% of control values ($n = 6$; Figure 3b). At a stimulus frequency of 0.1 Hz, the half-maximally inhibitory concentration of (±)-CP-96,345 was 18 ± 2 μM. TTX-sensitive sodium tail currents were also depressed by (±)-CP-96,345 at similar concentrations. As with the racemic mixture of CP-96,345, the NK_1-selective 2S3S diastereomer also depressed sodium currents to 58 ± 5% of control values at a concentration of 10 μM ($n = 5$).

Sodium currents were also reversibly depressed in a concentration-dependent manner by bath application of either lignocaine or TTX (Figure 3b). However at concentrations comparable to those required to half-maximally inhibit sodium currents by (±)-CP-96,345 (10 μM), lignocaine had no effect at low stimulus frequencies (101 ± 2% at 0.1 Hz; $n = 6$). The half-maximally inhibitory concentration of lignocaine was 1.3 ± 0.2 mM. In contrast, TTX was a potent antagonist of these voltage-sensitive sodium currents with an IC_{50} of 10 ± 2 nM.

Frequency-dependence of CP-96,345 and lignocaine

In previous studies, the actions of lignocaine and TTX on voltage-dependent sodium channels have been reported to be both voltage- and use-dependent (Baer *et al.*, 1976; Catterall, 1988; Grant *et al.*, 1989). Consequently, we have examined the frequency-dependence of the block produced by (±)-CP-96,345 and compared this to lignocaine and TTX.

In the present study, increasing the stimulus frequency accentuated the block of sodium currents by tetrodotoxin and lignocaine, as well as (±)-CP-96,345. Under control conditions the amplitude of sodium currents was minimally affected by raising the stimulus frequency to 10 Hz (Figure 4a). The ability of lignocaine to depress sodium currents was accentuated by increasing the frequency of stimulation from 0.1 to 10 Hz (Figure 4). Sodium currents in the presence of lignocaine (500 μM) were further depressed from 70% (0.1 Hz) to 40% (10 Hz) of control values over this frequency range. As with lignocaine, the depression of sodium currents by (±)-CP-96,345 (10 μM) at a stimulus frequency of 0.1 Hz (60%, $n = 6$) was also accentuated by raising the stimulus frequency to 10 Hz (37%, $n = 6$), an effect that was fully reversible upon resuming the control stimulus rate (Figure 5).

Effect of (±)-CP-96,345 on activation and inactivation kinetics

To test whether the depression of sodium currents by (±)-CP-96,345 was mediated by an effect upon sodium current activation or inactivation kinetics (for example a 20–30 mV positive shift in activation kinetics would account for the depression seen in Figure 3) the actions of a half-maximally effective concentration of (±)-CP-96,345 were examined on the conductance-voltage relationships, calculated as described in Figure 2.

The potentials required for half-maximal activation and inactivation of sodium currents in these neurones, 36 ± 2 mV

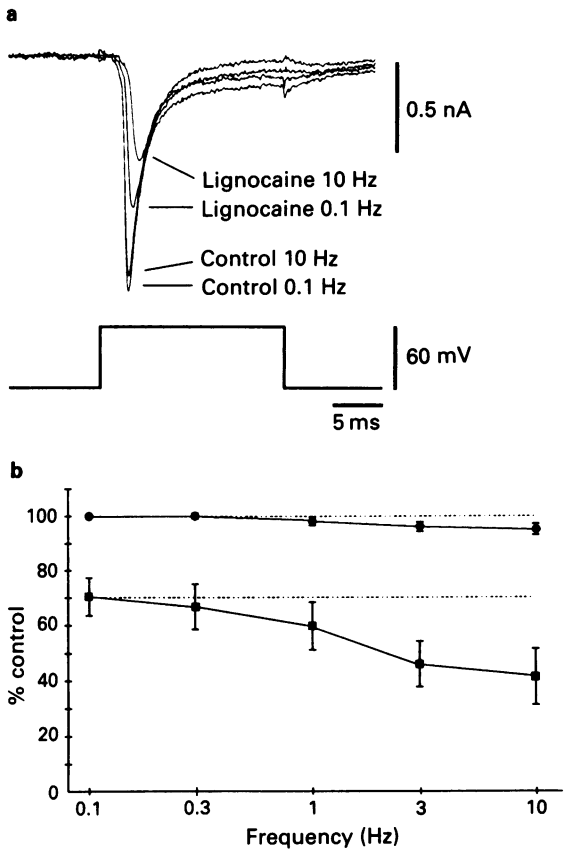


Figure 4 Frequency-dependent depression of Na^+ currents by lignocaine. (a) Depression of sodium currents by lignocaine (500 μM) was accentuated by raising the stimulus frequency from 0.1 Hz to 10 Hz. Currents were elicited by step depolarizations to − 20 mV. (b) Graph depicting the frequency-dependence of Na current amplitudes in the absence (●) and presence (■) of lignocaine (500 μM). At each stimulation frequency, values were determined from the 35th voltage step, and represent the mean ± s.e.mean of 6 cells. Data were normalized to the current amplitude at 0.1 Hz for each cell.

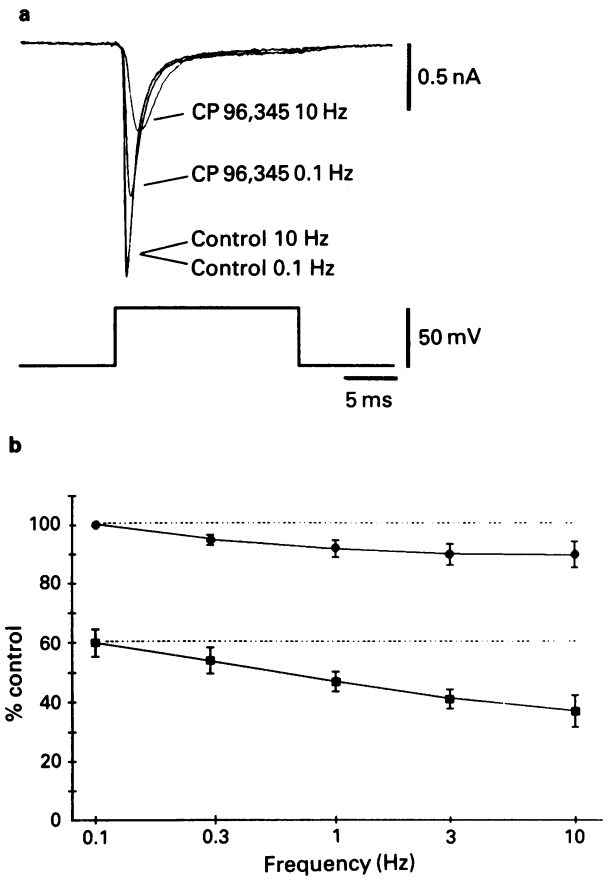


Figure 5 Frequency-dependent depression of Na^+ currents by (±)-CP-96,345 (10 μM). Experiments were performed as in Figure 4. (a) Depression of Na currents by (±)-CP-96,345 (10 μM) was accentuated by increasing the stimulus frequency from 0.1 Hz to 10 Hz. (b) Graph depicting the frequency-dependence of Na current amplitudes in the absence (●) and presence (■) of (±)-CP-96,345. At each stimulation frequency, values were determined from the 35th successive voltage step, and represent the mean ± s.e.mean of 6 cells. Data were normalized to the current amplitude at 0.1 Hz for each cell.

and 53 ± 2 mV respectively, were unaffected by perfusion with (±)-CP-96,345 (10 μM), 37 ± 2 mV and 56 ± 2 mV, respectively ($n = 6$; Figure 6). However, the extent of depression caused by (±)-CP-96,345 (10 μM) was greater upon the maximal conductance during the inactivation protocol (55 ± 5% depression, $n = 7$) than that of the activation protocol (39 ± 5% depression, $n = 5$).

The decay rate of macroscopic sodium currents in neurones is in part determined by the rate of inactivation of the underlying sodium channels. However, due to the similarities in the rates of both activation and inactivation, effects upon activation may also affect macroscopic current decay rates. This is exemplified by the voltage-dependence of activation rates, which imparts a voltage-dependence to current decay rates despite the voltage-insensitivity of channel inactivation rates (Bezanilla & Armstrong, 1977; Vandenberg & Horn, 1984). Nonetheless, agents such as batrachotoxin and deltamethrin which modulate channel inactivation, substantially slow the decay rates of macroscopic Na^+ currents (Lund & Narahashi, 1981; Tanguy & Yeh, 1988). Thus, in order to examine whether sodium current inactivation in neocortical neurones was modified by (±)-CP-96,345, the time constants of current decay rates were examined. However, in 19 neurones, the macroscopic current decay rate, 0.98 ± 0.07 ms (mean ± s.e.mean), was unaffected by a submaximal concentration of (±)-CP-96,345 (10 μM), 0.97 ± 0.07 ms.

Discussion

This study demonstrates that the non-peptide NK_1 receptor antagonist, CP-96,345, is a potent, use-dependent blocker of voltage-sensitive sodium currents in rat neocortical neurones. These data raise the possibility that effects seen in some studies, previously attributed to an action on NK_1 receptors, may in part be mediated by the non-specific actions of this drug.

Comparison of kinetics with other studies

Voltage-sensitive sodium currents in rat cortical neurones maintained in dissociated cell cultures exhibit biophysical characteristics which are similar to previous studies on acutely isolated neocortical neurones (Sah *et al.*, 1988; Huguenard *et al.*, 1989), and cultured neocortical cells (Huguenard *et al.*, 1988). These properties are similar to cloned type II sodium channels in a variety of cells (Noda *et al.*, 1986a,b; Stuhmer *et al.*, 1989; Gambale & Montal, 1990; Ahmed *et al.*, 1992). However, despite similarities in the macroscopic characteristics of sodium currents between issues, there are a number of structural and pharmacological differences between sodium channels located in neurones of the central and peripheral nervous system, and those of cardiac and skeletal muscle (Renaud *et al.*, 1983; Moczydlowski *et al.*, 1986; reviewed in: Catterall, 1988; Stuhmer & Parekh, 1992). This heterogeneity is reflected by the variation in potency of neurotoxins (e.g. tetrodotoxin), for different types of Na^+ channels in brain and heart cells (Renaud *et al.*, 1983; Roy & Narahashi, 1992). Similarly CNS neurones, unlike cardiac Na^+ channels, are only weakly affected by local anaesthetics such as lignocaine (Hille, 1977; Lee *et al.*, 1981; Grant *et al.*, 1989; Kaneda *et al.*, 1989; McDonald *et al.*, 1989). Consistent with this, the potency of lignocaine in the present study was found to be in the low millimolar range.

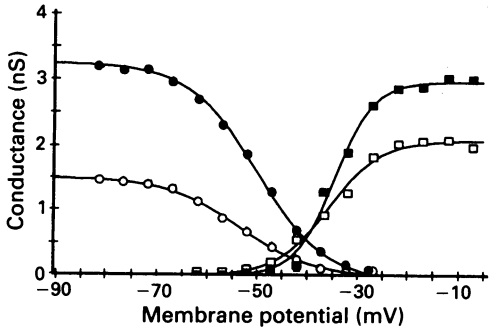


Figure 6 Activation and steady-state inactivation curves of Na^+ currents before (filled symbols) and during perfusion (open symbols) with (±)-CP-96,345 (10 μM). Experiments were performed as described in the legend to Figure 2. Bath application of (±)-CP-96,345 reduced the maximal conductance during both the activation and inactivation protocol without a shift in voltage-dependence. In this cell the potentials for half-maximal activation were − 39 mV and − 40 mV in the absence and presence of the drug. For the inactivation curve, the potentials for half maximal inactivation were − 55 mV and − 56 mV respectively.

Mechanism of block by CP-96,345

Lignocaine, like tetrodotoxin is believed to interact with the open state of voltage-sensitive sodium channels (reviewed in: Catterall, 1987; Grant & Wendt, 1992). However, despite subtle differences in their mechanism of action, the actions of these drugs are similar to open channel blockade rather than a modulating effect upon the kinetics of Na^+ channels such as those identified for alkaloids (e.g. batrachotoxin). The larger blockade of Na^+ currents in the inactivation protocols compared to the activation protocol (Figure 6) may be caused by a voltage-dependent action of (±)-CP-96,345 on sodium channels.

Inhibition of the macroscopic sodium currents in neocortical neurones by (±)-CP-96,345 was not distinguishable from that predicted for an open-channel blocker. The block was frequency-dependent, had minimal effect on the voltage-dependence of activation or inactivation and did not affect the macroscopic inactivation rates of sodium currents in these neurones. In addition, the ability of (±)-CP-96,345 to block sodium channels is not restricted to central Na^+ channels, but is also apparent at comparable concentrations in rat sciatic nerve (unpublished observations).

Implications for CP-96,345 pharmacology

It has been reported that the substance P antagonist, CP-96,345, has a binding affinity for NK_1 receptors in bovine caudate membrane of 0.6 nM (Snider *et al.*, 1991a). Furthermore, CP-96,345 antagonizes substance P induced neuronal activity *in vitro* and *in vivo*. For example, it blocks SP-induced potentials in the spinal cord (Lepre *et al.*, 1992) and locus coeruleus (McLean *et al.*, 1991; Seabrook *et al.*, 1992) *in vitro*. Physiological activity in the spinal cord *in vivo* evoked by substance P or noxious cutaneous stimulation can also be blocked by CP-96,345 (Radhakrishan & Henry, 1991; DeKoninck & Henry, 1991; Birch *et al.*, 1992). Despite its high affinity for NK_1 receptors, micromolar concentrations of CP-96,345 are required to antagonize some effects of SP or noxious stimulation in some nociceptive and inflammatory models *in vivo* (e.g. Lecci *et al.*, 1991; Xu *et al.*, 1992). The present study raises the possibility that effects, other than on NK_1 receptors, may contribute to the activities of this compound.

In addition to its effect on sodium currents, (±)-CP-96,345 shows other non-specific actions (±)-CP-96,345 interacts with the diltiazem and nitrendipine binding site on Ca^{2+} channels (Schmidt *et al.*, 1992) and blocks neuronal Ca^{2+} currents in neocortical neurones *in vitro* (Caeser & Kemp, 1993) and synaptic transmission in smooth muscle (Wang & Hakanson, 1992). Interestingly, bath applied (±)-CP-96,345 at micromolar concentrations also blocks muscarine-induced depolarizations in brain slices of the rat locus coerulus (Shen & North, 1992), but these effects may simply be attributable to ion channel blockade.

Most of the available studies on CP-96,345 have been

carried out using a racemic mixture of the compound. It has been shown that the binding of CP-96,345 on NK_1 receptors is stereoselective (Snider *et al.*, 1991a). Only the 2S3S form binds with a very high affinity on NK_1 receptors but not the 2R3R form. The inactive enantiomer 2R3R which fails to block SP-induced postsynaptic depolarizations in the locus coeruleus *in vitro* (McLean *et al.*, 1991) shows antinociceptive and antiinflammatory activity *in vivo* similar to the 2R3S enantiomer when applied at the micromolar range (Nagahisa *et al.*, 1992), suggesting that NK_1 receptor blockade may not account for all of the *in vivo* activities of the racemic compound.

In conclusion, this study demonstrates that CP-96,345 is a use-dependent antagonist of voltage-and TTX-sensitive sodium currents in rat neocortical neurones. Therefore, this effect, should be taken into consideration when studying the antinociceptive and anti-inflammatory effect of this compound.

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(Received December 21, 1992
Revised March, 11, 1993
Accepted March 15, 1993)

Br. J. Pharmacol. (1993), **109**, 925–932 © Macmillan Press Ltd, 1993

Effects of rubidium on responses to potassium channel openers in rat isolated aorta

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1 In a physiological salt solution (PSS) in which potassium (K) was replaced by rubidium (Rb), segments of rat aorta precontracted with 20 mM RbCl were fully relaxed by K-channel openers with an order of potency levcromakalim > cromakalim > aprikalim > RP 49356. These relaxations were inhibited by glibenclamide.

2 Segments of rat aorta bathed in normal PSS and precontracted with 20 mM KCl were also relaxed by these K-channel openers with an order of potency levcromakalim > cromakalim > aprikalim > RP 49356. These relaxations were glibenclamide-sensitive. However, the absolute potencies of the K-channel openers were approximately four times greater in normal PSS than in RbPSS.

3 In RbPSS, minoxidil sulphate relaxed segments of aorta precontracted with 20 mM RbCl by approximately 20% whereas in normal PSS it fully relaxed those contracted with 20 mM KCl.

4 In RbPSS, levcromakalim-induced relaxation of aortic segments precontracted with 20 mM RbCl was initially well-maintained but then faded by approximately 60% of the initial relaxation to a new, stable level. Subsequent exposure to RP 49356 or to higher concentrations of levcromakalim was without further relaxant effect. Similar changes were observed when RP 49356 was the initial relaxant and tissues were exposed to either RP 49356 or levcromakalim. In normal PSS, levcromakalim- or RP 49356-induced relaxation of contractions produced by 20 mM KCl was well-maintained.

5 In RbPSS, minoxidil sulphate-induced relaxation of aortic segments precontracted with 20 mM RbCl was well-maintained. Subsequent exposure to either levcromakalim or to RP 49356 produced further tissue relaxation.

6 In RbPSS, levcromakalim produced no detectable increase in either ^{86}Rb- or ^{42}K-efflux from rat aortic strips. In normal PSS, a significant increase in the exchange of both isotopes was detected.

7 Levcromakalim hyperpolarized segments of rat aorta bathed both in normal PSS and after depolarization by the addition of 20 mM KCl. Exposure to RbPSS depolarized the tissue and under these conditions, levcromakalim had no effect on membrane potential.

8 In Rb- and normal PSS, levcromakalim produced a similar degree of inhibition of the refilling of the noradrenaline-sensitive Ca store.

9 It is concluded that millimolar concentrations of Rb inhibit the plasmalemmal ATP-sensitive K-channels (K_{ATP}) which are the target of the K-channel openers. The relaxant actions of the K-channel openers in both normal and Rb-PSS and the inhibition of these effects by glibenclamide may reflect a functional interaction between these agents at ATP-binding sites associated with both K_{ATP} and with intracellular structures including Ca stores.

Keywords: ATP-sensitive K-channels; cromakalim; levcromakalim; RP 49356; aprikalim; minoxidil sulphate; rubidium; vascular smooth muscle; ^{42}K-efflux

Introduction

^{86}Rb has frequently been used as a marker to indicate changes in membrane potassium (K) permeability in studies on the mode of action of the K-channel opening vasorelaxant drugs. However, the use of this isotope may not always completely reflect the full extent of K-permeability changes in the cell under investigation. For example, patch clamp studies have shown that Ca-activated K-channels in mouse exocrine acinar cells (Gallacher *et al.*, 1984) and ATP-sensitive channels in rat pancreas (Ashcroft *et al.*, 1989) can discriminate between K^+ and Rb^+. Furthermore, in studies on smooth muscle tissues, the basal exchange of ^{86}Rb is approximately 80% of the ^{42}K basal efflux (Smith *et al.*, 1986; Bray & Weston, 1989).

Recent reports have suggested that the K-channels opened by K-channel openers such as cromakalim, pinacidil or RP 49356 can also distinguish between K^+ and Rb^+ ions. For example, relatively low concentrations of cromakalim induced a greater ^{42}K efflux compared with identical experiments in which ^{86}Rb was employed (Quast & Baumlin, 1988; Longmore *et al.*, 1991). Furthermore, Newgreen *et al.* (1990) reported that minoxidil sulphate could increase ^{42}K efflux from the rat aorta and portal vein, with no detectable effect on ^{86}Rb exchange. In addition, the magnitude of the ratio of ^{42}K:^{86}Rb efflux in the presence of cromakalim was found to be dependent on the cromakalim concentration (Quast & Baumlin, 1988). A similar result was also obtained for the action of pinacidil in the rat mesenteric artery (Videbaek *et al.*, 1988) and for cromakalim in the rabbit aorta (Bray & Weston, 1989). Such observations have led to the suggestion that K-channel openers like cromakalim and pinacidil open both Rb-permeable and Rb-impermeable K-channels, while the action of minoxidil sulphate is confined to Rb-impermeable K-channels (Newgreen *et al.*, 1990).

In addition to these quantitative differences between ^{42}K and ^{86}Rb efflux, several workers have shown that Rb itself may modulate the action of K-channel openers. For example, in dual isotope (^{42}K/^{86}Rb) experiments, Edwards & Weston (1989) reported that the ^{42}K efflux rate following exposure of

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rat portal vein to cromakalim was 15% lower than that obtained in the presence of ^{42}K alone. Furthermore, Foster *et al.* (1989) showed that cromakalim-induced inhibition of spontaneous activity in the guinea-pig urinary bladder was progressively attenuated when the tissue was bathed in Rb-containing PSS. A similar effect was also observed during a study of the ability of cromakalim to relax spontaneous- or histamine- induced tone in guinea-pig trachea (Morris & Taylor, 1989) and in a parallel investigation, the effects of cromakalim on $^{42}K/^{43}K$ efflux were completely blocked by millimolar concentrations of Rb (Foster, 1989).

The aim of the present investigation was to investigate the effects of cromakalim, levcromakalim, aprikalim, RP 49356 and minoxidil sulphate on rat aorta exposed to Rb-containing PSS. By use of this approach it was hoped to gain further insight into the mode of action of these agents by clarifying whether two types of K-channel were involved in the K-channel opener-induced relaxations.

Methods

Experiments were carried out on whole portal veins and segments of thoracic aorta isolated from male Sprague-Dawley rats (250–400 g) which were killed by stunning and exsanguination. All animals were supplied by the University of Manchester Biological Services Unit.

Tissue bath experiments

Tissue preparation and dosing protocols Each segment of aorta was cut into four rings approximately 0.5 cm long. Each ring was cut along its longitudinal axis and opened out to form a sheet. The endothelium was removed by rubbing the intimal surface with a cotton bud moistened with physiological salt solution (PSS). Threads attached to small pins were inserted through each longitudinally-cut edge and the preparation was mounted for isometric tension recording under a tension of 9.8 mN in a tissue bath containing PSS at 37°C and bubbled with 95% O_2/5% CO_2. Changes in isometric tension were recorded on a Rikadenki Flat-bed recorder. The tissue was washed with PSS every 15 min during a 60 min equilibration period.

The effect of PSS in which all K^+ had been replaced by Rb^+ (RbPSS) on the relaxant action of cromakalim, levcromakalim, RP 49356, aprikalim, minoxidil sulphate and nifedipine was tested on paired tissues. After an initial challenge with 20 mM KCl to establish tissue responsiveness, the PSS bathing the tissues was replaced with RbPSS with washes every 5 min for a 30 min period. RbCl (20 mM) was then applied to these tissues in RbPSS and when the increase in tension had reached a maximum, the effects of cromakalim, levcromakalim, RP 49356, aprikalim or minoxidil sulphate on the tone induced by RbCl (20 mM) in RbPSS were evaluated by use of a 5 min cumulative protocol. Parallel experiments were carried out in normal PSS using 20 mM KCl as the spasmogen. In some experiments, tissues bathed with normal PSS or RbPSS were pre-contracted with 100 nM noradrenaline and levcromakalim was then applied in increasing concentrations with use of a 5 min cumulative protocol.

Tachyphylaxis to relaxant responses in RbPSS In RbPSS, the ability of levcromakalim and RP 49356 to relax a RbCl (20 mM) contraction in rat aorta often exhibited fade. This was characterized by the partial reversal of the inhibitory response in the continued presence of levcromakalim or RP 49356. To characterize this phenomenon in the rat aorta, tissues bathed in RbPSS were precontracted with RbCl (20 mM) and then exposed to levcromakalim (1 μM), RP 49356 (5 μM) or minoxidil sulphate (10 μM). After 20 min when any fade of the inhibitory response had occurred, tissues were then exposed to increasing concentrations of either levcromakalim or RP 49356 with a 5 min cumulative protocol in the continuing presence of either levcromakalim (1 μM), RP 49356 (5 μM) or minoxidil sulphate (10 μM) Control tissues precontracted with RbCl (20 mM) were exposed to the appropriate vehicle for 20 min.

Effects of levcromakalim on the refilling of Ca stores In initial experiments, exposure of segments of rat aorta to either KCl 20 mM in Ca-free PSS or to RbCl, 20 mM, in Ca-free Rb-PSS failed to evoke a contraction. This presumably reflected the fact that such depolarizing stimuli evoke tension via a combination of Ca influx through the plasmalemma and subsequent Ca-induced Ca release (see Ganitkevich & Isenberg, 1992). Thus, to assess any effects of the K-channel openers on Ca stores, noradrenaline was used as the stimulus to release stored Ca and the protocol used was adapted from that described by Bray *et al.* (1991) for rabbit aorta. Briefly, strips of aorta were incubated in PSS (control group) or RbPSS (test group) for 1 h and were then contracted with 1 μM noradrenaline for 8 min. Exposure to noradrenaline was repeated until mechanical responses had stabilized after which the tissue was washed in normal PSS or RbPSS (control and test groups, respectively) to remove any noradrenaline and to refill noradrenaline-sensitive Ca stores. Tissues were then incubated in Ca-free normal PSS or Ca-free RbPSS containing EGTA for 15 min to remove extracellular Ca. The preparation was then contracted with noradrenaline (1 μM) for 8 min to deplete noradrenaline-sensitive Ca stores. The tissue was subsequently washed in Ca-free normal PSS or Ca-free RbPSS for 10 min to remove any noradrenaline and then exposed to Ca-containing normal PSS or Ca-containing RbPSS for 25 min to allow the refilling of noradrenaline-sensitive Ca stores.

The above procedure was repeated with levcromakalim (1 μM) included in the normal PSS and RbPSS (control and test groups, respectively) during the Ca-store refilling period. After a series of washes in EGTA-containing Ca-free normal PSS or Ca-free RbPSS to remove extracellular Ca and levcromakalim, the tissue was exposed to noradrenaline (1 μM) for 8 min. The magnitude of the resulting contraction was taken to indicate the extent to which levcromakalim had modified store refilling. The preparation was then washed several times in Ca-free normal PSS or Ca-free RbPSS for 10 min to remove noradrenaline and levcromakalim. Subsequently, tissue Ca stores were replenished by exposure to Ca-containing normal PSS and Ca-containing RbPSS for 25 min.

Results were calculated with the response to noradrenaline after refilling for 25 min in the presence of levcromakalim (1 μM) expressed as a percentage of the response to noradrenaline (1 μM) generated by tissues with full Ca stores in Ca-free normal PSS or Ca-free RbPSS.

$^{42}K/^{86}Rb$ efflux experiments

Initial experiments Endothelium-free segments of rat aorta were prepared and mounted on syringe needles attached to a perspex gassing manifold. After a 10 min equilibration period in normal PSS, tissues were loaded with ^{86}Rb by incubation in PSS containing $^{86}RbCl$ (5 μCi ml^{-1} for 90 min). The efflux of ^{86}Rb from the aortic segments was assessed by transferring the tissues into 3 ml aliquots of normal PSS or RbPSS at 4 min intervals. After 3 such 4 min periods tissues were exposed to levcromakalim or aprikalim at various concentrations for 20 min followed by a 12 min washout period; the tissues were then blotted and their ^{86}Rb content was determined along with that of the PSS in the respective collecting tubes by γ-counting.

Experiments with ^{42}K in depolarizing solutions In a further series of experiments the tissues were loaded with ^{42}K by incubation in $^{42}K_2CO_3$ (1.57 μCi ml^{-1}) for 3 h. To assess the effect of levcromakalim and aprikalim on ^{42}K-efflux rate from

depolarized tissues, aortic strips were incubated in either KCl (25 mM)-containing normal PSS or RbCl (25 mM)-containing RbPSS in which the [Na^+] had been reduced to maintain osmolarity. Tissues were then exposed to either levcromakalim or aprikalim for 20 min in a similar manner to the initial experiments. Efflux data were expressed in terms of the rate coefficient (fractional loss of tracer from the tissue standardised for a 1 min period) expressed as a percentage.

Electrophysiological experiments

A segment of rat aorta approximately 1 cm in length was excised and cut along its longitudinal axis. The tissue was fixed with fine pins to the Sylgard floor of a thermostatically-controlled recording chamber through which normal PSS at 37°C was flowing at a rate of 5 ml min^{-1}. The vascular endothelium was removed by gentle rubbing with a cotton bud and the preparation was then allowed to equilibrate for 1 h. Glass microelectrodes (resistance 40–80 MΩ) were inserted into the smooth muscle cells and when a stable membrane potential of at least 1 min duration had been obtained, levcromakalim (1 μM) was added to the reservoir of PSS perfusing the chamber. Recordings were then made over at least a 10 min period after which the microelectrode was deliberately withdrawn and the resulting voltage change was taken as a measure of membrane potential immediately prior to withdrawal.

After washout of levcromakalim for 30 min, the superfusing medium was changed to RbPSS or to normal PSS containing added 20 mM KCl. After 30 min, the tissue was re-impaled with a microelectrode and after a suitable penetration had been achieved, levcromakalim (1 or 10 μM) was added to the PSS reservoir. A second recording of membrane potential was then made in the manner already described for normal PSS.

Drugs and solutions

The following substances were used: cromakalim and levcromakalim (SmithKline Beecham); RP 49356 ((±)-N-methyl-2-(3-pyridinyl)-tetrahydro-thiopyran-2-carbothioamide-1-oxide) and aprikalim (Rhône-Poulenc Rorer); minoxidil sulphate (ICI); RbCl (Sigma); nifedipine (Sigma) glibenclamide (Sigma) and noradrenaline (Sigma). $^{86}RbCl$ was obtained from Amersham and $^{42}K_2CO_3$ was obtained from the University of London. Stock solutions were made by dissolving cromakalim, levcromakalim, RP 49356 and aprikalim in 70% v/v ethanol. Dilutions were made each day in distilled water. Minoxidil sulphate was dissolved in 70% ethanol each day. Noradrenaline prepared as a stock in 0.1 N HCl containing a small amount of ascorbic acid was diluted each day to the requisite concentration. Nifedipine stock solution was prepared in 70% ethanol under sodium lamp illumination due to the light-sensitive nature of the compound.

The normal PSS had the following composition (mM): NaCl 117, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.6, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.1. In the RbPSS, KCl and KH_2PO_4 were omitted and replaced by RbCl 5.9 mM and NaH_2PO_4 1.2 mM.

Ca^{2+}-free PSS was produced by omitting $CaCl_2$ and $MgSO_4$ and adding 20 mM $MgCl_2$ while the [NaCl] was reduced to 82 mM to maintain osmolarity (Quast & Baumlin, 1991). EGTA (0.1 mM) was added to chelate any excess Ca and this was made as a 100 mM stock by initially dissolving the EGTA in NaOH (3 M) and making up to volume with distilled H_2O. The pH of the Ca-free PSS was then corrected to 7.4 with HCl or NaOH.

The depolarizing solutions used in the ^{42}K-efflux experiments were of an identical composition to those above but with the [Na^+] reduced by an amount equal to the increase in [K^+].

Analysis of data

Data are expressed as the mean of n observations ± the standard error of the mean (s.e.mean). Statistical comparisons were made by an unpaired Student's t test with significance identified at $P<0.05$. IC_{50} values were calculated for each experimental concentration-effect curve and expressed as the mean of n experiments together with the appropriate 95% confidence interval.

Results

Effects of RbPSS on short-term exposure to relaxants

Replacement of normal PSS with RbPSS produced a slowly-developing increase in the baseline tension (0.2–2 mN) of aortic segments over a period of 15 min. Subsequent addition of 20 mM RbCl produced a further contraction of magnitude comparable to that seen in normal PSS after exposure to 20 mM KCl.

After precontraction with either 20 mM KCl (normal PSS) or 20 mM RbCl (RbPSS), relatively short-term (up to 15 min) exposure to levcromakalim, cromakalim, RP 49356 and aprikalim, typical of concentration-effect experiments using a cumulative protocol, produced complete relaxation of aortic segments (Figure 1). Although the *potency ratios* of the K-channel openers was not changed in RbPSS compared with those in normal PSS (levcromakalim > cromakalim > aprikalim > RP 49356; ratios: 1:1.2:2:2.7 respectively), the *absolute potencies* of all these agents were reduced approximately 4 fold in RbPSS (Table 1). Levcromakalim-induced

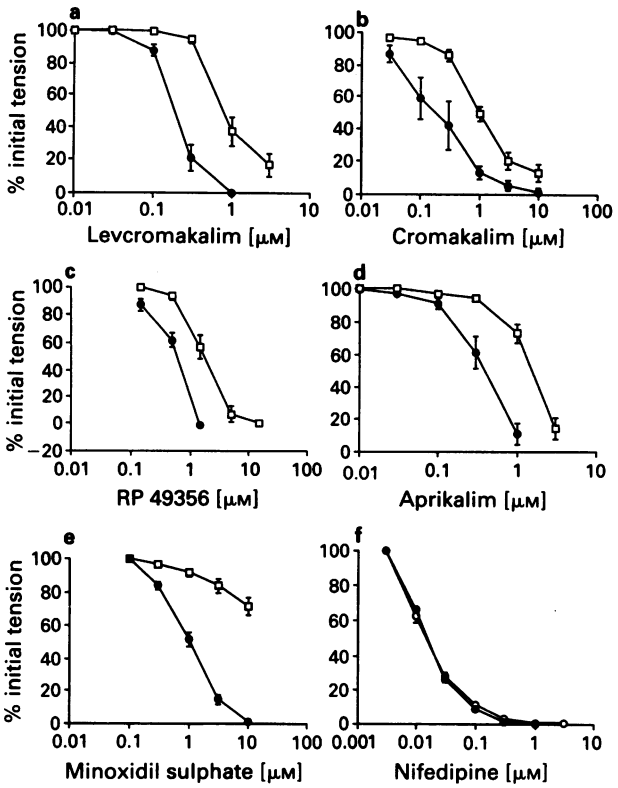


Figure 1 Relaxant effects following short-term exposure to (a) levcromakalim, (b) cromakalim, (c) RP 49356, (d) aprikalim, (e) minoxidil sulphate and (f) nifedipine. A cumulative concentration-response protocol was used and segments of rat aorta were pre-contracted with either 20 mM KCl in normal PSS (●) or 20 mM RbCl in RbPSS (□). Ordinate scale: % initial contraction produced by KCl (20 mM) of RbCl (20 mM) each defined arbitrarily as 100%; abscissa scale; concentration of relaxant on a logarithmic scale. Each point represents the mean ± s.e.mean, $n = 6–8$.

Table 1 Comparison of EC_{50} values for a series of K-channel openers and nifedipine in normal and RbPSS

Relaxant	*Normal PSS* mean EC_{50} (μM)	n	*RbPSS* mean EC_{50} (μM)	n
Levcromakalim	0.223 (0.17–0.28)	8	0.645 (0.51–0.81)	6
Cromakalim	0.123 (0.078–0.19)	5	0.79 (0.57–0.94)	5
RP 49356	0.52 (0.43–0.65)	5	1.7 (1.2–2.3)	8
Aprikalim	0.35 (0.23–0.51)	7	1.28 (1.09–1.51)	7
Minoxidil sulphate	1.07 (0.75–1.54)	8	4.4* (2.7–8.5)	8
Nifedipine	0.042 (0.033–0.057)	5	0.056 (0.051–0.062)	6

The values shown are the mean concentrations (with 95% confidence intervals in parentheses) necessary to produce 50% relaxation of the contraction produced by addition of 20 mM KCl to normal PSS or by addition of 20 mM RbCl to RbPSS as appropriate. *Concentration required to produce 20% relaxation of the contraction produced by addition of 20 mM RbCl to RbPSS.

relaxation of noradrenaline-contracted rat aorta was also similarly inhibited in RbPSS, a phenomenon previously reported for cromakalim by Bray & Quast (1991; data not shown).

The ability of glibenclamide to inhibit K-channel opener-induced relaxations of tissues pre-contracted with 20 mM KCl (normal PSS) or 20 mM RbCl (Rb-PSS) were examined by use of levcromakalim in cumulative concentration-effect experiments. Glibenclamide (0.1–1 μM) shifted the levcromakalim concentration-effect curves in both normal and Rb-PSS to the right in a concentration-dependent manner. The ratio of the levcromakalim mean EC_{50} value in normal and Rb-PSS was not changed in the presence of glibenclamide ($P > 0.05$, $n = 6$).

Although minoxidil sulphate fully reversed a 20 mM KCl-induced contraction in normal PSS, this agent could only relax rat aortic strips precontracted with 20 mM RbCl in RbPSS by approximately 20% (Figure 1e and Table 1). In contrast to the potassium channel openers, nifedipine was able to relax fully aortic strips precontracted with 20 mM KCl (normal PSS) or 20 mM RbCl (RbPSS) with no change in potency (Figure 1f; Table 1).

Effects of RbPSS on long-term exposure to relaxants

In normal PSS with added 20 mM KCl, the relaxations produced by all the tested K-channel openers were maintained in the continuing presence of these agents. However, in Rb-PSS with added 20 mM RbCl, the relaxations generated by levcromakalim or RP49356 were not maintained in the continuing presence of these openers (Figure 2). For example, the initial maximal relaxation produced by 1 μM levcromakalim was 64 ± 8% (of the response to 20 mM RbCl) which after 15–20 min faded to 34 ± 6% in the continuing presence of levcromakalim ($n = 13$). Such fade to a reduced level of relaxation was often accompanied by phasic tension changes, or was characterized by rhythmical oscillations in the level of tone which were also accompanied by phasic changes in

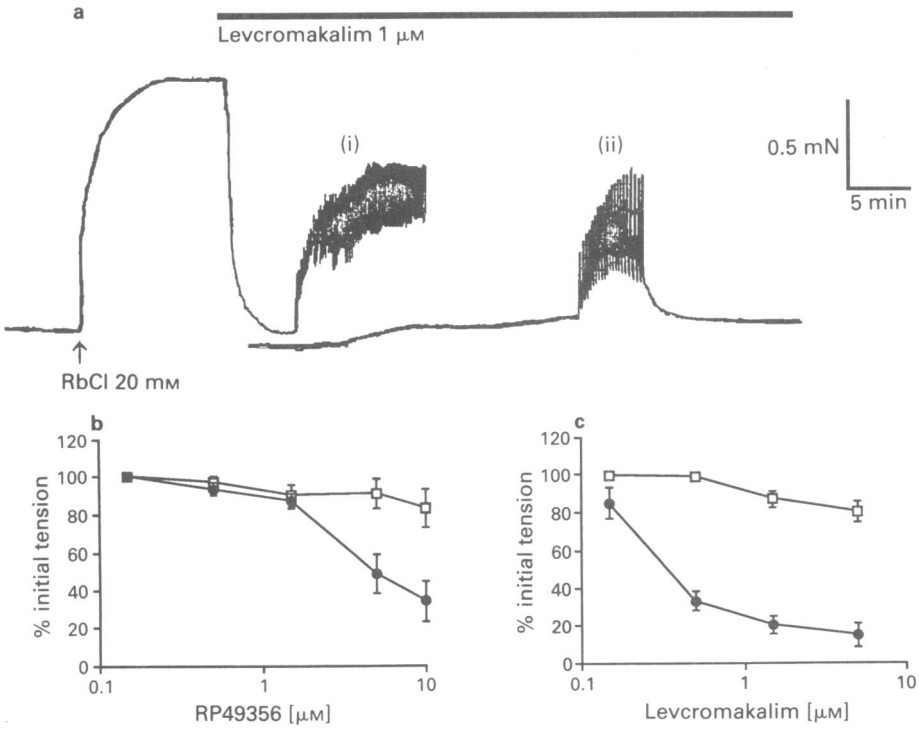


Figure 2 Long-term exposure to K-channel openers in Rb-PSS and the development of fade and tachyphylaxis. (a) Tension traces from individual tissues showing generation of the tone produced by addition of RbCl (20 mM), the inhibition of this by levcromakalim (1 μM) (horizontal bar) and the subsequent fade of the inhibition in the continuing presence of levcromakalim. The tension faded either to a relatively constant level with superimposed phasic changes (i) or exhibited rhythmic periods of fade (ii). In trace (ii), the initial exposure to 20 mM RbCl and relaxation by levcromakalim have been omitted for clarity. Similar effects were observed with 5 μM RP 49356. (b and c) When fade to levcromakalim (1 μM) or to RP 49356 (5 μM) of the type shown in (i) had developed, additional exposure to increasing concentrations of RP 49356 (b) or levcromakalim (c) had little relaxant effect (□), whereas in control tissues contracted with RbCl (20 mM) and then initially exposed to (b) levcromakalim- or (c) RP 49356-vehicle; RP 49356 (b) and levcromakalim (c) each produced a concentration-dependent relaxation (●). Ordinate scales % initial tension after exposure to vehicle (control) or after fade of the relaxant response to the initial challenge with the K-channel opener, each expressed arbitrarily as 100%. Points show mean values with ± s.e.mean, ($n = 6$).

tension (see Figure 2). In comparison, the relatively small relaxations of aorta precontracted with 20 mM RbCl in RbPSS by minoxidil sulphate were well-maintained on long-term exposure to this agent (data not shown).

The tone (relaxation fade) which developed in the continued presence of a maximally-effective concentration of levcromakalim (1 μM) in RbPSS could not be relaxed by increasing the concentration of levcromakalim and a similar tachyphylaxis was also observed in the parallel experiment using RP 49356 (Figure 2). Furthermore, cross-tachyphylaxis also occurred in that the tone which developed in the continuing presence of levcromakalim or RP 49356 could not be relaxed by additional exposure to either RP 49356 or levcromakalim, respectively (Figure 2). In RbPSS, pretreatment with the vehicle for either RP 49356 or levcromakalim had no effect on relaxations produced by cumulative application of levcromakalim or RP 49356, respectively.

In contrast, cumulative addition of either levcromakalim and RP 49356 (each 1 μM) relaxed the tone which remained after exposure of tissues to a maximally-effective concentration of minoxidil sulphate (10 μM) in RbPSS (data not shown).

Efflux studies

Initial studies were performed using ^{86}Rb as a marker of K-permeability with segments of aorta bathed in normal PSS or RbPSS. Both levcromakalim (5 μM) and aprikalim (5 μM) each produced an increase in efflux rate coefficient in normal PSS (1.4 ± 0.26 and 1.26 ± 0.15 fold $n = 5$, respectively) but neither agent could induce an increase in efflux rate from tissues bathed in RbPSS. The basal efflux rate from tissues bathed in RbPSS was 97 ± 0.07% ($n = 5$) greater than the basal efflux from tissues incubated in normal PSS.

A second series of experiments was then performed on aortic segments incubated in either 25.9 mM KCl-containing normal PSS or 25.9 mM RbCl-containing RbPSS in an attempt to mimic the conditions of the tissue bath experiments. In 25.9 mM KCl-containing normal PSS, levcromakalim (5 μM) produced a large increase in the ^{86}Rb efflux rate coefficient (4.38 ± 0.62 fold) within 5 min of the exposure to the drug (Figure 3a). In contrast levcromakalim (5 μM) did not induce any detectable increase in efflux rate coefficient compared to the vehicle from tissues bathed in 25.9 mM RbCl-containing RbPSS (Figure 3c). The basal ^{86}Rb efflux rate from tissues incubated in 25.9 mM RbCl-containing RbPSS was 37 ± 0.5% greater than the basal efflux from tissues incubated in 25.9 mM KCl-containing normal PSS.

To establish whether the lack of levcromakalim-induced increase in ^{86}Rb efflux rate in RbPSS was due to the opening of Rb-impermeable K-channels, aortic segments were incubated in normal PSS containing ^{42}K. Under these conditions, 10 μM levcromakalim induced a large and rapid increase in ^{42}K efflux rate coefficient (6.2 ± 0.6 fold, Figure 3b) from rat aortic strips bathed in 25.9 mM KCl-containing normal PSS which was greater than the increase induced by 1 μM levcromakalim (data not shown). In comparison, 10 μM levcromakalim failed to increase the ^{42}K-efflux rate coefficient when the tissues were incubated in 25.9 mM RbCl-containing RbPSS (Figure 3d). The basal efflux of ^{42}K from tissues incubated in 25.9 mM RbCl-containing RbPSS was 43 ± 0.4% higher compared with tissues incubated in 25.9 mM KCl-containing normal PSS.

Effects of levcromakalim on the refilling of Ca stores

Exposure of rat aortic strips incubated in Ca-free PSS to noradrenaline produced an initial, transient peak contraction which was followed by a maintained plateau phase (Figure 4). These changes in normal Ca-free PSS were not significantly different in magnitude from those produced in Ca-free RbPSS ($P > 0.05$). Thus, in seven experiments the

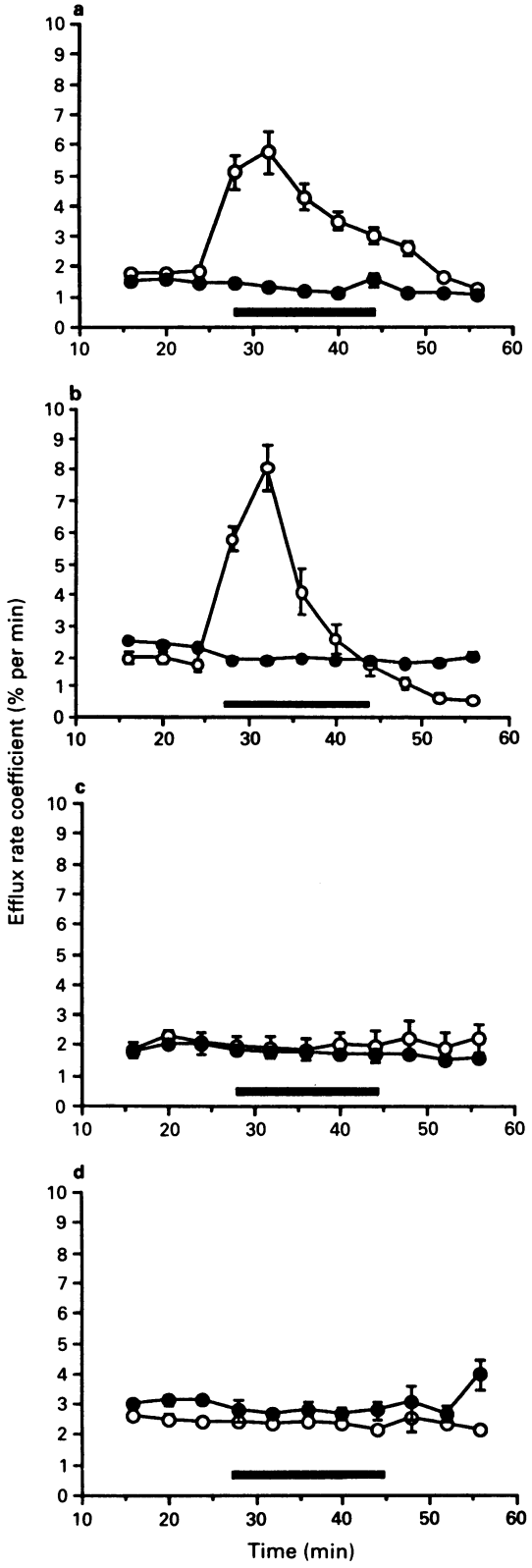


Figure 3 Changes in (a,c) ^{86}Rb- and (b,d) ^{42}K-efflux from rat aortic segments produced by (a) levcromakalim (5 μM) and (b) levcromakalim (10 μM) in normal PSS containing 25.9 mM KCl, and (c) levcromakalim (5 μM) and (d) levcromakalim (10 μM) in RbPSS containing 25.9 mM RbCl. Tissues were exposed to levcromakalim (○) or vehicle (●) during the period denoted by the horizontal bar. Ordinate scales: ^{86}Rb- or ^{42}K-efflux coefficient (% per min); abscissa scales; time (min) after the start of the efflux period. Each point represents the mean ± s.e.mean, $n = 5$.

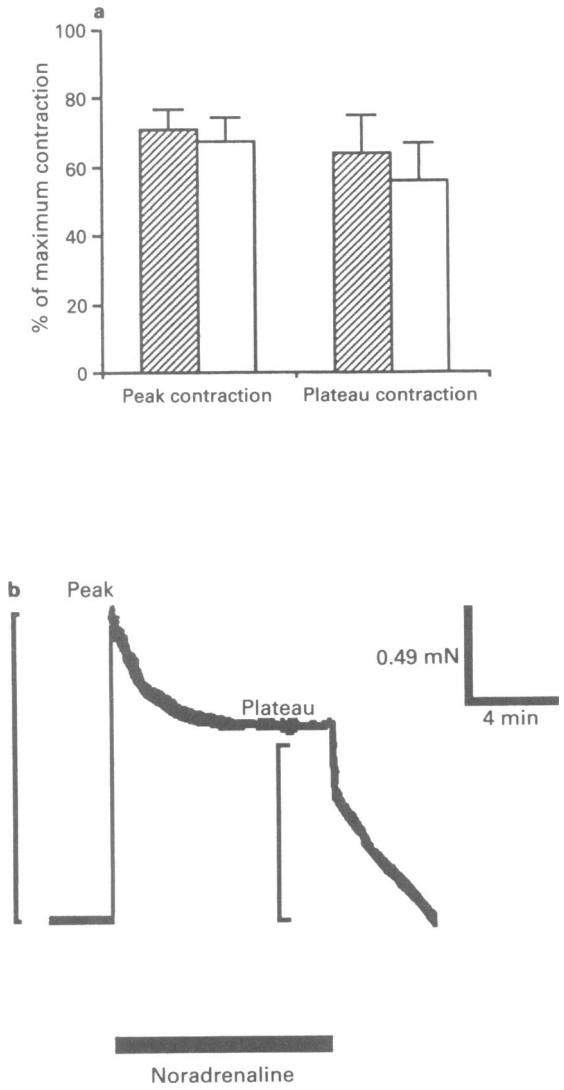


Figure 4 The effect of levcromakalim (1 μM) on the refilling of noradrenaline-sensitive Ca stores in rat aortic strips. (a) Comparison of effects of levcromakalim (1 μM) in Ca-free RbPSS (hatched columns) and in Ca-free normal PSS (open columns); (b) typical mechanical response in Ca-free PSS following exposure to noradrenaline (1 μM) showing the peak and plateau phases of contraction. Ordinate scale: % maximum contraction obtained in Ca-free PSS or Ca-free RbPSS; s.e.mean of 9 observations per group shown.

maximal contraction in Ca-free RbPSS was 1.65 ± 0.37 mN compared to 1.01 ± 0.15 mN, whilst the respective plateau phases were 0.83 ± 0.19 mN (Ca-free RbPSS) and 0.54 ± 0.12 mN. In rabbit aorta, Bray *et al.* (1991) reported that refilling of noradrenaline-sensitive stores was maximal after 25 min in Ca-containing PSS. In this study it was also found that exposure of the tissue to Ca-containing normal PSS or RbPSS for 25 min was also sufficient to refill calcium stores completely as indicated by the size of subsequent mechanical changes induced by 1 μM noradrenaline in either Ca-free RbPSS or Ca-free normal PSS.

Application of 1 μM levcromakalim during the refilling phase significantly inhibited both the initial peak and plateau phases of the contraction produced by 1 μM noradrenaline in Ca-free PSS (Figure 4). Thus, the mean peak contraction in normal Ca-free PSS following Ca-store refilling in the presence of 1 μM levcromakalim was 67.25 ± 7.4% of the control contraction, whilst the plateau phase was 56 ± 11% of the control plateau phase ($n = 9$ for each). Incubation of the tissue in RbPSS did not modify the ability of levcromakalim to inhibit the refilling of noradrenaline-sensitive stores. Noradrenaline applied to tissues bathed in Ca-free RbPSS following a refilling period in the presence of 1 μM levcromakalim produced a peak contraction which was 71 ± 6% of the control contraction, whilst the plateau phase was 64 ± 11% of the control plateau ($n = 9$ for each).

Microelectrode experiments

In normal PSS, the mean resting membrane potential of rat aorta smooth muscle cells was − 53 ± 2 mV ($n = 9$ preparations). After the initial equilibration period, the membrane potential remained stable and no fluctuations were observed. On exposure to levcromakalim (1 μM) a slowly-developing hyperpolarization was observed and this reached a mean maximum value of 16 ± 2 mV, (n = 9) in 6–8 min. After at least 30 min in either normal PSS containing added 20 mM KCl or in RbPSS, the mean basal membrane potential was − 40 ± 4 mV ($n = 6$) and − 41.5 ± 3 mV ($n = 4$), respectively. Addition of levcromakalim (1 μM) to the KCl-rich PSS superfusing the tissue produced a hyperpolarization of 9 ± 3 mV ($n = 6$). In Rb-PSS, however, essentially no change in membrane potential was seen after addition of levcromakalim, 10 μM (a maximal mean depolarization of 0.3 ± 1.5 mV, measured 10–15 min after exposure to levcromakalim, $n = 4$).

Discussion

K-channel opener-induced relaxations in RbPSS: lack of involvement of K-channels

In Rb-PSS, levcromakalim-induced relaxations of a RbCl (20 mM) contraction could be divided into two phases. The first of these consisted of a fairly rapid relaxation which was maintained for approximately 5 min. This was followed by a second phase during which the initial relaxation partially faded to a new level, often characterized by phasic mechanical fluctuations. No opener-induced ^{42}K- or ^{86}Rb-efflux could be detected even from tissues bathed in RbPSS containing 25.9 mM RbCl, experimental conditions which exactly mimicked those in which mechanical changes were measured. Furthermore, levcromakalim failed to hyperpolarize the tissue in RbPSS.

Thus, the present study does not support the view that a Rb-*permeable* K-channel is responsible for the relaxant effects of the K-channel openers in RbPSS (Morris & Taylor, 1989). Instead the data are consistent with those of two earlier brief reports (Foster, 1989; Bray & Quast, 1991). They strongly suggest that Rb (in millimolar concentrations) is a K-channel inhibitor and that neither of the relaxant phases associated with exposure to levcromakalim, cromakalim or aprikalim in Rb-PSS involves the opening of *plasmalemmal* K-channels. This inhibitory action of Rb does not seem to extend to micromolar concentrations of this ion. The finding of many previous workers that ^{86}Rb can be used as a tracer for K in studies of the K channel openers shows that low concentrations of Rb (typically 50 μM in Newgreen *et al.*, 1990) exert relatively little block of the K_{ATP} which is opened by the K-channel openers.

No inhibitory effect of Rb on the ATP-sensitive K-channels (K_{ATP}) which are one of the targets for the K-channel openers in vascular smooth muscle cells (Noack *et al.*, 1992a,b) has been reported. However, millimolar concentrations of Rb have been reported to block Ca-activated K-channels in secretory cells (Gallacher *et al.*, 1984), inwardly-rectifying K-channels in skeletal muscle cells (Standen & Stanfield, 1980) and K_{ATP} in pancreatic β-cells (Ashcroft *et al.*, 1989). Glibenclamide failed to contract the aorta in normal PSS (unpublished observations) from which it can be concluded that K_{ATP} channels in this tissue are probably closed under these conditions. Thus the contraction

seen on bathing tissues in Rb-PSS is likely to reflect the closure of K-channels other than K_{ATP}.

Fade and tachyphylaxis of K-channel opener-induced relaxations in Rb-PSS

In Rb-PSS, the relaxations produced by levcromakalim faded and under these conditions, the tissue became tachyphylactic to the effects of subsequent exposure to this agent or to RP49356. Similar effects were observed when RP 49356 was the initial relaxant with cross-tachyphylaxis to levcromakalim. In contrast, the relatively weak relaxations produced by minoxidil sulphate in Rb-PSS did not fade and in the continuing presence of this agent, both levcromakalim and RP 49356 were able to relax the tissue further.

These results could indicate that two basic mechanisms are involved in the relaxations produced by the K-channel openers in Rb-PSS. One of these would then underlie both the response to minoxidil sulphate and the reduced level of relaxation associated with prolonged exposure to the other openers. The second putative mechanism (which exhibits fade), would also be activated by levcromakalim and RP 49356, but not by minoxidil sulphate. Alternatively, minoxidil sulphate could be an agonist of low efficacy relative to that of levcromakalim and RP 49356. All three agents could then relax via the same mechanism which for some reason exhibits fade when maximally stimulated (by levcromakalim or RP 49356). Experiments are in progress to test these alternatives.

Intracellular Ca stores at a target for the K-channel openers

In guinea-pig aorta, Ito *et al.* (1991a) have shown that the addition of Ca^{2+} (0.1–2.5 mM) to a Ca-free depolarizing (KCl-rich) PSS induces a contraction, part of which is sensitive to ryanodine and requires full intracellular Ca-stores for maximum force development. Furthermore, Ganitkevitch & Isenberg (1992) have reported that the Ca-transient associated with depolarization of guinea-pig bladder myocytes results from both Ca-influx and from Ca-induced intracellular Ca-release. It thus seems reasonable to suggest that the RbCl-induced contractions of rat aorta elicited in the present experiments in Rb-PSS probably involve Ca release from internal stores. Thus, in the absence of any increase in ^{42}K efflux and membrane hyperpolarization, at least part of the K-channel opener-induced relaxation in Rb-PSS (present study) could be produced by inhibition of Ca-store refilling.

Consistent with this suggestion, levcromakalim produced an approximately 30% inhibition of agonist (noradrenaline)-sensitive calcium store refilling in RbPSS. Previously, Bray *et al.* (1991) have reported that cromakalim produces some inhibition of the noradrenaline-induced release of calcium from rabbit aorta in calcium-free (normal) PSS with a more substantial inhibitory effect on calcium store refilling. Cromakalim-induced interference with noradrenaline-mediated Ca mobilization in rat mesenteric beds was also described by Quast & Baumlin (1991). In addition, levcromakalim inhibits the loading of ^{45}Ca into inositol trisphosphate ($InsP_3$)-sensitive calcium stores in rabbit cultured airways smooth muscle (Chopra *et al.*, 1992) and antagonizes agonist-induced stimulation of $InsP_3$ formation (Ito *et al.*, 1991b; Challis *et al.*, 1992; Itoh *et al.*, 1992). Pinacidil and levcromakalim also reduce the noradrenaline-induced increase in $[Ca^{2+}]_i$ as determined by fura-2 epifluorescence techniques (Ito *et al.*, 1991b; Criddle *et al.*, 1992; Itoh *et al.*, 1992).

From the above it is clear that the K-channel openers can exert intracellular effects which directly or indirectly inhibit agonist-modulated intracellular Ca^{2+} release and sequestration. If such effects do make an important contribution to opener-induced relaxations, it could be argued that these agents should also be able to inhibit contractions produced by depolarization, a stimulus which induces Ca-induced Ca-release (Ito *et al.*, 1991a; Ganitkevitch & Isenberg, 1992). However, a diagnostic feature of the K-channel openers is their *inability* to relax depolarization-induced contractions produced by KCl (80 mM)-rich PSS.

A definitive explanation for this paradox cannot be given at present. However, it is known that KCl-induced contractions of smooth muscle are associated with a larger increase in $[Ca^{2+}]_i$ than tension changes of a comparable size generated by noradrenaline due to the ability of the adrenoceptor agonist to sensitize the contractile apparatus to $[Ca^{2+}]_i$ (Yanagisawa *et al.* 1989). Thus, the relatively massive increase in $[Ca^{2+}]_i$ *via* Ca^{2+} influx following exposure to 80 mM KCl may render ineffectual any inhibitory effects of the openers at Ca stores. Alternatively, additional mechanisms may be involved in the powerful relaxations produced by these agents in Rb-PSS.

The design of the present experiments did not allow any correlation to be established between the different phases of K-channel opener-induced relaxation in Rb-PSS or of the tachyphylaxis to these agents and any possible effects on Ca stores. These complex questions are currently under investigation.

Involvement of intracellular K-channels

Studies on internal Ca stores in skeletal muscle have shown that a countercurrent flow of K^+ into the store maintains electroneutrality during Ca release (Muallem *et al.*, 1985; Fink & Stephenson, 1987; Shah & Pant, 1988). Thus, the opening of such intracellular K-channels by the K-channel openers could modify the functioning of the store, an action which would be consistent with some of the affects of these agents in normal PSS (Bray *et al.*, 1991; Chopra *et al.*, 1992). However, if such a glibenclamide-sensitive channel is a target for the K-channel openers, the results of the present study in Rb-PSS suggest that this channel must differ from its plasmalemmal equivalent due to its lack of sensitivity to Rb.

Based on studies in portal vein, Noack *et al.* (1992a,b) concluded that the plasmalemmal effects of the K-channel openers on K_{ATP} could be most easily explained if these agents effectively reduced the supply of ATP to the ATP-regulatory sites associated with this channel. Furthermore, it has been proposed that glibenclamide can enhance ATP production by shifting the glycolytic pathway in the direction of glucose breakdown (see Caro, 1990). Thus, the observed antagonism between glibenclamide and the K-channel openers in RbPSS (and in normal solutions) could result from functional interactions at ATP regulatory sites. If so, the ability of the openers to produce glibenclamide-sensitive relaxations without the involvement of the plasmalemma (in Rb-PSS) could reflect the *ATP-dependency* of these events rather than the necessary involvement of K-channels *per se*. In support of this view, recent studies have established that the inositol trisphosphate receptor contains ATP-binding sites (see Ferris & Synder, 1992). If such loci are similar to the ATP regulatory site on plasmalemmal K_{ATP} (Noack *et al.*, 1992b; Edwards & Weston, 1993), the apparent intracellular interactions between agents like levcromakalim and glibenclamide at such sites would be self-explanatory.

Conclusions

This present study has extended the initial observations of Foster *et al.* (1989). It has shown that exposure of the rat aorta to RbPSS inhibits the plasmalemmal actions of levcromakalim (as determined by increased ^{42}K efflux and membrane hyperpolarization) whilst the mechano-inhibitory effects of this agent, as well as those of cromakalim, RP 49356 and aprikalim are only slightly affected. This suggests that in normal PSS, agents such as levcromakalim do not relax the rat aorta solely by the opening of plasmalemmal

K-channels and that non-plasmalemmal actions are also involved. In comparison, minoxidil sulphate-induced relaxations of precontracted aortic segments are more markedly inhibited in RbPSS, suggesting that under normal conditions the openings of plasmalemmal K-channels plays a more important role in the spasmolytic effects of this compound.

Evidence from the present study, together with data from recent investigations (Noack *et al.*, 1992b; Ibbotson *et al.*, 1993) is combining to suggest that the K-channel openers do not exert their effects *directly* on plasmalemmal and intracellular K-channels. Instead, a modification of ATP-binding to sites associated with both K-channels and intracellular structures such as Ca stores could provide a unifying explanation for the apparently diverse effects of these agents. Further experiments to test this hypothesis are currently in progress.

I.A.G. was supported by an SERC Case award in conjunction with SmithKline Beecham. We acknowledge helpful discussions with Dr T.C. Hamilton (SKB).

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(Received November 9, 1992
Revised February 25, 1993
Accepted March 15, 1993)

Br. J. Pharmacol. (1993), **109**, 933–940 © Macmillan Press Ltd, 1993

A mechanism underlying dopamine D_1 and D_2 receptor-mediated inhibition of dopaminergic neurones in the ventral tegmental area *in vitro*

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1 An intracellular recording study was performed to elucidate the mechanism underlying D_1 and D_2 receptor-mediated inhibition of neuronal activities of dopaminergic neurones in the ventral tegmental area (VTA) using slice preparations of the rat brain.

2 VTA neurones were classified into type I and type II neurones according to the shape of the action potential, which correspond to dopaminergic and non-dopaminergic neurones, respectively.

3 Addition of dopamine (10 μM) and quinpirole (1–100 μM) to the bath hyperpolarized the membrane of the type I neurones concomitantly with an increase in membrane conductance and an inhibition of action potentials which occurred spontaneously and were elicited by depolarizing pulses applied to the cell. However, quinpirole (10 μM) had no effect on the threshold for action potentials induced by a depolarizing pulse.

4 These quinpirole (10 μM)-induced effects were antagonized by simultaneous application of domperidone (5 μM), a D_2 receptor antagonist.

5 The amplitude of quinpirole (10 μM)-induced hyperpolarization was decreased by increasing the potassium concentration in the perfusing fluid or simultaneous application of tetraethylammonium (10 μM).

6 SKF 38393 (10 or 100 μM), a D_1 receptor agonist, had no effect on the resting membrane potential or action potential firing induced by a depolarizing pulse applied to the cell. However, when SKF 38393 (10 μM) was applied simultaneously with quinpirole (10 μM), the threshold for action potential generation was elevated by 5–6 mV, although there was no enhancement of hyperpolarization induced by quinpirole.

7 The elevation of the threshold for action potentials induced by SKF 38393 in the presence of quinpirole was antagonized by simultaneous application of SCH 23390 (5 μM), a D_1 receptor antagonist.

8 Dopamine (10 μM), quinpirole (10 or 100 μM) and SKF 38393 (10 or 100 μM) had no effect on the resting membrane potential or spontaneously occurring action potentials in type II neurones.

9 These findings suggest that activation of dopamine D_2 receptors of dopaminergic neurones in the VTA increases potassium conductance, thereby hyperpolarizing the membrane and eventually inhibiting neuronal activities. They also suggest that simultaneous activation of both D_1 and D_2 receptors enhances the D_2 receptor-mediated inhibitory effects by elevation of the threshold for action potential generation.

Keywords: Ventral tegmental area; intracellular recording *in vitro*; D_1 receptor; D_2 receptor; SKF 38393; quinpirole

Introduction

The ventral tegmental area (VTA) is rich in dopaminergic-neurones, which mainly project to the nucleus accumbens (Acc). Our previous studies using a microiontophoretic technique *in vivo* have demonstrated that dopamine, OPC-4392, which is a dopamine autoreceptor agonist (Kiuchi *et al.*, 1988; Yasuda *et al.*, 1988; Sasa *et al.*, 1988), and talipexole, a dopamine D_2 agonist, produce a dopamine D_2 receptor-mediated inhibition of neuronal activity of dopaminergic VTA neurones, which are identified by antidromic stimulation of the Acc, and by the shape of the extracellularly recorded action potentials (Momiyama *et al.*, 1990; 1991). Recently, an *in vitro* study has demonstrated that dopamine produces suppression of spontaneous firing, sometimes with concomitant hyperpolarization of the membrane in dopaminergic VTA neurones (Mueller *et al.*, 1988). On the other hand, dopamine and quinpirole, a D_2 receptor agonist, have been reported to inhibit spontaneous action potential firing and hyperpolarize the membrane of dopaminergic neurones in the substantia nigra pars compacta *in vitro*, resulting from an increase in membrane potassium conductance (Lacey *et al.*, 1987; 1990). Therefore, a further intracellular recording study on a brain slice preparation was performed to elucidate the ionic mechanism underlying the D_2 receptor-mediated inhibition of dopaminergic VTA neurones.

Previous autoradiographic studies have suggested the existence of both D_1 and D_2 receptors in the VTA (Boyson *et al.*, 1986; Dawson *et al.*, 1986; 1988; Cortes *et al.*, 1989), although conflicting results regarding the existence of D_1 receptors in the VTA have been reported in mRNA hybridization and autoradiographic studies (Alter & Hauser, 1987; Dearry *et al.*, 1990; Sunahara *et al.*, 1990; Weiner *et al.*, 1991). Nevertheless, we have found in a microiontophoretic *in vivo* study that a D_1 receptor agonist alone has no effect but that the combination with D_2 receptor agonist potentiates the D_2 receptor-mediated inhibition of the dopaminergic VTA neurones (Momiyama *et al.*, unpublished). Therefore, the effects of activation of D_1 receptors on dopaminergic VTA neurones in the presence and absence of a D_2 receptor agonist were examined to determine the mechanism underlying the potentiation of D_2 receptor-mediated effects by a D_1 agonist.

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Methods

Male Wistar rats weighing 100–200 g were used. After decapitation, the block containing the midbrain obtained from the brain which had been quickly removed was trimmed in fresh artificial cerebrospinal fluid (ACSF) at 1–4°C. Then, the block was cut into approximately 400 μm-thick slices in the horizontal plane with a microslicer DTK-1000 (Dosaka E.M., Kyoto, Japan) or a Vibratome (Oxford Laboratories, U.S.A.). The horizontal level of the slice was determined under a microscope according to the atlas of Paxinos & Watson (1986), and then the slice containing the VTA was cut into pieces of about 8 mm^2. The procedure was completed within 30 min. After at least 1 h of incubation, one slice was transferred to a recording chamber (volume: approximately 0.5 ml) which was continuously perfused with ACSF at a flow rate of 1.6–2.0 ml min^{-1} warmed to 33–35°C by a Peltier thermoelectric unit with a miniature ceramic module (Nihon Unicon, Tokyo, Japan). ACSF had the following composition (in mM): NaCl 125, KCl 2.5, $MgSO_4$ 1.3, NaH_2PO_4 0.92, $CaCl_2$ 2.5, $NaHCO_3$ 26.2 and glucose 11.0.

The intracellular recording was made from the VTA neurones with a glass microelectrode filled with 3 M KCl and with an electrical resistance of 50–100 MΩ. The depolarizing or hyperpolarizing pulse current was applied using a bridge circuit. The electrical signals amplified were monitored on a digital oscilloscope (Nihon Kohden, VC-11) and a thermal array recorder (Nihon Kohden, RTA-1100M). At the same time, they were stored on an analog data recorder (Sony, A-65) for data analysis.

The stock drug solutions were as follows: 10 mM dopamine hydrochloride (Sigma), 10 mM SKF 38393 (2,3,4,5-tetrahydro-1-phenyl H-3-benzapine-7,8-diol hydrochloride; RBI, pH 6.0), 10 mM quinpirole (LY171555; RBI, pH 5.5), 5 mM domperidone (Kyowa Hakko; dissolved in 1% lactate) and 5 mM SCH 23390 ((R)-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1 H-3-benzazepin-7-ol hemimaleate; Schering Corp., dissolved in 0.3% tartrate, pH 4.0). Their final concentrations were obtained by diluting these stock solutions with the ACSF just before each experiment. Tetraethylammonium chloride (TEA; Wako Pure Chemical) was dissolved in the ACSF just before each experiment. The standard perfusion fluid was replaced by the drug containing fluid using a separate inflow system following the control recording, and exchange of the solution in the chamber was completed within 30–60 s. Sodium metabisulphite (Wako Pure Chemical), a dopamine antioxidant (50 μM) without effects on the binding of the dopamine agonist and antagonist to their receptors, was added to the solution containing dopamine to prevent oxidation.

Statistical significance of differences between the values before and during drug application was determined by a paired Student's t test. Further details of the experimental procedure are similar to those described elsewhere (Akaike et al., 1987; 1988; Ishihara et al., 1991).

Results

Electrophysiological properties of neurones in the VTA

Forty-five neurones, which had a relatively stable resting membrane potential (RMP) of greater than −45 mV, were intracellularly recorded. The forty-five neurones were examined in the following studies.

As our previous *in vivo* studies (Momiyama et al., 1990; 1991) demonstrated, VTA neurones were classified into two types according to the form of action potentials which occurred spontaneously or were elicited by depolarizing pulses applied to the cell through the recording electrode; type I neurones had action potentials with a long duration (> 5 ms), low spontaneous firing rate (1.7 ± 0.6 Hz, $n = 35$) and prominent after-hyperpolarization (AHP) following the action potentials, while type II neurones showed a short duration (< 5 ms) and high spontaneous firing rate (34.9 ± 6.5 Hz, $n = 10$). Twenty-six of the 35 type I neurones did not fire action potentials spontaneously. Figures 1a and b show the typical action potentials that were recorded intracellularly in type I and type II neurones, respectively. There was no significant difference in RMP between the type I and type II neurones (Table 1). However, input resistance of type I neurones (40.8 ± 1.2 MΩ, $n = 35$) was significantly ($P < 0.01$) higher than that of type II neurones (32.0 ± 1.1 MΩ, $n = 10$) (Table 1).

Effects of dopamine and quinpirole on RMP and input impedance

The effects of dopamine (10 μM) on RMP and input impedance were examined in 7 type I neurones. Addition of dopamine at a dose of 10 μM to the bath hyperpolarized the membrane (range 4–10 mV) in 6 out of 7 type I neurones (Figure 2a), although the RMP of the remaining one neurone remained unaffected. Five of the 6 type I neurones, the membrane of which was hyperpolarized by dopamine, were not spontaneously active, but the remaining one neurone showed spontaneous action potentials (rate: 2.0 s^{-1}) which were completely inhibited by dopamine, concomitantly with the appearance of the hyperpolarization of the membrane. The input impedance was decreased by dopamine (10 μM) concomitantly with the hyperpolarization of the membrane in all 4 neurones tested (Figure 2a).

The effects of quinpirole at concentrations of 1, 10 and 100 μM were examined in 4, 20, and 4 type I neurones, respectively. Addition of quinpirole to the bath hyperpolarized the membrane in a dose-dependent manner (Figure 3). The mean amplitude of hyperpolarization induced by

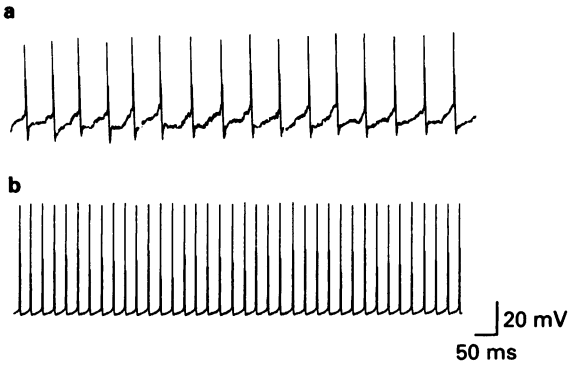


Figure 1 Spontaneously occurring action potentials of type I (a) and type II (b) neurones recorded intracellularly in the ventral tegmental area (VTA).

Table 1 Electrophysiological properties of type I and type II neurones in the ventral tegmental area (VTA)

	Type I neurone ($n = 35$)	*Type II neurone* ($n = 10$)
Resting membrane potential (mV)	− 52.5 ± 0.8	− 52.3 ± 1.2
Input resistance (MΩ)	40.8 ± 1.2*	32.0 ± 1.1
Spontaneous firing rate (s^{-1})	1.7 ± 0.6	34.9 ± 6.5

Each value represents the mean ± s.e.mean.
*$P < 0.01$, compared with Type II neurone.

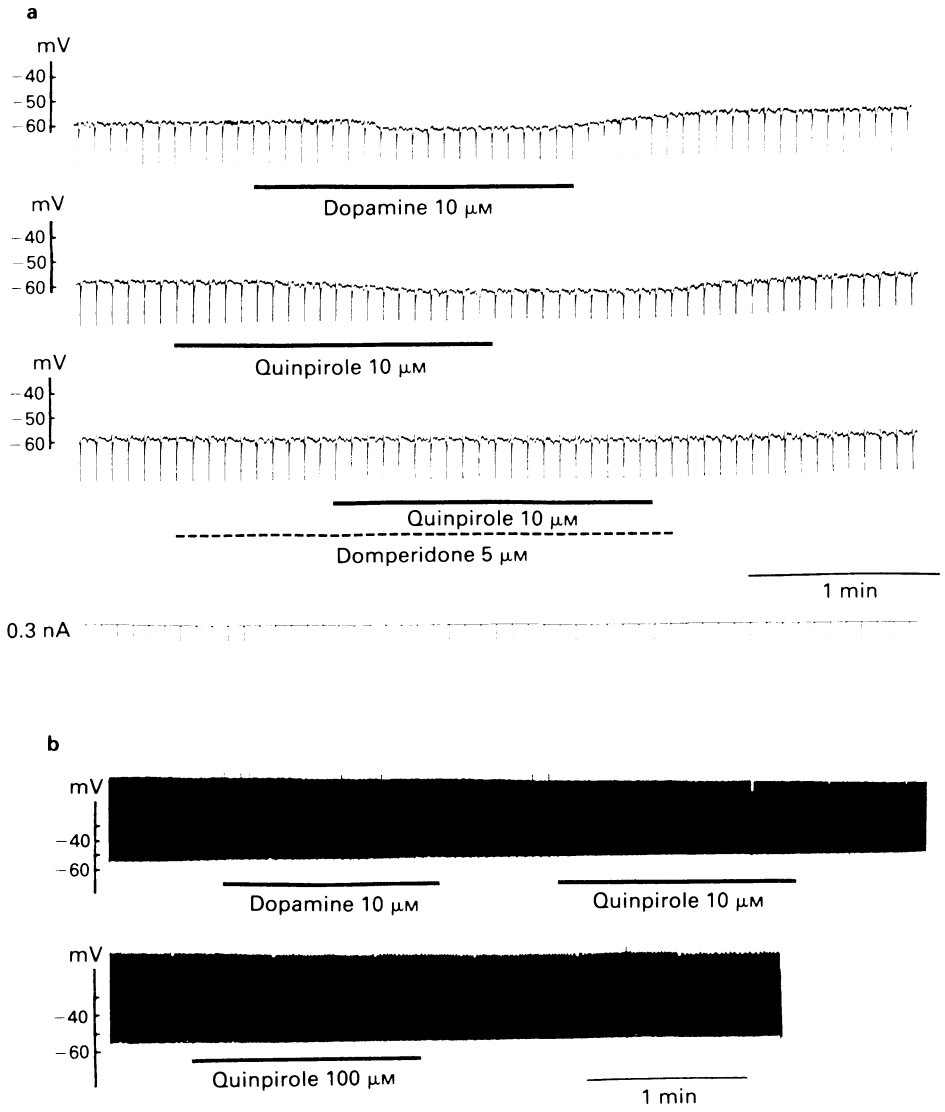
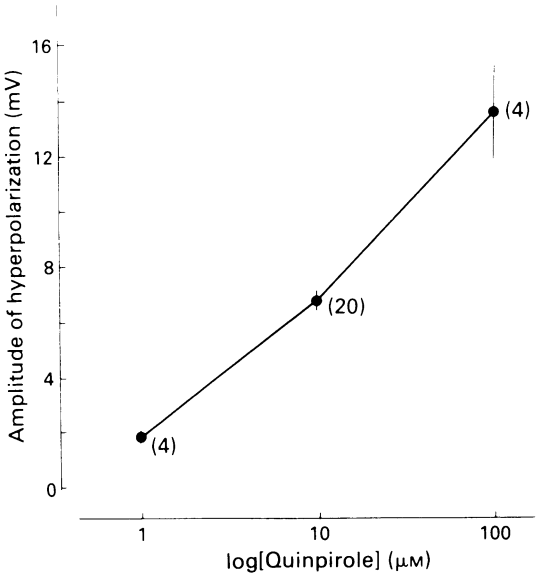


Figure 2 (a) Hyperpolarizing effects of dopamine and quinpirole at a concentration of 10 μM on the resting membrane potential of the same type I neurone in the ventral tegmental area (VTA), and antagonism by domperidone at a concentration of 5 μM against quinpirole (10 μM)-induced hyperpolarization of the membrane. The downward reflection on the resting membrane potential indicates the changes of membrane potential induced by applying a hyperpolarizing current of 0.3 nA to the cell through the recording electrode. The upper, middle and lower traces show the same neurone. (b) Lack of the effects of dopamine (10 μM) and quinpirole (10 and 100 μM) on the resting membrane potential and spontaneously occurring action potentials of a type II neurone in the VTA. The upper and lower traces indicate the same neurone. This neurone fired action potentials at about 50 Hz.



quinpirole at 1, 10 and 100 μM was 1.9 ± 0.1 ($n = 4$), 6.8 ± 0.3 ($n = 20$) and 13.6 ± 1.7 ($n = 4$) mV, respectively (Figure 3). Quinpirole at a concentration of 10 μM significantly ($P < 0.01$) decreased the input impedance in all 20 neurones tested. The mean input impedance was decreased by quinpirole (10 μM) from 40.8 ± 1.5 to 29.9 ± 1.3 MΩ ($n = 20$). In addition, the quinpirole (10 μM)-induced hyperpolarization of the membrane was antagonized by simultaneous application of domperidone (5 μM) in all 3 neurones examined, although domperidone alone had no effect on the RMP in any of the 3 neurones tested (Figure 2a). On the contrary, dopamine (10 μM) and quinpirole (10 and 100 μM) had no effects on the RMP or spontaneously occurring

Figure 3 Relationship between the concentration of quinpirole added to the bath and amplitude of hyperpolarization of the membrane potential in type I neurones (points are mean with s.e.mean). Number of neurones tested in parentheses.

action potentials in any of the 3 type II neurones tested (Figure 2b).

Effects of dopamine and quinpirole on depolarizing current-induced action potentials

Effects of dopamine (10 μM) and quinpirole (10 μM) on the action potentials elicited by the depolarizing current applied to the neurone were examined in 11 type I neurones which were not spontaneously active. In 10 of the 11 type I neurones, an inhibition of action potential and concomitant hyperpolarization (range 5–10 mV) were produced by both dopamine and quinpirole (Figure 4), although the action potentials and RMP in the remaining one neurone were unaffected by either dopamine or quinpirole. In all of the 10 type I neurones with action potentials inhibited by quinpirole, the action potential reappeared by application of a larger depolarizing current to elevate the RMP to the control threshold level (Figure 4), indicating that the threshold level was not affected by quinpirole. In addition, simultaneous application of domperidone (5 μM) with quinpirole antagonized the quinpirole-induced inhibition of action potentials and hyperpolarization in all 4 neurones tested (Figure 4).

The effect of various potassium concentrations in the perfusing fluid

When the potassium concentration in the perfusing fluid was increased from 2.5 to 10.0 mM, the RMP of 5 type I neurones was decreased by 3–6 mV. Therefore, a direct current was applied through the recording electrode to return the RMP to the original level. Under these conditions, quinpirole at a concentration of 10 μM was added to the bath, and the amplitude of quinpirole-induced hyperpolarization was measured under various external potassium concentrations. The net amplitude of quinpirole (10 μM)-induced hyperpolarization was reduced by increasing the potassium concentration in the perfusing fluid to 5.0 mM in all 5 neurones tested (Figure 5). When the potassium concentration was increased to 10.0 mM, the quinpirole-induced hyperpolarization was decreased to 1.13 ± 0.43 mV ($n = 4$) (Figure 5). There was a good correlation between the quinpirole-induced hyperpolarization and the external potassium concentration (Figure 5).

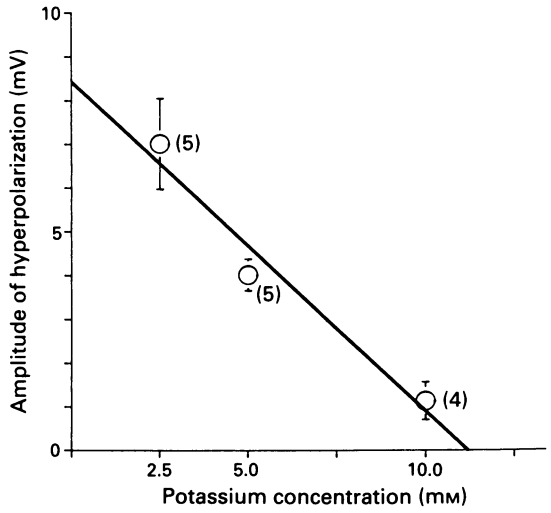


Figure 5 Relationship between potassium concentration in the perfusing fluid and the net amplitude of hyperpolarization of the membrane potential induced by quinpirole (10 μM) in type I neurones (points are mean with s.e.mean). Number of neurones tested in parentheses.

Effect of TEA on quinpirole-induced hyperpolarization

Figure 6 demonstrates the effect of TEA (10 mM) on quinpirole (10 μM)-induced hyperpolarization of the membrane and inhibition of spontaneously occurring action potentials in a typical type I neurone. TEA (10 mM) alone had no effects on the RMP or spontaneous firing in any of the 3 neurones tested. However, when TEA was applied simultaneously with quinpirole (10 μM), the quinpirole-induced hyperpolarization of the membrane and inhibition of spontaneously occurring action potentials were blocked completely in all 3 neurones examined. The mean amplitude of quinpirole-induced hyperpolarization in the 3 neurones was significantly ($P < 0.01$) reduced from 7.7 ± 1.8 mV to 1.1 ± 0.7 mV in the presence of TEA.

Furthermore, the effect of TEA on the depolarizing current-induced action potentials was examined in 3 type I neurones which did not fire action potentials spontaneously (Figure 7). When TEA (10 mM) was added at the same time as quinpirole (10 μM), the quinpirole-induced inhibition of action potentials elicited by a depolarizing current was completely blocked in all 3 neurones tested. However, TEA (10 mM) alone did not affect the depolarizing current-induced action potentials (Figure 7).

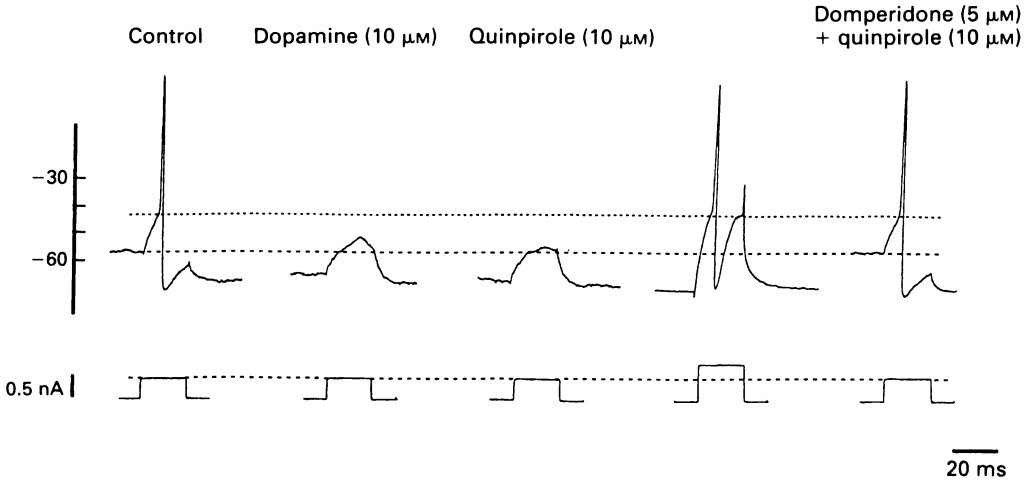


Figure 4 Effects of dopamine (10 μM) and quinpirole (10 μM) on action potentials elicited by depolarizing current in a type I neurone, and antagonism by domperidone (5 μM) against quinpirole-induced inhibition of the action potentials. Upper, middle and lower broken lines indicate the threshold for action potential generation, the resting membrane potential and the amplitude of depolarizing current in the control, respectively.

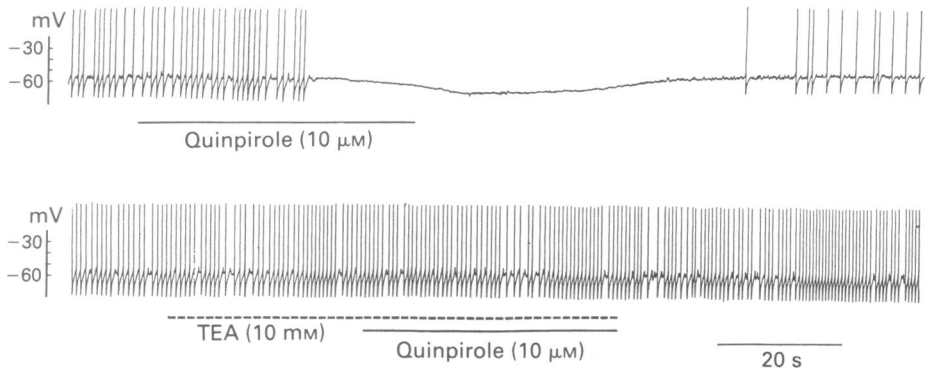


Figure 6 Effects of tetraethylammonium (TEA) at a concentration of 10 mM on quinpirole (10 μM)-induced hyperpolarization of the membrane potential and inhibition of spontaneously occurring action potentials in a type I neurone. The upper and lower traces are from the same neurone.

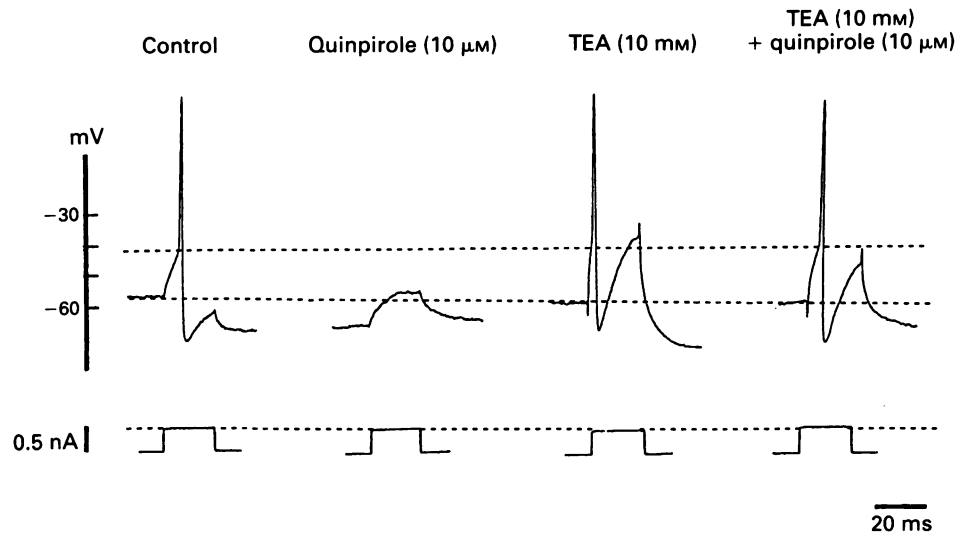


Figure 7 Effect of tetraethylammonium (TEA, 10 mM) on quinpirole-induced inhibition of action potentials elicited by a depolarizing current in a type I neurone. Upper, middle and lower broken lines indicate the threshold for action potential generation, the resting membrane potential and the amplitude of depolarizing current in the control, respectively.

Effect of SKF 38393 and SCH 23390 on neuronal activity in the VTA

Addition of SKF 38393, a selective D_1 agonist, to the bath at doses of 10 or 100 μM alone had no effect on the RMP or action potentials which occurred spontaneously or were elicited by depolarizing pulses applied to the cell in any of the 4 type I and 3 type II neurones tested (Figure 8a and b). When SKF 38393 (10 μM) was added simultaneously with quinpirole (10 μM), the effect on the RMP was not significantly different from that induced by quinpirole (10 μM) alone in any of the 10 type I neurones examined (Figure 8a and Table 2A). However, the action potential generation by depolarizing current was inhibited during application of both quinpirole (10 μM) and SKF 38393 (10 μM) even when a larger depolarizing current was applied to decrease the membrane potential to the control threshold level (Figure 8b). When the membrane potential was further decreased to more positive level than the original control threshold level, the action potential was obtained in the presence of both quinpirole and SKF 38393 (Figure 8b). This elevation of threshold level during application of both quinpirole and SKF 38393 was observed in all 6 type I neurones examined. The mean control RMP and threshold for action potential generation of these 6 type I neurones in the absence of SKF 38393 and quinpirole were -53.3 ± 1.3 mV ($n = 6$) and -45.8 ± 0.9 mV ($n = 6$), respectively (Table 2A and B). On simultaneous applications of SKF 38393 (10 μM) and quinpirole (10 μM), the mean threshold was significantly ($P < 0.01$) elevated to -40.3 ± 1.2 mV ($n = 6$), although the threshold of these neurones was unaffected by quinpirole (10 μM) alone (Table 2B). In addition, the SKF 38393-induced elevation of the threshold potential in the presence of quinpirole was completely blocked by simultaneous application of SCH 23390, a D_1 antagonist, at a dose of 5 μM in all 4 neurones examined (Figure 8b). The mean elevated threshold of these 4 neurones recovered to -45.3 ± 1.0 mV from -40.0 ± 0.8 mV ($n = 4$) in the presence of SCH 23390 (5 μM) (Table 2B).

Discussion

VTA neurones recorded intracellularly were classified into type I and type II, according to distinct electrophysiological characteristics such as the shape of spontaneously occurring action potentials and those elicited by depolarizing pulses applied to the cell. The type I and type II neurones recorded intracellularly in the present study are considered to corres-

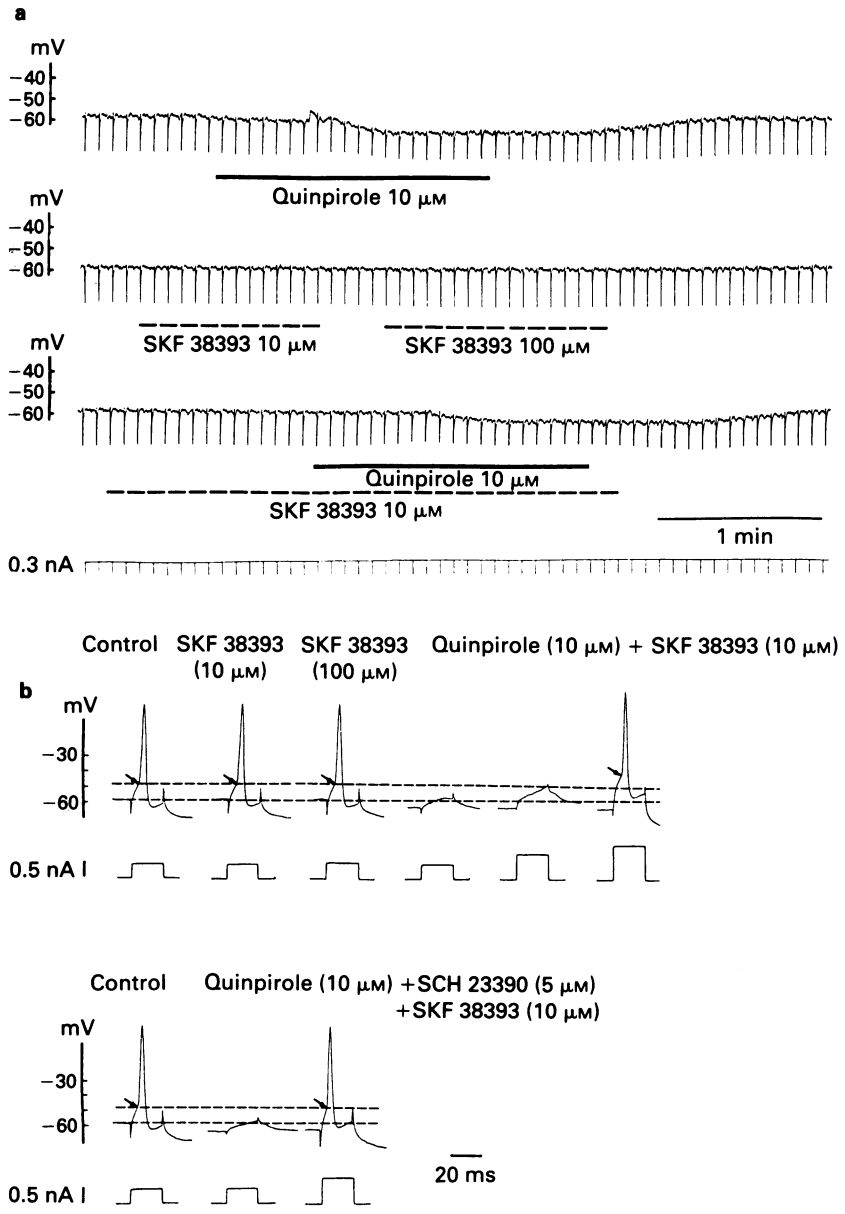


Figure 8 Effect of SKF 38393 at concentrations of 10 or 100 μM added alone or at a concentration of 10 μM simultaneously with quinpirole (10 μM) on the resting membrane potential (a), and depolarizing pulse-induced action potentials in the same type I neurone as well as antagonism by SCH 23390 at a concentration of 5 μM against SKF 38393-induced elevation of the threshold for action potential generation in the presence of quinpirole (b). Upper and lower broken lines in (b) indicate the threshold potential and resting membrane potential before the drug application. Arrows on each trace show the threshold potential of respective action potentials.

pond to the type I and type II neurones recorded extracellularly in our previous *in vivo* studies (Momiyama *et al.*, 1990; 1991), since type I neurones in both intracellular and extracellular studies have a low spontaneous firing rate and action potentials with a long duration of more than 5 ms, while type II neurones have a high spontaneous firing rate and action potentials with a short duration. Thus, type I and II neurones probably correspond to dopaminergic and non-dopaminergic neurones, respectively. Most of the electrophysiological properties of the type I neurones such as long duration action potential and prominent AHP following the action potential, are similar to those of dopaminergic neurones recorded in the substantia nigra (Lacey *et al.*, 1987; 1990; Grace & Onn, 1989) or ventral tegmental area (Mueller & Brodie, 1988; Johnson & North, 1992). However, the properties of type II neurones obtained in our study appear to be different from those of non-dopaminergic secondary cells reported by Johnson & North (1992) in terms of existence of spontaneous firing. This difference might be due to the different types of non-dopaminergic neurones in the VTA.

Addition of dopamine at a concentration of 10 μM to the bath hyperpolarized the membrane of the type I neurones concomitantly with a decrease in input resistance. A similar effect was seen with quinpirole, and the quinpirole-induced hyperpolarizing effect was dose-dependent. However, the effective concentration of quinpirole (10 μM) on our VTA dopaminergic neurones was higher than that reported on those in the substantia nigra (Lacey *et al.*, 1987), although the doses of dopamine on both neurones were comparable. This might have been attributed to the different sensitivity of dopaminergic neurones in the substantia nigra and the VTA to D_2 agonists. In addition, domperidone antagonized the hyperpolarizing effect of quinpirole. Therefore, these findings

Table 2 Effects of simultaneous application of SKF 38393 (SKF, 10 μM) and quinpirole (Q, 10 μM) on resting membrane potential (**A**) and threshold potential (**B**) in type I neurones, and antagonism by SCH 23390 (SCH, 5 μM)

A

Neurone no.	*Resting membrane potential* (-mV) *Control*	*SKF*	*Q*	*SKF + Q*	*SCH + SKF + Q*
1	50	50	56	56.5	–
2	55	55	62	62	–
3	50	50	55	55	55
4	52	52	56	57	57
5	55	55.5	63.5	63	63
6	58	58	64	64	64
7	60	60	66	66	–
8	60	60	64.5	65	–
9	50	50	56	56	–
10	51	51	57	57	–
mean	54.1	54.2	60.0*	60.2†	59.8
s.e.mean	1.3	1.3	1.4	1.3	2.2

B

Neurone no.	*Threshold for action potential generation* (-mV) *Control*	*SKF*	*Q*	*SKF + Q*	*SCH + SKF + Q*
1	44	44	44	37	–
2	49	49	49	45	–
3	44	44	44	39	44
4	44	44	44	39	43.5
5	46	46	46	39.5	45.5
6	48	48	48	42.5	48
mean	45.8	45.8	45.8	40.3*[1]	45.3*[2]
s.e.mean	0.9	0.9	0.9	1.2	1.0

Action potential was elicited by depolarizing pulse applied in the cell.
Neurone numbers (1–6) in **A** correspond to those (1–6) in **B**.
*$P<0.01$, compared with control.
†$P<0.01$, compared with control, but not significant compared with Q alone.
*[1] $P<0.01$, compared with control.
*[2] $P<0.01$, compared with simultaneous application of SKF and Q.

suggest that activation of D_2 receptors increases membrane conductance, thereby hyperpolarizing the membrane of dopaminergic neurones in the VTA. However, it was confirmed here that non-dopaminergic neurones were unaffected by dopamine or quinpirole, probably due to a lack of D_2 receptors, as found in *in vivo* studies (Momiyama *et al.*, 1990; 1991). Concomitantly with hyperpolarization of the membrane, dopamine and quinpirole also inhibited the action potentials which occurred spontaneously or were elicited by depolarizing pulses applied to the cell. However, the action potential was generated even during application of quinpirole when the membrane potential reached the original threshold level by application of a larger depolarizing current to the cell. Therefore, activation of the D_2 receptor in dopaminergic neurones does not affect the threshold level for action potential generation, and inhibition of the action potential generation by D_2 receptor activation is probably due to hyperpolarization of the membrane.

The net amplitude of quinpirole (10 μM)-induced hyperpolarization of the membrane with concomitant decrease in input resistance was decreased as the concentration of potassium in the perfusing fluid was increased. These findings suggest that quinpirole increases potassium conductance of dopaminergic neurones in the VTA, thereby hyperpolarizing the membrane and inhibiting neuronal activity. These findings are in agreement with those in the dopaminergic neurones in the substantia nigra pars compacta (Lacey *et al.*, 1987). In addition, tetraethylammonium (TEA, 10 mM) added simultaneously with quinpirole to the bath, completely, blocked quinpirole-induced hyperpolarization, again suggesting that D_2 receptor activation opens the potassium channel to induce hyperpolarization of the membrane and this potassium channel is sensitive to TEA at a concentration of 10 mM.

SKF 38393, a selective D_1 receptor agonist, at concentrations up to 100 μM alone had no effect on the RMP or action potential firing of dopaminergic neurones. This finding is in accordance with that obtained in our *in vivo* study (Momiyama *et al.*, unpublished) and that in dopaminergic neurones in the substantia nigra pars compacta (Lacey *et al.*, 1987). Furthermore, SKF 38393 (10 μM) applied simultaneously with quinpirole (10 μM) did not affect quinpirole-induced hyperpolarization, but potentiated the inhibition by quinpirole alone of depolarizing pulse-induced action potentials by elevating the threshold potential for generation of action potentials. This indicates that SKF 38393 elevates the threshold for action potential generation without affecting the RMP under the conditions in which quinpirole activates D_2 receptors. In addition, SCH 23390, a D_1 receptor antagonist, applied simultaneously with both quinpirole and SKF 38393, inhibited elevation of the threshold, which suggests that the SKF 38393-induced effect on the threshold is mediated by D_1 receptors. Therefore, it is suggested that activation of D_1 receptors in the VTA dopaminergic neurones elevates the threshold to inhibit the action potential generation under activation of D_2 receptors, thereby enhancing the D_2 receptor-mediated inhibition. However, the mechanism underlying the D_1 receptor-mediated elevation of the threshold observed in the presence of the D_2 receptor agonist, but not in the absence of the agonist, remains to be determined.

There are still controversial findings regarding the existence of D_1 receptors in dopaminergic neurones in the VTA. For instance, some autoradiographic studies have demonstrated the existence of D_1 receptors as well as D_2 receptors in the VTA (Boyson *et al.*, 1986; Dawson *et al.*, 1986; 1988; Cortes *et al.*, 1989), but in another similar study, D_1 receptors were not found there (Alter & Hauser, 1987). In recent *in situ* hybridization studies using mRNA of D_1 receptor clone, D_1 receptors have not been detected in the VTA dopaminergic neurones (Dearry *et al.*, 1990; Sunahara *et al.*, 1990; Weiner *et al.*, 1991). However, at least two subtypes of D_1 receptors (D_{1A} and D_{1B}) have been found in a recent cloning study (Tiberi *et al.*, 1991). The aforementioned 3 groups have used the same cDNA of D_1 receptor for their hybridization studies. Therefore, D_1 receptor subtypes different from their D_1 receptor might be present in the VTA dopaminergic neurones. Indeed, the figures in the report by Tiberi *et al.* (1991) shows the existence of D_{1B} receptors presumably in the VTA, although they did not discuss whether or not such receptors are present in the VTA.

The authors are grateful to Schering-Plough Corporation (Bloomfield, NJ, U.S.A.) and Kyowa Hakko Company (Tokyo, Japan) for the gifts of SCH 23390 and domperidone, respectively. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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(Received December 21, 1992
Revised March 8, 1993
Accepted March 12, 1993)

Br. J. Pharmacol. (1993), **109**, 941–945 © Macmillan Press Ltd, 1993

Comparative study of the control of basal acid output from rodent isolated stomachs

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1 Isolated, lumen-perfused, whole stomach preparations from mouse and immature rat produced a stable basal acid output which, although not blocked by histamine H_2-, acetylcholine M- or CCK_B/gastrin receptor antagonists, was almostly completely blocked by the H^+/K^+-ATPase inhibitor, omeprazole, and the metabolic inhibitor, sodium thiocyanate (NaSCN).

2 Fully-defined concentration-effect curves could be obtained on both assays with the phosphodiesterase inhibitor, isobutyl methylxanthine (IBMX) and with dibutyryl cyclic AMP.

3 On the rat stomach assay, histamine H_2-receptor blockade had no effect on the IBMX curve. In contrast, the IBMX response in the mouse was abolished by histamine H_2-receptor blockade. On both assays responses to dibutyryl cyclic AMP were resistant to H_2-receptor blockade.

4 In the absence of suprathreshold endogenous histamine, it is argued that H^+/K^+-ATPase mediated basal acid secretion from the mouse stomach assay is regulated by something other than cyclic AMP.

Keywords: Gastric acid; whole stomach preparation of mouse, rat; H^+/K^+-ATPase; phosphodiesterase inhibitors; adenylate cyclase; histamine

Introduction

The isolated, lumen-perfused stomach of the mouse is now a standard preparation for studying the pharmacology of oxyntic cell secretion (Wan, 1977; Angus & Black, 1979; Szelenyi & Vergin, 1980; Black & Shankley, 1985; Mahklouf & Schubert, 1990). Under basal conditions, this preparation lowers the pH of the unbuffered perfusate from about 6.0 to about 4.4. Treatment with the metabolic poison, sodium thiocyanate (NaSCN), abolished all but a few percent of this acidification suggesting a true basal secretion (Black & Shankley, 1985). That the oxyntic cells were the source of the secretion was implied by the abolition of this secretion by omeprazole, the H^+/K^+-ATPase inhibitor (Sachs & Wallmark, 1989).

The basal oxyntic cell secretion was not inhibited by tetrodotoxin, N-methylatropine or tiotidine thus eliminating the involvement of histamine or neurally-released acetylcholine in the activity. As gastrin (pentagastrin) stimulation is also completely suppressed by histamine H_2-receptor blockade by tiotidine, then presumably gastrin is not involved either. However, isobutyl methylxanthine (IBMX), a highly-selective inhibitor of the phosphodiesterase which hydrolyses adenosine 3':5'-cyclic monophosphate (cyclic AMP), was found to be as full an agonist as histamine in this preparation. Arguably, phosphodiesterase inhibition was potentiating the basal activity of adenylate cyclase (Black & Shankley, 1985). We concluded, therefore, that the basal oxyntic cell secretion was being driven by this basal activity of adenylate cyclase (Black & Shankley, 1985).

Earlier, Berglindh *et al.* (1980) found that IBMX was not only a full agonist in their preparation of rabbit isolated, gastric glands but also that this action could be abolished by histamine H_2-receptor antagonists. However, this suspension of gastric glands was found to have about 100 nM histamine in the supernatant (Soll & Berglindh, 1987). This pointed to the basal adenylate cyclase activity being subliminally driven by histamine in that preparation. Soll (1980) made similar observations on his suspensions of canine isolated oxyntic cells.

During the development of an isolated, lumen-perfused, stomach assay from the rat, a species which we have observed to be 3 fold less sensitive to histamine, we had occasion to re-examine and extend the analysis of the relationship between histamine, adenylate cyclase activity and basal gastric acid output.

Methods

Isolated, lumen-perfused stomach preparations

Gastric acid secretion was measured in isolated, lumen-perfused, stomach preparations essentially as described previously for the mouse (Black & Shankley, 1985). Young adult male mice (Charles River 22–26 g), fasted for 18 h prior to experimentation but with free access to water, and pre-weaned rat pups (Wistar 32–38 g) were used. Animals were killed by cervical dislocation, the abdomen opened and the oesophagus ligated close to the stomach. A polythene cannula (2 mm internal diameter) was inserted into the pylorus via the duodenal bulb, and a small incision made in the fundus through which the stomach contents were gently washed. A second cannula was tied into this incision. The stomachs were then transferred into a 40 ml organ bath containing buffered serosal solution (mM: NaCl 118, KCl 4.8, $MgSO_4$ 1.2, KH_2PO_4 1.14, $NaHPO_4$ 15.9, $CaCl_2$ 0.65, glucose 31.6) maintained at 37°C and gassed with 95% O_2 and 5% CO_2. The preparations were continuously perfused from the fundic through the pyloric cannulae (1 ml min^{-1}) with warmed, unbuffered, mucosal solution (mM: NaCl 135, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2$ 1.3, glucose 31.6) gassed with 100% O_2, and the perfusate passed over a pH-electrode system adjusted to provide 12 cmH_2O intragastric pressure.

Experimental design

Six preparations were studied simultaneously and, after a 60 min stabilisation period, any not showing stable basal responses were rejected (less than 5%). Thereafter, drugs were added to the serosal solution according to individual experimental protocols and the total vehicle volume did not exceed 1 ml.

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A randomized block design was used throughout for allocation of experimental treatments such that, as far as possible, each organ bath received each treatment within the course of an experiment.

Acid secretory responses were expressed as ΔpH, that is, the difference between basal pH, measured immediately prior to experimental intervention, and stimulated pH. Fully-defined, agonist concentration-effect curves were obtained by cumulative dosing at 0.5 $\log_{10}$ units when stable response plateaux were achieved.

Data analysis

Concentration-effect curve data from individual preparations were fitted by means of an iterative least squares minimisation programme to a general logistic function to provide estimates of the midpoint location ($\log[A_{50}]$), midpoint slope parameter (p) and upper asymptote (α) as described previously (Black & Shankley, 1985). For analysis and display purposes the individual computed parameter estimates for each treatment group were expressed as mean ± s.e.mean and single logistic curves stimulated and shown superimposed upon the experimental data.

Computed logistic curve-fitting parameters were compared by one-way ANOVA or Student's t test. Values of $P<0.05$ were considered significant. All errors quoted are s.e.mean.

Drugs

Histamine (Sigma Chemical Co. Ltd.) was dissolved in distilled water to give a 1 M stock which was back-neutralised with 25 μl 10 N NaOH ml^{-1}. Tiotidine (a gift from Imperial Chemical Industries Ltd.) was dissolved in dilute HCl to give a 0.2 mM stock solution; L365,260 (1,3-dihydro-1-methyl-3(**R**)-(3-methylphenyl)-amino-carbonylamino-6-phenyl-2H-1, 4-benzodiazepin-2-one; a gift from Dr Lotti, Merck Sharp & Dohme Research Labs.) and omeprazole (Research Biochemicals Inc.) were dissolved in 90% ethanol and SKF96356 (1-(2-methylphenyl)-4-(methylamino)-6-methyl-2,3-dihydropyrrolo[3,2-c]quinoline; a gift from SmithKline & Beecham) in 90% ethanol and 0.1 N HCl. Subsequent dilutions were made in distilled water. Isobutyl methylxanthine (IBMX) and dibutyryl cyclic AMP (db-cyclic AMP) (Sigma) and ICI 63,197 (2-amino-6-methyl-4-propyl-[1,2,4]triazolo[1,5-a]pyrimidin-5(4H) one; a gift from Imperial Chemical Industries Ltd.) were dissolved in distilled water. None of the vehicles concentrations and volumes used had an effect on basal acid output.

Results

Basal acid output

The lumen-perfusion solution for both preparations, being unbuffered, had a pH of approximately 6. Immediately after the preparations were established, the outflow pH in both preparations lay between 5 and 5.5. After 1 h of stabilization the outflow pH in the mouse was 4.64 ± 0.08 (n = 16) and in the rat was 4.86 ± 0.04 (n = 18). The outflow pH from the mouse remained stable within limits for at least 4 h although in the rat the pH tended to drift downwards after 4 h (data not shown). Blockade of histamine H_2-, acetylcholine (ACh) muscarinic (M)- and gastrin/CCK_B-receptors by tiotidine (0.1 mM), atropine (20 μM), L365,260 (1 μM), respectively, did not produce significant inhibition of the basal acid output in either preparation (Table 1). These concentrations were calculated to give dose-ratios in excess of 100 without losing receptor selectivity.

H^+/K^+-ATPase inhibition

Omeprazole (0.1 mM), an 'irreversible inhibitor of H^+/K^+-ATPase' (Fellenius *et al.*, 1981) and SKF96356 (0.1 mM), described as a 'reversible K^+-competitive inhibitor, of that enzyme (Leach *et al.*, 1992) produced significant inhibition of basal acid output in both assays (Figure 1). This inhibition attained a plateau after 60 min. We judged the concentrations of these ligands to be maximal because of the following facts. Omeprazole (10 μM) has been reported to produce greater than 90% inhibition of basal acid output in rat isolated stomachs (Coruzzi *et al.*, 1986) and we used a 10

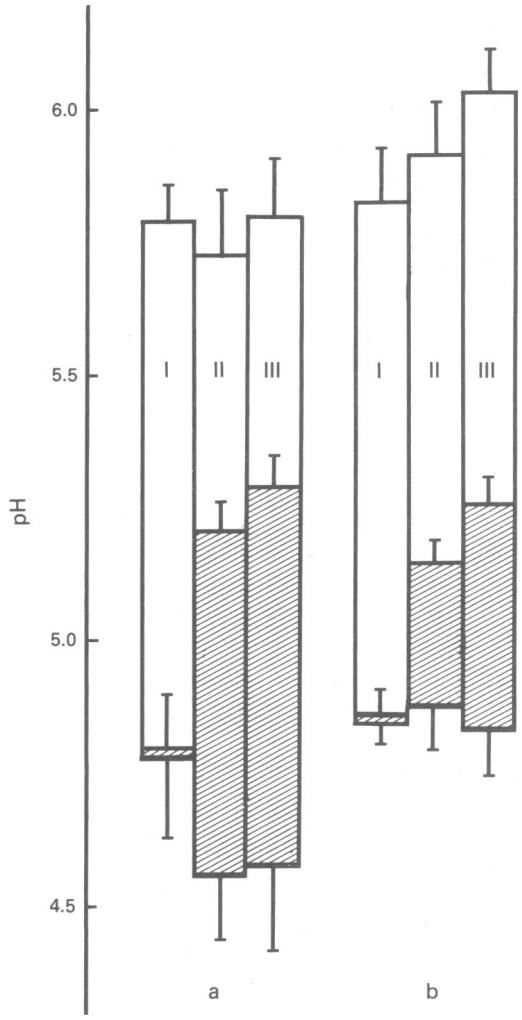


Figure 1 Inhibition of basal gastric acid secretion from isolated, lumen-perfused stomach assays of mouse (a) and immature rat (b). The histograms show the effect of (I) vehicle control followed by sodium thiocyanate (NaSCN, 30 mM), (II) 0.1 mM omeprazole followed by NaSCN (30 mM) and (III) 0.1 mM SKF96356 followed by NaSCN (30 mM). In each case the first treatment is shown as the hatched bar. All treatments were incubated for 120 min.

Table 1 The effect of tiotidine (0.1 mM), atropine (20 μM) and L365,260 (1 μM) on basal gastric acid output, expressed as ΔpH from mouse and immature rat isolated stomach assays

	Mouse	*Rat*
Control	0.04 ± 0.02	0.05 ± 0.02
Tiotidine	0.01 ± 0.03	0.02 ± 0.02
Control	0.06 ± 0.01	0.07 ± 0.02
Atropine	0.06 ± 0.01	0.05 ± 0.02
Control	0.04 ± 0.03	0.08 ± 0.02
L365,260	0.07 ± 0.04	0.08 ± 0.01

Values are given ± s.e.mean. n = 5/7.

fold higher concentration. This concentration of omeprazole abolishes the response to histamine (data not shown). A fully-defined inhibitory concentration-effect curve was obtained for SKF96356 on the rat assay which attained an asymptote at 0.1 mM (logistic curve-fitting parameters: $p[A_{50}] = 5.92 \pm 0.13$, $\alpha = 0.76 \pm 0.11$ pH, $p = 1.32 \pm 0.16$).

H^+/K^+-ATPase inhibition produced a significantly greater reduction (ΔpH) in the mouse than in the rat but Figure 1 shows that this was due to the lower starting pH in the mouse; the final equilibrium pH values of about 5.2 were not significantly different between compounds or assay species. These pH values were still significantly lower than the input pH (Figure 1). When expressed in terms of $[H^+]min^{-1}$, the acid output which was not blocked by omeprazole and SKF96356 was approximately 20% of the total basal output.

Effect of sodium thiocyanate

The residual acid output, following both omeprazole and SKF96356 treatment, was almost abolished (Figure 1) by 30 mM sodium thiocyanate (NaSCN). When NaSCN was tested on its own, two phases of inhibition were seen in most preparations. The first phase reduced the pH, after 60–90 min, to levels achieved with the maximal concentrations of the H^+/K^+-ATPase inhibitors (see Figure 2) and the second phase, beginning after about 120 min, abolished the secretion more or less completely (Figure 1).

Effects of histamine and phosphodiesterase inhibition

The above experiments established that the basal acid output was mainly due to the activity of the H^+/K^+-ATPase. This 'proton pump' can be driven by cyclic AMP leading to kinase activation (Wallmark & Sachs, 1989). Therefore, possible involvement of adenylate cyclase activity in driving basal acid output was assessed by inhibiting phosphodiesterase with isobutyl methylxanthine (IBMX) in both preparations and, additionally, by ICI63197 in the mouse preparation. Fully-defined concentration-effect curves were obtained to the compounds on both the rat and the mouse assays. These curves have been compared with histamine concentration-effect curves, which are believed (Scholes *et al.*, 1976) to be due to activation of the adenylate cyclase (Figure 3). Histamine was more potent on the mouse assay than on the rat but the midpoint slope of the concentration-effect curve in the rat was steeper (approximately double) than in the mouse. In contrast, IBMX was equipotent in both species although the midpoint slope in the rat was again approximately twice that in the mouse. Histamine and IBMX produced similar maximum responses in the mouse. However, in the rat, the maximum response to histamine was much smaller than that to IBMX. Dibutyryl cyclic AMP, the cell membrane penetrating analogue of cyclic AMP, produced a

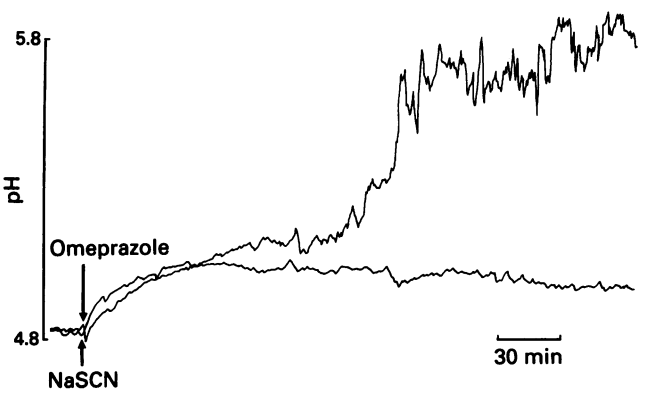


Figure 2 Examples of the time course of effect of 30 mM sodium thiocyanate (NaSCN) and 0.1 mM omeprazole on basal acid output from the lumen-perfused, immature rat stomach assay.

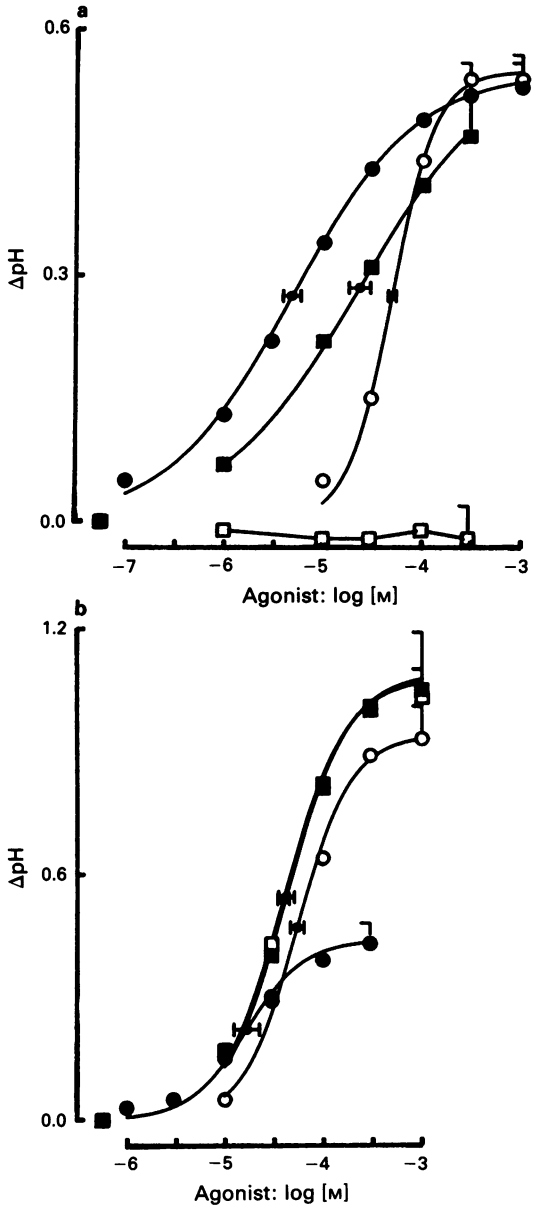


Figure 3 Concentration-effect curves to histamine (●) and dibutyryl cyclic AMP (db-cyclic AMP, ○), and to isobutyl methylxanthine (IBMX) in the absence (■) and presence (□) of 0.1 mM tiotidine obtained in the (a) mouse and (b) immature rat stomach assays. Logistic curve-fitting parameters as follows:

	n	$p[A_{50}]$	$\alpha(\Delta pH)$	*p*
Rat				
Histamine	7	4.78 ± 0.03	0.44 ± 0.05	1.48 ± 0.18
IBMX	6	4.40 ± 0.06	1.08 ± 0.14	1.38 ± 0.14
IBMX (+ tiotidine)	6	4.37 ± 0.08	1.09 ± 0.07	1.38 ± 0.19
db-cyclic AMP	6	4.26 ± 0.07	0.94 ± 0.08	1.59 ± 0.08
Mouse				
Histamine	5	5.32 ± 0.09	0.55 ± 0.04	0.70 ± 0.07
IBMX	5	4.67 ± 0.11	0.57 ± 0.09	0.63 ± 0.03
IBMX (+ tiotidine)	6	–	− 0.02 ± 0.04	–
db-cyclic AMP	6	4.37 ± 0.08	1.09 ± 0.07	1.38 ± 0.19

concentration-dependent stimulation of gastric acid output on both assays (Figure 3). The maximum response to dibutyryl cyclic AMP was not significantly different from that obtained with IBMX.

Effects of histamine H_2-receptor blockade

Tiotidine, was found to be a simple competitive antagonist of

histamine H_2-receptors in both mouse and rat preparations with pK_B values of 6.96 ± 0.11 (Black *et al.*, 1985) and 7.40 ± 0.05 (Welsh *et al.*, 1992), respectively. Tiotidine (0.1 mM) had no effect on the IBMX concentration-effect curves obtained in the rat stomach assay. However, in the mouse assay the IBMX responses were concentration-dependently inhibited by tiotidine and were abolished by 0.1 mM (Figure 3). Tiotidine (0.1 mM) had no significant effect on the dibutyryl cyclic AMP concentration-effect curves on either assay (data not shown).

Discussion

The isolated, lumen-perfused, stomach preparations from mouse and immature rat were developed for quantitative pharmacological analysis of the regulation of acid secretion (Black & Shankley, 1985). Therefore, the assay conditions were manipulated such that agonist responses could be measured from a stable basal acid secretion which was not driven by endogenous secretagogues as determined by the lack of effect on basal secretion of receptor antagonists of the natural stimulants of gastric acid secretion, histamine, gastrin and acetylcholine.

Previously, we reported that the H^+/K^+-ATPase inhibitor, omeprazole, and the metabolic inhibitor, sodium thiocyanate (NaSCN), produced similar levels of inhibition of basal acid ouput on the mouse assay (Black & Shankley, 1985). However, in this study, omeprazole produced only $58 \pm 5\%$ of the maximum pH change, equivalent to approximately 80% when the results are expressed as $[H^+]min^{-1}$, achieved by NaSCN on the mouse assay (Figure 1). The discrepancy between this result and the previous findings in the mouse preparation may be due to the biphasic timecourse of the inhibition by NaSCN. Previously the response was only studied for 60 min and the biphasicity was not identified. Consequently, the omeprazole response was compared with what we now believe to be the first plateau of the NaSCN response.

One possible explanation for the failure of omeprazole to abolish acid output would be if access to the H^+/K^+-ATPase were limited by a similar disposition mechanism to that envisaged for hormone receptor antagonists on these assays (Shankley *et al.*, 1988). On the mouse stomach assay, the underestimation of histamine H_2- and ACh M-receptor antagonist pK_B values was imagined to be due to the loss of the antagonists into the perfusate because the extent of underestimation was correlated with their log P values. Omeprazole is a relatively lipophilic compound (log $P = 2.1$ calculated by ClogP Medchem ver. 3.54, using a benzyl approximation) and therefore a candidate for such loss. However, the 'reversible' H^+/K^+-ATPase inhibitor, SKF96356, a far more lipophilic compound (log $P > 4$; C. Leach, personal communication), produced the same level of inhibition supporting the view that the inhibition of the proton pump was maximal. Moreover, in the rat assay, where we have previously shown that there is far less underestimation of antagonist affinity values (Welsh *et al.*, 1992), omeprazole and SKF96356 also produced the same submaximal inhibition of basal acid output.

Previously, we suggested that the basal acid output from the mouse stomach assay which was not blocked by omeprazole was of metabolic origin (Black & Shankley, 1985). If this assumption were correct then the biphasic nature of the NaSCN timecourse, observed in both species in this study (Figure 2), could reflect the two mechanisms of acid output inhibition by NaSCN proposed by Hersey *et al.* (1981), namely, the inhibition of oxyntic cell acid secretion which apparently occurs at lower concentrations (< 10 mM) and inhibition of mitochondrial respiration which requires higher concentrations (> 10 mM). It was as though the difference in effective concentration for the two processes were expressed in these intact assays as different rates of onset of action. However, this hypothesis may be flawed because the inhibition of mitochondrial activity in the gastric mucosa might be expected to increase rather than decrease the omeprazole-resistant basal acid output due to the release of metabolic acids. Clearly, further experimentation is required to determine the source of the NaSCN-sensitive but omeprazole-resistant basal acid output from the assays.

According to Wallmark & Sachs (1989), the oxyntic cell H^+/K^+-ATPase is driven by kinases which are activated by *inter alia*, cyclic AMP produced by adenylate cyclase. Previously, we provisionally concluded that the basal acid output from the mouse stomach preparation was related to basal activity of adenylate cyclase (Black & Shankley, 1985). The concentration-effect curves for IBMX and histamine obtained on the mouse assay had similar maxima and slopes (Figure 3). This suggested that IBMX was behaving like a post-receptor stimulant of the histamine pathway by mimicking histamine. We had assumed, in common with Soll & Berglindh (1987), that the reason Berglindh *et al.* (1980) found that the response to IBMX was abolished by histamine H_2-receptor antagonists on their assay of rabbit isolated, gastric glands was due to the presence of significant background endogenous histamine.

On the rat assay in this study, IBMX produced concentration-dependent stimulation of acid secretion which was not blocked by the histamine H_2-receptor antagonist tiotidine (Figure 3). Presumably, IBMX inhibits the phosphodiesterase-catalysed breakdown of cyclic AMP produced by the basal activity of adenylate cyclase (Figure 4). In the rat, although the curve slopes were not significantly different, the maximum response to IBMX was significantly greater than that to histamine (Figure 3). The response to IBMX was probably maximal because it was not significantly different from that obtained to dibutyryl cyclic AMP. The latter, by providing the maximum stimulus, presumably defines the maximum effect achievable via the adenylate cyclase pathway. So far as the secretory system is concerned, therefore, histamine behaves as a partial agonist in the rat with the response limitation lying between receptor occupancy and cyclic AMP production, which might be expected in view of the relatively low potency of histamine.

In this study, we have found that the response to IBMX in the mouse assay was abolished by tiotidine. That the secretagogue action of IBMX was due to phosphodiesterase inhibition was confirmed when the same result was obtained with the non-xanthine based inhibitor, ICI63197. Evidently, the IBMX stimulation of gastric acid secretion in the mouse involves the potentiation of histamine H_2-receptor-mediated acid secretion. However, if there had been any oxyntic cell adenylate cyclase activity contributing to basal acid secretion then phosphodiesterase inhibition would be expected to potentiate this and, as found in the rat assay, there would be a histamine H_2-receptor antagonist resistant component of the IBMX response. It is as though the mouse basal adenylate cyclase activity, although governed by histamine, is insufficient to produce a secretory response as judged by its being refractory to histamine H_2-receptor blockade. However, when the phosphodiesterase is inhibited, cyclic AMP can accumulate to levels which stimulate acid secretion (see Figure 4). The possibility that IBMX, by inhibiting phosphosidesterase, releases significant levels of endogenous histamine in the mouse (and rat) is excluded by the finding that db-cyclic AMP responses were refractory to histamine H_2-receptor blockade (Figure 3).

The difference in sensitivity to histamine H_2-receptor blockade of the IBMX response does not necessarily imply that the underlying biochemical regulation of basal acid secretion is fundamentally different between the species. In Figure 4, we illustrate a model which contains the minimum operators we believe are necessary to explain our results. We have shown in rectangles the three essential enzymes in the model, adenylate cyclase, phosphodiesterase and H^+/K^+-ATPase. The chemical inputs to these enzymes are shown in

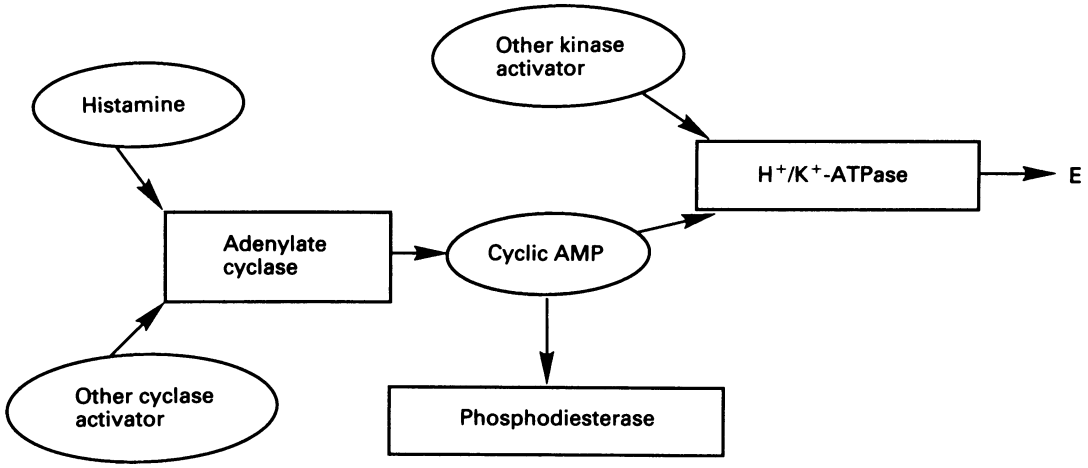


Figure 4 Schematic representation of the regulation of basal and histamine-stimulated gastric acid secretion in the mouse and immature rat stomach (see text for details).

ellipses. The H^+/K^+-ATPase is driven by both cyclic-AMP and another source of kinase activation. The adenylate cyclase can be driven by histamine and other activators not specified by our experiments. The level of cyclic AMP is determined by the ratio of the activities of adenylate cyclase and phosphodiesterase. In the rat, the inability of histamine H_2-receptor blockade to block the effects of phosphodiesterase inhibition implies that the cyclic AMP is being activated by some other factor not specified by these experiments. Similarly, the experiments do not allow us to distinguish whether cyclic AMP alone or in combination with some other source of kinase activation drives the H^+/K^+-ATPase. However, in the mouse, the fact that histamine H_2-receptor blockade abolishes the effects of phosphodiesterase inhibition but not the basal activity of the pump means that the adenylate cyclase is being driven by a level of histamine stimulation which produces a flux of cyclic AMP lower than the flux through phosphodiesterase. However, inhibition of phosphodiesterase now leads to accumulation of cyclic AMP and activation of the H^+/K^+-ATPase. The fact that histamine H_2-receptor block abolished the response to phosphodiesterase inhibition and yet had no effect on basal secretion can only happen if the adenylate cyclase is being driven solely by histamine but the pump is driven by another source of kinase activation.

This work was funded in part by a grant from Imperial Chemicals Industries Ltd. and MRC Grant No. 8702159. We thank Robert Hull for his criticism.

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(Received December 17, 1992
Revised March 8, 1993
Accepted March 16, 1993)

Br. J. Pharmacol. (1993), **109**, 946–952 © Macmillan Press Ltd, 1993

Selective inactivation of muscarinic M_2 and M_3 receptors in guinea-pig ileum and atria *in vitro*

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1 The role of muscarinic M_2 and M_3 receptors in ileal smooth muscle has been evaluated by use of selective receptor alkylation. The alkylating agents, 4-diphenylacetoxy-*N*-(2-chloroethyl)-piperidine (4-DAMP mustard) was studied for effects against (+)-*cis*-dioxolane, at muscarinic M_2 and M_3 receptors in guinea-pig atria or ileum, respectively. 4-DAMP mustard (10 nM, 40 min exposure) did not discriminate between these muscarinic receptors. In ileum, 4-DAMP mustard, at 100 nM, resulted in a large dextral shift (197 fold) and depression in maxima. In atria there was a smaller dextral shift (14 fold) but no depression in maxima.

2 The muscarinic antagonists, atropine (non-selective), methoctramine (M_2-selective) and *para*-fluoro-hexahydro-siladiphenidol (pFHHSiD; M_3 selective) were studied in protection studies against alkylation by phenoxybenzamine. Washout studies following equilibration of the tissues with atropine (30 nM), methoctramine (0.3 μM) or pFHHSiD (3 μM), showed the compounds to be reversible. No temporal changes in sensitivity to (+)-*cis*-dioxolane were observed.

3 Exposure, for 20 min, of atria and ileum to phenoxybenzamine (3 and 10 μM respectively) caused dextral shifts and depressions in the maxima of the concentration-response curve to (+)-*cis*-dioxolane. These effects were inhibited by prior equilibration with atropine (30 nM) and methoctramine (0.1 μM) in atria or atropine (30 nM) and pFHHSiD (3 μM) in ileum. Similar results in ileum were obtained when pilocarpine was used as the agonist.

4 These data were consistent with muscarinic M_2 receptors mediating responses in atria and M_3 receptors mediating responses in ileum. No evidence was provided for a direct role of muscarinic M_2 receptors in ileal contraction.

5 It is concluded that receptor protection by reversible antagonists for muscarinic M_2 or M_3 receptors provides a means to isolate pharmacologically a single subtype in a tissue possessing heterogeneous populations. This technique may prove useful in defining the role of the respective subtypes in smooth muscle contraction.

Keywords: Receptor protection; alkylation; muscarinic receptor subtypes; guinea-pig ileum; guinea-pig left paced atria; 4-DAMP mustard; phenoxybenzamine

Introduction

Muscarinic receptor subtypes, denoted as M_1, M_2, M_3 and M_4 can be differentiated both operationally and structurally (Hulme *et al.*, 1990). The proportion of muscarinic M_2 receptors outnumber the M_3 population by about 4:1 in numerous smooth muscles. These include human colon (Gomez *et al.*, 1992), guinea-pig ileum (Ford *et al.*, 1991), rat ileum (Candell *et al.*, 1990), rat colon (Gomez *et al.*, 1992), canine colon (Zhang & Buxton, 1991; Zhang *et al.*, 1991), oesophageal mucosa (Lad *et al.*, 1991), trachea (Yang, 1991) and bovine trachea (Roffel *et al.*, 1987). In these tissues, increases in isometric tension are mediated by muscarinic M_3 receptors (see Hulme *et al.*, 1990, for review) due, in part, to coupling of the M_3 receptor to a phosphoinositide-specific phospholipase C. The function of the predominant muscarinic M_2 receptor population, which characteristically couples to guanine nucleotide binding proteins G_i or G_K, may be to inhibit β-adrenoceptor-stimulated adenylyl cyclase activity and consequently oppose relaxation (Candell *et al.*, 1990; Fernandes *et al.*, 1992). In this manner, muscarinic receptors could modulate muscle contraction both directly and indirectly (Candell *et al.*, 1990).

Selective inactivation of either muscarinic M_2 or M_3 receptors would facilitate elucidation of their role in smooth muscle contraction. There are two means by which this can be achieved. First, use of subtype selective alkylating agents and second, receptor protection with a selective, reversible antagonist. It has been proposed that 4-DAMP mustard (4-diphenylacetoxy-*N*-(2-chloroethyl)-piperidine) preferentially alkylates muscarinic M_2 receptors over M_3 receptors (Barlow *et al.*, 1990; Thomas *et al.*, 1992; Birdsall *et al.*, 1993) although this has been disputed (Waelbroeck *et al.*, 1992). Few muscarinic receptor protection studies using selective reversible antagonists have been described. McLesky & Wojcik (1990) reported that both atropine and methoctramine protected against alkylation by [^{3}H]-propylbenzilylcholine mustard of M_2 receptor protein isolated from rat cerebellar granule cells. Thomas *et al.* (1992) demonstrated that AF-DX 116 protected against the alkylating effects of 4-DAMP mustard in rat submaxillary gland and heart. Contractile studies in smooth muscle, using selective muscarinic receptor protection have yet to be reported.

The aims of the present report were, firstly, to evaluate functionally the effects of 4-DAMP mustard at muscarinic M_2 and M_3 receptors in guinea-pig ileum and atria. Secondly, the effects of receptor protection against phenoxybenzamine were studied. The guinea-pig ileum was chosen to assess these two approaches since binding studies have demonstrated selective protection of muscarinic M_2 receptors by reversible antagonists from alkylation (Giraldo *et al.*, 1988). The effects have been compared to those in guinea-pig atria, a tissue which possesses a homogeneous muscarinic M_2 receptor population (Eglen *et al.*, 1992).

A preliminary account of some of these data has been reported previously at the Fifth International Symposium on Muscarinic Receptor Subtypes (Eglen & Harris, 1992).

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Methods

All tissues were isolated from male, Hartley guinea-pigs, previously killed by CO_2 asphyxiation. Portions of ileum, isolated 15 cm distal to the caecum, were removed and flushed with warm Krebs solution. The composition of the Krebs solution, in mM, was: NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 10.0, $NaHCO_3$ 25.0 and $CaCl_2$ 2.5. Left atria were removed and suspended between parallel platinum electrodes in Krebs solution, to allow for electrical pacing (threshold voltage + 10%, 15 ms pulse duration, 0.5 Hz). All preparations were allowed 60 min to equilibrate in Krebs solution, under 1 g tension, prior to construction of a concentration-response curve to (+)-*cis*-dioxolane. This agonist was chosen because of its high potency and lack of selectivity between muscarinic receptor subtypes (Eglen *et al.*, 1992). Concentration-response curves were constructed using incremental concentrations, at 0.5 log intervals.

4-DAMP mustard studies

To assess the putative selectivity of 4-DAMP mustard, the method used was similar to that described by Barlow *et al.* (1990) and Thomas *et al.* (1992). After 60 min equilibration, concentration-response curves to (+)-*cis*-dioxolane were established in atria (cumulatively) or ileum (non-cumulatively; 30 s exposure on a 5 min dose cycle). The tissues were then washed and equilibrated with 4-DAMP mustard at 10 or 100 nM for 40 min. (Each concentration and equilibration period was assessed on separate pieces of tissue.) The preparations were then washed, allowed to regain baseline tension and a second concentration-response curve constructed.

Receptor protection studies

In receptor protection studies, Furchgott (1966) has proposed that the protecting antagonist should be reversible and to study this, washout studies were undertaken in both ileum and atria. Thus, a concentration-response curve to (+)-*cis*-dioxolane was established in the absence and presence of atropine, methoctramine or pFHHSiD, following an equilibration period of 60 min. The tissues, were then washed at 10 min intervals and a third concentration-response curve established 60 min, 120 or 180 min after. In all experiments parallel control studies were performed to correct for temporal changes in sensitivity to the agonist.

The receptor protection studies were undertaken as follows: after construction of the initial concentration-response curve, tissues were washed and exposed to phenoxybenzamine (10 μM in ileum and 3 μM in atria) for 20 min. Tissues were then washed at 10 min intervals over the succeeding 120 min in atria or 180 min in ileum and a second concentration-response curve established. The affinity ($-\log K_A$) of the agonist was estimated by the method of Furchgott (1966) interpolating equiactive responses-agonist concentrations before and after inactivation by phenoxybenzamine. These were plotted in double reciprocal manner and the affinity estimated from the resulting straight line.

In separate studies, tissues were exposed to atropine (non-selective), methoctramine (M_2 selective) or pFHHSiD (M_3 selective) for 60 min prior to exposure to phenoxybenzamine. The concentrations of selective antagonists were determined to allow maximal occupancy of one receptor subtype with minimal occupation of the other (Table 1). The tissues were then exposed to phenoxybenzamine and experiments conducted as described above.

In ileum, protection studies were also undertaken with pilocarpine as the agonist. Since this has a low intrinsic efficacy (Furchgott, 1966), the concentration of phenoxybenzamine used was reduced from 10 to 3 μM. In all experiments parallel studies were performed in the absence of antagonist to correct for temporal changes in sensitivity. To determine the muscarinic receptor mediating responses to pilocarpine, concentration-response curves were constructed in the absence and presence of a single concentration of either atropine, methoctramine or pFHHSiD.

Table 1 Receptor occupancies of antagonists at muscarinic M_2 and M_3 receptor subtypes

Antagonist	*$-\log K_B$*	*Concentration*	*Occupancy (%)*
Muscarinic M_2 receptors - guinea-pig left atria			
Atropine	8.9	30 nM	95.8
Methoctramine	7.8	0.1 μM	86.2
pFHHSiD	6.0	0.1 μM	9.0
Muscarinic M_3 receptors – guinea-pig ileum			
Atropine	9.1	100 nM	99.2
Methoctramine	5.8	0.1 μM	5.9
pFHHSiD	7.8	3 μM	99.5

Apparent affinity values ($-\log K_B$) were estimated as described in the Methods.

Analysis of results

Responses were measured as gram changes in isometric tension and calculated as a percentage of the maximum response attained in the initial concentration-response curve. Agonist potency (defined as the $-\log EC_{50}$) was assessed by the relationship of Parker & Waud (1971), using non-linear iterative curve fitting procedures (RS1 software, BBN Corporation). Agonist affinities (defined as $-\log K_A$) were calculated according to the method of Furchgott (1966). Antagonist affinities (defined as the $-\log K_B$) were estimated by the method of Furchgott (1972). All statistically significant differences were determined by unpaired Student's t test, with $P<0.05$ being considered significant. All values quoted are expressed as mean ± s.e.mean, $n = 4$–8 animals.

Compounds used

4-DAMP mustard was generously donated by Dr R. Barlow, Univ. Bristol, UK. (+)-*cis*-dioxolane, racemic *para*-fluorohexahydro-siladiphenidol (pFHHSiD), methoctramine and phenoxybenzamine were purchased from Research Biochemicals Inc., Natick, MA, U.S.A. All remaining compounds were purchased from Sigma Chemical Co., St Louis, MO, U.S.A.

Results

Receptor characterization studies

The potency of (+)-*cis*-dioxolane at muscarinic receptors in atria and ileum of guinea-pig was similar ($-\log EC_{50}$ in atria = 7.8 ± 0.1 and in ileum = 7.6 ± 0.2). Receptor inactivation studies indicated that this was also true of the affinity ($-\log K_A$ in atria = 5.8 ± 0.2 and ileum = 6.0 ± 0.4). The potency ($-\log EC_{50} = 6.4 \pm 0.2$) of pilocarpine in ileum was less than that of (+)-*cis*-dioxolane, although the compound acted as a full agonist in this tissue with respect to (+)-*cis*-dioxolane. The affinity of pilocarpine, estimated from inactivation, was similar to its potency ($-\log K_A = 6.1 \pm 0.3$).

Muscarinic receptor subtypes mediating responses in guinea-pig atrium or ileum were characterized by determination of antagonist affinities. In atria, atropine (30 nM), methoctramine (0.1 μM) and pFHHSiD (10 μM) antagonized responses to (+)-*cis*-dioxolane with affinities ($-\log K_B$) of 8.9 ± 0.2, 7.8 ± 0.1 and 6.0 ± 0.3, respectively. In ileum these values against atropine, methoctramine, and pFHHSiD were 9.1 ± 0.2, 5.8 ± 0.4 and 7.8 ± 0.1, respectively. These experiments were repeated in ileum with pilocarpine as the

agonist and affinities ($-\log K_B$) for the antagonists were 9.1 ± 0.1, 6.0 ± 0.3 and 7.6 ± 0.2, respectively. In all studies, these antagonists dextrally shifted the concentration-response curve to (+)-*cis*-dioxolane in a parallel, surmountable fashion.

4-DAMP mustard studies

Equilibration of the tissues with 10 nM 4-DAMP mustard for 40 min resulted in small but significant dextral shifts (atria dose-ratio = 2.4 ± 0.3, ileum dose-ratio = 2.8 ± 0.4). However, equilibration with 4-DAMP mustard at 100 nM for 40 min dextrally shifted the concentration-response curves to (+)-*cis*-dioxolane in atria (dose-ratio = 14.2 ± 2.1) with no depression in maxima. In ileum, similar effects were seen although the dextral shift (dose-ratio = 197 ± 6) was larger and the maximum was significantly depressed.

Receptor protection studies

The reversibility of atropine, methoctramine and pFHHSiD were assessed in washout studies. In atria, following 60 min equilibration with atropine (30 nM), methoctramine (0.1 μM) or pFHHSiD (0.1 μM), control responses to (+)-*cis*-dioxolane were attained following washout at 10 min intervals for 120 min. In ileum, after exposure to similar concentrations of atropine and methoctramine and 3 μM pFHHSiD, control responses to the agonist were re-attained after a washout period of 180 min (data not shown).

In guinea-pig paced left atria (+)-*cis*-dioxolane elicited concentration-dependent decreases in isometric tension. There was no evidence for temporal changes in sensitivity to the agonist observed. After exposure to phenoxybenzamine (3 μM; 20 min), concentration-response curves were dextrally shifted and the maximal response reduced. Equilibration of the tissues with atropine (30 nM) or methoctramine (0.1 μM) inhibited these effects of phenoxybenzamine. In contrast, pFHHSiD (0.3 μM) failed to inhibit the effects of phenoxybenzamine since the effects after equilibration with this antagonist were similar to those seen with phenoxybenzamine alone. These data are shown in Figure 1.

In ileum, (+)-*cis*-dioxolane elicited concentration-dependent contractions and there were no significant temporal changes in the sensitivity. Phenoxybenzamine, reduced the maximal response and dextrally shifted the concentration-response curve. This effect was inhibited by atropine (30 nM) and pFHHSiD (3 μM) but not with methoctramine (0.1 μM). These data are shown in Figure 2. When pilocarpine was used as the agonist, a similar pattern was seen, in that atropine and pFHHSiD but not methoctramine protected against the effects of phenoxybenzamine. These data are shown in Figure 3.

Discussion

In ileal and tracheal smooth muscle, muscarinic M_3 receptors mediate contraction whereas M_2 receptors inhibit stimulation of adenylyl cyclase activity by β-adrenoceptors (Candell *et al.*, 1990). Selective alkylation of one muscarinic receptor subtype would facilitate understanding of the role of muscarinic M_2 and M_3 receptors in smooth muscle. This may be achieved either by the use of subtype selective alkylating agents or by selective protection by a reversible antagonist from a non-selective alkylating compound. In the present report these two approaches have been assessed in guinea-pig ileum.

The potency and affinity of (+)-*cis*-dioxolane at muscarinic M_2 and M_3 receptors were similar, suggesting that the agonist was not selective. Pilocarpine, although an agonist of lower intrinsic efficacy (Van Rossum *et al.*, 1960; Furchgott, 1966), was a full agonist. As expected, the potency of pilocarpine was similar to the affinity resulting in maximal occupancy at maximal responses. Muscarinic antagonist affinities were consistent with muscarinic M_2 receptors mediating negative inotropic responses in left atria and M_3 receptors mediating contractile responses in ileum (Eglen *et al.*, 1988b; 1990; Ford *et al.*, 1991).

4-DAMP mustard preferentially alkylated muscarinic M_3 receptors mediating contractions of guinea-pig ileum, compared with muscarinic M_2 receptors mediating negative inotropy. These data support functional (Barlow *et al.*, 1990) and radioligand binding data (Thomas *et al.*, 1992; Birdsall *et al.*, 1993). In the present study, however, exposure to 10 nM 4-DAMP mustard for 40 min resulted in only marginal antagonism. This is in contrast to data reported by Thomas *et al.* (1992), showing that 4-DAMP mustard (at 10 nM and 90 min exposure) resulted in a dose-ratio of 27 fold. An initial rapid, reversible antagonism due to formation of the aziridinium ion, has been shown in the antagonism of muscarinic M_3 receptors by 4-DAMP mustard (Barlow *et al.*, 1990), although the degree of inactivation remains constant after 30 min (Barlow *et al.*, 1990). This supports the use, in the present study, of 40 min exposure period. The selectivity observed at 100 nM in the present study and by other workers (Barlow *et al.*, 1990; Thomas *et al.*, 1992; Birdsall *et al.*, 1993) contrasts with data reported by Waelbroeck *et al.* (1992) who demonstrated that 4-DAMP mustard did not discriminate between muscarinic M_1–M_4 receptors. The reasons for these discrepancies require further study.

Overall, however, 4-DAMP mustard exhibited moderate selectivity between muscarinic M_2 and M_3 receptors (6.8 fold comparing muscarinic M_3 and M_2 receptor affinity; Thomas *et al.*, 1992). Consequently, its use in isolating pharmacologically the muscarinic M_3 receptor in tissues possessing heterogeneous populations is limited. It was therefore of interest to compare this approach with protection from alkylation by a reversible selective antagonist. One advantage of the latter technique is that reversible antagonists for muscarinic M_2 and M_3 receptors are available which are more selective than 4-DAMP mustard, including methoctramine (200 fold muscarinic M_2 selective) and pFHHSiD (63 fold muscarinic M_3 selective).

Furchgott (1966) has emphasized the importance of ensuring that the 'protecting' antagonist is reversible. In the present study, atropine, methoctramine and pFHHSiD acted reversibly since extensive washing allowed control responses to be re-attained. The longer time required to wash out pFHHSiD from ileum, in comparison to atria, was probably due to the high concentration employed in the latter. There was no significant change in sensitivity of the tissues to (+)-*cis*-dioxolane. These data provide confidence that dextral shifts in the concentration-response curves were attributable to the effects of the alkylating agent and thus inactivation of muscarinic receptors.

The ability of atropine to protect equally against alkylation in both atria and ileum, suggested that phenoxybenzamine, at the concentrations used, acted selectively to alkylate muscarinic receptors rather than through some non-specific action. In atria, atropine at 95% receptor occupancy, prevented receptor alkylation. Methoctramine, at 86% occupancy also inhibited the effects of phenoxybenzamine. A higher concentration of methoctramine was not used since, in ileum, it would occupy a higher proportion (>9%) of muscarinic M_3 receptors and act allosterically (Eglen *et al.*, 1988b). The inability of pFHHSiD, at 9% occupancy, to protect against alkylation also supports the presence of a preponderant muscarinic M_2 receptor population. In ileum, the converse was true since methoctramine, at a concentration that fully protected against alkylation in the atria, did not influence the effects of alkylation on contraction, whereas pFHHSiD fully protected against alkylation. This indicated that muscarinic M_3 receptors but not M_2 receptors participated in the contractile response to (+)-*cis*-dioxolane.

In ileum, similar protection was seen when pilocarpine was used as the agonist. This indicated that the technique could

be used against contractile responses associated with no receptor reserve. In bovine trachea, phosphoinositide metabolism stimulation by pilocarpine but not carbachol or oxotremorine (full and partial agonists, respectively) was sensitive to inhibition by β-adrenoceptor stimulated adenylyl cyclase activity (Offer *et al.*, 1991). These authors suggested that pilocarpine interacted with a muscarinic M_2 receptor that coupled to phosphoinositide hydrolysis and thus

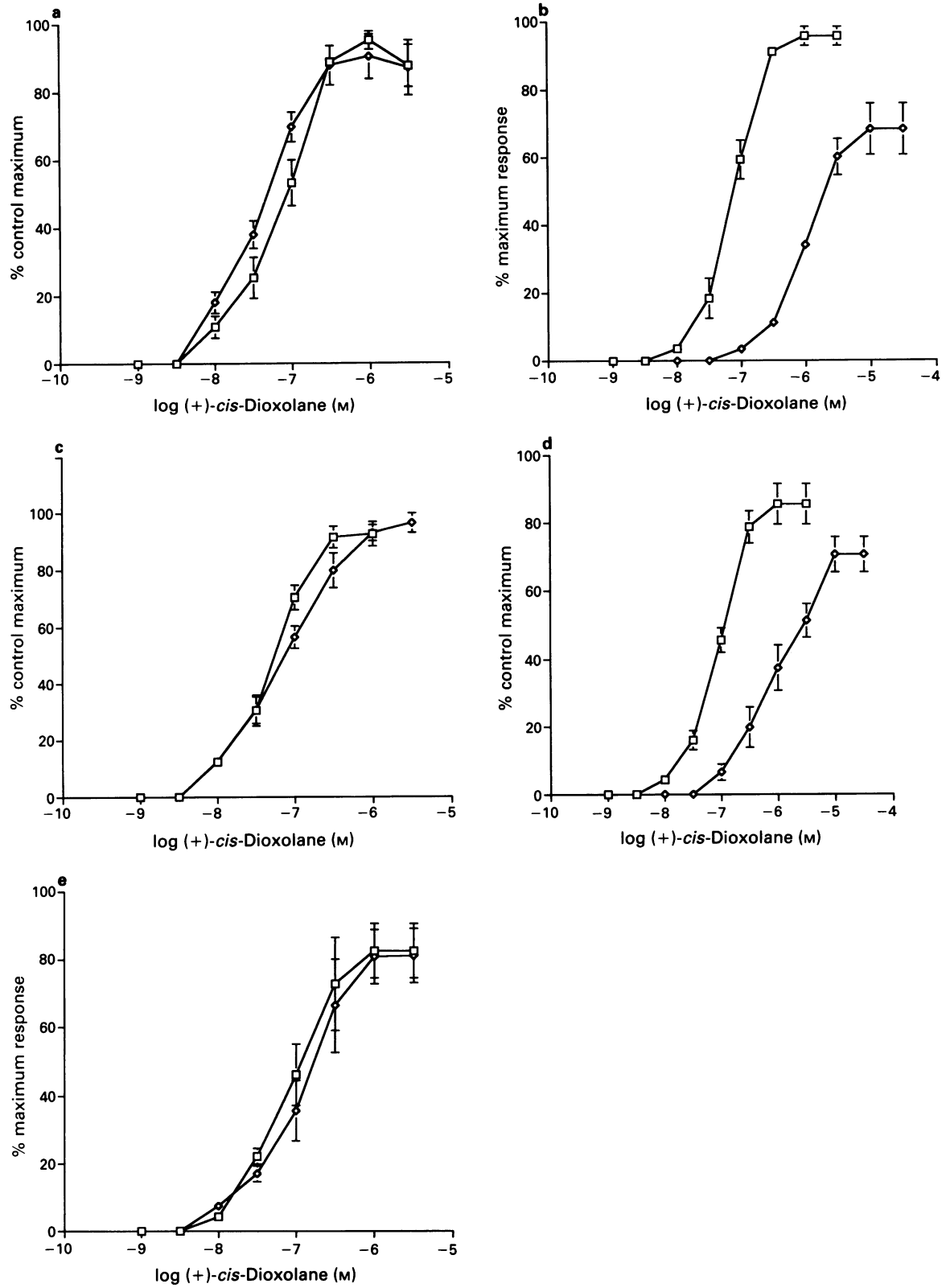


Figure 1 Effects of receptor protection on responses to (+)-*cis*-dioxolane in guinea-pig paced left atria. The time controls are shown in (a), effects of exposure to 3 μM phenoxybenzamine for 20 min in (b), effects of pre-equilibration with atropine (30 nM), pFHHSiD (0.1 μM) and methoctramine (0.1 μM) are shown in (c), (d) and (e), respectively. In these studies, tissues were washed at 10 min intervals for 120 min prior to construction of the second curve. Values are mean ± s.e.mean, from 4–8 animals. In this and subsequent figures, the control curve is denoted by (□) and the second curve established either in the presence of antagonist or time control is shown by (◇).

may mediate contraction. The antagonist affinity data and the effects of selectively inactivating muscarinic M_2 receptors does not support this contention in guinea-pig ileum.

A disadvantage of the use of phenoxybenzamine is that, at higher concentrations, it has the ability to depress smooth muscle contraction non-specifically. This did not occur in the present study as control responses were restored by muscarinic receptor blockade. After selective alkylation of muscarinic M_3 receptors, responses to either (+)-*cis*-dioxolane or pilocarpine were not abolished. It was possible that these

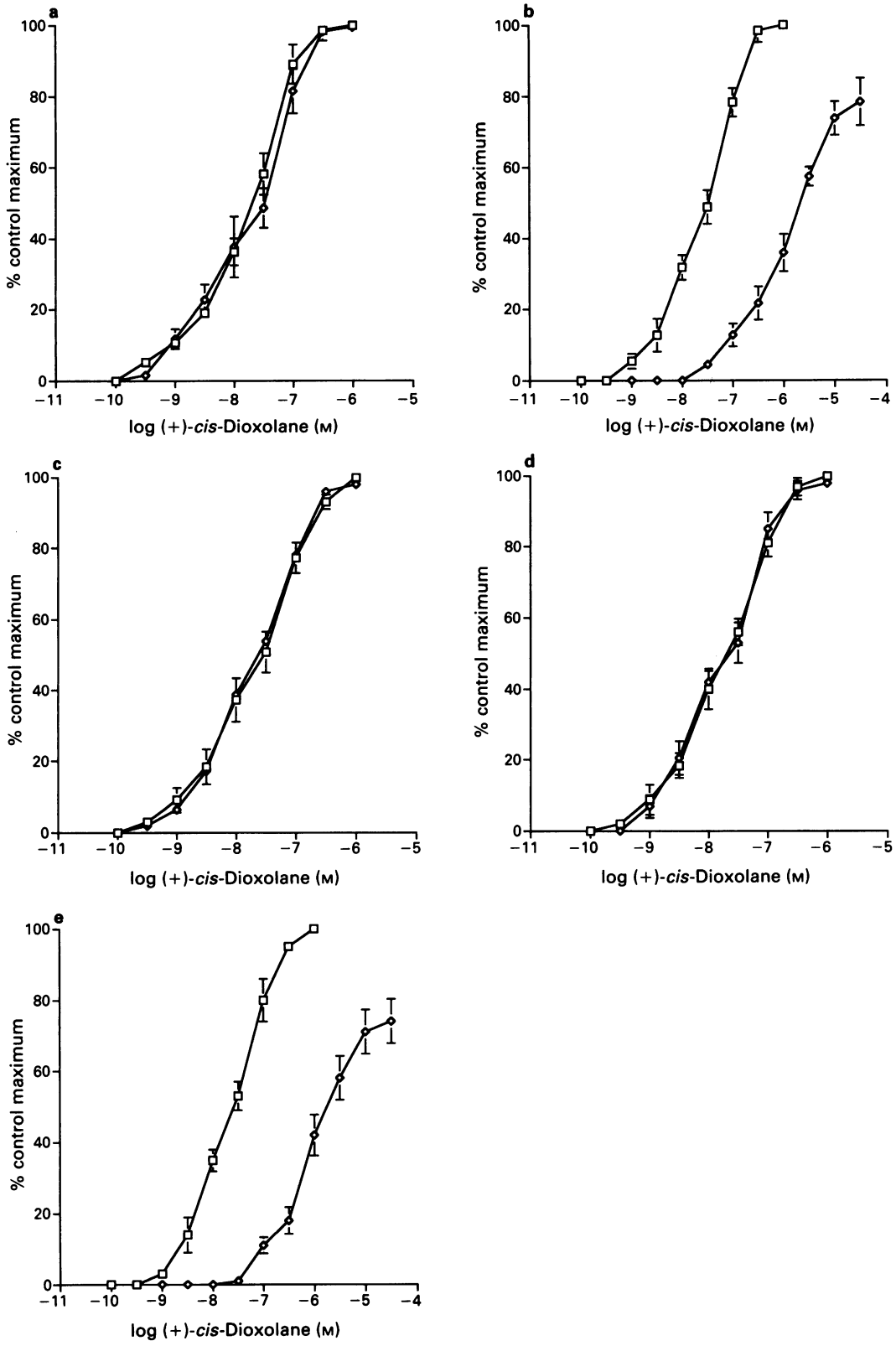


Figure 2 Effects of receptor protection on responses to (+)-*cis*-dioxolane in guinea-pig ileum. The time controls are shown in (a), effects of exposure to 10 μM phenoxybenzamine for 20 min in (b), effects of pre-equilibration with atropine (30 nM), pFHHSiD (3 μM) and methoctramine (0.1 μM) are shown in (c), (d) and (e), respectively. In these studies, tissues were washed at 10 min intervals for 180 min prior to construction of the second curve. Symbols as in Figure 1.

residual contractions were mediated by muscarinic M_2 receptors, as shown in guinea-pig uterus (Eglen *et al.*, 1992). Against this, these responses were antagonized by compounds with affinities consistent with activation of muscarinic M_3 receptors (Eglen & Harris, unpublished observations). It is probable that the muscarinic M_3 receptor population was incompletely alkylated by phenoxybenzamine under the conditions used in the present study. As the non-specific effects of this compound, described above, preclude its use at higher concentrations, additional experiments are required using alkylating agents such as propyl benzilyl choline mustard to ensure complete selective alkylation of muscarinic M_3 receptors.

Taken together, the data concur with the hypothesis that

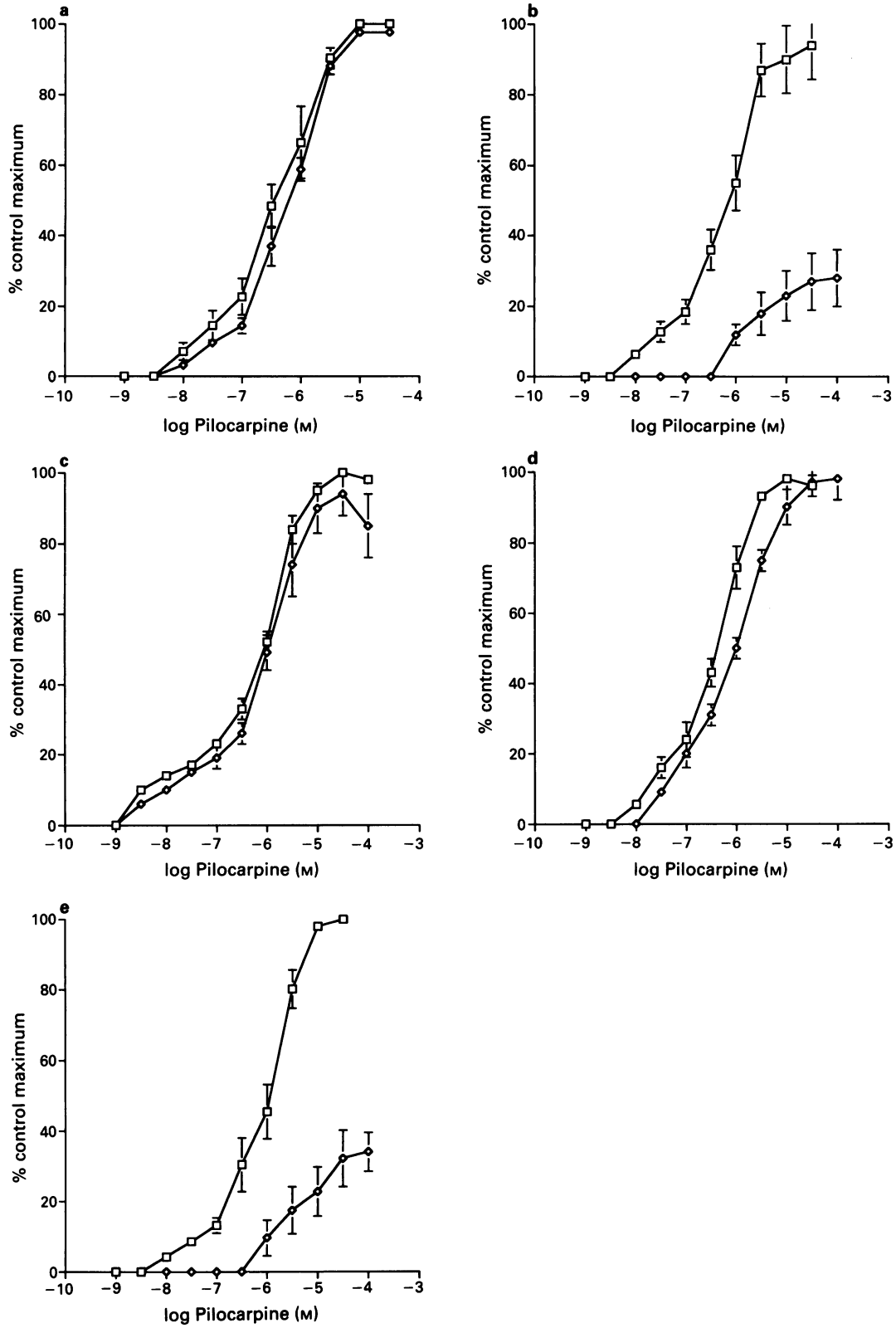


Figure 3 Effects of receptor protection on responses to pilocarpine in guinea-pig ileum. The time controls are shown in (a), effects of exposure to 10 μM phenoxybenzamine for 20 min in (b), effects of pre-equilibration with atropine (30 nM), pFHHSiD (3 μM) and methoctramine (0.1 μM) are shown in (c), (d) and (e), respectively. Symbols as in Figure 1.

muscarinic M_3 receptors mediate contractile responses in guinea-pig ileum even though muscarinic M_2 receptors predominate by a ratio of about 4:1 (Ford *et al.*, 1991). Evidence for a direct role of muscarinic M_2 receptors in ileal contraction was not found. This supports previous studies showing that contractions of guinea-pig ileum or canine colon to muscarinic agonists were unaffected by pertussis toxin (Eglen *et al.*,1988a; Zhang & Buxton, 1991), an agent that inhibits coupling of muscarinic M_2 receptors. The function of muscarinic M_2 receptors in ileal smooth muscle function thus remains to be clearly established.

It is concluded that, with appropriate care taken to establish conditions of antagonist reversibility and reproducibility of agonist responses, selective protection of either muscarinic M_2 or M_3 receptors in ileal smooth muscle can be achieved. In this respect, the approach may be preferable to the use of 4-DAMP mustard (Barlow *et al.*, 1990; Thomas *et al.*, 1992; Birdsall *et al.*, 1993) for which the M_2/M_3 selectivity is limited and has been questioned (Waelbroeck *et al.*, 1992). Selective protection enables muscarinic M_2 or M_3 receptors to be examined in isolation, potentially without requiring selective muscarinic M_2 or M_3 receptor agonists. Finally, by using other selective reversible antagonists such as pirenzepine, the approach permits selective alkylation of other muscarinic receptors, including the muscarinic M_1 or M_4 receptors, for which selective inactivation agents are unavailable (Baumgold *et al.*, 1992).

The authors thank Dr A.P.D.W. Ford for his advice in the preparation of the manuscript.

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(Received March 3, 1993
Accepted March 16, 1993)

Br. J. Pharmacol. (1993), **109**, 953–959 © Macmillan Press Ltd, 1993

Effect of extracellular ATP on contraction, cytosolic calcium activity, membrane voltage and ion currents of rat mesangial cells in primary culture

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1 The effects of extracellular ATP on contraction, membrane voltage (Vm), ion currents and intracellular calcium activity $[Ca^{2+}]_i$ were studied in rat mesangial cells (MC) in primary culture.

2 Addition of extracellular ATP (10^{-5} and 10^{-4} M) to MC led to a cell contraction which was independent of extracellular calcium.

3 Membrane voltage (Vm) and ion currents were measured with the nystatin patch clamp technique. ATP induced a concentration-dependent transient depolarization of Vm (ED_{50}: 2×10^{-6} M). During the transient depolarization ion currents were monitored simultaneously and showed an increase of the inward- and outward current.

4 In a buffer with a reduced extracellular chloride concentration (from 145 to 30 mM) ATP induced a depolarization augmented to -4 ± 4 mV.

5 ATP-γ-S and 2-methylthio-ATP depolarized Vm to the same extent as ATP, whereas α,β-methylene-ATP (all 10^{-5} M) had no effect on Vm.

6 The Ca^{2+} ionophore, A23187, depolarized Vm transiently from -51 ± 2 to -28 ± 4 mV and caused an increase of the inward current.

7 The intracellular calcium activity $[Ca^{2+}]_i$ was measured with the fura-2 technique. ATP stimulated a concentration-dependent increase of $[Ca^{2+}]_i$ (ED_{50}: 5×10^{-6} M). The increase of $[Ca^{2+}]_i$ was biphasic with an initial peak followed by a sustained plateau.

8 The $[Ca^{2+}]_i$ peak was still present in an extracellular Ca^{2+}-free buffer, whereas the plateau was abolished. Verapamil (10^{-4} M) did not inhibit the $[Ca^{2+}]_i$ increase induced by ATP.

9 The data indicate that extracellular ATP contracts MC and is able to increase $[Ca^{2+}]_i$ by the release of Ca^{2+} from intracellular stores and recruitment from the extracellular space. In addition ATP depolarizes Vm of MC by activating a Cl^- conductance. The ATP-induced depolarization is mediated by a P_{2y} receptor.

Keywords: Rat mesangial cell; P_{2y}-purinoceptor; contraction; Cl^- conductance; intracellular Ca^{2+} activity

Introduction

Extracellular adenosine 5'-triphosphate (ATP) has been shown to act as a co-transmitter and is able to modulate the function of a great variety of tissues via specific P_2 receptors (Drury & Szent-Györgyi, 1929; Burnstock, 1976; Gordon, 1986; von Kügelgen & Starke, 1991). In the kidney the release of a purinergic transmitter, probably ATP causes the vasoconstrictor response elicited by periarterial nerve stimulation (Schwartz & Malik, 1989). Therefore, ATP co-secreted with noradrenaline may also act on cells within the kidney. Indeed it has been recently shown that ATP increases $[Ca^{2+}]_i$ in glomerular epithelial- and endothelial cells (Marsden *et al.*, 1990; Pavenstädt *et al.*, 1992) and that mesangial cells respond to ATP in terms of phosphoinositol turnover via a P_2 receptor (Pfeilschifter, 1990a).

In pathological conditions such as glomerular injury, sources of ATP might be damaged cells (Born & Kratzer, 1984), endothelial cells and platelets, which contain high concentrations of ATP in their dense bodies (Gordon, 1986). Platelets are involved in the initial stage of glomerular injury and intraglomerular platelet activation may promote local proliferation of the mesangium by the release of vasoactive substances (Cameron, 1984; Klahr *et al.*, 1988). Proliferation of MC plays an important role in the inflammatory response to glomerular injury and is a prominent feature of most types of progressive glomerular diseases (Kashgarian & Sterzel, 1992). Recently it has been demonstrated that ATP stimulates proliferation of mesangial cells confirming that ATP might participate in inflammatory processes occurring in glomerulonephritis (Schulze-Lohoff *et al.*, 1992 and own observations).

Little is known about the ability of ATP to contract MC. Furthermore no data about an ATP-induced $[Ca^{2+}]_i$ response and a possible activation of ion currents by ATP are available. The present study investigates whether ATP is able to contract MC and to modulate $[Ca^{2+}]_i$ and ion currents in MC. In addition, the P_2 receptor involved in the ATP response has been characterized.

Methods

Cell culture

MC were cultured as previously described with few modifications (Pfeilschifter *et al.*, 1986). In brief, rat glomeruli were obtained by the sieve technique and incubated with collagenase IV (1 g l^{-1}, Sigma, Deisenhofen, Germany) for

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30 min. Glomeruli cores were then suspended in RPMI 1640 medium with 170 g l^{-1} foetal calf serum, L-glutamine 2.5 mM, sodium pyruvate 0.1 mM, penicillin 10^5 u l^{-1}, streptomycin 100 mg l^{-1}, non-essential amino acids 0.2% (all Seromed, Berlin, Germany) and insulin-transferrin-sodium selenite supplement 5 mg l^{-1} (Boehringer-Mannheim, Germany). Fifty glomeruli cm^{-2} were plated onto collagen-coated glass cover slips (Greiner, Nürtingen, Germany) and incubated at 37°C in a moist atmosphere containing 5% CO_2. MC outgrowth were characterized morphologically by phase-contrast microscopy, with positive staining for smooth muscle actin, desmin and vimentin. Cytokeratin and factor VIII did not stain, thus excluding glomerular epithelial- or endothelial cells. Furthermore MC contracted in response to angiotensin II. Therefore the cells studied showed the morphological, immunological and physiological properties as described by others for MC (Kreisberg & Karnovsky, 1983). All experiments were done between the 14th and the 30th day with MC in primary culture.

Contraction studies

For contraction studies, MC were kept at 37°C and perfused with a Ringer-like solution to which 5 mM D-glucose was added. MC were continuously observed under an inverted phase-contrast microscope (axiovert 10, Zeiss, Stuttgart, Germany). After addition of ATP, cell contraction was recorded by timed photographs.

Nystatin patch clamp experiments

The patch clamp methods used in these experiments have been described in detail in a previous paper by Greger & Kunzelmann (1991). MC were mounted in a bath chamber on the stage of an inverted microscope, kept at 37°C and perfused with a solution containing (in mM): NaCl 145, K_2HPO_4 1.6, KH_2PO_4 0.4, $CaCl_2$ 1.3, $MgCl_2$ 1, D-glucose 5, pH: 7.4. The patch pipettes were filled with a solution (in mM) containing K-gluconate 115, KCl 30, Na_2HPO_4 1.6, NaH_2PO_4 0.4, $CaCl_2$ 0.73, $MgCl_2$ 1.03, EGTA 1, D-glucose 5, pH 7.2, Ca^{2+} activity 10^{-7} M to which 10–100 mg l^{-1} nystatin was added. The patch pipettes had an input resistance of 5 MΩ. A flowing (10 µl h^{-1}) KCl (2 M) electrode was used as a reference. Vm was recorded continuously by a pen recorder (Gould, Seligenstadt, Germany). After achieving a GΩ seal, nystatin slowly permeabilized the cell-attached membrane by inducing a nonselective cation conductance (Marty & Finkelstein, 1975). In 20 experiments the cell attached membrane was only partially permeabilized (cell-attached nystatin = CAN experiments). In these experiments the input current was 0.33 ± 0.03 nS. In 101 experiments in the slow whole cell configuration (with a nystatin concentration > 50 mg l^{-1}) the cell membrane was permeabilized to a greater extent (slow whole cell = SWC configuration), the input current was 8.5 ± 0.9 nS. The Vm value was not different in all series. All experiments were done after a control period of about 5 min. The voltage (Vm) and the current (C) were followed before, during and after addition of the agonists. Currents were measured in the voltage clamp mode of the amplifier. Voltage steps of ± 40 mV (duration: 0.1 s) were applied to estimate cellular ion currents. In ion replacement experiments NaCl was replaced iso-osmotically by the substitution ion. To vary the free Ca^{2+} activity, the solutions were prepared according to established techniques with EGTA and NTA as Ca^{2+} buffers. The Ca^{2+} activity was calculated from a standard equation (Gögelein *et al.*, 1987) and was determined with a Ca^{2+} selective electrode (Radiometer, Copenhagen, Denmark).

$[Ca^{2+}]_i$ measurements

Measurement of $[Ca^{2+}]_i$ was performed with a set-up described recently by Nitschke *et al.* (1992). In brief, fura-2 fluorescence (excitation wavelengths: 340, 360, 380 nm, emission wavelength: 510 nm) was measured under an inverted fluorescence microscope (bath temperature: 37°C, bath solution changes within 1 s). The set up was based on a high speed filter wheel and a single photon tube (Hamamatsu H 3460-04, Hersching, Germany) allowing a time resolution of up to 200 Hz. MC were incubated with fura-2 (5×10^{-6} M) for 30 min at 37°C. Experiments were performed with a window including on average 10 cells. The measured noise and autofluorescence of the cells did not exceed 1% of the fura-2 fluorescence and was therefore not corrected. The calibration of the fura-2 fluorescence signal was attempted at the end of each experiment using the Ca^{2+} ionophore ionomycin (1 µM) and low and high Ca^{2+} buffers. $[Ca^{2+}]_i$ was calculated from the fluorescence ratio 340/380 nm according to the equation described by Grynkiewicz *et al.* (1985).

The following agents were used: adenosine 5'-triphosphate sodium salt (ATP), α,β-methylene-ATP, A23187, ionomycin, verapamil (Sigma Deisenhofen, Germany), ATP-γ-S tetrasodium salt (Boehringer-Mannheim, Mannheim, Germany), 2-methylthio-ATP tetrasodium salt (Biotrend, Köln, Germany).

Statistics

The data are given as mean values ± s.e.mean (n), where n refers to the number of experiments. Paired t test was used to compare mean values within one experimental series. A P value of <0.05 was accepted as indicating statistical significance.

Results

MC contraction in response to ATP

MC contracted within 30 s in response to ATP. In four different preparations for each ATP concentration, 10^{-5} M ATP contracted 20–40% and 10^{-4} M ATP 70–100% of approximately 40 observed MC in each experiment (Figure 1). The contraction took as long as 10 min to reach a maximum and was independent of extracellular Ca^{2+}.

Effect of ATP on Vm and ion currents of MC

In 101 experiments in the SWC configuration, a stable Vm of − 49 ± 1 mV and in 20 CAN experiments a stable Vm of − 48 ± 2 mV was obtained. Figure 2 shows the histogram of the frequency distribution of the Vm measurements in MC. Because the Vm values were not different in the SWC- or CAN configuration, the data from both series were pooled. ATP (10^{-5} M) led to depolarization of Vm from − 47 ± 1 mV to − 30 ± 2 mV (n = 65). The depolarization was transient, i.e. resting Vm was reached again after 1–2 min, although ATP was still present. Figure 3a shows an original recording and the concentration-response curve of the effect of ATP on Vm (Figure 3b). Like ATP, angiotensin II depolarized Vm of MC with an EC_{50} value of 10^{-9} M (n = 49, data not shown).

Effect of a reduced extracellular chloride concentration on the depolarization induced by ATP

The presence of a reduced extracellular Cl^- concentration (from 145 to 30 mM) did not change Vm of MC significantly (n = 9). Under these experimental conditions ATP (10^{-5} M) induced a significantly augmented depolarization of Vm to − 4 ± 4 mV (n = 9). Figure 4a shows an original recording of an experiment of the effect of ATP (10^{-5} M) on Vm in the absence and presence of a reduced extracellular Cl^- concentration. Figure 4b summarizes the data of the paired experiments.

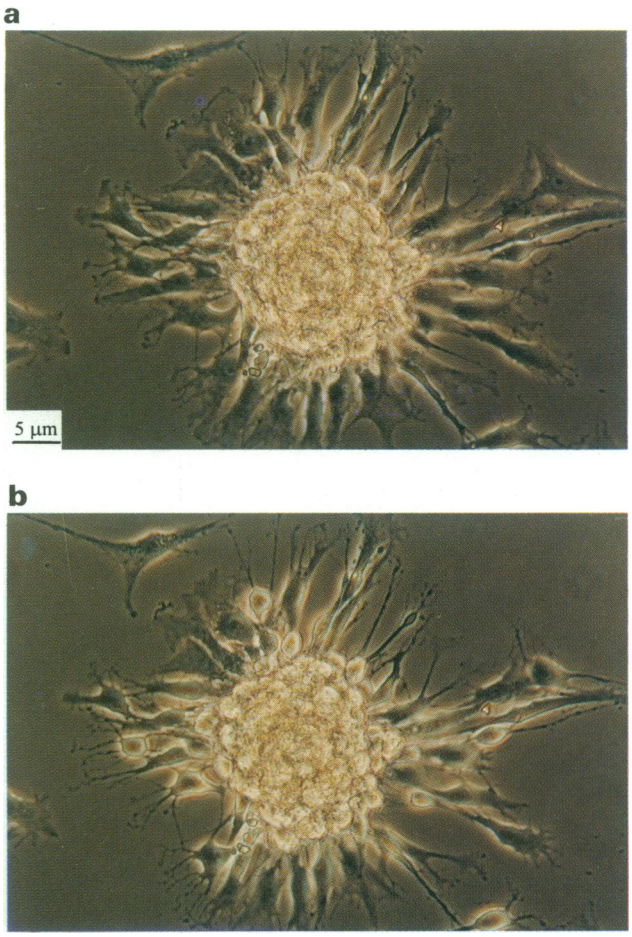


Figure 1 Phase-contrast microscopy of a glomerulus and rat mesangial cells in primary culture. (a) Cells in resting state; (b) cells 1 min after addition of ATP (10^{-4} M). Calibration bar, 5 µm.

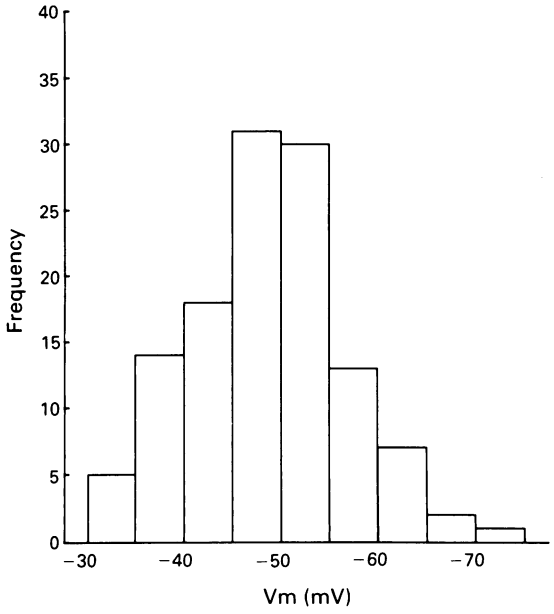


Figure 2 Frequency distribution of the membrane voltage of MC measured with the nystatin patch clamp technique. $\bar{x} = -49 \pm 1$ mV. $n = 121$.

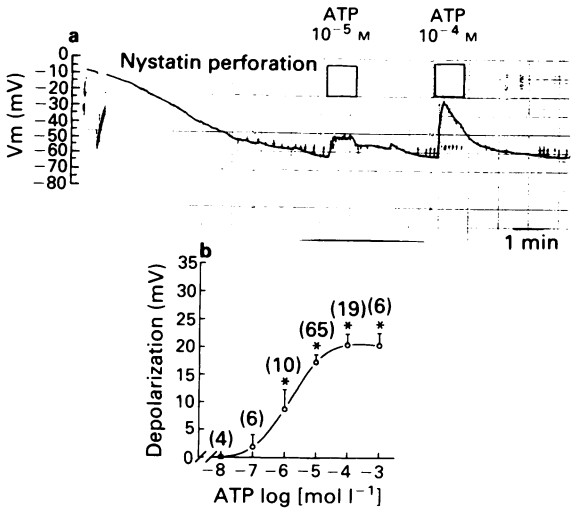


Figure 3 Effect of ATP on the membrane voltage (Vm) of MC. (a) Original recording of an experiment with ATP (10^{-5} and 10^{-4} M). Initially the voltage hyperpolarizes slowly to a steady state. This is caused by the incorporation of nystatin into the membrane patch. (b) Concentration-response curve for the depolarizing effect of ATP. *n* shown in parentheses.

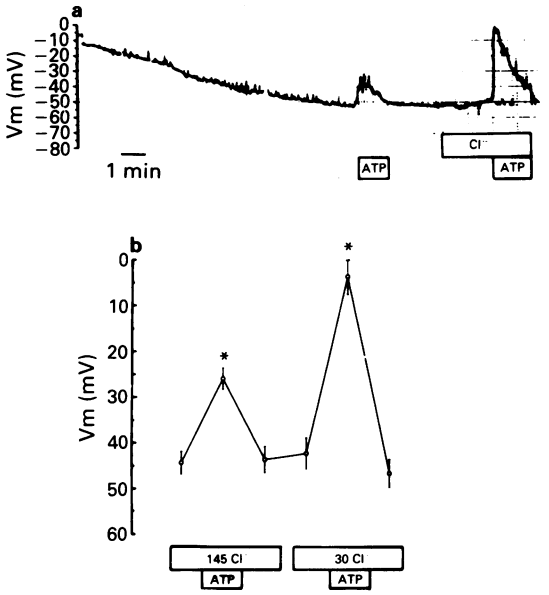


Figure 4 Effect of a reduction of the extracellular Cl^- concentration (from 145 to 30 nM) on Vm of MC in the absence and presence of ATP (10^{-5} M). (a) Original recording. (b) Summary of the experiments. Data points were collected at the peak of the depolarization induced by ATP. ATP depolarized significantly more in the presence of a low extracellular Cl^- concentration. $n = 9$.

Effect of ATP on ion currents in MC

In SWC experiments, ATP (10^{-5} M) induced a significant increase of the inward current by $18 \pm 3\%$ during the depolarization (cf. Figure 3a, peak). Thereafter, during the hyperpolarization phase (cf. Figure 3a, repolarization in the presence of ATP) the inward current collapsed. During this phase the outward current increased gradually to a maximum increment of $9 \pm 2\%$ (data not shown).

Characterization of the P_2 receptor

The depolarizing response of Vm to several ATP analogues was used to characterize the P_2 receptor of MC. ATP-γ-S and 2-methylthio-ATP (both 10^{-5} M) depolarized Vm to the same extent as ATP, whereas α,β-methylene-ATP (10^{-5} M) had no effect on Vm (all $n = 7$). In addition preincubation of MC with α,β-methylene-ATP (10^{-5} M, $n = 5$) did not inhibit the Vm-response to ATP. Figure 5 summarizes the data.

The Vm response to ATP is independent of extracellular Ca^{2+}

To examine whether a Ca^{2+} influx from the bath was necessary for the Vm response to ATP, ATP (10^{-5} M) was added to MC in the presence and absence of extracellular Ca^{2+}. Figure 6 summarizes the data: In the absence of extracellular Ca^{2+} ATP depolarized Vm to the same extent (by 25 ± 4 mV) as in the presence of extracellular Ca^{2+} (by 26 ± 4 mV, $n = 10$).

The Ca^{2+} ionophore A23187 stimulates a Cl^- conductance in MC

A23187 (10^{-6} M) depolarized Vm of MC (from -51 ± 2 to -29 ± 4 mV, $n = 20$). During the depolarization a marked increase of the inward conductance by $46 \pm 15\%$ was found (Figure 7a). In a buffer with a reduced extracellular Cl^- concentration (from 145 to 30 mM) the depolarization induced by A23187 was increased to -1.7 ± 2 mV, $n = 7$. Figure 7b summarizes the experiments of the effect of A23187 on Vm with normal and reduced extracellular Cl^- concentration.

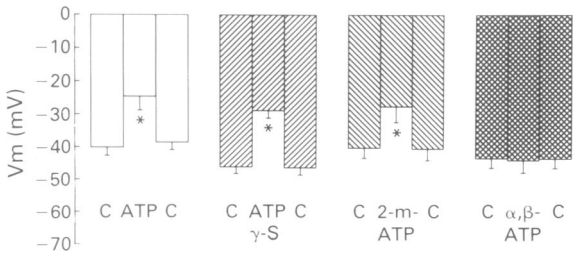


Figure 5 P_2 receptor characterization of MC. Summary of the Vm response to ATP and the synthetic ATP analogues ATP-γ-S, 2-methylthio-ATP (2-m-ATP), and α,β-methylene-ATP (α,β-ATP), all 10^{-5} M. Note that ATP, ATP-γ-S and 2-methylthio-ATP have a significant depolarizing effect, whilst α,β-methylene-ATP is devoid of any effect. C = control. $n = 7$–9.

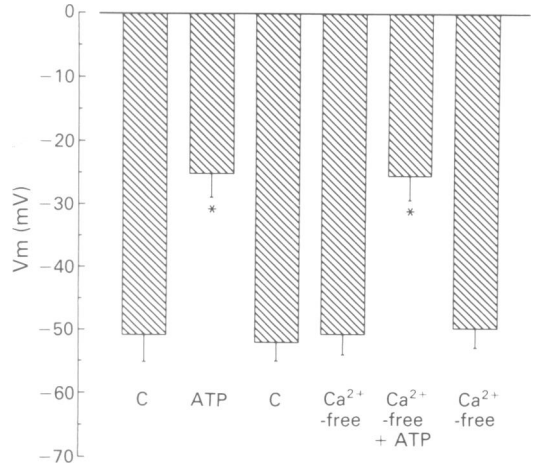


Figure 6 Summary of the effect of ATP (10^{-5} M) on Vm of MC in the presence and absence of extracellular Ca^{2+} (buffered with 1 mmol EGTA). Note that the ATP-induced depolarization occurs both in the absence and presence of extracellular Ca^{2+}. C = control.

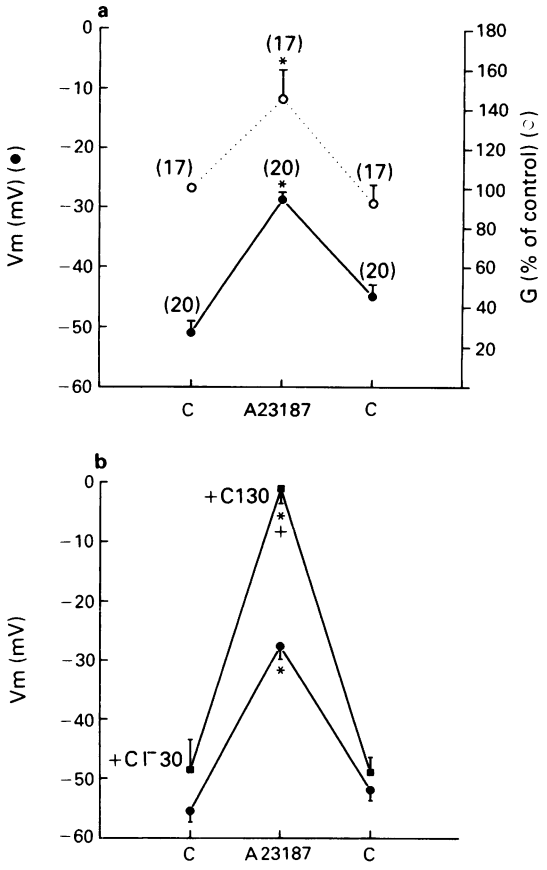


Figure 7 (a) Summary of the depolarizing response of Vm (●) and the increase in the inward current (○) of MC in response to A23187. A23187 depolarizes and increases the inward current significantly. (b) Summary of the effect of A23187 on Vm in a bath containing normal (145 nM) or reduced (30 nM) extracellular Cl^- concentration. Note that the depolarization induced by A23187 is augmented in the low Cl^- medium. $n = 6$.

ATP increases $[Ca^{2+}]_i$ in MC

Addition of ATP to single MC loaded with fura-2 resulted in a rapid and transient $[Ca^{2+}]_i$ peak followed by a plateau elevation above baseline values (ED_{50} about 5×10^{-6} M). Figure 8a and b show an original recording and the concentration-dependence of the $[Ca^{2+}]_i$ response to ATP. Like ATP, angiotensin II ($n = 9$), vasopressin ($n = 2$) and platelet derived growth factor ($n = 3$) led to an increase of $[Ca^{2+}]_i$ in MC (data not shown). In the absence of extracellular Ca^{2+}, the $[Ca^{2+}]_i$ peak induced by ATP was still present, whereas the plateau was reduced (Figure 9, $n = 9$). In addition, removal of the extracellular Ca^{2+} during the plateau phase of the ATP response led to a rapid decline of $[Ca^{2+}]_i$ towards basal levels. To examine whether a voltage-dependent Ca^{2+} channel was responsible for the ATP-induced plateau phase, the $[Ca^{2+}]_i$ response to ATP was examine in the presence and absence of verapamil (10^{-5} and 10^{-4} M, $n = 7$). Each experiment was performed in a paired fashion. Figure 10 shows that the $[Ca^{2+}]_i$ peak- and plateau induced by ATP was not reduced in the presence of verapamil.

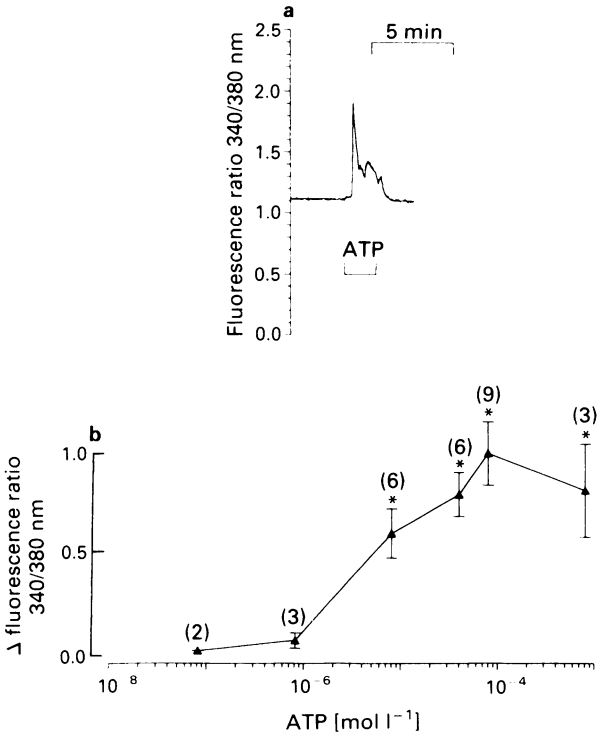


Figure 8 (a) Typical microfluorescence recording of the ATP-induced $[Ca^{2+}]_i$ increase in MC. The fluorescence ratio with 340/380 nm excitation is shown as a function of time. (b) Concentration-response curve of the effect of ATP on $[Ca^{2+}]_i$.

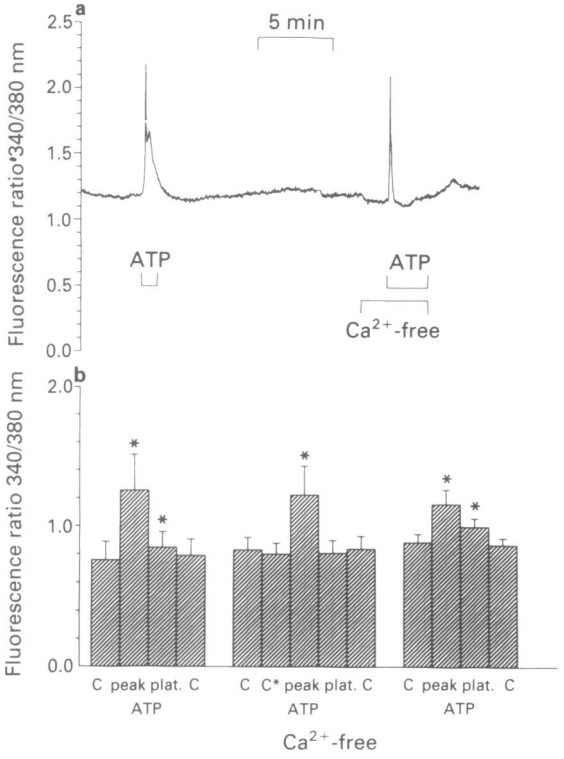


Figure 9 (a) Example of an experiment showing the effect of ATP on $[Ca^{2+}]_i$ in the presence and nominal absence of extracellular Ca^{2+}. Note that the ATP-induced peak is unaltered in the absence of extracellular Ca^{2+}. However, the sustained phase is missing. (b) Summary of the dependence of the ATP-induced $[Ca^{2+}]_i$ peak- and plateau on extracellular Ca^{2+} (C = control, C* = control without extracellular Ca^{2+}). Asterisks indicate significant differences between controls and ATP peak- and plateau values respectively. $n = 5$.

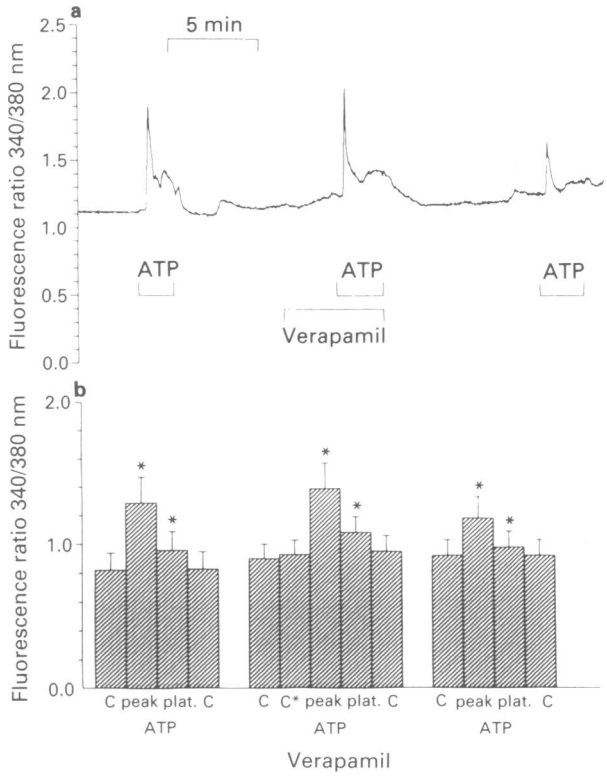


Figure 10 Verapamil did not inhibit the $[Ca^{2+}]_i$ increase induced by ATP. (a) Original experiment on the effect of ATP (10^{-5} M) on $[Ca^{2+}]_i$ in the absence and presence of verapamil (10^{-4} M). (b) Summary of the experiments (C = control, C* = control with verapamil). Asterisks indicate significant differences between controls and ATP peak and plateau values respectively. $n = 7$.

Discussion

The present study demonstrates that extracellular ATP exerts physiological effects, including contraction, stimulation of ion currents and increasing of $[Ca^{2+}]_i$ in MC. MC are smooth muscle-like cells, which contract in response to several agonists, like angiotensin II, vasopressin (Ausiello *et al.*, 1980), endothelin (Badr *et al.*, 1989), histamine (Sedor & Abboud, 1985) and platelet activating factor (Schlondorff *et al.*, 1984). The contraction induced by ATP was independent of the presence of extracellular Ca^{2+}. This fits well to data by others who demonstrated that the vasoactive agonist, angiotensin II, contracted MC independent of extracellular Ca^{2+} (Okuda *et al.*, 1986; Takeda *et al.*, 1988), whereas other groups showed a dependence on extracellular calcium of arginine vasopressin-induced contraction of MC (Venkatachalam & Kreisberg, 1985). The physiological consequences of MC contraction induced by ATP or other vasoactive substances still remains a matter of debate. On one hand, contraction of MC is considered to decrease the capillary loop diameter leading to a decrease of glomerular filtration rate, whilst on the other hand, biomechanical studies propose a more static role of mesangial cells (for review see Mene *et al.*, 1989; Kriz *et al.*, 1990).

The effect of ATP on Vm and ion currents was examined with a modified nystatin patch clamp technique, allowing for the simultaneous recording of Vm and ion currents (Greger & Kunzelmann, 1991). The Vm value of − 49 mV in MC measured with this technique is in agreement with Vm values measured by others with conventional glass microelectrodes or by fluorescence techniques (Okuda *et al.*, 1986; Kremer *et al.*, 1989). ATP led to a transient depolarization of Vm to − 30 mV, which is probably close to the Nernst equilibrium potential for Cl^-. When extracellular Cl^- concentration was

reduced, the depolarization was increased and Vm depolarized nearly to the new Nernst equilibrium potential for Cl^-. In addition, an increase of the inward current was found during the depolarization. This indicates that in MC the ATP induced depolarization is due to an activation of a Cl^- conductance. An activation of a Cl^- conductance by ATP was shown in epithelial cells (Kunzelmann *et al.*, 1992). In smooth muscle cells and other excitable cells ATP is known to activate cation channels (Benham & Tsien, 1987; Bean, 1992). A Cl^- channel activation in MC by angiotensin II and vasopressin has been reported previously (Okuda *et al.*, 1986; Kremer *et al.*, 1989).

The effect of extracellular ATP is mediated by P_2-purinoceptors, which are subdivided in P_{2x} and P_{2y} subtypes (Burnstock & Kennedy, 1985; Kenney, 1990). Since specific antagonists of the P_2 receptor subtypes are not available, the rank order of potency of different synthetic analogues of ATP was used to characterize the receptor. The rank order of potency of the agonists inducing a depolarization was ATP = ATP-γ-S = 2-methylthio-ATP > α,β-methylene-ATP = 0. Therefore, the receptor involved in the response to ATP showed similarities to the P_{2y} receptor (rank order of potency: 2-methylthio-ATP > ATP > α,β-methylene ATP). In addition, α,β-methylene-ATP, which was shown to desensitize the P_{2x} receptor in other tissues was not able to desensitize the P_2 receptor in MC (for review see Kennedy, 1990). A very similar P_2 receptor was recently described in long-term cultured MC (Pfeilschifter, 1990b). In contrast to our findings others have shown that ATP led to a proliferation of MC via a P_2 receptor different from the P_2 receptor observed in this study (ATP-γ-S > ATP > α,β-methylene-ATP > 2-methylthio-ATP = 0) (Schulze-Lohoff *et al.*, 1992). One explanation for these differences might be the fact that long-term effects of ATP on MC proliferation were examined in the latter study, whereas short-term effects of ATP on MC were studied in the present paper. Other explanations might be the existence of various P_2 receptors for different cellular functions in MC or a possible variability of the P_2 receptor in different subclones of long term cultured MC.

The ATP-induced activation of a Cl^- conductance shown here, was mimicked by the Ca^{2+} ionophore, A23187. Recently it has been shown that not only an increase of $[Ca^{2+}]_i$ but also the activaiton of protein kinase C by phorbol ester induced a Cl^- conductance in MC (Kremer *et al.*, 1992). Therefore, ATP may not only stimulate a Cl^- conductance in MC via an increase of $[Ca^{2+}]_i$ but also via an activation of protein kinase C. In fact, we also observed a marked depolarization of Vm upon stimulation of protein kinase C in MC with phorbol ester (own unpublished observations). In contrast to the ATP-induced depolarization, the phorbol ester-induced depolarization was not transient and lasted for minutes. Since the kinetics of the activation of the Cl^- conductance by ATP and A23187 were very similar, the ATP-induced increase of $[Ca^{2+}]_i$ may play the dominant role in activating a Cl^- conductance in MC. The effect of ATP on Vm was independent of the presence of extracellular Ca^{2+}, indicating that an intracellular $[Ca^{2+}]_i$ increase is mostly responsible for the depolarization induced by ATP. This observation fits well with studies revealing the effect of angiotensin II on Vm of MC (Okuda *et al.*, 1986).

To investigate further the cellular mechanisms of the ATP-induced Cl^- conductance $[Ca^{2+}]_i$ measurements were performed in MC. ATP produced a biphasic $[Ca^{2+}]_i$ increase with a transient peak and a sustained plateau. The plateau, but not the peak was dependent on extracellular Ca^{2+}. This indicates that ATP is capable of releasing $[Ca^{2+}]_i$ from intracellular stores, but also stimulates a calcium influx from the extracellular space. The intracellular component of the $[Ca^{2+}]_i$ increase is a possible result of an ATP-induced accumulation of inositol trisphosphate in MC which was recently reported (Pfeilschifter, 1990a). To determine whether the Ca^{2+} influx induced by ATP was due to an activation of the L-type voltage-dependent Ca^{2+} channel, experiments with verapamil were performed. The ATP-induced Ca^{2+} influx by ATP was not inhibited by verapamil. This indicates that not an L-type voltage-dependent Ca^{2+} channel, but a receptor-operated Ca^{2+} channel might be responsible for the Ca^{2+} influx induced by ATP. A Ca^{2+} influx through receptor-operated calcium channels in MC was recently postulated in response to endothelin and platelet-derived growth factor (Mene *et al.*, 1987; Simonson & Dunn, 1991), whereas the angiotensin II-induced Ca^{2+} influx in MC might be in part due to an activation of a voltage-dependent calcium channel (Takeda *et al.*, 1988).

In summary, ATP regulates important physiological effects such as contraction, activation of ion currents and stimulation of the Ca^{2+} signalling system in MC. Since endothelial cells, platelets and damaged cells are a source of ATP, ATP may be released during glomerular injury and participate in the initial pathogenic insult of MC.

We thank Mrs Monika von Hofer and Roswitha Weigel for their excellent technical assistance as well as Dr E. Schlatter for his helpful comments. Supported by DFG Pa 483/1-1.

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(Received December 7, 1992
Revised February 27, 1993
Accepted March 16, 1993)

Br. J. Pharmacol. (1993), **109**, 960–966 © Macmillan Press Ltd, 1993

Bronchodilatation of guinea-pig perfused bronchioles induced by the H_3-receptor for histamine: role of epithelium

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1 The influence of epithelium on the effects of H_3-histamine receptor agonist (**R**)α-methylhistamine [(**R**)α-MeHist] on airways was investigated on the guinea-pig perfused bronchioles.

2 In preparations under resting tone, removal of the bronchiolar epithelium or treatment with the cyclo-oxygenase inhibitor indomethacin (10^{-5} M) increased the constriction induced by histamine and acetylcholine in a concentration-dependent manner without an alteration of the K^+-induced contraction.

3 In this preparation (**R**)α-MeHist induced a concentration-dependent bronchodilatation which was antagonized in a competitive manner by thioperamide (an H_3-antagonist) with a pA_2 value of 8.6.

4 This bronchodilatation was reversed to a low concentration-dependent constriction after either removal of the epithelium or treatment with indomethacin (10^{-5} M) but was unaffected by both 10^{-5} M tranylcypromine (an inhibitor of PGI_2 synthesis) and 5×10^{-5} M N^G-nitro-L-arginine methyl ester (an inhibitor of NO synthesis).

5 It is suggested that, in guinea-pig perfused bronchioles (**R**)α-MeHist induces an epithelium-dependent relaxation via the release of metabolite(s) of arachidonic acid.

Keywords: Guinea-pig perfused bronchioles; epithelium; prostaglandins; (**R**)α-methylhistamine

Introduction

The airway epithelium is known to exert an influence on tracheal or bronchial smooth muscle reactivity. It has been reported that airway hyperresponsiveness can be induced following epithelial damage in animals to a variety of bronchoconstrictor agents such as histamine, acetylcholine, 5-hydroxytryptamine, substance P and adenosine (Flavahan *et al.*, 1985; Goldie *et al.*, 1986; Tschirhart & Landry, 1986; Hay *et al.*, 1987; Stuart-Smith & Vanhoutte, 1987; Advenier *et al.*, 1988; Munakata *et al.*, 1988; Morisson *et al.*, 1990). It was demonstrated that the removal of airway epithelium increases the responsiveness of smooth muscle, suggesting that the epithelial layer may be involved in the inhibitory effect on smooth muscle responses (Flavahan *et al.*, 1985; Barnes *et al.*, 1985; Hay *et al.*, 1987; Fernandes & Goldie, 1990).

The influence of epithelial cells on the responsiveness to bronchial smooth muscle relaxants is more complex. On guinea-pig trachea without epithelium, the relaxant activity of a β_2-selective agonist, isoprenaline, was either increased (Farmer *et al.*, 1986; Hay *et al.*, 1986) or unmodified (Goldie *et al.*, 1986; Aizawa *et al.*, 1988) while that of papaverine was unchanged (Farmer *et al.*, 1986). In similar experiments on canine or bovine isolated trachea deprived of epithelium, the airways responsiveness to a β_2-adrenoceptor agonist was reduced (Barnes *et al.*, 1985; Flavahan *et al.*, 1985; Stuart-Smith & Vanhoutte, 1987). Studies using a 'sandwich' preparation (Tschirhart & Landry, 1986) or coaxial bioassay system (Ilhan & Sahin, 1986) have provided direct evidence for the existence of airway epithelium-derived relaxing factor(s) (EpDRF). Although the phenomenon of potentiation of bronchoconstriction by removal of the epithelium is established, the mechanism underlying it is still a matter of debate. Several bioassay studies suggest that epithelium can secrete diffusible factors that relax bronchial or vascular smooth muscle (Tschirhart & Landry, 1986; Fernandes *et al.*, 1989). However, little is known about the nature of EpDRF. Some studies show that the EpDRF cannot be similar to the endothelium derived relaxing factor (EDRF) described by Furchgott & Zawadzki (1980). Then Morisson *et al.* (1990) and Munakata *et al.* (1990) demonstrated that EpDRF was pharmacologically different from nitric oxide. On the other hand, it is possible that EpDRF contains metabolites of arachidonic acid, in particular prostaglandins E_1 and E_2, but also nonprostanoid substances, according to different authors (Large *et al.*, 1969; Rosenthal *et al.*, 1971; Adaikan & Karim, 1979; Shore *et al.*, 1985; Douglas & Brink, 1987; Tschirhart *et al.*, 1987; Braunstein *et al.*, 1988).

In 1988, Guc *et al.* demonstrated that histamine induced an epithelium-dependent relaxation of guinea-pig tracheal smooth muscle via non-H_1- and non-H_2-histamine receptors.

Recently, we have shown that (**R**)α-methylhistamine [(**R**)αMeHist], a potent and selective H_3-agonist (Arrang *et al.*, 1987), induced a concentration-dependent relaxation on the resting tone of the guinea-pig perfused bronchioles (Burgaud *et al.*, 1992).

The first aim of the present study was to determine whether guinea-pig airway epithelium possesses the ability to decrease the responsiveness of smooth muscle. The second aim was to clarify whether (**R**)α-MeHist induces an epithelium-dependent relaxation on resting tone of guinea-pig perfused bronchioles.

Methods

Measurement of mechanical activity

We have modified the perfusion technique of Oudart *et al.* (1981) in the rabbit cerebral artery according to Burgaud *et al.* (1992) in guinea-pig perfused bronchioles. Hartley guinea-pigs of either sex weighing 400–500 g were anaesthetized with urethane (1.5 g kg^{-1}, i.p.) and bled. The lungs were quickly removed and placed in modified Krebs-Henseleit solution. This solution contained (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.6, KH_2PO_4 1.2, $NaHCO_3$ 24.9 and glucose 11, and was gassed with 5% CO_2/95% O_2 with pH of 7.3–7.45.

An intact bronchiolar segment of the pulmonary cardiac lobe (0.3 mm i.d., about 5 mm long) was cannulated with a short, polished hypodermic needle (0.4 mm o.d.). Before cannulation, in some bronchioles, the epithelium was removed by gently rubbing the luminal surface with a pipe cleaner.

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Then the cannulated bronchiole was placed in an organ bath at 37°C and perfused at a constant rate of 1.0 ml min^{-1} by means of a peristaltic pump. Perfusion pressure which was used as an index of contraction or relaxation was measured continuously via a T-connection placed just before the cannula and was set at 51 ± 2 mmHg ($n = 217$) and 56 ± 5 mmHg ($n = 84$) in the intact bronchioles with or without 10^{-5} M indomethacin respectively and 54 ± 4 mmHg ($n = 61$) in the bronchioles without epithelium. These values showed that treatments did not modify significantly the resting tone of the guinea-pig perfused bronchioles. This pressure had been determined in a preliminary study by measuring the maximal bronchoconstriction or bronchodilatation to a constant concentration of acetylcholine or theophylline respectively (Figure 1).

Protocol

The bronchiolar segments were allowed to equilibrate for over 90 min in the buffer solution before tests. During this time the basal tone increased a little to reach a steady plateau. Increasing concentrations of histamine, acetylcholine, potassium chloride or (R)α-MeHist were added to the perfusate reservoir in a cumulative manner. The interval between consecutive concentrations did not exceed 10 min. In some experiments, tissues were pretreated for 30 min with either the H_1-antagonist mepyramine (10 μM), the H_2-antagonist cimetidine (10 μM), the cyclo-oxygenase inhibitor, indomethacin (10 μM; Orehek *et al.*, 1975; Korbut *et al.*, 1981), the NO synthetase inhibitor L-NAME (1 and 50 μM; Rees *et al.*, 1990; Tucker *et al.*, 1990) or the prostacyclin synthetase inhibitor tranylcypromine (10 μM; Gryglewski *et al.*, 1975; Bakhle & Ferreira, 1985; Ea-Kim *et al.*, 1992). In our model, at these concentrations, the cyclo-oxygenase-, NO synthetase- and prostacyclin synthetase-inhibitors did not modify significantly the baseline pressure when added to the perfusate reservoir.

At the end of each test, 10^{-4} M papaverine was added to the perfusate reservoir to induce a maximal bronchodilation (27 ± 2 mmHg; $n = 103$) as an internal reference (Farmer *et al.*, 1986).

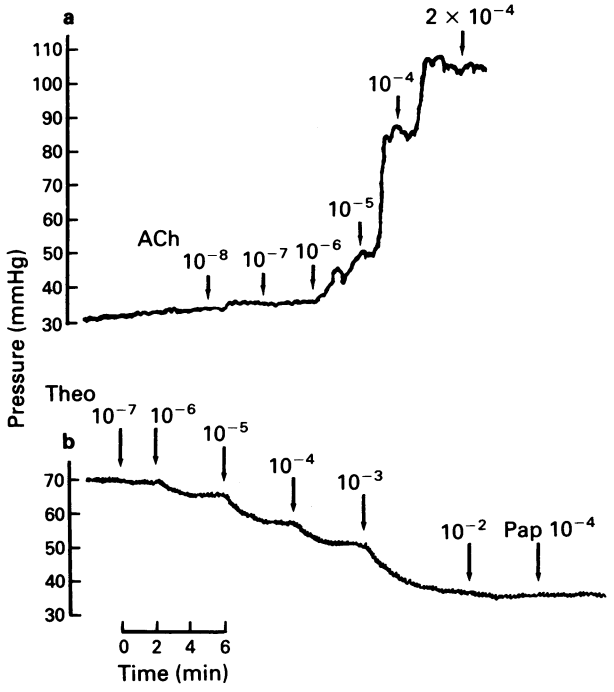


Figure 1 Representative tracings of effect of acetylcholine (ACh) (a) and theophylline (Theo) (b) in guinea-pig perfused bronchioles. Pap 10^{-4} M is papaverine (10^{-4} M). Traces are redrawn from originals.

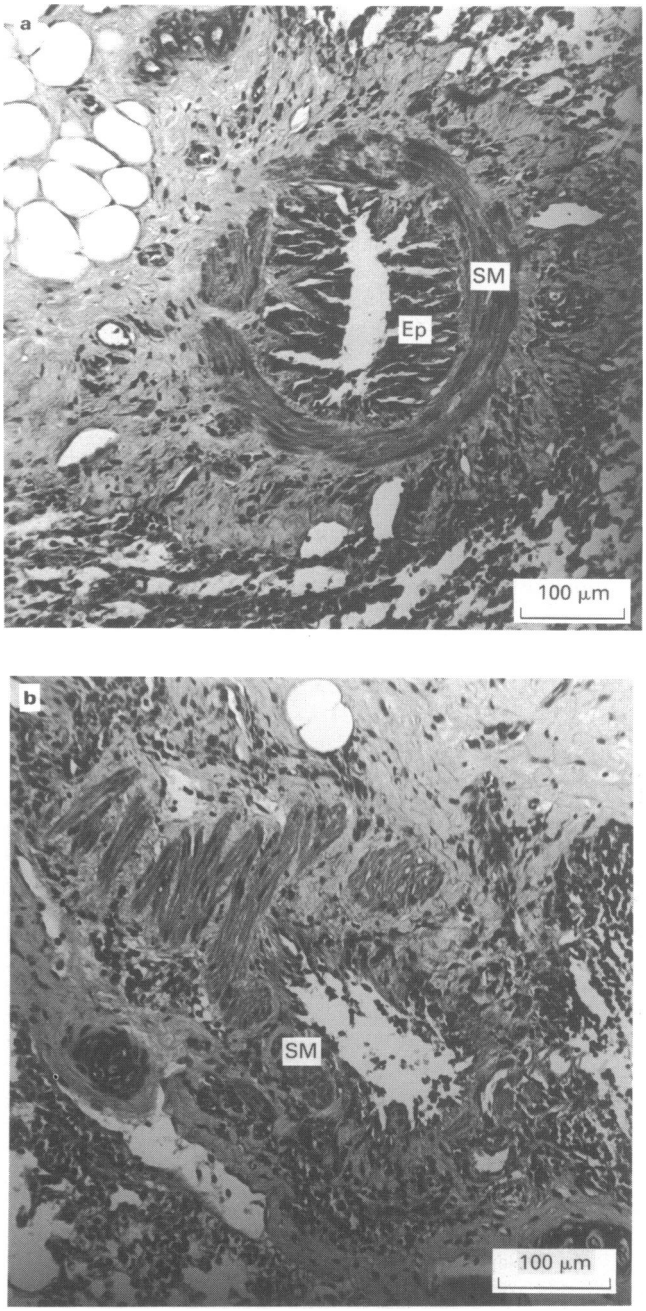


Figure 2 Histological sections (see Methods) demonstrating that gentle rubbing of the luminal surface of bronchioles (b) denudes the preparation of epithelial cells (control: a). Ep: epithelium; SM: smooth muscle.

Histological method

At the end of each experiment, the perfused bronchioles with or without epithelium were stored in Bouin solution for subsequent histological examination. Paraffin embedded specimens were stained with haematoxylin-eosin-safran and after sectioning at 4 μm, examined by light microscopy (× 32) (Bloom & Fawcett, 1968).

Data analysis

Pressure rises are expressed as a percentage of the maximum contraction (pressure rise) induced by each agonist, whereas relaxation is expressed as a percentage of the maximum relaxation induced by 10^{-4} M papaverine. The pD_2 were

established from the concentration-response curves plotted for each agonist in each segment. The slopes of the log concentration-response curves, pD_2 and E_{max} were compared by analysis of variance when we compared results obtained with different treatments (Wallenstein *et al.*, 1980). Differences were considered significant when $P<0.05$. Dispersion of values about the mean were indicated by the standard error of mean (s.e.mean).

Drugs

The drugs used were: histamine dihydrochloride, acetylcholine chloride, N^G-nitro-L-arginine methyl ester (L-NAME), tranylcypromine (Sigma, U.S.A.), (**R**)α-methylhistamine thioperamide (Bioprojet, France), papaverine hydrochloride (Méram, France), indomethacin (Merck Sharp et Dohme-Chibret), mepyramine, cimetidine (SmithKline Beecham). (**R**)α-MeHist was diluted in water and stored as aliquote (100 µl) at − 20°C and used as required. Thioperamide was dissolved in dimethylsulphoxide (DMSO) and stored at 0–5°C. Indomethacin was dissolved in ethanol.

Bouin solution had the following composition: picric acid, saturated solution 75 ml, formaldehyde (37% solution) 25 ml, acetic acid, glacial 5 ml.

Results

Histological results

Light microscopic examination of a paraffin section of bronchiolar segment (0.3 mm, i.d.) revealed the morphology of airways usually classified as terminal bronchioles. Bronchioles were a homogeneous preparation because a histological study shows that our bronchiolar segments contain only epithelial and smooth muscle cells unlike trachea and lung parenchymal strips which also contain chondrocytes, non muscular contractile cells and blood vessels. All these structures can react to the different substances and by contraction of relaxation. Figure 2a shows that the flow and the pressure of perfusion did not injure the pseudostratified epithelium during the experiment (3 h). The use of a pipe cleaner probe was found consistently to cause complete removal of epithelium without damaging other tissue structures (Figure 2b).

Response to histamine

Figure 3 shows that histamine caused a biphasic response in guinea-pig perfused bronchioles which resulted in a small relaxation response at low concentrations (3×10^{-11}–3×10^{-8} M) and a concentration-dependent contraction with higher concentrations (3×10^{-7}–10^{-4} M). The pD_2 value was equivalent to 5.73 ± 0.09 ($n = 7$).

Influence of epithelium and indomethacin (10^{-5} M)

The data presented in Figure 3 show that the rubbed preparations or those treated with indomethacin (10^{-5} M) exhibited an increased maximal response and sensitivity to histamine. The maximal response of intact preparations (49 ± 11 mmHg; $n = 7$) was significantly different from that obtained in rubbed tissues (110 ± 15 mmHg; $n = 10$; $P<0.05$) and in the presence of indomethacin (105 ± 14 mmHg; $n = 7$; $P<0.05$). The histamine sensitivity (pD_2 values) was also significantly different in these preparations [intact: 5.73 ± 0.09, rubbed: 6.2 ± 0.07; $P<0.05$, with indomethacin: 7.34 ± 0.19; $P<0.05$ and rubbed with indomethacin: 6.15 ± 0.06; $P<0.05$]. Table 1 shows that the removal of the epithelial wall increased the sensitivity to acetylcholine (10^{-8}–6×10^{-4} M) without an alteration in maximal response. However, there was no difference in the K^+-induced perfusion pressure increasing between the two preparations in the range (4.7×10^{-3}–1.2×10^{-1} M).

Effect of thioperamide

In the presence of 10^{-7} M thioperamide the concentration-response curve for histamine was shifted to the left with a significant change of the E_{max} (90 ± 7 mmHg; $n = 6$; $P<0.05$)

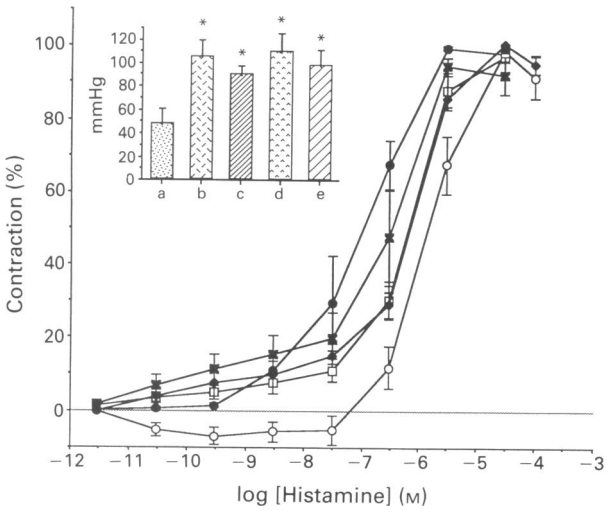


Figure 3 Concentration-response curves of guinea-pig perfused bronchioles to histamine: alone (○) ($n = 7$) or without epithelium (□) ($n = 10$), in presence of 10^{-7} M thioperamide (●) ($n = 6$) or in presence of 10^{-5} M indomethacin with (×) ($n = 8$) or without epithelium (◆) ($n = 6$). The ordinate scale represents the response as a % of the maximal response. Points represent the mean with s.e.mean. The inset represents the E_{max} (mmHg) for the response to histamine: alone (a), in presence of 10^{-5} M indomethacin (b), in presence of 10^{-7} M thioperamide (c) and without epithelium in absence (d) or in presence of 10^{-5} M indomethacin (e). Differences from control are considered significant when $^*P<0.05$.

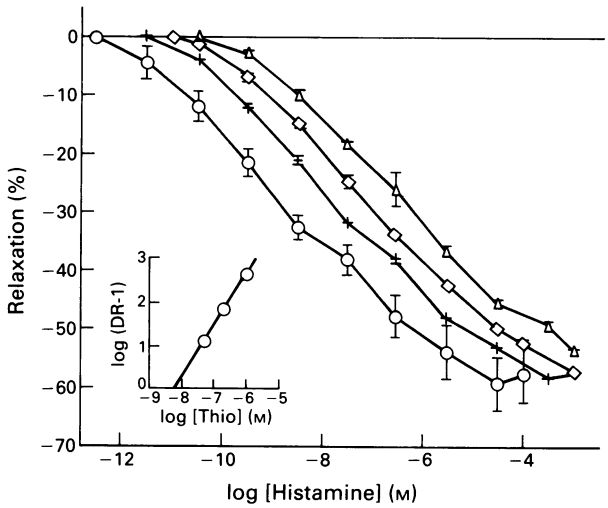


Figure 4 Effects of histamine in presence of 5×10^{-6} M mepyramine and 10^{-5} M cimetidine: alone (○) and with 5×10^{-8} M (+), 2×10^{-7} M (◇) or 10^{-6} M (△) thioperamide in guinea-pig perfused bronchioles. The pD_2 values were (9.1 ± 0.25), (7.77 ± 0.02), (7.12 ± 0.09) and (6.38 ± 0.17), respectively, The ordinate scale represents the response as a % of the maximal relaxation induced by 10^{-4} M papaverine. Each point represents the mean with s.e.mean, $n = 6$. The inset represents a Schild plot of the data, dose-ratio (DR) being determined from EC_{50} values for histamine. The coefficient of correlation was 1 and the pA_2 was 8.2.

Table 1 Effect of epithelium (Ep) removal on the sensitivity of guinea-pig perfused bronchioles to contractile drugs

	With Ep (control)		*Without Ep*	
	E_{max} (mmHg)	pD_2	E_{max} (mmHg)	pD_2
Acetylcholine	78.2 ± 12.9 ($n = 7$)	5.71 ± 0.09	77.8 ± 18 ($n = 8$)	6.73 ± 0.27*
K^+	147.3 ± 27.0 ($n = 14$)	1.62 ± 0.02	162 ± 31 ($n = 6$)	1.62 ± 0.03

Values show mean ± s.e.mean; n = number of experiments.
Differences/control are considered significant when $*P < 0.05$.

(Figure 3). The pD_2 of histamine in the presence of the H_3-antagonist was 6.76 ± 0.07 ($n = 6$) ($P < 0.05$). Furthermore we observed that the small relaxation induced by histamine (3×10^{-11}–3×10^{-8} M) was abolished.

Histamine in presence of 5×10^{-6} M mepyramine (H_1-antagonist) and 10^{-5} M cimetidine (H_2-antagonist) induced a concentration-dependent relaxation in the range (3×10^{-13}–10^{-4} M) reaching (57 ± 5)% of papaverine-induced maximal relaxation (Figure 4). The pD_2 value and slope of the regression line were 9.10 ± 0.25 and (−8.1 ± 0.8)%/log unit, respectively ($n = 6$). When 5×10^{-8} M, 2×10^{-7} M or 10^{-6} M thioperamide was present, the concentration-response curve for histamine-induced relaxation was shifted to the right without an alteration in E_{max}. The pD_2 of histamine in the presence of the H_3-antagonist (5×10^{-8} M) was 7.77 ± 0.02. Schild plot analysis indicated that antagonism by this compound was competitive, the slope for the regression of log (DR-1) versus log [thioperamide] was 1.15 ± 0.10 with a pA_2 value of 8.2 ($n = 6$).

Response to (R)α-methylhistamine

(R)α-MeHist (10^{-13}–10^{-5} M) induced a concentration-dependent relaxation of guinea-pig perfused bronchioles at resting tone reaching approximately (60 ± 2)% of papaverine-induced maximum dilatation with a pD_2 equivalent to 10.28 ± 0.29 (Figure 5). When 10^{-8} M, 10^{-7} M, 10^{-6} M or 10^{-5} M thioperamide was present, the concentration-response curve for (R)α-MeHist-induced relaxation was shifted to the right without change of the E_{max}. Schild plot analysis indicated that antagonism by this compound was competitive, the slope for the regression of log (DR-1) versus log [thioperamide] was 0.97 ± 0.15 with a pA_2 of 8.6. Thioperamide alone (10^{-10}–10^{-5} M) did not elicit any modification of the resting tone.

Effect of removal of epithelium

In the guinea-pig perfused bronchioles, removal of the epithelium reversed the relaxation induced by the H_3-agonist to a low concentration-dependent constriction (Figure 6) in the range 10^{-13}–10^{-7} M with an E_{max} of (21 ± 5.5)% (17.5 ± 4 mmHg) of the maximum 10^{-4} M acetylcholine-induced contraction and 10.0 ± 3.1% of the maximum 1.2×10^{-1} M K^+-induced contraction obtained at a concentration of around 3×10^{-7} M ($n = 10$).

Effect of indomethacin (10^{-5} M)

On the other hand, in the presence of 10^{-5} M indomethacin, the H_3-agonist induced a concentration-dependent constriction similar to the effect of (R)α-MeHist on guinea-pig perfused bronchioles without epithelium (Figure 6). The E_{max} was (27.7 ± 5.2)% of the maximum 10^{-4} M acetylcholine-induced contraction; it was obtained at a concentration of around 10^{-6} M ($n = 12$).

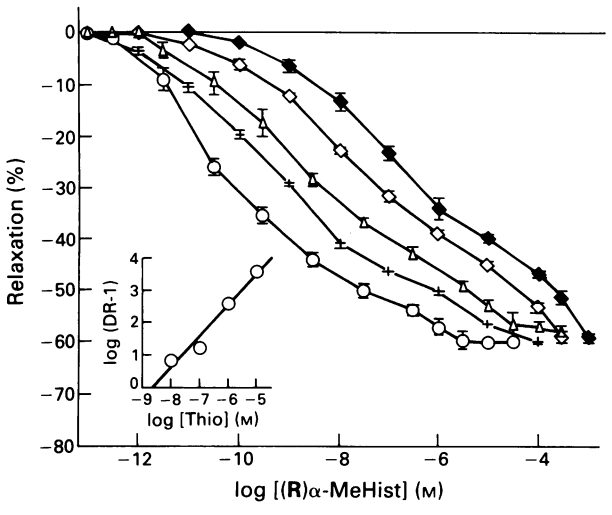


Figure 5 Effects of (R)α-methylhistamine [(R)α-MeHist] alone (○) ($n = 9$) and with 10^{-8} M (+) ($n = 6$), 10^{-7} M (△) ($n = 11$), 10^{-6} M (◇) ($n = 6$) or 10^{-5} M (◆) ($n = 6$) thioperamide in guinea-pig perfused bronchioles. The pD_2 values were (10.28 ± 0.29), (8.94 ± 0.01), (8.74 ± 0.15), (7.25 ± 0.05) and (6.38 ± 0.17), respectively. The ordinate scale represents the response as a % of the maximal relaxation induced by 10^{-4} M papaverine. Each point represents the mean and vertical bars the s.e.mean. The inset represents a Schild plot of the data, dose-ratio (DR) being determined from EC_{50} values for (R)α-MeHist. The correlation coefficient was 0.98 and the pA_2 was 8.6.

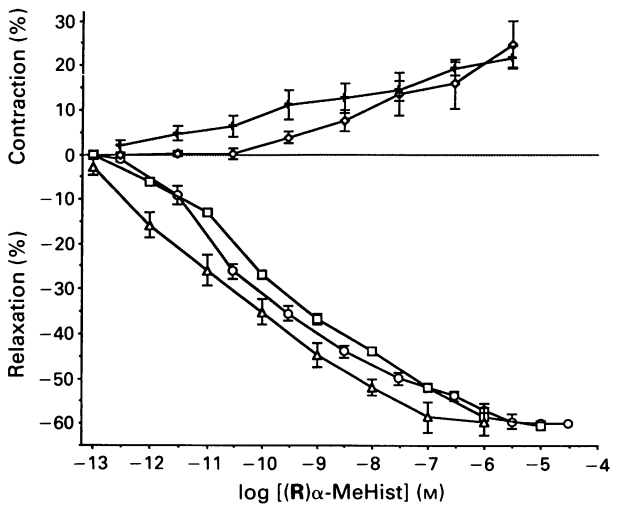


Figure 6 Responses of guinea-pig perfused bronchioles to (R)α-methylhistamine: alone (○) ($n = 9$), in presence of 10^{-6} M (△) ($n = 6$) or 5×10^{-5} M (□) ($n = 6$) of N^G-nitro-L-arginine methyl ester (L-NAME), in presence of 10^{-5} M indomethacin (◇) ($n = 12$) or without epithelium (+) ($n = 10$). Contractions were expressed as a % of the contraction induced by 10^{-4} M acetylcholine and relaxation expressed as a % of maximal relaxation induced by 10^{-4} M papaverine. Points represent the mean with s.e.mean.

Effect of L-NAME (10^{-6} M and 5×10^{-5} M)

L-NAME 10^{-6} M or 5×10^{-5} M did not modify the (R)α-MeHist-induced concentration-dependent relaxation in the range 10^{-13}–10^{-6} M. The E_{max}, pD_2 and slopes [(%/log unit) for the regression line of (R)α-MeHist] in absence or in presence of 10^{-6} M or 5×10^{-5} M L-NAME were (60 ± 2)%, 10.28 ± 0.29, − 8.0 ± 0.7; (59.5 ± 2.9)%, 10.68 ± 0.39, − 8.6 ± 1 and (60.5 ± 0.3)%, 9.64 ± 0.08, − 8.9 ± 0.1, respectively.

Effect of tranylcypromine (10^{-5} M)

Tranylcypromine, 10^{-5} M, failed to modify significantly the concentration-dependent relaxation induced by the H_3-agonist. The E_{max} (61 ± 1)%, pD_2 (9.99 ± 0.18) and slope (− 8.8 ± 0.8) were not significantly different from those obtained with (R)α-MeHist alone.

Discussion

Numerous pharmacological studies of the reactivity of distal airways *in vitro* have been, and still are, conducted on isolated lung parenchymal strips. Some authors have tested the effects of different agonists on the isolated bronchus with an isometric technique (Hooker *et al.*, 1977; Goldie *et al.*, 1986).

In our studies, we have used guinea-pig isolated bronchioles segments as a model of peripheral airway smooth muscle. The minor differences in sensitivity to contractile agents between the two preparations can well be explained by the morphological heterogeneity of the contractile elements in the airways (Breeze & Wheeldon, 1977; Evans & Adler, 1981).

In the present study, preparations contracted when histamine, acetylcholine and K^+ were applied. For these drugs, the maximum effects and pD_2 (Figure 3; Table 1) were obtained over a similar range of concentrations to those reported by other authors who worked with guinea-pig but with different airways generations and various methods (Advenier & Freslon, 1985; Farmer *et al.*, 1986; Touvay *et al.*, 1986; Braunstein *et al.*, 1988). The experimental system described in our study seems to be of a similar nature to that used by Munakata *et al.* (1988) but there are some differences which could be important. In our protocol, as opposed to that of other authors, we cannulated the bronchiolar segment with only one needle (opened system). In this way we can eliminate, in the steady state, endogenous agents which could react with different tested drugs because the inner perfusate was not recirculated during each protocol. In 1988, Munakata concluded that he could not distinguish between mechanisms involving an epithelial-dependent relaxing factor or a passive diffusion barrier, but the results suggested a strong protective role of epithelium for smooth muscle stimulation from the luminal surface. The qualitatively different effects of epithelium removal on responses to histamine, acetylcholine and KCl suggested that alterations in reactivity were not due to removal of a diffusion barrier or to damage of the smooth muscle. The contrasting results obtained may be explained by an anatomical difference, as the two studies examined different airway generations. Raeburn *et al.* (1986), using rabbit airways, indicated that the effects of removal of the epithelium on smooth muscle reactivity exhibit marked regional variation. The potency of histamine was 4 fold greater in epithelium-denuded perfused bronchioles than in intact preparations with a shift to the left (0.60 ± 0.01 log units) consistent with previous reports (Advenier *et al.*, 1988; Braunstein *et al.*, 1988) in guinea-pig trachea. We obtained similar results when we used 10^{-5} M indomethacin but with a greater shift to the left.

The epithelium removal, or 10^{-5} M indomethacin (data not shown), shifted to the left the concentration-response curve to acetylcholine (1.02 ± 0.1 log units) but did not affect the E_{max} (Table 1). In the two preparations, the effect of K^+ was not affected by epithelium removal which suggests smooth muscle function *per se* was unaltered (Table 1).

The implication of these results is that histamine induced the release of the EpDRF containing metabolites of arachidonic acid via cyclo-oxygenase (e.g. prostaglandins E_1, E_2) on intact bronchioles.

Guc *et al.* (1988) observed an epithelium-dependent relaxation of guinea-pig tracheal smooth muscle by histamine. Using the coaxial bioassay system, he showed that this relaxant response could be prevented by thioperamide but (R)α-MeHist was found ineffective in inducing a relaxation. It was concluded that the stimulation of non-H_1- and non-H_2-histamine receptors mediated the release of (a) relaxant factor(s).

Using intact bronchioles we demonstrated that histamine, in the presence either of mepyramine or cimetidine, induced a concentration-dependent relaxation which was antagonized in a competitive manner by thioperamide giving a pA_2 and a K_i value of 8.2 and 3.2 nM, respectively. These values were similar to data obtained by Arrang *et al.* (1987) and Hew *et al.* (1990). These results suggested that histamine induced a bronchorelaxation via an H_3-receptor.

In Figure 5, we showed that (R)α-methylhistamine, a potent and selective H_3-agonist, had intrinsic effect on the resting tone of the guinea-pig airways *in vitro*. The concentration-dependent (R)α-MeHist-induced relaxation was competitively antagonized by the H_3-antagonist, thioperamide. In our own tests, thioperamide did not induce any intrinsic relaxation when administered in the range 10^{-10}–10^{-5} M. The pA_2 value of the H_3-antagonist was very similar to its values for various responses mediated by H_3-receptors (Arrang *et al.*, 1987; 1988a,b). Moreover, it did not shift the concentration-response curve to fenoterol (data not shown). These data favour the hypothesis that an H_3-receptor dilator exists in guinea-pig bronchioles.

In the guinea-pig bronchioles, removal of epithelium reversed the concentration-dependent relaxation induced by (R)α-MeHist in a low concentration-dependent contraction.

Our hypothesis suggests that H_3-histamine receptors are localized in the epithelial wall and act on the smooth muscle. However, 10^{-5} M indomethacin reversed the relaxation induced by (R)α-MeHist into a concentration-dependent contraction suggesting that H_3-receptors were localized in the epithelium and that (R)α-MeHist induced a bronchodilatation via prostanoid(s) released by epithelial cells.

PGE_1 and prostacyclin (PGI_2), have been described as relaxant agents of basal tone (Sheard, 1968; Adaikan & Karim, 1979). PGE_2 is generally considered to be a relaxant substance, but its activity profile is complex. In our model (data not shown) PGE_2, sometimes increased and sometimes decreased basal tone as previously described by Gardiner (1986). Moreover 10^{-5} M tranylcypromine failed to abolish the relaxation induced by (R)α-MeHist.

We showed that 10^{-6} M or 5×10^{-5} M nitro-L-arginine methyl ester (L-NAME) had no effect on the concentration-response curve to (R)α-MeHist. These data demonstrated that the stimulation of the H_3-histamine receptor did not induce relaxation in the perfused bronchiole as in the rabbit middle cerebral arteries (Ea Kim *et al.*, 1992); (R)α-MeHist did not induce production of nitric oxide by the epithelial cells.

All these features clearly identify H_3-receptors as mediating the relaxation of guinea-pig perfused bronchioles increasing the release of EpDRF. EpDRF seems very unlikely to be the same as endothelium-derived relaxing factor (EDRF) or nitric oxide (NO) (Munakata *et al.*, 1990) but may be metabolite(s) of arachidonic acid.

We cannot demonstrate the total mechanism of transduction, indeed the factor may well act on other subepithelial structures (for example sensory nerves, mast cells), which in turn could modulate the responsiveness of the underlying smooth muscle.

The authors are grateful to Dr J.M. Arrang for his constructive comments during the preparation of this manuscript.

We are grateful for the kind assistance provided by the laboratory of anatomo-pathology (Pr. Cantanzano), C.H.U. Limoges France and to J. Javellaud for excellent technical assistance.

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(Received October 21, 1992
Revised March 11, 1993
Accepted March 16, 1993)

Br. J. Pharmacol. (1993), **109**, 967–971 © Macmillan Press Ltd, 1993

Evidence that a form of ATP uncomplexed with divalent cations is the ligand of P_{2y} and nucleotide/P_{2u} receptors on aortic endothelial cells

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1 The response of bovine aortic endothelial cells to adenosine 5'-triphosphate (ATP) is mediated by both P_{2y} and nucleotide/P_{2u} receptors. In order to determine which form of the nucleotide is the true ligand of these receptors, we have investigated the effects of divalent cations on ATP-, uridine 5'-triphosphate (UTP)- and 2 methylthioadenosine 5'-triphosphate (2MeSATP)-induced inositol phosphate accumulation in these cells.

2 Omisson of Mg^{2+} from a calcium-free incubation buffer caused a shift to the left of the ATP concentration-action curve.

3 In the presence of EDTA (1 mM), the basal level of inositol trisphosphate ($InsP_3$) was markedly increased and the absolute maximal response to ATP was decreased; however, the response to low concentrations of ATP was enhanced.

4 When the results were plotted in terms of calculated ATP^{4-} concentrations, the concentration-response curves obtained in the presence of 1.25 mM Mg^{2+} lay closer to the respective curves obtained when Mg^{2+} was omitted from the medium or when Mg^{2+} was omitted and EDTA (1 mM) was added. The curves became almost superimposable when the baseline value was subtracted.

5 A similar shift to the left of the concentrations-action curves was also observed with both UTP and 2MeSATP.

6 Our data provide evidence that a form of ATP uncomplexed with divalent cation is the preferential agonist of both the nucleotide/P_{2u} and the P_{2y} receptors expressed on bovine aortic endothelial cells.

Keywords: ATP; UTP; 2-methylthioadenosine 5'-triphosphate (2MeSATP); magnesium; calcium; purinoceptors; inositol phosphates; endothelium

Introduction

Extracellular adenosine 5'-triphosphate (ATP) plays an important role in the regulation of many biological processes including the control of vascular tone and haemostasis. The cellular responses to ATP are mediated by specific receptors called P_2 as opposed to P_1 receptors which interact preferentially with adenosine. According to the rank order of potency of several ATP analogues, a subclassification of P_2 purinoceptors into 2 major classes (P_{2x}, P_{2y}), both present on blood vessels, has been proposed by Burnstock & Kennedy in 1985. P_{2x} responses are characterized by the following order of potency: α,β-methylene ATP (APCPP) = β,γ-methylene ATP (APPCP) > ATP = 2-methylthioadenosine 5'-triphosphate (2-MeSATP). The P_{2y} subtype is more specific for 2MeSATP than for ATP and α,β-methylene ATP. Other P_2 purinoceptor subtypes have been described (Gordon, 1986). The P_{2t} type is present on platelets and megakaryocytes and responds only to ADP. The P_{2z} subclass is expressed on mast cells and macrophages, where it is coupled to histamine release and an increase in membrane permeability (Cockcroft & Gomperts, 1980; Tatham *et al.*, 1988; Steinberg & Silverstein, 1987). In addition to these four subclasses of purinoceptors (P_{2x}, P_{2y}, P_{2t}, P_{2z}), the existence of another receptor which is called P_{2u} or nucleotide receptor has been recently proposed (Seifert & Schultz, 1989; O'Connor *et al.*, 1991). This receptor has a high affinity for both ATP and UTP and a low affinity for 2MeSATP. The existence of nucleotide/P_{2u} receptors is supported by studies on calcium mobilization in human polymorphonuclear neutrophils (Walker *et al.*, 1991), pituitary cells (Davidson *et al.*, 1990), human fibroblast (Fine *et al.*, 1989) and NCB-20 cells (Garritsen *et al.*, 1992), on prostacyclin production in bovine pulmonary artery endothelial cells (BPAE) (Lustig *et al.*, 1992), and on inositol phosphate accumulation in renal mesangial cells (Pfeilschifter, 1990), human epithelial cells (Brown *et al.*, 1992) and bovine aortic endothelial cells (BAEC) (Motte *et al.*, 1993).

ATP^{4-} forms complexes with the cations Mg^{2+}, Ca^{2+}, Na^+, K^+ and H^+, the percentage distribution of these various forms is dependent upon the ionic composition of the solution, its pH, the temperature and the concentration of ATP (Bartfai, 1979). Using dissociation constants published in the literature (Fabiato, 1981), Fine *et al.* have calculated that in a physiological extracellular medium (140 mM Na^+, 4 mM K^+, 1 mM Ca^{2+}, 1 mM Mg^{2+}, pH 7.4) the distribution is as follows: $MgATP^{2-}$ (71%), $CaATP^{2-}$ (22%), $NaATP^{3-}$ (3%), $KATP^{3-}$ (0.8%), $HATP^{3-}$ (1.2%) and ATP^{4-}(2.4%) (Fine *et al.*, 1989). Similar values have been reported by Lustig *et al.* (1992). Which of these forms constitutes the true or preferential agonist has not been determined for all classes of P_2 receptors. It is now well established that the P_{2z} receptors selectively recognize the tetrabasic form of ATP: these receptors have indeed been called 'ATP^{4-} receptors' (Cockcroft & Gomperts, 1980; Tatham *et al.*, 1988). ATP^{4-} also seems to be the best ligand for the nucleotide/P_{2u} receptors identified on human fibroblasts (Fine *et al.*, 1989), pituitary cells (Davidson *et al.*, 1990), neutrophils (Walker *et al.*, 1991) and BPAE (Lustig *et al.*, 1992), as well as for P_{2x} receptors on vas deferens smooth muscle (Fedan *et al.*, 1990). The primary biological event coupled to receptor activation induced by ATP in BAEC has been characterized in our

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laboratory as the hydrolysis of phosphatidylinositol bisphosphate by phospholipase C resulting in a rapid accumulation of inositol trisphosphate ($InsP_3$) (Pirotton *et al.*, 1991). We have recently established that $InsP_3$ accumulation in BAEC in response to ATP involved both P_{2y} and nucleotide/P_{2u} receptors (Motte *et al.*, 1993).

In order to determine whether the P_{2y} receptor preferentially recognizes ATP^{4-} or a form of ATP complexed with monovolent cations, as does the nucleotide/P_{2u} receptor, we have evaluated the effects of changes in Mg^{2+} concentration and the effect of the Ca^{2+} and Mg^{2+} chelator ethylenediaminetetraacetic acid (EDTA) on the accumulation of $InsP_3$ induced by ATP, UTP and 2MeSATP in BAEC.

Methods

Cell incubation

BAEC were obtained by collagenase digestion of the aorta excised from a freshly slaughtered cow and cultured as previously described (Pirotton *et al.*, 1991). Subconfluent cells were labelled for 24 h in inositol-free Dulbecco's Modified Eagle's Medium (80%, v/v), with Ham's F_{12} (20%, v/v), foetal-calf serum (5%, v/v), penicillin 100 units ml^{-1}, streptomycin 100 $\mu g\ ml^{-1}$ and amphotericin B 2.5 $\mu g\ ml^{-1}$, supplemented with *myo*-D-[^{3}H]-inositol 10 $\mu Ci\ ml^{-1}$.

The cells were washed twice with buffer containing Mg^{2+} and Ca^{2+} (see Materials) and incubated in this medium for 30 min. Before addition of the agonist, cells were washed twice with Ca^{2+}-free buffer containing various concentrations of Mg^{2+} or EDTA and then stimulated in the same buffer containing the agonist.

The inositol phosphates were extracted as described previously (Pirotton *et al.*, 1991). Briefly, the medium was rapidly replaced by 1 ml of $HClO_4$ (3%, w/v). The dishes were rinsed with 1 ml of $HClO_4$ (1%) and the lysate was neutralized with KOH (0.765 M) and HEPES (0.375 M).

The inositol phosphates were separated on Dowex AG1X8 columns (0.8 ml wet bed volume) as previously described (Pirotton *et al.*, 1991).

Calculation of ATP^{4-} concentration

We have assumed that the predominant forms of ATP under our experimental conditions were: $MgATP^{2-}$, $CaATP^{2-}$, $NaATP^{3-}$, $HATP^{3-}$, $KATP^{3-}$ and ATP^{4-}. Therefore, the relevant equation is as follows: ATP total = ATP^{4-} + $MgATP^{2-}$ + $CaATP^{2-}$ + $NaATP^{3-}$ + $HATP^{3-}$ + $KATP^{3-}$. The equilibrium involved in the formation of complex of ATP and a cation (C) may be expressed as follows:

$$[C]_{free} + [ATP^{4-}]_{free} \rightleftharpoons [CATP]$$

$$K_C = \frac{[CATP]}{[C]_{free} \times [ATP^{4-}]_{free}}$$

Moreover, the ionic concentration may be described by: $[C]_{total} = [C]_{free} + [CATP]$. We have assumed that $[Na]_{free}$ and $[K]_{free}$ approximate $[Na]_{total}$ and $[K]_{total}$ respectively, as these cations were in large excess over ATP. When $MgSO_4$ and $CaCl_2$ were omitted from the bathing solution, the actual concentrations of Mg^{2+} and Ca^{2+} were measured by a colorimetric method using methylthymol blue and calmagite as indicators for Ca^{2+} and Mg^{2+} respectively (BioMérieux, Marcy-l'Etoile, France). Mg^{2+} concentrations were 45 $\mu M \pm 15$ and 52 $\mu M \pm 35$ (mean ± s.d.), and Ca^{2+} concentrations were 5 $\mu M \pm 2$ and 9 $\mu M \pm 4$, respectively before and after incubation with the cells. Finally, the following association constants were taken from the literature: K_{Mg}: $10^{4.28}\ M^{-1}$, K_{Ca}: $10^{3.94}\ M^{-1}$, K_H: $10^{6.95}\ M^{-1}$, K_{Na}: $5.82\ M^{-1}$, K_K: $5.30\ M^{-1}$ (Taqui Khan & Martell, 1966; Fine *et al.*, 1989). As $[ATP]_{total}$, $[Mg^{2+}]_{total}$, $[Ca^{2+}]_{total}$, $[Na^+]$, $[K^+]$ and $[H^+]$ were known, we derived by substitution the following third-degree polynomial which was used to determine ATP^{4-} concentration:

$$K_{Mg} \times K_{Ca} \times [ATP^{4-}]^3 \times (1 + K_H \times [H^+] + K_{Na} \times [Na^+] + K_K \times [K^+]) + [ATP^{4-}]^2 \times (K_{Mg} + K_{Ca} + K_{Mg} \times K_{Ca} \times [Mg^{2+}]_{total} + K_{Ca} \times K_{Mg} \times [Ca^{2+}]_{total} + K_H \times K_{Mg} \times [H^+] + K_H \times K_{Ca} \times [H^+] + K_{Na} \times K_{Mg} \times [Na^+] + K_{Na} \times K_{Ca} \times [Na^+] + K_K \times K_{Mg} \times [K^+] + K_K \times K_{Ca} \times [K^+] - K_{Mg} \times K_{Ca} \times [ATP]_{total}) + [ATP^{4-}] \times (1 + K_{Mg} \times [Mg^{2+}]_{total} + K_{Ca} \times [Ca^{2+}]_{total} + K_H \times [H^+] + K_{Na} \times [Na^+] + K_K \times [K^+] - K_{Mg} \times [ATP]_{total} - K_{Ca} \times [ATP]_{total}) - [ATP]_{total} = 0$$

ATP^{4-} concentrations were calculated, according to the polynomial, by successive iterations.

Materials

Collagenase type IA and ATP (disodium salt) were from Sigma Chemical Co., St. Louis, MO, U.S.A.; trypsin was from Flow Laboratories, Bioggio, Switzerland; all the other culture media and reagents were purchased from GIBCO, Grand Island, NY, U.S.A. EDTA (disodium salt) was from Boehringer Mannheim, Germany. Dowex AG1X8 (formate form) was from Bio-Rad Laboratories, Richmond, CA, U.S.A. The radioactive product *myo*-D-[2-^{3}H]-inositol (10–20 Ci $mmol^{-1}$) was from Amersham, Gent, Belgium. UTP (disodium salt) was from Sigma; 2 MeSATP was from RBI, Natick, MA, U.S.A. The basic composition of the buffer used for the incubation of the cells was (mM): NaCl 124, KCl 5, $MgSO_4$ 1.25, $CaCl_2$ 1.45, KH_2PO_4 1.25, D-glucose 8 and Na-HEPES 25, pH 7.4. In most experiments, the cells were stimulated in a medium from which $CaCl_2$ was omitted, and which contained $MgSO_4$ (1.25 or 5 mM) or no $MgSO_4$, or no $MgSO_4$ plus EDTA (1 mM).

Results

The major form of ATP in a physiological medium is $MgATP^{2-}$: therefore the most crucial experiment was to evaluate the effect of Mg^{2+} omission from the incubation buffer on the cell responsiveness to ATP. Since preliminary results indicated that the maximal response to ATP was maintained in a calcium-free buffer (data not shown), we chose like others (Steinberg & Silverstein, 1987) to perform these experiments in a calcium-free buffer. As shown in Figure 1a, omission of Mg^{2+} from the incubation buffer caused a shift to the left of the ATP concentration-action curve without altering the maximal response. The magnitude of the response to 200 μM ATP was unaffected (1.5 ± 0.5 versus 1.6 ± 0.3 c.p.m. $\times 10^3$/dish in Mg^{2+}-free medium and in medium with 1.25 mM Mg^{2+} respectively; mean ± s.d. of 3 independent experiments). When the results were plotted in terms of calculated ATP^{4-} concentration, the concentration-response curve obtained in the presence of 1.25 mM Mg^{2+} lay closer to the one obtained when Mg^{2+} was omitted from the medium (Figure 1b). A similar shift of the concentration-response curve was observed with ATPγS, which is not hydrolyzed by aortic endothelial cells (Cusack *et al.*, 1983) (data not shown).

In order to prevent the complexing of ATP by Ca^{2+} or Mg^{2+} released from the endothelial cells, the chelator EDTA was used. Although EDTA itself had a significant effect on the basal level of $InsP_3$ and on the absolute maximal response, it appears clearly from Figure 2 that the endothelial cells remained responsive to ATP in the presence of EDTA. Furthermore, the response to a low concentration of ATP was enhanced by EDTA. Plotting the results against calculated ATP^{4-} rather than total ATP brought closer to each other the concentration-action curves obtained in the presence of 1.25 mM Mg^{2+} or in the absence of Mg^{2+} plus EDTA, respectively (Figure 2b). When the baseline value was subtracted, the two curves became superimposable (Figure 2c).

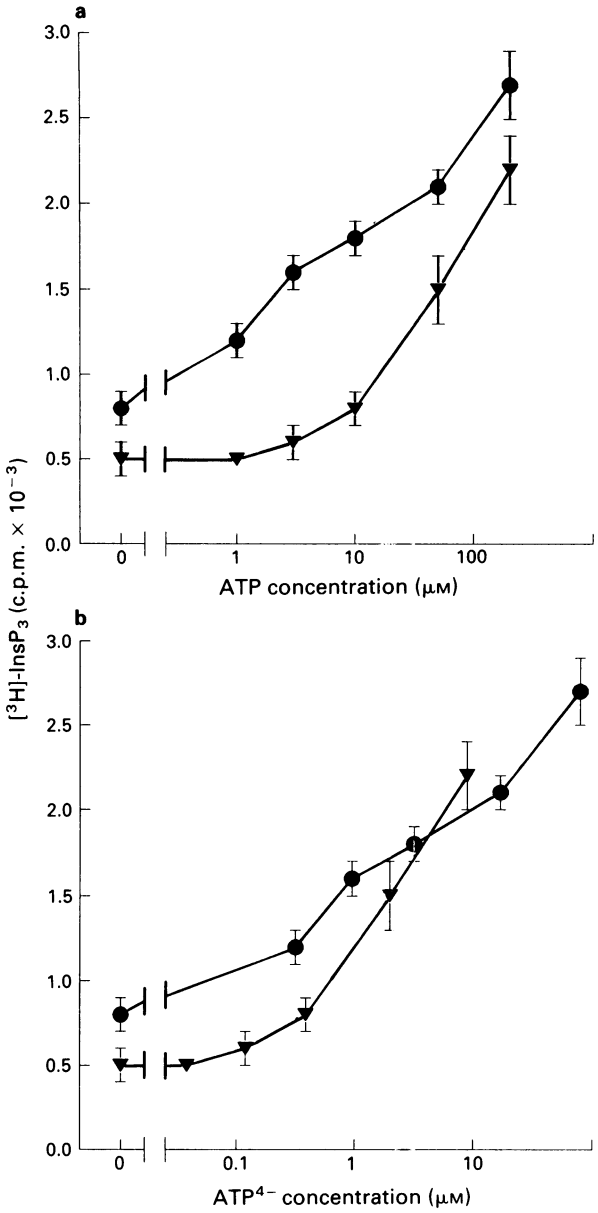


Figure 1 [^{3}H]-inositol-labelled BAEC were washed twice with 1 ml Ca^{2+}-free buffer with 1.25 mM Mg^{2+} (▼) or without Mg^{2+} (●). Cells were then challenged for 15 s in the same medium, with various concentrations of ATP as indicated. Results represent the radioactivity in the inositol trisphosphate ($InsP_3$) fraction (mean ± s.d. of triplicate determinations in one representative experiment out of two). (a) Data plotted in terms of the total concentration of ATP; (b) data plotted in terms of calculated ATP^{4-} concentration as described in the Methods section.

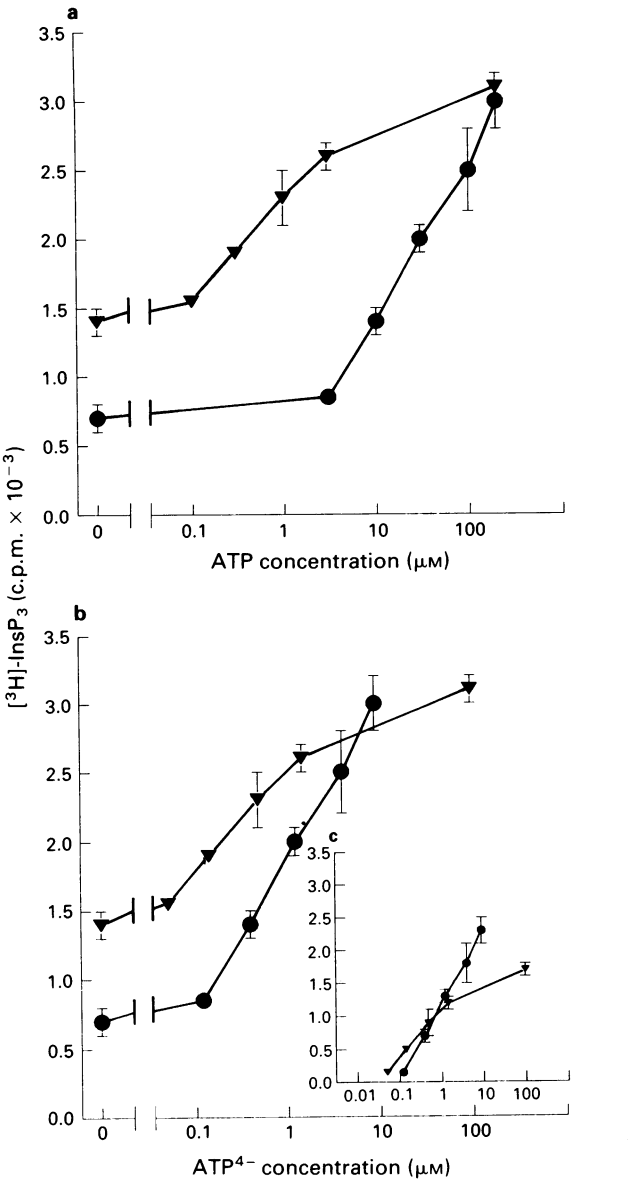


Figure 2 [^{3}H]-inositol-labelled cells were washed twice with 1 ml Ca^{2+}-free buffer containing 1.25 mM Mg^{2+} (●) or 1 mM EDTA (▼). Cells were then challenged for 15 s in the same medium containing ATP. Data are expressed as means ± s.d. of triplicate determinations in one representative experiment out of three. (a) Data plotted in terms of the total ATP concentration; (b) data plotted in terms of the calculated ATP^{4-} concentration; (c) data from (b) expressed as the $InsP_3$ production above the basal level.

Finally, omission of Mg^{2+} from the incubation fluid produced a similar leftward shift of the UTP (Figure 3) and 2 MeSATP (Figure 4) concentration-action curves.

Discussion

Omission of $MgSO_4$ and $CaCl_2$ from the incubation fluid enhanced the responsiveness of endothelial cells to low concentrations of ATP. However, such a medium still contains some Mg^{2+} (± 50 μM). Solving the equation derived in the methods section shows that the distribution of the predominant forms of total ATP (50 μM) is as follows: ATP^{4-} (34%), $MgATP^{2-}$ (25%), $NaATP^{3-}$ (25%) and $HATP^{3-}$ (12%). The endothelial cells remained responsive to ATP in a Ca^{2+}- and Mg^{2+}-free medium (no addition of $CaCl_2$ and $MgSO_4$, 1 mM EDTA). In these conditions, ATP^{4-} represents 48% of total ATP (50 μM), $NaATP^{3-}$ 34% and $HATP^{3-}$ 17%. The interpretation of concentration-response curves in the presence of EDTA is somewhat complicated because the basal level of $InsP_3$ was notably increased and the absolute maximal response was reduced. This might explain why the curve replotted in terms of ATP^{4-} in the presence of Mg^{2+} is not superimposable on that obtained in the presence of EDTA (Figure 2b). When the data were expressed as the absolute $InsP_3$ production above the basal level, the curves were almost superimposable (Figure 2c). These effects on the basal level and on the absolute maximal response probably result from the fact that chelation of extracellular divalent cations may affect the intracellular levels of Mg^{2+} and Ca^{2+}

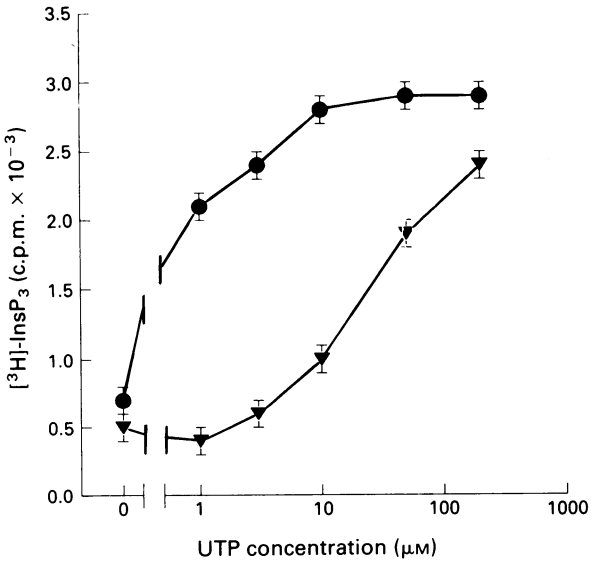


Figure 3 [^{3}H]-inositol-labelled BAEC were washed twice with 1 ml Ca^{2+}-free buffer with 5 mM Mg^{2+} (▼) or without Mg^{2+} (●). Cells were then challenged for 15 s in the same medium, with various concentrations of UTP as indicated. Results represent the radioactivity in the inositol trisphosphate ($InsP_3$) fraction (mean ± s.d. of triplicate determinations in one representative experiment out of three).

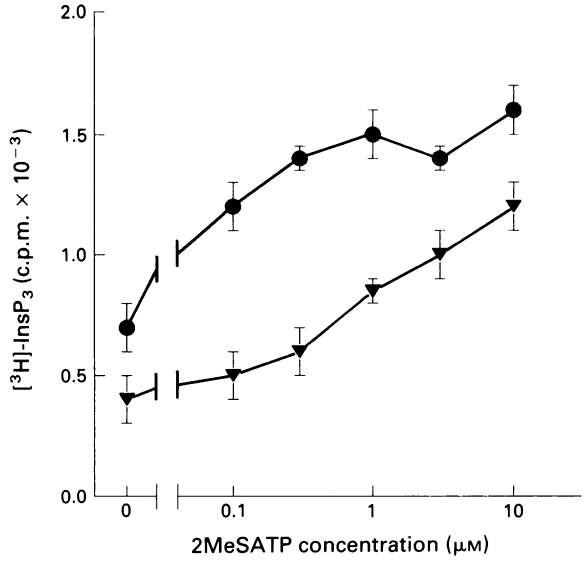


Figure 4 [^{3}H]-inositol-labelled BAEC were washed twice with 1 ml Ca^{2+}-free buffer with 5 mM Mg^{2+} (▼) or without Mg^{2+} (●). Cells were then challenged for 15 s in the same medium, with various concentrations of 2-methylthioadenosine-5′-triphosphate (2MeSATP) as indicated. Results represent the radioactivity in the inositol trisphosphate ($InsP_3$) fraction (mean ± s.d. of triplicate determinations in one representative experiment out of four).

which are both involved in several steps of phosphoinositides metabolism (Abdel-Latif, 1986). For example, Lambert *et al.* (1986) studying the bradykinin stimulation of inositol phosphate production in aortic endothelial cells have observed differences between basal and stimulated values in calcium-free versus calcium-supplemented buffer, although the degree of stimulation was not different. Nevertheless, our results indicate that the active agonist which induces phosphoinositide hydrolysis in BAEC is neither $MgATP^{2-}$, the form present in highest concentration in physiological conditions, nor $CaATP^{2-}$. The active species might thus be ATP^{4-} or a form of ATP complexed with monovalent cations. We have excluded the possibility that the shift of the concentration-response curve in the absence of Mg^{2+} is due to a reduced hydrolysis of ATP by Mg^{2+}-dependent ecto-ATPase, since the same shift was observed with ATPγS, which is not a substrate of endothelial ecto-ATPase (Cusack *et al.*, 1983). Interestingly, a similar shift was observed with both UTP and 2MeSATP; it appears therefore that both P_{2y} and nucleotide/P_{2u} receptors, recently shown to coexist on aortic endothelial cells (Motte *et al.*, 1993), recognize a form of ATP uncomplexed with bivalent cations. One implication of these studies is that the affinity of endothelial P_2-receptors for their true ligand is much larger than the value which can be derived from the concentration-action curve obtained in physiological medium. It has been initially assumed that the role of ATP forms uncomplexed with divalent cations as receptor agonists was restricted to the P_{2z} receptors expressed on mast cells and macrophages (Gordon, 1986). Recent data indicate that this situation is far more general. First, studies have shown that Mg^{2+} removal enhanced the ATP responses mediated by the nucleotide/P_{2u} receptor. Examples include the ATP-induced $InsP_3$ production in pituitary cells (Davidson *et al.*, 1990), the effects of ATP and UTP on Ca^{2+} translocation in human fibroblasts (Fine *et al.*, 1989) and in neutrophils (Walker *et al.*, 1991), and the PGI_2 production in BPAE (Lustig *et al.*, 1992). Our data regarding UTP further extend this concept to $InsP_3$ production in BAEC. Second, Fedan *et al.* (1990) have shown that ATP^{4-} was the active agonist of the P_{2x} purinoceptors of the smooth muscle cells of the guinea-pig vas deferens. Finally, our data regarding 2MeSATP further extend these observations to the P_{2y} subtype purinoceptor and suggest that all the P_2 subtype ATP-receptors, including the nucleotide/P_{2u} receptor, are preferentially activated by ATP^{4-} or a form of ATP complexed with a monovalent cation rather than by $MgATP^{2-}$ or $CaATP^{2-}$. In that respect, the behaviour of ATP receptors is thus basically different from that of enzymes such as ATPases and kinases, for which the physiological substrate is $MgATP^{2-}$.

This work was made possible by a contract of the Ministère de la Politique Scientifique (Sciences de la Vie B 10/04) and was supported by a grant of the Fonds de la Recherche Scientifique Médicale (F.R.S.M.). S.M. is a fellow of the Fondation Erasme and is supported by the Therabel Research Fellowship. S.P. is an 'Aspirant' of the Fonds National de la Recherche Scientifique (F.N.R.S.). We are grateful to Mr Delobbe for assistance with Ca^{2+} and Mg^{2+} measurements and to Dr S. Swillens for his assistance in numeric iteration. We thank Mrs N. Galand for her excellent technical assistance in performing the experiments and Mrs E. Ledent and C. Piesen for typing the manuscript.

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(Received November 26, 1992
Revised March 2, 1993
Accepted March 16, 1993)

Br. J. Pharmacol. (1993), **109**, 972–979 © Macmillan Press Ltd, 1993

The effects of evening primrose oil on nerve function and capillarization in streptozotocin-diabetic rats: modulation by the cyclo-oxygenase inhibitor flurbiprofen

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1 The aims of this study were first, to examine whether deficits in nerve conduction in streptozotocin-diabetic rats could be reversed by a 10% dietary supplement of evening primrose oil. Second, to determine the time-course of reversal, and third, to assess whether the effects could be blocked by the cylco-oxygenase inhibitor flurbiprofen (5 mg kg^{-1} day^{-1}).

2 One-month diabetes produced 20% and 15% deficits in sciatic motor and saphenous sensory conduction velocity respectively, which were maintained over 2 months diabetes.

3 The effect of 1-month evening primrose oil treatment on abnormalities caused by an initial month of untreated diabetes was examined. Motor and sensory nerve conduction velocity were restored to the non-diabetic level.

4 Resistance to hypoxic conduction failure was investigated for sciatic nerve trunk *in vitro*. The 80% conduction failure times were 29% and 55% prolonged by 1- and 2-month diabetes respectively. Evening primrose oil did not reverse the increased hypoxic resistance following 1-month untreated diabetes.

5 Sciatic nerve endoneurial capillary density was not significantly affected by diabetes, but was 16% increased in diabetic rats with reversal by evening primrose oil treatment for 1 month compared to 2-month untreated diabetes.

6 Serial motor conduction velocity measurement after 3-month untreated diabetes revealed complete normalization by evening primrose oil within 4 days. Cessation of treatment resulted in a rapid decline in conduction velocity over 24 h.

7 In a preventive study of 2-month duration, 6 groups of rats were used. These comprised non-diabetic controls, diabetic rats, and evening primrose oil-treated diabetic rats, both with and without flurbiprofen treatment. Flurbiprofen had no significant effect in non-diabetic rats, but produced an 11% worsening of motor conduction velocity and a 21% reduction of sciatic capillary density in diabetic rats. Evening primrose oil prevented the decreases in conduction velocity and increased hypoxic resistance with diabetes, and caused a 23% increase in capillary density. Flurbiprofen completely blocked the effect of evening primrose oil on conduction velocity, resistance to hypoxia, and capillarization.

8 Six main conclusions were reached. First, evening primrose oil rapidly reverses conduction deficits in diabetic rats. Second, the effects of treatment may be very short-lived, suggesting a primary metabolic action. Third, evening primrose oil cannot reverse established changes in hypoxic resistance over 1-month treatment. Fourth, long-term treatment causes angiogenesis, suggesting a vascular action. Fifth, products of cyclo-oxygenase-mediated metabolism are necessary for maintaining vasa nervorum integrity in diabetic rats. Sixth, evening primrose oil probably acts by providing substrate for vasodilator prostanoid synthesis by vasa nervorum.

Keywords: Neuropathy; nerve conduction; ischaemia; angiogenesis; evening primrose oil; essential fatty acids; eicosanoids; streptozotocin-induced diabetes; cyclo-oxygenase

Introduction

Nerve conduction velocity (NCV) is reduced in experimental diabetes mellitus and also in diabetic patients even in the absence of clinical neuropathy (Eliasson, 1964; Gregerson, 1968). This is accompanied by increased resistance to ischaemic/hypoxic conduction failure (RHCF) (Seneviratne & Peiris, 1969; Low *et al.*, 1986; Masson *et al.*, 1988). Although strict metabolic control can improve NCV in patients, the best current non-experimental approaches to glycaemic control do not sufficiently mimic the normal physiological mechanisms to prevent long-term nerve damage and dysfunction (Zeigler *et al.*, 1991). Thus, there is great interest in alternative treatments for diabetic complications.

A rational therapeutic approach demands a knowledge of the mechanisms underlying diabetic neuropathy; however, these are not fully known in detail. A plausible (but arguable) current view is that vascular dysfunction, which causes decreased nerve blood flow and results in endoneurial hypoxia sufficient to damage nerve fibres (Newrick *et al.*, 1986; Low *et al.*, 1987; Cameron *et al.*, 1991b; Tesfaye *et al.*, 1992), provides the most important contribution to the aetiology of diabetic neuropathy.

In diabetes, there is a deficit in Δ-6 desaturation of ω-6 series essential fatty acids (Horrobin, 1988), thus, conversion of linoleic acid to γ-linolenic acid is impaired. Previous studies have demonstrated that dietary supplementation with evening primrose oil (EPO), which is relatively rich in γ-linolenic acid (8–10%), prevents the development of nerve conduction deficits and increased RHCF in streptozotocin-diabetic rats (Julu, 1988; Tomlinson *et al.*, 1989; Julu & Mutamba, 1991; Cameron *et al.*, 1991c). Preliminary reports of clinical trials also suggest an improvement in nerve function (Gamma-linolenic acid multicentre trial group, 1992).

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The mechanism of EPO's action is not known but several ideas have been advanced, including increases in membrane fluidity (Julu, 1988), increased availability of arachidonic acid/diacylglycerol to stimulate nerve Na^+-K^+-ATPase activity and compensate for the deficit in this enzyme with diabetes (Tomlinson *et al.*, 1989; Julu & Mutamba, 1991), or by acting as a substrate for production of vasodilator prostanoids such as prostacyclin to improve nerve perfusion (Cameron *et al.*, 1991c). In support of the latter notion, Ward *et al.* (1989) noted that prostacyclin synthesis by sciatic nerve and perineurial preparations was markedly reduced in experimental diabetes. Further evidence for a vascular action of EPO comes from the finding that chronic treatment of diabetic rats caused angiogenesis in nerve and muscle (Cameron *et al.*, 1991c).

The aims of this investigation were to examine the effects of EPO on nerve conduction in streptozotocin-diabetic rats in more detail, particularly regarding reversibility of an established NCV deficit and its timecourse, and to test what facets of the response to preventive EPO treatment depend on cyclo-oxygenase-mediated prostanoid metabolism by blocking the actions of this enzyme with flurbiprofen.

Methods

Animals and diabetes induction

Male Sprague-Dawley rats (Aberdeen University breeding colony), 19 weeks old at the start of the study were used. Non-diabetic animals acted as onset controls. Others were given streptozotocin (Sigma) at 40–45 mg kg^{-1} in 20 mmol l^{-1} sodium citrate buffer, pH 4.5, i.p. Diabetes was verified 24 h later by estimating hyperglycaemia and glycosuria (Visidex II and Diastix; Ames, Slough, UK). Diabetic rats were tested weekly, and weighed daily. They were rejected if blood glucose concentration was <20 mmol l^{-1} or if they showed a consistent increase in body weight over 3 days. Samples for plasma glucose measurement with a standard test kit (GOD-Perid method; Boehringer Mannheim, Mannheim, Germany) were taken from the tail vein on the day of final experiments.

Diabetic rats were used in three studies, along with non-diabetic controls. The first study concerned the ability of EPO to reverse an established NCV deficit. Groups employed were 1 month and 2 month diabetic controls, and a reversal group treated with EPO (Scotia Pharmaceuticals, Guildford, Surrey) using a 10% w/w dietary supplement for 1 month, following 1 month of untreated diabetes. The second study examined the timecourse of reversal by EPO and the re-establishment of NCV deficits following cessation of treatment, using serial measurement of NCV in motor nerve fibres supplying tibialis anterior muscle after 3 months untreated diabetes.

The third study used control, diabetic control, and preventative EPO-treated diabetic groups. These groups were divided in two, and half the rats also received flurbiprofen (Sigma) added to drinking water over a 2 month period such that the effective dose received by all animals, irrespective of changes in the volume consumed due to diabetes, was approximately 5 mg kg^{-1} day^{-1}. This dose is similar to that recommended for the treatment of rheumatoid arthritis in patients (Payan & Shearn, 1989) and has been demonstrated to reduce *in vivo* prostaglandin production by several tissues in rats when given orally (Brogden *et al.*, 1979). There was no evidence of adverse side effects of flurbiprofen. The mortality rate was zero in treated control rats, and did not exceed that attributable to streptozotocin alone ($\leqslant 10\%$) in treated diabetic rats. Growth curves for control rats treated with flurbiprofen approximated those normally found for untreated rats. Similarly, weight loss curves in diabetic rats were unaffected by flurbiprofen treatment. This suggests that there was no appreciable incidence of adverse gastrointestinal side effects which are occasionally seen in patients treated with NSAIDs (Payan & Shearn, 1989), as any interference with appetite, digestion or absorption would be expected to affect body weight, particularly for diabetic rats.

Acute measurements of nerve conduction velocity and resistance to hypoxic conduction failure

In final experiments, rats were anaesthetized with urethane (1–1.5 g kg^{-1}, i.p.). Methods have been described in detail previously (Cameron *et al.*, 1989a; 1991c). Briefly, the sciatic nerve was exposed and bipolar stimulating electrodes were placed close to it at the sciatic notch and knee. A concentric bipolar electrode was inserted into either tibialis anterior (peroneal sciatic division) or triceps surae (tibial sciatic division) muscle to monitor evoked EMG activity. Potentials from each stimulating site were averaged 8 times. NCVs for tibialis anterior and triceps surae were calculated by dividing the distance between stimulating electrodes by the average latency difference between the onset of EMG potentials evoked from the two sites. The results of the two motor nerve measurements were averaged and taken to be representative of sciatic motor NCV (Cameron *et al.*, 1991b). Nerve temperature was monitored with a thermocouple probe, and maintained in the range of 36.5–37.5°C by radiant heat. Body temperature was also maintained around 37°C using radiant heat and monitored by a rectal probe. NCVs were also measured between groin and ankle for the saphenous nerve.

The nerve trunk, between sciatic notch and knee, was then removed to a tissue bath filled with Krebs solution gassed with 95% O_2:5% CO_2 (pH 7.35) at 35°C. The Krebs solution contained 40 mmol l^{-1} glucose for nerves from diabetic rats, and 5.5 mmol l^{-1} for nerves from non-diabetic rats. They were left to equilibrate for approximately 30 min, then the chamber was refilled with mineral oil pregassed with 100% N_2 for 1 h and N_2 gassing continued. Myelinated fibres in nerves were stimulated with just supramaximal pulses (1 Hz, 0.05 ms width, 10 mA) and compound action potential amplitude was monitored at 2 min intervals until it fell below 10% of its initial value.

Serial measurements of sciatic peroneal nerve conduction velocity

A sub-group of 8 diabetic rats was used to investigate changes in NCV in tibialis anterior muscle over an 8 day EPO treatment period following 12 weeks untreated diabetes, and for a subsequent 3 days from cessation of treatment. Under halothane anaesthesia (2–5% in air), sterile bipolar needle stimulating electrodes (2 mm separation of tips) were inserted through the skin to stimulate sciatic nerve at the sciatic notch and popliteal fossa. A sterile concentric bipolar recording electrode was inserted into tibialis anterior muscle. Leg skin temperature was monitored by a thermistor probe and kept in the range 36–38°C by radiant heat. EMGs evoked from both stimulation sites were averaged 8 times and latencies of the first inflections were measured. The sciatic nerve between the two stimulating electrodes takes a fairly straight course, and interelectrode distances were used to calculate conduction velocity. NCV values obtained by this serial method are in good agreement with those found in acute measurements (Cameron *et al.*, 1989a).

Endoneurial capillary density measurements

At the end of the experiments a length of sciatic nerve between the notch and knee was removed (2.5 cm), cleared of surrounding tissues and divided into approximately 0.5 cm pieces. These were then mounted on cork discs using skeletal muscle as support tissue, and frozen in isopentane which had been prechilled in liquid nitrogen. Three 10 μm thick transverse sections, each 90 μm apart, were cut on a cryostat at −20°C and capillary endothelium was stained for alkaline

phosphatase by the method of Ziada *et al.* (1984). Capillaries in all nerve fascicles were counted with the assistance of a projection microscope. Fascicle outlines were traced and areas measured with a digitizing pad linked to a microcomputer.

Statistical analysis

Results are expressed as mean ± s.e.mean. Data were subjected to one-way analysis of variance. Where statistical significance was attained ($P<0.05$), Student's t tests were performed between groups to assign probability levels and the Bonferroni correction for multiple comparisons was applied (Instat, GraphPad, San Diego, Ca, U.S.A.). For serial measurements of NCV, which involved within-animal comparisons, data were analyzed by paired Student's t tests.

Results

Investigation 1: Removal of nerve conduction deficits by evening primrose oil treatment

Table 1 gives body weights and plasma glucose concentrations for the groups used in this study. Diabetes resulted in marked hyperglycaemia, and progressive body weight loss. EPO treatment in the reversal group had no effect on plasma glucose concentration or body weight loss.

Results for motor and sensory NCV are illustrated in Figure 1a and b respectively. With diabetes there was a 20% reduction in average motor NCV after 1 month ($P<0.001$), which was maintained over the subsequent month in untreated diabetic controls. Sensory saphenous NCV was also 15% reduced by diabetes. In the EPO-treated reversal group, motor and sensory conduction deficits were largely corrected; the degree of amelioration compared to 1 month diabetic controls being 99% ($P<0.001$) and 85% ($P<0.01$) respectively. There were no significant differences in motor or sensory NCV values for the EPO-treated reversal group compared to non-diabetic controls.

The durations of hypoxia necessary for an 80% reduction in sciatic nerve compound action potential amplitude (T_{80}) are shown in Figure 2. There was a progressive increase in T_{80} with increasing diabetes duration compared to controls, which was 28.6% after 1 month ($P<0.05$) and 54.6% after 2 months ($P<0.001$). In the EPO-treated reversal group the increase in T_{80} (35.1%) was similar to that for 1 month diabetic group, significantly elevated compared to controls ($P<0.001$), and not significantly different from the 2 month diabetic group ($P=0.052$, single comparison, two-tailed).

Table 1 also shows sciatic trunk endoneurial capillary density revealed by alkaline phosphatase staining. Compared to the control group, diabetes did not have a significant effect on capillary density. In the reversal EPO-treated group, capillary density was 16% increased compared to 2 month untreated diabetic controls ($P<0.05$). However, capillary density was not significantly elevated compared to the non-diabetic control group.

Investigation 2. Timecourse of the effects of evening primrose oil treatment after 3 months chronic diabetes

For the 8 rats in this investigation, body weight declined from 470 ± 12 g to 364 ± 13 g over the 3 month period of

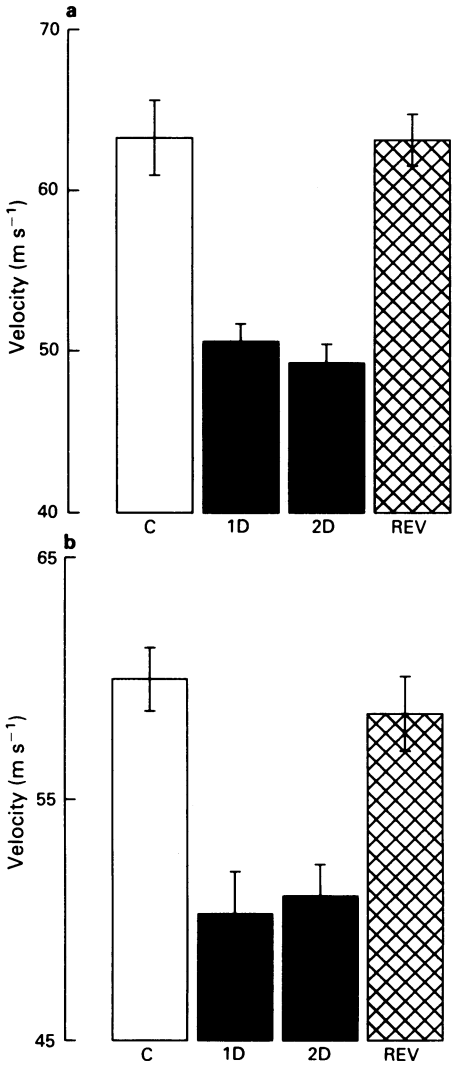


Figure 1 Conduction velocity in sciatic motor and saphenous sensory nerves for control, 1 and 2 month diabetic and evening primrose oil (EPO)-treated diabetic in the reversal study. (a) Motor; (b) sensory. Columns show means ± s.e.means. Controls (open columns), C, ($n=14$); diabetic controls (solid columns), 1-month 1D, ($n=10$), 2-month, 2D, ($n=12$); EPO-treated reversal diabetic group (cross-hatched columns), REV, ($n=12$). For motor and sensory nerves, values were significantly reduced by 1- and 2-month untreated diabetes compared to non-diabetic controls ($P<0.001$, all comparisons). Conduction velocity was restored to non-diabetic values by treatment ($P<0.001$, motor; $P<0.01$, sensory).

Table 1 Body weights, plasma glucose levels, and sciatic nerve capillary density in the reversal study (investigation 1)

Group	n	*Body weight* (g)		*Plasma glucose* (mmol l^{-1})	*Capillary density* (mm^{-2})
		Start	Finish		
Control	14	482 ± 16	–	6.5 ± 0.5	58.4 ± 1.9
Diabetic					
1 month	10	461 ± 6	413 ± 12[a]	32.6 ± 3.1[b]	57.0 ± 2.0
2 month	12	514 ± 12	377 ± 11[b]	40.1 ± 2.0[b]	55.2 ± 1.5
EPO-treated reversal	12	486 ± 11	402 ± 12[a]	35.9 ± 2.2[b]	64.2 ± 2.5[c]

Data are expressed as group means ± s.e.mean.
[a]$P<0.01$, [b]$P<0.001$ versus control group; [c]$P<0.05$ versus 2 month diabetic group.

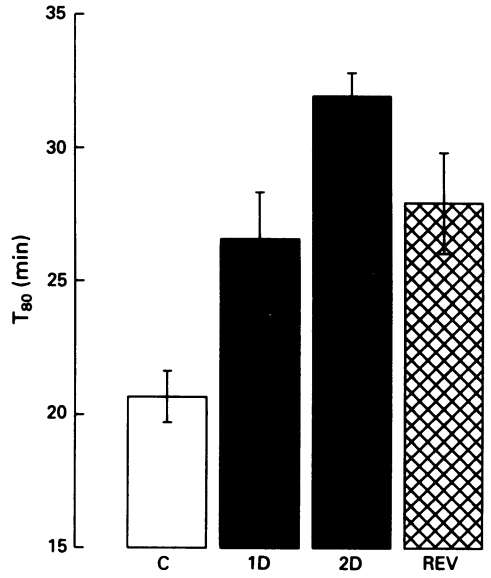


Figure 2 Durations of hypoxia necessary for an 80% reduction in sciatic nerve compound action potential amplitude (T_{80}) in the reversal study. Columns show means ± s.e.means. Control (open columns), C, ($n = 14$); diabetic controls (solid columns), 1-month, 1D, ($n = 10$), 2-month, 2D ($n = 12$); evening primrose oil (EPO)-treated reversal diabetic group (cross-hatched column), REV, ($n = 12$). Untreated diabetes caused a progressive increase in T_{80} ($P < 0.05$ for 1-month group, $P < 0.001$ for 2-month group compared to controls). Treatment with EPO resulted in T_{80} values which were not significantly different from 1- or 2-month diabetes, but were increased compared to controls ($P < 0.01$).

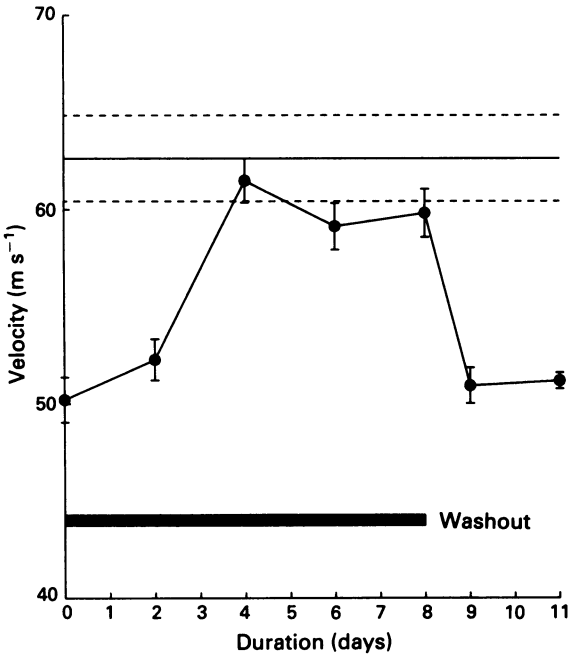


Figure 3 Serial measurements of sciatic tibialis anterior motor conduction velocity changes with 8 days evening primrose oil (EPO) treatment following 3 months of untreated diabetes, and the effects of discontinuing treatment over a 3 day washout period. The treatment period is indicated by the thick horizontal bar. The upper horizontal line flanked by dashed lines represent the mean ± s.e.mean for non-diabetic control tibialis anterior conduction velocity. Symbols and error bars represent the mean ± s.e.mean of a group ($n = 8$) of 3-month diabetic rats, in which EPO treatment commenced after the initial conduction velocity measurement on day 0. Conduction velocity was significantly elevated at day 4 ($P < 0.0001$) and remained so until treatment was withdrawn after measurement on day 8. Over the next 24 h, conduction velocity returned to a level not significantly different from that measured on day 0.

untreated diabetes, and plasma glucose was 46.8 ± 1.9 mmol l^{-1}. Figure 3 shows the results of serial NCV measurements on the motor sciatic branch supplying tibialis anterior muscle during 8 days of EPO treatment, and over 3 days following cessation of treatment. At the start of treatment, NCV was similar to that seen for the tibialis anterior component for the average motor measurement following 2 months diabetes in investigation 1. With treatment NCV increased ($P < 0.001$) towards non-diabetic control levels (dashed lines mean ± s.e.mean for control tibialis anterior from investigation 1) and were not significantly different from them on day 4. This was maintained as the treatment period was extended to 8 days. When EPO treatment was terminated, motor NCV returned to untreated diabetic levels within 24 h ($P < 0.0001$).

Investigation 3. Effects of flurbiprofen treatment on nerve function and capillarization in control, diabetic and evening primrose oil-treated diabetic rats

Table 2 gives body weights and plasma glucose values for control, diabetic and EPO-treated diabetic groups, with and without flurbiprofen treatment. Body weights were reduced with diabetes by an average of 20% over 2 months, and this was not significantly affected by EPO or flurbiprofen treatment. Controls given flurbiprofen showed a 14% increase in body weight, which is in line with the normal growth pattern over this period (Cameron *et al.*, 1989a). Plasma glucose concentrations were elevated by diabetes, and this was unaffected by treatment.

Figure 4 illustrates the results for motor and sensory NCV measurements, which were reduced 21% ($P < 0.001$) and 12% ($P < 0.001$) respectively by 2 months diabetes compared to controls. EPO treatment prevented the decreases in motor ($P < 0.001$) and sensory ($P < 0.001$) NCV with diabetes; the resulting values were not significantly different from the con-

Table 2 Body weights and plasma glucose levels in the evening primrose oil – flurbiprofen preventive study (investigation 3)

Group	n	*Body weight* (g)		*Plasma glucose* (mmol l^{-1})
		Start	Finish	
Control	20	485 ± 11	–	7.6 ± 0.3
Control + flurbiprofen	10	499 ± 15	568 ± 13	6.9 ± 0.3
Diabetic groups				
Diabetic control	20	496 ± 11	369 ± 10^{ab}	39.2 ± 1.8^{ab}
Diabetic + flurbiprofen	21	490 ± 11	409 ± 13^{ab}	34.6 ± 2.1^{ab}
Diabetic + EPO	13	487 ± 13	382 ± 11^{ab}	37.9 ± 3.4^{ab}
Diabetic + EPO + flurbiprofen	23	468 ± 7	391 ± 7^{ab}	33.8 ± 0.8^{ab}

Data are expressed as group means ± s.e.mean.
$^{a}P < 0.001$ versus control group, $^{b}P < 0.001$ versus flurbiprofen-treated control group.

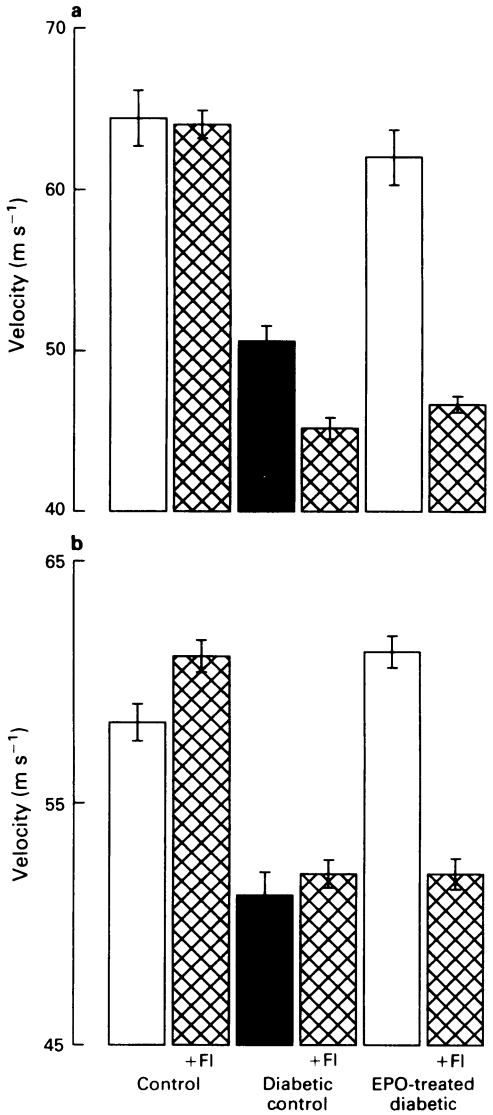


Figure 4 Preventive evening primrose oil (EPO) treatment and the effects of flurbiprofen on sciatic motor and saphenous sensory conduction velocity. (a) Motor; (b) sensory. Columns show means ± s.e.means. Untreated diabetic controls are shown by the solid column ($n = 20$); untreated control ($n = 20$), and EPO-treated diabetic groups ($n = 13$) are shown by open columns. Control ($n = 10$), diabetic ($n = 21$), and EPO-treated diabetic ($n = 23$) groups treated with flurbiprofen (5 mg kg^{-1} day^{-1}), + Fl, are shown by cross-hatched columns. Diabetes caused reductions in motor and sensory conduction velocity ($P < 0.001$) which were prevented by EPO treatment ($P < 0.001$). Flurbiprofen treatment had no significant effect on motor or sensory conduction velocity in non-diabetic controls. It had no effect on sensory conduction in diabetic rats, but reduced motor conduction velocity ($P < 0.01$). Flurbiprofen completely blocked the effects of EPO treatment on motor and sensory conduction velocity ($P < 0.001$).

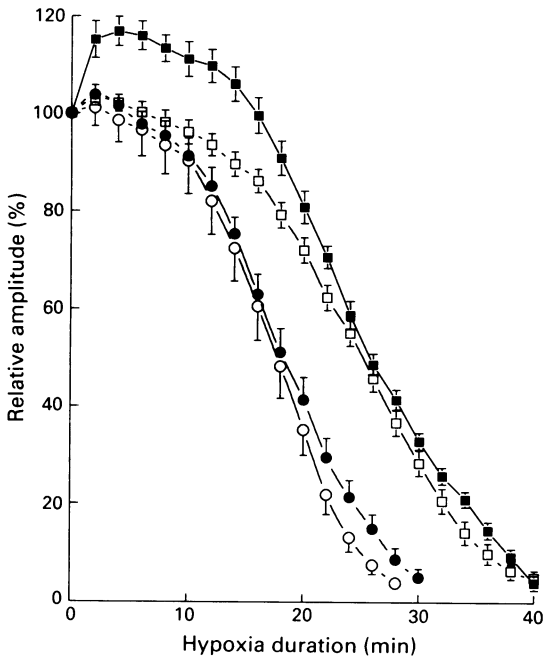


Figure 5 Decline in sciatic compound action potential amplitude with hypoxia for control, diabetic, evening primrose oil (EPO) treated diabetic, and joint evening primrose oil and flurbiprofen-treated diabetic groups. Data points show means ± s.e.means. Control, (●), ($n = 20$); diabetic control, (□), ($n = 20$); EPO-treated diabetic, (●), ($n = 12$); EPO and flurbiprofen-treated diabetic group, (■), ($n = 11$). Data points for diabetic and EPO and flurbiprofen-treated diabetic groups were significantly different ($P < 0.05$) from controls for 16 and 2 min and beyond respectively. Data points from the EPO-treated diabetic group were significantly different ($P < 0.05$) from those for diabetic control and the EPO and flurbiprofen treated group from 16 and 12 min and beyond respectively. There were no significant differences between control and EPO-treated diabetic groups, and the diabetic control and EPO and flurbiprofen treated groups were only significantly different ($P < 0.05$) at 2 and 4 min.

trol group. Flurbiprofen treatment did not have a significant effect on motor or sensory NCV in control rats. For diabetic rats, however, flurbiprofen treatment caused a worsening of motor NCV by 11% ($P < 0.01$), although there was no significant effect on sensory NCV. When EPO and flurbiprofen treatments were combined in diabetic rats, the effect of EPO was completely blocked ($P < 0.001$); the resultant motor and sensory NCV values were not significantly different from diabetic control values.

Figure 5 shows data from *in vitro* measurement of resistance to hypoxic conduction failure (RHCF) for control, diabetic control, EPO-treated diabetic, and joint EPO-flurbiprofen-treated diabetic groups. On initial exposure to hypoxia, sciatic compound action potential amplitude tended to increase, as previously noted (Seneviratne & Peiris, 1969), particularly in the EPO-flurbiprofen-treated diabetic group, and then it declined progressively; the decline being more rapid for control than diabetic nerves. EPO-treated diabetic nerves, however, showed a decline similar to controls, whereas when EPO and flurbiprofen treatment were combined the decline strongly resembled that of untreated diabetic nerves. The decline is summarized for all groups in Figure 6 in terms of the durations of hypoxic exposure necessary for an 80% reduction (T_{80}) in compound action potential amplitude. Compared to controls, T_{80} was increased by approximately 50% with diabetes. This was not significantly affected by flurbiprofen treatment (41% increase). EPO treatment limited the increase in T_{80} to around 6% ($P < 0.001$), which was not significantly different from the control group. The EPO-flurbiprofen-treated diabetic group had a T_{80} prolongation of around 56%, not significantly different from diabetic controls, but greater than for EPO treatment alone ($P < 0.001$).

Figure 7 shows the results of measurements of sciatic endoneurial capillary density. This was not significantly affected by flurbiprofen treatment in control rats. It was also not significantly affected by untreated diabetes. However, in the flurbiprofen-treated diabetic group, capillary density was reduced by 21% ($P < 0.001$ vs non-diabetic and diabetic controls) indicating regression of the vascular bed. EPO treatment increased capillary density by 23% compared to non-diabetic and diabetic controls ($P < 0.001$). However, for the EPO-flurbiprofen-treated diabetic group the increase in capillary density was completely prevented, and there was a

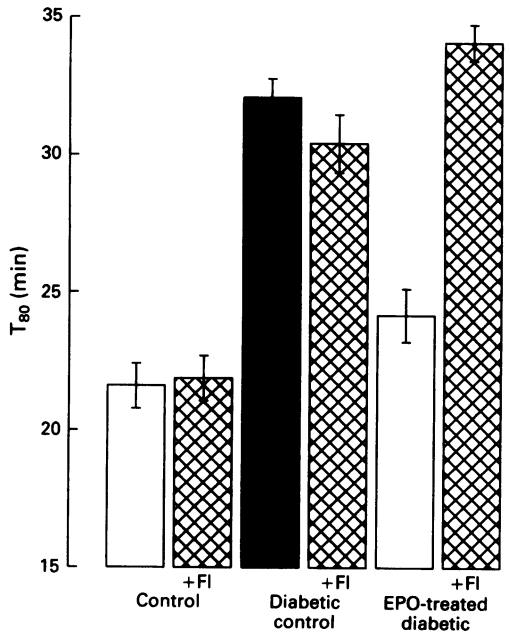


Figure 6 Preventive evening primrose oil treatment (EPO) and the effects of flurbiprofen on the durations of hypoxia necessary for an 80% reduction in sciatic nerve compound action potential amplitude (T_{80}). Columns show means ± s.e.means. Untreated diabetic controls are shown by the solid column ($n = 20$); untreated control ($n = 20$), and EPO-treated diabetic groups ($n = 12$) are shown by open columns. Control ($n = 10$), diabetic ($n = 9$), and EPO-treated diabetic ($n = 11$) groups treated with flurbiprofen (5 mg kg^{-1} day^{-1}), + Fl, are shown by cross-hatched columns. Diabetes increased T_{80} ($P < 0.001$) compared to controls. This was prevented by EPO treatment ($P < 0.001$). Flurbiprofen had no significant effect on T_{80} for control or diabetic groups but blocked the effect of EPO on diabetic T_{80} ($P < 0.001$).

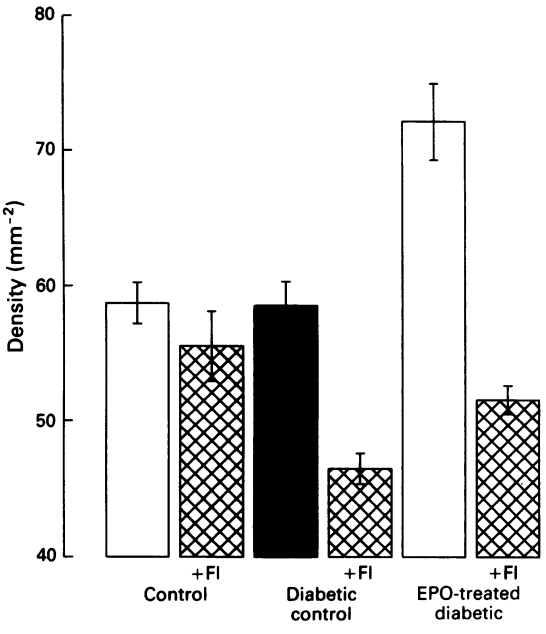


Figure 7 Effect of evening primrose oil (EPO) and flurbiprofen-treatment on sciatic endoneurial capillary density. Columns show means ± s.e.means. Untreated diabetic controls are shown by the solid column ($n = 20$); untreated controls ($n = 20$), and EPO-treated diabetic groups ($n = 12$) are shown by open columns. Control ($n = 10$), diabetic ($n = 21$), and EPO-treated diabetic ($n = 23$) groups treated with flurbiprofen (5 mg kg^{-1} day^{-1}), + Fl, are shown by cross-hatched columns. Diabetes had no effect on capillary density compared to controls, whereas it was increased by EPO treatment ($P < 0.001$). Flurbiprofen treatment blocked the effect of EPO ($P < 0.001$), and also reduced capillary density in diabetic rats ($P < 0.001$).

small (12%) but significant reduction compared to both non-diabetic and diabetic controls ($P < 0.05$).

Discussion

The data demonstrate that the main effects of chronic experimental diabetes on peripheral nerve function, a reduction in NCV and increased RHCF, may be prevented by EPO treatment. This is in agreement with several previous reports for NCV, and one for RHCF (Julu, 1988; Tomlinson *et al.*, 1989; Julu & Mutamba, 1991; Cameron *et al.*, 1991c). The changes in RHCF consequent on 1-month untreated diabetes were not reversed by EPO treatment. Instead, there was a numerical trend for further progression to be blunted, which did not reach statistical significance. This is in agreement with the effects seen for both prazosin and aldose reductase inhibitor treatments (Cameron *et al.*, 1991a; Cameron & Cotter, 1992b), although in these cases attenuation of further RHCF increases was statistically significant. The NCV deficit also appears to be readily and rapidly reversible by EPO treatment, at least during the first 3–4 months of diabetes. The time-course of reversal is similar to that found for chronic electrical nerve stimulation (Cameron *et al.*, 1989b), and for vasodilator treatment with lisinopril (Cameron, Cotter & Robertson, unpublished observations). The washout of EPO effects on NCV was also extremely rapid, with a return to diabetic control levels within 24 h. This suggests that EPO's action relies in the first instance on fairly rapidly synthesized metabolic products, rather than depending on structural changes of nerve fibres or vasa nervorum.

EPO has a complex chemical composition, with only 8–10% being the putative active ingredient γ-linolenic acid, which may act to bypass the deficit in Δ-6 desaturation of linoleic acid in diabetes (Horrobin, 1988). It is likely that this interpretation is essentially correct because previous work has shown that a dietary corn oil supplement which contains a similar proportion of the major EPO constituent linoleic acid (approximately 70%) was completely ineffective in preventing NCV changes (Cameron *et al.*, 1991c), whereas pure γ-linolenic acid, at a dose which matched that contained in the EPO treatment regime used in the present investigation, prevented the development of diabetic NCV deficits (Cotter *et al.*, 1992).

The clear-cut effect of 5 mg kg^{-1} day^{-1} flurbiprofen in completely blocking the long term consequences of EPO treatment on NCV, RHCF, and sciatic nerve capillarization suggests that cyclo-oxygenase-mediated metabolism is crucial to its role in ameliorating nerve dysfunction in experimental diabetes. Major metabolites of potential vascular importance are prostaglandin E_1 (PGE_1), PGE_2, prostacyclin and thromboxane A_2. Treatment of diabetic rats with a PGE_1 analogue, TFC 612, has been demonstrated to ameliorate NCV deficits in diabetic rats (Yasuda *et al.*, 1989). However, it is not clear that this is pertinent to the effects of EPO; at the dose of PGE_1 analogue employed, sciatic nerve blood flow was elevated and it is known that vasodilators which increase nerve perfusion but do not necessarily act via prostanoid metabolism also correct NCV (Cameron *et al.*, 1991b; Hotta *et al.*, 1992). Two further findings also argue against PGE_1 as a mediator of EPO's action. First, PGE_1 analogues cause an increase in sciatic nerve Na^+-K^+-ATPase activity in diabetic rats (Sonobe *et al.*, 1991), whereas EPO appears to have an inhibitory effect on the Na^+-K^+ pump (Lockett & Tomlinson, 1992). Second, admixture of ω-3 essential fatty acids (derived from fish oil) with EPO reduces the effectiveness of the latter in preventing changes in NCV, and RHCF and increasing capillarization in diabetic rats (Cameron *et al.*, 1991c). This formulation promotes the synthesis of PGE_1 from dihomo-γ-linolenic acid (Horrobin, 1988), at the expense of arachidonic acid production, in accordance with the antagonistic effect of ω-3 components such as eicosapentaenoic acid on ω-6 essential fatty acid metabolism (Lands,

1992). Several observations, therefore, do not support the importance of PGE_1 as a major mediator of EPO's actions on diabetic nerve. Thus, the main cyclo-oxygenase metabolite(s) likely to underlie EPO's actions are prostacyclin and/or PGE_2, although data in this investigation and the results of studies published to date are not sufficient to allow a definite judgement on their relative importance to be made. However, Ward et al. (1989) demonstrated a 60% deficit in epi/perineurial vessel synthesis of prostacyclin in experimental diabetes, which depended on lack of substrate availability. As EPO normalizes plasma arachidonic acid levels in diabetic rats (Cameron et al., 1991c), it is plausible that it also acts to restore vasa nervorum prostacyclin production and improves nerve perfusion. Another action of EPO, which may depend on its complex composition as opposed to any single ingredient, is a reduction in thromboxane A_2 (TxA_2) synthesis (Horrobin & Manku, 1990). This is a potentially beneficial action as increased platelet aggregation may contribute to elevated blood viscosity (Barnes et al., 1977) in diabetes which, in turn, could be partly responsible for reduced endoneurial blood flow (Low et al., 1987). Thus, EPO may affect nerve conduction indirectly by partly restoring normal endothelial vasodilator production and the prostacyclin/TxA_2 ratio, a view that is entirely consistent with the effects of vasodilators on NCV and RHCF in experimental diabetes (Cameron et al., 1991a,b; 1992).

Data on sciatic nerve capillary density confirm a previous finding (Cameron et al., 1991c) of angiogenesis with chronic EPO treatment. This was less apparent for the reversal study, which suggests that either insufficient treatment time was given for vessel growth at the dose of EPO used, or that chronic diabetes blunts the ability of vessels to grow. The former seems to be the case, as a reversal study with prazosin over a similar time period produced greater capillary growth (Cameron et al., 1991a). Thus, comparing prevention and reversal studies, the similar effect of EPO on NCV in both cases contrasts with differential effects on capillarization, suggesting that angiogenesis is a result of changes in nerve vascular dynamics that also improve NCV, rather than angiogenesis being a necessary condition for NCV normalization. In other tissues, notably heart and skeletal muscle, the mechanical effect of increased blood flow on endothelium leads to angiogenesis (Hudlická & Tyler, 1986), and it is possible that this also applies to sciatic nerve in diabetic rats.

Flurbiprofen treatment exacerbated the sciatic motor NCV deficit with diabetes, and this was accompanied by a reduction in endoneurial capillary density. Prevention of the effect of EPO on NCV was also paralleled by a somewhat lesser loss of capillaries. The effect on capillary density suggests regression of vasa nervorum which, on an hypothesis linking the size of the vascular bed with flow levels (Hudlická & Tyler, 1986), further suggests that sciatic endoneurial blood flow may have chronically decreased below the diabetic level. The fact that there was not a similar regression of capillaries in flurbiprofen-treated control rats, or a deterioration of NCV, indicates that there are probably other abnormalities of vascular/endothelium function found in diabetes that may normally be important for maintaining the integrity of the vascular bed. A large number of cardiovascular deficits have been demonstrated in experimental diabetes, including a reduction in nitric oxide production (Tomlinson et al., 1992; Cameron & Cotter, 1992a), and abnormalities in the thrombolytic system (Maiello et al., 1992). It is tempting to speculate that multiple endothelial dysfunction in diabetes reduced the capacity of the vascular system to compensate for the further insult of flurbiprofen treatment, compared to the situation in control rats.

A previous report (Parry & Kohzu, 1990) on the effect of picroxicam (5 mg kg^{-1} day^{-1}) on motor NCV in diabetic rats did not detect significant worsening with treatment. However, this study was carried out using the sciatic tibial-interosseous nerve preparation in younger rats. In this preparation, the NCV deficit with diabetes is relative to age-matched rather than onset controls, and reflects a substantial component of reduced axon growth compared with the nerve branches examined in the present study (Cameron et al., 1986; 1989a). It is possible that this may explain the discrepancy between the effects of cyclo-oxygenase inhibition in the present study and that of Parry & Kohzu (1990); certainly in our hands the tibial-interosseous preparation is atypical of sciatic nerve motor fibres in response to other treatments such as aldose reductase inhibitors (Cameron et al., 1989a; Cameron & Cotter, 1992b).

In conclusion, EPO prevented and rapidly reversed the NCV deficits found in diabetic rats. It prevented but did not reverse the RHCF abnormalities, at least over 1 month of treatment. Changes in nerve capillarisation suggest a vascular action. All EPO effects were completely blocked by flurbiprofen treatment, and both motor NCV and sciatic capillary density in diabetic rats were compromised by flurbiprofen. This suggests that cyclo-oxygenase mediated metabolites such as prostacyclin are vital for EPO's actions, and are necessary for maintaining the integrity of sciatic vasa nervorum in diabetic rats.

This work was supported in part by a grant from the British Diabetic Association. K.C.D. was supported by a research studentship from Scotia Pharmaceuticals. D.C. was supported by a vacation studentship from the Scottish Home and Health Department.

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(Received October 13, 1992
Revised February 17, 1993
Accepted March 16, 1993)

Br. J. Pharmacol. (1993), **109**, 980–986 © Macmillan Press Ltd, 1993

Vasoreactivity and prostacyclin release in streptozotocin-diabetic rats: effects of insulin or aldose reductase inhibition

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1 Alterations in vasoreactivity and endothelial cell function could underlie some of the vascular abnormalities in diabetes. To examine aspects of these phenomena we studied the effects of 4– 6 weeks streptozotocin-induced diabetes in the rat on basal and angiotensin II (AII)-stimulated prostacyclin release from isolated lung, perfused at constant flow. In addition, pressure was monitored throughout the lung perfusion as an index of vasomotor tone.

2 The experiment also included lungs from groups of diabetic rats treated with either insulin or an aldose reductase inhibitor (imirestat), to determine whether these treatments influenced the development of any defects seen in untreated diabetes.

3 Despite some indication of a trend towards reduced prostacyclin release in lungs from diabetic rats, neither the basal nor AII-stimulated release was significantly different from that seen in tissues from control animals. There were no significant differences between groups in the average basal perfusion pressure and in either the absolute pressure response to AII or the time of this peak.

4 The area under the perfusion pressure curve during AII infusion was greater in lungs from diabetic animals than in controls indicating a prolonged vasoconstrictor response. This increased pressor response may indicate increased sensitivity of diabetic tissue to AII or a reduced production of vasodilators in response to the vasoconstriction.

5 Whichever mechanism was responsible, this alteration was prevented by insulin treatment but not by aldose reductase inhibition, implicating mechanisms probably unrelated to exaggerated polyol pathway flux.

Keywords: Prostacyclin; diabetes; aldose reductase inhibition; insulin; angiotensin II (AII); lung

Introduction

Microvascular complications are a major cause of morbidity and mortality in Type I and Type II diabetes mellitus and encompass changes in both the macro- and microvasculature (Tooke, 1989). Microangiopathy is characterized by capillary basement membrane thickening and endothelial cell proliferation, which occurs in most tissues of diabetic patients and animals (Williamson & Kilo, 1983). Alterations in haemorheology (McMillan, 1983) and endothelial function (Stout, 1987) have been implicated in the aetiology of diabetes-related microvascular changes but the cause may well be multifactorial. There is evidence to suggest that, with the possible exception of the kidney where early increases in prostaglandin production may mediate early increases in renal blood flow (Craven & De Rubertis, 1989), the production of the vasodilator and anti-platelet agent, prostacyclin, is reduced in experimental and clinical diabetes (Silberbauer *et al.*, 1979; Rosenblum & Hirsh, 1984; Carreras *et al.*, 1980). Conversely, the production of the vasoconstrictor and platelet aggregating agent, thromboxane A_2, is increased (Lagarole *et al.*, 1980; Ziboh *et al.*, 1979; Rosenblum & Hirsh, 1984). The production of the potent vasoconstrictor endothelin may also be increased in diabetic animals (Takeda *et al.*, 1991) and patients (Takahashi *et al.*, 1990) while endothelium-dependent relaxation is reduced in diabetic animals (Durante *et al.*, 1988; Hattori *et al.*, 1991) possibly as a consequence of a reduced production of nitric oxide. In addition, vascular smooth muscle from diabetic animals appears to be more sensitive to vasoconstrictors and less sensitive to vasodilators (Gebremedhin *et al.*, 1988; Abebe & MacLeod, 1991; Mayhan *et al.*, 1991). Although not all studies have confirmed these findings (Rosen & Schror, 1980), the presence of one or more of the above abnormalities might provoke ischaemia. These phenomena may, therefore, be causative factors in the development of microvasculopathy.

The development of microangiopathy is associated with both poor control of blood glucose and long duration of diabetes (Pirart, 1978a,b), although genetic background and the presence of other complicating factors may play a role (Jennings & Barnett, 1988). However, optimal diabetic control remains a practical impossibility in many diabetics and there is, therefore a requirement for supplementary therapies. These must develop from a knowledge of the biochemical and cellular basis of the microvascular changes. Exaggerated flux of glucose through the polyol or sorbitol pathway has been implicated in a range of diabetic complications including retinopathy, nephropathy and neuropathy (Dvornik, 1987). Aldose reductase, the first enzyme of this pathway, has been identified in endothelial cells from several sources (Kern & Engerman, 1982; Hawthorne *et al.*, 1989; Yorek & Dunlap, 1989) and it is thus logical to examine its role in diabetes-associated aspects of endothelial cell dysfunction.

To examine aspects of both vasoreactivity and endothelial cell function we have studied the effects of streptozotocin-induced diabetes in the rat on perfusion pressure and prostacyclin release from isolated perfused lungs under basal and angiotensin II (AII)-stimulated conditions. We have also studied tissues from diabetic rats treated with either insulin or an aldose reductase inhibitor.

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Methods

Experimental organisation and treatment regimes

Male Wistar rats (weight range 310–380 g) were randomly assigned to four groups. Three groups were made diabetic by a single intra-peritoneal injection of 65 mg kg^{-1} streptozotocin (STZ) in sterile physiological saline; the remaining group served as controls. Three days later blood samples were obtained by tail prick from the STZ-injected rats and blood glucose concentration determined by a glucose oxidase strip-operated reflectance meter (Reflolux II; Boehringer Mannheim, U.K.). All these rats had blood glucose concentrations >15 mM and were included in the study. One group of diabetic rats remained untreated while another group was given the aldose reductase inhibitor imirestat (Hoe 843; 2,7-difluorospiro[fluorene-9,4'-imidazolidine]-2',5'-dione; Hoechst AG, Frankfurt/Main, Germany) at a dose of 1 mg kg^{-1} day^{-1} by gavage. The remaining diabetic group were maintained on a sub-cutaneous insulin treatment regime (Willars *et al.*, 1987). In addition to our previous regime, on Friday afternoons any rat registering blood glucose $\geqslant 10$ mM, received 2 iu 100 g^{-1} of a long-acting heat-treated Ultralente preparation (Novo Industri A/S, Copenhagen, Denmark; see Rasch, 1979), any with blood glucose <10 mM, but $\geqslant 5$ mM received 1 iu 100 g^{-1}. If blood glucose was <5 mM then no insulin was given until Monday morning. In the third week of the study a 24 h blood glucose profile was obtained by determining the blood glucose levels at approximately 2 h intervals over two periods separated by 1 day.

Determination of perfusion pressure and prostacyclin release

Lung preparation Four to six weeks following STZ injection, diabetic and control rats were studied in mixed batches. Blood samples were obtained for determination of glucose concentration and rats were anaesthetized with sodium pentobarbitone (Sagatal, May and Baker Ltd., England: 120 mg kg^{-1} i.p.). After administration of heparin (Multiparin, C.P. Pharmaceuticals, England: 100 iu kg^{-1}) via the vena cava, the abdominal aorta was cut and the pulmonary artery cannulated via the heart's right ventricle. Lungs were perfused at a constant flow of approximately 5 ml min^{-1}, without recirculation, with Krebs-Henseleit bicarbonate-buffered saline solution (KHBBS; composition mM: NaCl 118, KCl 4.8, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 11.1, $CaCl_2$ 2.5), pH 7.4, containing 100 unit ml^{-1} heparin (CP Pharmaceuticals Ltd. Wrexham, U.K.) and 1% bovine serum albumin (BSA, essential fatty acid free, fraction V, Sigma Chemical Co. Ltd., England). The perfusate was gassed (95%O_2/5%CO_2) and at room temperature. The left atrium was cut to allow perfusate outflow and the trachea cannulated; the preparation was then excised and suspended in a heated chamber. The perfusate line was switched to non-heparinised KHBBS at 37°C and gassed (95%O_2/5%CO_2) with a disc oxygenator. Lungs were inflated via the tracheal cannula. Perfusion pressure, immediately proximal to the lungs, was monitored throughout with a Bell and Howell (England) pressure transducer. The analog output was amplified, digitized, captured and analysed on computer.

Protocol for lung perfusion Lungs were perfused for a 30 min 'equilibration' period during which perfusate was collected in 10 min samples. This was followed by a 3 min 'basal' period and a 5 min 'stimulation' period of AII infusion, both with 1 min perfusate collections. AII (5×10^{-5} M in KHBBS) was infused immediately proximal to the lung at 0.125 ml min^{-1}. Following the stimulation period, perfusion was continued for a further 15 min 'recovery' period with 3 min collections. Perfusate samples were stored at −20°C until radioimmunoassay of 6-keto-prostaglandin F_1, the stable metabolite of prostacyclin. Following perfusion, lungs were dissected free from other tissue, blotted dry, weighed, finely minced and portions taken for determination of protein content (Lowry *et al.*, 1951) and for dry weight by freeze drying.

Sciatic nerve sugar and polyol content

Portions of right sciatic nerve were removed from rats and stored at −20°C until determination of glucose, sorbitol and fructose levels by capillary gas-chromatographic detection of their trimethylsilyl derivatives using α-methyl(D)mannoside as an internal standard (Mayer & Tomlinson, 1983).

Statistical analysis

Data were analysed by one-way analysis of variance and where the F ratio gave $P<0.05$, comparisons between individual group means were made by Duncan's multiple range tests at significance levels of $P=0.05$ and $P=0.01$.

Results

Body weight, final blood glucose and nerve content of glucose, sorbitol and fructose

All rats lost weight immediately following STZ injection (Figure 1). Diabetic rats, whether untreated or treated with imirestat, showed progressive weight loss over the study period. Insulin-treated diabetic rats gained weight at a similar rate to control animals although, due to the initial weight loss, their final weights did not reach control values. Final blood glucose levels were considerably greater in the untreated and imirestat-treated diabetic rats compared to both control and insulin-treated diabetic animals (Table 1). Typical blood glucose values during a 24 h period for the insulin-treated rats are shown in Figure 2.

Levels of glucose, sorbitol and fructose were significantly elevated in sciatic nerves from diabetic rats compared to those from controls (Table 1). These elevations were markedly reduced by insulin treatment and, in the cases of sorbitol and fructose, by imirestat.

Lung perfusion pressures and prostacyclin release

Despite the absence of differences in lung dry weights, following perfusion, the wet:dry weight ratio was significantly elevated in the diabetic group compared to that of controls (Table 2). This was prevented by treatment with insulin but not imirestat.

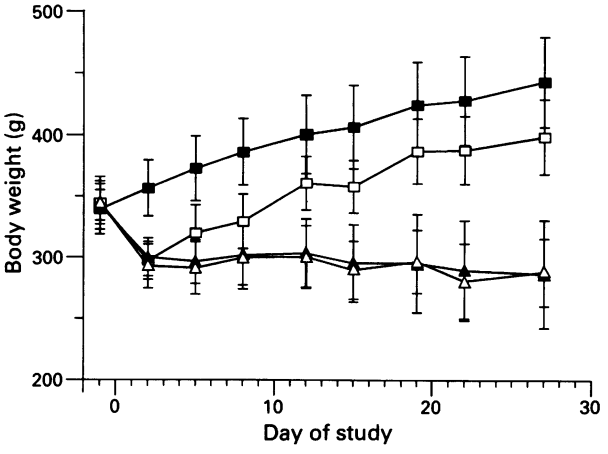


Figure 1 Body weights of all animals throughout the experimental period. Data are means ± 1 standard deviation of 9 or 10 rats per group; (■) control; (▲) diabetic; (□) diabetic plus insulin; (△) diabetic plus imirestat.

Table 1 Final blood glucose and sciatic nerve levels of sugars and polyols

Group	n	*Final blood glucose* (mM)	n	*Sciatic nerve* (nmol mg^{-1} nerve wet weight) *Glucose*	*Sorbitol*	*Fructose*
Control	9	5.2 ± 0.83	8	0.56 ± 0.39[a]	0.14 ± 0.12[a]	1.01 ± 0.21[a]
Diabetic	10	>27.7	10	14.51 ± 4.68[b]	5.39 ± 2.09[b]	10.79 ± 2.38[b]
Diabetic insulin-treated	9	7.9 ± 5.46	6	2.67 ± 2.31[c]	0.29 ± 0.18[c]	2.04 ± 1.66[c]
Diabetic imirestat-treated	9	>27.7	8	19.84 ± 3.27[d]	0.39 ± 0.19[e]	2.83 ± 0.66[d]

Data are mean ± 1 standard deviation
Blood glucose values were not subject to statistical analysis. Sugar and polyol levels were analysed by oneway analysis of variance with Duncan's multiple range tests at $P = 0.05$ and $P = 0.01$: a *vs* b; d and b *vs* c; d,e and c *vs* d; $P < 0.01$, a *vs* e $P < 0.05$.

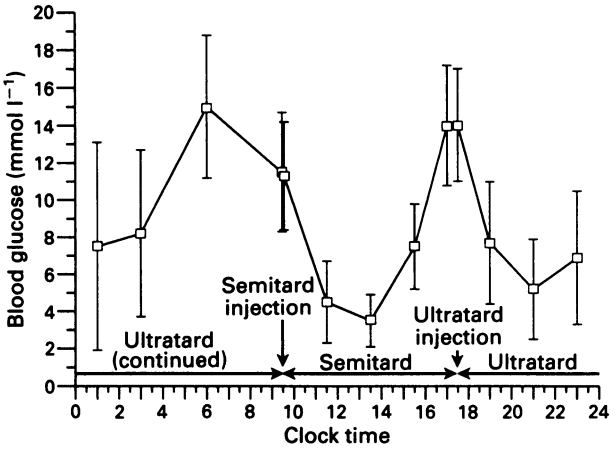


Figure 2 Blood glucose profile throughout a 24 h period in insulin-treated diabetic rats. Data are means ± 1 standard deviation of 9 rats.

Figure 3 shows a typical perfusion pressure trace and profile of protacyclin release. In response to AII infusion there was a sharp rise in perfusion pressure which returned to basal levels within the period of infusion. However, the stimulation of prostacyclin release was still apparent during the early period of the recovery phase.

There were no significant differences between groups in average basal perfusion pressure (mean for all lungs ± s.d. = 5.1 ± 3.3 mmHg, $n = 33$) and in either pressure response above zero to AII (mean ± s.d. = 22.3 ± 6.1 mmHg, $n = 33$) or time of this peak (mean ± s.d. = 59 ± 20s, $n = 33$). However, areas under perfusion pressure curves during AII infusion, both above zero and above mean basal pressure, were greater in lungs from untreated diabetic rats compared to those from controls (Figure 4), although this did not reach significance when measured above zero. These increases were ameliorated by the insulin treatment but not by imirestat.

There was considerable within group variability in prostacyclin release from lung. However, there was some indication of a trend toward both reduced basal release and absolute release during AII stimulation in the untreated and imirestat-treated diabetics compared to controls and insulin-treated diabetics (Figure 5). Differences in mean basal release, peak when measured either above zero or basal levels

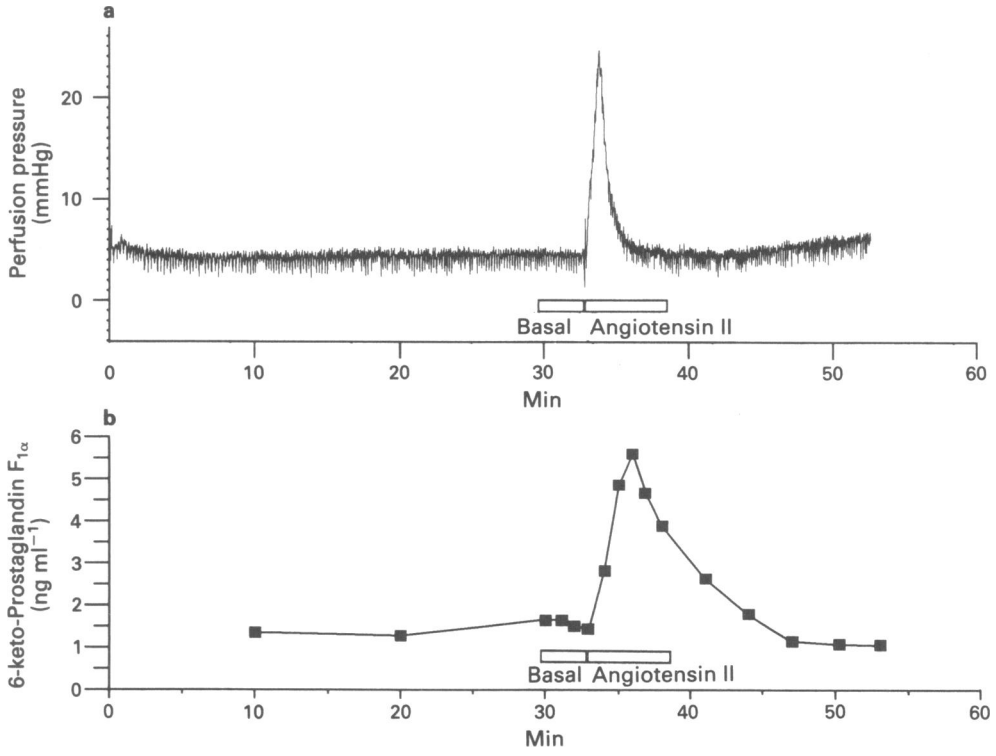


Figure 3 Typical profiles of (a) perfusion pressure and (b) prostacyclin (measured as 6-keto-prostaglandin $F_{1\alpha}$) release during lung perfusion from a single control rat. The boxes represent basal and angiotensin II stimulation periods as indicated.

Table 2 Lung wet and dry weights, wet:dry weight ratios and protein contents following perfusion

Group	n	Lung: *Wet weight* (g)	*Dry weight* (g)	*Wet:dry weight ratio*	*Protein content* (mg)
Control	9	1.53 ± 0.28	0.265 ± 0.039	5.76 ± 0.63[a]	129.5 ± 18.7
Diabetic	10	1.92 ± 0.70	0.224 ± 0.036	8.38 ± 2.09[b]	130.6 ± 45.2
Diabetic insulin-treated	9	1.57 ± 0.28	0.238 ± 0.023	6.61 ± 0.83[c]	122.6 ± 21.7
Diabetic imirestat-treated	9	1.61 ± 0.27	0.211 ± 0.075	7.47 ± 1.84[d]	110.8 ± 21.3

Data are mean ± 1 standard deviation
Data were anlysed by oneway analysis of variance with Duncan's multiple range tests at $P = 0.05$ and $P = 0.01$; a *vs* b $P < 0.01$; b *vs* c and a *vs* d $P < 0.05$.

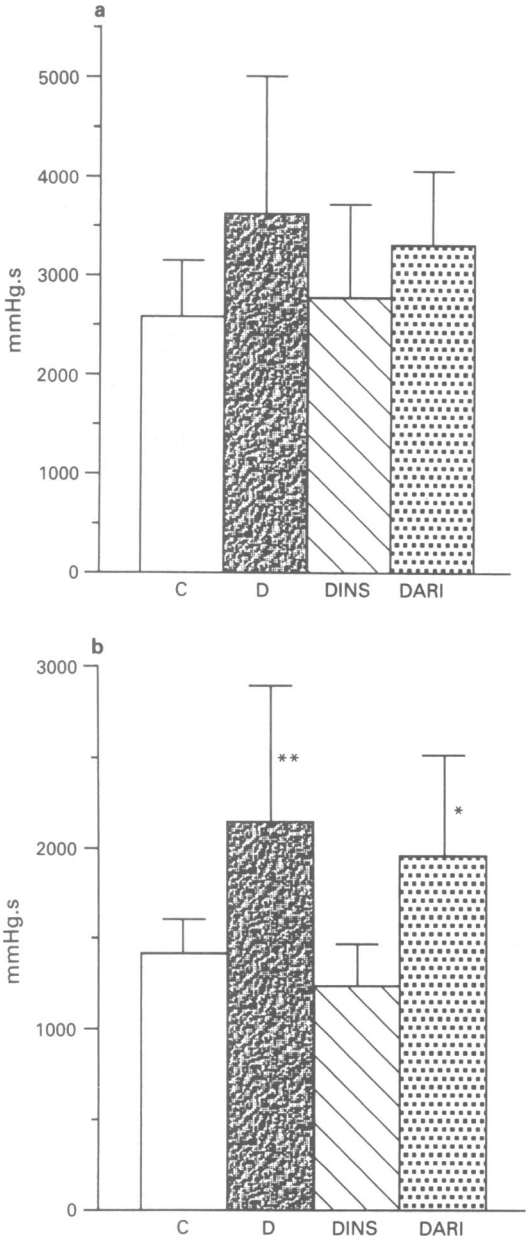


Figure 4 Areas under the perfusion pressure curves during angiotensin II (AII) infusion both (a) above zero and (b) above mean basal perfusion pressure. Key: C = control, D = diabetic, DINS = diabetic + insulin, DARI = diabetic + imirestat. Data are means + 1 standard deviation for 7–9 rats per group and were analysed by oneway analysis of variance with Duncan's multiple range tests at $P = 0.05$ and $P = 0.01$: for $*P < 0.05$ and for $**P < 0.01$ versus control and diabetic + insulin groups.

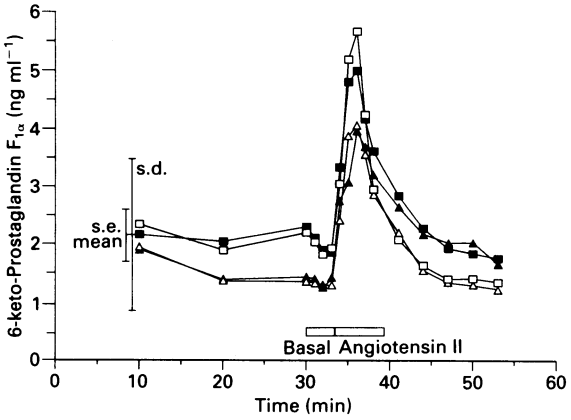


Figure 5 Prostacyclin (measured as 6-keto-prostaglandin $F_{1\alpha}$) release profiles during lung perfusion. The boxes represent basal and angiotensin AII stimulation periods as indicated. Data are means of 8–10 rats per group. For clarity, only an example of the error bars are shown. (■) Control; (▲) diabetic; (□) diabetic plus insulin; (△) diabetic plus imirestat.

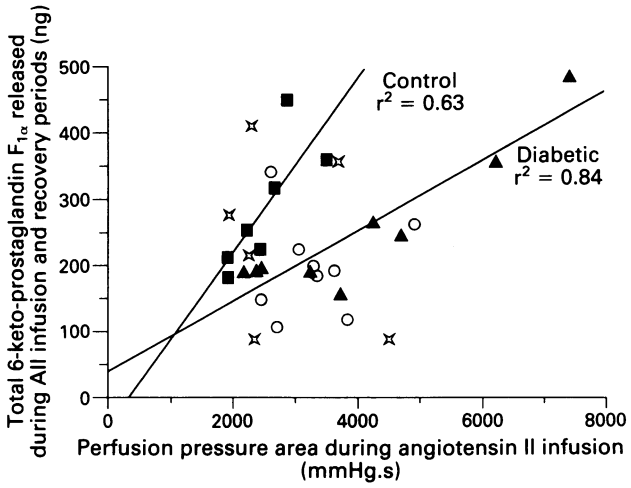


Figure 6 Areas under the lung perfusion pressure curves, above zero, during angiotensin II (AII) infusion plotted against total prostacyclin (measured as 6-keto-prostaglandin $F_{1\alpha}$) release during the AII infusion and the recovery period. (■) Control; (▲) diabetic; (□) diabetic plus insulin; (○) diabetic plus imirestat.

during AII infusion and total prostacyclin released during stimulation and recovery periods were not statistically significant. Lung protein content and dry weight were not significantly different (Table 2) and expressing prostacyclin release in relation to either unit of tissue mass did not reveal any significant differences (data not shown).

We examined the relationship between AII-stimulated prostacyclin release and pressor response by scatter plot (Figure 6). Regression lines of best fit (determined by the least squares method) were significant for control ($P = 0.03$) and untreated diabetic ($P = 0.0005$) animal groups, though comparison of slopes by coregression analysis did not reach statistical significance. Nonetheless, this plot does indicate a deficit in prostacyclin release in preparations from diabetic rats. The points derived from preparations from insulin- and imirestat-treated diabetic rats were more scattered and did not give significant regression; in general, those of the former group fell closer to the control regression, whilst the latter approximate to the regression of untreated diabetic preparations.

Discussion

The insulin treatment regime was judged to be effective on the basis of parallel weight increases in control and insulin-treated groups, low blood glucose values and prevention of exaggerated nerve levels of sugars and polyols compared to untreated diabetic tissues. Imirestat treatment was effective at inhibiting aldose reductase, at least in peripheral nerve, as judged by the marked prevention of sorbitol and fructose accumulation of sciatic nerve. It should be noted that we cannot be sure that this inhibitor entered vascular cells to inhibit aldose reductase.

Following perfusion, the wet:dry weight ratio of lungs was significantly elevated in tissues from diabetics compared to those from controls. This was prevented by insulin, but not imirestat treatment. The increased wet weights of lungs from untreated and imiresat-treated diabetic rats may have developed during perfusion and therefore may reflect alterations in permeability barriers previously reported in diabetes (Williamson *et al.*, 1985). Raised glucose content of these tissues might be expected to provide an adequate driving force for fluid uptake either *in vivo* or *in vitro*.

Tissues from patients and animals with different forms of diabetes mellitus have been reported to produce less prostacyclin *in vitro* than tissues from their non-diabetic controls (Watts *et al.* 1982; Lubawy & Valentovic, 1982; Roth *et al.*, 1983). We were therefore surprised by the lack of marked effect of diabetics on prostacyclin release from perfused lung in the present study. Others have also noted a large within-group variability in protacyclin release from isolated lung (Watts *et al.*, 1982) and, against such a background, it is difficult to resolve differences between experimental groups. Previous studies have demonstrated reduced arachidonic acid (AA)-stimulated release of prostacyclin from perfused lungs of diabetic rats (Lubawy & Valentovic, 1982), albeit with a recirculating perfusion system which amplified differences. In another study using the lung, basal prostacyclin release was reduced, but AA-stimulated release, although numerically reduced, was not significantly different from control (Watts *et al.*, 1982). That study also indicated large differences in body weight which highlights the importance of selection of an appropriate unit of tissue reference, as alterations in tissue amount contributing to prostacyclin release may artifactually influence results.

Prostacyclin release from isolated perfused hearts of diabetic rats has been reported to be both increased (Rosen & Schror, 1980) and decreased (Rosen *et al.*, 1983). In the former study, compared to controls, prostacyclin release was increased in response to AA whereas in the latter it was decreased in response to challenge by bradykinin. Choice of agonist may, therefore, influence the outcome. Thus, while a low dose of AA increased prostacyclin release in diabetic hearts, there was no such difference at a higher dose (Roth *et al.*, 1983). Direct comparison between studies which have examined the effects of diabetes on prostacyclin release is, therefore, often difficult and extrapolation to the physiological condition is fraught with difficulties.

The use of AA has other implications in assessment of the nature of any alterations in prostanoid production in diabetes. Provision of this substrate has the potential to reveal deficits in cyclo-oxygenase or subsequent metabolic steps, whereas agonists which liberate AA from membrane phospholipids by activation of a phospholipase have the potential to reveal both receptor and post-receptor defects. Although there are many reports of reduced prostacyclin production under basal conditions in tissues from diabetic sources (Watts *et al.*, 1982; Lubawy & Valentovic, 1982), results in the presence of AA have been equivocal. With the possible exception of Rosen *et al.* (1983), few studies have examined the release of prostacyclin via a receptor-linked agonist and there is, therefore, little direct evidence of the integrity of receptor and early post-receptor events.

The present study suggests no marked alterations in basal or AII-stimulated release of prostacyclin from lungs of diabetic rats. However, consideration should be given to the mechanism(s) by which AII provoked prostacyclin release. While some have argued that this agonist is able to stimulate endothelial cells directly to produce prostacyclin (Dusting *et al.*, 1981), others have suggested release due to mechanical stimulation of the endothelium brought about by contraction of the adjacent smooth muscle (Nakagawa *et al.*, 1987). Indeed AII receptors have been reported to be absent from endothelial cells (Ody *et al.*, 1983), but present on smooth muscle cells (Gunther *et al.*, 1979). This raises the possibility that prostacyclin release was provoked, at least in part, by the mechanical consequences of vasoconstriction. The scatter plot (Figure 5) indicates a positive correlation between perfusion pressure and prostacyclin release, in response to AII, although it must be remembered that this does not imply cause and effect. It is, however, important to note that the mean area under the perfusion pressure curve during AII stimulation was greater in lungs of diabetic animals compared to those of controls. The magnitude of the stimulus promoting prostacyclin release may therefore have differed between experimental groups and the greater pressure response in the diabetics could mask any potential reductions in prostacyclin release. The scatter plot indicates that, for a given perfusion pressure, prostacyclin release would be reduced in lungs from untreated diabetic rats, especially in the upper ranges of pressure responses to AII. Data were obtained under constant flow conditions and with a single submaximal dose of AII. Alternative experiments, using constant pressure with possible variations in flow and endothelial shearing forces may reveal more marked differences in prostacyclin release between groups. Differences might also be revealed with a full range of AII doses.

The increased response of diabetic tissue to AII may have resulted from an increased tissue sensitivity to the agonist but could also arise due to reduced release of other vasodilators such as nitric oxide in response to the vasoconstriction. Indeed it has been reported that in some vessels, nitric oxide, but not cyclo-oxygenase products, modulate the contractile response to AII (Gruetter *et al.*, 1988). Both an increased sensitivity to a vasoconstrictor and reduced production of nitric oxide are in accord with previous reports (Durante *et al.*, 1988; Abebe & MacLeod, 1991).

Insulin treatment of diabetic rats prevented many of the alterations seen in untreated animals, including increased pressure response to AII and increased wet:dry weight ratio. Imirestat, however, had no effect on these parameters suggesting an aetiology unrelated to excessive polyol pathway flux. Although aldose reductase activity has been reported in lung (Ghahary *et al.*, 1991) in many respects the lack of an imirestat effect is consistent with a lack of aldose reductase

immunoreactivity reported in lung capillaries, albeit in the non-diabetic dog (Kern & Engerman, 1982). Although the relevance to the initiation of complications is unclear, it is of interest that the level of aldose reductase activity in lung is not increased in diabetes. In tissues such as the lens, nerve, retina and kidney, in which secondary complications of diabetes occur, there is increased enzyme activity with the development of diabetes (Ghahary *et al.*, 1991). There have been few other reports on the effects of aldose reductase inhibition on prostacyclin release. In one study, unstimulated prostacyclin release was reduced by about 70% by diabetes. Aldose reductase inhibition had a significant ameliorative effect on this reduction but release was still about 50% lower than that seen in controls (Wagasugi *et al.*, 1991).

In summary, we have seen little difference in the ability of lung to release prostacyclin under basal or AII-stimulated conditions, although evidence indicates an increased vasoconstriction in diabetic tissue to AII. If the magnitude of vasocontriction contributes significantly to prostacyclin release this may, therefore, have had the effect of masking a deficit in release in lungs from diabetic animals because of unequal challenges. Whatever the reason for the increased pressure response to AII, whether increased tissue sensitivity or reduced nitric oxide production, in diabetic lung this was probably not related to exaggerated polyol pathway flux.

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(Received October 22, 1992
Revised February 25, 1993
Accepted March 16, 1993)

Br. J. Pharmacol. (1993), **109**, 987–991 © Macmillan Press Ltd, 1993

Bacterial endotoxin rapidly stimulates prolonged endothelium-dependent vasodilatation in the rat isolated perfused heart

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1 The effects of bacterial lipopolysaccharide (*Escherichia coli* 0111-B4; LPS) on coronary vascular tone were examined in the isolated perfused heart of the rat. The role of nitric oxide and/or prostaglandin products of the cyclo-oxygenase pathway in mediating the actions of LPS were also investigated.

2 Coronary vascular tone was raised and maintained by a continuous perfusion of the thromboxane-mimetic U46619 (5 nM). LPS perfusion ($0.1-100\,\mu g\,ml^{-1}$) caused a concentration-dependent fall in coronary tone without any significant change in the force of cardiac contractility.

3 At $5\,\mu g\,ml^{-1}$, LPS reduced perfusion pressure by 38 ± 9 mmHg. This effect was rapid in onset, maximal within the first 5 min and sustained for 90 ± 10 min ($n = 6$).

4 The vasodilatation induced by LPS was dependent on the presence of an intact endothelium and abolished following endothelial damage caused by air embolism.

5 N^G-nitro-L-arginine methylester (L-NAME; 50 μM) or N^G-nitro-L-arginine (L-NOARG; 50 μM) blocked the vasodilatation induced by LPS ($5\,\mu g\,ml^{-1}$). The inhibition caused by these arginine analogues was partially reversed by 1 mM L- but not D-arginine.

6 The vasodilator action of LPS was also completely blocked by the glucocorticoid, dexamethasone (10 μM) but unaffected by indomethacin (10 μM).

7 These results suggest that LPS evokes rapid release of nitric oxide (NO) in the microvasculature of the rat isolated heart presumably via activation of the constitutive L-arginine-NO pathway in the endothelium. Furthermore, the lack of effect of indomethacin suggests that prostaglandins released via the cyclo-oxygenase pathway are not involved in mediating this action of LPS.

Keywords: Rat heart; coronary microcirculation; bacterial lipopolysaccharide; nitric oxide; L-arginine; N^G-nitro-L-arginine methylester; N^G-nitro-L-arginine; dexamethasone; indomethacin; bradykinin

Introduction

Experimental models of endotoxin shock have shown that administration of bacterial lipopolysaccharide (LPS) to animals *in vivo* results in a characteristic biphasic fall in systemic blood pressure in which the first phase of the response occurs rapidly following administration of LPS and the second phase develops as a gradual progressive fall in blood pressure after a lag phase of about 30 min (Thiemermann & Vane, 1990; Wright *et al.*, 1992). The development of the sustained hypotensive response (second phase) is currently attributed mainly to nitric oxide (NO) (Kilbourn *et al.*, 1990; Thiemermann & Vane, 1990; Wright *et al.*, 1992), derived from the cationic amino acid L-arginine (Palmer *et al.*, 1988a; Schmidt *et al.*, 1988). Synthesis of NO under these conditions involves induction of the Ca^{2+}/calmodulin-independent NO synthase previously identified in macrophages (Stuehr & Marletta, 1987; Marletta *et al.*, 1988). Expression of this enzyme is time-dependent and involves *de novo* protein synthesis which can be inhibited by the protein synthesis inhibitor, cyclohexamide and by the glucocorticoid, dexamethasone (Knowles *et al.*, 1990; Radomski *et al.*, 1990; Rees *et al.*, 1990a). Once induced the activity of the enzyme can be blocked by inhibitors of NO synthase including N^G-monomethyl-L-arginine (L-NMMA) (Kilbourn *et al.*, 1990; Radomski *et al.*, 1990; Rees *et al.*, 1990a; Thiemermann & Vane, 1990, Wright *et al.*, 1992).

In addition to the inducible enzyme there is also a constitutive Ca^{2+}/calmodulin-dependent NO synthase which generates NO in the endothelium under normal physiological conditions. NO released via this pathway regulates blood flow and is thought to maintain the cardiovascular system in a constant state of vasodilatation (Rees *et al.*, 1990b; see Moncada *et al.*, 1991 for review). At present it is not clear whether, in the vasculature, this enzyme is also activated by LPS and whether NO released via the constitutive pathway plays any role in maintaining the deleterious actions of LPS either *in vivo* or *in vitro*. In this context it is of interest that in rats, both the initial and sustained phases of the vasodilator response to LPS are attenuated by L-NMMA (Thiemermann & Vane, 1990), suggesting that NO may be released acutely by LPS in the earlier stages of endotoxic shock. This hypothesis however remains to be substantiated, since in similar studies L-NMMA did not inhibit the initial action of LPS in lowering arterial blood pressure in rabbits (Wright *et al.*, 1992).

In the present study we have investigated the effects of LPS in the isolated perfused heart of the rat with the aim of establishing whether coronary tone can be altered acutely by a direct action of LPS on the microvasculature. Using inhibitors of nitric oxide synthase, N^G-nitro-L-arginine (Moore *et al.*, 1990; Ishii *et al.*, 1990) and N^G-nitro-L-arginine methylester (Rees *et al.*, 1990b), we have also examined whether NO is released acutely in response to LPS and whether this may mediate the effects of LPS in the microvasculature of the rat heart. Furthermore, since LPS is known to activate phospholipase A_2 (Liu & Takeda, 1982) and thereby elevate prostaglandin synthesis we have assessed whether inhibition of the cyclo-oxygenase pathway influenced the actions of LPS in the rat heart.

Methods

Isolation and perfusion of the rat heart

Hearts obtained from male Sprague-Dawley rats (200–350 g) were perfused by a modified Langendorff technique as described previously (Baydoun & Woodward, 1991). Briefly,

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hearts were perfused at a constant rate of 10 ml min^{-1} with Krebs-Henseleit buffer maintained at 37°C and equilibrated with 95% O_2 and 5% CO_2. Coronary perfusion pressure was monitored continuously with a pressure transducer attached to an aortic cannula. The force of cardiac contraction was measured with a tension transducer attached to the apex of the heart. Each heart was allowed to equilibrate for a minimum period of 20 min prior to beginning an experimental protocol.

In some experiments, coronary tone was raised and maintained with the thromboxane-mimetic, 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619; 5 nM). When applied, *E. coli* lipopolysaccharide (LPS) was dissolved in Krebs buffer and perfused continuously through the heart. Maximal doses of bradykinin (BK, 1 nmol) and sodium nitroprusside (SNP; 10 nmol), used in a previous study (Baydoun & Woodward, 1991), were applied as bolus injections into the perfusate, 3 cm proximal to the aortic cannula. All other agents, including indomethacin (10 μM), dexamethasone (10 μM), N^G-nitro-L-arginine (L-NOARG; 50 μM) and N^G-nitro-L-arginine methylester (L-NAME; 50 μM) were added to the buffer reservoir and perfused through the heart for a minimum period of 30 min before obtaining responses to LPS (5 μg ml^{-1}). In other experiments dexamethasone (10 μM) or L-NAME (50 μM) was administered after the vasodilator response to LPS (5 μg ml^{-1}) had reached a maximum. The dependency of the vasodilator action of LPS on an intact endothelium was investigated after damaging the endothelium by injecting 1 ml bolus of air into the coronary circulation as previously described (Baydoun *et al.*, 1989).

Materials

Bradykinin, sodium nitroprusside, *Escherichia coli* lipopolysaccharide (serotype: 0111 B4), indomethacin, dexamethasone, 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$, N^G-nitro-L-arginine, N^G-nitro-L-arginine methylester, L- and D-arginine were obtained from Sigma (Poole, Dorset). All other reagents were of analytical grade and purchased from British Drug House (Poole, Dorset).

Statistical analysis

Results are representative of observations in at least three separate hearts and are expressed as mean ± s.e. of n experiments. Statistical significance was assessed by Student's t test and values of $P<0.05$ were considered significant.

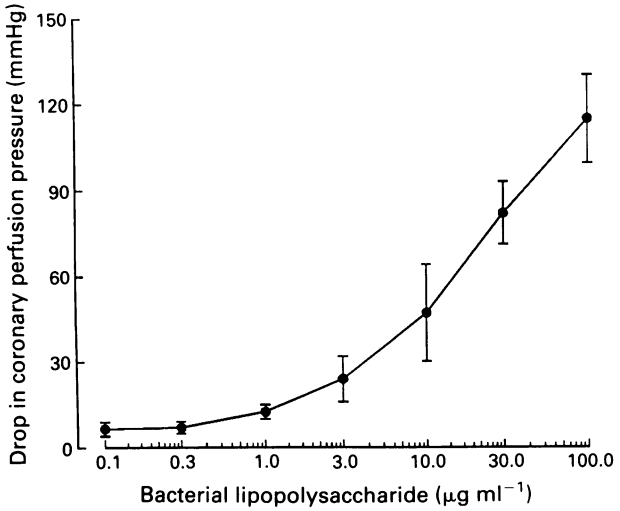


Figure 1 Concentration-response curve to bacterial lipopolysaccharide (LPS) in the rat isolated perfused heart. The graph shows the mean with s.e.mean ($n = 3$).

Results

Vasodilator action of bacterial lipopolysaccharide

Coronary tone was raised in each experiment prior to the administration of LPS by continuous perfusion of the thromboxane-mimetic, U46619. At 5 nM U46619 increased perfusion pressure from a basal value of 44 ± 6 mmHg to 120 ± 15 mmHg ($n = 9$). Cumulative application of LPS (0.1–100 μg ml^{-1}) caused a rapid, concentration-dependent decrease in coronary tone (Figure 1). At 5 μg ml^{-1}, the lowest concentration required to initiate a reproducible sustained response, LPS induced a maximum fall in perfusion pressure of 38 ± 9 mmHg ($n = 6$). This response was maximal within 5 min and reversible upon the removal of LPS from the perfusate. Subsequent administration of LPS to the same tissue, after a 50 min washout period, resulted in a marked reduction in the response compared with that previously observed (Figure 2).

During continuous infusion of LPS (5 μg ml^{-1}; Figure 3), the vasodilatation induced was sustained for 30 ± 2 min before recovering, requiring a further 60 ± 10 min to return to the pre-LPS level ($n = 3$). Cardiac contractility was not altered over this period (Figure 3). Furthermore, continued infusion of LPS after this period did not result in any additional decrease in perfusion pressure nor did it cause a loss in the responsiveness to the vasoconstrictor U46619 (5 nM). Following recovery, coronary tone was maintained at 120 ± 14 mmHg ($n = 3$) for a further 3 h in the continued presence of both LPS and U46619.

Effects of NO synthase inhibitors on LPS-induced vasodilatation

L-NAME (50 μM) or L-NOARG (50 μM) produced a maintained rise in perfusion pressure, increasing basal coronary

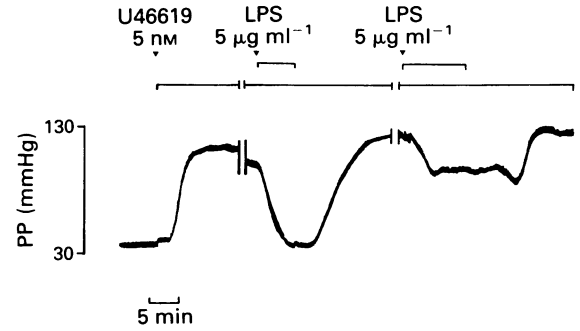


Figure 2 Representative tracing of the vasodilatation induced in the rat isolated heart by acute perfusion of bacterial lipopolysaccharide (LPS). PP denotes coronary perfusion pressure.

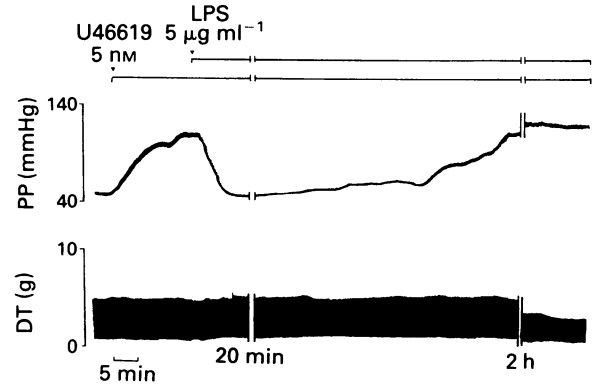


Figure 3 Representative tracing of the vasodilatation induced in the rat isolated heart by prolonged perfusion of bacterial lipopolysaccharide (LPS). PP denotes coronary perfusion pressure and DT the developed tension.

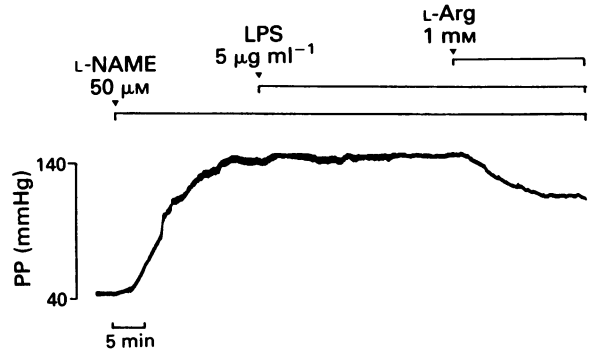


Figure 4 Inhibition of the vasodilator action of bacterial lipopolysaccharide (LPS) by N^G-nitro-L-arginine methylester (L-NAME) and the partial reversal of the inhibition by L-arginine (L-Arg).

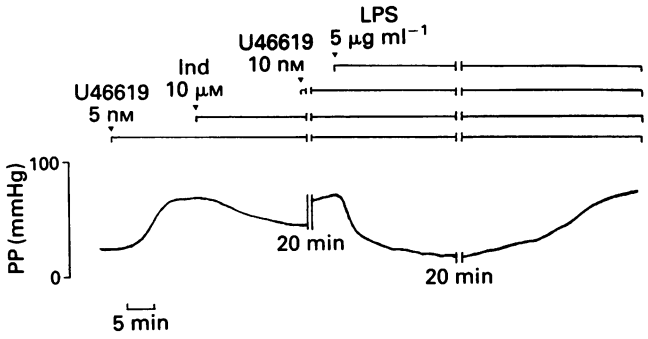


Figure 6 Effects of idomethacin (Ind) on coronary perfusion pressure (PP) and bacterial lipopolysaccharide (LPS)-induced vasodilatation in the rat isolated perfused heart.

tone by 81 ± 19 mmHg ($n = 6$) and 75 ± 5 mmHg ($n = 3$) respectively. The vasonconstriction caused by either compound was partially reversed by L- but not D-arginine (1 mM), and appeared to be endothelium-dependent as it was abolished by the passage of air through the heart. Perfusion of L-arginine (1 mM) had no significant effect on coronary tone in the absence of either L-NAME or L-NOARG but reduced the vasoconstriction caused by these compounds by 18 ± 3 mmHg ($n = 5$) and 15 ± 2 mmHg ($n = 3$) respectively.

Infusion of L-NAME (50 μM) through hearts 30 min prior to the application of LPS (5 μg ml^{-1}) completely blocked the vasodilatation induced by LPS (Figure 4) in four, and reduced the response by 50 and 75% respectively in two out of six hearts. In the experiments where complete inhibition was not achieved, the vasodilator response to LPS was only short-lived in that the fall in pressure returned to control levels within 8 min. In addition, L-NAME (50 μM) reversed the vasodilatation caused by LPS when added to the perfusate after the response to LPS had reached a maximum. Similar results were also obtained with L-NOARG (50 μM) which completely blocked the vasodilator action of LPS in 3 separate experiments. The inhibition caused by either of these arginine analogues was partially reversed by L-arginine (1 mM).

The above findings suggest that nitric oxide may be released acutely in the microvasculature of the rat heart in response to LPS. We therefore investigated whether the gradual recovery of the vasodilator action of LPS (Figure 3) was due to the depletion of L-arginine, the substrate for nitric oxide biosynthesis. In these experiments, addition of L-arginine (1 mM) to the buffer reservoir after the vasodilatation to LPS had returned to its pre-LPS level failed to elicit a drop in coronary perfusion pressure. Furthermore, inclusion of L-arginine (1 mM) in the perfusate at the start of LPS infusion failed to prolong the vasodilator action of LPS (data not shown).

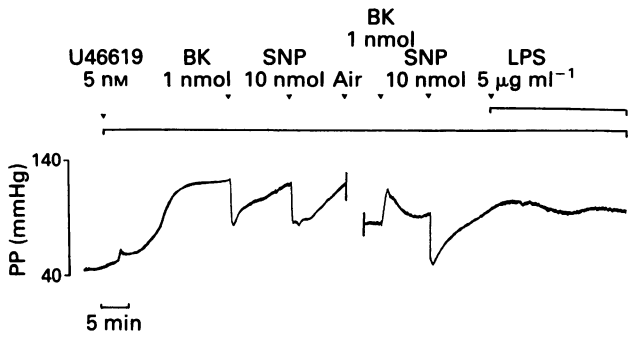


Figure 5 Effect of perfusion of air (1 ml) through the coronary circulation of the rat isolated heart on the vasodilator responses to bolus injections of bradykinin (BK) and sodium nitroprusside (SNP) and to continuous perfusion of bacterial lipopolysaccharide (LPS).

Endothelium-dependent vasodilator action of LPS

Continuous perfusion of the rat isolated heart with bradykinin (BK) results in a rapid desensitization, and thus it could only be applied in bolus doses (unpublished observations). Administration of either BK (1 nmol) or sodium nitroprusside (SNP; 10 nmol) to the intact perfused rat heart, with tone raised by U46619 (5 nM), produced a marked fall in coronary tone, reducing perfusion pressure by 68 ± 5 mmHg ($n = 5$) and 66 ± 3 mmHg ($n = 5$) respectively. The responses to these agents were characteristically different from that obtained with LPS in that they were rapid in onset, reaching a maximum within 30 s.

Application of air (1 ml) to the intact heart had no significant effect on the maximal vasodilator action of SNP but completely abolished the vasodilatation produced by BK and LPS. When administered to hearts after air embolism, BK (1 nmol) initiated a marked vasoconstrictor response, increasing perfusion pressure by 45 ± 3 mmHg (Figure 5; $n = 4$).

Effects of indomethacin on LPS-induced vasodilatation

Perfusion of rat isolated hearts with indomethacin (10 μM) caused a marked drop in coronary tone (Figure 6), reducing perfusion pressure from 102 ± 12 mmHg to 58 ± 4 mmHg ($n = 3$). To maintain the coronary tone at its previous elevated level the concentration of U46619 was raised to 10 nM.

In three separate experiments indomethacin (10 μM) did not significantly modify the vasodilator actions of LPS. Under these conditions both the magnitude (43 ± 9 mmHg; $n = 3$) and duration (94 ± 15 min; $n = 3$) of the LPS vasodilator response was maintained even though hearts had been perfused with indomethacin for 30 min prior to and during the LPS challenge (Figure 6).

Effects of dexamethasone on LPS-induced vasodilatation

In contrast to indomethacin, infusion of dexamethasone (10 μM) 30 min before LPS administration completely blocked the vasodilator action of LPS. Interestingly, when added to the perfusate after the response to LPS had reached a maximum, dexamethasone failed to reverse the vasodilatation induced by LPS (data not shown).

Discussion

The data obtained in this study show that LPS is a potent vasodilator agent in the microvasculature of the rat isolated heart, causing an acute drop in coronary tone which was rapid in onset and sustained over a prolonged period. Following recovery, perfusion pressure was maintained at its

pre-LPS levels for period of up to 3 h without any loss of vascular tone even though hearts were continually perfused with LPS and U46619. This lack of hyporeactivity to U46619, following prolonged exposure to LPS, is in contrast to findings in rat isolated aortic rings in which exposure to LPS for 4 h results in a hyporeactive response to phenylephrine (Rees *et al.*, 1990a) and noradrenaline (Fleming *et al.*, 1990; Julou-Schaeffer *et al.*, 1991). This effect has been attributed to induction of NO biosynthesis, as it could be potentiated by L-arginine and abolished by L-NMMA (Fleming *et al.*, 1990; Rees *et al.*, 1990a; Julou-Schaeffer *et al.*, 1991).

Lack of a sustained loss in coronary tone under our experimental conditions indicates that induction of the Ca^{2+}/calmodulin-independent NO synthase may not occur in the microvasculature of the rat heart *in vitro*. It is likely however that under *in vivo* conditions LPS could induce NO synthase in the coronary vessels of the rat. Indeed in other studies, vasoconstrictor responses of rabbit isolated perfused hearts to U46619 were significantly reduced if the animals were pre-exposed to LPS for 4 h prior to isolation of the hearts (Smith *et al.*, 1991). The fact that large blood vessels (aorta) can be induced *in vitro* whereas resistance vessels could not, perhaps reflects a difference between macrovascular and microvascular tissues. Alternatively, it is possible that in the rat heart, marked desensitization to LPS occurs following the initial vasodilator response. This would explain the recovery of vascular tone after ~90 min and the inability of continued LPS administration to sustain the vasodilatation. Furthermore, since L-arginine did not maintain the LPS-mediated response, this suggests that depletion of cellular L-arginine did not limit production of NO.

The inhibition of LPS-induced vasodilatation caused by L-NAME and L-NOARG, both competitive inhibitors of NO synthase (Ishii *et al.*, 1990; Moore *et al.*, 1990; Rees *et al.*, 1990b), strongly suggests that NO is a mediator of this effect. More importantly, it indicates that LPS can cause NO release acutely in the microvasculature of the rat isolated heart. The rapidity in onset of the response suggests that under these conditions, synthesis and release of NO does not involve induction of the Ca^{2+}/calmodulin-insensitive NO synthase but instead the activation of the constitutive pathway in endothelium. This finding is further substantiated by recent studies showing that exposure of cultured endothelial cells to LPS results in the rapid release (within 1 min) of a NO-like factor which is capable of inhibiting platelet aggregation (Salvemini *et al.*, 1990). In addition, it also lends support to the hypothesis that acute release of NO *in vivo* may in part mediate the early hypotensive response observed following administration of LPS to rats (Thiemermann & Vane, 1990).

In our studies both L-NAME and L-NOARG caused a marked increase in perfusion pressure, in the absence of U46619, indicating that NO is released basally in the rat coronary microcirculation. This finding is consistent with studies in other tissues *in vitro* (Palmer *et al.*, 1988b; Amezcua *et al.*, 1989; Rees *et al.*, 1989a; 1990b; Moore *et al.*, 1990) and *in vivo* (Aisaka *et al.*, 1989; Rees *et al.*, 1989b; 1990b; Gardiner *et al.*, 1990) and further strengthens the belief that basal release on NO modulates vascular tone (Rees *et al.*, 1990b; Amrani *et al.*, 1992; see Moncada *et al.*, 1991).

In the vasculature, constitutive NO synthase is localized entirely in the endothelial layer (Knowles *et al.*, 1990). Our observation that the vasodilator action of LPS was endothelium-dependent and abolished following perfusion of air through the coronary circulation further supports the concept that LPS activates constitutive NO synthase in endothelium. Under these conditions the vasodilator action of bradykinin, an endothelium-dependent vasodilator, was abolished whereas responses to the endothelium-independent vasodilator, sodium nitroprusside were maintained. Thus, following air embolism endothelial function is impaired with the smooth muscle layer remaining virtually intact. The underlying mechanism for the vasoconstrictor action of bradykinin following endothelial damage is as yet unclear but may involve a direct action of this peptide on the vascular smooth muscle.

The data from the present study implicate the constitutive, rather than the inducible, L-arginine-NO pathway in the actions of LPS in the rat isolated heart. It is therefore paradoxical that the vasodilatation caused by LPS within 5 min of administration could be inhibited by pretreatment with the glucocorticoid, dexamethasone. In other studies dexamethasone blocks induction of the Ca^{2+}/calmodulin-independent NO synthase but does not affect the activity of either the constitutive or the induced enzyme (Radomski *et al.*, 1990; Rees *et al.*, 1990a). The mechanism by which dexamethasone exerts its inhibitory action in our study is yet to be established.

Dexamethasone has a wide spectrum of activity and, in addition to preventing the expression of inducible NO synthase, can also block metabolism of membrane phospholipid to arachidonic acid and thus inhibit production of vasoactive prostanoids including prostacyclin. If prostayclin were involved in mediating the effects of LPS, then inhibition of its synthesis would account for the actions of dexamethasone. However, our data obtained with indomethacin suggest that cyclo-oxygenase products are not released in the rat heart following administration of LPS. Indeed it is questionable whether vasodilator products from this pathway are involved in regulating vascular tone in the rat heart *in vitro*. In previous studies we could not detect significant changes in prostacyclin levels in the effluent collected from rat hearts stimulated with bradykinin (Baydoun & Woodward, 1991). The fact that perfusion with indomethacin (Figure 6), or flurbiprofen (Baydoun *et al.*, 1990), results in a marked fall in coronary perfusion pressure suggests that vasoconstrictor prostaglandins are released basally in the rat heart and contribute to the maintenance of vascular tone.

One possible mechanism by which dexamethasone can exert its inhibitory action would be by preventing the binding of LPS to its recognition sites on the endothelium. This hypothesis is supported by recent studies in mouse peritoneal macrophages in which binding of tritium-labelled LPS was strongly blocked by dexamethasone, over the same concentration range as used in our study (Tahri-Jouti & Chaby, 1990). The fact that dexamethasone could not reverse a vasodilatation induced by LPS suggests that LPS may avidly bind to its recognition sites on the endothelium and may not be easily displaced.

In summary, the data obtained from the present study show that LPS is a potent vasodilator agent in the microvasculature of the rat isolated heart. This action of LPS is endothelium-dependent, rapid in onset and maintained over 90 min. Furthermore, it is unaffected by indomethacin but susceptible to inhibition by NO synthase inhibitors. This inhibition by the L-arginine analogues is partially reversed by L-arginine, suggesting that the vasodilator action of LPS in the fine resistance vessels of the rat heart may be mediated by NO synthesized by the Ca^{2+}/calmodulin-dependent NO synthase. More importantly, it delineates a crucial pathogenic role for NO, synthesized via the constitutive pathway, in the initial hypotension observed in the earlier stages of endotoxic shock.

We gratefully acknowledge the support of the British Heart Foundation (Grant number 19/0755). We thank Professor J.D. Pearson for his helpful comments on this manuscript. R.D.F. was the recipient of a summer BSc studentship awarded by the Biomedical Sciences Division, King's College.

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(Received January 21, 1993
Revised March 9, 1993
Accepted March 16, 1993)

Br. J. Pharmacol. (1993), **109**, 992–997 © Macmillan Press Ltd, 1993

Acetylcholine-induced endothelium-independent relaxations in monkey isolated superior and inferior caval veins

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1 We examined the effects of acetylcholine (ACh), isoprenaline (Isop) and Ca-ionophore, A23187 on monkey isolated superior (SCV) and inferior caval veins (ICV) with and without intact endothelium, which had been partially contracted by 2×10^{-6}–5×10^{-6} M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$).

2 Low concentrations of ACh (10^{-10}–10^{-9} M) produced a dose-dependent relaxation in the precontracted venous segments with endothelium. ACh at concentrations more than 10^{-7} M elicited a transient contraction followed by a relaxation in these segments.

3 An addition of 5×10^{-7} M A23187 induced about 60% of maximum relaxation produced by 10^{-5} M sodium nitroprusside (SNP) in each venous segment with endothelium.

4 Isop (10^{-10}–10^{-5} M) caused a dose-related relaxation in the precontracted caval veins with intact endothelium.

5 Removal of endothelium caused no significant effect on the ACh-induced dual responses but a significant inhibition of the A23187-induced relaxation.

6 Pretreatment with atropine antagonized competitively the ACh-induced relaxations in the endothelium-intact and endothelium-denuded caval veins. The Schild plot analysis showed that the pA_2 values of the segments with and without endothelium were 9.72 ± 0.14 ($n = 5$) and 10.01 ± 0.23 ($n = 6$) in the ICV; and 9.95 ± 0.20 ($n = 5$) and 9.70 ± 0.10 ($n = 5$) in the SCV, respectively.

7 Pretreatment with 5×10^{-5} M aspirin, 3×10^{-5} M N^G-nitro-L-arginine methylester, 1 mM tetraethylammonium, or 3×10^{-6} M glibenclamide caused no significant effect on the basal tone, ACh-induced transient contraction, and ACh-induced relaxation in the precontracted venous segments with and without endothelium.

8 Pretreatment with 10^{-5} M methylene blue produced a significant reduction of the ACh- and SNP-induced relaxations in the precontracted venous segments with and without endothelium. The pretreatment with the same concentration of methylene blue, however, caused no significant effect on the Isop-induced relaxation in venous segments with endothelium.

9 The results suggest that ACh acts directly on the venous smooth muscle cells via a high-affinity muscarinic receptor subtype to accumulate cellular cyclic GMP producing endothelium-independent relaxation in the monkey caval veins.

Keywords: Acetylcholine; high-affinity muscarinic receptor; endothelium-independent relaxation; venous smooth muscle; aspirin; nitro-L-arginine methylester; methylene blue; cyclic GMP; tetraethylammonium; glibenclamide

Introduction

Numerous studies of acetylcholine (ACh)-induced endothelium-dependent relaxation in arteries have been reported since the original description by Furchgott & Zawadzki (1980). The degree of endothelium-dependence of relaxation induced by ACh apparently depends on the vascular bed from which the arteries are isolated (Furchgott, 1983). Although most of the preparations studied have been large conduit arteries, experiments carried out in small arteries or in microvascular beds indicate that ACh may also induce endothelium-mediated relaxations in resistance vessels (Pohl *et al.*, 1987; Kaley *et al.*, 1989). It has also become evident that the endothelium-dependent responses to ACh are heterogeneous between systemic arteries and veins (Vanhoutte & Miller, 1985; Furuta & Shigei, 1987; Yang *et al.*, 1991). For example, ACh causes relaxations in most systemic arteries of the dog but minimal or no decrease of tension in the corresponding veins (De Mey & Vanhoutte, 1982). This difference between arteries and veins could be related to the release of different amounts of endothelium-derived nitric oxide or other endothelium-derived relaxing factors, a different vascular responsiveness to the relaxing factors, or a different release of endothelium-derived contracting factors in the two blood vessels (De Mey & Vanhoutte, 1982; Lüscher *et al.*, 1988; Miller & Vanhoutte, 1989; Yang *et al.*, 1991). The exact mechanisms underlying the smaller endothelium-dependent response to ACh in veins are still unknown.

On the other hand, several studies suggest that ACh can produce an endothelium-independent relaxation in the rabbit and cat extracerebral cephalic arteries (Brayden & Bevan, 1985; Brayden & Large, 1986) and in the rat intrapulmonary veins (Chen & Suzuki, 1989). Thus in the cat posterior auricular arteries, ACh released from perivascular nerves can act directly on the smooth muscle cells to cause relaxation via a muscarinic receptor (Brayden & Bevan, 1985). In the rat intrapulmonary veins, ACh causes hyperpolarization of the venous smooth muscles by increase in potassium permeability, with no relationship to the endothelial cells, and then produces a marked relaxation in the precontracted veins (Chen & Suzuki, 1989).

By use of the monkey isolated caval veins, therefore, we have undertaken to re-evaluate whether or not ACh has the capacity to influence venomotor tone in a manner similar to that in which it affects arterial tone. A specific aim of the present studies was also to determine whether any response evoked by ACh in the veins was dependent on the functional integrity of the endothelium. In addition, to begin to characterize this relationship, we compared venomotor responses produced by ACh, Isop and the Ca-ionophore, A23187, a substance known to produce endothelium-dependent relaxation of both arteries and veins (Furchgott, 1984).

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Methods

Tissue preparation

Thirty-four adult macaque monkeys of either sex, weighing 3–6 kg, were anaesthetized with ketamine hydrochloride (50 mg kg^{-1}, i.m.) and exsanguinated from the common carotid arteries. Superior and abdominal inferior caval veins were rapidly dissected out and cleaned of surrounding tissues. Ring segments (4 mm in width) were cut from the middle portion of the superior caval veins or the portion of the inferior caval veins located between the renal veins and the common iliac veins. The other tissues from these animals were used in other studies.

Measurement of mechanical activity

Each segment was suspended vertically in a 10 ml organ bath and perfused at a constant rate (4 ml min^{-1}) with Krebs solution, which was maintained at 37 ± 0.5°C and aerated with a gas mixture of 95% O_2:5% CO_2 to give a pH of 7.3–7.4. The composition of the Krebs solution (mM) was as follows: NaCl 120.0, KCl 5.9, $NaHCO_3$ 25.0, NaH_2PO_4 1.2, $CaCl_2$ 2.5, $MgCl_2$ 1.2, and glucose 5.5. The high potassium Krebs solution (80 mM K^+) was prepared by replacing NaCl with equimolar amounts of KCl and then was loaded for 5–10 min to obtain maximal contraction in each venous segment before starting the experiment. The short-time loading of the high potassium solution was confirmed to produce no chemical denudation of the venous endothelial cells and no significant effect on mechanical responses of the venous segments to drugs used in the experiments.

Two thin silk strings were run through the lumen of a ring segment and formed into two rings. The lower end of the segment was connected to the bottom of the bath. The upper end was connected to the lever of a force-displacement transducer (Shinko Tsushin, UL-10). The circumferential isometric tension detected by the transducer was amplified and recorded on a direct-writing oscillograph (Sanei Sokki, 8K). The resting tension was set at 0.5–0.8 g throughout the experiment, which was optimal for obtaining maximal contractile response to 80 mM KCl solution in each venous segment. All segments were allowed to equilibrate for 60 min in the oxygenated bathing medium before the start of the experiment.

Protocols

Some ring segments were everted, and endothelial cells were mechanically removed by rubbing the intimal surface with Krebs solution-wetted filter paper (Toyo Advantec). In rubbed and unrubbed venous segments, the absence and presence of endothelial cells were confirmed histologically by the silver staining procedure (Poole *et al.*, 1958).

The effects of ACh were examined in caval veins that had been partially precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 2×10^{-6}–5×10^{-6} M). The $PGF_{2\alpha}$-induced contraction was about 50% of the contraction induced by 80 mM KCl in each segment. Concentration-response curves for ACh (10^{-10} to 10^{-5} M) or Isop (10^{-10} to 10^{-5} M) were obtained by adding the agent directly to the bathing media in cumulative concentrations. At the end of each series of experiments, the Ca-ionophore, A23187 (5×10^{-7} M) and sodium nitroprusside (SNP, 10^{-5} M) were added to attain an endothelium-dependent response and maximum relaxation, respectively. The venous segments were pretreated with atropine (10^{-9}, 10^{-8} M), aspirin (5×10^{-5} M), N^G-nitro-L-arginine methylester (L-NAME, 3×10^{-5} M), methylene blue (10^{-5} M), glibenclamide (3×10^{-6} M), or tetraethylammonium (TEA, 1 mM) perfused for 60 min, and then concentration-response curves for ACh were obtained. The dose-response curves for Isop were also produced in the presence of 10^{-5} M methylene blue. Controls were always run with no agent to observe time-dependent changes in the sensitivity of the venous segments to ACh or Isop.

A linear relation was obtained when the molar concentrations of the antagonist were plotted as abscissae on logarithmic graph paper against the dose-ratio of ACh minus unity (Arunlakshana & Schild, 1959). The dose-ratio, i.e., the mean effective dose (ED_{50}) of ACh obtained from the dose-response curve in the presence of atropine divided by that in the control dose-response curve, was determined at different concentrations of atropine. The slope of a straight line is 1.0, and the intersection of the line and abscissae gives the value of pA_2, i.e., the antagonist concentration at which the dose-ratio is equal to 2. This value represents the blocking potency of the antagonist (Kenakin, 1984).

Drugs

The following drugs were used: ACh chloride (Daiichi Seiyaku), atropine sulphate (Tanabe Seiyaku), $PGF_{2\alpha}$ (Ono Yakuhin), isoprenaline hydrochloride (Sigma), glibenclamide (Shionogi Seiyaku), aspirin (Sigma), TEA bromide (Sigma), L-NAME hydrochloride (Sigma), Ca-ionophore, A23187 (Sigma), methylene blue (Merck), and SNP (Merck). The doses of the drugs were expressed in terms of the base and at the final organ bath concentrations.

Statistics

Experimental values in the text and Figures are expressed as means ± s.e.mean. For comparison of statistical difference between two groups, Student's paired or unpaired t tests were used. For multiple comparison, one-way analysis of variance followed by Dunnett's test was used. The difference in means was considered significant at $P < 0.05$.

Results

Effects of ACh in monkey caval veins with and without endothelium

The addition of ACh in concentrations ranging from 10^{-10} to 10^{-5} M caused dual responses in monkey isolated superior caval vein (SCV) with intact endothelium that had been partially contracted with 5×10^{-6} M $PGF_{2\alpha}$ (Figure 1a). Low concentrations of ACh (up to 10^{-9} M) produced a dose-dependent relaxation. On the other hand, ACh at concentrations more than 10^{-7} M induced a transient contraction followed by a marked relaxation. Addition of Ca-ionophore, A23187 (5×10^{-7} M) also elicited about 60% of maximum relaxation produced by 10^{-5} M SNP (Figure 1a). The mechanical rubbing of the endothelium caused no significant effect on the ACh-induced dual responses but produced a significant inhibition of the A23187-induced relaxation in the precontracted venous segment (Figure 1b). Figure 2 summarizes the effects of ACh and A23187 on monkey isolated superior (SCV) and inferior caval veins (ICV) with and without intact endothelium. ACh caused dose-related relaxations in the venous segments with and without endothelium. No significant difference in the threshold concentration and pD_2 value, $-\log[ED_{50}]$ in which ED_{50} is the concentration of agonists causing 50% of each maximum response, of the dose-response curves for ACh between the SCV with and without intact endothelium was observed (pD_2 value; 6.72 ± 0.16 with endothelium vs 6.76 ± 0.14 without endothelium, $n = 12$ in each case). An almost maximal relaxation was achieved by 10^{-6} M ACh in both venous segments with and without endothelium. The ACh-induced maximal relaxations were 47.6 ± 7.7% and 34.8 ± 3.7% of the maximum relaxation in each venous segment with and without intact endothelium, respectively (100%; 1.40 ± 0.17 g with endothelium vs 1.35 ± 0.20 g without endothelium, $n = 12$ in each case). The A23187-induced relaxations in the precontracted SCV,

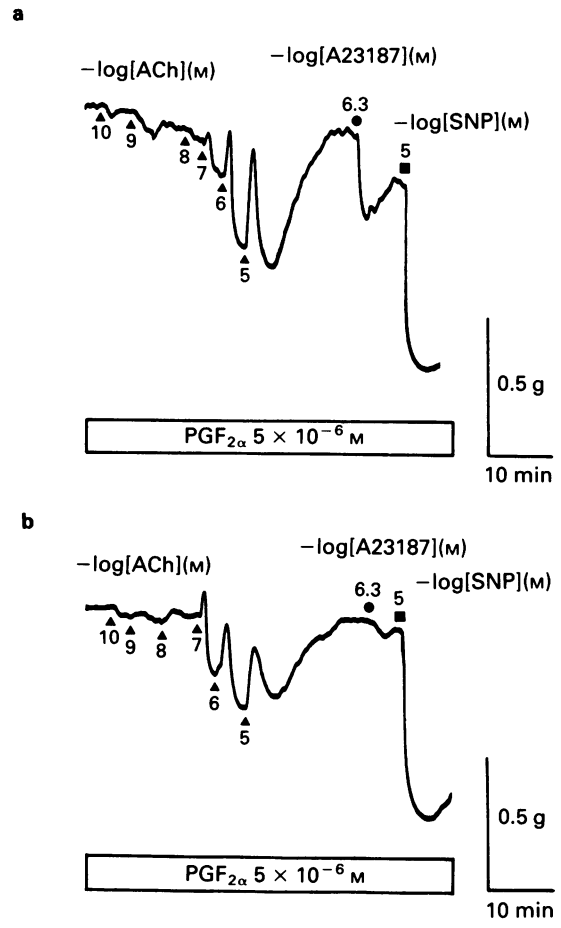


Figure 1 Typical responses to acetylcholine (ACh), Ca-ionophore A23187, and sodium nitroprusside (SNP) in monkey isolated superior caval veins with (a) and without (b) endothelium, precontracted by 5×10^{-6} M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). All concentrations in this figure are given as negative logs. The triangles, circles and squares demonstrated the points at which ACh, A23187, and SNP respectively, were directly added to the organ bath. The bath was perfused at a constant rate with Krebs solution.

however, were significantly reduced by removal of the endothelium (61.0 ± 8.8% with endothelium vs 18.8 ± 5.0% without endothelium, $n = 9$ in each case, $P < 0.001$) (Figure 2b).

There was no significant difference in the above-mentioned responses to ACh and A23187 between monkey SCV and ICV. Thus, the pD_2 values in the ICV with and without endothelium were 6.74 ± 0.11 ($n = 18$) and 6.58 ± 0.10 ($n = 18$), respectively (Figure 2c). The A23187-induced relaxation in the precontracted ICV with and without endothelium were 62.4 ± 4.9% ($n = 18$) and 11.4 ± 2.3% ($n = 18$), respectively (Figure 2d).

Effects of atropine on ACh-induced relaxations in precontracted monkey caval veins with and without endothelium

Figure 3 shows the effects of atropine (10^{-9}, 10^{-8} M) on the cumulative dose-response curves for ACh in precontracted monkey ICV with (Figure 3a) and without (Figure 3b) intact endothelium. The pretreatment with atropine caused no effect on the basal tone of the venous segments with and without endothelium. Atropine, however, caused a parallel shift to the right of the dose-response curves for ACh. Schild plot analysis showed that the slope for these antagonisms in the ICV with and without endothelium were 1.17 ± 0.13 ($n = 5$) and 0.98 ± 0.13 ($n = 6$), respectively, which did not differ from unity and that the pA_2 values in the segments with and

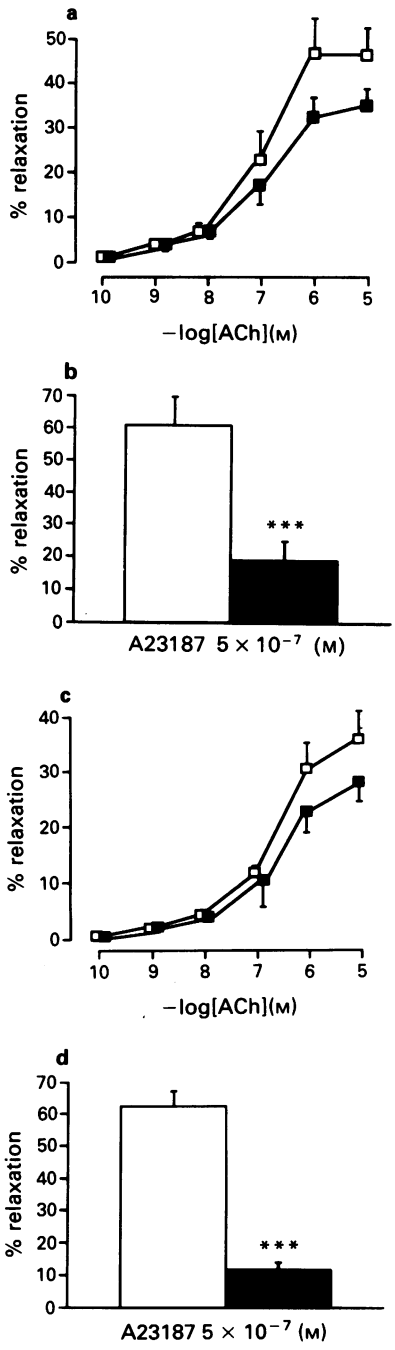


Figure 2 (a and c) Cumulative dose-response curves for acetylcholine (ACh) in the precontracted superior (a, $n = 12$) or inferior caval veins (c, $n = 18$) with (□) and without (■) endothelium. The ordinate scale shows the extent of relaxation expressed as a percentage of maximum relaxation produced by 10^{-5} M sodium nitroprusside (SNP) in each segment. The abscissa scale is concentration of ACh (in M) on a logarithmic scale. (b and d) Ca-ionophore, A23187 (5×10^{-7} M)-induced relaxations in the precontracted superior (b, $n = 9$) or inferior (d, $n = 12$) caval veins with (open column) and without (solid column) endothelium. Extent of relaxation is expressed as a percentage of maximum relaxation induced by 10^{-5} M SNP in each segment. ***, Significantly different from the control (open column in (b) and (d), with intact endothelium), $P < 0.001$.

without endothelium were 9.72 ± 0.14 ($n = 5$) and 10.01 ± 0.23 ($n = 6$), respectively.

Similar results were also obtained in Schild plot analysis of the isolated SCV with and without intact endothelium. Thus, the slope and pA_2 values in the venous segments with and without endothelium were 1.20 ± 0.07 and 9.95 ± 0.20 ($n = 5$), and 1.28 ± 0.08 and 9.70 ± 0.10 ($n = 5$), respectively.

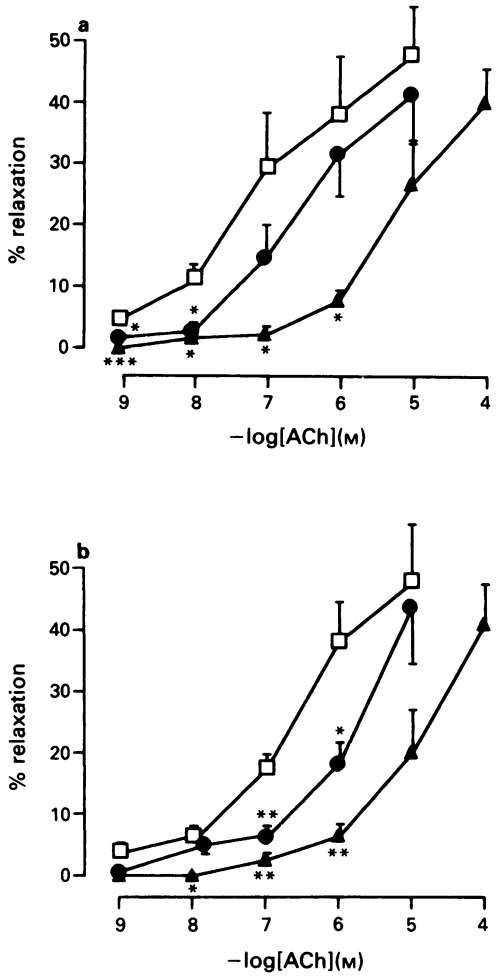


Figure 3 Effects of 10^{-9} M (●) and 10^{-8} M (▲) atropine on relaxant responses to acetylcholine (ACh) in the precontracted inferior caval veins with (a, $n = 5$) and without (b, $n = 6$) endothelium. The ordinate and abscissa scales are the same as those in Figure 2a. Significantly different from the control (□), $*P<0.05$ and $**P<0.01$.

Atropine (10^{-9}, 10^{-8} M) also shifted dose-dependently the dose-contractile response curves for ACh downwards and to the right in the precontracted monkey caval veins with and without endothelium.

Effects of aspirin and L-NAME on ACh-induced relaxations in precontracted monkey caval veins with and without endothelium

The effects of aspirin (5×10^{-5} M), an inhibitor of cyclo-oxygenase, and L-NAME (3×10^{-5} M), an inhibitor of production of endogenous nitric oxide via L-arginine, on the cumulative dose-response curves for ACh were examined in precontracted monkey caval veins with and without intact endothelium, respectively. In contrast to atropine, pretreatment with 5×10^{-5} M aspirin or 3×10^{-5} M L-NAME caused no significant inhibition of the basal tone and the ACh-induced relaxations in the venous segments with and without endothelium, respectively. The pretreatment with aspirin and L-NAME also produced no significant effect on the less than 10^{-6} M ACh-induced transient contractions in the same segments with and without endothelium.

Effects of methylene blue on ACh- or Isop-induced relaxations in precontracted monkey caval veins with and without endothelium

The ACh-induced relaxations were significantly inhibited by pretreatment with methylene blue (10^{-5} M) in the precontracted caval veins with and without endothelium. The addition of methylene blue produced a slight or no increase of the basal tone in the caval veins with and without endothelium. Figure 4 shows the effects of methylene blue on the ACh-induced relaxations in the precontracted caval veins with (Figure 4a, $n = 6$) and without endothelium (Figure 4b, $n = 9$). Treatment with methylene blue caused a significant reduction of the ACh-induced relaxations in the venous segments with and without endothelium. The threshold concentrations for the relaxations were increased more than 10 times by pretreatment with methylene blue. The relaxations produced by 10^{-5} M ACh in the presence of methylene blue (10^{-5} M) in the venous segments with and without endothelium were significantly reduced to about 36% and 28% of those in the control (Figure 4), respectively. The pretreatment with methylene blue caused a marked reduction of the SNP-induced relaxation ($92.2 \pm 1.0\%$, $n = 11$, in the control vs $85.6 \pm 1.3\%$, $n = 11$, with methylene blue, $P<0.001$), but a slight or no inhibition of the ACh-induced transient contraction in the precontracted caval veins with and without endothelium.

Figure 5 shows the dose-response curves for Isop in the precontracted caval veins with intact endothelium in the presence ($n = 5$) and absence ($n = 5$) of 10^{-5} M methylene blue. The addition of Isop in concentrations ranging from 10^{-10} to 10^{-5} M produced dose-related relaxations in the isolated caval veins with intact endothelium that had been partially contracted with 2×10^{-6}–5×10^{-6} M $PGF_{2\alpha}$. The pretreatment with methylene blue caused no significant reduction of the Isop-induced relaxations in the precontracted caval veins with endothelium (Figure 5).

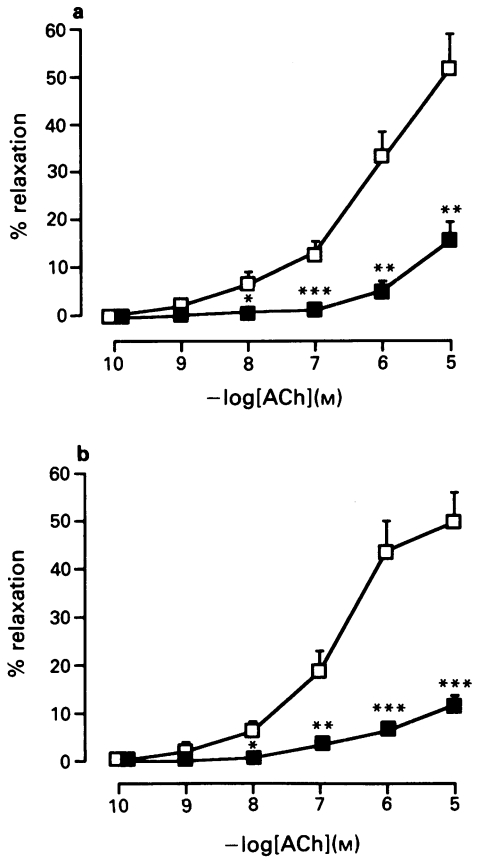


Figure 4 Effects of 10^{-5} M methylene blue (■) on relaxant responses to acetylcholine (ACh) in precontracted caval veins with (a, $n = 6$) and without endothelium (b, $n = 9$). The ordinate and abscissa scales as in Figure 2a. Significantly different from the control (□) in (a) and (b) $*P<0.05$; $**P<0.01$, and $***P<0.001$.

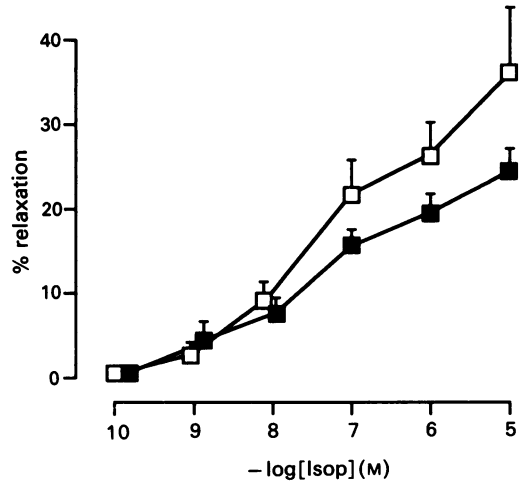


Figure 5 Dose-response curves for isoprenaline (Isop) in precontracted caval veins with endothelium in the absence of (□, $n = 5$) and presence (■, $n = 5$) of 10^{-5} M methylene blue. The ordinate and abscissa scales as in Figure 2a.

Effects of glibenclamide and TEA on ACh-induced relaxations in precontracted monkey caval veins with and without endothelium

The effects of glibenclamide (3×10^{-6} M), a selective inhibitor of ATP-sensitive K channel, and TEA (1 mM), a nonselective inhibitor of K channel (Cook, 1988), on the cumulative dose-response curves for ACh were examined in precontracted monkey caval veins with and without intact endothelium. Pretreatment with 3×10^{-6} M glibenclamide or 1 mM TEA caused no significant reduction of the basal tone and ACh-induced relaxations in the venous segments with and without endothelium, respectively. Pretreatment with glibenclamide or TEA also produced no significant effect on the ACh-induced transient contraction followed by relaxation in the precontracted caval veins with and without endothelium.

Discussion

ACh-induced endothelium-independent relaxaton in monkey caval veins

In the present study, we concentrated on demonstrating clearly the endothelium-independent relaxation in model preparations of monkey caval veins precontracted by $PGF_{2\alpha}$. A cumulative concentration-response curve for ACh was constructed in paired endothelium-intact and endothelium-denuded segments of the caval veins. ACh (10^{-10} to 10^{-5} M) produced concentration-dependent relaxations in the precontracted venous segments with intact endothelium. In the same venous segments, the Ca-ionophore, A23187 (5×10^{-7} M) caused about 130% of the ACh-induced maximum relaxation. When the endothelium was mechanically removed, the A23187-induced relaxation was significantly reduced. The finding suggests that a significant denudation of the endothelium is confirmed pharmacologically in the precontracted venous segments. The absence of endothelial cells was also confirmed histologically by the silver staining procedure. In the endothelium-denuded venous segments, ACh (10^{-10} to 10^{-5} M) caused similar concentration-dependent relaxations to those obtained with the endothelium-intact venous segments. The mechanical rubbing of endothelium also caused no significant effect on the ACh-induced transient contraction followed by relaxation in the precontracted monkey caval veins. These results suggest clearly that ACh can produce an endothelium-independent relaxation in the monkey superior and inferior caval veins.

The relaxations seem to be mediated via muscarinic receptors, because they were competitively inhibited by atropine (Figure 3b). The Schild plot showed a pA_2 value of 10.01 ± 0.23 ($n = 6$) in the ICV or 9.70 ± 0.10 ($n = 5$) in the SCV ($n = 5$). Thus the ACh-induced relaxation in the monkey caval veins without endothelium may be mediated by a high-affinity muscarinic receptor subtype. In contrast, in isolated rings of rabbit thoracic aorta and dog femoral artery, the ACh-induced endothelium-dependent relaxations are mediated by a low-affinity muscarinic receptor subtype only (pA_2; 8.4–8.8) (Eglen & Whiting, 1985). On the other hand, in dog isolated thoracic ducts, ACh stimulates the release of nitric oxide or a related compound from the endothelial cells by acting on a high-affinity muscarinic receptor subtype (pA_2; 10.4) (Ohhashi & Takahashi, 1991). Thus it may be a characteristic feature of monkey caval veins that the high-affinity muscarinic receptor subtype is related to the ACh-induced endothelium-independent relaxation. In the present study, the Schild plot analysis for this antagonism in the precontracted venous segments with intact endothelium also demonstrated a pA_2 value of 9.72 ± 0.14 ($n = 5$) in the ICV or 9.95 ± 0.20 ($n = 5$) in the SCV, being quite similar to that obtained with each endothelium-denuded venous segment. The finding supports strongly our conclusion that ACh acts directly on the venous smooth muscle cells to produce relaxation via a high-affinity muscarinic receptor subtype. The conclusion contrasts with the experimental evidence that ACh has an excitatory role on muscarinic receptors of vascular smooth muscles (Shepherd & Vanhoutte, 1975). On the other hand, our conclusion is compatible with the previous study in cat posterior auricular arteries, that there are inhibitory muscarinic receptors located directly on the arterial smooth muscle cells (Brayden & Bevan, 1985). The relaxant response of this artery to the Ca-ionophore, A23187, was endothelial cell-dependent, which is completely consistent with present experimental findings (Figure 2).

The mechanisms of ACh-induced endothelium-independent relaxation in monkey caval veins

It is well known that the endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980) and prostacyclin (Moncada & Vane, 1978; Toda & Miyazaki, 1981) are released from vascular endothelial cells and cause vascular smooth muscle cells to relax. In the present experiments, pretreatment with 5×10^{-5} M aspirin, a concentration known to inhibit significantly cyclo-oxygenase in isolated blood vessels (Toda & Miyazaki, 1981) did not affect the ACh-induced relaxations in endothelium-intact and endothelium-denuded venous segments, suggesting that prostacyclin and the other vasodilator prostaglandins did not play an important role in the ACh-induced endothelium-independent relaxation in the monkey caval veins. In addition, pretreatment with 3×10^{-5} M L-NAME, a concentration that inhibits significantly the production of endothelium-derived nitric oxide (Moncada *et al.*, 1991), also caused no significant reduction of the ACh-induced relaxations in the precontracted monkey caval veins with and without intact endothelium. The findings suggest that the ACh-induced relaxation is not mediated through release of nitric oxide or its related compounds from the endothelial cells in the monkey caval veins. All these findings also support strongly our conclusion that there are inhibitory muscarinic receptors located directly on venous smooth muscle cells.

On the other hand, the ACh-induced endothelium-independent relaxation in the precontracted monkey caval veins was markedly suppressed by pretreatment with 10^{-5} M methylene blue, an inhibitor of guanylate cyclase (Ignarro & Kadowitz, 1985). In fact, pretreatment with methylene blue caused a significant reduction of the SNP-induced relaxations in precontracted venous segments with and without endothelium. In addition, in the precontracted caval veins with

intact endothelium the Isop-induced relaxation was unaffected by the same concentration of methylene blue. The findings suggest that ACh activates smooth muscle guanylate cyclase through the stimulation of high-affinity muscarinic receptors producing an accumulation of cellular guanosine 3', 5'-cyclic monophosphate (cyclic GMP), which results in the relaxation of monkey caval veins without endothelium. The conclusion may be, in part, related to a recent interesting article showing that nitric oxide may participate in the signal transduction of a neurogenic, cyclic GMP-mediated, and endothelium-independent relaxation in bovine mesenteric arteries, although no evidence for an involvement of L-arginine pathway in the generation of nitric oxide could be found (Ahlner *et al.*, 1991).

Another possible mechanism of the ACh-induced endothelium-independent relaxation in the monkey caval veins may be related to recent articles showing that the cholinergic endothelium-independent vasodilatation in the rabbit lingual artery is accompanied by hyperpolarization (Brayden & Large, 1986) and that exogenous ACh causes an increase of potassium permeability, hyperpolarization of smooth muscle cells, and then a relaxation in rat intrapulmonary vein (Chen & Suzuki, 1989). In the present study, pretreatment with TEA, a nonselective inhibitor of potassium channels, or glibenclamide, a selective inhibitor of the ATP-sensitive potassium channel (Cook, 1988), caused no significant inhibition of the ACh-induced endothelium-independent relaxation in the monkey caval veins. Thus, another mechanism, the increase of potassium permeability and hyperpolarization of vascular smooth muscles, does not seem to contribute to the ACh-induced endothelium-independent relaxation in monkey caval veins.

In conclusion, in isolated superior and inferior caval veins of the monkey ACh acts directly on the venous smooth muscles via a high-affinity muscarinic receptor subtype to increase cellular cyclic GMP which results in relaxation of the veins. This may be the first demonstration that exogenous ACh, but not ACh released from cholinergic nerve endings, is able to produce an endothelium-independent and methylene blue-sensitive relaxation of vascular smooth muscles, which may be independent of hyperpolarization of the smooth muscle cells by increasing potassium permeability.

This study was supported by a Grant-in-aid for Scientific Research (04557039, 04670047) from the Japanese Ministry of Education, Science and Culture.

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(Received December 8, 1992
Revised March 11, 1993
Accepted March 16, 1993)

Br. J. Pharmacol. (1993), **109**, 998–1007 © Macmillan Press Ltd, 1993

Heterogeneity of muscarinic receptors in lamb isolated coronary resistance arteries

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1 *In vitro* experiments in a microvascular myograph were designed to characterize postjunctional muscarinic receptors producing contraction both in the presence and absence of the endothelium in coronary resistance arteries (normalized diameter of 150–450 μm), isolated from the left ventricle of hearts from 3–6 month old lambs. Preferential muscarinic receptor antagonists were used to determine the receptor subtype: pirenzepine (M_1 receptor), AFDX 116 (M_2 receptor), 4-DAMP and pFHHSiD (M_3 receptor).

2 The rank order of potency for muscarinic agonist-induced increases in tension in endothelium-intact preparations was oxotremorine-M = methacholine = acetylcholine (ACh) > carbachol. Removal of the endothelium increased the potency of ACh, but this procedure did not change either the sensitivity or maximal response to carbachol.

3 The contractile response to ACh was reproducible. Incubation with 3×10^{-7}–3×10^{-6} M pirenzepine induced non-parallel rightward shifts and depressed the maximum of the concentration-response curve to ACh in endothelium-intact arteries. The slope by Schild analysis was 2.9 ± 0.8 ($P < 0.05$, $n = 7$). Atropine, AFDX 116, 4-DAMP and pFHHSiD produced parallel rightward shifts of the curves to ACh and the slopes of the Schild plots were not significantly different from unity. The pK_B values for the antagonists from plots constrained to unity in endothelium-intact segments were: atropine (9.4), 4-DAMP (9.0), pFHHSiD (7.9) and AFDX 116 (6.2).

4 In endothelium-denuded arteries, pirenzepine, AFDX 116 and pFHHSiD caused concentration-dependent, parallel rightward displacements of the concentration-response curves to ACh and the slopes of the Schild plots were not significantly different from unity. The plots constrained to a slope of unity gave the following pK_B values: pFHHSiD (8.7), pirenzepine (7.5) and AFDX 116 (6.2).

5 In the presence of the endothelium, low concentrations of pirenzepine (10^{-9}–10^{-7} M) produced leftward shifts of the ACh concentration-response curves. This potentiating effect of pirenzepine was reversed by endothelial cell removal. In preparations precontracted with the thromboxane-mimetic, U46619, the putative M_1-selective agonist, McN-A-343, induced a biphasic relaxation with log IC_{50} of 8.53 ± 0.14 and 5.02 ± 0.08 for the first and second phase of the relaxation, respectively, and maximal relaxations of $22.8 \pm 4.3\%$ and $41.1 \pm 5.4\%$ ($n = 16$). McN-A-343 relaxed the vessels in the presence of 10^{-7} M pFHHSiD and 3×10^{-7} M AFDX 116, but not after incubation with 10^{-9} M pirenzepine.

6 It is concluded from the pK_B values for the antagonists used, that contraction induced by ACh in lamb coronary resistance arteries, in either the presence or the absence of the endothelium, is mediated via the M_3 subtype of muscarinic receptors, while muscarinic receptors of another subtype at the endothelium seem to modulate the contractile response to ACh.

Keywords: Coronary resistance arteries (lamb); acetylcholine; muscarinic receptors

Introduction

The cholinergic innervation is thought to play an important role in the distribution of myocardial blood flow, even though large interspecies differences have been reported (Kalsner, 1985). Vagal stimulation produced coronary vasoconstriction in the rat heart (Van Charldorp *et al.*, 1987; Bognar *et al.*, 1990), and periarterial electrical stimulation of cattle isolated perfused heart (Kalsner, 1979) or human myocardial slabs *in vitro* induced an atropine-sensitive vasoconstriction (Kalsner, 1989). In contrast, efferent stimulation of the vagal nerves or activation of reflex mechanisms in the dog produced coronary vasodilatation (Feigl, 1969; Hackett *et al.*, 1972; Ito *et al.*, 1985; Van Winkle & Feigl, 1989).

Infusion of acetylcholine (ACh) *in vivo* in the coronary circulation revealed that ACh has a dual action, causing vasodilatation at low and vasoconstriction at high doses in the baboon (Van Winkle & Feigl, 1989; Knight *et al.*, 1991), cattle and man (Hodgson & Marshall, 1989; Knight *et al.*, 1991), vasodilatation in dogs (Van Winkle & Feigl, 1989; Knight *et al.*, 1991), and only vasoconstriction in the porcine coronary circulation (Kawamura *et al.*, 1989; Cowan & McKenzie, 1990).

In vitro studies of isolated coronary resistance arteries, which excluded the influence of myocardial oxygen consumption and metabolic regulation of coronary blood flow, show the same species differences to cholinergic stimulation as *in vivo* studies. ACh relaxes preconstricted rat, rabbit and dog coronary resistance arteries (Nyborg, 1990; Angus *et al.*, 1991; Simonsen *et al.*, 1992), while it induces atropine-sensitive contraction of small porcine arteries *in vitro* (Nakayama *et al.*, 1988; Angus *et al.*, 1991; Tschudi *et al.*, 1991). In human small coronary arteries isolated from the atrial appendage, ACh induces contraction, even in arteries with a functional endothelium, assessed by a relaxation to substance P (Angus *et al.*, 1991).

Although it has been recently demonstrated that muscarinic receptor subtypes mediate myocardial blood flow redistribution (Pelc *et al.*, 1988), there are only a few studies concerned with muscarinic receptor characterization, and

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most of these have been carried out in large coronary arteries. Genomic cloning studies have identified 5 distinct human gene products for muscarinic receptors, named from m_1-m_5 (Buckley *et al.*, 1989). In functional pharmacological studies, the muscarinic receptors have been classified into three subtypes M_1, M_2 and M_3, which can be differentiated on the basis of their affinity for different antagonists (Eglen & Whiting, 1990; Hulme *et al.*, 1990). In large epicardial bovine and pig coronary arterial segments, the contractile response to cholinergic stimulation was found to be mediated through M_3-receptors located on the smooth muscle (Duckles, 1988; 1990; Van Charldorp & Van Zwieten, 1989; Duckles & Garcia-Villalon, 1990; Entzeroth *et al.*, 1990; Brunner *et al.*, 1991b), and the endothelium-dependent relaxation of bovine coronary arteries was shown also to be mediated through an M_3-receptor (Brunner *et al.*, 1991a).

The aim of the present study was to characterize, by the use of preferential receptor antagonists, the receptor subtype mediating the contractile response to ACh in coronary resistance arteries in a species, the lamb, where this contractile response predominates.

Methods

Dissection and mounting

Hearts from 3–6 month old lambs were obtained at the local slaughterhouse. The hearts were excised immediately after the lambs were killed, and placed in ice cold physiological salt solution (PSS) to reduce cardiac metabolism. Throughout the subsequent dissection, the hearts were bathed in cold PSS (4°C) of the following composition (mmol l^{-1}): NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $CaCl_2$ 1.5, ethylenediaminetetraacetic acid (EDTA) 0.026 and glucose 5.5. The solution was gassed with 5% CO_2 in O_2 to maintain pH at 7.4.

Third to fourth order small subepicardial arteries of the left anterior descending coronary artery (LAD), located close to the apex of the left ventricle were dissected as previously described for rat and rabbit distal coronary arteries (Prieto *et al.*, 1991; Simonsen *et al.*, 1992). Segments (ca. 2 mm long) of the small vessels were subsequently mounted as ring preparations on two 40 μm wires on an isometric double myograph by fixing one of the wires to a force transducer and the second wire to a length displacement device (Mulvany & Nyborg, 1980). The vessels were allowed to equilibrate in PSS, 37°C, pH 7.4 for about 30 min. The relation between resting wall tension and internal circumference was determined, and from this the internal circumference L_{100} corresponding to a transmural pressure of 100 mmHg for a relaxed vessel *in situ* was calculated (Mulvany & Halpern, 1977). The vessels were set to the internal circumference L_1, given by $L_1 = 0.9 \times L_{100}$. Preliminary experiments showed that the force development is close to maximal at this internal circumference. The effective internal lumen diameter was determined as $l_1 = L_1/\pi$.

Experimental procedure

The following procedure was performed for each small coronary artery to ensure functional integrity of the vessel segments: (1) After normalization, the contractile ability of the vessels was tested by stimulating the arterial rings with K-PSS (equivalent to PSS but NaCl exchanged with KCl on an equimolar basis giving a final concentration of 123.7 mM K^+) until reproducible responses were recorded. (2) The presence of spontaneous tonus was measured as the difference between the baseline just after normalization compared to the baseline obtained after exchange of PSS for Ca^{2+}-free PSS (similar to PSS except that $CaCl_2$ was omitted and replaced with 10^{-4} M EGTA) or application of 10^{-4} M papaverine as the last step of the protocol. (3) Intact endothelium was evaluated by inducing a stable contraction either with 3×10^{-7} M ACh or $1-3 \times 10^{-7}$ M U46619 and then adding either $3-5 \times 10^{-6}$ M calcium-inophore, A23187, or 3×10^{-8} M substance P. Relaxation greater than 50% was taken as evidence for endothelial integrity and segments with a relaxation of less than 50% were discarded.

All the responses to ACh were evoked in normal PSS, and control experiments (see Results) showed no tachyphylaxis of the preparation to this drug. Preliminary experiments were performed to test whether the nicotonic antagonist, hexamethonium, the inhibitor of acetylcholinesterase, neostigmine, the α-adrenoceptor antagonist, phentolamine, or the β-adrenoceptor antagonist, propranolol, altered the response to ACh in the coronary resistance arteries.

Cumulative concentration-response curves to various cholinoceptor agonists were obtained by increasing the organ bath concentration in half-log increments. To obtain comparable parameters among vessel segments the maximum response of the cholinoceptor agonists was expressed relative to the maximal contractile capacity, E_{max}, for each vessel. E_{max} was determined by measuring the increase in wall tension when the vessel is maximally contracted with K-PSS to which 10^{-5} M ACh was added. This procedure was performed either at the beginning or end of the experiment.

In another set of experiments, a possible relaxant effect of ACh was examined by inducing a tone with $1-3 \times 10^{-7}$ M U46619 and after a plateau was reached, a concentration-response curve to ACh ($10^{-9}-10^{-7}$ M) was constructed in vessels in which endothelial cell integrity was confirmed by relaxation to $1-5 \times 10^{-6}$ M of the calcium ionophore, A23187.

In experiments examining the effects of the muscarinic antagonist, atropine ($10^{-10}-10^{-8}$ M), pirenzepine ($3 \times 10^{-7}-3 \times 10^{-6}$ M), AFDX 116 ($3 \times 10^{-7}-3 \times 10^{-6}$ M), 4-DAMP ($3 \times 10^{-9}-3 \times 10^{-8}$ M), and pFHHSiD ($3 \times 10^{-8}-3 \times 10^{-7}$ M), a first, cumulative concentration-response curve to ACh in the absence of antagonist was obtained. This served as a control curve for the following curves. After washing, the vessel was incubated with the antagonist for 30 min and a new concentration-response curve to ACh was constructed in the presence of the antagonist. The ACh concentration-response curves were repeated in the presence of three different concentrations of antagonist for each preparation, since previous reproducibility studies revealed no significant differences among four consecutive concentration-response curves to ACh.

The role of the vascular endothelium on the effect of both muscarinic agonists (ACh and carbachol) and antagonists (pirenzepine, AFDX 116 and pFHHSiD) was studied in parallel experiments where the endothelium was mechanically removed with the arterial segment *in situ* in the myocardium, by passing a human hair through the vessel lumen and then rubbing it back and forth across the lumen several times. Thereafter, mounting, normalization and agonist and antagonist studies were performed as described above for endothelium-intact segments. Lack of functional endothelium was confirmed by the abolition of relaxation to 3×10^{-8} M substance P, and less than 10% relaxation to $3-5 \times 10^{-6}$ M A23187 in preconstricted vessels.

In addition, experiments were performed to clarify whether inhibitory muscarinic receptors were located on the endothelium. In a first set of experiments with endothelium-intact segments, two cumulative concentration-response curves to ACh were obtained; the first in the absence and the second in the presence of a low concentration ($10^{-9}-10^{-7}$ M) of pirenzepine. In a second set of experiments, a cumulative concentration-response curve to ACh was constructed in vessels with intact endothelium, the functional presence of which was checked by preconstricting with 3×10^{-7} M ACh and relaxing with $1-5 \times 10^{-6}$ M A23187. The endothelium was mechanically removed as described above, the vessels were allowed to equilibrate in PSS for 20 min and were then stimulated twice with K-PSS. After washout, two consecutive

concentration-response curves for ACh were constructed, one in the absence and one in the presence of a low single dose of pirenzepine. This protocol was carried out for 10^{-9} and 10^{-7} M pirenzepine.

Furthermore, a possible relaxant effect for the M_1-selective muscarinic agonist McN-A-343 was examined by inducing a tone with $1-3 \times 10^{-7}$ M U46619, and once a plateau was reached a concentration-response curve to McN-A-343 ($10^{-9}-3 \times 10^{-4}$ M) was constructed in vessels with intact endothelium. The specificity of this relaxant effect was checked by incubation of the preparation for 30 min with a single dose of either pirenzepine (10^{-9} M), AFDX 116 (3×10^{-7} M) or pFHHSiD (10^{-7} M) before a new concentration-response curve was repeated.

Analysis of results

The mechanical responses of the vessels were measured as force and expressed as active wall tension, δT, which is the increase in measured force, δF, divided by twice the segment length (Mulvany & Halpern, 1977). Using a computer programme (GraphPad, Institute for Scientific Information, San Diego, California, U.S.A.), the concentration-response curves to the cholinoceptor agonists were fitted to the classical 'Hill-equation': $R/R_{max} = A(M)^n/(A(M)^n + EC_{50}(M)^n)$, where R/R_{max} is the relative response to the effective concentration of drug, A(M), and EC_{50}(M) is the concentration of agonist required to give half maximal vessel response (R_{max}), when A(M) and EC_{50}(M) are given in molar concentrations. n is a curve fitting parameter or 'Hill-coefficient'. The same computer programme was used for the calculation of IC_{50} values for ACh and McN-A-343 in precontracted vessels. EC_{50} values are expressed as the negative log molar concentration, $pD_2 = -\log (EC_{50})$. The relative potency, Rp, of the cholinoceptor agonists was calculated as the ratio of equiactive concentrations taking the response to ACh in endothelium-intact preparations as reference.

For the assessment of the effect of the cholinoceptor antagonists at least three different concentrations of the antagonist were examined. Since four consecutive concentration-response curves to ACh could be performed in one vessel (see Results), a maximum of three concentrations of the antagonist were studied per coronary ring and the results compared to those obtained in a first concentration-response curve to ACh performed in the absence of antagonist. The pA_2 values of the competitive antagonists, defined as the negative logarithm of the molar antagonist concentration, in the presence of which twice the original agonist concentration is needed for the original effect, was determined by plotting log (CR-1) against log antagonist concentration, where CR is the concentration-ratio of the agonist in the absence and presence of antagonist (Arunlakshana & Schild, 1959). Concentration-ratios (CR) were calculated at the EC_{50} level. The slope and the pA_2 value were determined by least square fitting of a regression line to the points. Antagonism was considered to be competitive if there was not more than a 10% reduction in the maximum response and if the 95% confidence limits for the slope of the Schild plot overlapped unity. Providing that the regression of the Schild plot is linear and the slope is not significantly different from unity, the antagonist is in equilibrium with the receptor and the pA_2-value, which is the intercept along the abscissa scale of the Schild plot is equal to the negative logarithm of the equilibrium dissociation constant for the antagonist: $pA_2 = -\log K_B = pK_B$, and in this case the points were constrained to a slope of unity and a more precise value of the pK_B was calculated (Tallarida & Muray, 1984).

Drugs

Acetylcholine HCl (Merck); AFDX 116 (11-(2-((diethylamino)methyl)-1-piperidinylacetyl)-5,-11-dihydro-6H-pyrido-(2.3-b)-(1.4)-benzodiazepin-6-one) (Dr Karl Thomae GmbH); atropine sulphate (Merck); carbachol (carbamoylcholine HCl, Sigma); methacholine (acetyl-β-methylcholine, Sigma); 4-DAMP (4-diphenyl-acetoxy-*N*-methyl piperidine methiodide) (courtesy of Dr R.B. Barlow, Bristol, U.K.); hexamethonium bromide (Serva); McN-A-343 ((4-hydroxy-2-butynyl)-1-trimethylammonium *m*-chlorocarbimilate chloride, Sigma); oxotremorine-M (ICN Biochemicals), pFHHSiD (*para*-fluoro-hexahydro-sila-diphenidol, Research Biochemicals Incorporated, UK); pirenzepine HCl (Dr Karl Thomae GmbH); calcium-ionophore (A23187), phentolamine HCl, (±)-propranolol HCl and substance P (Sigma). Most drugs were prepared daily in distilled water except for the calcium-ionophore, A23187, where a 10^{-3} M stock solution was prepared in dimethylsulphoxide and further diluted in PSS. Previous experiments showed that the solvents had no effect on the preparations at the concentrations applied. Concentrations of drugs were expressed as the final organ chamber concentrations (M).

Statistical analysis

The results are expressed as means ± s.e.mean. Significant differences between means were assessed by Student's two-tailed *t* test. pD_2 values of the agonists were analysed according to one-way analysis of variance and Bonferroni method as an *a posterio* test (Wallenstein *et al.*, 1980). Probability levels under 5% were considered significant.

Results

The endothelium-intact lamb coronary resistance arteries investigated in the present study had an internal effective lumen diameter, $l_i = 314 \pm 10$ μm (172 vessels from 86 animals). Initially, successive stimulations with K-PSS resulted in increasing contractions, but the responses to the third and fourth stimulation with K-PSS were not significantly different, being 0.82 ± 0.19 Nm^{-1} and 0.86 ± 0.22 Nm^{-1} ($n = 6$), respectively. Therefore, the contractile ability of the vessels was tested by three stimulations with K-PSS at the beginning of each experiment. All the lamb

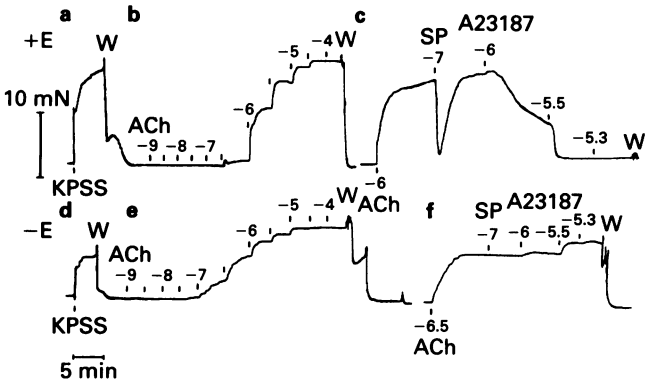


Figure 1 Isometric force recordings showing concentration-dependent contractions to acetylcholine (ACh) in a lamb isolated coronary resistance artery with an effective lumen diameter, $l_i = 248$ μm. (a–c) Endothelium-intact segment stimulated with 125 mM potassium (K-PSS) (a), response to increasing cumulative concentrations of ACh (b), and relaxation to 10^{-7} M substance P (SP), and $1-5 \times 10^{-6}$ M calcium-ionophore A23187 (c). (d–f) The same vessel after removal of the endothelium with a 40 μm thick wire (see Methods). (d) The response to K-PSS of the artery is apparently diminished if the increase in basal myogenic tone induced by endothelial denuding is not considered (see Results). (e) The vessel responds to lower concentrations of ACh, and (f) the relaxations to substance P and A23187 are abolished. Numbers show the point at which each drug addition was made and the corresponding cumulative concentration of the drug in the tissue bath, expressed as the log molar concentration. W, wash.

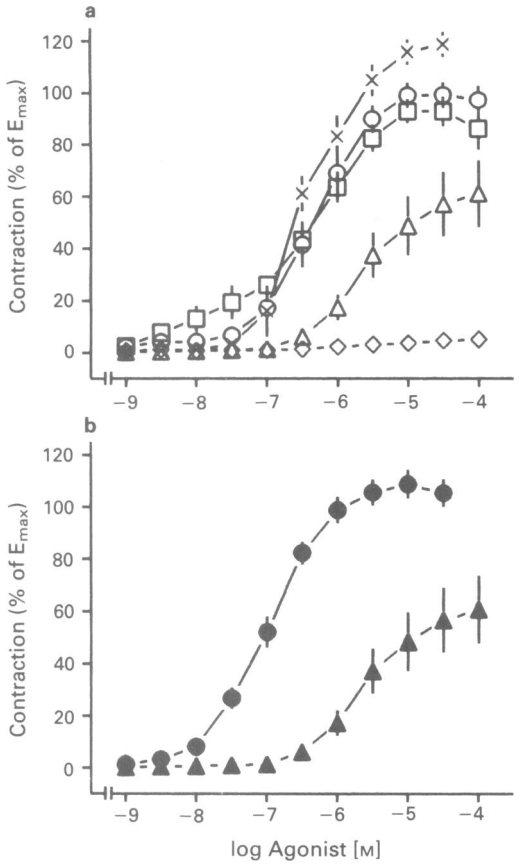


Figure 2 Concentration-response curves to cholinoceptor agonists inducing contraction in lamb coronary resistance arteries. (a) Concentration-response curves in endothelium-intact segments to acetylcholine (○), methacholine (□), carbachol (△), McN-A-343 (◇), and oxotremorine M (×) are shown. (b) Concentration-response curves to acetylcholine (●) and carbachol (▲) in endothelium-denuded preparations. The contractile effect is expressed as a percentage of the response evoked by stimulation with 10^{-5} M ACh in K-PSS (E_{max}). Values are means ± s.e.mean ($n = 6-14$ preparations).

coronary resistance arteries considered as endothelium-intact and preconstricted with 10^{-6} M ACh relaxed transiently to $3 \times 10^{-8}-10^{-7}$ M of substance P ($n = 8$, Figure 1), while $3-5 \times 10^{-6}$ M of the calcium-ionophore, A23187, produced a sustained relaxation with a maximum of 79.80 ± 2.08% ($n = 172$) of the preconstriction (Figure 1). Spontaneous contraction measured as the relaxation over the base-line tension either after addition of 10^{-4} M papaverine or by exchange of the bath solution, PSS, for calcium-free PSS was 0.21 ± 0.02 Nm^{-1} in the endothelium-intact coronary resistance arteries. The maximal contractile capacity, E_{max} (see Methods), of the endothelium-intact vessels was 2.67 ± 0.16 Nm^{-1} ($n = 54$).

In 31 lamb coronary resistance arteries with an internal diameter $l_1 = 321 \pm 17$ μm, the endothelium was mechanically removed, evaluated as an abolished relaxation to substance P ($n = 24$) and a maximum relaxation of less than 10% to A23187 ($n = 31$). This procedure did not alter the response to the third stimulation with K-PSS, being 1.67 ± 0.16 Nm^{-1} ($n = 31$) compared to endothelium-intact preparations: 1.51 ± 0.08 Nm^{-1} ($n = 172$). The spontaneous myogenic tone measured by the application of 10^{-4} M papaverine was 0.35 ± 0.07 Nm^{-1} ($n = 18$, $P < 0.05$ compared to endothelium-intact segments, Student's t test). E_{max} of the endothelium-denuded arteries was 2.44 ± 0.37 Nm^{-1} ($n = 14$).

The response of coronary resistance arteries to ACh

ACh ($10^{-8}-10^{-4}$ M) induced a concentration-dependent contraction in endothelium-intact lamb coronary resistance arteries (Figure 1) with a maximum response which corresponded to the E_{max} of the vessel (Figure 2). In a set of experiments where the preparations were preconstricted with $1-3 \times 10^{-7}$ M U46619, ACh induced relaxation only in 3 out of 18 lamb coronary resistance arteries examined, with a half maximal inhibition log $IC_{50} = 8.43 \pm 0.09$ ($n = 3$) and a maximal relaxation of 49 ± 17% of the preconstriction induced with U46619, and constriction in the other 15 vessels. All the 18 vessel segments relaxed to $1-5 \times 10^{-6}$ M A23187, with a maximum relaxation of 73 ± 9% ($n = 18$). There was no correlation between normalized internal diameter (150–800 μm), the A23187-induced relaxation and the effect of ACh in the preconstricted resistance arteries.

Agonist study

The order for the relative potency of the muscarinic agonist-induced increases in tension was oxotremorine-M = methacholine = acetylcholine > carbachol, while, apart from oxotremorine-M, there were no significant differences in the maximal responses to these agonists in endothelium-intact preparations (Table 1). The muscarinic agonist McN-A-343 alone did not evoke any significant response (Figure 2), but in the presence of 10 mM K^+ in PSS it did evoke a contraction that corresponded to 23.3 ± 5.6% of the E_{max} (Figure 3a).

Table 1 Contractile responses to acetylcholine compared to other cholinoceptor agonists in endothelium-intact (+E) and endothelium-denuded (−E) lamb coronary resistance arteries

	n	pD_2	T (Nm^{-1})	*Slope*	*Rp*	l_1 (μm)
+E						
Acetylcholine	11	6.31 ± 0.11	2.14 ± 0.24	1.42 ± 0.09	1.0	276 ± 25
Methacholine	7	6.49 ± 0.19	2.33 ± 0.30	0.78 ± 0.10	1.0	281 ± 30
Carbachol	12	5.75 ± 0.20[a,b]	1.70 ± 0.39	1.16 ± 0.15	6.7	323 ± 19
McN-A-343[+]	9	4.93 ± 0.09[a,b,c]	0.59 ± 0.17[a,b,c]	1.70 ± 0.27	248	381 ± 32
Oxotremorine M	6	6.33 ± 0.11[c,d]	3.04 ± 0.34[a,c,d]	1.34 ± 0.11	0.6	276 ± 23
−E						
Acetylcholine	14	7.03 ± 0.07*	2.61 ± 0.37	1.1 ± 0.06	0.2	296 ± 27
Carbachol	8	5.78 ± 0.33	1.42 ± 0.19	2.3 ± 0.4	4.9	316 ± 27

Values are mean ± s.e.mean. n, number of vessels from at least 3–4 animals. T is the maximal response obtained with the respective substance. The slope as determined from the Hill equation. l_1, normalized internal diameter of the coronary arterial segments at which the experiments were performed (see Methods). The relative potency, Rp is the ratio of equiactive concentrations, using the response to acetylcholine in endothelium-intact arteries as reference. [+]The concentration-response curves to McN-A-343 were performed in presence of 10 mM K^+ in PSS.

Significantly different parameter, tested by the Bonferroni Method: [a]$P < 0.05$, versus acetylcholine; [b]$P < 0.05$ versus methacholine; [c]$P < 0.05$ versus carbachol; [d]$P < 0.05$ versus McN-A-343.

*Significantly different parameter compared to endothelium-intact preparations ($P < 0.05$, Student's t test).

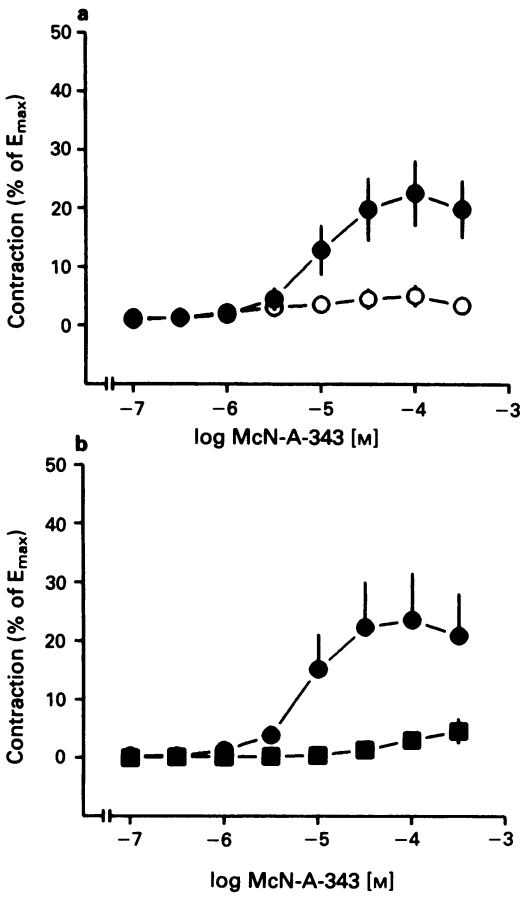


Figure 3 Cumulative concentration-response curves to the M_1-selective cholinoceptor agonist, McN-A-343, in endothelium-intact preparations. (a) McN-A-343 added alone does not produce significant contraction (○), but in presence of 10 mM K^+ in PSS it induces a contractile response (●). (b) The contractile response to McN-A-343 in 10 mM K^+ in PSS (●) is blocked by 10^{-7} M pFHHSiD, an M_3-selective antagonist (■). The contractile effect is expressed as a percentage of the response evoked by stimulation with 10^{-5} M ACh in K-PSS (E_{max}). Each point is the mean ± s.e.mean of 5–14 vessels.

Mechanical removal of the endothelium significantly shifted to the left the concentration-response curves to ACh, while the maximum response to ACh was unaltered expressed relative to E_{max}. The endothelium did not change significantly either the sensitivity or the maximal contraction to carbachol in lamb coronary resistance arteries, although the relative potency for this agonist was increased (Table 1).

The generation of four consecutive concentration-response curves at 40 min intervals did not reveal tachyphylaxis to ACh. The pD_2 values for the first, second, third and fourth concentration-response curves were: 6.56 ± 0.14, 6.68 ± 0.20, 6.81 ± 0.25, and 6.56 ± 0.23, respectively, and the maximal responses were: 2.31 ± 0.38, 2.33 ± 0.37, 2.40 ± 0.39, 2.29 ± 0.40 Nm^{-1}, respectively ($n = 7$). Prior incubation with either the nicotinic antagonist, hexamethonium (10^{-4} M), the α-adrenoceptor antagonist, phentolamine (10^{-6} M), the β-adrenoceptor antagonist, propranolol (10^{-5} M), or the inhibitor of acetylcholinesterase, neostigmine (10^{-7} M) did not alter the contractile concentration-dependent responses to ACh. However, the muscarinic antagonist, atropine (10^{-10}–10^{-8} M) competitively antagonized the response to ACh. Schild analysis for atropine against ACh gave a straight line with a slope, which was not significantly different from unity (Table 2), and the constrained plot revealed an intercept with the abscissa, $pK_B = 9.46 \pm 0.09$ ($n = 3$).

Antagonism of the contractile response to ACh with selective muscarinic antagonists

In endothelium-intact preparations, the M_1-selective antagonist, pirenzepine (3×10^{-7}–3×10^{-6} M) inhibited, in a non-competitive manner, the concentration-response curves to ACh in lamb coronary resistance arteries, producing non-parallel shifts of the curves and depressing the maximal response to ACh (Figure 4a). The Hill slope for the ACh concentration-response curve in absence of pirenzepine was 1.20 ± 0.06 ($n = 8$), and in presence of 3×10^{-7} M, 10^{-6} M and 3×10^{-6} M pirenzepine the slopes were 0.80 ± 0.13 ($P < 0.05$, paired t test), 0.78 ± 0.14 ($P < 0.05$) and 0.65 ± 0.14 ($P < 0.05$), respectively. Schild analysis gave a straight line with a slope which significantly differed form unity: 2.87 ± 0.78 ($P < 0.05$, $n = 8$). Non-competitive antagonism evoked by pirenzepine was not agonist-dependent, since 3×10^{-7} M–3×10^{-6} M pirenzepine also displaced the concentration-response curve for methacholine in endothelium-intact segments in a non-competitive manner, giving a straight line with a slope significantly different from unity: 2.33 ± 0.10 ($P < 0.05$, $n = 6$). The regression line intersected the abscissae at 6.41 ± 0.05. However, after mechanical removal of the endothelium determined by a lack of relaxation to A23187, pirenzepine produced parallel rightwards shifts of the ACh concentration-response curves and Schild analysis gave a slope not significantly different from unity (Table 2, Figure 4b,c). The line of the plot intersected the abscissa at $pA_2 = 7.49 \pm 0.15$ ($n = 13$).

Table 2 Potencies of various antagonists in endothelium-intact (+E) and endothelium-denuded (−E) lamb coronary resistance arteries isolated from the left ventricle, determined by their ability to inhibit the acetylcholine-induced contraction of vascular segments

	n	pA_2	pK_B	*Slope*	l_1 (μm)
+E					
Atropine	3	9.43 ± 0.17	9.46 ± 0.09	1.06 ± 0.18	212 ± 40
Pirenzepine	8	6.58 ± 0.04[a]	———	2.9 ± 0.8*	320 ± 28
AFDX 116	6	6.04 ± 0.25	6.15 ± 0.10	1.12 ± 0.25	273 ± 40
4-DAMP	6	9.11 ± 0.15	9.01 ± 0.08	0.91 ± 0.18	295 ± 25
pFHHSiD	7	7.56 ± 0.08	7.85 ± 0.05	1.33 ± 0.19	382 ± 24
−E					
Pirenzepine	13	7.49 ± 0.15	7.51 ± 0.06	1.02 ± 0.12	361 ± 22
AFDX 116	6	6.14 ± 0.16	6.18 ± 0.05	1.03 ± 0.14	291 ± 34
pFHHSiD	6	8.24 ± 0.19	8.65 ± 0.08	1.33 ± 0.20	300 ± 50

The values are expressed as means ± s.e.mean. n, number of vessels. pA_2, intercept at the abscissae of the regression line in Schild plots. pK_B, the negative logarithm of the equilibrium dissociation constant of antagonist-receptor complex, calculated from a plot constrained to a slope of unity.

[a]Apparent pA_2 value (not real) due to the non competitive nature of the antagonism calculated for comparative interest. l_1, normalized internal diameter of the coronary arterial segments at which the experiments were performed (see Methods).

*Slopes of the Schild plots significantly different from unity ($P < 0.05$).

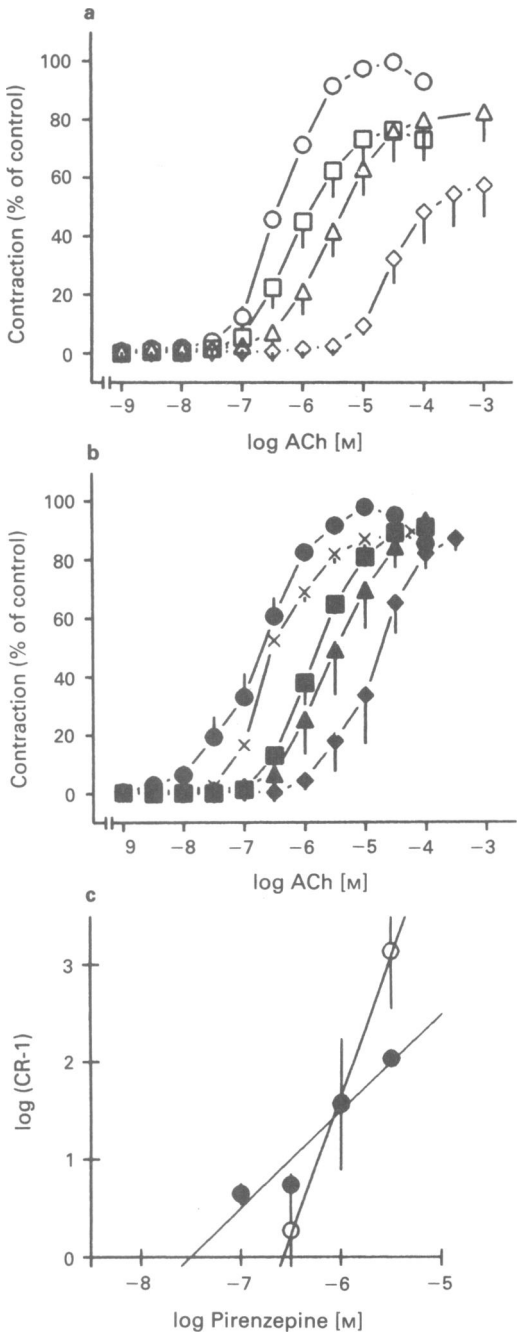


Figure 4 Cumulative concentration-response curves to acetylcholine (ACh) in lamb coronary arteries: (a) with (+E, open symbols) and (b) without (−E, closed symbols) endothelium in absence (○,●) and presence of increasing concentrations of the M_1-selective muscarinic antagonist, pirenzepine: 10^{-7} M (×), 3×10^{-7} M (□,■), 10^{-6} (△,▲), and 3×10^{-6} M (◇,◆). (c) The Schild regressions for the effect of pirenzepine on concentration-response curves to ACh in endothelium-intact (○) and endothelium-denuded (●) lamb coronary arteries.

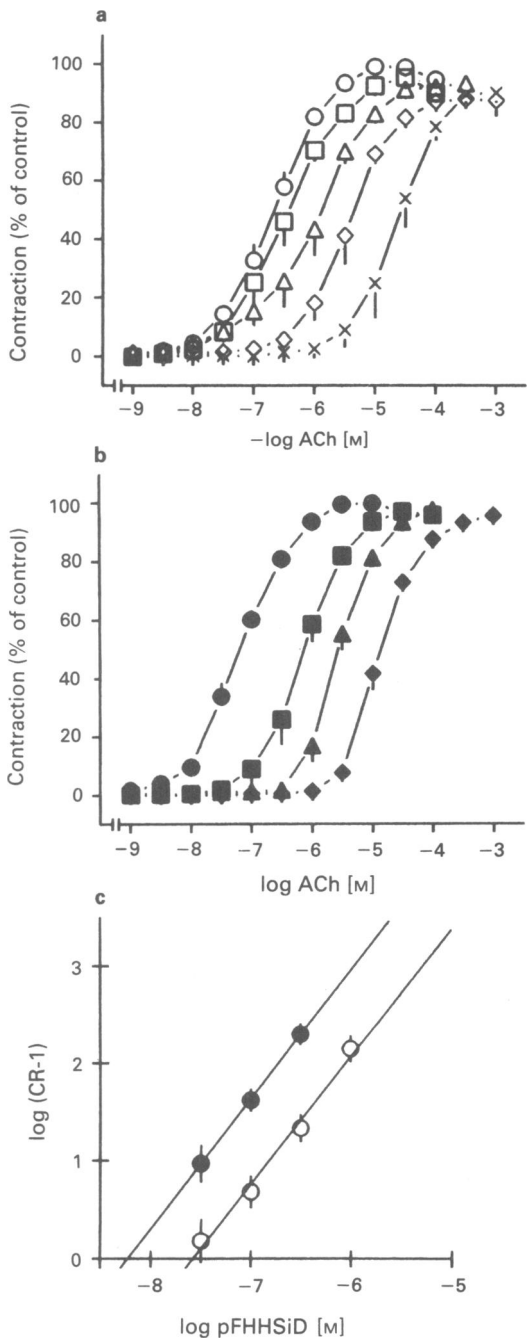


Figure 5 Cumulative concentration-response curves to acetylcholine (ACh) in lamb coronary arteries: (a) with (+E, open symbols) and (b) without (−E, closed symbols) endothelium in absence (○,●) and presence of increasing concentrations of the M_3-selective muscarinic antagonist, pFHHSiD: 3×10^{-8} M (□,■), 10^{-7} M (△,▲), 3×10^{-7} M (◇,◆), and 10^{-6} M (×). (c) The Schild regressions for the effect of pFHHSiD on concentration-response curves to ACh in endothelium-intact (○) and endothelium-denuded (●) lamb coronary arteries. The pA_2 values in endothelium-intact and denuded vessels are 7.56 ± 0.08 ($n = 6$) and 8.24 ± 0.19 ($n = 6$), respectively.

Table 3 Low concentrations of pirenzepine (10^{-9}–10^{-7} M) caused leftward shifts in acetylcholine concentration-response curves in endothelium-intact (+E), but not in endothelium-denuded (−E) lamb coronary resistance arteries

	+E			−E		
	pD_2 with antagonist			pD_2 with antagonist		
	Absent	*Present*	*log shift*	*Absent*	*Present*	*log shift*
10^{-9} M	6.29 ± 0.09(6)	7.30 ± 0.25(6)*	1.01 ± 0.21(6)	6.58 ± 0.16(4)	6.37 ± 0.13(4)	− 0.21 ± 0.08(4)
10^{-8} M	6.16 ± 0.20(6)	6.69 ± 0.20(6)*	0.53 ± 0.11(6)	—	—	—
10^{-7} M	6.22 ± 0.15(6)	8.18 ± 0.60(6)*	1.96 ± 0.52(6)	6.93 ± 0.19(5)	6.30 ± 0.12(5)*	− 0.63 ± 0.22(5)

Values are mean ± s.e.mean. (*n*), number of vessels examined.
*Statistically significant shift compared to control of the concentration-response curve to acetylcholine caused by pirenzepine ($P < 0.05$, paired *t* test).

In endothelium-intact coronary resistance arteries, the M_2-selective antagonist, AFDX 116, and the M_3-selective antagonists, 4-DAMP and pFHHSiD competitively antagonized the response to ACh producing a parallel shift to the right of the ACh concentration-response curve (Figure 5, Table 2). Schild plot of the three antagonists to the ACh concentration-response curves gave straight lines with slopes not different from unity, and the plots were constrained to unity giving pK_B-values for AFDX 116 6.15 ± 0.10, 4-DAMP 9.01 ± 0.08 and pFHHSiD 7.85 ± 0.05 (Table 2). pFHHSiD (10^{-7} M) also displaced the contractile response evoked by McN-A-343 in the presence of 10 mM K^+ in PSS (Figure 3b).

Endothelial cell removal did not alter the parallel rightward displacements induced by AFDX 116 on the concentration-response curves for ACh (Table 2), but shifted to the left the Schild plot for the M_3-selective antagonist, pFHHSiD (Figure 5c).

Endothelium-dependent modulation of the contractile response to ACh

In endothelium-intact preparations incubation with low concentrations of pirenzepine (10^{-9}–10^{-7} M) induced leftward shifts of the contractile concentration-response curves compared to a first ACh concentration-response curve in the absence of antagonist (Table 3).

In a second set of experiments to clarify whether inhibitory muscarinic receptors are present on the endothelium (see Methods), mechanical endothelial cell removal caused a leftwards shift ($\delta pD_2 = 0.57 \pm 0.12$, $n = 9$, $P < 0.05$, paired t test) of the concentration-response curve to ACh. Incubation with 10^{-9} M or 10^{-7} M pirenzepine did not further potentiate the response to ACh of endothelium-denuded segments (Table 3).

In lamb coronary resistance arteries preconstricted with 3×10^{-7} M U46619, the putative M_1-selective agonist, McN-A-343, produced a biphasic relaxation (Figure 6). From 10^{-9}–10^{-7} M McN-A-343 produced a weak relaxation with an extrapolated log $IC_{50} = 8.53 \pm 0.14$ and a maximum relaxation of 22.8 ± 4.3%, and from 3×10^{-6}–10^{-4} M, it relaxed the vessel further with an extrapolated log $IC_{50} = 5.02 \pm 0.08$ and a maximum relaxation of 41.1 ± 5.4% ($n = 16$). In endothelium-denuded segments McN-A-343 only produced further constriction ($n = 2$). The relaxation induced by McN-A-343 was not abolished by incubation with either the M_3-selective antagonist, pFHHSiD (10^{-7} M, $n = 6$), or the M_2-selective antagonist, AFDX 116 (3×10^{-7} M, $n = 4$), in concentrations supposed to block the M_3 and M_2-receptors, respectively. However, the presence of a low concentration of pirenzepine (10^{-9} M) inhibited the relaxation to McN-A-343 ($n = 5$, Figure 6), while 5×10^{-6} M A23187 relaxed the vessels by 92.3 ± 1.3% ($n = 5$).

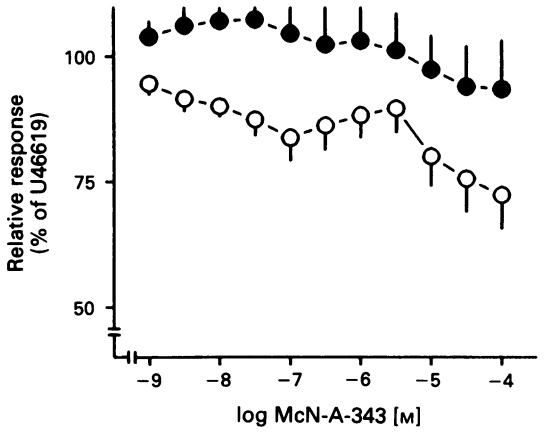


Figure 6 Concentration-response curves to the M_1-selective cholinoceptor agonist, McN-A-343, in endothelium-intact lamb coronary arterial segments contracted with 3×10^{-7} M U46619. The concentration-response curves were performed in the absence (○) and the presence (●) of 10^{-9} M pirenzepine. Each point is the mean ± s.e.mean of 5–16 vessels.

Discussion

The contractile response to ACh predominates in lamb coronary arteries, as has been previously reported for the proximal parts of the coronary circulation in this species (Kalsner, 1985). However, the findings that ACh relaxed a small percentage of precontracted preparations and endothelial cell removal potentiated the contractile response to ACh (Figure 1, Table 1) suggest that the endothelium might modulate the contractile response to ACh in lamb coronary resistance arteries.

The present study was designed to characterize the contractile postsynaptic muscarinic receptor, using agonists and subtype selective antagonists. The agonists used in the present experiments, with the exception of McN-A-343 acted as full agonists in producing contraction of the coronary resistance arteries. ACh was equipotent with metacholine and oxotremorine-M, but more potent than carbachol in inducing contractile responses in both endothelium-intact and denuded lamb coronary resistance arteries. These results agree with other studies on muscarinic receptors suggesting that an M_3-subtype of receptor is involved in ACh-induced contraction of the bovine coronary artery (Duckles, 1988; Duckles & Garcia-Villalon, 1990). McN-A-343 alone did not induce any motor response in the coronary resistance arteries, a finding similar to that reported for large coronary arteries (Duckles, 1988). However, in the presence of a low concentration of potassium in PSS, it elicited a weak motor response with a potency ($pD_2 = 4.9$) similar to that found for relaxation of the rabbit ear artery (Duckles, 1988). McN-A-343 was proposed as a selective M_1 agonist (Hammer & Giachetti, 1982; Jokisch-Mehrling *et al.*, 1989), but it exhibits variable responses at all muscarinic receptor subtypes, depending upon the receptor reserve of the preparation (Eglen *et al.*, 1987). Thus, the activity of McN-A-343 was reported to vary from no agonist activity up to a partial agonism at M_2 and M_3 receptors (Eglen *et al.*, 1987; Jokisch-Mehrling *et al.*, 1989). The weak agonism at M_2 and M_3 receptors might exclude the M_1 subtype for mediating the contractile response to ACh. However, the lack of reliable selective muscarinic agonists questions the use of agonists for receptor identification. Therefore, based on our results with agonists, it is not convenient to define the subtype of muscarinic receptors mediating contraction in the lamb coronary resistance arteries.

In endothelium-denuded lamb coronary resistance arteries all the muscarinic antagonists investigated acted as pure competitive antagonists of the ACh-induced contraction. Moreover, in the absence of the endothelium the slopes of Arunlakshana-Schild regression lines did not significantly differ from unity, which suggests that the contraction to ACh is mediated by a homogeneous muscarinic receptor population at the medial smooth muscle.

In endothelium-denuded coronary resistance arteries the affinity for pirenzepine ($pK_B = 7.5$) is not comparable to its high affinity (7.7–8.1) at M_1 cortical binding sites or to the low affinity (6.5–6.7) at M_2 cardiac receptors, but it is similar to the intermediate affinity (6.8–7.5) reported for the M_3-subclass of receptors in visceral and vascular smooth muscle and glands (Eglen & Whiting, 1985; Delmendo *et al.*, 1989; Hulme *et al.*, 1990; Dauphin & Hamel, 1990; Brunner *et al.*, 1991b). This suggests that M_1 muscarinic receptors are not mediating the contractile response to ACh in lamb coronary resistance arteries. Furthermore, the weak potency of AFDX 116 does not support a role for the M_2 receptor subtype in the contractile response to ACh, but rather a site pharmacologically coincident with an M_3 muscarinic receptor. The potent parallel displacement of the concentration-

response curves to ACh in the presence of the M_3 receptor antagonists, 4-DAMP and pFHHSiD favours the M_3 subtype for mediating the contraction. Although 4-DAMP was shown to possess equal affinity for the M_1 and M_3 receptor subtypes (Delmendo *et al.*, 1988; Hulme *et al.*, 1990), pFHHSiD showed high affinity with good correlation for a M_3 receptor subtype (7.8–8.2; Lambrecht *et al.*, 1988; Eglen *et al.*, 1990; Duckles, 1990).

Therefore, both the affinities and the order of potency of the muscarinic antagonists (pFHHSiD > pirenzepine > AFDX 116) in endothelium-denuded preparations suggest that the muscarinic receptor mediating the contractile effect of ACh in lamb coronary resistance arteries seems to belong to the M_3-subclass. This finding agrees with other functional *in vitro* studies on bovine (Duckles, 1990; Duckles & Garcia-Villalon, 1990; Brunner *et al.*, 1991b) and porcine large coronary arteries (Van Charldorp & Van Zwieten, 1989), and also with *in vivo* studies on the canine coronary circulation, where AFDX 116 showed low affinity, and pirenzepine intermediate affinity for the muscarinic receptors mediating vasoconstriction to infused ACh (Pelc *et al.*, 1988).

In the presence of the endothelium, both the responses to muscarinic agonists and the action of the antagonists, were rather different from those obtained in endothelium-denuded arteries. ACh was less potent in producing contraction in the presence than in the absence of the endothelium, while the response to carbachol was unaltered. Similar differential changes in the contractile response to ACh and carbachol upon removal of the epithelium were observed in the guinea-pig trachea, where these resutls were ascribed to differences in the abilities of these choline esters to diffuse to the muscarinic receptor sites in the epithelium intact preparations (Morrison & Vanhoutte, 1992). The endothelium might act as a barrier for ACh in lamb coronary arteries. If the barrier capability of the endothelium became saturated at higher concentrations of agonist, then the equilibrium state of agonist, antagonist and receptor population would be changed and the slope of the Arunlakshana-Schild plot would be expected to be less than unity (Kenakin, 1987). However, in the presence of the endothelium, the slope of the Arunlakshana-Schild plot for pirenzepine was greater than unity and antagonism produced by AFDX 116 on the ACh-induced contraction was unaltered by removal of the endothelium. This suggests that the leftwards shift of the contractile response to ACh in absence of the endothelium cannot be ascribed to the endothelium functioning as a diffusion barrier for ACh.

In some aspects, however, the present results could be compared with those recently published by Alosachie & Godfraind (1988) on the role of the vascular endothelium in the interaction of α-adrenoceptor agonists and antagonists in the rat aorta. These authors reported that α_1-adrenoceptor antagonists acted as non-competitive antagonists against noradrenaline in the presence of endothelium and as competitive antagonists after endothelial cell removal. In contrast, the α_2-adrenoceptor antagonist, yohimbine, and the mixed antagonist, phentolamine, acted as competitive antagonists both in the presence and absence of the endothelium, although the pA_2 values for the latter two substances were lower in vessels with intact endothelium. In the present study, pirenzepine acted as a non-competitive antagonist against ACh in the presence of the endothelium and as a competitive antagonist when the endothelium was removed, while both AFDX 116 and pFHHSiD acted as competitive antagonists in arteries both with and without endothelium, although the pK_B for pFHHSiD was underestimated in endothelium-intact preparations (Figure 5). Alosachie & Godfraind (1988) suggested that their findings might be ascribed to the continuous basal release of EDRF acting as an endogenous functional antagonist, reducing the apparent efficacy of the adrenoceptor agonists and hence leading to the non-competitive antagonism of prazosin against noradrenaline in endothelium-intact preparations. We have likewise observed in the present study an increase in basal tension after endothelial cell removal which might indicate abolition of the basal release of EDRF, as earlier demonstrated for coronary resistance arteries (Prieto *et al.*, 1991; Tschudi *et al.*, 1991; Simonsen *et al.*, 1992).

However, an alternative explanation of the present results might be that ACh is activating a heterogeneous muscarinic receptor population with opposing effects in the lamb coronary resistance arteries, contractile M_3 receptors at the smooth muscle and inhibitory muscarinic receptors producing endothelium-dependent relaxation. Thus, there might be a receptor-mediated release of EDRF that functions as an endogenous antagonist, reducing the efficacy of ACh and leading to the non surmountable antagonism of pirenzepine against the contractile response to ACh in endothelium-intact preparations. Indeed, the contractile response to ACh is potentiated in the presence of low concentrations of pirenzepine, an effect which is reversed by endothelial cell removal (Table 3). The latter finding supports the concept that ACh might be activating receptors with opposing effects, since occupation by pirenzepine of a small part of the inhibitory receptors produces a significant leftward shift of the concentration-response curve to ACh. The lack of a change in the Schild graphs for AFDX 116 in coronary arteries with and without endothelium, despite the evidence for a heterogeneous population of muscarinic receptors obtained from the results with pirenzepine, might be ascribed to the low selectivity of this antagonist for M_1 and M_3 receptors. However, further investigation is needed to clarify the leftward shift in the Schild plot and the unmasking of an underestimated pK_B value for pFHHSiD against ACh, induced by endothelial cell removal in the coronary resistance arteries. This finding could be due to several possible factors such as a temporal disequilibilium of the antagonist-receptor complex with the endothelium as a diffusion barrier to the compound, or that pFHHSiD possess affinity for membrane sites other than muscarinic receptors in the presence of endothelium, which could result in an artifactually low pK_B value (Kenakin, 1987). Finally, this shift might also be caused by removal of either basal or of agonist-induced EDRF release, in the latter case implicating the presence of a heterogeneous muscarinic receptor population in endothelium-intact vessels.

Additional evidence for a heterogeneous muscarinic receptor population comes from the relaxation induced by the putative M_1-selective agonist, McN-A-343, in lamb coronary resistance arteries. This relaxation to McN-A-343 was inhibited in the presence of a low concentration of the M_1-selective antagonist, pirenzepine but not by pFHHSiD or AFDX 116, known to block M_3 and M_2 receptors, respectively. The concentration of pirenzepine inhibiting the relaxation to McN-A-343 was coincident with that inducing potentiation of the ACh concentration-response curves (Table 3). These findings, together wtih the demonstration of a contractile effect of McN-A-343, suggest that ACh is not the only agonist activating a heterogeneous population of muscarinic receptors with opposing effects, but also that McN-A-343 is able to produce both relaxation and contraction in lamb coronary arteries. The present results therefore suggest that an inhibitory muscarinic receptor population at the endothelium, probably of the M_1-subclass, modulates the contractile response to ACh elicited through the M_3-receptors at the smooth muscle layer in the lamb coronary resistance arteries.

The present findings that the endothelial receptors might belong to the M_1-subclass and the contractile receptor at the smooth muscle of the lamb coronary resistance arteries to the muscarinic M_3-subclass agree with *in vivo* studies in the canine coronary circulation (Pelc *et al.*, 1988). In contrast, the endothelial receptor that mediates relaxation in bovine large coronary arteries seems to belong to the M_3-subtype (Brunner *et al.*, 1991a). However, apart from species differences, there might be muscarinic receptor heterogeneity

not only between the endothelial layer and smooth muscle layer in the distal parts of the circulation, but also between proximal and distal parts of the coronary vascular tree (Nyborg *et al.*, 1991).

In summary, the present study suggests that coronary resistance arteries have heterogeneous populations of muscarinic receptors located at both the smooth muscle and endothelium causing contraction and the release of EDRF, respectively. The affinities of the muscarinic antagonists for the receptor at the smooth muscle layer suggest that ACh-induced contraction is mediated through M_3-receptors, while muscarinic receptors probably of another subtype or subtypes at the endothelium seem to modulate the contractile response to ACh.

We thank Elena Ordaz and Manolo Perales for technical assistance and Dr Miguel Isla for helpful discussions. The authors wish to express their thanks to Dr Barlow, Bristol, U.K., for the gift of 4-DAMP and Dr Karl Thomae, GmbH, for the gift of AFDX 116, and the Madrid Municipal Slaughterhourse for kindly supplying fresh tissue. U.S. was supported by a grant from Universidad Complutense de Madrid. Participating laboratories in Eurad.

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(Received September 11, 1992
Revised February 26, 1993
Accepted March 17, 1993)

Br. J. Pharmacol. (1993), **109**, 1008–1013 © Macmillan Press Ltd, 1993

Tachykinin receptors mediating responses to sensory nerve stimulation and exogenous tachykinins and analogues in the rabbit isolated iris sphincter

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1 We have used selective tachykinin receptor agonists and antagonists to investigate the nature of the receptors mediating responses to endogenous and exogenous tachykinins in the rabbit iris sphincter preparation *in vitro*.

2 The NK_1-selective agonist, substance P methyl ester, induced contraction with a pD_2 of 9.16 indicating the presence of NK_1 receptors. In confirmation, the NK_1-selective antagonist, GR82334, competitively antagonized responses to substance P methyl ester with high affinity (pK_B 7.46).

3 NK_3 receptors also mediate contraction since NK_3-selective agonists exhibited high potency, e.g. the pD_2 of [Me-Phe^7]-neurokinin B was 9.67, and their responses were not inhibited by GR82334 (10 μM).

4 NK_2 receptor activation does not seem to contribute to contraction since the NK_2-selective agonist [β-Ala^8]-neurokinin A(4-10) had relatively low potency (pD_2 6.43), and the NK_2-selective antagonists MEN10207 (1 μM) and L-659,877 (10 μM) were inactive or had low affinity, respectively.

5 GR82334 (1 μM) significantly inhibited responses to electrical field-stimulation of non-adrenergic non-cholinergic sensory nerves (3, 10 and 30 Hz), and caused a rightward shift of the log concentration-response curve to bradykinin (lateral shift *ca.* 1000 fold). Higher concentrations of GR82334 (10 μM) significantly attenuated responses to capsaicin (1–60 μM) whilst completely abolishing responses to field-stimulation (3, 10 and 30 Hz) and bradykinin (1 nM– 3 μM).

6 In conclusion, NK_1 and NK_3 receptor activation results in contraction of the rabbit iris sphincter. The contractile response following sensory nerve stimulation by bradykinin, capsaicin and electrical field stimulation results from NK_1 receptor activation.

Keywords: Tachykinin receptors; neurokinin; iris sphincter; bradykinin; capsaicin; substance P; sensory nerves; NK_1 antagonist; GR82334; NANC responses.

Introduction

Sensory, non-adrenergic non-cholinergic (NANC), nerve stimulation of the rabbit iris sphincter *in vitro* by electrical field-stimulation, capsaicin or bradykinin, results in contraction of the smooth muscle. Functional studies suggest these responses are caused by nerve-released tachykinins (Butler & Hammond, 1980; Muramatsu *et al.*, 1987), although the identity of the tachykinins released by each of the sensory nerve stimulants remains unclear. Our previous work with selective tachykinin receptor agonists (Hall *et al.*, 1991) and antagonists (Hall *et al.*, 1992b) indicated that activation of NK_1 and NK_3 postjunctional receptors results in contraction in this preparation; while radioligand binding studies have demonstrated specific binding sites for substance P in iris sphincter muscle membrane preparations (Too *et al.*, 1988; Denis *et al.*, 1991). In principle, therefore, an interaction of neuronally-released tachykinins with either or both NK_1 and NK_3 receptor types could contribute to contractile responses following sensory nerve stimulation.

The aim of the present study was two fold. First, to confirm the nature of the tachykinin receptors in this preparation, by use of recently available agonists and antagonists that are selective for the individual tachykinin receptors. Second, to investigate the effect of NK_1-selective antagonists on responses to NANC electrical field-stimulation, and to chemo-stimulation by capsaicin and bradykinin. Preliminary accounts of this work have been published (Hall *et al.*, 1992b; 1993).

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Methods

Tissue preparation and recording arrangements

Male New Zealand albino rabbits (2.5–3.0 kg) were killed by an overdose of i.v. pentobarbitone sodium (Sagatal). The eyes were enucleated immediately after death and opened by an incision 2–3 mm dorsal to the limbus, followed by excision of the iris from the ciliary margin. The sphincter pupillae muscles were dissected free of dilator muscle and mounted intact, either on stainless-steel tissue-holders or between parallel platinum electrodes, in 2 ml silanised glass organ baths in Krebs solution at 37°C. The preparations were attached to isometric Grass FT03B force-displacement transducers under an initial resting tension of 150 mg. Mechanical activity was recorded on Grass model 7E polygraphs. Electrical field stimulation was applied by Scientific Research Instruments Limited (SRI 6051) stimulators.

The composition of the Krebs solution was (mM): Na^+ 140, K^+ 5.9, Cl^- 104.8, $H_2PO_4^-$ 1.2, HCO_3^- 24.9, Ca^{2+} 2.6, Mg^{2+} 1.15, SO_4^{2-} 1.15, glucose 10. The Krebs solution was maintained at pH 7.4 by constant bubbling with 95% O_2: 5% CO_2. In all experiments the Krebs solution contained hexamethonium (100 μM), mepyramine, cimetidine, guanethidine, atropine and ibuprofen (all 1 μM).

Experimental protocols

An equilibration period of 60 min was allowed before commencing regular dosing, after which time the maximal response to carbachol was established. Concentration-response curves for bradykinin were determined at the start, and for substance P at the end, of experiments and a concentration-response curve to carbachol was obtained in each prepara-

tion. Cumulative concentration-response curves were established to stimulants with 20 min allowed between agonists, and when several different tachykinins and analogues were used they were applied in a randomized order between preparations. Antagonist incubation time was 5 min, since increasing this to 15 min had no effect on affinity estimates for GR82334 and MEN10207. Electrical field-stimulation was carried out with square-wave pulses of 0.3 ms duration at 50 V for 15 s with 15–20 min between each stimulation period.

Effect of tachykinin receptor agonists and antagonists

The receptor selectivity of the tachykinin analogues used in this study is as follows: substance P methyl ester and GR73632 (NK_1 agonists); [β-Ala^8]-NKA(4-10) and GR64349 (NK_2 agonists); senktide and [Me-Phe^7]-NKB (NK_3 agonists), GR82334 (NK_1 antagonist), MEN10207 and L-659,877 (NK_2 antagonists). In all experiments, paired irises from the same animal were used. Full concentration-response curves were obtained for bradykinin, mammalian tachykinins and analogues in each series either in the absence (concurrent control), or the presence (test) of the antagonist (1, 3.2 or 10 μM). For substance P methyl ester control concentration-response curves were obtained followed by those obtained in the presence of increasing concentrations of GR82334 (1, 3.2 and 10 μM; test tissues), or no antagonist (concurrent time-control tissues). Full concentration-response curves for carbachol and [Arg^8]-vasopressin were obtained in the absence and presence of GR82334 (30 μM).

Effect of GR82334 on responses to electrical field-stimulation and capsaicin

One paired preparation acted as a concurrent control, with the other as a test preparation. Responses to carbachol, a submaximal concentration of substance P methyl ester (10 nM), and electrical field-stimulation (3, 10 and 30 Hz) were obtained, and these were then repeated in the presence of 1 μM GR82334, and then 10 μM GR82334. Finally, a concentration-response curve was established for capsaicin in the presence of GR82334 (10 μM). The concurrent control preparation received an identical dosing protocol, but in the absence of GR82334 throughout. In a further series of experiments, the effect of a higher concentration of GR82334 (30 μM) was tested against responses to capsaicin only.

Source of agents used

Agents were obtained as follows: Sagatal (RMB Animal Health Ltd, U.K.), carbachol, capsaicin, hexamethonium bromide, ibuprofen, phosphoramidon (Sigma, U.K.), mepyramine maleate (May & Baker, U.K.), guanethidine sulphate (CIBA, U.K.), substance P, substance P methyl ester, senktide (Succ-[Asp^6,Me-Phe^8]-substance P(6-11)), neurokinin A, neurokinin B, [Arg^8]-vasopressin, and bradykinin (Peninsula Laboratories, Europe; or Bachem. U.K.), [Me-Phe^7]-neurokinin B ([Me-Phe^7]-NKB; Nova Biochem, U.K.), cyclo(Gln,Trp,Phe,Gly,Leu,Met) (L-659,877, Cambridge Research Biochemicals, U.K.), δ-amino valeryl-[L-Pro^9,*N*-MeLeu^{10}]-substance P(7-11) (GR73632), [Lys^3, Gly^8-R-γ-Lactam,Leu^9]-neurokinin A(3-10) (GR64349) and [D-Pro^9 [Spiro-γ-Lactam] Leu^{10}, Trp^{11}] - physalaemin(1-11) (GR82334) (Penninsula Laboratories, Europe; or Glaxo Group Research, U.K.), [β-Ala^8]-neurokinin A(4-10); ([β-Ala^8]-NKA(4-10), A. Menarini Pharmaceuticals, Italy; or Bachem, U.K.), [Tyr^5,D-Trp^{6,8,9},Arg^{10}]-neurokinin A(4-10) (MEN10207) (A. Menarini Pharmaceuticals, Italy; or Peninsula Laboratories, Europe). All salts were of analytical grade and were obtained from B.D.H., U.K.

L-659,877, capsaicin, neurokinin B, [Me-Phe^7]-NKB, senktide and [β-Ala^8]-NKA(4-10) were dissolved in dimethylsulphoxide to 1 mM stock. Ibuprofen was dissolved in 5% Na_2CO_3. All other agents, and all dilutions, were made up in distilled water and peptides were stored at −20°C. Dimethylsulphoxide (maximum amount injected during experiments) did not affect basal tension *per se*, nor concentration-response curves to neurokinin A ($n = 4$, data not shown).

Expression of results and statistical analysis

For agonists, responses are expressed in terms of % maximal response to carbachol in individual preparations, determined at the start of experiments. Data for individual experiments were combined and analysed in two ways. To allow conventional graphical display, responses were calculated for each concentration of test agonist in individual experiments (control tissues from all the antagonist experiments described below) and plotted as mean responses ± s.e.mean. For the purposes of obtaining objective slope and EC_{50} estimates, individual agonist concentration-response relationships were analysed by determining the least-squares fit of data to the logistic function $y = y_{max}\ A^p/(A^p + A_{50}{}^p)$, where y is the response (expressed in terms of maximum responses to carbachol), y_{max} is the estimated maximum response to that tachykinin, A is the tachykinin concentration, A_{50} is the concentration producing 50% of that agonist's maximum response (the EC_{50}), and p is a slope factor (equivalent to the Hill Slope). These curve-fitting procedures were carried out using Sigma Plot 4.1 computer software (Jandel Scientific, CA, USA).

For antagonists, the pK_B estimates were determined from individual dose-ratios using the Gaddum-Schild equation, $-\log K_B = pK_B = \log (x\text{-}1) - \log [A]$, where x is the dose-ratio and [A] the applied antagonist concentration. Affinities are described as pK_B values when Schild plot analysis showed no significant departure from unity slope, or as apparent pK_B values when such analysis could not be carried out because a limited antagonist concentration-range was used (see Results). Dose-ratios were estimated between the responses in test and concurrent-control tissues, except in experiments to estimate the effect of GR82334 against responses to substance P methyl ester. In all cases *n* represents data obtained from 4–17 animals and refers to either the number of estimates (pK_B) or the number of preparations (all other estimates). Error estimates are given as s.e.mean. Tests for statistical significance were carried out with paired or unpaired *t* tests, or analysis of variance, as appropriate.

Results

Agonist potencies

Agonist potencies are shown in Table 1. The NK_1-selective agonist substance P methyl ester and the NK_3-selective agonist [Me-Phe^7]-NKB had high potency, whereas the NK_2-selective agonist [β-Ala^8]-NKA(4-10) was *ca.* 1000 fold less active. The mammalian tachykinins substance P, neurokinin A and neurokinin B were of similar potency (pD_2 estimates were 8.45, 8,64 and 8.88 respectively). All tachykinins and analogues reached similar maximal responses, which did not significantly differ from the maximal response obtained for carbachol ($P > 0.05$) (Table 1 and Figure 1). Estimates of the logistic slope factor (p) varied significantly ($P < 0.05$) between tachykinins as a whole; but after exclusion of the apparently non-sigmoidal [β-Ala^8]-neurokinin A(4-10) curve, there were no other significant differences ($P > 0.05$) in useful parameter comparisons by analysis of variance.

Antagonist studies

The NK_1-selective antagonist GR82334 (1, 3 and 10 μM), caused a parallel shift of the log concentration-response

Table 1 Activities of tachykinins and analogues in contracting the rabbit iris sphincter preparation

Tachykinin	n	*Slope*	*Maximum* (%)	pD_2	EC_{50} [nM]
Substance P	13	0.73 ± 0.10	92.1 ± 4.3	8.45 ± 0.26	3.5
Neurokinin A	9	0.84 ± 0.10	106.4 ± 5.7	8.64 ± 0.20	2.3
Neurokinin B	6	0.75 ± 0.11	106.5 ± 4.2	8.88 ± 0.17	1.3
Substance P methyl ester	17	0.62 ± 0.04	100.4 ± 4.8	9.16 ± 0.10	0.7
[Me-Phe^7]-neurokinin B	4	0.43 ± 0.04	87.7 ± 4.7	9.67 ± 0.21	0.2
[β-Ala^8]-neurokinin A (4-10)	5	1.12 ± 0.18	97.2 ± 6.0	6.43 ± 0.12	370.7

Experiments were carried out in the presence of hexamethonium (100 μM), guanethidine, mepyramine, cimetidine, atropine and ibuprofen (all 1 μM). Logistic parameter estimation procedures are described in Methods. Maxima are expressed in terms of % carbachol maximum, and none differed significantly from 100% ($P > 0.05$). Slopes overall differed significantly between tachykinins ($P < 0.05$).

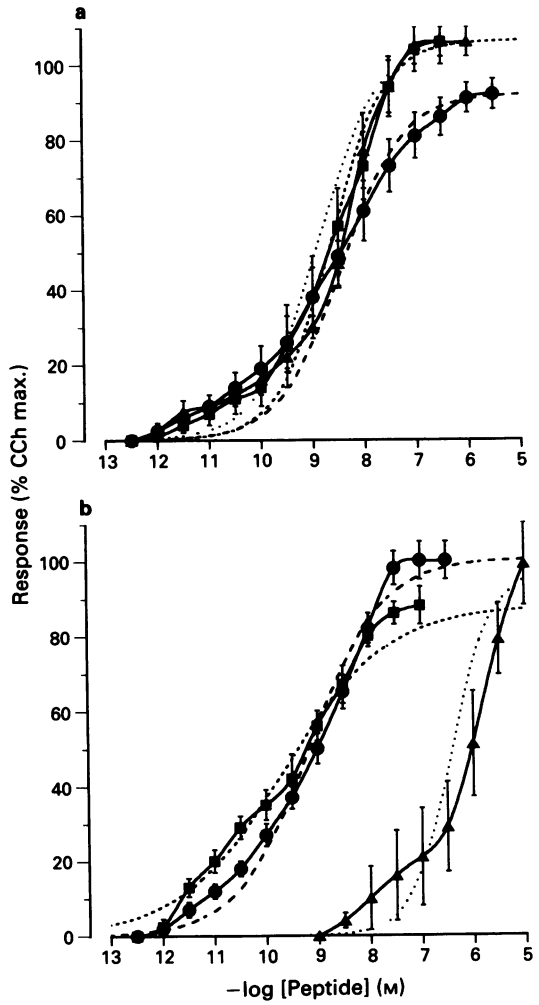


Figure 1 Log concentration-response curves for tachykinins and analogues in rabbit iris sphincter. In (a) responses to the mammalian tachykinins substance P (●), neurokinin A (■) and neurokinin B (▲) and in (b), responses to the NK_3-selective agonist [Me-Phe^7]-neurokinin B (■), the NK_1-selective agonist substance P methyl ester (●) and the NK_2-selective agonist [β-Ala^8]-neurokinin A(4-10) (▲). Responses are shown as % maximal response to carbachol (CCh) and each point shows the mean (± s.e.mean) obtained from 4–17 individual preparations. The broken lines represent meaned logistic fit log concentration-response curves calculated from the parameters in Table 1. Note some deviations from the logistic model (see text).

curve to the NK_1-selective agonist substance P methyl ester, and the Schild regression did not differ significantly from unity (0.87 ± 0.11 $P > 0.05$), compatible with a competitive antagonism at a single site, for which the pK_B estimate was 7.46 ± 0.14 ($n = 12$). However, when tested against neurokinin A, GR82334 (10 μM) showed antagonism (in 4 out of 6 preparations) only of the lower portion of that agonist's log concentration-response curve, from which an apparent pK_B estimate of 6.25 ± 0.23 could be calculated (data not shown). GR82334 (3 μM) did not antagonize responses to substance P and senktide (i.e. apparent $pK_B < 5.5$), carbachol or [Arg^8]-vasopressin data not shown).

The NK_2-selective antagonist MEN10207 (1 μM) did not inhibit responses to [β-Ala^8]-NKA(4-10), GR64349 and substance P. The potent NK_2-preferring antagonist, L-659,877, inhibited responses to GR64349 and GR73632 but with low affinity (apparent pK_B values 6.29 ± 0.29, $n = 5$, and 6.37 ± 0.34, $n = 7$, respectively), and was inactive (10 μM, $n = 4$) against responses to substance P.

Effect of GR82334 on responses to sensory nerve stimulation

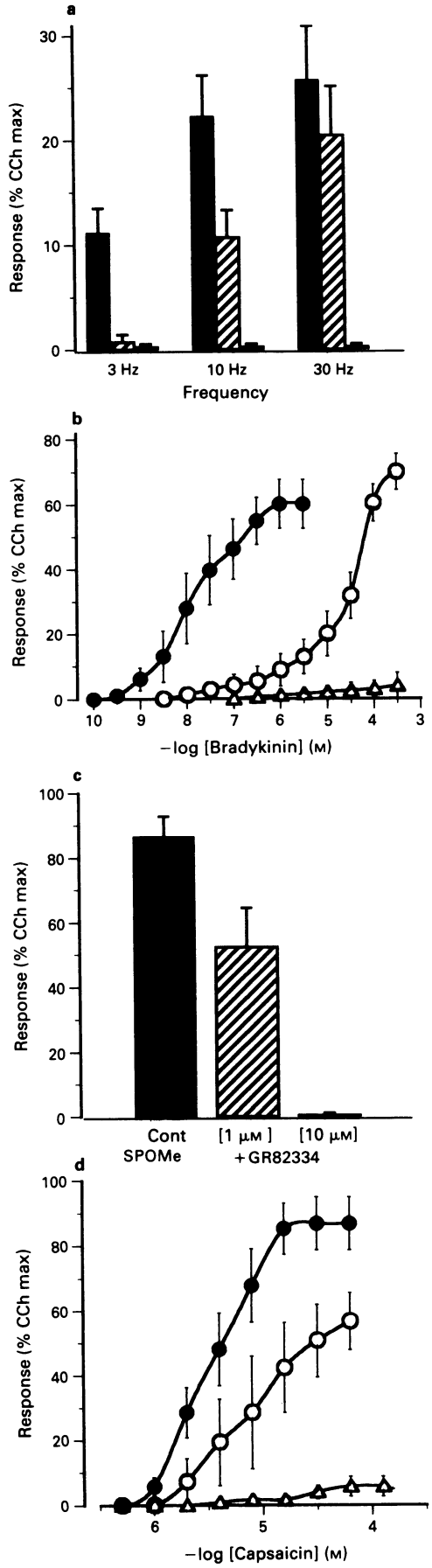
The NK_1-selective antagonist GR82334 (1 μM) attenuated responses to sensory NANC field-stimulation, (Figure 2a), bradykinin (Figure 2b), and submaximal responses to substance P methyl ester used as a control (Figure 2c). At 10 μM, GR82334 attenuated responses to capsaicin (Figure 2d), abolished responses to both bradykinin (Figure 2b) and to substance P methyl ester (Figure 2c), and reversibly abolished responses to electrical field-stimulation (Figure 2a). At a higher concentration (30 μM), GR82334 abolished responses to capsaicin (Figure 2d).

Discussion

Our findings in this study indicate a role for NK_1 and NK_3, but not NK_2, receptors mediating tachykinin-evoked contractile responses in the rabbit isolated sphincter preparation. Further, our observations with the NK_1 antagonist, GR82334, suggest a role for NK_1 receptors in mediating contractions following tachykinin-release evoked from sensory NANC neurones either by electrical field-stimulation or chemo-stimulation by bradykinin and by capsaicin.

Tachykinin receptor types in the rabbit iris sphincter

Results from this study using agonists selective for the NK_1, NK_2 and NK_3 receptor types support our earlier findings (see Introduction) suggesting that a mixed NK_1 and NK_3 receptor population mediates smooth muscle contraction to exogenous tachykinins and analogues in the isolated iris sphincter preparation of the rabbit. Thus, in agreement with our previous studies (Hall *et al.*, 1991) using the novel NK_1-selective agonist GR73632 (Hagan *et al.*, 1991b), the established NK_1-selective agonist substance P methyl ester (Watson *et al.*, 1983) has relatively high potency (pD_2 9.16). This suggested presence of NK_1 receptors was confirmed with the NK_1-selective antagonist GR82334 (Hagan *et al.*, 1991a). Schild plot analysis of the antagonism by GR82334 of res-



ponses to substance P methyl ester, indicated a competitive interaction with an NK_1-receptor population, yielding a pK_B estimate of 7.46. This value is similar to estimates of the affinity of this antagonist at NK_1 receptors in the guinea-pig ileum (pK_B 7.64, Hagan *et al.*, 1991a; pK_B 7.42, Hall *et al.*, 1992a), guinea-pig trachea (pK_B 7.23; Hagan *et al.*, 1991a) and guinea-pig proximal urethra (pK_B 7.31; Maggi & Patacchini, 1992). Our affinity estimate for GR82334 (pK_B 7.46) is, however, higher than that reported by Beresford *et al.* (1992) in the rabbit iris (pK_B 6.11): the reason for this discrepancy is not known.

Activation of NK_3-receptors also appears to cause contraction of the iris sphincter since the NK_3-selective agonists senktide (Hall *et al.*, 1991) and [Me-Phe^7]-NKB (Drapeau *et al.*, 1987) (pD_2 9.67) have high potency. However, it should be noted that the evidence for the existence of NK_3-receptors is the high activity of NK_3-selective agonists, together with the absence of antagonism of these responses by GR82334. Confirmation through the use of NK_3-selective receptor antagonists is required.

With respect to NK_2 receptors, the NK_2-selective agonist [β-Ala^8]-NKA(4-10) (Rovero *et al.*, 1989) had low potency (pD_2 6.43), in agreement with our earlier studies (Hall *et al.*, 1991) with the NK_2-selective agonist GR64349 (Hagan *et al.*, 1991b). In view of the low potencies of NK_2-selective agonists compared to those seen in defined NK_2 receptor systems (Rovero *et al.*, 1989; Hagan *et al.*, 1991b; Hall *et al.*, 1992a), it seems most likely that at the high concentrations necessary to obtain responses, these agonists are interacting with non-NK_2 receptors. This interpretation is supported by our demonstration of the lack of antagonism by the NK_2-selective antagonist, MEN 10207 (Maggi *et al.*, 1990; Rovero *et al.*, 1990), and the low affinity of the NK_2-selective antagonist, L-659,877 (McKnight *et al.*, 1991) when tested against NK_2-selective agonists. The latter antagonist also inhibited responses to the NK_1-selective agonist, GR73632 when used at these high concentrations. In fact, a low affinity of L-659,877 for NK_1 or NK_3 receptors has previously been demonstrated (Williams *et al.*, 1988). We therefore conclude that NK_2 receptors are not present in rabbit iris sphincter.

Effect of GR82334 on responses to mammalian tachykinins and sensory NANC nerve stimulation

Both neurokinin A and substance P are released from peripheral termini of mammalian primary afferent neurones (see Holzer, 1988). In the iris sphincter, the NK_1-selective antagonist, GR82334, inhibited some part of responses to neurokinin A, though not to substance P. The apparent affinity estimate for GR82334 obtained with neurokinin A as the agonist, was much lower than when tested against substance P methyl ester. However, since neurokinin A shows little selectivity between tachykinin receptor types, and is

Figure 2 The effect of GR82334 on responses to sensory nerve stimulation. Mean responses (± s.e.mean) are shown for 7 preparations. In (a) responses to sensory non-adrenergic non-cholinergic electrical field-stimulation at 3, 10 and 30 Hz was determined in the absence (solid columns) and presence of GR82334 at 1 μM (diagonally hatched columns) and 10 μM (horizontally hatched columns). In (b) log concentration-response curves to bradykinin (●) control; and in the presence of (○) 1 μM, and (△) 10 μM GR82334. In (c) control submaximal responses to substance P methyl ester (SPOMe) are shown (solid column), and after GR82334 treatment (diagonally hatched column) 1 μM, and (third column) 10 μM. In (d) (●) control log concentration-response curves for capsaicin; and in the presence of GR82334 at 10 μM (○), and 30 μM (△). Experiments were carried out in Krebs solution containing hexamethonium (100 μM), atropine, mepyramine, cimetidine, guanethidine and ibuprofen (all 1 μM). Panels (a), (b) and (d) are reproduced from Hall *et al.* (1993) with permission from the Publishers.

active at NK_3 receptors it seems likely that the relative lack of antagonism is due to an interaction of this agonist with NK_3 receptors and that this component of the neurokinin A response is resistant to antagonism by GR82334. This suggestion of an action of neurokinin A at two sites is supported by the finding that in most preparations GR82334 (10 μM) caused a parallel shift of only the lower portion of the log concentration-response curve. However, with substance P, the lack of any demonstrable antagonism of responses was unexpected because, although this agonist is not particularly selective between the three tachykinin receptors, it is thought to show a preference for the NK_1 type (see Hall *et al.*, 1991; 1993). However, one explanation for this discrepancy comes from preliminary data suggesting that the predominant component of the action of substance P is through an interaction with a further 'atypical' site from which the offset kinetics are very slow (Hall *et al.*, 1993): however, after phenoxybenzamine pretreatment this site is no longer evident, and GR82334 now antagonizes substance P with the affinity that might be expected at NK_1 receptors (Hall *et al.*, 1993).

GR82334 inhibited responses to three different modes of sensory nerve stimulation, namely by bradykinin, sensory NANC electrical field stimulation and by capsaicin. GR82334 (1 μM) attenuated responses to 3, 10 and 30 Hz electrical field-stimulation and shifted the log concentration-response curve for bradykinin *ca.* 1000 fold to the right. At a higher concentration (10 μM), GR82334 abolished responses to bradykinin and to electrical field-stimulation. These results suggest that both bradykinin and electrical field-stimulation contract the iris sphincter through the release of tachykinins that act on NK_1 receptors, but argue against a major role of substance P contributing to these responses (see above). GR82334 (10 μM) inhibited contractile responses to capsaicin, and at still higher concentrations (30 μM) abolished responses to this sensory nerve stimulant. The higher concentration of GR82334 required for total inhibition of responses to capsaicin may be a consequence of the different mechanism of excitation by this agent, differential access to transmitter pools and amounts of released peptide, (Håkanson *et al.*, 1987; Wang & Håkanson, 1992a; Hall *et al.*, 1993). Though it is of interest that the potent non-peptide NK_1 antagonist (±)-CP-96,345 also inhibits responses to capsaicin and electrical field-stimulation in this preparation (Håkanson *et al.*, 1991; Wang & Håkanson, 1992b), it does so at relatively high concentrations (e.g. pIC_{50} 5.4 for nerve field-stimulation; Wang & Håkanson, 1992c), when the established non-specific actions of the antagonist in this and other preparations (Hall *et al.*, 1992a; Wang & Håkanson, 1992c) complicates interpretation. It is of interest that GR82334 was able to inhibit completely responses to bradykinin, capsaicin, and electrical field-stimulation which suggests that under these conditions only NK_1-receptors are involved in mediating contractile responses. A similar conclusion has been drawn previously (Wang & Håkanson, 1992b).

In conclusion, NK_1 receptors are present in the rabbit isolated iris sphincter preparation, and their activation results in contraction of the smooth muscle. NK_3 receptor stimulation also leads to contraction. The contractile responses seen following sensory nerve-stimulation by electrical field stimulation, bradykinin and capsaicin are mediated via NK_1-receptor activation.

We wish to thank Dr C.A. Maggi, A. Menarini Pharmaceuticals, Italy, for the gifts of [β-Ala^8]-NKA(4-10) and MEN10207, and Drs R.M. Hagan and S.J. Ireland, Glaxo Group Research, UK, for GR82334, GR73632 and GR64349. This study was supported by the Wellcome Trust.

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(Received January 8, 1993
Revised February 16, 1993
Accepted March 17, 1993)

Br. J. Pharmacol. (1993), **109**, 1014–1019 © Macmillan Press Ltd, 1993

Effects of beraprost on the transmembrane potentials of guinea-pig ventricular muscles during normoxia and hypoxia-reoxygenation

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1 The present study was performed to determine whether beraprost, a new stable analogue of prostacyclin, may exert beneficial effects on the transmembrane action potentials during normoxia and hypoxia-reoxygenation in isolated right ventricular muscles of the guinea-pig.

2 Under normal oxygenation, beraprost (0.01– 100 μmol^{-1}) had no effects on the electrophysiological parameters.

3 Hypoxic conditions induced a decrease in action potential duration (APD) without affecting other action potential parameters. Beraprost inhibited this hypoxia-induced decrease in APD. However, beraprost had no effect on the decrease in contractile force induced by hypoxia, whereas it significantly improved the recovery of contractile force after reoxygenation.

4 Pinacidil-induced shortening of APD was not antagonized by beraprost.

5 Hypoxia significantly decreased the myocardial adenosine triphosphate (ATP) level, which was also prevented by beraprost.

6 These results suggested that beraprost may inhibit the hypoxia-induced shortening of APD by some mechanisms which contribute to the maintenance of muscle ATP level.

Keywords: Beraprost; prostacyclin; normoxia; hypoxia; adenosine triphosphate; cardioprotection

Introduction

It is well established that prostacyclin has a potent inhibitory effect on platelet aggregation (Whittle *et al.*, 1978) and also has vasodilator effects (Moncada *et al.*, 1976; Bunting *et al.*, 1976). Furthermore, cytoprotective effects of prostacyclin on various tissues, including those of the cardiovascular system have been suggested (Ogletree *et al.*, 1979). However, its rapid metabolic degradation *in vivo* has limited its therapeutic application. Beraprost (sodium ((±)-1***R****, 2***R****, 3a***S****, 8b***S****)-2,3,3a,8b-tetrahydro-2-hydroxyl-1-[(*E*)-(3*S*)-3-hydroxy-4-methyl-1-octen-6-ynyl]-1*H*-cyclopenta [b] benzofuran-5-butyrate) is a chemically stable prostacyclin analogue which can be administered orally and has been demonstrated to possess a similar pharmacological profile to prostacyclin, i.e., potent antiplatelet aggregation effect *in vitro* and *ex vivo* in various animal species (Umetsu *et al.*, 1987; Nishio *et al.*, 1988), antithrombotic effect on arterioles in the hamster cheek pouch (Sim *et al.*, 1985) and vasodilator effects in the dog (Akiba *et al.*, 1986). Prostacyclin has been reported to possess a cardioprotective effect during acute myocardial ischaemia and reperfusion in *in vitro* and *in vivo* animal models (Lefer *et al.*, 1978; Ogletree *et al.*, 1979; Jugdult *et al.*, 1981; Melin *et al.*, 1983; Berti *et al.*, 1990). A number of preliminary clinical studies with prostacyclin have demonstrated haemodynamic benefits in heart failure (Yui *et al.*, 1982; 1984; Auinger *et al.*, 1989). However, the precise mechanisms for this cardioprotective effect are unclear. In the present study we examined the effects of beraprost on the transmembrane potentials of guinea-pig ventricular muscles during normoxia and hypoxia-reoxygenation.

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Methods

Action potential and tension recordings

Briefly, the heart was removed from open chest male guinea-pigs, weighing 380–480 g, anaesthetized with pentobarbitone sodium. Ventricular free wall strips and papillary muscles were dissected out from the right ventricle.

For the electrophysiological experiments, the free wall preparation was pinned down horizontally on a silicon block placed in an organ bath containing physiological salt solution (PSS) of the following composition (mmol l^{-1}): NaCl 135, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 15 and glucose 5.5. The bathing solution was bubbled and circulated with a 95% O_2:5% CO_2 mixture at 36.5 ± 0.5°C. Hypoxic conditions were produced by introducing a nitrogen/carbon dioxide gas mixture for 30 min followed by reoxygenation with the normal gas mixture for 15 min. Conventional microelectrode penetrations were made into the endocardial surface with glass microelectrodes filled with 3 mol l^{-1} KCl. The output of a microelectrode preamplifier (Nihon Kohden, model MEZ-8201) with high input impedance and capacity neutralization was fed into a dual beam cathode ray oscilloscope (Nihon Kohden, VC-11) and displayed to monitor the action potentials. Preparations were driven by external electrical stimulation using bipolar platinum electrodes and rectangular current pulses (5 ms duration) at a constant frequency (1 Hz) that was generated by an electronic stimulator (Nihon Kohden, SEM-3201).

The following action potential variables were analyzed: action potential amplitude (APA), maximum rate of rise of the action potential ($+\dot{V}_{max}$) and 50% action potential duration (APD_{50}:time required for 50% repolarization) and 90% APD (APD_{90}).

For the mechanical studies, papillary muscle preparations were suspended vertically in an organ bath containing 50 ml PSS of the same composition as described above, and the bathing solution was bubbled with the same gas mixture at 36.5 ± 0.5°C. Isometric contractions were measured with a

force displacement transducer (Nihon Kohden, TB-611T) by applying a baseline tension of about 0.5 g. They were recorded on an ink-writing chart recorder (Nihon Kohden, RM-6100). Electrical field stimulation of 5 ms duration (1.5 × threshold strength) was applied through bipolar platinum plate electrodes at a frequency of 1 Hz.

Experimental protocol

Beraprost was given under either normoxic or hypoxic conditions. To determine the effects of beraprost under normoxic conditions, control action potentials in normoxic PSS were recorded before the drug addition, and the preparation was incubated with normoxic PSS containing beraprost for 15 min; at the end of this period, various action potential parameters were measured.

To determine the effects of beraprost during hypoxia or reoxygenation, the preparation was made hypoxic for 30 min, which was followed by the reoxygenation with normal gas mixture for 15 min. To test the effects of beraprost, it was added at 15 min after the start of hypoxic conditions and was present throughout the experiments until the end of the reoxygenation experiments.

ATP levels were determined at time 0 and 15 min after the treatment with beraprost during normoxia. ATP measurements in hypoxia or reoxygenation experiments were made at the end of the period of hypoxia and at the end of reoxygenation.

Tissue ATP determination

Guinea-pig right ventricular free wall strips in which ATP level was to be determined were immediately freeze-clamped with aluminium tongs kept in liquid nitrogen (temperature: −80°C) as described by Wollenberger *et al.* (1960). The frozen muscle was then pulverized and homogenized; ATP was assayed by the luciferin-luciferase method (Herring, 1978).

Drugs and statistical analysis

Beraprost, prepared in the chemical laboratory of Toray Industries, Inc., was dissolved in distilled water to make a series of test solutions. Pinacidil was dissolved in dimethyl sulphoxide. The data were expressed as mean ± s.e.mean of 4 to 8 experiments. Statistical analysis was performed by Dunnett's test applied after ANOVA analysis of variance.

Results

Effects of beraprost under normoxic conditions

The effects of beraprost in normoxic conditions are summarized in Table 1. In this experiment, the preparation was incubated in normoxic PSS containing beraprost at concentrations between 0.01 and 100 μmol l^{-1} for 15 min. Beraprost induced no changes in the action potential variables namely APA, $+\dot{V}_{max}$ and APD.

Effects of beraprost during hypoxia-reoxygenation

Changes in action potential configuration produced by hypoxia are shown in Figure 1a. Hypoxia itself gradually shortened APD without affecting other parameters of the action potential (Table 2, Figure 1a). In control preparations (without treatment), APD_{50} and APD_{90} were significantly shortened ($P<0.05$) after 30 min of hypoxia. In the same hypoxic conditions, beraprost added at 15 min after the start of hypoxia concentration-dependently inhibited the decrease in APD (Table 2). For example, in 0.1 μmol l^{-1} beraprost-treated preparations, the shortening of APD_{50} and APD_{90} after 30 min hypoxia was significantly smaller than that observed in the non-treated control preparations (Figure 1b, Table 2).

At the same concentration, beraprost had no effect on the decreased contractile force of papillary muscles produced by hypoxia (Figure 2). In control and beraprost-treated preparations, contractile force was decreased to 0.128 ± 0.023 g and 0.134 ± 0.018 g, respectively, after 30 min of hypoxia. However, after 15 min reoxygenation, though beraprost had no significant effect on the action potential (APD_{50}; 134.2 ± 13.2 ms in control, 141.8 ± 13.6 ms in 0.1 μmol l^{-1} beraprost treatment), it significantly improved the recovery of contractile force; it recovered to 90.3 ± 4.4% of the pre-hypoxia value in beraprost-treated group as compared with 72.4 ± 6.3% in the control group (Figure 2), the difference being statistically significant ($P<0.05$).

Effects of beraprost on pinacidil-induced action potential shortening

Pinacidil markedly shortened APD in guinea-pig right ventricular wall preparations stimulated at 1.0 Hz. After 15 min exposure to 50 μmol l^{-1} pinacidil, APD_{50} and APD_{90} shortened to 52.0 ± 6.7% and 57.3 ± 5.9% from the control values of 154.2 ± 15.7 ms and 177.5 ± 14.7 ms, respectively. Beraprost (0.1 μmol l^{-1}) did not antagonize pinacidil-induced shortening of APD (Table 3).

Table 1 Effects of beraprost on action potential parameters under normoxic conditions

		APA (mV)	$+\dot{V}_{max}$ (V s^{-1})	APD_{50} (ms)	APD_{90} (ms)
Control	pre	111.3 ± 3.0	310.3 ± 10.6	152.5 ± 4.8	179.2 ± 4.4
	post	111.3 ± 3.0	310.7 ± 10.6	152.5 ± 4.8	179.2 ± 4.4
Beraprost					
10^{-8} mol l^{-1}	pre	104.3 ± 2.4	245.0 ± 9.0	154.2 ± 11.5	179.6 ± 13.2
	post	104.5 ± 2.8	238.7 ± 15.2	150.9 ± 12.4	177.5 ± 15.2
10^{-7} mol l^{-1}	pre	110.5 ± 2.4	285.7 ± 19.8	153.7 ± 10.8	178.3 ± 11.5
	post	109.7 ± 2.7	270.3 ± 16.2	145.4 ± 10.0	169.2 ± 10.8
10^{-6} mol l^{-1}	pre	109.8 ± 4.0	260.7 ± 18.1	146.7 ± 12.7	180.0 ± 14.7
	post	109.7 ± 3.0	248.7 ± 16.9	130.8 ± 11.7	162.1 ± 14.2
10^{-5} mol l^{-1}	pre	107.2 ± 3.4	290.0 ± 14.3	156.7 ± 11.5	179.6 ± 11.1
	post	106.7 ± 3.2	276.0 ± 16.7	130.8 ± 13.9	156.3 ± 13.4
10^{-4} mol l^{-1}	pre	104.5 ± 1.4	241.7 ± 19.2	150.4 ± 12.6	179.2 ± 12.4
	post	106.7 ± 2.6	244.5 ± 18.4	139.6 ± 13.8	167.1 ± 13.5

Action potentials were recorded from guinea-pig ventricular free wall preparations under normoxic conditions before and 15 min after the addition of each concentration of beraprost; in control preparations, vehicle (PSS) was given instead of beraprost. APA: action potential amplitude, $+\dot{V}_{max}$: maximum rate of rise of the action potential, APD_{50}:50% action potential duration, APD_{90}:90% action potential duration. Values are means ± s.e.mean of six experiments.

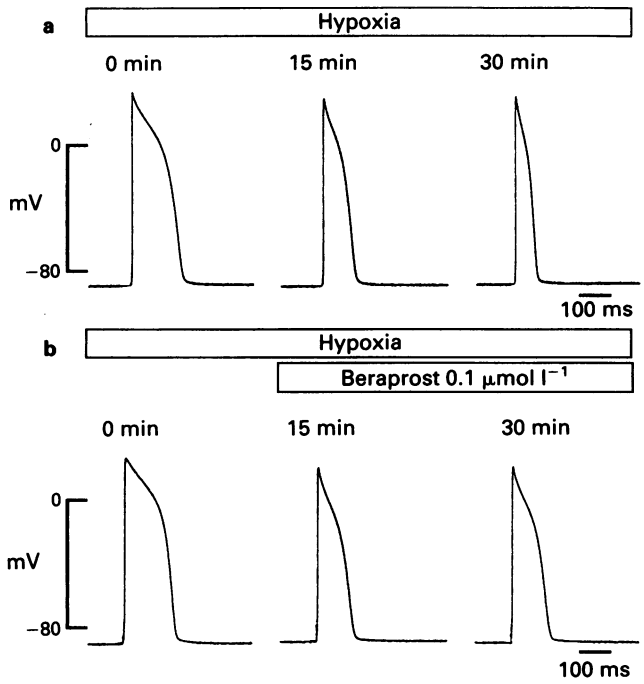


Figure 1 Changes in action potential configuration in guinea-pig isolated ventricular muscles in the absence (a) and presence of (b) beraprost (0.1 μmol l^{-1}) during exposure to hypoxia. Note that beraprost-treated preparations are resistant to the action potential shortening under hypoxic conditions.

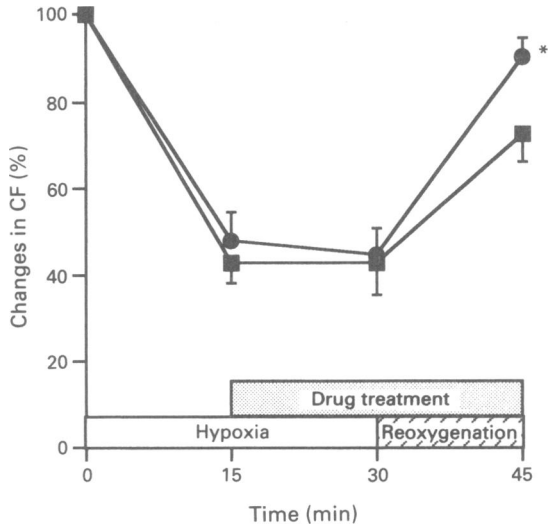


Figure 2 Effect of beraprost on contractile force in guinea-pig isolated right ventricular papillary muscles. Ordinate scale: contractile force (CF) expressed as a percentage of the control contractile force before the state of hypoxia. Value in beraprost (0.1 μmol l^{-1})-treated preparations (●); control preparations (■). Each point represents the mean ± s.e.mean of six experiments. $*P<0.05$ from the value in control group.

Table 2 Effects of beraprost on action potential parameters during hypoxia

		APA (mV)	$+\dot{V}_{max}$ (V s^{-1})	APD_{50} (ms)	APD_{90} (ms)
Control	pre	112.8 ± 4.4	251.3 ± 23.8	153.2 ± 8.9	178.6 ± 9.1
	15′	110.8 ± 3.9	249.7 ± 26.8	98.7 ± 12.3	128.1 ± 9.8
	30′	109.2 ± 4.6	270.0 ± 22.8	79.3 ± 12.1	106.5 ± 12.1
Beraprost					
10^{-9} mol l^{-1}	pre	105.7 ± 2.4	269.0 ± 26.2	150.8 ± 9.4	172.5 ± 9.1
	15′	106.0 ± 3.4	254.3 ± 25.0	93.8 ± 7.5	115.8 ± 9.3
	30′	103.3 ± 2.9	267.3 ± 26.4	81.7 ± 9.5	105.8 ± 10.9
10^{-8} mol l^{-1}	pre	113.3 ± 3.0	263.3 ± 15.8	153.8 ± 12.8	180.3 ± 14.0
	15′	110.2 ± 4.0	263.3 ± 17.8	101.7 ± 4.2	128.8 ± 5.3
	30′	109.3 ± 4.2	267.7 ± 9.5	90.0 ± 7.2	115.5 ± 5.9
10^{-7} mol l^{-1}	pre	110.7 ± 2.3	278.7 ± 10.8	158.7 ± 21.2	181.7 ± 21.7
	15′	106.5 ± 2.0	258.0 ± 12.8	102.3 ± 12.2	127.3 ± 12.7
	30′	108.3 ± 2.0	265.7 ± 14.3	102.5 ± 9.5*	126.7 ± 9.6*
10^{-6} mol l^{-1}	pre	110.8 ± 3.0	286.7 ± 15.7	159.0 ± 12.3	184.8 ± 13.4
	15′	111.2 ± 2.4	288.2 ± 12.5	100.0 ± 5.0	128.0 ± 6.4
	30′	111.3 ± 2.7	274.0 ± 8.2	89.0 ± 3.2	113.0 ± 2.9

Action potentials were recorded from guinea-pig ventricular free wall preparations under hypoxic conditions. APA: action potential amplitude, $+\dot{V}_{max}$: maximum rate of rise of the action potential, APD_{50}:50% action potential duration, APD_{90}:90% action potential duration. Pre: values under normoxic conditions before the exposure to hypoxia, 15′ and 30′: values obtained 15 and 30 min after the start of hypoxia, respectively. Beraprost was given 15 min after the start of hypoxia. Values are means ± s.e.mean of six experiments. $*P<0.05$, statistically significant difference from the values of control action potentials at the same time.

Table 3 Effects of beraprost on pinacidil-induced action potential shortening

		APA (mV)	$+\dot{V}_{max}$ (V s^{-1})	APD_{50} (ms)	APD_{90} (ms)
Pinacidil (5×10^{-5} mol l^{-1})					
alone	pre	110.2 ± 3.0	290.3 ± 18.7	154.2 ± 15.7	177.5 ± 14.7
	post	108.5 ± 4.0	296.7 ± 8.6	82.5 ± 16.3	103.3 ± 16.2
Pinacidil (5×10^{-5} mol l^{-1})					
+ beraprost (10^{-7} mol l^{-1})	pre	119.5 ± 2.1	313.0 ± 19.7	159.4 ± 9.9	181.9 ± 7.7
	post	118.5 ± 2.6	313.0 ± 22.9	76.3 ± 17.7	93.8 ± 17.4

Action potentials were recorded from guinea-pig ventricular free wall preparations under normoxic conditions before and 15 min after the addition of pinacidil (50 μmol l^{-1}) without berapost and with beraprost (0.1 μmol l^{-1}). APA: action potential amplitude, $+\dot{V}_{max}$: maximum rate of rise of the action potential, APD_{50}:50% action potential duration, APD_{90}:90% action potential duration. Values are means ± s.e.mean of four or six experiments.

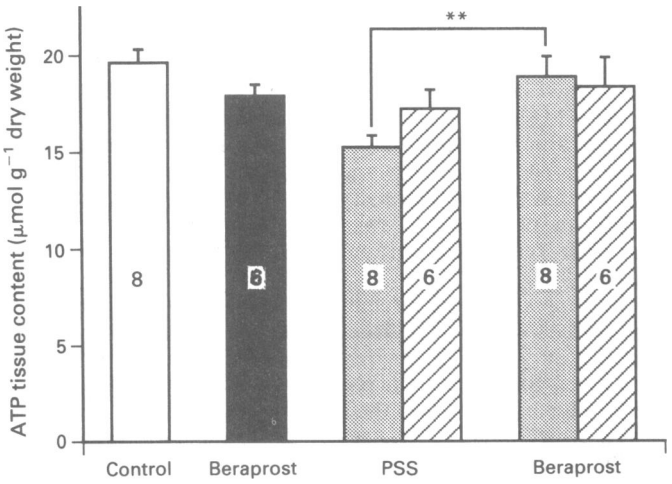


Figure 3 Effect of beraprost (0.1 μmol l^{-1}) on tissue ATP content. PSS indicates physiological salt solution. Columns show: aerobic condition (open column); 15 min normoxia (solid column); 30 min hypoxia (stippled columns); and 30 min hypoxia + 15 min reoxygenation (hatched columns). Each column represents mean ± s.e. mean. Figure in each column shows the number of experiments. $^{**}P<0.01$ (vs corresponding PSS control).

Effects of beraprost on tissue ATP level

Beraprost at the concentration used in this study (0.1 μmol l^{-1}) had no effect on the ATP content of the heart muscle during normoxia; ATP content was 19.6 ± 0.7 and 17.9 ± 0.9 μmol g^{-1} dry wt. before and after beraprost-treatment, respectively. In control hearts, (physiological salt solution (PSS)-treated) after the hypoxic condition has been maintained for 30 min to induce a depletion of endogenous ATP, the myocardial ATP content decreased from 19.6 ± 0.7 to 15.3 ± 0.6 μmol g^{-1} dry wt (Figure 3). However, the ATP level at the end of 30 min of hypoxia was significantly higher in the preparation treated with beraprost (0.1 μmol l^{-1}) than in the PSS-treated preparations (Figure 3). But after reoxygenation, the ATP level in beraprost-treated muscle was comparable to that in PSS-treated muscle.

Discussion

Prostacyclin has been shown to have a cardioprotective effect during acute myocardial ischaemia and reperfusion (Lefer *et al.*, 1978; Ogletree *et al.*, 1979; Araki *et al.*, 1980; Jugdutt *et al.*, 1981; Schror *et al.*, 1981; Melin *et al.*, 1983; Berti *et al.*, 1990), although the exact mechanism for this cardioprotective effect is still unclear. The present study suggests that, like prostacyclin, beraprost may have cardioprotective effects with respect to both action potential and contractile force in guinea-pig ventricular muscles during hypoxia-reoxygenation. Cytoprotective effects of beraprost were also shown in a variety of organs such as the endothelium (Kainoh *et al.*, 1992).

Although the concentrations of beraprost (0.1 to 1 μmol l^{-1}) which induced significant cardioprotective effects seem to be higher than those of prostacyclin released during acute myocardial ischaemia determined in the blood from coronary vein (Coker *et al.*, 1981), the concentration in the ischaemic tissue might be much higher. However, it cannot be denied that the effects of beraprost were only demonstrated at pharmacological concentrations and this does not suggest a physiological role for prostacyclin in the ischaemic heart, which confirms the original findings of Tanonaka *et al.* (1991).

We confirmed that, in the concentration-range of this study, beraprost did not produce any change in normal action potential parameters (Table 1). Although prostacyclin was shown by Alloatti *et al.* (1991) to prolong APD without affecting other action potential parameters, the concentration they used was as high as 5 μmol l^{-1}. A stable prostacyclin analogue, 7-oxo-prostacyclin, was shown only to accelerate the repolarization phase at high concentrations in guinea-pig right ventricular papillary muscles (Kecskemeti, 1987). In addition, it was shown that prostacyclin does not produce any direct inotropic effect on cat electrically driven papillary muscles (Lefer *et al.*, 1978). These reports are in good agreement with our present results showing the lack of effect of a stable prostacyclin analogue, beraprost, on normal heart electrophysiology at the 0.01 to 100 μmol l^{-1} concentration-range.

ATP content in normoxic hearts did not show any change after treatment with beraprost (Figure 3). In the same condition, beraprost had no effect on the creatine phosphate level (data not shown). Thus, at the concentrations used in the present study, beraprost is not considered to affect the energy metabolism of normal heart.

It has long been known that hypoxia and metabolic blockade produce a marked shortening of cardiac action potentials (e.g., Carmeliet, 1978). Two hypotheses have been proposed to explain the APD shortening produced by hypoxia or metabolic inhibitors, i.e., a decrease in a slow inward current (McDonald *et al.*, 1973; Schneider *et al.*, 1974) and an increase in a potassium outward current (Vleugels *et al.*, 1980; Isenberg *et al.*, 1983). Since the discovery of ATP-sensitive K^+ (K^+_{ATP}) channels in cardiac muscle (Noma, 1983), it has been assumed that activation of K^+_{ATP} channels plays a major role in the APD shortening during hypoxia or metabolic inhibition (Noma & Shibasaki, 1985; Fosset *et al.*, 1988; Sanguinetti *et al.*, 1988; Friedrich *et al.*, 1990) and probably during myocardial ischaemia *in vivo*.

In beraprost (0.1 μmol l^{-1})-treated hypoxic preparations, APD_{50} and APD_{90} were maintained significantly longer than those in non-treated hypoxic preparations, and the ATP level was also kept significantly higher. It is not clear at present whether beraprost acted directly on the slow inward current channels. In this connection, Moffat (1987) described the possible involvement of the slow inward current in mediating some of the effects of prostcyclin in guinea-pig isolated hearts during ischaemia. With respect to inotropic effects of prostacyclin, contradictory results have been reported. Lefer *et al.* (1978) showed that prostacyclin does not produce any direct inotropic effect on electrically driven papillary muscles of the cat but Fassina *et al.* (1983) described positive inotropic effects of prostacyclin, on guinea-pig isolated atria.

This was also confirmed by the fact that the shortening of APD induced by 50 μmol l^{-1} pinacidil was not antagonized by beraprost. Pinacidil, 100 μmol l^{-1}, significantly increases the opening probability of K^+_{ATP} channels in the presence of 0.3 μmol l^{-1} ATP, which is shown in whole cell clamp (Arena & Kass, 1989a) and single channel recording studies (Arena & Kass, 1989b). In the present study, 50 μmol l^{-1} pinacidil produced an action potential shortening in guinea-pig muscles, probably resulting from activation of K^+_{ATP} channels. The lack of effect of beraprost on pinacidil-induced shortening of APD suggests that it does not directly inhibit K^+_{ATP} channels. On the other hand, it has been assumed that activation of K^+_{ATP} channels plays a major role in the APD shortening during hypoxia (Noma & Shibasaki, 1985). From these considerations, our present study suggests that beraprost indirectly inhibits K^+_{ATP} channels probably through the maintenance of ATP.

Coenzyme Q_{10}, α_1-adrenoceptor blocking agents and antiarrhythmic agents are reported to improve the recovery of cardiac function after hypoxia (Takeo *et al.*, 1987; 1988; 1989). However, no drug was shown to prevent ATP decrease during hypoxia. Beraprost at the concentration of 0.1 μmol l^{-1} significantly improved the recovery of contractile force after 15 min reoxygenation (Figure 2). In addition, we demonstrated the preservation of ATP content by beraprost during hypoxia (Figure 3), which may contribute to the

improvement of the recovery of cardiac function. Furthermore, even under conditions with severe damage to cardiac contractile function and metabolism, treatment with beraprost was shown to be beneficial for post-hypoxic recovery of cardiac function (Tanonaka *et al.*, 1991), although these workers only investigated the release of ATP metabolites. Therefore, the cardioprotective effects of beraprost during reoxygenation may have resulted from some mechanisms which spare ATP consumption during hypoxia. Maintenance of ATP content may in turn prevent the loss of phospholipid species in the sarcolemma as was suggested with prostacyclin (Ogletree *et al.*, 1979; Schrör *et al.*, 1981). Thus, stabilization of the sarcolemma would contribute to the maintenance of its physiological role as an impermeable barrier to ions such as calcium particularly during ischaemia (e.g., prevention of calcium paradox).

Prostacyclin was shown to diminish the ischaemia-induced release of endogenous catecholamines from adrenergic nerve terminals by several authors (Lefer *et al.*, 1978; Schrör *et al.*, 1981). They postulated that the cardioprotective effects of prostacyclin were due to inhibition of noradrenaline release in the ischaemic areas, thereby reducing the acceleration of metabolism in the ischaemic areas and resulting in diminished damage. Additionally, the increase in myocardial cyclic AMP produced by catecholamines may be related to reperfusion arrhythmias. It was suggested that prostacyclin antagonized these deleterious effects by its proposed membrane-stabilizing activity. Beraprost may have similar effects to prostacyclin, which may contribute to the cytoprotective effects of beraprost during hypoxia and reperfusion.

In summary, beraprost inhibited hypoxia-induced decreases in APD. However, beraprost had no effect on the decrease in contractile force induced by hypoxia, whereas it significantly improved the recovery of contractile force after reoxygenation. Hypoxia significantly decreased the myocardial ATP level, which was also prevented by beraprost. It was also suggested that the inhibition of the hypoxia-induced shortening of APD by beraprost might result from some mechanisms which preserve muscle ATP level.

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(Received January 8, 1993
Revised March 16, 1993
Accepted March 17, 1993)

Br. J. Pharmacol. (1993), **109**, 1020–1023 © Macmillan Press Ltd, 1993

Existence of rolipram-sensitive phosphodiesterase in rat megakaryocyte

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1 The effect of rolipram (ME3176) on ADP- and IP_3-induced repetitive $I_{K(Ca)}$ in rat megakaryocyte was investigated by use of the nystatin perforated patch and conventional whole-cell patch-clamp techniques.

2 The ADP-induced $I_{K(Ca)}$ was depressed by treatment with rolipram in a concentration-dependent manner. The inhibition by rolipram disappeared after treatment with a cyclic nucleotide-dependent protein kinase inhibitor, H-8. The inhibition of $I_{K(Ca)}$ was also observed in the presence of cyclic AMP accumulating agents such as forskolin and isobutylmethylxanthine (IBMX).

3 Rolipram enhanced the inhibitory action of forskolin, suggesting that rolipram facilitates the accumulation of cyclic AMP by blocking its breakdown. Similar results was obtained with adenosine, an endogenous adenylate cyclase activator.

4 Intracellular application of inositol trisphosphate (IP_3) induced repetitive $I_{K(Ca)}$ in megakaryocytes. The induced $I_{K(Ca)}$ was also inhibited by rolipram and by other cyclic AMP accumulating agents.

5 It was concluded that megakaryocytes possess rolipram-sensitive phosphodiesterase (PDE), which was not detected in platelets, but plays a distinct modulatory role in megakaryocytes for generating ADP-induced $I_{K(Ca)}$.

Keywords: Rolipram; phosphodiesterase; cyclic AMP; rat megakaryocyte; nystatin perforated patch recording; calcium

Introduction

Recent studies on the biochemical and pharmacological properties of cyclic nucleotide phosphodiesterase (PDEs) have revealed that there are at least five isoforms (type I–V) of PDEs with different tissue distributions (Nicholson *et al.*, 1991). As PDE inhibitors may be useful as modulators of cellular functions, many drugs have been developed with the aim of therapeutic use (Simpson *et al.*, 1988; Murray *et al.*, 1990; Schudt *et al.*, 1991). The platelet is one of the major targets for these chemicals, since the elevation of cyclic nucleotides in platelets causes remarkable inhibition of agonist-induced responses (Packham & Mustard, 1980). Many lines of evidence suggested that in platelets there are types I, II, III and V PDEs, but not type IV PDE (Nicholson *et al.*, 1991; Simpson *et al.*, 1988). In this connection, a type IV PDE inhibitor, rolipram, failed to inhibit platelet aggregations (Holbrook & Coker, 1991). Although platelets are produced by cleavage of the progenitor cells, megakaryocytes, we have little information about the isoforms or roles of PDE in megakaryoctes.

Owing to the newly developed nystatin perforated whole-cell patch-clamp technique (Horn & Marty, 1988), it has become possible to investigate electrophysiological responses in various kinds of isolated cells with minor disturbances of the intracellular milieu. By the use of this technique, we found that megakaryoctes responded to externally applied purine nucleotides such as ATP and ADP and showed repetitive K^+ current activation, reflecting oscillation of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) (Uneyama *et al.*, 1992; 1993).

In the present study, we have found that the oscillatory response in this preparation was negatively regulated by adenosine 3':5'-cyclic monophosphate (cyclic AMP). We also have investigated the effects of rolipram and other cyclic AMP-accumulating agents on these responses in megakaryocytes and have clarified a regulatory role for rolipram-sensitive PDE in these responses.

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Methods

Preparation

The isolation technique for megakaryocytes was described previously (Uneyama *et al.*, 1993). Adult male and female rats of Wistar strain were decapitated under ether anaesthesia. Femoral bones were isolated from each animal and the bone marrow was washed out with standard external solution. The solution containing bone marrow cells was transferred to the recording chamber (Falcon, primaria tissue culture dish, diameter 35 mm). The chamber was left for a few hours at room temperature until the cells settled on the base of the chamber. With a phase-contrast inverted microscope, megakaryocytes could be clearly distinguished from other bone marrow cells by their large size (40–50 μm in diameter).

Solutions

The ionic composition of the standard external solution was (in mM): NaCl 150, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, N-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) 10 and glucose 10. The pH was adjusted to 7.4 with tris[hydroxymethyl]aminomethane (Tris)-OH. The composition of the perforated patch-pipette solution was (in mM): KCl 150 and HEPES 10. The pH was adjusted to 7.2 with Tris-OH. Conventional patch-pipette solution contained (in mM): KCl 150, ATP-Mg 2 and HEPES 10. The pH was adjusted to 7.2 with Tris-OH. Nystatin was dissolved in acidified methanol (adjusted with 1 N HCl to pH 2.0). Immediately after dissolving nystatin, the pH was adjusted to 7.2 with 1 N KOH (nystatin stock solution; 10 mg ml^{-1}). The stock solution was dissolved in the perforated patch-pipette solution at a final concentration of 50 μg ml^{-1} immediately before use.

Electrical measurements

Electrical measurements were performed with nystatin perforated patch recording (Horn & Marty, 1988). Some experiments were done with the whole-cell mode (Hamill *et al.*, 1981). The pipettes were pulled from 1.5 mm capillary glass

(Narishige) in two stages on a vertical pipette puller (Narishige, PB-7). The pipette tip was fire polished before use. The resistance between the recording electrode filled with internal solution and reference electrode in external solution was 4–6 MΩ. The current and voltage were measured with a patch-clamp amplifier (List Medical, EPC-7), and were monitored on both a storage oscilloscope (Tektronix, R5113) and a pen recorder (San-ei, RECTI-HORIZ-8K), and were stored on video tapes after digitization with a PCM processor (Nihon Kohden, PCM501ESN). All experiments were performed at room temperature (20–22°C).

Drugs

The drugs used in the present study were nystatin, forskolin, isobutylmethylxanthine (IBMX) and adenosine (Sigma), D-*myo*-inositol-triphosphate (sodium salts) (Dojindo) and 4-**RS**-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone (rolipram, ME3167) (Meiji Seika) and N-[2-(methylamino)ethyl]-5-isoquinotinesulphonamide dihydrochloride (H-8) (Seikagaku Corporation). All drugs were dissolved in the pipette or standard external solution just before use. Drugs were applied by a rapid application method termed the 'Y-tube' method, as described elsewhere (Murase *et al.*, 1990). With the use of this technique, the solution surrounding a megakaryocyte could be exchanged within 20 ms.

Statistical analysis

Current traces were subsequently integrated with respect to the baseline for 40 s during evoking K^+ current oscillation. Effects of drugs were shown as a percentage change of the integrated current in the presence or absence of drugs (% of control). Experimental values are presented as mean ± standard error of the mean (s.e.mean). Results were analyzed by Student's *t* test (unpaired or paired comparison), and a *P* value of less than 0.05 was considered to be significant.

Results

In all the following experiments, megakaryocytes were voltage-clamped at a holding potential (V_H) of −40 mV. At first, we confirmed that the drugs used here did not affect the Ca^{2+}-activated K^+ current ($I_{K(Ca)}$) by using A23187-permeabilized cells (Figure 1). A 23187, a Ca^{2+} ionophore, transports Ca^{2+} as a lipid soluble complex through cell membranes and increases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Application of A23187 (3 μM) irreversibly elicited the K^+ current in the presence of external 2 mM Ca^{2+}. The K^+ current activated by A23187 was abolished by the removal of Ca^{2+} in the external solution, suggesting that the K^+ current was $I_{K(Ca)}$, reflecting an increase in $[Ca^{2+}]_i$. By observing the effect of drugs on the K^+ current, we ascertained whether the drugs affected the $I_{K(Ca)}$, or not. Consequently, all drugs used here including rolipram had no observable effects on $I_{K(Ca)}$.

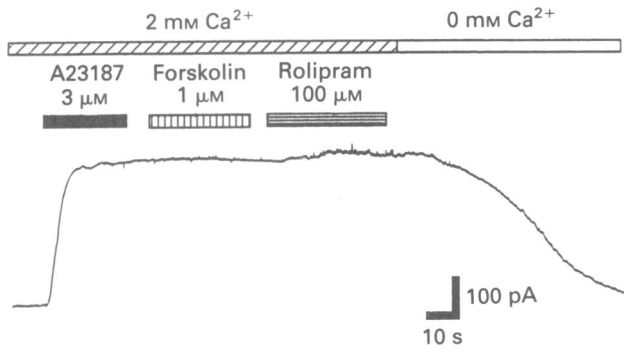


Figure 1 Effects of rolipram and other drugs on A23187-induced $I_{K(Ca)}$ current. Megakaryocyte was voltage-clamped at a V_H of −40 mV. External application of A23187 (3 μM) evoked a sustained $I_{K(Ca)}$ showing dependence on external Ca^{2+}. Rolipram and other drugs had no remarkable effects on the $I_{K(Ca)}$. Each drug was applied for 80–90 s. This result is typical of four reproducible observations.

Effects of rolipram and other cyclic AMP-accumulating agents on ADP-induced Ca^{2+} oscillation

Megakaryocytes show repetitive K^+ currents in response to external application of ADP under voltage-clamp conditions at a holding potential (V_H) of −40 mV. The oscillatory currents were induced only during application of ADP, disappeared immediately after washing out of ADP and were reproducible in the same megakaryocyte. This current is carried by K^+ and abolished completely by intracellular Ca^{2+}-chelating agents such as BAPTA or EGTA (Kawa, 1990; Uneyama *et al.*, 1992). The detailed analysis on the ionic channels of megakaryocytes was investigated previously (Kawa, 1990), and only the Ca^{2+}-activated K^+ current ($I_{K(Ca)}$) could be seen in our experimental conditions (Ikeda *et al.*, 1992; Uneyama *et al.*, 1992). Thus we regarded the current as reflecting the changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). As a next step in investigating the existence of type IV PDE, the effects of rolipram and other cyclic AMP-elevating agents on the ADP-induced repetitive $I_{K(Ca)}$ of the megakaryocyte were examined. External application of rolipram (ME3176) reversibly depressed the ADP-induced repetitive $I_{K(Ca)}$ in a concentration-dependent manner (Figure 2). The IC_{50} value for rolipram was 5×10^{-5} M. Rolipram also lowered the frequency of ADP-induced $I_{K(Ca)}$, and sometimes decreased the amplitude of $I_{K(Ca)}$ (Figures 2a and 3a). In these cases, rolipram had little influence on the first transient peak of $I_{K(Ca)}$ but the drug depressed the subsequent repetitive $I_{K(Ca)}$. Rolipram could not inhibit the first transient $I_{K(Ca)}$ even at a concentration of 1 mM. The nonspecific PDE inhibitor, isobutylmethylxanthine (IBMX) and adenylate cyclase activator, forskolin inhibited the $I_{K(Ca)}$ oscillation evoked by ADP (Figure 3a). The inhibitory action of IBMX was rever-

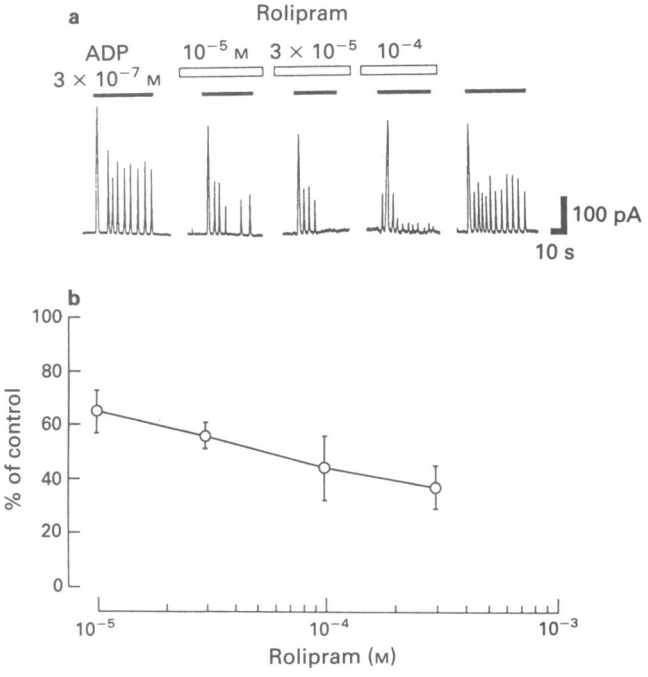


Figure 2 Effect of rolipram on ADP-induced $I_{K(Ca)}$. (a) Current traces from the same cell. V_H was −40 mV. Drugs were continuously applied for the period shown by the bars above each response. Rolipram was pretreated for 1 min. (b) Concentration-inhibition curve for rolipram. Each current trace was subsequently integrated with respect to the baseline to obtain an index of the size of the response. Each point and vertical bar represent mean ± s.e.mean from 5–7 different observations.

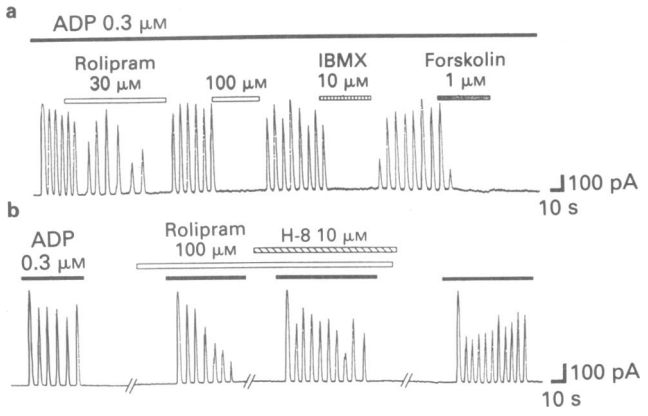


Figure 3 Effects of cyclic AMP-elevating agents on ADP-induced $I_{K(Ca)}$ oscillation. V_H was −40 mV. Drugs were continuously applied for the period shown by the bars above each response. (a) Effects of rolipram (30 and 100 μM), IBMX (10 μM) and forskolin (1 μM). After the $I_{K(Ca)}$ oscillation was induced by a continuous application of ADP, the effect of each drug on the $I_{K(Ca)}$ was examined. The results are typical of three other reproducible observations. (b) Effect of H-8. Pretreatment time for rolipram (100 μM) and H-8 (10 μM) was 1 and 4 min, respectively. Each current trace is typical of five to seven reproducible observations.

sible but that of forskolin was almost irreversible during the time of observation (20–30 min). The inhibitory action of rolipram was reversed by pretreatment with a cyclic nucleotide-dependent protein kinase inhibitor, H-8 (Figure 3b). $I_{K(Ca)}$ was depressed by rolipram (64 ± 7% of control, $n = 6$) and the depression by rolipram was significantly ($P < 0.05$) reversed by pretreatment with 10 μM H-8 (90 ± 16% of control, $n = 5$). The effect of H-8 combined with rolipram was also reversible. These results strongly suggest that rolipram, through the inhibition of PDE, depressed ADP-induced $I_{K(Ca)}$ by potentiating the production of cyclic AMP and by subsequent activation of cyclic nucleotide-dependent protein kinase.

Potentiation by rolipram of the actions of adenylate cyclase activators

$I_{K(Ca)}$ was little influenced by treatment with a low concentration of forskolin (0.1 μM) (95 ± 4% of control, $n = 3$). Application of rolipram at 100 μM, moderately depressed $I_{K(Ca)}$ (65 ± 12% of control, $n = 3$). Co-administration of forskolin and rolipram, however, potently inhibited the ADP-induced $I_{K(Ca)}$ (25 ± 8% of control, $n = 3$). A typical current trace obtained from the same cell is shown in Figure 4a. Similar results were obtained with adenosine when used instead of forskolin (Figure 4b). Adenosine is one of the endogenous anti-aggregatory agonists which improve adenylate cyclase activity in human platelets (Paul *et al.*, 1990). In the case of rat megakaryocytes, application of 0.1 μM adenosine remarkably depressed ADP-induced $I_{K(Ca)}$ (Figure 4b(i)). The adenosine response was reversed either by pretreatment with H-8 (10 μM) or with 7-chloro-theophylline (100 μM) (data not shown, $n = 3$), which is a non-specific adenosine receptor antagonist. The inhibition of the $I_{K(Ca)}$ by adenosine might be due to enhancement in the production of cyclic AMP *via* activation of receptor/adenylate cyclase coupling. Pretreatment with rolipram combined with adenosine potently augmented the inhibitory effect of adenosine (Figure 4b(i)). Figure 4b(ii) summarizes the results quantitatively; the inhibition by 0.1 μM adenosine or 100 μM rolipram alone was incomplete, but co-administration of adenosine and rolipram at the same concentrations abolished $I_{K(Ca)}$ oscillation completely.

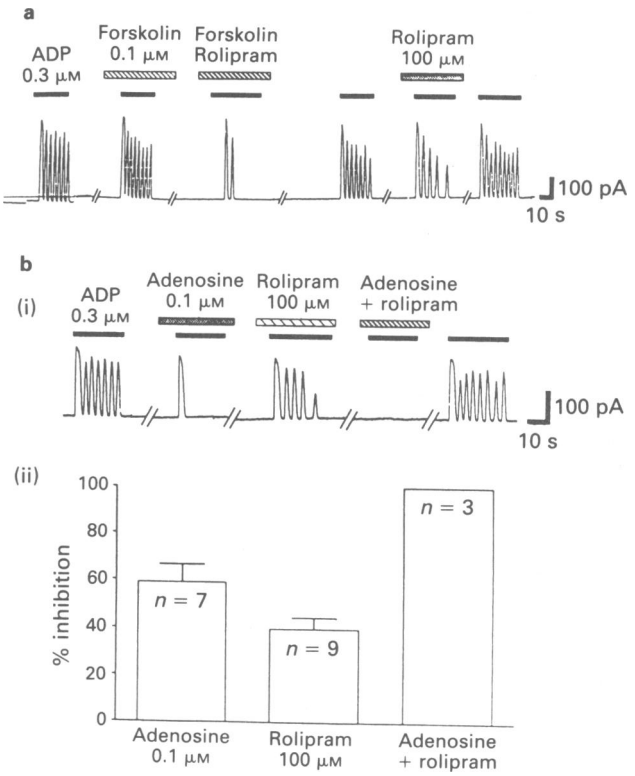


Figure 4 Rolipram augments the effect of adenylate cyclase activators. V_H was −40 mV. Drugs were applied for the period shown by the bars above each response. (a) The inhibitory effect of forskolin is potentiated by simultaneous application of rolipram. Each current trace was obtained from the same cell and is typical of five reproducible experiments. (b) Rolipram potentiated the inhibitory action of adenosine. Typical current traces are shown in (i). The summary of results is shown in (ii). The number of cells tested is indicated in each column.

Effect of rolipram on inositol trisphosphate (IP_3)-induced Ca^{2+} oscillation

To investigate the effects of rolipram and other cyclic AMP-accumulating agents on $I_{K(Ca)}$ currents without the involvement of ADP-receptors, we have applied IP_3 directly to the cell to increase the internal Ca^{2+} concentration to evoke $I_{K(Ca)}$ (Uneyama *et al.*, 1993). We used a pipette solution containing 10 μM IP_3 in the conventional whole-cell mode to introduce IP_3 into the cell. Intracellular application of IP_3 immediately evoked repetitive $I_{K(Ca)}$, but the amplitude of $I_{K(Ca)}$ was depressed in the presence of 100 μM rolipram as well as after an application of 1 μM forskolin (Figure 5). IBMX (10 μM) also inhibited the IP_3-induced $I_{K(Ca)}$ (data not shown, $n = 3$).

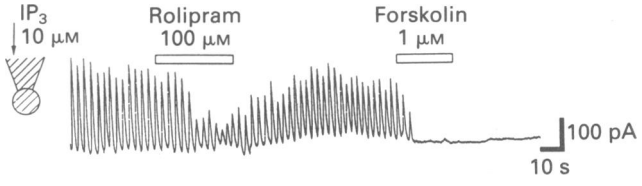


Figure 5 Effect of rolipram on inositol trisphosphate (IP_3)-induced $I_{K(Ca)}$ oscillation. Records were obtained by conventional whole-cell mode. The recording pipette was filled with internal solution containing 10 μM IP_3. Arrow indicates the start of internal perfusion with IP_3 containing pipette solution. Drugs were applied extracellularly during a period indicated by horizontal bars. The current trace is typical of five reproducible observations.

Discussion

We found that the type IV PDE specific inhibitor, rolipram, inhibited ADP-induced $I_{K(Ca)}$ oscillation in rat megakaryocytes (Figure 2). The inhibitory action of rolipram was synergistic and/or additive with those of an adenylate cyclase activator (Figure 4). H-8, an inhibitor of cyclic nucleotide-dependent protein kinase, reversed the effect of rolipram. Rolipram was also effective for inhibition of the IP_3-induced response (Figure 5). All these properties were similarly observed with the non-specific PDE inhibitor, IBMX, and with an adenylate cyclase activator, forskolin. Thus, these results can be explained by an increase in the intracellular cyclic AMP concentration. As shown in Figures 1 and 5, involvement of other nonspecific actions of rolipram seems to be minor with the present conditions. Based on these, it is most likely that the megakaryocyte possesses rolipram-sensitive (type IV) PDE and that the inhibitory action of rolipram on the ADP-induced $I_{K(Ca)}$ derives from its PDE-inhibiting activity in the cell.

Megakaryocytes, as progenitor cells of platelets, have been considered to share many functional properties with platelets such as phagocytic activity (Fedorko, 1977b), uptake of 5-hydroxytryptamine (Fedorko, 1977a), responsiveness to bioactive substances (Leven & Nachmias, 1982; Miller, 1983), and proteoglycan composition (Schik *et al.*, 1988). Megakaryocytes might also have the same types of PDEs as platelets. But unexpectedly, PDEs in platelets were reported as type I, (II), III and V (Simpson *et al.*, 1988), and type IV inhibitors such as rolipram were shown to be ineffective in platelets (Holbrook & Coker, 1991). Type IV PDE, which is cyclic AMP-specific and cyclic GMP-insensitive, are widely distributed in kidney, lung, trachea and leukocytes, but not in platelets (Nicholson *et al.*, 1991). It might be conceivable that platelets are produced from a specific part of the megakaryocyte excluding type IV PDE. This may explain why the platelet does not have type IV PDE. It is interesting to note that other bone marrow-derived cells such as neutrophils and eosinophils have predominantly type IV PDE (Wright *et al.*, 1990; Dent *et al.*, 1991; Meskini *et al.*, 1992). Although this speculation needs further clarification, it may provide a novel view for considering megakaryocyte function and platelet differentiation, with special relevance to rolipram-sensitive phosphodiesterase in these processes.

This study was supported by Grants-in-Aid for Scientific Research (Nos. 040444029 and 04404023) to N.A. from The Ministry of Education, Science and Culture, Japan.

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(Received February 1, 1993
Revised March 15, 1993
Accepted March 17, 1993)

Br. J. Pharmacol. (1993), **109**, 1024–1028 © Macmillan Press Ltd, 1993

Metabolism of endothelin-1 and big endothelin-1 by recombinant neutral endopeptidase EC.3.4.24.11

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1 Inhibitors of neutral endopeptidase EC.3.4.24.11 (NEP) have been shown to attenuate the hypertensive effect of big-endothelin-1 (BET-1) in rats. To determine whether NEP converts BET-1 to endothelin-1 (ET-1), the effect of a recombinant NEP (rNEP) on BET-1 and on ET-1 was assessed *in vitro*.

2 Incubation of [^{125}I]-ET-1 with 1 $\mu g\,ml^{-1}$ of rNEP resulted in degradation of the peptide within minutes. Increase in the amount of rNEP to 10 $\mu g\,ml^{-1}$ led to total cleavage of [^{125}I]-ET-1 within seconds.

3 Phosphoramidon (10 μM) or SQ-28,603 (100 μM) totally suppressed the degradation of [^{125}I]-ET-1 by rNEP.

4 The degradation of [^{125}I]-BET-1 by either 1 or 10 $\mu g\,ml^{-1}$ of rNEP was much slower than that of [^{125}I]-ET-1. Again, both phosphoramidon and SQ 28,603 protected the peptide from degradation.

5 Intact [^{125}I]-ET-1 was not observed when [^{125}I]-BET-1 was incubated with rNEP.

6 These data show that neutral endopeptidase EC.3.4.24.11 is *not* an endothelin converting enzyme.

Keywords: Endothelin-1; big-endothelin-1[1-38]; neutral endopeptidase inhibitors; recombinant neutral endopeptidase; SQ-28,603; phosphoramidon; h.p.l.c.

Introduction

Endothelin-1, a 21 amino-acid peptide with potent vasoconstrictor properties, is derived from a 39 amino acids (38 amino acids in man) precursor named big-endothelin-1 (BET-1). This cleavage is performed by an unidentified protease called 'endothelin-converting enzyme' (ECE) (Yanagisawa *et al.*, 1988), the nature of which has not been established. Several studies have detected ECE activity in endothelial cells (Matsumura *et al.*, 1990a; Okada *et al.*, 1990), renal epithelial cells (Takada *et al.*, 1992), pulmonary membrane preparations (Kundu & Wilson, 1992) and human leucocytes (Kaw *et al.*, 1992). ECE activity has been attributed to a wide range of enzymes, including: aspartic protease (Ikegawa *et al.*, 1990a; Wu-Wong *et al.*, 1990), metalloendopeptidase (Matsumura *et al.*, 1990b; Okada *et al.*, 1990; McMahon *et al.*, 1991; Pollock & Opgenorth, 1991), cathepsin-D (Sawamura *et al.*, 1990a; Takaoka *et al.*, 1990), elastase (Kaw *et al.*, 1992) and chymase (Wypij *et al.*, 1992). These enzymes are not likely to be the major physiologically relevant ECE, since many of them are known to be non-specific. In addition, there is strong evidence against their involvement in the conversion process (Sawamura *et al.*, 1990b; McMahon *et al.*, 1991; Wypij *et al.*, 1992; Yamaguchi *et al.*, 1992).

In vitro studies have shown that the secretion of ET-1 by cultured endothelial cells is inhibited by the metalloendopeptidase inhibitor phosphoramidon, while the secretion of BET-1 was increased (Ikegawa *et al.*, 1990b; Matsumura *et al.*, 1991). Moreover, phosphoramidon attenuated the hypertensive effect of BET-1, but not of ET-1, in rats (McMahon *et al.*, 1991; Pollock & Opgenorth, 1991). These results suggest that a phosphoramidon-sensitive metalloprotease is responsible for the *in vivo* conversion of BET to ET. Metalloproteases are a large family of enzymes, and from the current data it is impossible to designate the specific enzyme responsible for this reaction. A prominent metalloprotease which is known to be very sensitive to phosphoramidon is neutral endopeptidase EC.3.4.24.11 (NEP) (Turner, 1987). This is a ubiquitous enzyme, expressed by many tissues, including endothelial cells (Llorens-Cortes *et al.*, 1992). Recently, Gardiner and colleagues (1992) reported that the selective neutral endopeptidase inhibitor, SQ-28,603 attentuated the pressor effects of BET-1, but not of ET-1. In addition, McMahon and colleagues (1991) reported that thiorphan, another NEP inhibitor, decreased the hypertensive effects of BETE-1. The authors postulated that ECE is a form of neutral endopeptidase. The present study was designed to examine whether neutral endopeptidase EC.3.4.24.11 converts BET-1 to ET-1, using recombinant neutral endopeptidase (rNEP).

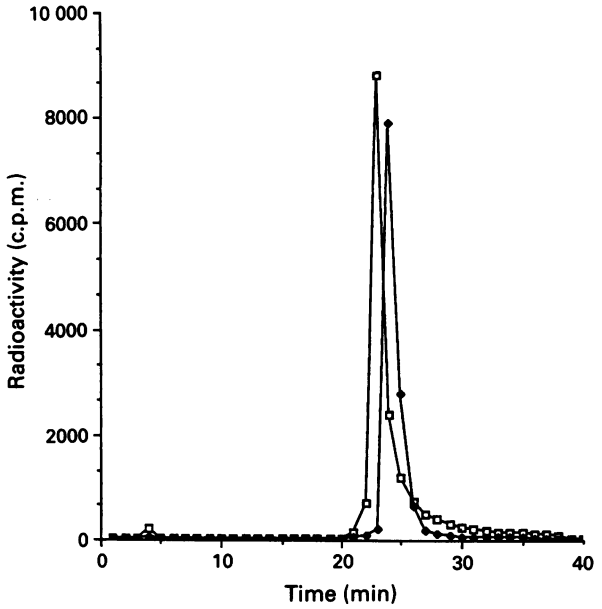


Figure 1 Reversed-phase h.p.l.c. analysis of synthetic ^{125}I-labelled endothelin-1 (■) and ^{125}I-labelled big-endothelin-1 (□).

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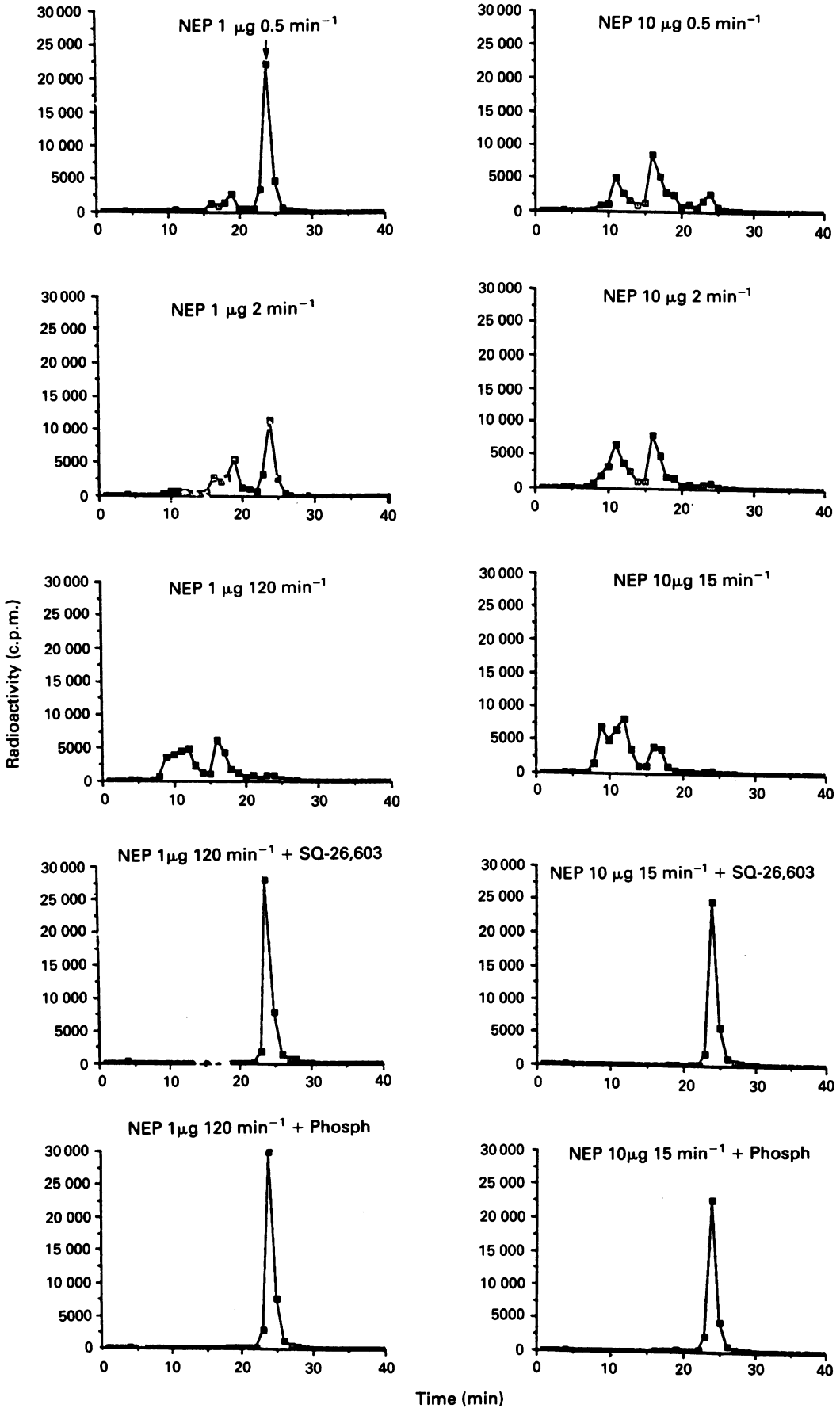


Figure 2 Reversed-phase h.p.l.c. radioactive flow monitoring analysis of the fragmentation of ^{125}I-labelled endothelin by human recombinant neutral endopeptidase. The figure depicts typical radio-chromatograms of reaction mixtures of 0.33 μCi of [^{125}I]-endothelin-1 (2200 Ci mmol^{-1}) with either 1 μg or 10 μg of recombinant neutral endopeptidase incubated for different time periods (30 s–120 min), with or without phosphoramidon (10 μM) or SQ-28,603 (100 μM). The arrow indicates the elution time of intact ^{125}I-labelled endothelin-1 (24 min). The experiment was performed 3 times, with reproducible results.

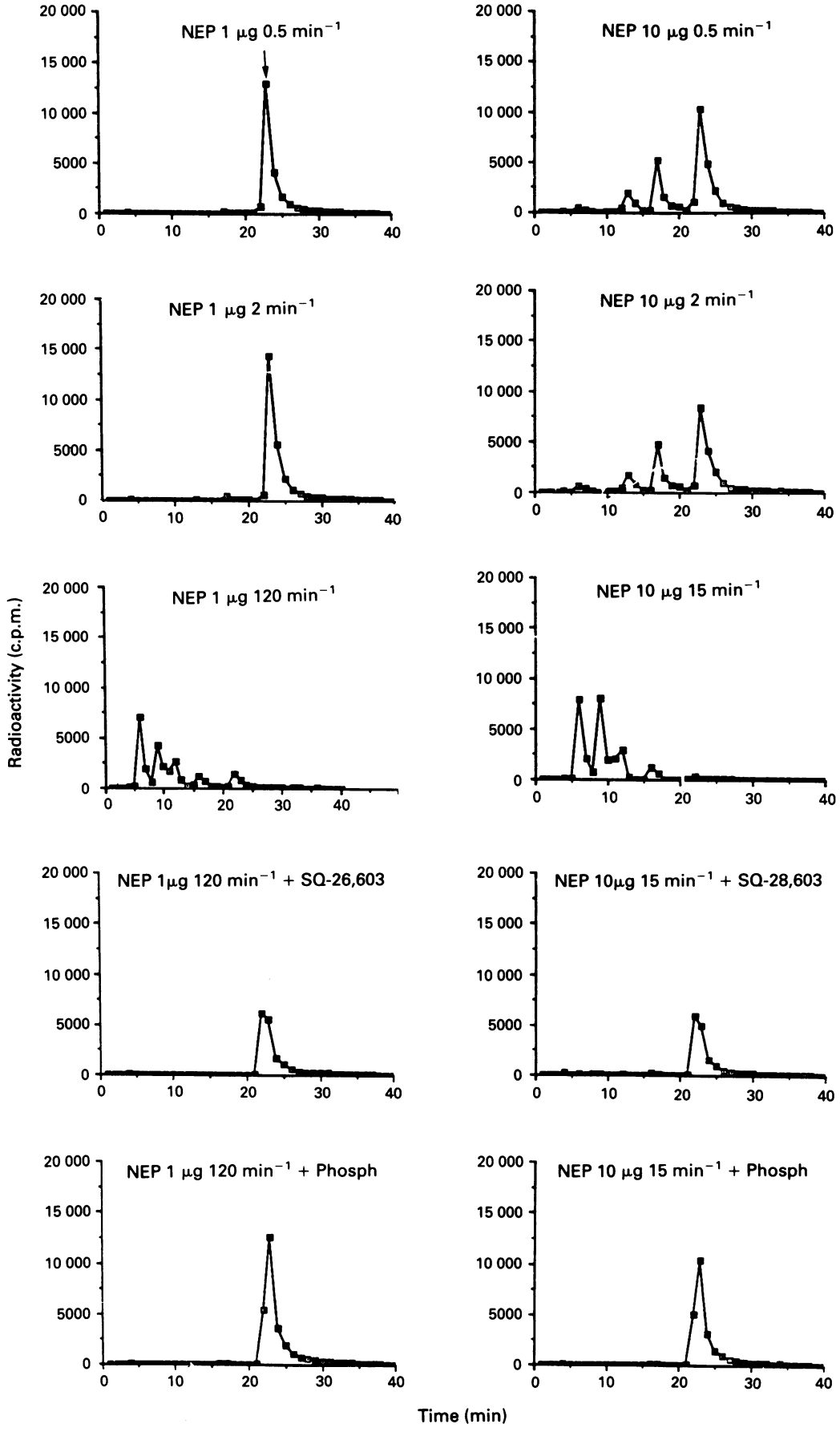


Figure 3 Reversed-phase h.p.l.c. radioactive flow monitoring analysis of the fragmentation of ^{125}I-labelled big-endothelin by human recombinant neutral endopeptidase. The figure depicts typical radio-chromatograms of reaction mixtures of 0.33 μCi of [^{125}I]-big-endothelin (2200 Ci mmol^{-1}) with either 1 μg or 10 μg of recombinant neutral endopeptidase incubated for different time periods (30 s–120 min), with or without phosphoramidon (10 μM) or SQ-28,603 (100 μM). The arrow indicates the elution time of intact ^{125}I-labelled big-endothelin-1 (23 min). The experiment was performed 3 times, with reproducible results.

Methods

The full length cDNA coding for human neutral endopeptidase EC.3.4.24.11 was cloned originally from a human placental cDNA library (Malfroy *et al.*, 1988). Transfection of mammalian cells with an expression plasmid containing this cDNA resulted in the production of the enzymatically active protein (Gorman *et al.*, 1989), which was purified by a modification of a standard procedure and quantified in terms of protein catalytic activity. The active enzyme is a glycoprotein, of approximately 90 kilodaltons.

Either ^{125}I-labelled BET-1(1-38, labelled at Tyr_{13} and Tyr_{31}) or ^{125}I-labelled ET-1 (labelled at Tyr_{13}, 0.33 μCi, 2200 Ci mmol^{-1}, NEN, Boston, MA, U.S.A.), was incubated with 1 and 10 μg of the rNEP at 37°C, in 1 ml solution of 50 mM HEPES, pH = 7.4, 50 mM NaCl, for different times (30 s–120 min). It was previously demonstrated (Hecker *et al.*, 1992), that both labelled and unlabelled BET-1 are converted to ET, although their catabolism is somewhat different. Either phosphoramidon (Peptides International, Louisville, KY, U.S.A.) or SQ-28,603 (N-[2-(mercaptomethyl)-1-oxo-3-phenylpropyl]-< b-alanine; Bristol-Myers Squibb Pharmaceuticals, Princeton, NJ, U.S.A.) was added to the solution prior to the addition of the enzyme, at concentrations of 10 and 100 μM, respectively. Each reaction was terminated by the addition of acetic acid to a final pH of 4.0, and heating at 60°C for 3 min. Fifty microliters of each reaction mixture were analysed by reverse phase high performance liquid chromatography (h.p.l.c.) (C_{18} μ bondapak, 3.9 × 300 mm column, Waters, Milford, MA, U.S.A.), using a linear gradient of 10–60% acetonitrile in 0.1% trifluoroacetic acid (TFA) over 40 min, at a flow rate of 1 ml min^{-1}. The column was calibrated with ^{125}I-labelled BET-1 and ^{125}I-labelled ET-1. Radioactivity eluting from the column was continuously monitored with a gamma counter (Beckman Instruments, Irvine, CA, U.S.A.).

Results

Radioactively labelled BET-1 and ET-1 eluted with retention times of 23 and 24 min, respectively. They were separable by the h.p.l.c. column described, with 30–35% overlap in the area under the peaks (Figure 1).

Incubation of ET-1 with 1 μg ml^{-1} rNEP resulted in degradation of the peptide within minutes, whereas its incubation with 10 μg ml^{-1} of the enzyme resulted in its total cleavage within seconds. Both phosphoramidon (10 μM) and SQ-28,603 (100 μM) fully protected ET-1 from degradation by NEP (Figure 2).

The degradation of BET-1 by rNEP was much slower than that of ET-1. Even after incubation with 10 μg ml^{-1} of the enzyme for 2 min, most of the radioactive counts in the sample eluted as intact peptide. Phosphoramidon (10 μM) and SQ-28,603 (100 μM) fully protected BET-1 from degradation by rNEP, identical to the effect on ET-1. At no time point during the incubation of BET-1 with rNEP, was ET-1 found in the reaction mixture (Figure 3).

Discussion

The present study demonstrates that recombinant neutral endopeptidase EC.3.4.24.11 does not convert [^{125}I]-BET-1 to [^{125}I]-ET-1, thus ECE and NEP are different enzymes. These findings agree with a recent *in vivo* study (Pollock & Opgenorth, 1991), in which it was demonstrated that the nonspecific metalloprotease inhibitor, phosphoramidon, blocked renal and haemodynamic effects of BET, while the more specific NEP inhibitor, thiorphan, did not. Pons and colleagues (1992) reported that phosphoramidon totally blocked the bronchoconstrictor and pressor effects of BET, while thiorphan inhibited these responses only partially. In addition, an *in vitro* study (Matsumura *et al.*, 1991) showed that phosphoramidon, but not thiorphan, inhibited the conversion of BET-1 to ET-1 by a membrane fraction of aortic endothelial cells. Furthermore, we showed that rNEP degrades both ET-1 and BET-1, confirming previous findings obtained, both in membrane preparations (Sokolovsky *et al.*, 1990; Vijayaraghavan *et al.*, 1990; Dickinson *et al.*, 1991; Fagny *et al.*, 1991), and *in vivo* (Abassi *et al.*, 1992b). Since ECE is the key enzyme in the formation of active ET, it would be most unusual for the same enzyme to be responsible for both its formation and degradation.

The following reservations should be pointed out concerning the interpretation of these results: (a) The labelling of BET by ^{125}I at position Tyr_{31} might cause a steric interference with ECE activity, since it was found (Okada *et al.*, 1991) that the adjacent residues 32-37 of BET are important for ECE activity. (b) The elution times of BET and of ET are rather close, and some overlap between the peaks is observed, so that production of small amounts of ET could have been missed. (c) Since the metabolism of ET-1 is much more rapid than that of BET-1, small amounts of ET could possibly be formed and rapidly degraded, without being detected by our method. Thus, we cannot exclude the possibility that rNEP possesses some degree of ECE activity. However, since we were not able to detect ET in the reaction mixture at any time after the addition of rNEP, we conclude that ECE activity is not a major function of rNEP 24.11.

Previous studies on the effects of NEP were performed with enzymes extracted from renal and pulmonary membrane preparations, tissues known to be rich in the enzyme (Olins *et al.*, 1987; Sokolovsky *et al.*, 1990; Vijayaraghavan *et al.*, 1990; Yamaguchi *et al.*, 1992). However, these preparations probably contain more than one enzyme. The use of a recombinant enzyme in this study enabled us to determine specifically its effects on BET and ET. rNEP exhibited a similar activity to that in membrane preparations.

This study shows that rNEP is very sensitive to inhibition by both phosphoramidon and SQ-28,603. The latter is a novel drug, designed to block specifically the activity of NEP EC.3.4.24.11. It was found to be an effective hypotensive and natriuretic agent in the treatment of hypertension and heart failure (Sybertz, 1990; Seymour *et al.*, 1991; Abassi *et al.*, 1992a). The mechanism underlying these effects of NEP inhibitors is still not fully explained. It has been attributed to the protective effect of the drug on atrial natriuretic factor (ANF) (Sybertz, 1990; Seymour *et al.*, 1991). However, the response to ANF in these conditions is attentuated, and even pharmacological doses of exogenous ANF fail to exert similar effects to those of the NEP inhibitors (Abassi *et al.*, 1991; Abouchacra *et al.*, 1992). Therefore, it is likely that additional mechanisms are involved in the beneficial effects of the inhibitor. The observation by Gardiner and colleagues (1992), that SQ-28,603 not only inhibits NEP, but also suppresses the activity of ECE, taken together with our demonstration that NEP is not ECE, raises the possibility that some of the therapeutic effects of SQ-28,603 are due to inhibition of ECE, rather than inhibition of NEP.

We thank Bristol-Myers Squibb for the gift of SQ-28,603.

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(Received January 4, 1993
Revised March 8, 1993
Accepted March 17, 1993)

Br. J. Pharmacol. (1993), **109**, 1029–1037 © Macmillan Press Ltd, 1993

Mechanism of carbachol-evoked contractions of guinea-pig ileal smooth muscle close to freezing point

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1 The effect of lowering the temperature to near freezing-point upon the contractions and [^{3}H]-inositol phosphate responses to carbachol were investigated in longitudinal smooth muscle from the guinea-pig ileum.

2 The peak amplitude of the contraction to a single application of 100 μM carbachol was the same at 37°C and temperatures near freezing-point. However, the sensitivity to carbachol was reduced upon lowering the temperature and the time to peak contraction was increased from 5–10 s to 2–10 min. Even when the temperature was maintained near freezing-point, washing off carbachol produced a relaxation and eventual return of tension to basal levels.

3 Incubating the tissue in 140 mM K^+, calcium-free solution or in calcium channel antagonists significantly reduced the carbachol-induced contraction to 10–30% of the control at 37°C and also at 3°C. Thus the majority of the activator calcium required for contraction entered the tissue via voltage-dependent calcium channels (VDCs) at both 37°C and 3°C.

4 The contractions produced by high potassium solutions were less at temperatures close to freezing-point than those at 37°C suggesting that voltage-dependent calcium entry was inhibited as the temperature was lowered.

5 A small part of the contractile response to 100 μM carbachol was resistant to the removal of extracellular calcium at both 37°C and 3°C and this component was increased under depolarizing conditions. This suggests that the release of stored calcium contributes to a minor degree to contraction at both 37°C and 3°C.

6 Although 100 μM carbachol produced a statistically significant rise in several [^{3}H]-inositol phosphate isomers at both 37°C and 3°C, the production of [^{3}H]-inositol phosphates was less at 3°C than at 37°C and the increase in their production caused by carbachol was much slower.

7 These results suggest that the carbachol-induced contraction at 3°C utilizes both calcium entry through VDCs and calcium release from intracellular stores, as at 37°C. The components of the responses dependent upon intracellular calcium release at 37°C and at temperatures near freezing-point were similar. However, the production of [^{3}H]-inositol phosphates, including the calcium-mobilizing second messenger inositol (1,4,5) trisphosphate (Ins(1,4,5)P_3), is reduced at such low temperatures.

Keywords: Muscle, smooth; muscle contraction; inositol phosphates; temperature

Introduction

The mammalian intestine normally functions throughout life at temperatures very close to body temperature except under very exceptional circumstances. It was therefore surprising to find that large contractions in response to activation of muscarinic receptors could be evoked at temperatures (<1°C) just above freezing in longitudinal smooth muscle strips from the guinea-pig ileum (Blackwood & Bolton, 1991). We were curious about the mechanisms involved in these contractions. The ionic gradients across the smooth muscle cell membrane decline severely at 4°C: for example the potassium content of the muscle falls to about one quarter (Freeman-Narrod & Goodford, 1962). However, since the relationship between the potassium content and equilibrium potential is roughly exponential, a low membrane potential might be maintained even after several hours at temperatures close to freezing as indeed was found by Tomita (shown in Buck & Goodford, 1966): in his experiments a membrane potential of about −25 mV was found in taenia of the guinea-pig caecum.

It is known that a rapid contraction can be obtained upon suddenly chilling smooth muscle, an event suggested to be caused by a discharge of stored calcium within the cell into the cytoplasm (Sakai & Kurihara, 1974). If so, contractions to receptor activation which depend on the release of stored calcium ought subsequently to be reduced or lost. However, moderate cooling also triggers the discharge of action potentials in taenia of the guinea-pig caecum (Magaribuchi *et al.*, 1973) and such discharge is an additional cause of cold contraction.

In guinea-pig taenia caeci, contraction at 35°C to carbachol acting via muscarinic receptors is bought about by calcium entry into the muscle via increased action potential discharge and, at higher concentrations where depolarization block of this discharge occurs, by the entry of calcium via voltage-dependent calcium channels (VDCs). Entry of calcium through voltage-dependent rather than via receptor-operated channels (Bolton, 1979) seems crucial for the maintenance of carbachol-induced tension (Brading & Sneddon, 1980). There is, however, a transient tension response to high concentrations (100 μM) of carbachol which is resistant to calcium entry blockers; this may result from the direct action of calcium discharged from internal stores on the contractile proteins (Brading & Sneddon, 1980).

The present experiments were carried out in order to discover whether the sources of calcium for contraction close to 0°C were the same as those available at 37°C.

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Methods

Preparation of muscle strips and isometric tension recording

Muscle strips approximately 5 mm wide and 2 cm long containing Auerbach's plexus were prepared from the distal portion of the ileum longitudinal smooth muscle of 300–350 g female guinea-pigs by the method of Paton & Zar (1968). Strips were subjected to 0.5 g tension and suspended by thread in a small, insulated vertical tube perfused at a flow rate of 1.1 ml min^{-1} with bicarbonate-buffered physiological salts solution (PSS). They were allowed to equilibrate at 37°C for at least 30 min, during which time the tension declined to between 0.2 to 0.4 g. Subsequently the basal tension levels at 37°C declined by less than 10%. Isometric tension increases were recorded with a force transducer (Gould Statham) coupled with a bridge amplifier (SC1105, Gould Statham), the output being displayed on an ink-writing chart recorder (BD41, Kipp & Zonen).

Temperature control

Solutions were equilibrated at 37°C with 95% O_2:5% CO_2 and pumped over the tissue. Tubing carrying solution to the tissue was wound in several turns inside a glass jacket filled with an anti-freeze solution and connected to a thermocirculator (Churchill). The temperature produced in the organ bath was measured with a thermistor (Bailey Instruments Inc.) which had a sensitivity of 0.1°C. Tissue temperature was lowered from 37°C to 1–3°C over 30–40 min and was then maintained to ± 0.5°C during the course of an experiment. The pH of the PSS did not change significantly at low temperatures.

Solutions

The composition of PSS used was (mM): NaCl 120, KCl 5.9, $NaHCO_3$ 15.4, NaH_2PO_4 1.2, $CaCl_2$ 2.5, $MgCl_2$ 1.2, glucose 11.5. pH was 7.15.

K^+ solutions of 35, 70 and 140 mM were prepared by substituting K^+ for Na^+. Ca^{2+}-free solution was prepared by omitting $CaCl_2$ and adding 1 mM EGTA.

Concentration-effect

Increasing concentrations of carbachol were applied by changing the perfusion solution pumped over the tissue. Each concentration was applied for 1 min after recovery of spontaneous activity at 37°C. At 3°C concentrations were applied only when tension had returned to its previous basal level.

Studies of the effect of pharmacological agents

Tension increases were measured in g and expressed as a % of the control response in the same strip at 37°C; 0% tension was taken as the pre-existing basal tension. At 37°C when there was spontaneous contractile activity the basal level was taken as the peak level of these spontaneous contractions.

Contractile responses to a 1 min application of 100 μM carbachol were recorded under various conditions at 37°C and 3°C and compared with the response in normal PSS in the same tissue. Longer applications of the agonist did not affect the peak amplitude of contraction, merely the duration of the response.

Because the contractile response to a second application of 100 μM carbachol was significantly reduced at 3°C, after each application of carbachol at this temperature the tissue was re-warmed over 40 min to 37°C, where it was equilibrated for 15 min, before re-cooling. The second response was then tested in the appropirate test solution. In normal PSS the second response was 114 ± 6.5% at 37°C and 95.4 ± 18.3% at 3°C of the original responses.

Strips were perfused with a calcium-free solution containing 1 mM EGTA at 37°C or 3°C until the response to 140 mM K^+ was abolished. Tissues were then returned to a calcium-containing medium at 37°C until spontaneous contractions resumed. Responses to carbachol in the same tissue were then measured after perfusion with calcium-free medium for the same period of time. Within strips the time taken in calcium-free solution to lose the response to high potassium was very consistent (in one strip $t_{1/2} = 2 \pm 0.3$ min) and always shorter than the time taken to lose the response to carbachol (in the same strip $t_{1/2} = 4 \pm 0.3$ min). However, variability between strips in the time taken to lose the response to high potassium was much greater ($t_{1/2}$ varied between 1 and 6 min). In 4/10 preparations the response to 140 mM K^+ could not be completely abolished even after incubation in calcium-free medium for 12 min; in these cases the effects of removing calcium on the carbachol contractions were not examined.

The calcium channel antagonists and receptor antagonists were added at 37°C and perfused for at least 15 min before examining their effect upon the carbachol response or before lowering the temperature to 3°C.

K^+ solution (140 mM) was added at 37°C. Responses to carbachol were then measured once the tension had reached a constant level at both 37°C and 3°C. This usually took 15–20 min.

Measurement of radio-labelled inositol phosphates

Eight strips of the muscle prepared as described previously were cut into approximately 1 mm × 1 mm fragments with forceps and incubated in freshly oxygenated PSS at 37°C for 20 min. Excess solution was pipetted off and the tissue fragments incubated with 1 ml of PSS containing 50 μCi [^{3}H]-inositol for 3 h at 37°C with occasional agitation under an atmosphere of 95% O_2:5% CO_2. After this period the fragments were washed with 3 × 10 ml PSS previously equilibrated at the required temperature, as determined with a thermistor, and resuspended in 1 ml of the same. The fragments were divided into two 0.5 ml portions (approximately 4–6 mg protein each) and allowed to equilibrate at the appropriate temperature for 15 min, after which 0.5 ml of 200 μM carbachol-containing or control PSS was added. Temperatures of 3°C were maintained throughout this period, when required, by incubation in an insulated ice bucket. Reactions were terminated with 1 ml ice-cold 20% trichloroacetic acid. The supernatant was removed and the acid extracted with 4 × 10 ml water-saturated diethyl ether. The lower phase was retained, the residual ether being removed by evaporation under N_2 and was brought to pH 7.0 with NaOH for subsequent high performance liquid chromatography (h.p.l.c.) analysis.

H.p.l.c. was performed on a Partisil SAX 10 column (Whatman) and inositol phosphates were eluted with water and increasing concentrations of ammonium formate (pH 3.7 with phosphoric acid) essentially as described by Salmon & Bolton (1988). Fractions (0.5 ml) were collected, mixed with 4 ml of HiSafe 3 scintillant (LKB) and counted on a liquid scintillation counter (LS6800, Beckman). ^{32}P-labelled turkey and human erythrocyte inositol phosphate standards, prepared as described by Downes *et al.* (1982), were run previously on this system, to aid with the identification of the [^{3}H]-inositol-labelled compounds by comparing their elution times. A commercial [^{14}C]-inositol(1)monophosphate (Ins(1)P) standard was also employed for this reason. The mass of protein in the acid insoluble material was determined by the method of Lowry *et al.* (1951). Radiolabelled inositol phosphate measurements were expressed as the number of d.p.m. per mg protein in a particular peak of radioactivity eluting from the column. Stimulated and control values of inositol phosphate isomers obtained in each experiment on a single guinea-pig were compared.

Statistics

Because of variability between strips from different guinea-pigs, statistical analysis of the results was performed by the paired Student's t test.

Drugs and chemicals

Carbachol chloride, histamine dihydrochloride, atropine sulphate and caffeine were from Sigma Chemical Co. Verapamil hydrochloride was from Abbott Laboratories Ltd. Mepyramine maleate was from May & Baker Ltd. Ryanodine was from Calbiochem Ltd. [^{3}H]-inositol (19 Ci mmol^{-1}) and [^{14}C]-inositol (1) phosphate were from Amersham Ltd. All other reagents were AnalaR grade from BDH Ltd.

Results

Carbachol

Strips of separated longitudinal muscle from guinea-pig ileum developed basal tone and spontaneous activity when bathed in PSS solution at 37°C. The form of the contraction to carbachol depended on the concentration applied: at lower concentrations (usually less than 1 μM) there was a rapid increase in tension, maintained until the drug was washed out, after which the tissue recovered its spontaneous activity rapidly. At higher concentrations, carbachol produced an initial phasic contraction followed by a partial relaxation of tension. A second more sustained component then followed, which was maintained for some time after washing (Figure 1a) and spontaneous activity recovered more slowly.

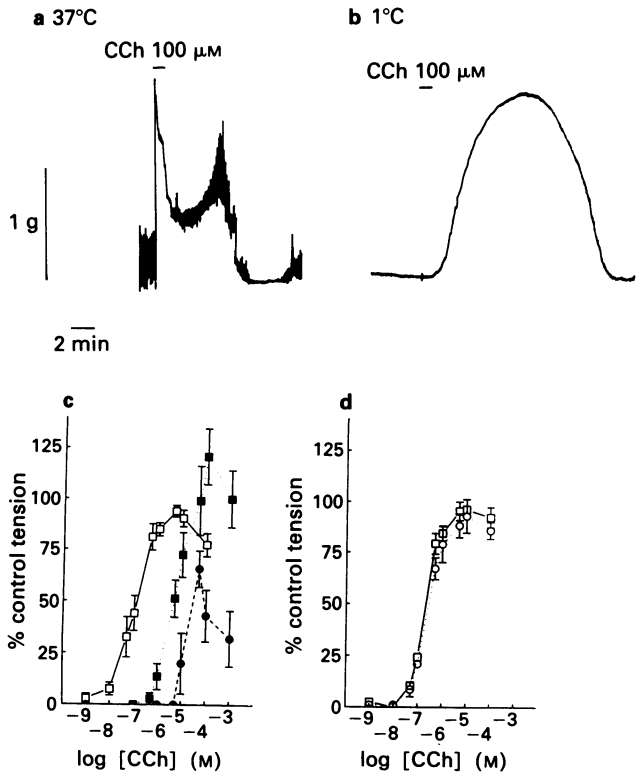


Figure 1 Effect of temperature on contractions to carbachol (CCh). Contractions to 100 μM carbachol at (a) 37°C and (b) 1°C. Ascending dose-response curve for the tension increases caused by carbachol (c) at 37°C (□), at 3°C (■) and at 1°C (●) (d) first (□) and second (○) dose-response curves to carbachol at 37°C on the same tissue. Each point is the mean ± s.e.mean of the maximum tension increase expressed as % of maximum response at 37°C. (c) 100% = 1.13 ± 0.22 g; $n = 8$: (d) 100% = 1.89 ± 0.14 g; $n = 3$.

Slowly lowering the temperature of the solution perfusing the strip produced a rise in tension which was greatest at about 20–15°C. Thereafter a fall in tension and a loss of spontaneous activity occurred. This contraction, which was seen on cooling, corresponds to the 'cooling contracture' observed by others (e.g. Magaribuchi *et al.*, 1973; Sakai & Kurihara, 1974).

Basal tension at 1°C was lower than at 37°C, typically falling to levels only observed in the absence of calcium at 37°C (<0.1 g). At 1°C all effective concentrations of carbachol produced a much slower increase in tension than at 37°C and an even slower relaxation (Figure 1b). The time taken to reach maximum tension at 1°C or 3°C was between 2 and 10 min compared to less than 30 s for the phasic response at 37°C.

At 37°C there was only a small effect on the concentration-effect curve if a second series of carbachol applications was made, 40 min after the first (Figure 1d). In contrast, if the second series of carbachol applications was made at 1°C the concentration-effect curve was shifted to the right and the maximum response significantly ($P<0.05$; $n = 5$) reduced compared with that at 37°C (Figure 1c). However, if a single application of 100 μM carbachol was made, without prior addition of lower concentrations, the tension developed was not significantly different from tension at 37°C (1.44 ± 0.34 g at 1°C; 1.28 ± 0.37 g at 37°C; $n = 3$). Indeed, the full response to 100 μM carbachol at low temperatures was restored if the tissue was warmed to 37°C before re-cooling and applying a second dose. In a second series of experiments done at 3°C a similar shift in the concentration-effect curve was observed, although there was no significant difference in the maximum response (Figure 1c). As at 1°C there was no significant difference between the size of the contractions produced by a single application of 100 μM carbachol (1.18 ± 0.23 g at 37°C; 1.23 ± 0.23 g at 3°C; $n = 8$).

At both temperatures the responses to carbachol were completely inhibited by 5 μM atropine, a muscarinic receptor antagonist.

High potassium solution

High concentrations of potassium (140 mM K^+) caused a rapid phasic contraction followed by a, usually lower, sustained component at 37°C. More slowly developing contraction and relaxation occurred at 3°C (Figures 2a and b).

Figure 2c shows a comparison of the maximum tension developed at 37°C and 3°C in response to three different extracellular K^+ concentrations (35, 70 and 140 mM). It can be seen that the response to 35 mM K^+ was almost abolished at 3°C whilst the response to 70 mM K^+ was also significantly reduced. The response to 140 mM K^+ was also less, although this was not statistically significant ($0.05 < P < 0.1$). Contractions to two consecutive stimulations with these solutions at 37°C were not significantly different (Figure 2d). If high potassium-containing solutions exert their effect by depolarizing the membrane and hence opening VDCs, these data suggest that at 3°C the channels can still be opened by depolarization.

When high potassium solutions were applied in 5 μM atropine, the contractions to 35 mM and 70 mM K^+ at 3°C remained less than those at 37°C ($P<0.05$; $n = 4$). This indicates that the decrease observed when the temperature was lowered was not because acetylcholine release from nerves was inhibited.

Calcium-free solution

Tissues were incubated in calcium-free solution to determine whether a contractile response remained when calcium entry was abolished. At 37°C omission of calcium from the bathing solution caused a transient increase in tension followed by a decline in basal tension to initial levels or below (Figure 3). Carbachol responses were obtained after a period in calcium-

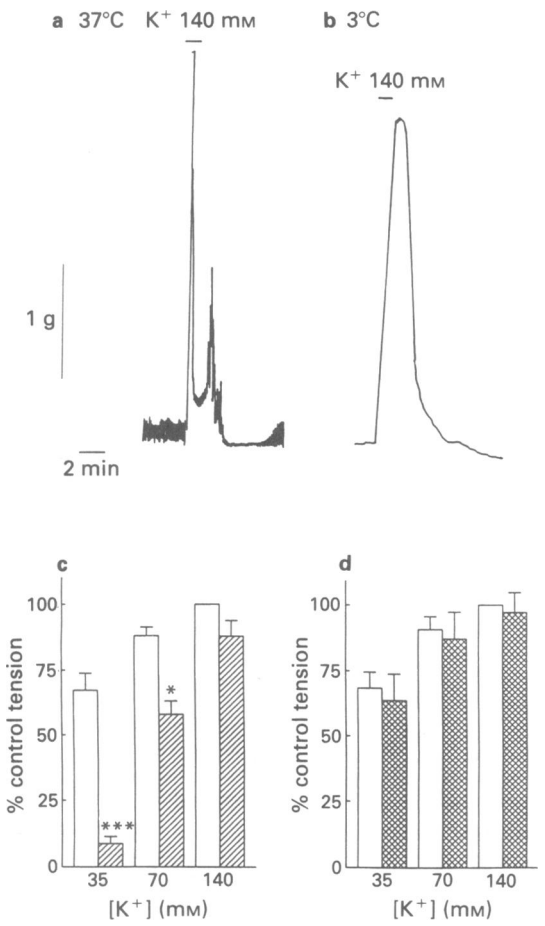


Figure 2 Effect of temperature on contractions to high potassium solutions. Contractions to 140 mM potassium (a) at 37°C and (b) at 3°C. Effect of cooling to 3°C on peak contractions elicited by different external K^+ concentrations. Columns show the mean ± s.e.mean of the maximum tension increase expressed as % 140 mM K^+ response at 37°C on the same tissue. (c) Open columns represent response at 37°C; hatched columns at 3°C. (d) Open columns represent first response at 37°C; cross-hatched columns second response at 37°C. (c) 100% = 1.63 ± 0.22 g; $n = 11$: (d) 100% = 1.73 ± 0.25 g; $n = 5$. $*P < 0.05$, $***P < 0.005$ when compared with the values of the initial response to 140 mM K^+ at 37°C.

free solution which was shown to abolish any response to 140 mM K^+. The phasic response to 100 μM carbachol was reduced to 11.2 ± 1.8% ($n = 6$) of its original level (Figure 5). This was significantly less than the control response suggesting that most of the contraction evoked by carbachol was due to calcium influx, although some reduction could also be due to store depletion.

At 3°C the response to 100 μM carbachol in calcium-free solution was still a gradual increase and decline in tension, although the decline was more rapid than when calcium was present in the bathing solution (Figure 3b). The contraction was significantly smaller than the control but not significantly different from the response in calcium-free medium at 37°C (Figure 5).

Depolarization with high potassium solutions

Tissues were depolarized with a high potassium solution in order to investigate the component of the contraction to carbachol which is independent of electro-mechanical coupling. At both 37°C and 3°C changing the bathing medium to a solution containing 140 mM K^+ produced an increase in tension which gradually declined to a steady level much higher than that in the normally polarized strip. When a steady resting level of tension in the high potassium solution was achieved, stimulation with 100 μM carbachol at 37°C induced an additional increase in tension (Figure 3a). This was followed by a large relaxation upon returning to drug-free solution as observed previously (Bolton & Clark, 1982). In 2 of 13 preparations the tension actually rose again and then returned to a lower level demonstrating that the sustained phase of the contraction was not completely abolished in the depolarized tissue. At 37°C carbachol produced an initial transient increase in tension in high potassium solution much smaller than those seen in normal PSS (Figure 5). The smaller size of the phasic response in high K^+ presumably represents the loss of the voltage-dependent component of calcium entry brought about by carbachol.

At 3°C, 140 mM K^+ also produced an increase in tension which gradually declined to a steady level, although this was significantly lower than that at 37°C. The contraction to 100 μM carbachol at 3°C in high K^+ was similar in shape but significantly smaller than that in normal PSS (Figures 3 and 5), suggesting a potential-dependent mechanism is involved in the response. In high K^+ solution the increase in tension to 100 μM carbachol was significantly greater at 3°C than at 37°C (Figure 5).

Calcium-free solutions on depolarized strips

Tissues were depolarized in order to load up the calcium stores and then extracellular calcium removed in order to investigate further the component of the contractile response remaining in calcium-free medium. At 37°C, incubation in calcium-free solution containing 1 mM EGTA decreased the tension response to 100 μM carbachol in 140 mM K^+ compared with the response in 2.5 mM Ca^{2+}, 140 mM K^+ solution. At 3°C, calcium-free solution also caused a decrease in the carbachol response in high K^+ (Figures 3 and 5). This decrease indicates that either some potential-independent calcium entry is involved in the receptor-evoked response or that some calcium is lost from the internal stores during incubation in the calcium-free medium.

At both temperatures, there was a significantly larger response to carbachol in depolarized tissue during perfusion with calcium-free solution, compared with normally polarized strips. This may be because depolarization causes loading of the internal calcium stores. Furthermore, contraction to 100 μM carbachol in calcium-free high K^+ solution was always larger at 3°C than at 37°C. In depolarized tissues incubated in calcium-free medium there should be no calcium influx; hence there is a component of the response to carbachol that is independent of extracellular calcium. This component can still operate at 3°C and actually appears to be enhanced at this low temperature in depolarized tissues.

Calcium channel antagonists

Two different calcium channel antagonists, verapamil and nifedipine, were used to investigate further the contribution of voltage-dependent calcium entry at the two temperatures. Their presence markedly inhibited the spontaneous contractions and decreased the basal tone of the guinea-pig ileal smooth muscle (Figure 4) presumably by blocking the voltage-dependent calcium channels (Bolton, 1979). Verapamil (10 μM) reduced the 140 mM K^+ contraction to 1.8 ± 1.1% and 8.5 ± 1.7% ($n = 4$) of the control response at 37°C and 3°C respectively. Lower concentrations were not so effective, 1 μM only reducing the response to 140 mM K^+ to 52.8 ± 7.6% of the control at 37°C. The dihydropyridine, nifedipine, appeared to be more potent at blocking VDCs in this smooth muscle preparation, since 0.5 μM reduced the responses to 140 mM K^+ to 2.4 ± 1.4% and 0% of the control response at 37°C and 3°C respectively.

The phasic and sustained response to 100 μM carbachol at 37°C were significantly decreased by 10 μM verapamil. Nifedipine (0.5 μM) also decreased the phasic response to this

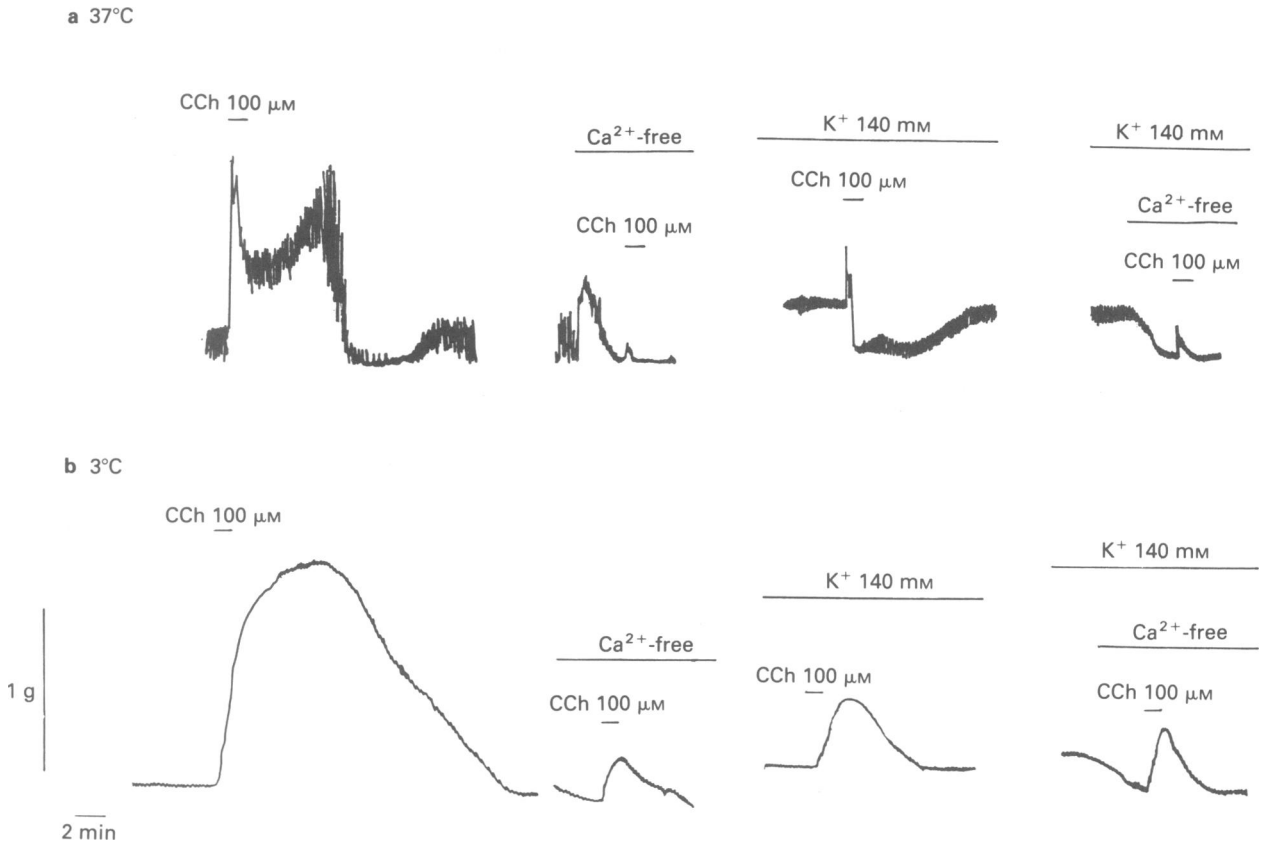


Figure 3 Effect of calcium-free and high potassium solutions on the responses to (a) 100 μM carbachol (CCh) at 37°C and (b) 100 μM carbachol at 3°C. Responses shown are from the same tissue. At 37°C the tissue was incubated in PSS until spontaneous activity was recovered before testing each response. The tissue was re-warmed to 37°C until spontaneous activity resumed and then re-cooled before testing each response at 3°C.

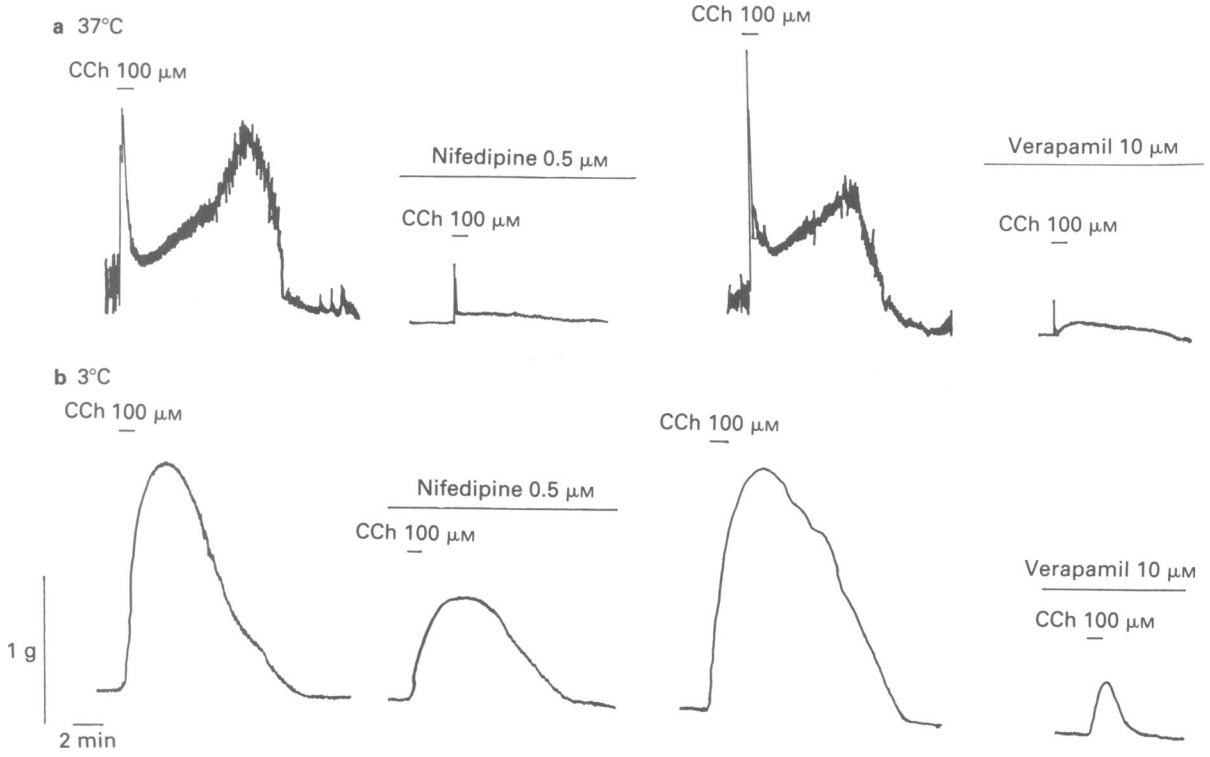


Figure 4 Effect on the responses to 100 μM carbachol (CCh) at (a) 37°C and (b) 3°C of nifedipine and verapamil. Responses shown are from two different tissues with similar responses to 100 μM carbachol, one incubated in verapamil and one in nifedipine.

agonist, although less so than 10 μM verapamil, and almost completely blocked the sustained component (Figure 4). However, at 37°C the contractions to 100 μM carbachol in 10 μM verapamil or 0.5 μM nifedipine were always significantly greater than the responses to 140 mM K^+ in the same conditions (Figure 5).

At 3°C the contractions to 100 μM carbachol in 10 μM verapamil or in 0.5 μM nifedipine were also significantly greater than the contractions to 140 mM K^+ in either of the calcium channel antagonists.

Inositol phosphate measurements at 37°C and 3°C

The inositol phosphate pathway was examined by labelling the cells with [^{3}H]-inositol and analysing the acid soluble extracts by h.p.l.c. The 3 h labelling period was not long enough to produce equilibrium labelling of the inositol lipids (Prestwich & Bolton, 1991), but allowed an investigation of flux through the pathway. A typical profile of such an h.p.l.c. analysis is shown in Figure 6. The inositol phosphates were identified by a comparison of their retention times with that of radiolabelled turkey and human erythrocyte standards. It was assumed that the majority of counts in each peak was incorporated in the isomer stated.

Tissue fragments were incubated in 20 mM Li^+ for the final hour of labelling. This is reported to inhibit the dephosphorylation of inositol mono- and bis-phosphates and should therefore enhance the amount of radioactivity in inositol phosphates and reduce recycling into phosphoinositide lipids (Chilvers *et al.*, 1990). Under these conditions the cells were stimulated with carbachol for 5 s at 37°C and for 1 min and 5 min at 3°C. These time points were chosen since they corresponded to the period when tension was still developing

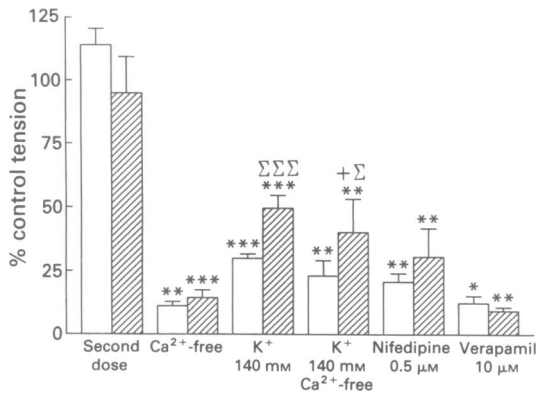


Figure 5 Effect of calcium-free, high potassium solutions and calcium channel antagonists on the responses to 100 μM carbachol. Columns show the mean ± s.e.mean of the maximum tension increase expressed as % control response in PSS at 37°C on the same tissue. Open columns represent response at 37°C; hatched columns at 3°C. The tissue temperature was returned to 37°C after each response at 3°C. The second dose columns show the tension developed in response to a second application of agonist after one such warming and cooling cycle. The Ca^{2+}-free columns show the responses to 100 μM carbachol after incubation in Ca^{2+}-free 1 mM EGTA solution for a time which abolished the responses to 140 mM K^+. The 140 mM K^+ columns show the responses to 100 μM carbachol after incubating in 140 mM K^+ solution until a steady tension level was achieved. (a) For 140 mM K^+ 100% at 37°C = 1.64 ± 0.20 g; $n = 13$. For Ca^{2+}-free and 140 mM K^+ Ca^{2+}-free responses 100% at 37°C = 1.59 ± 0.37; $n = 6$. For 10 μM verapamil, 100% at 37°C = 2.06 ± 0.39 g; $n = 4$. For 0.5 μM nifedipine, 100% at 37°C = 1.80 ± 0.27 g; $n = 4$. $^*P < 0.05$; $^{**}P < 0.001$, $^{***}P < 0.005$ when compared with the values of the control response at the same temperature. $\dagger P < 0.05$ when compared with the values of the response in Ca^{2+}-free solution, in normaly polarized strips at the same temperature. $^{\Sigma}P < 0.05$, $^{\Sigma\Sigma\Sigma}P < 0.005$ when compared with the 37°C response under the same conditions.

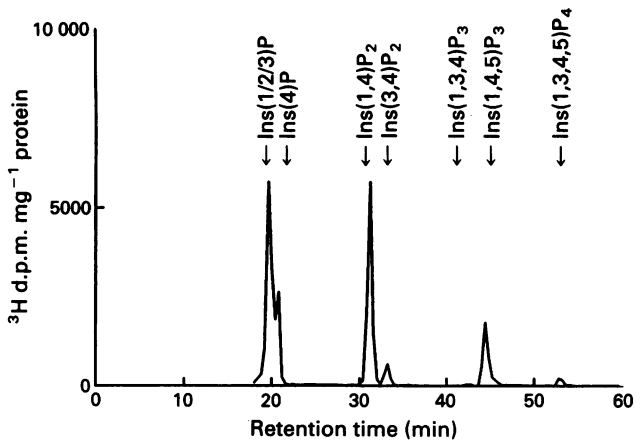


Figure 6 (a) A typical h.p.l.c. profile of [^{3}H]-inositol-labelled acid soluble extract from longitudinal smooth muscle fragments of guinea-pig ileum. The elution positions of radiolabelled turkey and human erythrocyte standards in this gradient system are shown.

at each temperature; that is when second messenger generation would be expected to be occurring.

After a 5 s stimulation with 100 μM carbachol at 37°C there were statistically significant increases in all the inositol phosphates studied (Figure 7) as has previously been reported at 22°C (Salmon & Bolton, 1988). Inositol (1,4,5)trisphosphate ($Ins(1,4,5)P_3$) rose to 248 ± 23% of its control value. However, a 1 min stimulation with the same concentration of carbachol at 3°C produced only very small changes. There is no increase in $Ins(1,4,5)P_3$ (98.5 ± 6.9% of control values) although statistically significant increases in $Ins(1,3,4)P_3$ and $InsP_4$ were observed. After 5 min of stimulation with 100 μM carbachol, increases were still small compared with those at 37°C. Although labelling in $Ins(1,4,5)P_3$ was increased (127.4 ± 10.2% of control values) this was not statistically significant ($P < 0.1$). However, there were statistically significant increases in the total inositol phosphates, $Ins(1,4)P_2$, $Ins(1,3,4)P_3$ and $InsP_4$. In contrast, 1 mM histamine produced no increases in labelling of any inositol phosphate isomer after 5 min stimulation at 3°C.

These results suggest that at 3°C, 100 μM carbachol causes an increase in flux through the phosphoinositide pathway, although this is much less than that observed at 37°C.

Discussion

It is believed that the contraction of guinea-pig ileal smooth muscle to an excitatory agonist is brought about by the opening of ROCs and the activation of phospholipase C (PLC). The former causes an influx of cations and the consequent depolarization opens VDCs in the membrane (Bolton, 1979). The latter results in the cleavage of phosphatidylinositol (4,5)bisphosphate to produce $Ins(1,4,5)P_3$ which binds to and opens calcium channels in the sarcoplasmic reticulum. In both cases the rise in $[Ca^{2+}]_{int}$ activates myosin light chain kinase, causing phosphorylation of myosin light chain and hence contraction. There appears to be an interaction between the two pathways since Pacaud & Bolton (1991) have shown that a rise in $[Ca^{2+}]_{int}$ increases the probability of ROC opening and hence the degree of depolarization and subsequent calcium entry. Other mechanisms, less thoroughly investigated, may also play a role in agonist-induced contractions, such as calcium-induced calcium release (CICR) from the sarcoplasmic reticulum and modulation of the sensitivity to calcium of the contractile proteins.

This study investigated the initially surprising phenomena of agonist-induced contractions and [^{3}H]-inositol phosphate

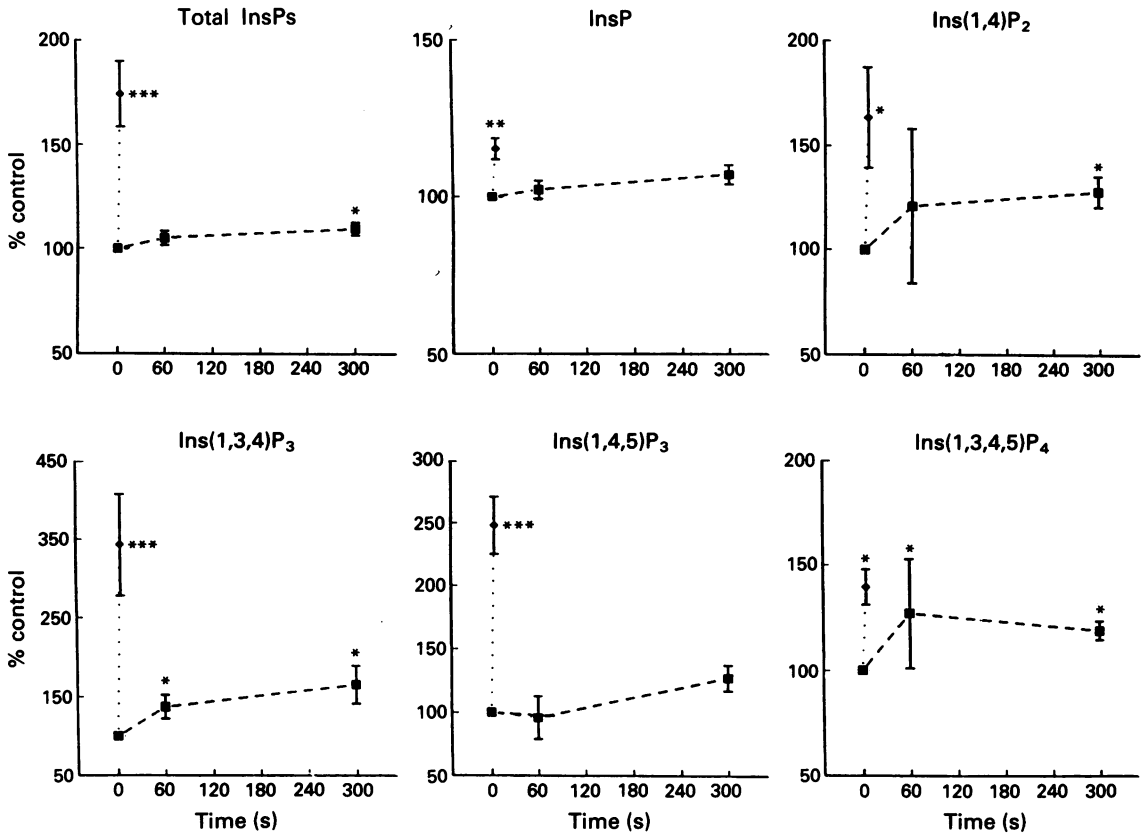


Figure 7 A comparison of [^{3}H]-inositol phosphate production in response to carbachol at 37°C and 3°C. Fragments of longitudinal smooth muscle labelled with [^{3}H]-inositol for 3 h and pre-incubated with 20 mM Li^+ for the final hour of labelling, were exposed to 100 μM carbachol for 5 s at 37°C and for 1 or 5 min at 3°C. Points show the mean ± s.e.mean of the % control; (◆) at 37°C and (■) at 3°C in response to 100 μM carbachol. Statistical analysis was performed by the paired Student's *t* test on the data expressed in d.p.m. mg^{-1} protein. $*P<0.05$, $**P<0.01$, $***P<0.005$ when compared with control values. (At 37°C control values of d.p.m. mg^{-1} protein in total InsPs = 47188 ± 6867; InsP = 27365 ± 1345; $Ins(1,4)P_2$ = 17108 ± 5053; $Ins(1,3,4)P_3$ = 193 ± 47; $Ins(1,4,5)P_3$ = 1249 ± 260; $InsP_4$ = 493 ± 61; $n = 4$. At 3°C, 1 min control values of d.p.m. mg^{-1} protein in total InsPs = 44842 ± 4165; InsP = 38942 ± 3658; $Ins(1,4)P_2$ = 4926 ± 1584; $Ins(1,3,4)P_3$ = 69 ± 39; $Ins(1,4,5)P_3$ = 279 ± 95; $InsP_4$ = 165 ± 50; $n = 6$: 5 min control values of d.p.m. mg^{-1} protein in total InsPs = 40937 ± 7458; InsP = 34985 ± 6128; $Ins(1,4)P_2$ = 4621 ± 1082; $Ins(1,3,4)P_3$ = 126 ± 17; $Ins(1,4,5)P_3$ = 441 ± 102; $InsP_4$ = 247 ± 41; $n = 5$).

production at temperatures near freezing point. It was hoped that the changes produced by cooling might also provide some insight into the mechanisms responsible for contraction and their interactions under physiological conditions.

The peak amplitude of a contraction to 100 μM carbachol was unimpaired upon lowering the temperature to between 3 and 1°C (Figure 1). However, the appearance of the contraction was very different from that at 37°C. Instead of an initial rapid phasic increase in tension, there was a smooth, slow rise which lasted many minutes. This is presumed to be partly due to the failure of action potentials to occur or to spread through the tissue at low temperatures, because of depolarization of the membrane (Buck & Goodford, 1966). The more gradual increase in tension seen at low temperatures may indicate that the contraction was the result of each cell being stimulated by agonist directly. However, the lack of effect of temperature on the peak amplitude of contraction suggests that the number of contracting cells may be the same in each case. The significant slowing of the response is in agreement with the relatively high temperature-dependence of the calcium current reported in other tissues (in guinea-pig ventricular myocytes Q_{10} = 2.5–2.9, Cavalie *et al.*, 1985).

Upon removal of carbachol, the tissue relaxed and tension levels returned to basal, even when the temperature was maintained near freezing point. This indicated that the pumps controlling ion movements were still functioning, although much more slowly than at 37°C. Indeed, calcium extrusion by Na^+/Ca^{2+} exchange in conjunction with accumulation of calcium into the sarcoplamic reticulum by a Ca^{2+}-ATPase has been shown to cause relaxation at temperatures of 1°C in guinea-pig ventricular myocytes (Bers *et al.*, 1989).

Decreasing the temperature caused a rightward shift of the ascending concentration-response curves to carbachol (Figure 1c), as previously observed at temperatures down to 9°C (Cheng & Ochillo, 1989). Since single applications of maximally effective concentrations of carbachol produced the same rise in tension regardless of temperature, there was no reduction in the ability of the muscle to respond to carbachol at the lower temperatures. The reduction in the maximum response seen at 1°C when ascending concentrations of carbachol were applied with washout between, suggests an increasing delay in recovery as the temperature falls. This may be due to the reduced ability of the muscle to extrude its sodium load at low temperatures (Himpens *et al.*, 1991).

The decrease seen in the peak amplitude of contraction to high K^+ solutions at 3°C is consistent with the idea that lowering the temperature depolarizes the membrane (Buck & Goodford, 1966) possibly inactivating some of the VDCs and inhibiting voltage-dependent calcium entry. It is also consistent with the decrease in the calcium selectivity of VDCs seen in other tissues upon lowering the temperature (e.g. heart, Suleiman & Chapman, 1990).

In contrast, it appears from the results obtained with calcium channel antagonists, and with calcium-free medium,

that a similar proportion of the contraction at 37°C and 3°C is the result of calcium entry through VDCs. Furthermore, this proportion accounts for the major part of the contractile response at both temperatures. The difference between the effects of temperature on the 140 mM K^+- and on the carbachol-induced contractions suggests that some other mechanism, in addition to depolarization, plays a part in the response to carbachol.

At temperatures close to 0°C, ROCs still appear to be activated by carbachol as VDC opening is dependent on the depolarization that ROCs produce. In their ability to function at low temperatures the channels resembled nicotinic acetylcholine-operated cation channels which have a low temperature dependence ($Q_{10} = 1.3$) and are still functional at 5°C (Quartararo & Barry, 1988).

There was a component of the carbachol-induced contraction remaining in the presence of calcium channel antagonists or in calcium-free solution. This accounted for between 10–30% of the initial contraction at 37°C and paired comparisons revealed that the proportion of the control responses remaining was not different at 3°C. This can be ascribed to the release of internal calcium or sensitization of the myofilaments to calcium.

The greater inhibition of the response to carbachol in tissues incubated in verapamil compared with those incubated with nifedipine, could reflect the lack of specificity of verapamil. It has been reported that at the concentrations employed, verapamil also inhibits calcium store release in this tissue (Kobayashi *et al.*, 1991). Thus both the experiments in verapamil and those in calcium-free medium, in which the calcium stores are depleted, would produce an underestimation of the involvement of calcium store release in the carbachol-evoked response. This is consistent with the results presented.

Evidence that release of stored calcium occurs in response to carbachol at both temperatures is also provided by the effects of depolarizing the muscle strips. It has been reported that the increase in $[Ca^{2+}]_{int}$ caused by depolarization causes an increase in the activity of the sarcoplasmic reticulum Ca^{2+}-ATPase and hence loading of more calcium than normal into the internal stores (Casteels & Droogmans, 1981). Such a proposal is supported by the fact that the response to carbachol in calcium-free medium is significantly enhanced when the tissue has previously been depolarized for several minutes at both 37°C and 3°C (Figure 5). This result provides further evidence that the component of the response remaining in calcium-free medium involves intracellular calcium release and thus suggests that calcium may still be accumulated into the intracellular stores at low temperatures. In fact, Bers *et al.* (1989) have demonstrated that the sarcoplasmic reticulum Ca^{2+}-ATPase of guinea-pig ventricular myocytes is still active at 1°C whilst low temperatures have also been reported to decrease the passive efflux of calcium from the sarcoplasmic reticulum (Yoshioka & Somlyo, 1987). Paired comparisons showed that the responses of depolarized strips to carbachol in both calcium-free and in calcium-containing medium were always higher at 3°C than at 37°C. This may indicate that more calcium can be released from the stores by carbachol under depolarized conditions at 3°C than at 37°C. Such a finding was initially surprising since at such low temperatures the rate of $Ins(1,4,5)P_3$-induced calcium release is reduced (Smith *et al.*, 1985) and calcium sensitization of the myofilaments may be decreased as in rabbit heart (Harrison & Bers, 1989).

These data suggest that carbachol causes a release of internal calcium from the sarcoplasmic reticulum at 3°C and that this is as important for contraction as at 37°C. However, the measurements of radiolabelled inositol phosphate accumulation in response to 100 μM carbachol at 3°C indicate that the production of $[^3H]$-$Ins(1,4,5)P_3$, although still occurring, is significantly reduced compared with that at 37°C. This method may underestimate the increases in inositol phosphates produced because of changes in the specific activity of the precursor lipids during stimulation (Nahorski & Challiss, 1991). However, the use of relatively short time courses of stimulation, of Li^+ to inhibit the conversion back to inositol and, most significantly, the much larger carbachol-induced response at 37°C suggests that at 3°C, carbachol causes a greatly reduced production of all the inositol phosphates, including the calcium-mobilizing second messenger $Ins(1,4,5)P_3$. It is possible that the amount of inositol phosphates produced at 37°C are in a large excess to the amount required for calcium release. Indeed it has been reported that in airway smooth muscle a maximum contraction to cholinoceptor agonists is produced when there is less than 30% of maximum inositol phosphate production (Grandordy *et al.*, 1986). The affinity of the $Ins(1,4,5)P_3$ receptor for $Ins(1,4,5)P_3$ may also be increased at low temperatures as demonstrated in liver cells (Mauger *et al.*, 1989). Alternatively another mechanism may be responsible for intracellular calcium release at 3°C. That CICR may be able to cause a contraction at 3°C is supported by the fact that the response to 30 mM caffeine, an agent causing CICR, is not significantly different at 37°C and 3°C (Blackwood & Bolton, unpublished observations).

In conclusion, this study demonstrates that when the temperature of ileal smooth muscle is very close to freezing point, it is still able to respond to high concentrations of the muscarinic agonist, carbachol, by contracting. The source of the activator calcium for this contraction appears to be very similar to that at 37°C involving mainly calcium entry through VDCs. Under conditions where this mechanism is inhibited, it appears that carbachol can release similar amounts of calcium from internal stores at both temperatures. As a similar proportion of the contraction remained in calcium-free solution at the two temperatures, a smaller amount of $Ins(1,4,5)P_3$ seems to be effective in generating tension in the muscle near freezing-point than at 37°C.

This work is supported by the MRC. A.M.B. held an MRC studentship.

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(Received September 10, 1992
Revised March 5, 1993
Accepted March 18, 1993)

Br. J. Pharmacol. (1993), **109**, 1038–1045 © Macmillan Press Ltd, 1993

Calcium modulatory properties of 2,6-dibutylbenzylamine (B25) in rat isolated vas deferens, cardiac and smooth muscle preparations

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1 In rat isolated vas deferens the new compound 2,6-dibutylbenzylamine (B25) evoked a series of repeating rhythmic contractions. Concentration-response curves constructed for this effect were bell-shaped, indicating a biphasic effect for this compound. By contrast, B25 depressed heart contractility without any visible positive inotropic or chronotropic activity.

2 Experiments with tetrodotoxin, reserpine, capsaicin, α-adrenoceptor blocking compounds and other agents permit us to exclude a release of neuromediators or a direct stimulation of post-synaptic receptors to account for the rhythmic effect of B25 in the rat vas deferens.

3 In the same tissue, the increase in $^{45}Ca^{2+}$ uptake, the voltage-dependency as well as the dependence of the B25-induced rhythmic activity upon the external calcium concentration indicate a direct activation of voltage-sensitive calcium channels (VSCC).

4 Verapamil paradoxically stimulated the rhythmic effect of B25 in the rat vas deferens. La^{3+} was inactive while nifedipine was a weak inhibitor. By contrast Ni^{2+} and Mn^{2+} ions were good inhibitors ($IC_{50} < 10^{-4}$M), suggesting that a possible opening of T-type VSCC underlies the rhythmic effect of B25.

5 In radioligand binding studies competition experiments with [^{3}H]-nitrendipine indicated that only at high concentrations was B25 able to interact with dihydropyridine-sensitive binding sites of heart and vas deferens smooth muscle.

6 B25 (3-30 μM) counteracted the inhibitory effects of ω-conotoxin GVIA in field-stimulated rat vas deferens.

Keywords: 2,6-Dibutylbenzylamine; rat vas deferens; guinea-pig ileum; isolated heart; voltage-sensitive calcium channels (VSCC)

Introduction

Smooth muscle contractile responses induced by various agonists are found to depend on the concentration of ionized cytoplasmic calcium within the cell. As in many other mammalian smooth muscles, in the rat vas deferens, a preparation often used to study muscular functions, the contractile responses induced by different agonists can be divided into two components: an initial fast 'phasic' response followed by a second 'tonic' one which are considered to be directly related to the activation of different diffusible calcium pools (Hurwitz & Suria, 1971; Chang & Triggle, 1973; Golenhoffen & Weston, 1975; Triggle *et al.*, 1979). In this preparation, however, a third component consisting of a series of repetitive, irregular muscular contractions has been described. For example, characteristic rhythmic contractions superimposed on phasic and tonic components have been observed following the exposure of both epididymal and prostatic portions of rat vas deferens to noradrenaline (Ashoori & Tomita, 1983). A similar phenomenon has also been observed in the whole vas deferens with fenfluramine, methoxamine, 5-hydroxytryptamine, kassinin, KCl and $BaCl_2$ (Mottram & Wadhwani, 1977; Hay & Wadsworth, 1979; Hay & Wadsworth, 1983; Maggi *et al.*, 1987; Mishra *et al.*, 1988).

Although rhythmic activity has been reported for many years, few studies on the molecular mechanism underlying this effect are available. Regardless of what agonist has been used, this rhythmic activity has proved to be closely dependent upon external calcium since it was completely prevented in $CaCl_2$-free Krebs solutions (Ashoori & Tomita, 1983; Hay & Wadsworth, 1979; 1983). More recently Langton & Huddard (1987) have shown that in the rat vas deferens, the rhythmic activity induced by slight depolarizing KCl solutions could be selectively mediated by rapidly inactivating voltage-sensitive calcium channels (VSCC) in the membrane. These results are interesting because they indicate that controlled K^+ depolarizations or other procedures inducing rhythmic contractions in the vas deferens could be a useful tool for the selective study of VSCC in the smooth muscle.

During a programme designed to investigate the pharmacological profile of a series of benzylamine derivatives synthesized as potential semicarbazide-sensitive amine oxidase (E.C.1.4.3.6.) inhibitors (Bertini *et al.*, 1985; 1988) we observed that 2,6-dibutylbenzylamine (B25) induced in the rat isolated vas deferens a series of rapid contractions strongly resembling the rhythmic effect previously described by various authors.

To clarify the nature of this effect we decided to assess concentration-response relationship by measuring the mean tension and frequency of B25-induced rhythmic responses.

Isolated preparations of rat vas deferens, guinea-pig ileum and some rat and guinea-pig cardiac tissues were selected for this study to allow comparison of the effects of B25 in different muscular systems.

Methods

Rat isolated vas deferens, guinea-pig ileum and heart preparations

Experiments were carried out on the vas deferens of Wistar rats weighing 200–250 g. Vasa deferentia were separated

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from connective tissue and suspended in 10 ml organ baths containing Krebs solutions of the following composition (mM): KCl 4.7, KH_2PO_4 1.2, NaCl 118, $CaCl_2$ 2.5, $MgSO_4$ 0.6, $NaHCO_3$ 25, and glucose 11.1. The medium was maintained at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2. The resting tension of the preparation was maintained at 500 mg and, after an equilibration period of 60 min, contractions were recorded isotonically with a Basile 7050 Unirecord.

Guinea-pig isolated ileum was used according to the method described by Paton (1955) in the presence of 10^{-7} M atropine sulphate in order to avoid spontaneous activity of the preparation.

In some experiments contractile responses of vas deferens were elicited by field stimulation (FS). Square wave pulses (0.1 Hz, 20 V, 0.1 ms of duration) were delivered by a tension constant MARB stimulator Mod. 25/2/50.

In unstimulated vas deferens, the rhythmic effect was evaluated by adding B25 cumulatively in a volume of 10 to 100 µl to the organ bath. Concentration-response curves were constructed by measuring both the frequency (number of contractions min^{-1}) and the amplitude (developed tension) during the last 5 min of a 30 min infusion time.

To see the effects of various pharmacological treatments some compounds were applied before or more frequently after an established rhythmic response. In this case, control preparations showed that the response evoked by B25 did not vary appreciably during the 120–180 min of the experiments. Some experiments were performed in vas deferens excised from reserpine (1.0 mg kg^{-1} daily, i.p. for 5–7 days) or capsaicin pretreated rats (50 mg kg^{-1}, s.c. 3–8 days before experiments) according to the methods described by Lee *et al.* (1975) and Maggi *et al.* (1987), respectively. From 3 to 6 experiments were performed with each compound.

To investigate the dependence on external calcium of the B25-induced rhythmic activity, the effects of a 10^{-5} M concentration of this compound in normal Krebs solutions were recorded and then the compound was washed out several times until complete relaxation was obtained. At this time the Krebs solution was changed to one with the same composition but without $CaCl_2$. After a 30 min equilibration time the next dose of B25 was tested. This experimental procedure was repeated by increasing progressively the $CaCl_2$ concentration in the bath fluid.

Chronotropic and inotropic effects of B25 were studied on spontaneously beating isolated atria and on electrically stimulated ventricular strips of rat and guinea-pig hearts, respectively, according to methods previously described (Ledda *et al.*, 1981). The contractions were measured by means of an isometric transducer and d.c. amplifier on a chart recorder. Ventricular strips were driven at 150 beats min^{-1}.

The calcium antagonist activity of B25 was evaluated by its ability to decrease the contractile tension in isolated ventricular strips incubated in a K^+-rich medium (22 mM) in which the contractility was restored by the addition of 10^{-5} M histamine as transmembrane calcium promoter. Details of these methods have been reported previously (Mantelli *et al.*, 1981).

$^{45}Ca^{2+}$ uptake

$^{45}Ca^{2+}$ influx was determined according to Sakai *et al.* (1989) in 5–10 mg of prostatic portions of rat vas deferens. Each sample (5 portions of muscle tissue) was incubated in 1 ml of non-radioactive HEPES solution containing (mM): Na^+ 118, K^+ 4.7, Ca^{2+}1.5, Mg^{2+} 1.2, Cl^- 125.7, SO_4^{2-} 1.2, glucose 11.5 and HEPES 7.8. The samples were gassed with 100% O_2 and the pH of the solution was adjusted to 7.3 with NaOH 2 N. After an equilibration period of 60 min the tissue samples were exposed to an experimental solution containing $^{45}CaCl_2$ (0.5 µCi ml^{-1}, New England Nuclear) for 180 min. The tissue was pretreated with each antagonist for 15 min before exposure to B25 10^{-5} M or K^+ (80 mM) depolarizing solutions for 30 min.

The tissues were then exposed to ice cold Ca^{2+}-free HEPES buffer containing 2 mM EGTA for 45 min to remove extracellular $^{45}Ca^{2+}$. At the end of the experiment the tissues were blotted, weighed and incubated overnight in 3 ml of 5 mM EDTA. Counting was carried out as described by Meisheri *et al.* (1981).

Displacement of [^{3}H]-nitrendipine binding

Sprague Dawley rat heart membranes were obtained from ventricular homogenates according to Matucci *et al.* (1992). In some experiments 100–200 µg of membrane protein was used and displacement of 0.05 nM [^{3}H]-nitrendipine binding (New England Nuclear 70 Ci $mmol^{-1}$) was obtained by increasing concentrations of cold nifedipine or B25.

Incubations were carried out for 60 min in Tris HCl 50 mM buffer, pH 7.4, at 25°C to achieve a final volume of 2 ml. Incubation samples were then diluted with 3 ml of ice cold buffer and filtered under vacuum through Watman GF/B filters; filters were washed three times with 5 ml ice-cold buffer, dried overnight at 40°C and placed in a scintillation cocktail (Insta-gel, Packard). The samples were counted in a TRICARB 1500 (Packard) at an efficiency of about 50%.

Similar experiments were also performed with 100,000 *g* microsomal rat vas deferens membranes obtained according to Yousif *et al.* (1984). In this case the determination of [^{3}H]-nitrendipine displacement was essentially the same as that used for the heart membranes.

Non-specific binding was determined by the addition of 10^{-6} M unlabelled nifedipine according to Bolger *et al.* (1983) and accounted for 15–30% of the total binding.

Because of the photolability of the dihydropyridines, binding studies were always carried out under a sodium lamp. The binding data were analyzed with the aid of a computer curve-fitting programme 'LIGAND' (Munson & Rodbard, 1980) to obtain the final estimates. The computer programme 'ALL-FIT' (De Lean *et al.*, 1978) was also used for the analysis of binding curves.

Drugs used included: tetrodotoxin (TTX), ω-conotoxin GVIA (ω-CT), capsaicin, verapamil HCl, α,β methylene ATP, reserpine, $MnCl_2$, $NiCl_2$, $LaCl_3$, atropine sulphate, prazosin HCl, pyrilamine maleate, nifedipine, haloperidol, noradrenaline bitartrate were obtained from Sigma Chemical Company (St. Louis, Mo, U.S.A.); phenoxybenzamine HCl and yohimbine HCl were purchased from RBI Natick U.S.A., methysergide from Sandoz; [^{3}H]-nitrendipine (70 Ci $mmol^{-1}$) and $^{45}CaCl_2$ (14 mCi mg^{-1}) were obtained from New England Nuclear, (Boston, MA, U.S.A.).

Results are given as mean ± s.e.mean. Student's *t* test was used for statistical analysis.

Results

Among a series of several dialkyl substituted benzylamines 2,6-dibutylbenzylamine (B25) was found to be the most potent in inducing (usually 2 min after the addition to the bath fluid, Figure 1b), a typical response consisting of a series of repetitive, irregular muscular contractions which reached their peak frequency (24.1 ± 2.6 peaks min^{-1}) and tension (540 ± 35 mg) about 30 min after the activity began. The amplitude of contractions increased between 5×10^{-7} and 2.5×10^{-5} M concentrations of B25 (ED_{50} $9.0 \pm 1.4 \times 10^{-6}$ M) whereas the active concentrations for frequency ranged between 5×10^{-7} and 5×10^{-5} M (ED_{50} $1.7 \pm 0.2 \times 10^{-5}$ M). Conversely at above 5×10^{-5} M, B25 progressively attenuated its own contractile effect. As shown in Figures 1c and 2 the resulting concentration-response curves were bell-shaped.

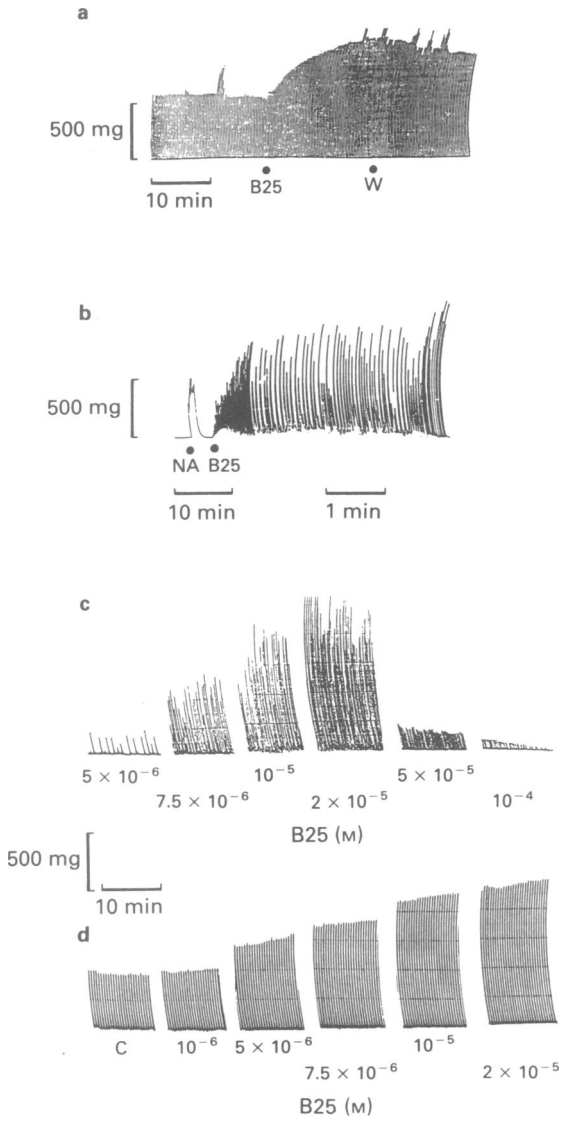


Figure 1 Representative isotonic recording showing: (a) the potentiation of electrically-induced contractions evoked by B25 10^{-5} M and (b) the rhythmic effect induced in unstimulated rat vas deferens by the same concentration of this compound. Changes in the rhythmic effect (c) and twitch tension in field stimulated preparations (d) obtained with cumulative applications of B25 (The last 10 min recordings are shown for each concentration compound). W = washing, NA = noradrenaline 10^{-6} M.

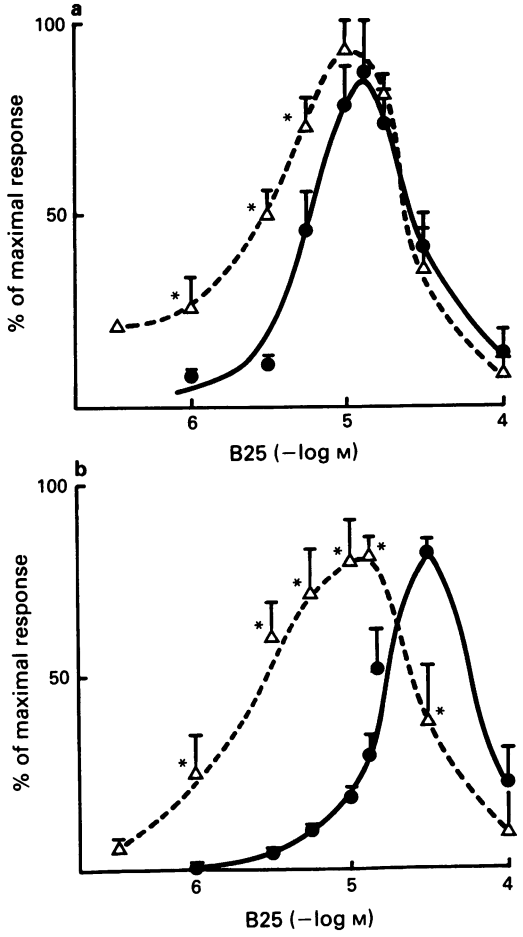


Figure 2 Mean concentration-response curves for the rhythmic effect induced in the rat vas deferens by B25 both for amplitude (a) or frequency (b) of contractions. The effect of B25 in normal (●) and in partially depolarizing (Δ) (K^+ = 20 mM) Krebs solutions are shown. Each point represents the mean ± s.e.mean of 8–12 experiments.

After washing out the B25 present at inhibitory concentrations, a complete recovery of the rhythmic activity was observed which declined exponentially within 30 min of the washout ($t_{½}$ = 10 min). B25 also induced rhythmic contractions in guinea-pig isolated ileum. The ED_{50} values both for frequency and amplitude of this effect were $4.6 \pm 1.9 \times 10^{-6}$ M and $2.3 \pm 1.9 \times 10^{-6}$ M respectively. Like that observed in the rat vas deferens, this contractile effect was followed by a depression of the contractions upon exposure to B25 concentrations above 5×10^{-5} M.

In the rat vas deferens the rhythmic effect was not prevented by pretreatment with phenoxybenzamine at concentrations (10^{-6} M) which irreversibly antagonized the contractile effects of noradrenaline. Similar results were also obtained in desensitization experiments with α,β methylene ATP (3×10^{-4} M) as well as with vas deferens preparations obtained from capsaicin or reserpine-pretreated rats. Moreover no significant modifications were observed when prazosin (10^{-6} M), yohimbine (10^{-6} M), atropine (10^{-6} M), methysergide (10^{-6} M) or haloperidol (10^{-5} M) and pyrilamine were applied during established mechanical activity. Finally tetrodotoxin (TTX) 3×10^{-6} M or ω-CT 3×10^{-8} M were found to be inactive against B25-induced rhythmic effects while this concentration completely inhibited electrically evoked contractions in this preparation.

Effects of B25 in isolated heart preparations

Unlike the effects observed in smooth muscle, B25 did not induce any measurable positive inotropic effect in rat and guinea-pig heart preparations.

However, concentration-dependent negative chronotropic (an inhibition by 64 ± 6% of the control values at 10^{-4} M concentration) and inotropic (an inhibition by 62 ± 8% of controls) effects both in atria and in electrically stimulated ventricular contractility were observed. At higher concentrations both atrial frequency and ventricular inotropism were blocked irreversibly. Similar results were obtained when B25 was tested in electrically driven ventricular strips exposed to a K^+-rich medium in which the contractility was restored by addition of 10^{-5} M histamine as calcium promoter (Figure 3).

Effects of B25 on electrically stimulated rat vas deferens

In the rat vas deferens, supramaximal field stimulation (FS) produced twitch contractions which were totally suppressed

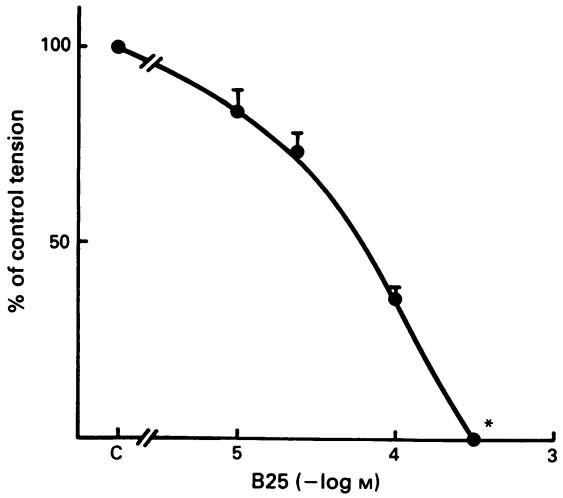


Figure 3 Effect of B25 on the contractile activity restored by 10^{-4} M histamine in K^+-depolarized rat ventricular preparations stimulated at 12 Hz min^{-1}. Cumulative concentrations of B25 were added at 30 min intervals. Note that at 5×10^{-5} M B25 (*) the ventricle contractility was irreversibly blocked. Each point is the mean ± s.e.mean of 4 experiments. C = control.

by TTX 3×10^{-6} M or ω-CT 3×10^{-8} M, thereby indicating their neurogenic origin. As shown in Figure 1 a–d, B25 concentration-dependently enhanced (92.4% over basal values at 10^{-5} M) the twitches evoked by electrical stimulation. The ED_{50} ($4.6 \pm 0.2 \times 10^{-6}$ M) values in these experiments were similar to those obtained in unstimulated preparations.

After complete inhibition induced by 3×10^{-8} M ω-CT, exposure of the preparations to B25 provided a concentration-related recovery (ED_{50} $1.3 \pm 1.0 \times 10^{-5}$ M) of contractile activity (Figure 4). However B25 was unable to antagonize the inhibitory effects induced in the vas deferens by TTX.

Voltage-dependency of B25-induced rhythmic effect

The voltage-dependency of the contractile effect induced by B25 was studied in unstimulated vas deferens incubated in bathing solutions in which external K^+ was increased to 20 mM in order to obtain a partial depolarization of the tissue without any significant mechanical response (6 ± 3% of the maximal contraction generated by 80 mM KCl). With this procedure, cumulative concentration-response curves to B25 were shifted to the left (Figure 2). The ED_{50} both for frequency and amplitude ($1.4 \pm 0.1 \times 10^{-6}$ M and $2.9 \pm 0.07 \times 10^{-6}$ M respectively) were significantly lowered ($P < 0.005$) compared to those obtained in normal Krebs solutions.

Calcium-dependence of the B25 induced rhythmic effect

The rhythmic activity induced by B25 in unstimulated rat vas deferens slowly disappeared when this compound was eliminated from the bathing medium. However, by washing the preparations with Ca^{2+}-free Krebs solutions the contractions were abruptly suppressed, suggesting a strong dependence of the phenomenon on external calcium. This hypothesis was further investigated by comparing the motility induced by 10^{-5} M B25 in normal Krebs solutions to the effect obtained when the same preparation was incubated in bathing solutions containing different amounts of $CaCl_2$. As shown in Figure 5, the rhythmic effect of B25 did not occur in Ca^{2+}-free medium. However, when Ca^{2+} was increased to 0.5 mM the contractile response appeared and became progressively greater by a further rise in external calcium.

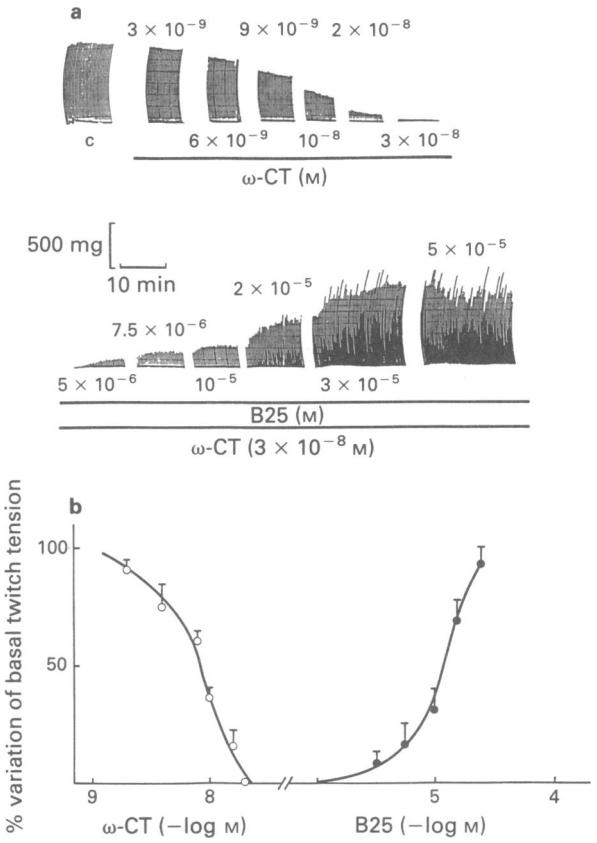


Figure 4 The effect of B25 on the inhibition induced by ω-conotoxin (ω-CT) in the field-stimulated rat vas deferens. Typical tracing (a) of a single experiment. ω-CT was applied cumulatively at 15 min intervals; when complete inhibition was obtained increasing concentrations of B25 were added at 15 min intervals. (b) Mean concentration-response curves for ω-CT (○) or 3×10^{-8} M ω-CT plus increasing concentrations of B25 (●). The results are the means ± s.e.mean of 8 experiments. C = control.

Modulation by calcium channel blockers of the effect of B25

In the rat vas deferens, nifedipine (IC_{50} $0.9 \pm 0.13 \times 10^{-7}$ M) induced a concentration-dependent decrease in amplitude of the rhythmic effect evoked by a maximally active concentration of B25 (Figure 6). The frequency of the responses were more resistant that the amplitude to the inhibitory effects of nifedipine since only 61.7% inhibition was obtained at 5×10^{-7} M concentration of this compound (experiments not shown). In contrast, verapamil at concentrations above 5×10^{-7} M, stimulated the rhythmic response of the preparation. For frequency and amplitude the maximal increase was 80.6 ± 13.3 and 198.0 ± 16.5% over controls, respectively, at 10^{-5} M and 5×10^{-5} M verapamil. A further increase in verapamil above 10^{-4} M attenuated its own potentiating effect.

In Figure 6 the effects of some inorganic calcium channel blockers are also shown. Among the ions used, the most potent in inducing a concentration-dependent inhibition of rhythmic activity were Ni^{2+} and Mn^{2+} (for the amplitude the IC_{50} was $1.8 \pm 0.04 \times 10^{-5}$ M and $1.2 \pm 0.8 \times 10^{-4}$ M respectively). The rhythmic activity induced by B25 was resistant to La^{3+} concentrations ranging from 5×10^{-6} M to 5×10^{-4} M.

Effect of B25 on the $^{45}Ca^{2+}$ uptake

We also investigated the effect of B25 on $^{45}Ca^{2+}$ influx into rat vas deferens preparations. In controls the $^{45}Ca^{2+}$ uptake was 388.7 ± 10.8 nmol g^{-1} wet weight ($n = 6$) and was

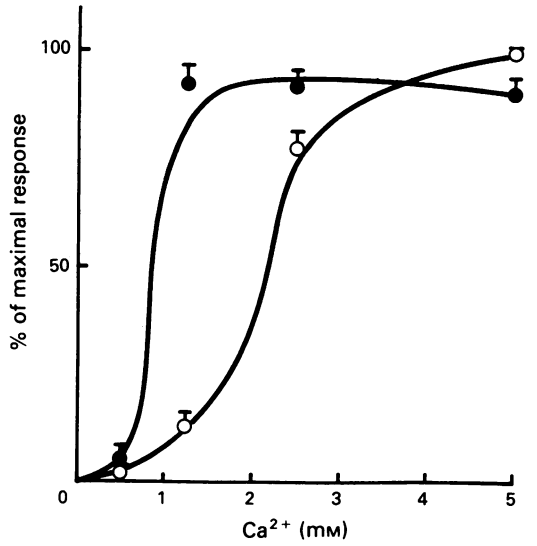


Figure 5 Dependence on external calcium of the rhythmic effect of B25 in unstimulated rat vas deferens. Mean concentration-response curves for amplitude (○) and frequency (●) of contractions are shown. Each point is the mean ± s.e.mean of 6 experiments.

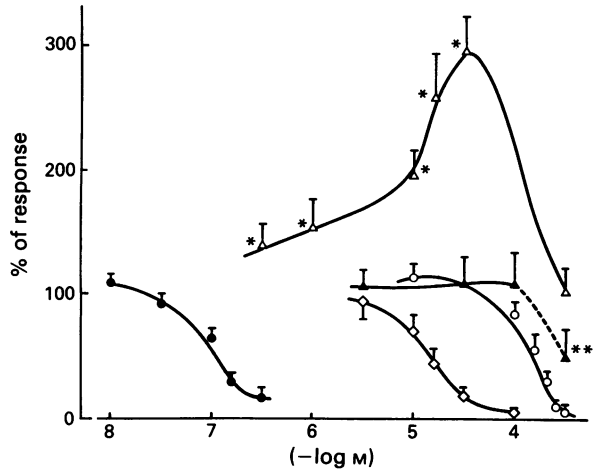


Figure 6 Influence of nifedipine (●), verapamil (△), Ni^{2+} (◇), Mn^{2+} (○) and La^{3+} (▲), on the rhythmic effect induced in unstimulated rat vas deferens by B25. Each curve represents the effect of cumulatively applied compounds during an established rhythmic effect (30 min after addition of 10^{-5} M B25). Precipitation (**) occurs in the nutrient solution at 5×10^{-4} M La^{3+} concentrations. Note that verapamil markedly potentiated. *($P<0.05$) the activity of B25 as compared to the control responses. Means ± s.e.mean of at least 6 experiments are shown for each antagonist.

significantly increased ($P<0.05$) after treatment of the samples with 80 mM KCl (613.2 ± 9.3 nmol g^{-1}) or 10^{-5} M B25 (500.7 ± 5.7 nmol g^{-1}). As shown in Figure 7 the increase of calcium uptake induced by B25 was unaffected by 10^{-5} M verapamil HCl while this effect was significantly ($P<0.05$; $n=8$) inhibited by 10^{-5} M nifedipine.

Displacement of [^{3}H]-nitrendipine binding

High affinity binding of [^{3}H]-nitrendipine was determined in rat ventricular membranes from heart and microsomal membranes from rat vas deferens. Scatchard analysis of saturation experiments in the heart preparations indicated B_{max} and K_d of 244.4 ± 27.2 fmol mg^{-1} protein and 0.48 ± 0.04 × 10^{-9} M, respectively. The binding parameters in vas deferens membranes (B_{max} = 332.2 ± 26 fmol mg^{-1} protein; K_d 0.88 ± 0.23 × 10^{-9} M) were comparable to the values described by Yousif *et al.* (1984).

Like nifedipine, B25 inhibited [^{3}H]-nitrendipine binding in a concentration-dependent fashion and was able to displace the ligand completely from its binding sites (Figure 8). These results suggest a pattern of activity of B25 similar, although at thousand-fold higher molar concentrations, to those of the dihydropyridines. Figure 8 also shows the effect of verapamil

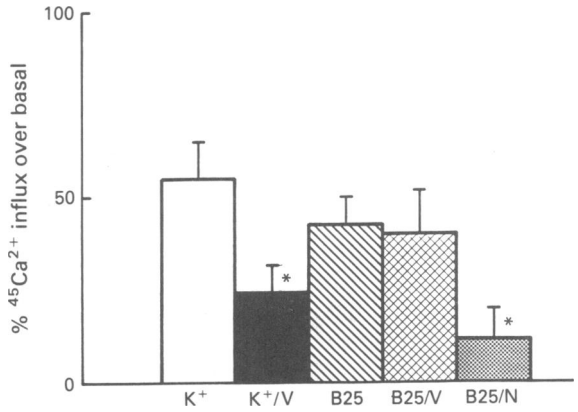


Figure 7 Stimulation of $^{45}Ca^{2+}$ uptake induced in prostatic portions of rat vas deferens by K^+ (80 mM) and B25 (10^{-5} M). The influence of preincubation with 10^{-5} M verapamil (/V) and 10^{-5} M nifedipine (/N) is also shown. Each column represents the mean ± s.e.mean of 4 to 8 experiments. *Statistically significant ($P<0.05$) effects as compared (paired Student's t test) to the response obtained in the absence of antagonists.

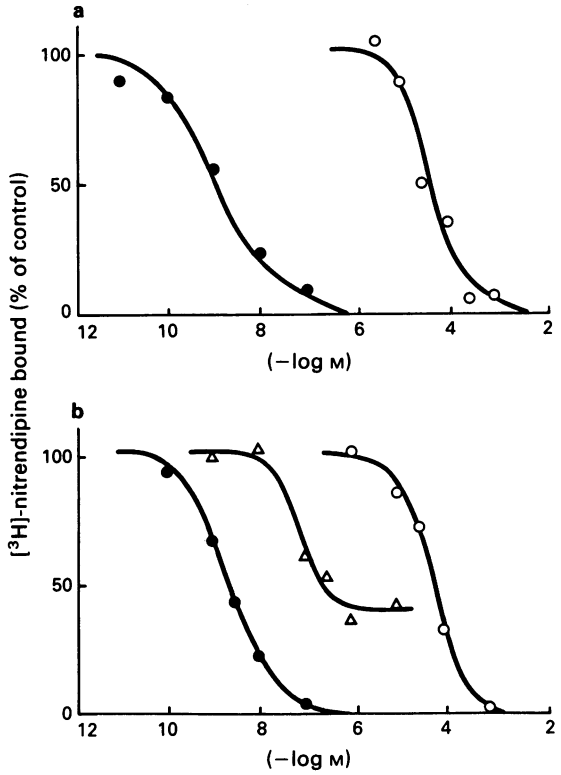


Figure 8 Displacement of [^{3}H]-nitrendipine binding (0.05 nM) induced in vas deferens microsomal membranes (a) and in the heart preparations (b) by increasing concentrations of nifedipine (●), verapamil (△) or B25 (○). The data are the results from at least 3 experiments, each performed in triplicate. Specific [^{3}H]-nitrendipine binding is expressed as a percentage of control binding in the absence of competing agent.

which is known to interact with binding sites distinct from those of dihydropyridines. The IC_{50} calculated for nifedipine and B25 in the heart membranes ($1.96 \pm 0.48 \times 10^{-9}$ M and $58.8 \pm 11.0 \times 10^{-6}$ M, respectively) were comparable to those values obtained in vas deferens ($0.87 \pm 0.4 \times 10^{-9}$ M and $33.1 \pm 9.7 \times 10^{-6}$ M).

Discussion

The present study indicates that B25 (2,6-dibutylbenzylamine) has contractile properties in smooth muscle and in cardiac preparations linked to its peculiar calcium modulatory properties.

In unstimulated vas deferens this compound produced a characteristic rhythmic activity which was also detectable in guinea-pig ileum. In field-stimulated preparations, B25 also potentiated the contractility, showing ED_{50} values similar to those observed in unstimulated preparations. One of the aims of this study was to clarify the mechanism of action of this compound.

It is well known that neurogenic stimulation induces the release of a variety of neurotransmitters in the vas deferens. Among them noradenaline, acetylcholine and ATP, have been demonstrated to possess contractile activity in this preparation (Knoll *et al.*, 1972; French *et al.*, 1983; Bell *et al.*, 1984). Stimulatory effects of substance P and other neurokinins have also been described (Maggi *et al.*, 1987; Nakazawa *et al.*, 1990). In our experiments the rhythmic activity induced by B25 was unaffected by TTX or ω-CT, by pretreatment of rats with capsaicin, reserpine or by blockade of membrane receptors known to mediate contractile activity in the vas deferens. It is therefore reasonable to rule out the possibility that this compound could act by releasing endogenous mediators or by directly stimulating known postsynaptic membrane receptors in smooth muscle.

The rhythmic activity induced in the rat vas deferens by B25 appeared to be very similar to the contractile effect obtained in this preparation with different compounds (Hay & Wadsworth, 1979; 1983; Ashoori & Tomita, 1983; Mishra *et al.*, 1988). In these reports the effects of some procedures affecting calcium translocation indicate that rhythmic contractions were highly dependent upon the activation of VSCC in the membrane. These observations are in agreement with other studies in which spontaneous rhythmic activity was observed in the rat vas deferens (which is normally quiescent) during supersensitivity obtained with surgical or chemical denervation (Westfall *et al.*, 1972; Lee *et al.*, 1975) or in streptozocin-induced diabetes (Sakai & Honda, 1987). It is well known that in these experimental conditions, the Ca^{2+} channel activity in the membrane could be increased to a different extent (Sakai & Honda, 1987; Sakai *et al.*, 1989; Ferrante & Triggle, 1990). Recently Langton & Huddard (1987) studied the effects of different calcium antagonists on the rhythmic activity developed in the rat vas deferens in response to slight depolarizing K^+ solutions. Attempts to antagonize this effect with the L channel antagonist, verapamil and La^{3+} ions have been unsuccessful. Since this effect was inhibited by Mn^{2+} and was markedly potentiated by the application of tetraethylammonium HCl (TEA), these authors concluded that it was dependent on a 'selective involvement of fast calcium channel activity' in the preparation.

Consistent with these observations, most of the effects of B25 described in the present paper, i.e. the increase in Ca^{2+} uptake, the dependence of rhythmic activity upon external calcium and the shift to the left of concentration-response curves in partially depolarizing solutions, indicate that an influx of Ca^{2+}, probably dependent on direct activation of VSCC, underlies the development of this response.

Verapamil at concentrations ranging from 10^{-5} M to 5×10^{-4} M significantly potentiated the rhythmic effect of B25. We cannot explain this effect on the basis of the results shown here. However, a number of studies have shown a potentiation of contractility induced by verapamil in vas deferens (Moritoki *et al.*, 1987; Rae & Calixto, 1989; Suzuki & Gomi, 1989; Morishita & Furukawa, 1989). Interestingly this effect has also been described for rhythmic activity induced by methoxamine and $BaCl_2$ (Hay & Wadsworth, 1979; 1983). For this paradoxical stimulatory property, a weak calcium agonist and/or a membrane depolarizing activity of verapamil has been suggested.

In concentrations ranging from 10^{-6} M to 2.5×10^{-5} M, B25 evoked contractile effects in smooth muscle attributable to its Ca^{2+} agonistic properties while with higher doses ($> 5 \times 10^{-5}$ M) inhibitory effects were observed both in smooth muscle and cardiac tissues. Conversely, in the rat vas deferens, by removing B25 present at inhibitory concentrations, a complete recovery of rhythmic activity was obtained, which in turn was removed by further washing out (especially in Ca^{2+}-free solutions). Similar biphasic effects are a common observation with many calcium modulatory compounds (Schwartz *et al.*, 1984; Tomas *et al.*, 1984; Dube' *et al.*, 1985; Scott & Dolphin, 1987; Bechem *et al.*, 1988; Scott *et al.*, 1988; Brown & Birnbaumer, 1988). To explain these observations the following have been proposed: (i) the coexistence of binding sites mediating VSCC activation together with inhibitory receptors which mediate channel blockade, (ii) a modulation of Ca^{2+} channel gating mode into an opening (agonist) or inactivated (antagonist) state depending on the membrane potential and (iii) a direct stimulation of calcium channel currents through a pertussis toxin-sensitive G protein.

The results of this study seem to be in agreement with some of these hypotheses. A dissociation from the active sites resulting from two different kinetic constants may be the reason for the biphasic response of B25. However, the following observations should be considered in order to clarify the mechanism of action of this compound: (a) We did not obtain in competition experiments with [^{3}H]-nitrendipine any significant evidence for two dihydropyridine binding sites in the rat vas deferens membranes. Our results were comparable with the binding parameters previously described by Yousif *et al.* (1985) in the same preparation. (b) Both the amplitude and frequency of the rhythmic effect induced by B25 were not inhibited by verapamil at concentrations known to affect L-type calcium channels in the rat vas deferens (Hay & Wadsworth, 1982). The dihydropyridine derivative, nifedipine, had relatively little influence while La^{3+} ions were inactive. In contrast, Ni^{2+} and Mn^{2+} ions, which are reported to inhibit Ca^{2+} conductance through T-type VSCC and to suppress the fast component of the contractile response in smooth muscle associated with the opening of rapidly inactivating VSCC (Brading *et al.*, 1983; Hofmann *et al.*, 1987), strongly inhibited the rhythmic activity in the vas deferens. These results are in good agreement with the previous observations of Langton & Huddard (1987). (c) A correlation exists between the IC_{50} values obtained in competition experiments and the B25 inhibitory concentrations in isolated heart and rat vas deferens. This effect is particularly significant in ventricular preparations bathed in K^+-rich medium where histamine-restored myocardial contractility is known to be related only to the activation of L-type VSCC in the muscle.

Taken as a whole our results may suggest that low concentrations of B25 induce rhythmic contractions in rat vas deferens which may be due to opening of dihydropyridine-insensitive (T type VOCs?) calcium channels in the smooth muscle. However, it has to be pointed out that an involvement of L-type VSCC cannot be completely excluded from this effect. Additional electrophysiological and binding studies, are needed to gain more insight into this point. However, higher (inhibitory) concentrations interact with dihydropyridine-sensitive sites both in vas deferens and heart membranes.

The contractile responses evoked by field stimulation in rat

vas deferens were completely and irreversibly inhibited by 3×10^{-8} M ω-CT. As shown in previous studies, this effect is essentially prejunctional and indicate that N-type VSCC play a major role in coupling neural excitation with neurotransmitter release (Keith *et al.*, 1989).

B25 dose-dependently counteracted the inhibitory activity of ω-CT without reversing TTX effects. This observation indicates that this compound could also act as a calcium agonist in neurones, probably opening Ca^{2+} channels different from ω-CT-sensitive N-type VSCC. Regarding this point, T-type VSCC, ω-CT resistant N or L-type VSCC and other calcium exchange processes have been postulated to explain the presence in neurones of ω-CT and dihydropyridine-insensitive K^+-evoked noradrenaline release (Rasmussen & Barrett, 1984; Miller, 1987; Keith *et al.*, 1989). Another finding of the present study is that the recovery of contractions in electrically stimulated rat vas deferens pretreated with ω-CT occurs at lower B25 concentrations than those required to induce the rhythmic effect in the same preparation (Figure 4a). Because of this it clearly appears that two distinct effects characterize the contractile activity of B25, suggesting that this compound could act as a calcium agonist with a higher affinity for neural than smooth muscle tissue.

To conclude, the rhythmic effect evoked in rat vas deferens (and guinea-pig ileum) is a part of the smooth muscle functional response which, in our opinion, has been underestimated so far and could be a useful tool for evaluating VSCC activity in some preparations. In the present paper the rhythmic effect of a new smooth muscle and neurally active compound showing interesting calcium modulatory properties has been described.

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(Received November 2, 1992
Revised March 8, 1993
Accepted March 18, 1993)

Br. J. Pharmacol. (1993), **109**, 1046–1052 © Macmillan Press Ltd, 1993

The effects of α_2-adrenoceptor antagonists on the inhibition of 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI)-induced head shakes by 5-HT_{1A} receptor agonists in the mouse

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1 8-Hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), gepirone, buspirone and ipsapirone dose-dependently antagonized the head-shakes induced by 1-(2,5-dimethoxy 4-iodophenyl)-2-amino propane hydrochloride (DOI) (1.0 mg kg^{-1}) in mice, when these agents were given i.p. 10 min beforehand.

2 *para*-Chlorophenylalanine (pCPA) abolished the effect of 8-OH-DPAT (0.1 mg kg^{-1}) and of buspirone (1.0 mg kg^{-1}). (±)-Pindolol (5.0 mg kg^{-1}) also antagonized the effect of 8-OH-DPAT(0.1 mg kg^{-1}).

3 The α_2-adrenoceptor antagonists, RX811059 (1.0 mg kg^{-1}), idazoxan (0.5 mg kg^{-1}), yohimbine (1.0 mg kg^{-1}) and 1-(2-pyrimidinyl)-piperazine (1-PP) (2.0 mg kg^{-1}) i.p. prevented the antagonistic effect of 8-OH-DPAT (0.1 mg kg^{-1}) on DOI-head-shakes.

4 Orally-administered buspirone, given 60 min beforehand, only reduced DOI-head-shakes at doses of 60 mg kg^{-1} and above. However, when buspirone (1.0 mg kg^{-1}) was administered orally twice daily for 21 days, DOI-head-shakes were significantly reduced when tested 60 min after the first daily dose on days 5, 12 and 21 and 48 h after withdrawal.

5 A single oral dose of buspirone (1.0 mg kg^{-1}) strongly antagonized DOI-head-shakes when given 24 h after the last of 4 daily doses of 1-PP (2.0 mg kg^{-1}, p.o.) but had no effect on DOI-head-shakes 24 h after the last of 4 daily doses of water (p.o.).

6 A single oral dose of 1-PP (2.0 mg kg^{-1}) abolished the inhibitory effect of i.p. buspirone (1.0 mg kg^{-1}) on DOI-head-shakes in mice which had received water (p.o.) daily on the 4 previous days but not in mice which had received 1-PP (2.0 mg kg^{-1}, p.o.) on these days.

7 The ability of 5-HT_{1A} receptor agonists to antagonize DOI-head-shakes may be due to an effect at presynaptic 5-HT receptors. It is suggested that 1-PP, formed from buspirone, may act at α_2-adrenoceptors to prevent acutely administered oral buspirone from antagonizing DOI-head shakes, but that tolerance occurs to this effect of 1-PP, thus revealing the inhibitory effect of buspirone when the latter is given repeatedly.

Keywords: 5-HT_{1A} receptors; $5HT_2$ receptors; α_2-adrenoceptor antagonists; head-shakes; buspirone; ipsapirone, gepirone; 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI); 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH DPAT); 1-(2-pyrimidinyl)-piperazine (I-PP)

Introduction

The finding that some behaviours, thought to be mediated by one particular population of 5-hydroxytryptamine (5-HT) receptors, may be modulated by a different population of 5-HT receptors, is relatively novel and intriguing. A number of studies indicate the possibility of interactions between 5-HT_{1A} and 5-HT_2 receptors. The 5-$HT_{2/1C}$ receptor antagonist, ritanserin (Leysen *et al.*, 1985), enhanced hyperactivity in rats induced by the 5-HT_1 receptor agonist, RU24969 (Goodwin & Green, 1985). Ritanserin, ICI 170,809 (Blackburn *et al.*, 1988) and ketanserin (Leysen *et al.*, 1981) enhanced the behavioural syndrome induced by 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) and 5-methoxy-*N*,*N*-dimethyl-tryptamine (5-MeODMT) (Backus *et al.*, 1990). Effects of 5-HT_{1A} receptor agonists on 5-HT_2-receptor related behaviour are more controversial. Shaking behaviour in rodents, such as head-shakes (HS) and wet dog shakes, appears to be mediated by 5-HT_2 rather than 5-HT_{1C} receptors (Kennett & Curzon, 1991). The 5-HT_{1A} receptor agonists, 8-OH-DPAT, buspirone, gepirone and ipsapirone inhibited quipazine-induced HS in the rat (Yocca *et al.*, 1990); because this inhibition was seen at low doses, it was speculated that the 5-HT_{1A} receptor involved may be presynaptic with respect to 5-HT neurones. Mescaline-induced HS in mice were also antagonized by these agents (Bristow *et al.*, 1991). In contrast, buspirone, ipsapirone and gepirone increased mouse HS induced by 5-MeODMT but not those induced by 5-hydroxytryptophan (5-HTP) (Goodwin *et al.*, 1986; Moser, 1990). 1-(2,5-Dimethoxy 4-iodophenyl)-2-amino propane hydrochloride (DOI) is a 5-HT_2/5-HT_{1C} receptor agonist with a very high selectivity (Glennon, 1987). 8-OH-DPAT has been widely reported to antagonize DOI-HS in both rats and mice (Arnt & Hyttel, 1989; Heaton & Handley, 1989; Berendsen & Broekkamp, 1990; Darmani *et al.*, 1990) but the effects of other 5-HT_{1A} receptor agonists have not been investigated.

1-(2-Pyrimidinyl)-piperazine (1-PP) is the major common metabolite of the azapirones buspirone, gepirone and ipsapirone (Bianchi *et al.*, 1988). It is a potent α_2-adrenoceptor antagonist with negligible binding to 5-HT_{1A} receptors, dopamine receptors or α_1-adrenoceptors (Caccia *et al.*, 1986; Rimele *et al.*, 1987; Fozard *et al.*, 1987).

α_2-Adrenoceptor antagonists can potentiate HS caused by intracerebrally injected 5-HT and conversely, α_2-adrenoceptor agonists inhibit them (Handley & Brown, 1982) by a mechanism throught to be postsynaptic with respect to noradrenergic neurones (Bednarczyk & Vetulani, 1978).

In the present experiments, the 5-HT_{1A} receptor ligands, 8-OH-DPAT, gepirone, buspirone, ipsapirone (Hoyer, 1988), MDL 73005EF (Moser *et al.*, 1990), and the mixed 5-$HT_{1A/1B}$ receptor and β-adrenoceptor antagonist, (±)-pindolol (Nahorski & Willcocks, 1983; Hoyer, 1988), have been inves-

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tigated for their effects on HS induced in the mouse by DOI (DOI-HS). The α_2-adrenoceptor antagonists, 2-ethoxy-idazoxan (RX811059), idazoxan, yohimbine (Doxey *et al.*, 1985; Mallard *et al.*, 1991) and 1-PP (Fozard *et al.*, 1987; Bianchi & Garratini, 1988) have been examined for their effects on the ability of 8-OH-DPAT to inhibit DOI-HS. The effect of the 5-HT depleting agent *p*-chlorophenylalanine (pCPA) on this interaction has been investigated in the case of 8-OH-DPAT and buspirone. The potential contribution of 1-PP to the interaction of buspirone with DOI has been investigated.

Methods

Animals

Experiments were carried out on Aston bred male MFI mice weighing between 20–30 g, fed a breeding diet (Pilsbury's Ltd., Birmingham) and tap water *ad libitum*. The animals were exposed to an 08 h, 00 min–20 h 00 min light-dark cycle.

Behavioural tests

For head shakes determinations, groups of 3 or 4 mice (Boulton & Handley, 1973) were taken at random from stock cages which had been housed in a room adjacent to the experimental room for at least 5 days and held in a further stock cage in the experimental room overnight with free access to food and water. The experimental room was maintained at 21 ± 1°C. All the behavioural experiments were carried out between 09 h 00 min and 19 h 00 min. Mice were habituated to the observation cages (33 × 23 × 20 cm clear plastic lined with 2 cm sawdust) for 60 min before DOI injection. Both control and test mice received a submaximal (Heaton & Handley, 1989) dose of DOI (1.0 mg kg^{-1}, i.p.) 5 min before videotape recording for 6 min and head shakes were counted from videotapes. Doses were randomized between groups such that at least one mouse in each group received saline-distilled water (concurrent control) and experiments were repeated to give at least 5 animals/dose of test agent.

Test agents were given by i.p. administration (10.0 ml kg^{-1}) or by oral gavage (p.o.). Pretreatment period was 10 min for i.p. administered 8-OH-DPAT, gepirone, buspirone and ipsapirone, 30 min for i.p. idazoxan, RX811059, yohimbine, (±)-pindolol and 1-PP and 60 min for p.o. buspirone and MDL 73005EF. For chronic experiments, buspirone was administered p.o. twice daily for 21 days and DOI-HS examined in naive mice on days 5, 12 and 21, 60 min after the first daily dosing, and also on day 23, 48 h after withdrawal. Depletion of brain 5-HT was achieved by administering 3 doses of pCPA (300 mg kg^{-1}, i.p.) 24, 48 and 72 h before the experiments concerned.

Determination of 5-HT

Mice were killed by cervical dislocation. Whole brains were dissected, removed immediately, homogenized in a Potter-glass homogenizer (1 part wet tissue plus 9 parts ice-cold buffer [0.32 M sucrose, 0.1 M phosphate buffer pH 7.4, 1 mM 2-mercaptoethanol and 1 mM EDTA] and centrifuged (1000 *g*; 15 min; 4°C). The supernatants were frozen at −20°C until analysed for 5-HT content by radioimmunoassay (Gow *et al.*, 1987; Dursun & Handley, 1992).

Determination of noradrenaline (NA)

Mice were killed by cervical dislocation, whole brains were removed and frozen at −70°C until assayed for noradrenaline content by high performance liquid chromatography – electrochemical detection (h.p.l.c. – e.c.d.) as described by Marsden & Joseph (1986).

Statistics

All results are presented as means and standard errors of the means (s.e.mean). Drug effects relative to controls were analysed by *t* test, analysis of variance (ANOVA) or 2 × 2 factorial ANOVA followed by Dunnett's *t* test where necessary (Linton & Gallo, 1975). Drug potency was expressed as the ID$_{50}$ dose (dose producing 50% inhibition relative to control) together with 95% confidence limits obtained by regression analysis of the log dose-response relationship.

Drugs

Drugs were obtained from the following sources: MDL 7300-5EF (8-[2-(2,3-dihydro-1,4-benzodioxin-2-yl-methyl amino) ethyl]-8-azaspiro[4,5]decane-7,9-dionemethyl sulphonate (Merrell Dow Research Institute), DOI and 8-OH-DPAT (Research Biochemicals Inc.), buspirone and gepirone (Bristol Myers Ltd.), 1-PP (Aldrich Chemicals), idazoxan and RX811059 (α-ethoxyidazoxan; Reckitt & Colman), yohimbine and pCPA (Sigma), ipsapirone (Troponwerke) were dissolved in 0.9% (w/v) saline. (±)-Pindolol (Sigma) was dissolved in water to which 1-2 drops of glacial acetic acid had been added.

Results

Effects of 8-OH-DPAT, gepirone, buspirone and ipsapirone on DOI-HS after i.p. administration

8-OH-DPAT (0.001–5 mg kg^{-1}) dose-dependently reduced DOI-HS with an ID$_{50}$ of 0.014 (0.00035–0.51) mg kg^{-1}; similar effects were seen with buspirone (0.1–4.0 mg kg^{-1}, ID$_{50}$ = 0.659 (0.206–2.086) mg kg^{-1}); gepirone (0.1–4.0 mg kg^{-1}, ID$_{50}$ = 0.54 (0.22–1.28) mg kg^{-1}) and ipsapirone (0.1–10 mg kg^{-1}, ID$_{50}$ = 0.84 (0.19–3.72) mg kg^{-1}). None of these doses of any of these compounds produced signs of the '5-HT syndrome' except for flat body posture which was

Table 1 Effect of α_2-adrenoceptor antagonists on the ability of 8-OH-DPAT to inhibit DOI head-shakes

	Number of head-shakes				
Agents	*Sal + Sal + DOI*	*Agent + Sal + DOI*	*Sal + 8-OH-DPAT + DOI*	*Agent + 8-OH-DPAT + DOI*	*2-way ANOVA Interaction term*
RX811059 (1.0 mg kg^{-1})	17.4 ± 1.4	22.8 ± 1.9*	4.3 ± 0.7	19.8 ± 0.4**	F (1,44) = 13.51; $P<0.01$
Idazoxan (0.5 mg kg^{-1})	17.2 ± 1.8	21.2 ± 2.0	4.5 ± 1.9	15.1 ± 1.7**	F (1,36) = 4.25; $P<0.05$
1-PP (2.0 mg kg^{-1})	18.3 ± 1.6	21.5 ± 2.1	5.4 ± 0.7	16.6 ± 0.9**	F (1,44) = 6.98; $P<0.05$
Yohimbine (1.0 mg kg^{-1})	17.7 ± 1.4	21.3 ± 0.4	4.9 ± 0.8	16.5 ± 1.2**	F (1,44) = 9.30; $P<0.01$

*Significantly different from Sal + Sal + DOI ($P<0.05$), **significantly different from Sal + 8-OH-DPAT + DOI ($P<0.0001$) (Dunnett's *t* test).
Values are means ± s.e.mean.

noted after higher doses (8-OHDPAT 1.0 and 5.0 mg kg^{-1}; buspirone 4.0 mg kg^{-1}; gepirone 4.0 mg kg^{-1} and ipsapirone 5 and 10 mg kg^{-1}) only.

Effects of pCPA on the inhibition of DOI-HS by 8-OH-DPAT and buspirone

Pretreatment with pCPA did not affect DOI-HS but prevented the inhibitory effect of 8-OH-DPAT (0.1 mg kg^{-1}, i.p. 10 min before DOI) and buspirone (1.0 mg kg^{-1}, i.p. 10 min before DOI) (F interaction$_{(1,24)}$: 4.51, $P<0.05$; F interaction$_{(1,16)}$: 11.64, $P<0.01$ respectively, 2-way-ANOVA) (Figure 1). In both cases, the effect of the agonist in the presence of pCPA was not significantly different from the effect of pCPA alone ($P>0.05$). In a parallel experiment, pCPA depleted whole brain 5-HT by 71.5% (vehicle controls 712.8 ± 38.7 ng g^{-1} wet weight; pCPA treated animals 201.4 ± 38.7 ng g^{-1} wet weight); noradrenaline was not altered (controls: 396.1 ± 11.2, pCPA pretreated animals: 369 ± 12.6 ng g^{-1} wet weight).

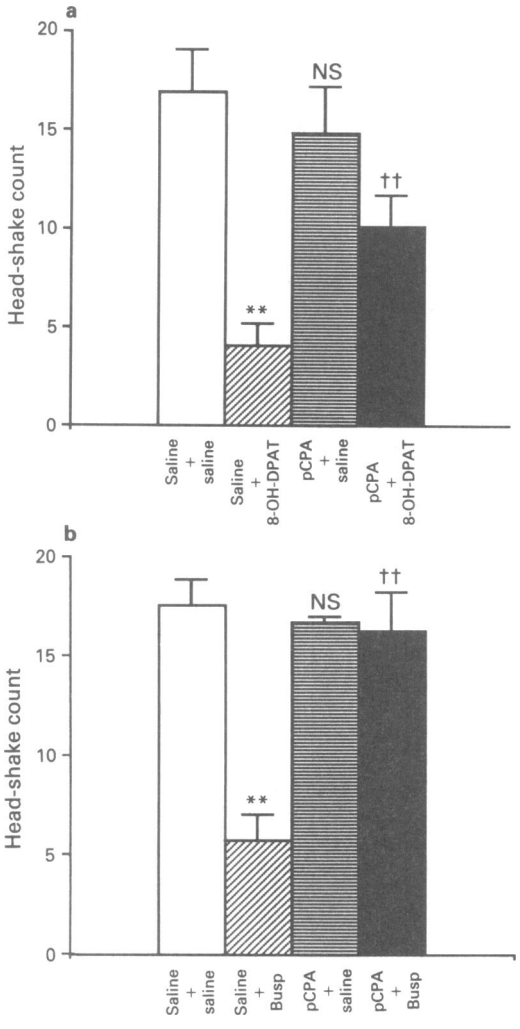


Figure 1 Effect of pCPA on the inhibition of DOI-head shakes by 8-OH-DPAT and buspirone administered i.p. pCPA 300 mg kg^{-1} or saline was administered daily for 3 days as described under Methods. All mice received DOI (1.0 mg kg^{-1}, i.p.) 10 min after saline, 8-OH-DPAT (0.1 mg kg^{-1}, i.p.) or buspirone (Busp 1.0 mg kg^{-1}, i.p.). $n=11$ per group. Values are mean ± s.e.mean. Significantly different from saline/saline (**$P<0.01$, NS, $P>0.05$). Significantly different from 8-OH-DPAT (a) or buspirone (b) alone (††$P<0.01$), (Dunnett's t test after significant 2-way ANOVA). For abbreviations, see text.

Effect of (±)-pindolol on the inhibition of DOI-HS by 8-OH-DPAT

(±)-Pindolol (5.0 mg kg^{-1}, i.p., 40 min before DOI) significantly reduced DOI-HS but also abolished the inhibitory effect of 8-OH-DPAT (0.1 mg kg^{-1}, i.p., 10 min before DOI), (F interaction$_{1,16}$: 7.91, $P<0.05$, 2-way-ANOVA, Figure 2).

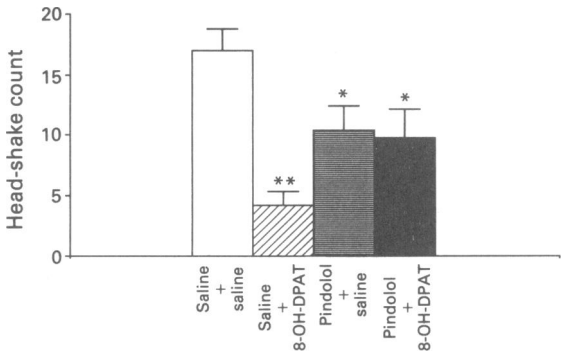


Figure 2 Effect of (±)-pindolol on the inhibition of DOI-head-shakes by 8-OH-DPAT administered i.p. (±)-Pindolol (5.0 mg kg^{-1}, i.p.) or saline was administered i.p., 30 min before 8-OH-DPAT (0.1 mg kg^{-1}, i.p). All mice received DOI (1.0 mg kg^{-1}, i.p.) 10 min later. $n=5$ per group. Values are mean ± s.e.mean. Significantly different from saline/saline (*$P<0.05$, **$P<0.01$), (Dunnett's t test after significant 2-way ANOVA).

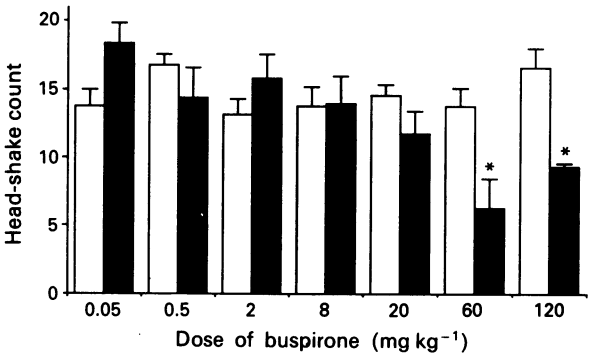


Figure 3 Effect of buspirone administered orally on DOI-head-shakes. Buspirone (solid columns) or saline (open columns) was given p.o., 60 min before DOI (1.0 mg kg^{-1}, i.p.). $n=6$ per group. Significantly different from paired control group (*$P<0.025$, Student's t test).

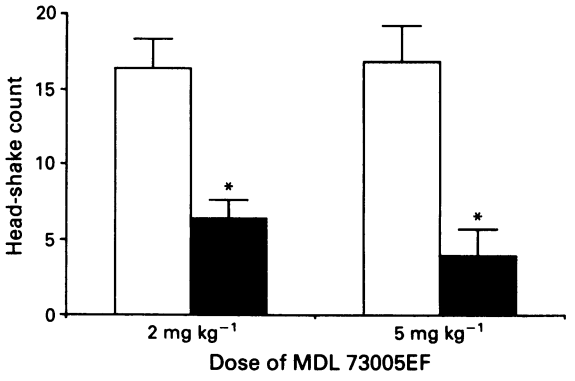


Figure 4 Effect of MDL 73005EF administered orally on DOI-head-shakes. MDL 73005EF (solid columns) or saline (open columns) was given 60 min before DOI (1.0 mg kg^{-1}, i.p.). $n=6$ per group. Values are mean ± s.e.mean. Significantly different from saline (*$P<0.005$, Student's t test).

Effects of RX811059, idazoxan, 1-PP and yohimbine on the inhibition of DOI-HS by 8-OH-DPAT

As shown in Table 1, the α_2-adrenoceptor antagonists RX811059 (1.0 mg kg^{-1}, i.p.), idazoxan (0.5 mg kg^{-1}, i.p.), 1-PP (2.0 mg kg^{-1}, i.p.) and yohimbine (1.0 mg kg^{-1}, i.p.) all potently reduced the ability of 8-OH-DPAT (0.1 mg kg^{-1}, i.p., 10 min before DOI) to suppress DOI-HS when they were administered 30 min before DOI. The significant interaction terms after 2-way ANOVA (Table 1) show that these effects were not accounted for the slight tendency of these doses of α_2-adrenoceptor antagonists to potentiate DOI-HS frequency. A range of doses of RX811059 and 1-PP were tested alone for their effects on DOI-HS. When administered i.p. 30 min before DOI, RX811059 (0.1–10 mg kg^{-1}) produced a dose-dependent increase in DOI-HS with a shallow slope. The ID_{200} value (dose to double HS frequency) was estimated by projection of the log dose-response curve obtained from linear regression to be 67 mg kg^{-1}. 1-PP (0.1–10.0 mg kg^{-1} 30 min before DOI) also weakly potentiated DOI-HS with a projected ID_{200} of 53 mg kg^{-1}. In both cases, the shallow slopes and need to project the log dose-response curves precluded calculation of confidence limits.

Effects of oral buspirone and MDL 73005EF on DOI-HS

Oral administration of buspirone at doses of 0.05–20 mg kg^{-1} (60 min before DOI) did not alter DOI-HS but 60 and 120 mg kg^{-1} buspirone reduced DOI-HS ($P<0.025$) as shown in Figure 3. Doses of 2.0 and 5.0 mg kg^{-1} (p.o.) of MDL 73005EF 60 min before DOI reduced DOI-HS (Figure 4).

Effects of chronic oral administration of buspirone on DOI-HS

Chronic administration of buspirone (1.0 mg kg^{-1} p.o. twice daily for up to 21 days) to groups of mice, as described in Methods, reduced DOI-HS relative to controls at 5, 12 and 21 days of drug administration and 48 h after the last dose on day 21 (Figure 5). DOI was administered 60 min after the first of the daily doses of buspirone. A different group was treated after each time interval, so that all groups were naive to DOI.

Effect of subchronic oral 1-PP

Water or 1-PP (2 mg kg^{-1}) was administered p.o. daily for four days. Twenty four hours after the last water pretreatment, a single low dose of buspirone, (1.0 mg kg^{-1}, p.o. 60 min before DOI), failed to antagonize DOI-HS (Figure 6), while twenty four hours after the last 1-PP (2.0 mg kg^{-1}) pretreatment, the same dose of buspirone significantly antagonized DOI-HS (Figure 6).

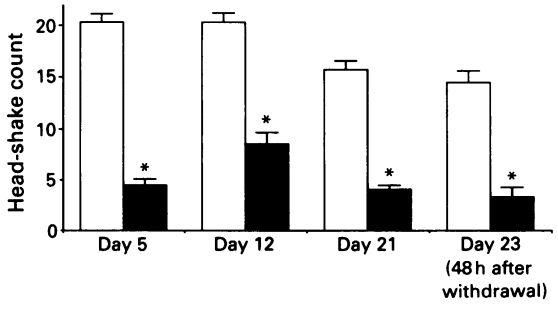


Figure 5 Effect of chronic oral administation of buspirone on DOI-head-shakes. Buspirone (1.0 mg kg^{-1}) (solid columns) or water (open columns) was given orally twice daily for 21 days. Groups of 6 naive mice received DOI (1.0 mg kg^{-1}, i.p.) on the days stated, 60 min after the 1st dose of buspirone. Values are mean ± s.e.mean. Significantly different from water on the same day (*$P<0.0005$, Student's t test).

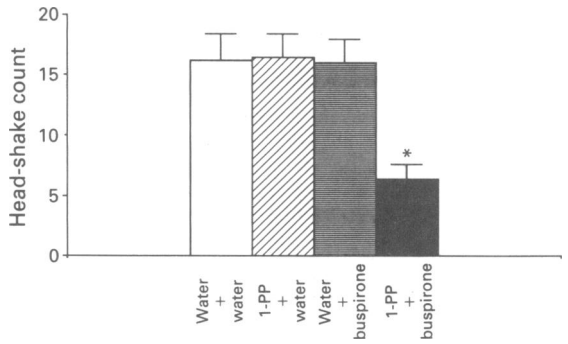


Figure 6 Effect of subchronic oral treatment with 1-PP on the ability of acute oral buspirone to antagonize DOI-head-shakes. 1-PP (2.0 mg kg^{-1}) or water was administered p.o. once daily for 4 days. On day 5, mice received water or buspirone (1.0 mg kg^{-1}, p.o.) and 10 min later DOI (1.0 mg kg^{-1}, i.p.). $n=7$ per group. Values are mean ± s.e.mean. Significantly different from water/water (*$P<0.05$, Dunnett's t test after significant 2-way ANOVA).

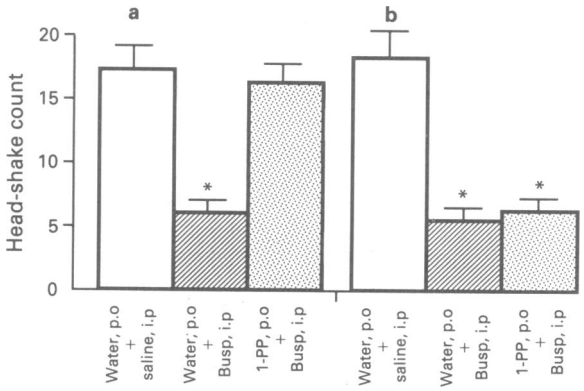


Figure 7 Effect of subchronic oral treatment with 1-PP on the ability of acute oral 1-PP to antagonize the effect of buspirone (Busp) (i.p.) on DOI-head-shakes. Water (a) or 1-PP (2.0 mg kg^{-1}, p.o.) (b) was administered once daily for 4 days. On day 5, mice received either water p.o. followed 60 min later by saline i.p. and 10 min later by DOI (1.0 mg kg^{-1}, i.p.) (open columns); water p.o. followed 60 min later by buspirone (1.0 mg kg^{-1}, i.p.) and 10 min later by DOI (1.0 mg kg^{-1}, i.p.) (hatched columns) or 1-PP (2.0 mg kg^{-1}, p.o.) followed 60 min later by buspirone (1.0 mg kg^{-1}, i.p.) and 10 min later by DOI (1.0 mg kg^{-1}, i.p.) (stippled columns). $n=6$ per group. Values are mean ± s.e.mean. Significantly different from water/water/saline/DOI (*$P<0.05$, Dunnett's t test after significant 1-way ANOVA).

In a further experiment, water or 1-PP (2 mg kg^{-1}) was again administered p.o. daily for 4 days. On the 5th day, 24 h after the last treatment, each of these groups was subdivided into 3 further groups, receiving water p.o. followed 60 min later by saline, i.p., water p.o. followed 60 min later by buspirone 1 mg kg^{-1}, i.p. or 1-PP p.o. followed 60 min later by buspirone 1 mg kg^{-1} i.p. All groups received DOI 10 min later. As shown in Figure 7, in the groups which received 4 days water pretreatment (Figure 7a), a single dose of 1-PP p.o. abolished the inhibitory effect of i.p. buspirone on DOI-HS. However, in the groups which had received 4 days 1-PP pretreatment (Figure 7b), a further oral dose of 1-PP on day 5 did not alter the inhibitory effect of buspirone.

Discussion

In the present study, 8-OH-DPAT, buspirone, gepirone and ipsapirone, agents which demonstrate selective 5-HT$_{1A}$ recep-

tor agonist activity, (Middlemiss & Fozard, 1983; Hoyer, 1988) dose-dependently attenuated DOI-HS when administered i.p. with a short pretreatment time. ID_{50} values obtained were in agreement with the potency of these agents against quipazine-induced head shakes in the rat (Yocca *et al.*, 1990). Antagonism of DOI-HS was not likely to have been due to occurrence of the '5-HT syndrome', since the only sign of this syndrome observed was flat body posture at doses well above the ID_{50} values for inhibition of DOI-HS.

Pretreatment with (±)-pindolol alone produced a significant inhibition of DOI-HS. This effect could have been mediated by stimulation of either 5-HT_{1A} or 5-HT_{1B} receptors since (±)-pindolol has little affinity for 5-HT_2 binding sites (Engel *et al.*, 1986) but acts as a partial agonist at both 5-HT_{1A} and 5-HT_{1B} receptors (Engel *et al.*, 1986; Yocca *et al.*, 1990). (±)-Pindolol also reduced the inhibition of DOI-HS by 8-OH-DPAT to the level produced by (±)-pindolol alone. This effect is likely to be due to the 5-HT_{1A} receptor antagonist action of (±)-pindolol, since 8-OH-DPAT has negligible affinity for 5-HT_{1B} receptors (Hoyer, 1988).

Thus, all the 5-HT_{1A} ligands tested strongly inhibited 5-HT_2-receptor-mediated head shakes. The antagonism of the buspirone and 8-OH-DPAT effect by pCPA supports the hypothesis that this effect of 5-HT_{1A} receptor agonists is mediated by the inhibitory 5-HT_{1A} autoreceptors which reside on 5-HT cell bodies (Yocca *et al.*, 1990). However, why 5-HT depletion with pCPA did not itself reduce the frequency of DOI-HS remains enigmatic.

In pursuit of a mechanism by which an apparently presynaptic drug effect on 5-HT_{1A} receptors could influence a response thought to be due to postsynaptic 5-HT_2 receptors (Heal *et al.*, 1986; Kennett & Curzon, 1991), it seemed worthwhile to investigate the possibility of a noradrenergic link. There is considerable evidence for anatomical (Fuxe, 1965; Baraban & Aghajanian, 1981) biochemical (Gothert & Huth, 1980; Gothert *et al.*, 1981; Limberger *et al.*, 1986) and behavioural (Handley & Brown, 1982; Nimgaonkar *et al.*, 1983; Heal *et al.*, 1986) interactions between 5-HT and noradrenaline systems.

RX811059 and 1-PP appeared to have only weak ability to potentiate DOI-HS. This contrasts with other findings where a low dose of 1-PP (2 mg kg^{-1}, i.p.) quadrupled the response to 5-MeODMT (Moser, 1990); the same dose however had no effect on the response to 5-HTP (Moser, 1990). At doses which were without significant effect on DOI-HS when given alone, the α_2-adrenoceptor antagonists, idazoxan, 1-PP and yohimbine reduced the ability of 8-OH-DPAT to suppress DOI-HS. RX811059 also antagonized 8-OH-DPAT but in this case the dose chosen slightly but significantly potentiated DOI-HS in this experiment. However, for all four α_2-adrenoceptor antagonists, the significant interaction terms after analysis of variance indicated that their ability to antagonize 8-OH-DPAT was not accounted for by any ability to increase DOI-HS when given alone. α_2-Adrenoceptors therefore appear to influence this 5-HT_{1A}/5-HT_2 receptor interaction. The location of these α_2-adrenoceptors is unknown. The inhibitory effect of α_2-adrenoceptor agonists against 5-HTP HS and wet-dog shakes in the rat was not prevented by noradrenaline depletion, indicating that the receptors involved may be postsynaptic to noradrenaline neurones (Bednarczyk & Vetulani, 1978). There is also a noradrenergic input to dorsal raphe nucleus (DRN) and it has been shown that α_2-adrenoceptor agonists decrease, while the corresponding antagonists increase DRN 5-HT neuronal firing (Svensson *et al.*, 1975; Garratt *et al.*, 1991). Conversely, the DRN supresses locus coeruleus (LC) neurones by a 5-HT_2-receptor dependent mechanism, although these 5-HT_2 receptors are not located in LC (Gorea *et al.*, 1991).

Injection route and/or pretreatment interval had a major effect on the ability of buspirone to reduce DOI-HS. When given by the i.p. route 10 min beforehand, buspirone powerfully reduced DOI-HS with an ID_{50} of 0.66 mg kg^{-1}, but when given orally, with a 60 min pretreatment interval, buspirone produced a significant effect only at doses of 60 mg kg^{-1} and above. However, by the 5th day of treatment with oral buspirone 1.0 mg kg^{-1} twice daily, DOI-HS were strongly inhibited and this effect was maintained throughout 21 days of buspirone treatment and 48 h of buspirone withdrawal.

Buspirone, like gepirone and ipsapirone, is extensively metabolized to 1-PP in both man and rats (Caccia *et al.*, 1985; 1986; Bianchi *et al.*, 1988). After 1.0 mg kg^{-1} buspirone was given orally to rats, buspirone was not detectable in plasma and brain, but maximal concentrations of 1-PP (0.15 nmol ml^{-1} in plasma and 0.70 nmol ml^{-1} in brain) were found 15–30 min after dosing and the brain: plasma ratio was 5:1 (Caccia *et al.*, 1986). In view of the ability of 1-PP and other α_2-adrenoceptor antagonists to inhibit the effect of 8-OH-DPAT, it appeared possible that the ineffectiveness of acute oral buspirone was due to the formation of 1-PP. The ability of chronic, but not acute, oral buspirone to inhibit DOI-HS could further have been due to the development of tolerance to 1-PP. These hypotheses were therefore investigated.

Twenty four hours after the last of 4 daily doses of oral 1-PP administration, a previously ineffective oral dose of buspirone (1.0 mg kg^{-1}) now induced potent inhibition of DOI-HS. That this was likely to have been due to the development of tolerance to 1-PP was established by a further experiment. It was confirmed that a single oral dose of 1-PP abolished the ability of i.p. buspirone to antagonize DOI-HS in a control group receiving four days subchronic oral water pretreatment. In contrast, the same oral dose of 1-PP did not alter the inhibitory effect of i.p. buspirone in mice which had received daily doses of 1-PP on the four previous days. Thus, subchronic 1-PP induced tolerance to the ability of 1-PP to antagonize the effects of both oral and i.p. buspirone. In addition, orally administered MDL 7300EF, which is not metabolized to 1-PP (Moser *et al.*, 1990) inhibited DOI-HS after acute treatment.

It thus appears likely that the initial resistance of DOI-HS to oral buspirone was indeed due to formation of 1-PP, and that tolerance developed to the effect of 1-PP during chronic oral administration of buspirone. Once developed, the inhibitory effect of oral buspirone was maintained from the 5th to the 21st day of treatment and 48 h into buspirone withdrawal. It would seem that either the 5-HT_{1A} receptors responsible for this effect did not down-regulate, or the 5-HT_2 receptors responsible for DOI-HS did down regulate. These two possibilities are not mutually exclusive. The antagonism of 8-OH-DPAT and buspirone by pCPA suggests that that inhibitory effect of 5-HT_{1A} receptor agonists on 5-HT_2 receptor mediated head shakes requires intact 5-HT transmission. This implies that the 5-HT_{1A} receptors involved may be located presynaptically. It is controversial whether or not 5-HT_{1A} cell-body autoreceptors adapt to repeated treatment with 5-HT_{1A} receptor agonists. Electrophysiological experiments have suggested that the ability of 5-HT_{1A} receptor agonists to silence raphe firing has disappeared following 14 days treatment with gepirone (Blier & de Montigny, 1987), ipsapirone (Schechter *et al.*, 1990) or tandospirone (Godbout *et al.*, 1991). However, *in vivo* dialysis indicated that 5-HT release was still reduced after chronic treatment with 8-OH-DPAT, ipsapirone or buspirone (for review, see Sharp & Hjorth, 1992).

The ability of 5-HT_{1A} receptor agonists to inhibit mouse HS may depend on the inducing agent used. Thus, there is a clear difference in the inhibitory effects of these agents on HS induced by DOI (present results) or by mescaline (Bristow *et al.*, 1991) and this ability to potentiate HS induced by 5-MeODMT (Goodwin *et al.*, 1986; Moser, 1990). While it is possible that this difference may reflect different pretreatment protocols used (Goodwin *et al.*, 1986, used 75 min i.p. and Moser 30 min s.c. pretreatment), the reasons for the discrepancy remain unclear.

The present findings indicate that 5-HT_{1A} agonists inhibit HS

induced by DOI and that the receptors involved may be presynaptic. The effects of α_2-adrenoceptor antagonists in blocking this interaction may have implications for the mechanism of the anxiolytic and antidepressant (Goldberg & Finnerty, 1979; Schweizer *et al.*, 1986) actions of 5-HT_{1A} agonists which are metabolized to 1-PP, particularly since this group of agents is marked by a delay in the appearance of their clinical effects (Schweizer & Rickels, 1991).

We thank the Tourette Syndrome Association of America for financial support. We thank Merrell Dow, Bristol Myers, Reckitt & Colman and Troponwerke for gifts of drugs.

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(Received February 8, 1993
Revised March 17, 1993
Accepted March 18, 1993)

Br. J. Pharmacol. (1993), **109**, 1053–1058 © Macmillan Press Ltd, 1993

β_1- and β_2-adrenoceptor-mediated relaxation in human internal mammary artery and saphenous vein: unchanged β- and α-adrenoceptor responsiveness after chronic β_1-adrenoceptor blockade

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1 We have recently reported that patients taking β_1-adrenoceptor-selective antagonists exhibit marked sensitization of β_2-adrenoceptor responses but unaltered β_1-adrenoceptor responses in the heart, both *in vitro* and *in vivo*. We therefore investigated β_1- and β_2-adrenoceptor-mediated relaxant responses in rings of human internal mammary artery and saphenous vein without endothelium, taken from β_1-blocked and non-β-blocked patients undergoing coronary artery bypass graft surgery, for comparison. We also examined α_1-adrenoceptor-mediated contraction in these vessels, to determine whether β_1-blockade had any cross-regulatory effect.

2 Following α-blockade with 10 μM phenoxybenzamine, both noradrenaline and adrenaline produced concentration-dependent relaxations in both blood vessels, their effects being mediated predominantly through β_2-adrenoceptors; a lesser β_1-adrenoceptor component to relaxation was also found in internal mammary artery and a minor β_1-adrenoceptor component was present in saphenous vein. No differences were found in β_1- or in β_2-adrenoceptor-mediated vasorelaxation between β_1-blocked and non-β-blocked patients.

3 Methoxamine produced concentration-dependent contractions in both blood vessels, and the potency and efficacy were not significantly different between vessels from β_1-blocked and from non-β-blocked patients.

4 These findings indicate that, in these tissues, which possess a relatively minor β_1-adrenoceptor component in contrast to myocardial tissue, chronic β_1-blocker treatment does not alter either β_1- or β_2-adrenoceptor responses. Likewise, in such tissues, α_1-adrenoceptor responses are unaffected by prior β_1-blockade.

Keywords: β_1-adrenoceptor antagonism; α_1-adrenoceptors; β-adrenoceptor subtypes; human internal mammary artery; human saphenous vein

Introduction

Human heart expresses both β_1- and β_2-adrenoceptors, and stimulation of either of these receptor subtypes can mediate increases in heart rate (Hall *et al.*, 1989) and contractility (Gille *et al.*, 1985; Zerkowski *et al.*, 1986; Kaumann & Lemoine, 1987; Lemoine *et al.*, 1988). When compared with other species, human heart is unusual in possessing a relatively high percentage of β_2-adrenoceptors, approximately 30% of the total β-adrenoceptor population (Buxton *et al.*, 1987). We and others have recently shown, both *in vitro* and *in vivo*, that myocardial β_2-adrenoceptor responses are markedly enhanced in patients and also in healthy volunteers receiving long term β_1-selective antagonists; this β_2-adrenoceptor sensitization occurs with no associated change in β_1-adrenoceptor responses (Motomura *et al.*, 1990; Hall *et al.*, 1990; 1991; 1993; Ferro *et al.*, 1991). At present, the cellular mechanism of this adaptive response is not clear, but an enhanced coupling of the β_2-adrenoceptor to adenylyl cyclase has been suggested (Hall *et al.*, 1990). There is an increase in the density of β_1-adrenoceptors, with no change in β_2-adrenoceptor density, in β_1-blocked patients (Michel *et al.*, 1988; Brodde *et al.*, 1992). The apparent affinity of β_2-adrenoceptors for the partial agonist salbutamol is unaltered in β_1-blocked patients; also, the sensitivity of right atrial strips to the adenosine 3':5'-cyclic monophosphate (cyclic AMP) analogue, dibutyryl cyclic AMP is not different following β_1-blockade (Hall *et al.*, 1990). Thus, a likely explanation for this phenomenon is a change in the amount or function of the guanine nucleotide-binding proteins (G-proteins) that couple the β_2-adrenoceptor to adenylyl cyclase.

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Human internal mammary artery (IMA) and saphenous vein (SV) can both undergo β-adrenoceptor-mediated vasorelaxation. Other workers have suggested that the β-adrenoceptors in both of these tissues are predominantly, if not exclusively, of the β_2 subtype (Ikezono *et al.*, 1987; Molenaar *et al.*, 1988; He *et al.*, 1989). Our prediction was that no sensitization by β_1-blockade should occur in these tissues in the absence of functional β_1-adrenoceptors, since there would be no cross-talk between the two receptor subtypes. However, IMA has recently been shown to possess a significant population of β_1-adrenoceptors by autoradiography (Dashwood, M., personal communication). We therefore investigated β-adrenoceptor-mediated vasodilatation in IMA and SV, in both β_1-blocked and non-β-blocked patients, firstly to determine the functional contributions of β_1- and β_2-adrenoceptors to vasodilatation, and secondly to ascertain whether β_2-adrenoceptor sensitization occurs in response to β_1-blockers.

Methods

Patients

Samples of left IMA and SV were obtained from patients undergoing coronary artery bypass graft surgery. Premedication was with papaveretum and hyoscine; anaesthesia was induced with midazolam, fentanyl and propofol, with pancuronium as muscle relaxant. Propofol infusion was used for maintenance of anaesthesia, and papaveretum infusion and heparin were added following the institution of cardiopul-

monary bypass. All patients suffered from ischaemic heart disease, of varying severity, confirmed by coronary angiography, and some had coexisting valvular (mitral or aortic) disease. For the purposes of analysis, patients were divided into 2 groups, namely those taking β_1-blocker therapy (atenolol, metoprolol or bisoprolol) and those not taking β-blockers. The 2 groups were evenly matched in terms of age, sex, other drug therapy (nitrates, calcium channel antagonists, diuretics, angiotensin converting enzyme inhibitors, and oral hypoglycaemics) and clinical assessment of myocardial function. All drugs, including β-blockers, were taken until and including day of operation.

Preparation of rings of internal mammary artery and saphenous vein

The inferior portion of the IMA, including its bifurcation, was excised at the time of its anastomosis to the diseased coronary artery at operation; the excised segment was placed in an oxygenated modified Krebs solution of the following composition (mM): Na^+ 125, K^+ 5, Ca^{2+} 2.25, Mg^{2+} 0.5, Cl^- 98.5, SO_4^{2-} 0.5, HCO_3^- 32, HPO_4^{2-} 1, and EDTA 0.04. Where SV grafts were inserted, that portion of vein which remained unused at the end of the operation was similarly collected and placed in oxygenated modified Krebs solution (composition as above).

The tissues were dissected within 1 h of collection, the surrounding fat and connective tissue being removed. The endothelium was rubbed off by passing a metal rod up and down the vessel lumen gently. Both the IMA and the SV were then cut precisely into 2 mm-long rings.

Each vessel ring was placed over two L-shaped hooks, the upper hook being attached to a Swema SG4-45 strain-gauge transducer, and mounted in a 50 ml organ-bath maintained at 37°C and containing a modified Krebs solution as above, supplemented with (mM): Na^+ 15, fumarate 5, pyruvate 5, L-glutamate 5, and glucose 10. This bathing medium was constantly bubbled with 95% O_2 and 5% CO_2.

Rings were stretched to a tension of 20 mN and left to equilibrate. Their viability was then assessed after 30 min by measuring the contractile response to 45 mM KCl. Repeated contractions to 45 mM KCl were elicited, with washouts in between, until stable reproducible contractions were obtained.

Relaxant responses of internal mammary artery and saphenous vein rings to catecholamines

Rings were incubated with phenoxybenzamine 10 μM for 2 h, in order to block α-adrenoceptors irreversibly and to inhibit uptake of catecholamines. Tissues were then washed several times, and left to incubate overnight; they were then incubated with one of the following combinations of β-adrenoceptor antagonists: (a) 300 nM CGP 20712A (a selective β_1-adrenoceptor antagonist); (b) 50 nM ICI 118551 (a selective β_2-adrenoceptor antagonist); (c) both; (d) neither. The concentrations of CGP 20712A and ICI 118551 were chosen to produce selective blockade of β_1- and β_2-adrenoceptors, respectively (Kaumann & Lemoine, 1987; Hall *et al.*, 1990). Incubation with these blockers was for 1 h, in order to achieve equilibrium blockade. Submaximal contraction was then elicited with 25 mM KCl, prior to determining a relaxation concentration-effect curve to either noradrenaline or adrenaline. Thus, concentration-effect curves to catecholamines were determined, either with no β-blockade or with subtype-selective β-blockade (β_1- or β_2-adrenoceptors or both). At the end of the catecholamine concentration-effect curve, sodium nitroprusside (SNP) 0.1 mM was added, in order to assess maximum vasorelaxation. Catecholamine-induced relaxations were determined in terms of absolute force, and then expressed as a percentage of SNP-induced relaxation.

Responses of internal mammary artery and saphenous vein rings to methoxamine

Cumulative concentration-effect curves were determined to the selective α_1-adrenoceptor agonist, methoxamine. Contractile responses to this drug were determined in terms of absolute force, and then expressed as a percentage of the contractile responses to 45 mM KCl for each blood vessel ring.

Statistics

All data are expressed as mean ± s.e.mean. Concentration-effect curves to catecholamines in the presence of the different combinations of β-adrenoceptor antagonists were analysed by determining the logarithm of the molar concentration of catecholamine which produced 20% of the response to 0.1 mM SNP ($-\log EC_{20}$), and comparing these by analysis of variance. Concentration-effect curves for methoxamine in vessels from β_1-blocked and non-β-blocked patients were compared by calculating the logarithm of the methoxamine concentration required to produce half-maximal response ($-\log EC_{50}$), and also the maximum contraction (E_{max}) obtained (expressed as a percentage of the contraction elicited by 45 mM KCl); these were compared by unpaired Student's t test. Concentration-effect curves for adrenaline, mediated through β_2-adrenoceptors, in vessels from β_1-blocked and non-β-blocked patients were compared by calculating the $-\log EC_{50}$ for adrenaline in the presence of CGP 20712A, and also the maximum relaxation (E_{max}) obtained (expressed as a percentage of the relaxation induced by 0.1 mM SNP); again, these were compared by unpaired Student's t test. In all cases, a P value of <0.05 was taken as statistically significant.

Materials

CGP 20712A (1-[2{3-carbamoyl-4-hydroxyphenoxy}-ethylamino]-3-[4-{1-methyl-4-trifluoromethyl 2-imidazolyl}phenoxy]-2-propanol methane sulphonate) was from CIBA-Geigy Pharmaceuticals, and ICI 118551-HCl (erythro[±]-1-[7-methylindan-4-yloxy]-3-isopropylamino-butan-2-ol) from ICI Pharmaceuticals. Phenoxybenzamine HCl was from Smith, Kline & French Laboratories Ltd. All other chemicals were from Sigma Chemical Company Ltd. The catecholamines used (noradrenaline and adrenaline) were the (−)-isomers and bitartrate salts.

Results

Responses of internal mammary artery and saphenous vein to catecholamines

Figure 1 shows the decrease in tone in two rings of IMA, in response to half log unit incremental concentrations of adrenaline in the presence of the β_1-adrenoceptor antagonist, CGP 20712A and of noradrenaline in the presence of the β_2-adrenoceptor antagonist, ICI 118551. It is noteworthy that there was a considerable amount of resting tone, as the catecholamines and SNP produced relaxation in the vessel rings which was greater than the contraction induced by KCl, so that the tone fell below baseline. Concentration-effect curves to adrenaline and to noradrenaline were determined in the presence or absence of CGP 20712A and ICI 118551 (Figures 2 and 3). The mean concentration-effect curve to adrenaline (for both IMA and SV) was not shifted by CGP 20712A, whereas there was approximately a 2.5 log unit increase in the EC_{20} by ICI 118551; both antagonists in combination produced a shift to the right that was not significantly greater than that to ICI 118551 alone. Noradrenaline relaxed both IMA and SV with significantly lower potency and efficacy than adrenaline. Each of the

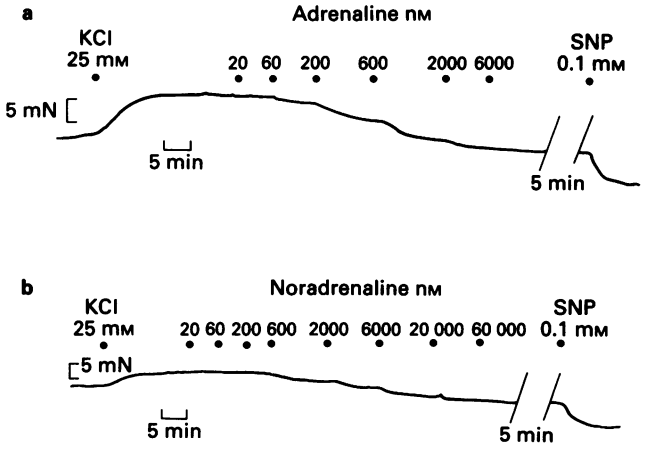


Figure 1 Tracings of developed tension in rings of internal mammary artery. The vessel rings contract following addition of 25 mM KCl, and subsequently there is a concentration-dependent relaxation in response to increasing concentrations of catecholamines. Concentration-effect curve to (a) adrenaline in the presence of 300 nM CGP 20712A, (b) noradrenaline in the presence of 50 nM ICI 118551. At the end of each experiment, there is a further relaxation following the addition of 0.1 mM sodium nitroprusside.

β-adrenoceptor subtype-selective antagonists caused a right shift in the concentration-effect curve for IMA, with a significant further shift being achieved by the combination of both blockers ($P = 0.006$); for SV, there was no significant right-shift by CGP 20712A, but there was a right-shift by ICI 118551, and a considerably larger shift by both antagonists in combination ($P = 0.034$). Table 1 shows the EC_{20} values for the catecholamines in the presence of the various combinations of β-adrenoceptor subtype-selective antagonists, in IMA and SV.

Mean $-\log EC_{50}$ and mean E_{max} values for adrenaline in the presence of CGP 20712A in IMA and SV, for non-β-blocked and for β_1-blocked patients, are displayed in Table 2. There was no significant difference in β_2-adrenoceptor responses between these groups of patients, in either IMA or SV. Nor was any significant difference found in β_1-adrenoceptor responses (i.e. in mean $-\log EC_{50}$ and mean E_{max} values for noradrenaline in the presence of ICI 118551) in either tissue between the two groups of patients (results not shown).

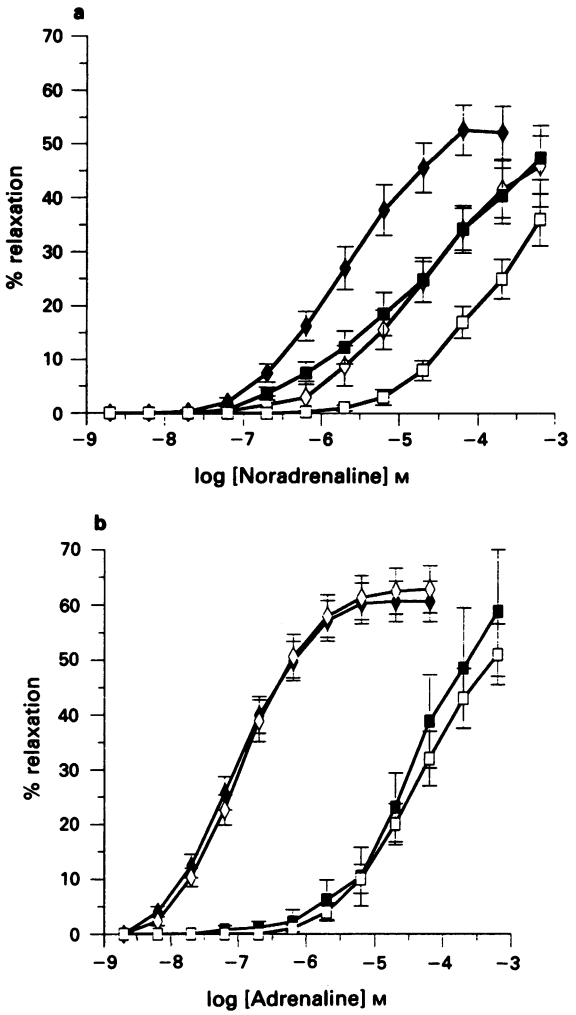


Figure 2 Relaxant effects of (a) noradrenaline and (b) adrenaline on isolated rings of human internal mammary artery, from control patients and patients chronically treated with β_1-adrenoceptor selective antagonists. Values plotted are mean ± s.e.mean. Concentration-effect curves are shown in the presence of no antagonist (◆) ($n = 15$ for noradrenaline, $n = 15$ for adrenaline), in the presence of CGP 20712A (◇) ($n = 5$ for noradrenaline, $n = 17$ for adrenaline), in the presence of ICI 118551 (■) ($n = 17$ for noradrenaline, $n = 5$ for adrenaline), and in the presence of both antagonists (□) ($n = 10$ for noradrenaline, $n = 10$ for adrenaline).

Table 1 Relaxant potencies of catecholamines and effects of subtype-selective β-blockers

		$-\log EC_{20}$ (M)		
Internal mammary artery	*+ 0*	*+ CGP 20712A* (300 nM)	*+ ICI 118551* (50 nM)	*+ Both*
Noradrenaline	6.1 ± 0.1	5.0 ± 0.2*	5.1 ± 0.2*	4.1 ± 0.1*†
(*n*)	(15)	(5)	(17)	(10)
Adrenaline	7.4 ± 0.1	7.3 ± 0.1	4.9 ± 0.2*	4.8 ± 0.1*
(*n*)	(15)	(17)	(5)	(10)
Saphenous vein	*+ 0*	*+ CGP 20712A* (300 nM)	*+ ICI 118551* (50 nM)	*+ Both*
Noradrenaline	6.1 ± 0.1	6.1 ± 0.1	5.4 ± 0.3*	3.9 ± 0.3*†
(*n*)	(13)	(6)	(13)	(10)
Adrenaline	7.6 ± 0.1	7.5 ± 0.1	5.3 ± 0.5*	4.9 ± 0.4*
(*n*)	(12)	(18)	(9)	(10)

Values are mean ± s.e.mean.
*$P < 0.05$ as compared with values in absence of antagonists.
†$P < 0.05$ as compared with values in presence of 50 nM ICI 118551 alone.

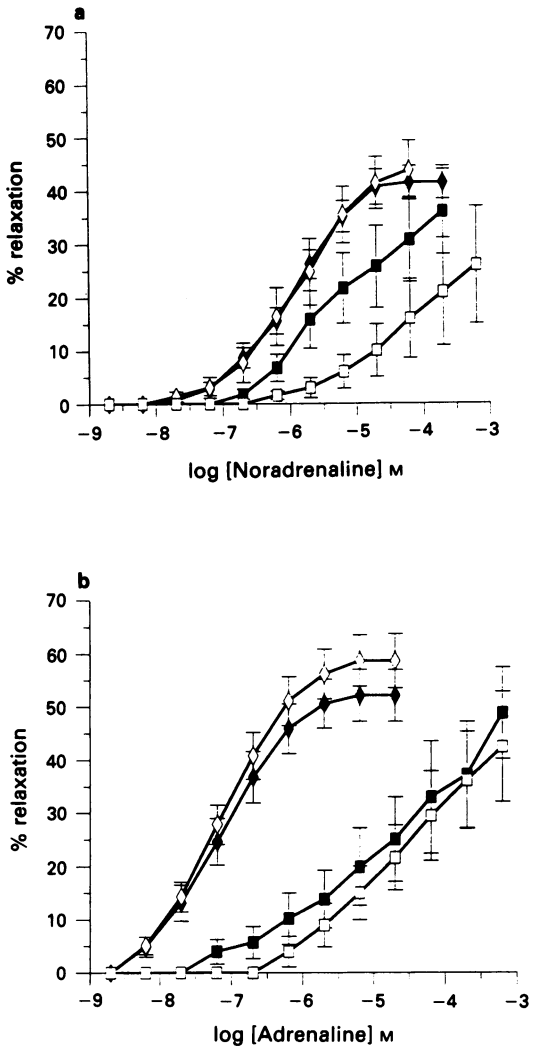


Figure 3 Relaxant effects of (a) noradrenaline and (b) adrenaline on isolated rings of human saphenous vein, from control patients and patients chronically treated with β_1-adrenoceptor selective antagonists. Values plotted are mean ± s.e.mean. Concentration-effect curves are shown in the presence of no antagonist (◆) (n = 13 for noradrenaline, n = 12 for adrenaline), in the presence of CGP 20712A (◇) (n = 6 for noradrenaline, n = 18 for adrenaline), in the presence of ICI 118551 (■) (n = 13 for noradrenaline, n = 9 for adrenaline), and in the presence of both antagonists (□) (n = 10 for noradrenaline, n = 10 for adrenaline).

Table 2 Relaxant potencies and efficacies of adrenaline, in the presence of 300 nM CGP 20712A

Internal mammary artery	*− log EC_{50}* (M)	*E_{max} (% 0.1 mM SNP)*
Non-β-blocked (n = 8)	6.9 ± 0.1	61.1 ± 7.6
β_1-blocked (n = 9)	6.8 ± 0.1	64.2 ± 4.7
Saphenous vein	*− log EC_{50}* (M)	*E_{max} (% 0.1 mM SNP)*
Non-β-blocked (n = 7)	7.0 ± 0.1	66.7 ± 9.7
β_1-blocked (n = 11)	7.1 ± 0.1	55.7 ± 6.2

Values are mean ± s.e.mean.
All values were not significantly different between β_1-blocked and non-β-blocked patients.

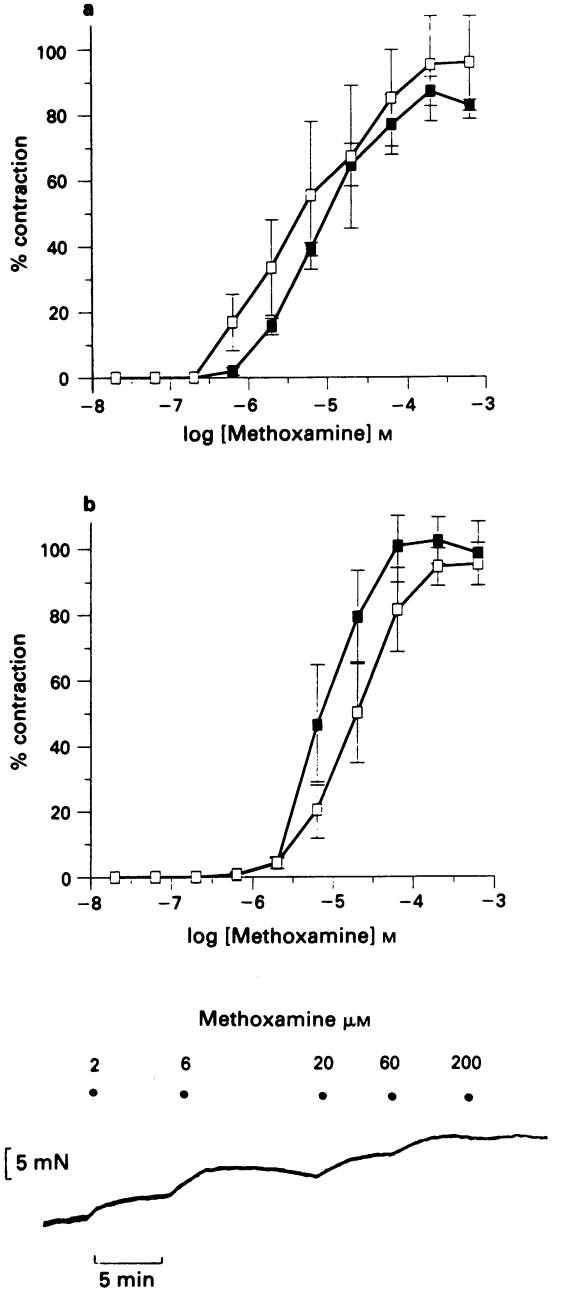


Figure 4 Contractile effects of methoxamine on isolated rings of (a) internal mammary artery (IMA) and (b) saphenous vein (SV), from control patients and patients chronically treated with β_1-adrenoceptor selective antagonists. Values plotted are mean ± s.e.mean. Concentration-effect curves are shown in control patients (■) (n = 5 for IMA, n = 7 for SV) and in β_1-blocked patients (□) (n = 5 for IMA, n = 12 for SV). Inset: Tracing of developed tension in a ring of IMA. The vessel contracts in a concentration-dependent manner in response to increasing concentrations of methoxamine.

Responses of internal mammary artery and saphenous vein to methoxamine

Concentration-effect curves to methoxamine for vascular rings from non-β-blocked and from β_1-blocked patients were compared (Figure 4). For both IMA and SV, there was no significant difference in the concentration-effect curve for non-β-blocked patients as compared with β_1-blocked patients. Table 3 shows the mean − log EC_{50} and mean E_{max} for methoxamine in IMA and SV, for non-β-blocked and for β_1-blocked patients.

Table 3 Contractile potencies and efficacies of methoxamine

Internal mammary artery	$-\log EC_{50}$ (M)	E_{max} (% 45 mM KCl)
Non-β-blocked ($n = 5$)	5.0 ± 0.1	85.1 ± 1.7
β_1-blocked ($n = 5$)	5.0 ± 0.3	87.5 ± 14.7
Saphenous vein	$-\log EC_{50}$ (M)	E_{max} (% 45 mM KCl)
Non-β-blocked ($n = 7$)	4.5 ± 0.2	100.0 ± 7.1
β_1-blocked ($n = 12$)	4.4 ± 0.1	97.7 ± 6.5

Values are mean ± s.e.mean.
All values were not significantly different between β_1-blocked and non-β-blocked patients.

Discussion

In both IMA and SV, we have found that β_2-adrenoceptor stimulation causes vasorelaxation; in addition, β_1-adrenoceptor stimulation can cause vasorelaxation in IMA, and to a lesser extent in SV. In both blood vessels, the concentration-effect curves to adrenaline in the presence of CGP 20712A (β_2-adrenoceptor responses) were not different for β_1-blocked and non-β-blocked patients. Furthermore, no significant differences in α_1-adrenoceptor vasoconstriction could be detected between the two groups of patients, as shown by the concentration-response curves to methoxamine, or in β_1-adrenoceptor responses, as demonstrated by the concentration-response curves to noradrenaline in the presence of ICI 118551. From these results, we may infer that the cross-sensitization by long term β_1-blockade on β_2-adrenoceptor responses which we have previously observed in heart is not a general phenomenon in all tissues, probably being limited to those with a dominant β_1-adrenoceptor population.

In several previous studies, both in the organ-bath and *in vivo*, we have found potentiation by β_1-blockade of β_2-adrenoceptor responses in atrial tissue (Hall *et al.*, 1990; 1991; 1993; Ferro *et al.*, 1991). We did not, therefore, repeat these observations on parallel atrial strips from the patients whose vascular tissue was obtained in the present study, as the supply of tissue is limited.

In our previous studies, strips of right atrium, taken from patients on treatment with β_1-adrenoceptor-selective antagonists, exhibited significantly enhanced β_2-adrenoceptor responses, with no concomitant alteration in β_1-adrenoceptor responses to agonists. The sensitivity of the contractile response to adrenaline was increased, and both the sensitivity (EC_{50}) and the maximum response to salbutamol were increased (Hall *et al.*, 1990). Because it is the coupling of receptor occupation to contraction which appears to be enhanced, we are currently investigating whether the β_2-adrenoceptor sensitization effect of β_1-blockade occurs by means of changes at the G-protein level, with a change in either the proportion of Gs/Gi or the subtypes within these two classes of G-protein. So far, however, we have shown no significant changes in either Gsα or Giα (Brown *et al.*, 1992) or their corresponding messenger RNA levels (Jia *et al.*, 1993), in atria from β_1- blocked patients as compared with those from non-β-blocked patients.

In order for β_2-adrenoceptor sensitization to occur at the cellular level in β_1-blocked patients, the cell should possess both β_1- and β_2-adrenoceptors; were the cell to express β_2-adrenoceptors alone, no such adaptive response would be expected. β_2-Adrenoceptor sensitization would not be predicted to occur in tissues where β_1-adrenoceptors are absent, or in tissues expressing a relatively low β_1/β_2 ratio. We selected human IMA and SV as two such tissues, which were believed to possess functional β_2- but not β_1-adrenoceptors. Our objectives were firstly to determine the relative contributions of β_1- and β_2-adrenoceptors to catecholamine-induced vasorelaxation, and secondly to establish whether or not the β_2-adrenoceptor response is sensitized in β_1-blocked as compared with non-β-blocked patients. Our results suggest that there is a β_1-adrenoceptor component to noradrenaline-induced vasorelaxation in IMA, but the β_2-adrenoceptor component is more important, especially for adrenaline. In the case of SV, there is a β_1-adrenoceptor component, but it is smaller by comparison. Furthermore, neither vessel exhibits β_2-adrenoceptor sensitization in response to long term β_1-blockade.

β_1- and β_2-adrenoceptors are known to coexist in individual human cardiomyocytes (del Monte *et al.*, 1993). However, it is not known whether these two receptor subtypes coexist in vascular smooth muscle cells. It is possible that, in human IMA and SV, β_1- and β_2-adrenoceptors are expressed in different myocytes, since the cells nearer the adventitia are more likely to be activated by noradrenaline released from sympathetic nerve endings, whilst cells nearer the intima may be activated preferentially by circulating adrenaline. In this case, receptor cross-sensitization would not be expected in blood vessels following long-term β_1-blockade.

Our finding of the presence of both β_1- and β_2-adrenoceptor-mediated vasorelaxation in these blood vessels is novel, and one may speculate as to the physiological relevance of having both receptor subtypes within the same vessel. The affinity of noradrenaline is much higher, by a factor of approximately 20, at the β_1- as compared with the β_2-adrenoceptor, whilst the affinity of adrenaline is the same for β_1- and β_2-adrenoceptors, as measured in human ventricle (Kaumann *et al.*, 1989). By contrast, at least in IMA and SV, adrenaline exerts its effects predominantly through the β_2-adrenoceptor, with very little if any activity on the β_1-adrenoceptor; this contrasts with the situation in human atrium from non-β-blocked patients, where adrenaline is equipotent at both receptor subtypes (Hall *et al.*, 1990; Kaumann, 1991). Thus, the β_1-adrenoceptor may have a role in mediating vasorelaxation under sympathetic nervous control, whereas the β_2-adrenoceptor may mediate vasodilatation under conditions of high circulating adrenaline, for example stress. It is possible, therefore, that β_1-adrenoceptors are innervated whereas β_2-adrenoceptors are not.

The role of neural β_1-adrenoceptor-mediated vasorelaxation is likely, however, to be minor physiologically. Firstly, the density of β-adrenoceptors in the smooth muscle of IMA and SV is much less than that in the heart as assessed by autoradiography, and those that are present are predominantly of the β_2 subtype (Molenaar *et al.*, 1988); it is therefore unlikely that there are spare β_1-adrenoceptors for noradrenaline in these vessels, by contrast with the situation in the human heart (Kaumann *et al.*, 1989; Hall *et al.*, 1990). Consistent with this is our finding that noradrenaline in the presence of the β_2-adrenoceptor antagonist, ICI 118551, failed to produce maximum relaxation even at concentrations as high as 0.6 mM. Secondly, there is no consistent evidence in animal models that sympathetic nerve stimulation produces β-adrenoceptor-mediated vasodilatation (Vatner & McRitchie, 1976; Russel & Moran, 1980); there is, however, evidence of cholinergically-mediated vasodilatation and α-adrenoceptor-mediated vasoconstriction by sympathetic nerves in various vessels (Glick *et al.*, 1967; Burnstock *et al.*, 1970; Bevan & Purdy, 1973; Russell & Moran, 1980). Finally, the concentration of noradrenaline in vascular tissue is less than that found in cardiac tissue; we have previously examined noradrenaline levels in feline tissues, and have found them to be approximately four fold greater in cat left ventricle as compared with femoral artery (Ashby, M.J. & Brown, M.J., unpublished results).

Our results demonstrate that β_2-adrenoceptor sensitization in response to chronic β_1-blockade is a tissue-specific phenomenon. It occurs in cardiac tissue, but not in IMA or

SV. This argues against the possibility that the β_2-adrenoceptor sensitization is a systemic phenomenon (e.g. secondary to hormonal changes in response to β-blockade), since this would be expected to affect β_2-adrenoceptors throughout the body similarly. Rather, this strengthens the view that the mechanism is a local one occurring at the cellular level, involving cross-talk between β-adrenoceptor subtypes. The precise mechanism remains to be elucidated.

We thank the Theatre staff and cardiothoracic surgeons at Papworth Hospital for the supply of tissues. This work was supported by the Medical Research Council, U.K. A.F. is an M.R.C. Training Fellow.

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(Received August 21, 1992
Revised March 18, 1993
Accepted March 19, 1993)

Br. J. Pharmacol. (1993), **109**, 1059–1063 © Macmillan Press Ltd, 1993

Contraction of the rat isolated spleen mediated by adenosine A_1 receptor activation

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1 A series of adenosine receptor agonists of varying degrees of selectivity induced concentration-dependent contraction of the rat isolated spleen. With the exception of the response to the selective A_{2A} receptor agonist, 2-[*p*-(2-carboxyethyl)phenylethylamino]-5'-*N*-ethylcarboxamidoadenosine (CGS 21680), responses to each ligand were blocked surmountably and to a broadly similar extent by 8-*p*-sulphophenyltheophylline (10^{-5} M).

2 There was a significant correlation between the pEC_{50} values obtained on the spleen and the binding affinities (pK_D; measured with [^{3}H]-NECA) for the A_1 receptor of pig striatum ($r = 0.98$, $P < 0.001$) but not the A_{2A} receptor ($r = 0.14$, NS).

3 The antagonist potencies of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) and 9-chloro-2-furyl [1,2,3]triazolo[1,5-C]quinazoline-5-amine (CGS 15943) were measured against the prototype selective A_1 receptor agonist, **R**-N^6-phenylisopropyladenosine (**R**-PIA). The resulting pK_B values of 8.67 and 7.70, respectively are consistent with the A_1 receptor subtype mediating splenic contraction.

4 The response to **R**-PIA was unaltered in the presence of a concentration (10^{-7} M) of CGS 21680 which is 6 fold its K_D concentration at the A_{2A} binding site in pig striatum but below the threshold for causing contraction *per se*; thus, A_{2A} receptors inhibitory to contraction appear to be absent.

5 The response to **R**-PIA was resistant to blockade by prazosin (10^{-7} M) and by nifedipine (10^{-6} M) but partially blocked by indomethacin (10^{-6} M).

6 The results show that the rat isolated spleen responds to adenosine receptor agonists with contraction. Both the relative potencies of agonists and the effects of antagonists indicate mediation by the A_1 receptor subtype. α_1-Adrenoceptor activation is not involved in contraction but a role for products of cyclo-oxygenase and calcium from a source not dependent on entry through L-channels is implicated.

Keywords: Adenosine A_1 receptors; smooth muscle; spleen

Introduction

Adenosine and several closely related analogues induce contraction of both vascular and non-vascular smooth muscle including the resistance vessels of rat kidney (Kenakin & Pike, 1987) and cat lung (Neely *et al.*, 1991), guinea-pig trachea (Advenier *et al.*, 1982; Caparrotta *et al.*, 1984; Ghai *et al.*, 1987; Farmer *et al.*, 1988), myometrium (Smith *et al.*, 1988) and aorta (Stoggall & Shaw, 1990) and both the muscularis mucosa and the longitudinal muscle of the rat colon (Bailey & Hourani, 1990; Bailey *et al.*, 1992). Evidence obtained with both agonists and antagonists implicates the adenosine A_1 receptor in these responses although such analyses have been complicated by the presence of other purinoceptor subtypes mediating both contraction and relaxation in these tissues.

In the present paper, we describe a robust contractile response to adenosine and its analogues in the rat isolated spleen. Pharmacological analysis with both selective agonists and antagonists indicates that the adenosine A_1 receptor subtype mediates the response.

Methods

General

Male Sprague-Dawley rats (Biological Research Laboratories, Füllinsdorf, Switzerland) weighing approximately 250 g were killed by a blow to the head and exsanguination and their spleen removed. The spleens were divided along the short and long axes to give four preparations per spleen and set up for recording isometric contractile responses in Krebs-Henseleit solution at 37°C and bubbled with 5% CO_2:95% O_2. The Krebs-Henseleit solution had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, dextrose 10. The resting tension was maintained at approximately 700 mg. Because in a number of experiments comparisons were made with the effects of the α_1-adrenoceptor agonist, phenylephrine, propranolol (1 μM) and desipramine (0.3 μM) were added routinely to the Krebs-Henseleit solution to minimize interference due to direct or indirect effects on adrenoceptors. Preliminary experiments established that the sensitivity of the tissue to the representative A_1 receptor agonist, **R**-PIA, was unaffected by these agents.

Experimental procedures

Following an initial settling down period of 60–90 min during which the bath fluid was changed at 15 min intervals, tissues were stimulated with a supramaximal concentration of potassium chloride (90 mM, Figure 1). Following repeated

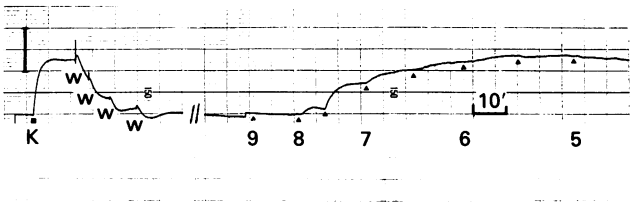


Figure 1 Representative experimental record showing contractile effects of **R**-PIA on rat isolated spleen. K = KCl 90 mM; W = wash; (▲) addition of **R**-PIA at concentrations indicated (– log M). Vertical scale 100 mg.

washing and recovery to baseline tension, the experimental procedure was begun.

Agonists were added cumulatively to generate full concentration-response curves, the concentrations being increased when the preceding response has reached a plateau. Only one agonist concentration-response curve was generated per tissue. Responses were expressed as a percentage of the response to potassium chloride. Antagonists were added to the incubation medium 20 min before the start of the cumulative addition of agonists. In all cases responses in the presence of antagonists were compared with control responses generated in spleen segments from the same animal run in parallel. EC_{50} values were interpolated from individual concentration-response curves. pK_B values for antagonists were calculated according to the formula (Furchgott, 1972):

$$\log (CR-1) = [B] - \log K_B$$

where CR is the ratio of concentration of agonist giving an equal response in the presence and in the absence of the concentration of the antagonist, B.

Radioligand binding

Membranes were prepared from pig striatum and binding studies carried out by the method previously described (Bruns *et al.*, 1986). [^{3}H]-5'N-ethylcarboxamidoadenosine ([^{3}H]-NECA), a non-selective adenosine receptor agonist was used at a concentration (4 nM) which selectively labels the A_1 and A_{2A} receptors. IC_{50} values were derived from the agonist displacement curves by weighted non-linear least-square curve fitting to the Langmuir equation and pK_D values calculated.

In both functional and radioligand binding studies, means (± s.e.mean) are presented throughout.

Drugs and solutions

N^6-cyclopentyladenosine (CPA), N^6-cyclohexyladenosine (CHA), N^6-cyclopentyl-2-chloroadenosine (CCPA), **R**- and **S**-N^6-phenylisopropyladenosine (**R**-PIA; **S**-PIA), 5'-N-ethylcarboxamidoadenosine (NECA), adenosine, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), (±)-propranolol hydrochloride, desipramine hydrochloride, (−)-phenylephrine hydrochloride and indomethacin were purchased from Sigma, Buchs, Switzerland. N^6-[IS, *trans*)-2-hydroxycyclopentyl]adenosine (HCPA), N^6-[2-hydroxy-3-(naphthyl-1-oxy) propyl]adenosine (HNOPA), 2-[*p*-(2-carboxyethyl)phenylethylamino]-5'-N-ethylcarboxamidoadenosine (CGS 21680) and 8-*p*-sulphophenyltheophylline (8-SPT) were synthesized by F. Gadient at Sandoz Pharma, Basel, Switzerland. 9-Chloro-2-(2-furyl) [1,2,3]triazolo[1,5-C]quinazoline-5-amine (CGS 15943) was a gift from Ciba-Geigy, Summit, N.J., U.S.A. Nifedipine and prazosin hydrochloride were obtained from Bayer, Leverkusen, Germany and Pfizer, Sandwich, Kent, UK, respectively. Stock solutions of all drugs were made up in dimethylsulphoxide (DMSO) with subsequent dilutions being made in distilled water. [^{3}H]-NECA, 23–26 Ci mmol^{-1}, was obtained from Amersham, Bucks, U.K.

Results

General aspects of the contractile response to adenosine receptor agonists of rat isolated spleen

Adenosine receptor ligands induced sustained contractile responses of rat isolated spleen (illustrated for the representative A_1 receptor agonist, **R**-PIA, in Figure 1). The concentration-response curves for a selection of agonists with differing adenosine receptor affinities and selectivities are shown in Figure 2. Curves were generally of similar shape, although that to the selective A_{2A} receptor agonist, CGS 21680, was noticeably less steep than those of the other ligands. The maximum response to CHA (88 ± 10% of that to 90 mM potassium chloride) was somewhat higher than those of the majority of the agonists (range 53–73%); in contrast, that of CGS 21680 reached only 34 ± 4% of the response to potassium chloride at 3×10^{-5} M (Figure 2, Table 1).

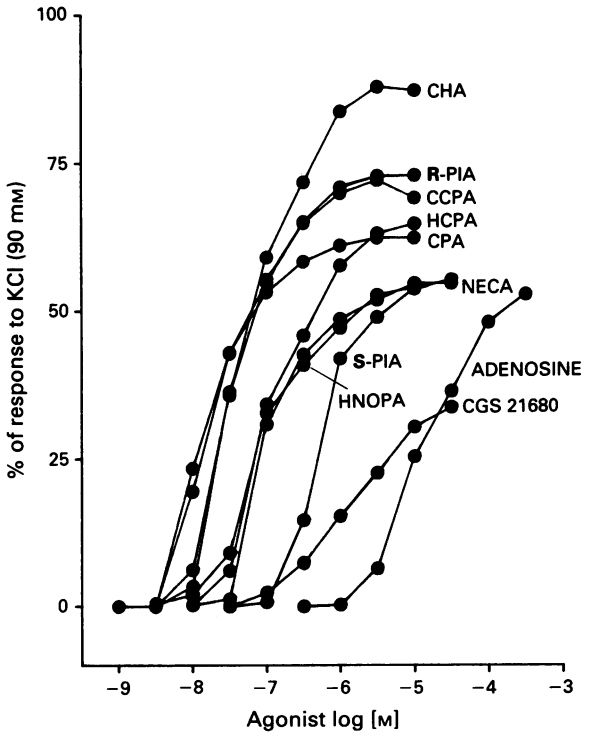


Figure 2 Concentration-response curves for the contractile effects of adenosine receptor agonists on rat isolated spleen. Curves represent mean values from the number of experiments detailed in Table 1. Standard errors have been omitted for clarity; an estimate of mean variation can be gained from the standard errors of the mean pEC_{50} and maximum response values shown in Table 1. For explanation of abbreviations, see Methods.

The receptor mediating the contractile response to adenosine receptor agonists of rat isolated spleen

Concentration-response curves to the prototype adenosine receptor agonists were (with the notable exception of CGS 21680) shifted in parallel to the right by exposure to the non-selective adenosine A_1/A_2 receptor antagonist, 8-SPT at 10^{-5} M (illustrated with respect to **R**-PIA in Figure 3a); the pK_B values derived from these data ranged from 5.54 (for NECA) to 6.16 (for **R**-PIA) (Table 1). At 10^{-5} M, 8-SPT did not affect the response to the α_1-adrenoceptor agonist, phenylephrine (data not illustrated). The effects of the A_{2A}-selective antagonist, CGS 15943, and the A_1-selective antagonist, DPCPX, were evaluated against **R**-PIA. At 10^{-7} M and 3×10^{-7} M, CGS 15943 induced a parallel rightward shift of the **R**-PIA concentration-response curve (Figure 3b) to yield a pK_B value of 7.70 ± 0.12 (mean of 10 values generated from tissues from 5 animals). DPCPX proved a particularly potent antagonist of **R**-PIA; concentrations of 3×10^{-9} and 3×10^{-8} M were effective (Figure 3c) yielding a pK_B value of 8.67 ± 0.10 (mean of 8 values generated from tissues from 4 animals).

The possibility that A_{2A} receptors are present in the spleen which might modulate the contractile response arising from A_1 receptor stimulation was tested by establishing the concentration-response curve to **R**-PIA in the presence of a concentration (10^{-7} M) of CGS 21680 which is 6 fold the K_D

Table 1 Contractile effect of adenosine receptor agonists on rat isolated spleen: susceptibility to blockade by 8-SPT and comparison of potency with A_1 and A_2 receptor binding affinities

	Radioligand binding[b]				*Spleen*		
Substance[a]	A_1 (pK_D)	A_{2A} (pK_D)	pEC_{50} *agonist*	*Maximum*[c] *response*	n	pK_B[d] *8-SPT*	n
CPA	9.13 ± 0.05	6.03 ± 0.05	7.77 ± 0.04	62 ± 6	6	5.86 ± 0.14	4
CCPA	9.00 ± 0.10	5.98 ± 0.04	7.68 ± 0.14	72 ± 17	5	5.90 ± 0.19	5
R-PIA	8.99 ± 0.03	6.34 ± 0.06	7.48 ± 0.03	73 ± 4	23	6.16 ± 0.09	8
CHA	8.91 ± 0.04	5.90 ± 0.06	7.36 ± 0.05	88 ± 10	7	5.89 ± 0.19	7
HCPA	8.46 ± 0.02	5.30 ± 0.06	7.09 ± 0.11	65 ± 17	6	6.15 ± 0.27	6
HNOPA	8.34 ± 0.06	5.26 ± 0.07	7.08 ± 0.10	55 ± 15	6	5.73 ± 0.17	6
NECA	8.25 ± 0.08	7.64 ± 0.05	7.02 ± 0.07	55 ± 6	9	5.54 ± 0.11	5
S-PIA	7.69 ± 0.05	5.57 ± 0.06	6.32 ± 0.04	55 ± 6	10	5.64 ± 0.05	6
CGS 21680	5.36 ± 0.10	7.65 ± 0.09	5.93 ± 0.14	34 ± 4	15	no blockade	4
Adenosine	e	e	4.90 ± 0.07	53 ± 5	6	5.61 ± 0.08	4

[a]For full chemical name see text (Drugs and solutions).
[b]Method of Bruns *et al.* (1986); mean values of 4–8 experiments.
[c] Expressed as % of the response to KCl, 90 mM.
[d]Antagonist potency of 8-SPT; see text.
[e]Cannot be estimated accurately in radioligand binding assay.

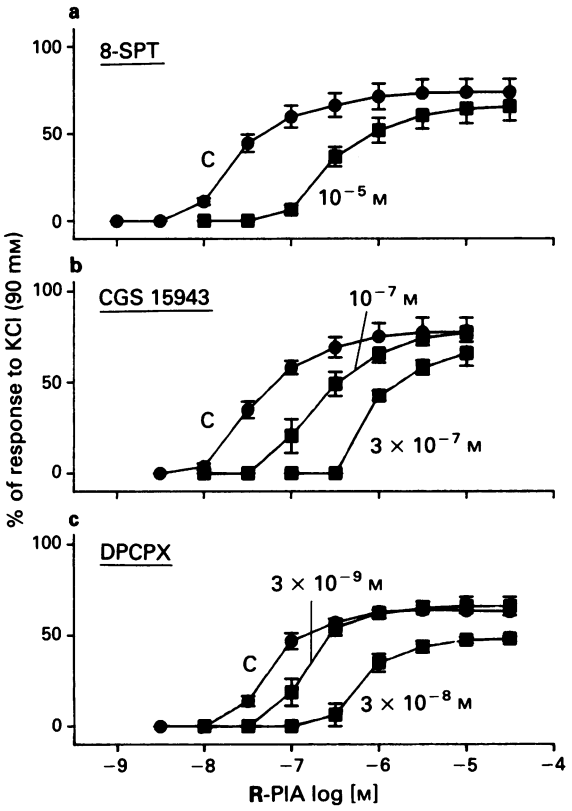


Figure 3 Effects of adenosine receptor antagonists on the response of rat isolated spleen to R-PIA. Concentration-response curves were established in the absence (C) or presence of the antagonist specified at the concentrations indicated. Points represent mean values (± s.e.mean) of the number of individual experiments. $n = 8$ in (a), 5 in (b) and 4 in (c).

concentration at A_{2A} binding sites (Table 1) but below that threshold for causing contraction *per se*. CGS 21680 had no effect on the concentration-response curve to R-PIA ($n = 4$ each for control and CGS 21680).

In Table 1, the adenosine receptor agonists are arranged in order of their contractile potencies on the spleen with, for comparison, their affinities for brain A_1 and A_{2A} binding sites measured in the radioligand binding assay. Omitting adenosine (no binding data available) and CGS 21680 (8-SPT-insensitive response) there was a significant correlation between the functional pEC_{50} values and binding affinities to the A_1 receptor ($r = 0.98$, $P < 0.001$) but not the A_{2A} receptor ($r = 0.14$, NS).

Mechanism of the contractile responses to R-PIA of rat isolated spleen

R-PIA was used in experiments to explore the cellular mechanisms underlying splenic contraction and comparisons were made with the selective α_1-adrenoceptor agonist, phenylephrine. The prototype α_1-adrenoceptor antagonist, prazosin at a concentration (10^{-7} M) which produced substantial blockade of the response to phenylephrine (Figure 4a) did not significantly affect the response to R-PIA (Figure

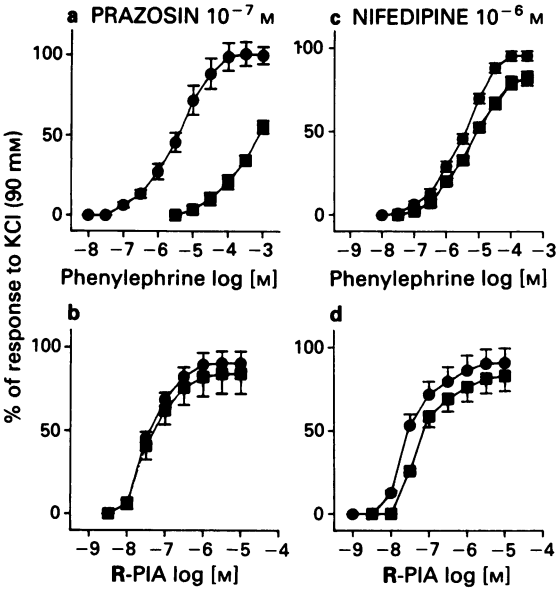


Figure 4 Effects of prazosin and nifedipine on responses of rat isolated spleen evoked by R-PIA or phenylephrine. Agonist concentration-response curves were established in the absence (●) or presence (■) of the modifying agent at the concentrations indicated. Points represent the mean values (± s.e.mean) of the number of individual experiments; $n = 4$ in (a) (b) and (d); $n = 8$ in (c).

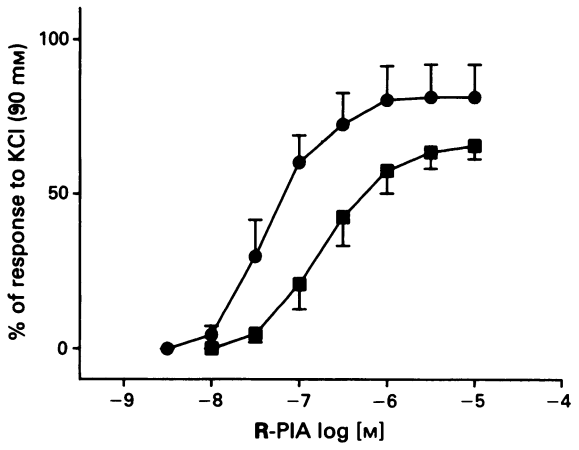


Figure 5 Effects of indomethacin on the response of rat isolated spleen to **R**-PIA. Concentration-response curves were established in the absence (●) or presence (■) of indomethacin, 10^{-6} M. Points represent mean values (± s.e.mean) of 4 individual experiments. for further details see text.

4b). Incubation with a high concentration (10^{-6} M) of the calcium entry blocking agent, nifedipine, induced only minimal inhibition of the responses to **R**-PIA and phenylephrine (Figure 4c and d). In the presence of the cyclo-oxygenase inhibitor, indomethacin (10^{-6} M), the concentration-response curve to **R**-PIA was shifted to the right and the maximum response was reduced (Figure 5).

Discussion

The present data document, for the first time, robust contractile responses induced by adenosine receptor agonists on rat isolated spleen. The effects are not the result of a direct or indirect interaction with the α_{1B}-adrenoceptors of the splenic smooth muscle (Minneman, 1988) since responses to the representative ligand, **R**-PIA, were unaffected by a high concentration of prazosin. Moreover, blockade by the broad spectrum adenosine receptor agonist, 8-SPT, at a concentration which did not affect responses to the α_1-adrenoceptor agonist, phenylephrine, provides direct evidence of a role for adenosine receptors in the response.

Several pieces of evidence suggest that splenic contraction results from activation of the adenosine A_1 receptor subtype. Firstly, there was a significant correlation between the agonist potencies to contract the spleen and affinities for the A_1 receptor defined by radioligand binding in pig brain; it bears emphasis that the highly selective A_{2A} receptor agonist, CGS 21680, was only weakly active in contracting the spleen and then only by a mechanism which was 8-SPT-insensitive. Secondly, the antagonist data clearly implicate the A_1 receptor in the response. For example CGS 15943 shows pA_2 values between 9.2 and 10.8 against a variety of A_{2A} receptor-mediated responses (Ghai *et al.*, 1987; Balwierczak *et al.*, 1991; Croning *et al.*, 1992) but is substantially less active against responses known to be mediated by A_1 receptors; for the negative inotropic and chronotropic responses in guinea-pig atria, for example, the pA_2 values were 7.4 (Ghai *et al.*, 1987) and 7.6 (Collis, 1990), respectively. In the spleen, the pK_B value for CGS 15943 against **R**-PIA was 7.70 and thus entirely consistent with involvement of the A_1 receptor. By contrast, DPCPX is a more potent antagonist at the A_1 receptors of guinea-pig heart (pA_2/pK_B values 8.1–8.9; Collis 1990; Martin, 1992) than in classical A_2 containing tissues such as aorta or trachea (pA_2 values 6.5–6.6; Collis 1990; Martin, 1992). Clearly the pK_B value of 8.67 from the antagonism of **R**-PIA by DPCPX in the spleen implies mediation by the A_1 receptor. Finally, there are reports of A_1 receptor-mediated contractile responses in smooth muscle of guinea-pig myometrium (Smith *et al.*, 1988) trachea (Farmer *et al.*, 1988), and aorta (Stoggall & Shaw, 1990) and rat colon muscularis mucosa (Bailey *et al.*, 1992) and longitudinal muscle (Bailey & Hourani, 1990). The existence in the rat spleen of a contractile A_1 receptor is, therefore, consistent with this general pattern.

The intracellular events involved in A_1 receptor-mediated splenic contraction remain to be defined. Classically, the A_1 receptor is associated with inhibition of adenylate cyclase (Linden, 1991), but in the absence of induced cyclase tone such a mechanism would seem unlikely. Moreover, a clear dissociation between contraction of guinea-pig myometrial smooth muscle induced by adenosine A_1 receptor stimulation and modulation of adenylate cyclase has been reported (Smith *et al.*, 1989). Subsequently Schiemann *et al.* (1991a,b) provided convincing evidence that the A_1 receptor can couple to phosphoinositide turnover in guinea-pig myometrium and that contraction is most likely a consequence of increased internal $[Ca^{2+}]$ following increased generation of D-*myo*-inositol 1,4,5-trisphosphate. The concept receives further support from the finding that the A_1 receptor may couple directly to phospholipase C and activate inositol phospholipid hydrolysis in DDT_1-MF_2 cells, a smooth muscle cell line from hamster vas deferens (Gerwins & Fredholm, 1992; Schachter & Wolfe, 1992; White *et al.*, 1992). Such a mechanism in the splenic capsular smooth muscle would provide a plausible explanation for the contractile response in the present study; it would also be entirely consistent with the observation that suppression of calcium influx through L-calcium channels had minimal effects on contraction induced by **R**-PIA (Figure 4d).

In cat lung and guinea-pig uterus and trachea, responses mediate by A_1 receptor stimulation are markedly attenuated by inhibitors of cyclo-oxygenase (Caparrotta *et al.*, 1984; Neely *et al.*, 1991; Schiemann *et al.*, 1991a) suggesting that smooth muscle contraction in these tissues may be secondary to the synthesis of prostaglandins from arachidonic acid. In contrast to these findings, a substantial component of the contraction of the spleen induced by **R**-PIA was resistant to blockade by indomethacin (10^{-6} M), a concentration sufficient to inhibit cyclo-oxygenase (Tolman & Partridge, 1975). The result indicates that at least part of the response involves cyclo-oxygenase products; whether the remaining response involves a direct action or is indirect via other mediators (see White *et al.*, 1992) remains to be determined.

We are grateful to Dr F. Gadient for synthesizing several of the compounds used in this study, to Mrs M. Nagy and Miss S. Gilgen for expert technical assistance and to Ms E. Born for manuscript preparation.

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(Received January 4, 1993
Revised March 19, 1993
Accepted March 22, 1993)

Br. J. Pharmacol. (1993), **109**, 1064–1070 © Macmillan Press Ltd, 1993

Modulation of opioid antinociception by CCK at the supraspinal level: evidence of regulatory mechanisms between CCK and enkephalin systems in the control of pain

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1 Much evidence in the literature supports the idea that cholecystokinin (CCK) interacts with opioids in pain mechanisms. In this work, we have investigated the supraspinal interactions between enkephalins and CCK, using the hot plate test in mice.

2 Intracerebroventricular (i.c.v.) administration of BDNL (a mixed CCK_A/CCK_B agonist) induced dose-dependent antinociceptive responses on both paw lick and jump responses. In contrast, using the same test, the i.c.v. injection of BC 264 (a selective CCK_B agonist) induced a hyperalgesic effect, which was restricted to paw licking and occurred only at a high dose of 2.5 nmol.

3 In addition, i.c.v. administration of BDNL potentiated the antinociceptive effects of the mixed inhibitor of enkephalin degrading enzymes, RB 101 and of the μ-agonist, DAMGO, while BC 264 reduced these effects.

4 Furthermore, at a dose where it interacts selectively with δ-opioid receptors, the opioid agonist BUBU reversed the hyperalgesic responses of BC 264 (2.5 nmol) but was unable to modify the effects induced by BDNL.

5 Taken together, these results suggest the existence of regulatory mechanisms between CCK and enkephalin systems in the control of pain. These regulatory loops could enhance the antinociceptive effects of morphine allowing the opiate doses used to be reduced and thus, possibly, the side-effects to be minimized.

Keywords: CCK system; opioid system; mouse; hot plate test; selective agonists; mixed enkephalin-metabolizing enzyme inhibitor, RB 101

Introduction

Cholecystokinin (CCK) was first recognized as a gastrointestinal hormone and later discovered in very high concentrations in the brain. Several forms of CCK have been detected, including the sulphated octapeptide C terminal, CCK_8, which is the most predominant form in the central nervous system (CNS) (Vanderhaeghen *et al.*, 1975). The unsulphated form of CCK_8 as well as the metabolites CCK_5 and CCK_4 are also found and may also have physiological roles. Analysis of the binding properties of these compounds has provided evidence for the existence of at least two CCK receptor types: CCK_B receptors, the predominent form found in the brain, and CCK_A receptors abundant in peripheral tissues (Moran *et al.*, 1986). CCK_A binding sites have also been found in a few discrete regions of the rat brain and CCK_B receptors have been shown to resemble closely gastrin receptors (Moran *et al.*, 1986; Hayward *et al.*, 1991). CCK_8 interacts at nanomolar affinity with both types of CCK receptors, while the other smaller fragments have selectivity for CCK_B receptors (Innis & Snyder, 1980; Gaudreau *et al.*, 1983; Durieux *et al.*, 1988).

Anatomical studies have shown that enkephalins and CCK_8 have a strikingly similar distribution within many regions of the CNS. This overlapping distribution of both the peptides and their respective receptors in the brain and spinal cord (Stengaard-Pedersen & Larsson, 1984; Crawley, 1985; Gall *et al.*, 1987; Pohl *et al.*, 1990), has focused attention on the role of CCK in nociception. Several studies have reported a naloxone reversible antinociceptive effect of CCK_8 or its analogues in several antinociceptive tests, such as the hot plate, writhing, and tail-flick tests (Zetler, 1980; Jurna & Zetler, 1981; Barbaz *et al.*, 1986; Hill *et al.*, 1987; Pittaway *et al.*, 1987; Hong & Takemori, 1989; Baber *et al.*, 1989).

However, it has also been suggested that CCK_8 has anti-opioid activity (Itoh *et al.*, 1982; Faris *et al.*, 1983). Faris *et al.* (1983) found that CCK reduced the antinociceptive effects produced by the release of endogenous opioids, and did not modify non-opiate responses induced by hind paw foot shock. In addition, numerous studies have shown that peripherally administered CCK antagonists potentiate opioid antinociceptive responses, confirming a functional antagonism of the opioid system by the CCK system (Watkins *et al.*, 1985; Hendrie *et al.*, 1989; Dourish *et al.*, 1990; Wiesenfeld-Hallin *et al.*, 1990; Lavigne *et al.*, 1992). It has been hypothesized that CCK down-regulates opioid effects through activation of CCK_B receptors. However, MK329, a selective CCK_A antagonist, also potentiated the analgesic effects of opiates (Rattray *et al.*, 1988; Hendrie *et al.*, 1989).

The purpose of the present study was to understand more fully the supraspinal interactions between the opioid and CCK systems. With this aim in mind, the effects induced by the CCK_B agonist, BC264 and by mixed CCK_A/CCK_B agonist, BDNL were studied after i.c.v. administration in the hot plate test in mice. In addition, the influence of these CCK agonists on the responses induced by selective opioid-agonists such as DAMGO (μ-agonist) and BUBU (δ-agonist) were determined. RB 101 (N-[(R,S)-2-benzyl-3[(S)(2-amino-4-methylthio)butyldithio]-1-oxopropyl]-L-phenylalanine benzyl ester, Fournié-Zaluski *et al.*, 1992), a mixed inhibitor of enkephalin-degrading enzymes, was also used. This systemically active molecule completely protects the endogenous enkephalins from degradation (Ruiz-Gayo *et al.*, 1992a), allowing their actions with the CCK system to be studied.

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Methods

Animals

Male Swiss mice (20–22 g) (Charles River, France) were used. Animals were housed in groups of 20 for at least two days before the experiments were started, and food and water were available *ad libitum*. Each animal was used only once. Antinociceptive measurements were recorded between 14 h and 20 h.

Administration procedures

Intracerebroventricular (i.c.v.) injections: BC264, BDNL or saline were injected free hand into the left lateral ventricle of a mouse with a modified Hamilton μl syringe in a volume of 10 μl per mouse according to the method of Haley & McCormick (1957).

Systemic injections: DAMGO, BUBU, RB101 or control vehicles were slowly administered intravenously (i.v.) in a volume of 0.1 ml 10 g^{-1}. Naloxone and naltrindole were injected subcutaneously (s.c.) in a volume of 0.1 ml 10 g^{-1}.

The jump responses induced by the thermal nociceptive stimulus were measured at various times, due to the differences in the maximal time-effect of the compounds studied. Thus the dose-response curves of BDNL and BC 264 were determined 15 min after i.c.v. administration. In studies of possible interactions between opioids and CCK-related compounds, BDNL and BC 264 were injected i.c.v. 5 min before i.v. administration of BUBU or 10 min before i.v. administration of DAMGO. Controls were treated with vehicle at the corresponding times. The hot plate test was performed as before, i.e. 15 min after i.c.v. injection of the CCK peptides. In the experiments with RB 101, the CCK agonists were injected i.c.v. 20 min before the hot plate test (i.e. 10 min before i.v. injection of the mixed inhibitor). A longer period (i.e. 10 min instead of 5 min) between the administration of RB 101 and the CCK agonists was required to eliminate a slight 'hypolocomotion' induced by the vehicle used to solubilize the mixed inhibitor.

Hot-plate test

The test was based on that described by Eddy & Leimbach (1953). A glass cylinder (16 cm high, 16 cm diameter) was used to keep the mouse on the heated surface of the plate which was maintained at a temperature of 55 ± 0.5°C using a thermoregulated water circulating pump. The latencies of both forepaw licking (cut-off time: 30 s) and jumping (cut-off time: 240 s) were measured for each animal with a stopwatch. Dose-response curves were established by expressing the data as a percentage of control using the following equation: % of control = (test latency − control latency)/(cut-off time − control latency) × 100.

Chemicals

BDNL (Boc-Tyr(SO_3H)-Nle-Gly-Trp-Nle-Asp-Phe-NH_2, Ruiz-Gayo *et al.*, 1985), BC264 (Boc-Tyr(SO_3H)-gNle-*m*Gly-Trp-(NMe)-Nle-Asp-Phe-NH_2, Charpentier *et al.*, 1988), BUBU (Tyr-D-Ser(O-Tert-Butyl)-Gly-Phe-Leu-Thr(O-Tert-Butyl, Gacel *et al.*, 1988), RB101 (H_2N-CH(CH_2-CH_2-S-CH_3)-CH_2-S-S-CH_2-CH($CH_2\Phi$)-CONH-CH($CH_2\Phi$)-$COOCH_2\Phi$, Fournié-Zaluski *et al.*, 1992) and naltrindole (NTI, 17-cyclopropylmethyl-6,7-dehydro-4,5α-epoxy-3,14-dihydroxy-6, 7,2′,3′-indolomorphinan, Portoghese *et al.*, 1988) were synthesized in the laboratory following previously reported methods. DAMGO (Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol) was purchased from Bachem AG (Switzerland) and naloxone from Mallet SA (France). BDNL, BC264, DAMGO, naloxone and naltrindole were dissolved in saline and BUBU (L-lysine salt) in distilled water. RB101 was dissolved in the following vehicle: ethanol (10%): cremophor EL (10%): distilled water (80%). Cremophor EL was purchased from Sigma (France).

Statistical analysis

Data were analysed with a one-way analysis of variance (ANOVA). *Posthoc* comparisons were made with Dunnett's *t* test for dose-response curves or Newman-Keuls test for multiple comparisons. The level of significance was set at $P<0.05$.

Results

Effects induced by i.c.v. administration of the mixed CCK_A/CCK_B agonist (BDNL) and of the selective CCK_B agonist (BC264) in the hot plate test in mice

As shown in Table 1, BDNL produced significant antinociceptive effects in both paw lick and jump responses ($F(3,28) = 10.88$, $P<0.01$ for the lick; $F(3,28) = 17.07$, $P<0.01$ for the jump) 15 min after i.c.v. administration.

In contrast, i.c.v. injection of the selective CCK_B agonist, BC264, only at a dose of 2.5 nmol, induced a significant decrease in the paw lick latency, 15 min after administration ($F(4,45) = 3.40$, $P<0.05$). No modification of the jump response ($F(4,45) = 0.85$, $P>0.05$) was observed after i.c.v. administration of BC264 (Table 1).

Effects of BC264 (i.c.v.) on DAMGO-induced analgesia (i.v.)

DAMGO administered i.v. (2 mg kg^{-1}) 5 min before the hot plate test induced antinociceptive effects on both paw lick (Figure 1a) and jump (Figure 1b) latencies. The increases in the threshold were diminished, but not suppressed, by prior injection of BC264 (15 min before testing) at a dose which

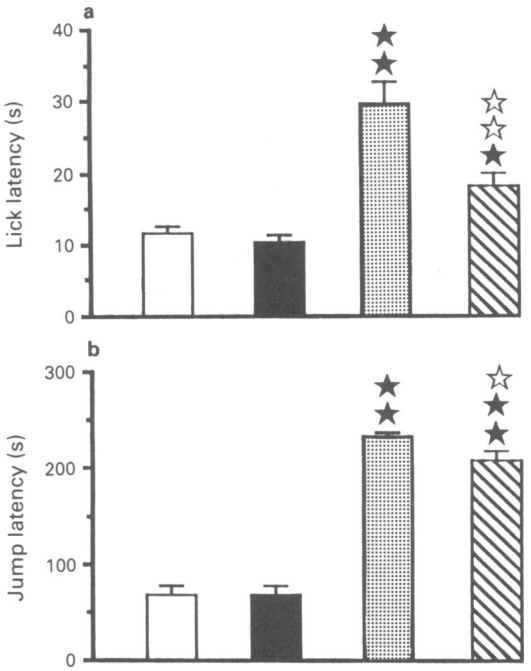


Figure 1 Effects of DAMGO (2 mg kg^{-1}, i.v., 5 min before the test), BC264 (1 nmol, i.c.v., 15 min before testing), and a combination of the two drugs in the hot plate test in mice. (a) Paw lick latencies. (b) Jump latencies. Control, open column; BC264, solid column; DAMGO, stippled column; and DAMGO + BC264, hatched column. The results are expressed as means ± s.e.mean. ($n = 8–10$ mice for each group). ★$P<0.05$; ★★$P<0.01$ as compared to control group, and ☆$P<0.05$; ☆☆$P<0.01$ as compared to DAMGO-treated group (Newman Keuls test).

Table 1 Hyperalgesic or antinociceptive effects observed 15 min after i.c.v. administration of BC264 or BDNL in the hot plate test in mice

	Paw licking		*Jumping*	
	Latency (s)	*% of control*	*Latency* (s)	*% of control*
BC264				
Control	9 ± 0.9		69.7 ± 8.9	
0.1 nmol	8.4 ± 0.8	− 2.8 ± 3.9	68.5 ± 6.2	− 0.7 ± 3.7
1 nmol	7.4 ± 0.9	− 7.6 ± 4.1	81.4 ± 12.6	6.9 ± 7.4
2.5 nmol	5.9 ± 0.7*	− 14.8 ± 3.3	72.1 ± 4.6	1.4 ± 2.7
5 nmol	7.9 ± 0.8	− 5.5 ± 4.0	60.9 ± 7.1	− 5.1 ± 4.2
BDNL				
Control	8.1 ± 1.0		83.1 ± 5.4	
0.05 nmol	8.1 ± 0.9		92.3 ± 14.2	5.9 ± 9.0
0.1 nmol	11.0 ± 0.9	13.2 ± 4.3	84.7 ± 8.2	1.0 ± 5.2
0.2 nmol	20.0 ± 3.4**	54.3 ± 15.5	150.7 ± 9.1**	43.1 ± 5.8
0.5 nmol	26.8 ± 4.1**	85.4 ± 18.8	153.7 ± 14.0**	45.0 ± 8.9

Values are mean ± s.e.mean.
*$P<0.05$; **$P<0.01$ as compared to control group (Dunnett's test).

had no effect on either paw lick or jump latency (1 nmol) ($F(3,30) = 22.45$, $P<0.0001$ and ($F(3,30) = 93.79$, $P<0.0001$ for paw lick and jump latencies, respectively).

Effects of BC264 (i.c.v.) on RB101-induced analgesia (i.v.)

As shown in Figure 2b, the mixed inhibitor RB101 (20 mg kg^{-1}) produced a significant increase in jump latency, 10 min after i.v. injection. This antinociceptive response observed was diminished, but not fully antagonized, by prior administration of BC264 (1 nmol, i.c.v.), 20 min before the hot plate test ($F(3,29) = 15.3$, $P<0.0001$). On the paw lick latency no modifications were observed after administration of RB101, or the CCK_B agonist BC264 or after co-administration of the two drugs (Figure 2a) ($F(3,29) = 1.19$, $P>0.05$).

Effects of BUBU (i.v.) on BC264-induced hyperalgesia (i.c.v.)

As shown in Figure 3a, i.c.v. administration of the highly selective CCK_B agonist, BC264, administered at 2.5 nmol, 15 min before the hot plate test, decreased the threshold of the paw lick latency, as compared to controls. This hyperalgesia was antagonized by BUBU administered 10 min before the hot plate test at a dose (1 mg kg^{-1}) reported to be δ-selective (Figure 3a) ($F(3,33) = 3.90$, $P<0.01$). On the jump response, no modifications were observed either after administration of the agonists alone, or after their co-administration (Figure 3b) ($F(3,33) = 0.58$, $P>0.05$).

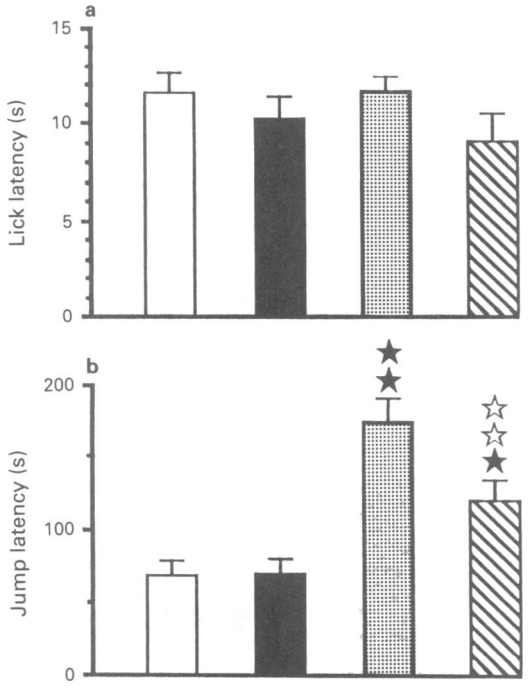


Figure 2 Effects of RB101 (20 mg kg^{-1}, i.v., 10 min before the test), BC264 (1 nmol, i.c.v., 20 min before testing), and a combination of the two drugs in the hot plate test in mice. (a) Paw lick latencies. (b) Jump latencies. Control, open column; BC264, solid column; RB101, stippled column; and RB101 + BC264, hatched column. The results are expressed as means ± s.e.mean. ($n = 8-9$ mice for each group). ★$P<0.05$; ★★$P<0.01$ as compared to control group, and ☆☆$P<0.01$ as compared to RB101-treated group (Newman Keuls test).

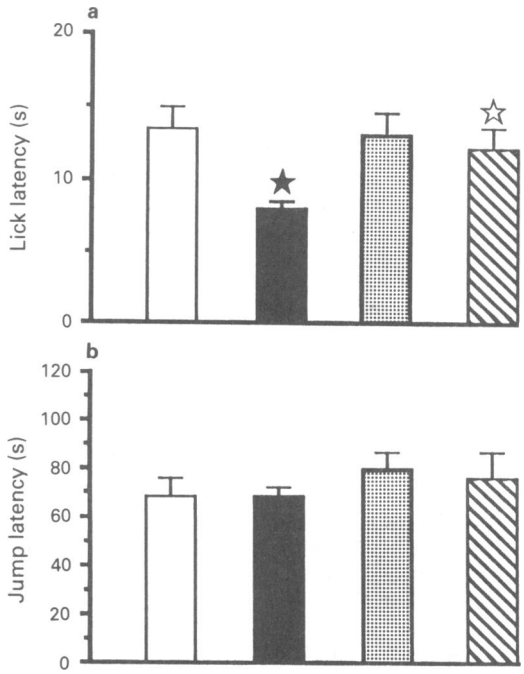


Figure 3 Effects of BUBU (1 mg kg^{-1}, i.v., 10 min before the test) on the responses observed after the i.c.v. administration of BC264 (2.5 nmol, 15 min before testing) in the hot plate test in mice. (a) Paw lick latencies. (b) Jump latencies. Control, open column; BC264, solid column; BUBU, stippled column; and BUBU + BC264, hatched column. The results are expressed as means ± s.e.mean. ($n = 9-10$ mice for each group). ★$P<0.05$ as compared to control group, and ☆$P<0.05$ as compared to BC264-treated group (Newman Keuls test).

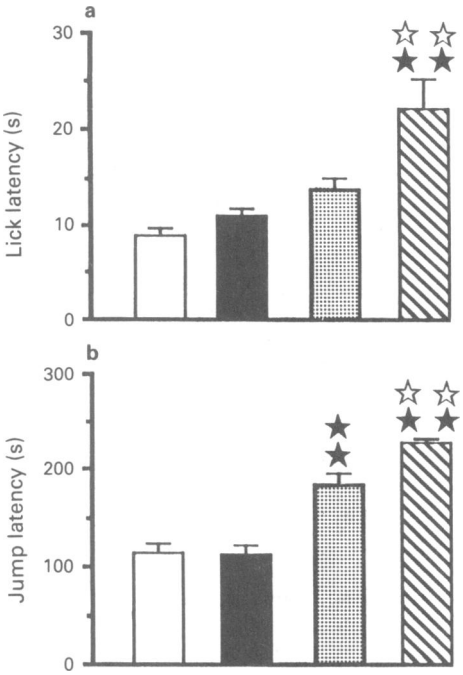


Figure 4 Effects of DAMGO (0.5 mg kg^{-1}, i.v., 5 min before the test), BDNL (0.1 nmol, i.c.v., 15 min before testing), and a combination of the two drugs in the hot plate test in mice. (a) Paw lick latencies. (b) Jump latencies. Control, open column; BDNL, solid column; DAMGO, stippled column; and DAMGO + BDNL, hatched column. The results are expressed as means ± s.e.mean. ($n = 9-11$ mice for each group). ★★$P<0.01$ as compared to control group, and ☆☆$P<0.01$ as compared to DAMGO-treated group (Newman Keuls test).

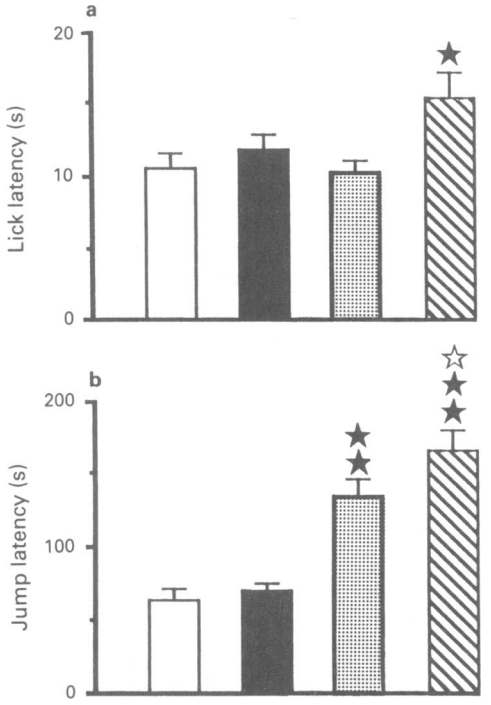


Figure 5 Effects of RB101 (10 mg kg^{-1}, i.v., 10 min before the test), BDNL (0.1 nmol, i.c.v., 20 min before testing), and a combination of the two drugs in the hot plate test in mice. (a) Paw lick latencies. (b) Jump latencies. Control, open column; BDNL, solid column; RB101, stippled column; and RB101 + BDNL, hatched column. The results are expressed as means ± s.e.mean. ($n = 9-11$ mice for each group). ★$P<0.05$; ★★$P<0.01$ as compared to control group, and ☆$P<0.05$ as compared to RB101-treated group (Newman Keuls test).

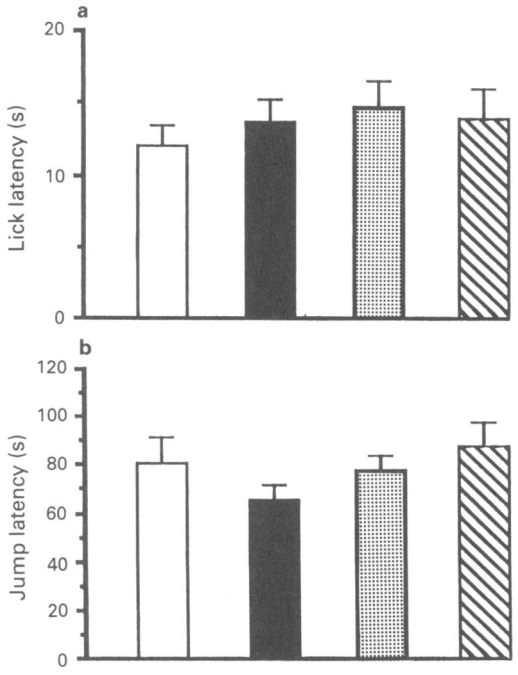


Figure 6 Effects of BUBU (1 mg kg^{-1}, i.v., 10 min before the test), BDNL (0.1 nmol, i.c.v., 15 min before testing), and a combination of the two drugs in the hot plate test in mice. (a) Paw lick latencies. (b) Jump latencies. Control, open column; BDNL, solid column; BUBU, stippled column; and BUBU + BDNL, hatched column. The results are expressed as means ± s.e.mean. ($n = 8-10$ mice for each group).

Effects of BDNL (i.c.v.) on DAMGO-induced analgesia (i.v.)

DAMGO, administered 5 min before the hot plate test (0.5 mg kg^{-1}, i.v.), increased the latency of the jump response (Figure 4b). This antinociceptive effect was enhanced by i.c.v. administration of BDNL (0.1 nmol) 15 min before the hot plate test (Figure 4b) ($F(3,37) = 42.92$, $P<0.0001$). Moreover, although neither BDNL nor DAMGO had any effect on the paw-lick latency, co-administration of the two agonists led to a significant increase in the threshold latency as compared to the control group (Figure 4a) ($F(3,37) = 10.00$, $P<0.0001$).

The antinociceptive effects induced by co-administration of DAMGO and BDNL were significantly antagonized by naloxone (0.1 mg kg^{-1}, s.c.) ($F(5,42) = 7.3$ and ($F(5,42) = 26.36$ for paw lick and jump latencies, respectively) but not by the selective δ-antagonist, naltrindole (0.1 mg kg^{-1}, s.c.) ($F(5,42) = 7.9$ paw lick, ($F(5,42) = 22.75$ jump) (data not shown).

Effects of BDNL (i.c.v.) on RB101-induced analgesia (i.v.)

As shown in Figure 5b, RB101 (10 mg kg^{-1}, i.v.) administered 10 min before the hot plate test, increased the jump latency threshold. This increase was enhanced by prior i.c.v. administration of BDNL (0.1 nmol), 20 min before the test ($F(3,37) = 25.80$, $P<0.0001$). On the paw lick response, i.v. administration of RB101 (10 mg kg^{-1}, 10 min before the test) had no effect. However, co-administration of RB101 and BDNL (0.1 nmol, i.c.v., 20 min before testing) led to an enhancement of the latency as compared to the control group (Figure 5a) ($F(3,37) = 3.58$, $P<0.05$).

These effects were antagonized by naloxone (0.1 mg kg^{-1}, s.c.) ($F(5.42) = 2.469$, paw lick; $F(5,42) = 20.835$, jump) but not by NTI ($F(5,42) = 5.269$ and $F(5,42) = 28.284$ for paw lick and jump responses, respectively) (data not shown).

Effects of co-administration of BDNL (i.c.v.) and BUBU (i.v.)

Co-administration of BDNL (0.1 nmol, i.c.v. 15 min before the hot plate test) and BUBU (1 mg kg^{-1}, i.v. 10 min before the test) did not modify either paw lick or jump latencies (Figure 6) ($F(3,32) = 0.41$, $P > 0.05$ and $F(3,32) = 1.68$, $P > 0.05$, for paw lick and jump responses, respectively).

Discussion

Intracerebroventricular administration of BDNL, a peptidase-resistant CCK_8 analogue which has mixed CCK_A/CCK_B agonist activity, significantly increased both paw lick and jump latencies in the hot plate test in mice, confirming previous reports suggesting that CCK has antinociceptive properties (review in Pittaway & Hill, 1987). In contrast, with the same test, i.c.v. injection of the selective CCK_B agonist, BC264 at a single dose of 2.5 nmol, induced a slight hyperalgesic effect, restricted to paw lick. Derrien *et al.* (1993) have demonstrated that the antinociceptive effects of BDNL involve activation of CCK_A binding sites, and result from an indirect involvement of μ-opioid receptors. The present results could indicate that the CCK_A agonist activity of BDNL is much greater than its CCK_B activity.

In order to investigate the role of the endogenous opioid peptides and which type of opioid receptor (μ or δ) is involved in both paw lick and jump latencies changes induced by the CCK agonist, BDNL and BC264 were administered to mice at non-active doses to avoid possible additive effects. This was followed by i.v. injection of either the μ-agonist, DAMGO, or the mixed inhibitor, RB101, at doses giving weak antinociceptive effects. Under these conditions, BDNL potentiated the antinociceptive effects of RB101 and DAMGO, while BC264 reduced them. Furthermore, the use of high doses of DAMGO (2 mg kg^{-1}, i.v.) or RB101 (20 mg kg^{-1}, i.v.) which elicit strong antinociceptive responses showed that BC264 does not completely antagonize the opioid antinociceptive effects. Moreover, the potentiation of the effects of DAMGO or RB101 by BDNL were fully antagonized by prior injection of naloxone, but not by the δ-selective antagonist NTI. Taken together, these results suggest the occurrence of regulatory mechanisms between CCK and opioid systems in the control of thermal nociception, and provide evidence that CCK can differentially modulate nociceptive perception in mice through at least two types of CCK receptors.

Schematically, activation of CCK_B receptors by BC264 could reduce the levels of endogenous enkephalins released into the extracellular space, while the reverse situation could occur by stimulation of CCK_A binding sites by BDNL, as previously suggested (Cesselin *et al.*, 1984; Hagino *et al.*, 1991). This is in agreement with a previous study, which showed that the antinociceptive effects of CCK_8 were both naloxone-reversible and potentiated by simultaneous administration of the peptidase inhibitors bestatin, thiorphan and captopril (Hill *et al.*, 1987) suggesting that the octapeptide produces its antinociceptive effects in an indirect manner by increasing the release of endogenous opioids.

Furthermore, several electrophysiological studies have demonstrated the existence of regulatory mechanisms between opioid and CCK systems in the control of pain. Indeed, CCK_8 has been found to prevent the inhibition of C-fibre evoked activity of dorsal horn nociceptive neurones resulting from injection of DAMGO (Magnuson *et al.*, 1990; Kellstein *et al.*, 1991) but had no effect on δ-mediated inhibition induced by δ-opioid agonists (Magnuson *et al.*, 1990). Thus, stimulation of CCK_B receptors by BC264, leading to a decrease in the antinociceptive responses observed after μ-opioid receptor activation by DAMGO or RB101, could be related to a diminution of the inhibition of C-fibre firing. The reverse situation could occur after administration of the mixed CCK_A/CCK_B agonist, BDNL.

Another possibility is that CCK peptides produce an allosteric change in the opioid receptors leading to a post-receptor change which counters the opioid effector system. Indeed, Wang & Han (1990) have shown in *in vitro* studies that CCK_8 modifies the binding of μ-opioid agonists to their receptors. This result is in agreement with behavioural studies reported by the same authors, showing that the analgesia produced by injection of μ-agonists is markedly antagonized by injection of CCK_8 (Wang *et al.*, 1990).

If stimulation of CCK sites is capable of modulating the opioid system, this system could in turn regulate the release of CCK peptides. In particular, μ-opioid receptor stimulation could modulate the release of endogenous CCK. In agreement with this, the stimulation of μ-opioid receptors has an inhibitory influence on the K^+-evoked release of CCK-like material (CCKLM) at spinal and supraspinal levels (Rattray & De Belleroche, 1987; Rodriguez & Sacristan, 1989; Benoliel *et al.*, 1991; 1992). Thus, the decrease in the opioid antinociceptive responses observed after administration of BC264, could be explained by a decrease in endogenous CCK release.

On the other hand, recent studies *in vitro* have shown that δ-opioid agonists enhance the K^+-evoked release of CCKLM from slices of rat substantia nigra and spinal cord (Benoliel *et al.*, 1991; 1992). Also, the activation of δ-opioid receptors either by BUBU, a highly selective δ-agonist (Gacel *et al.*, 1990), or by endogenous enkephalins, reduces the *in vivo* binding of the CCK_B-selective agonist [^{3}H]-pBC264, following increases in extracellular levels of endogenous CCK (Ruiz-Gayo *et al.*, 1992b). The results obtained in this study have shown that central injections of BDNL and BC264 produce opposing effects on the opioid system through stimulation of CCK_A and CCK_B receptors, respectively. Thus, it could be speculated that the antagonism of the hyperalgesia observed after administration of BC264 by the δ-opioid agonist BUBU, could be due to an increase in endogenous CCK release which could counterbalance the hyperalgesic effects of BC264 through activation of the different receptor types.

In summary, the results obtained provide evidence that the CCK system can modulate pain perception through activation of at least two receptors in thermal nociception leading to opposing effects. This observation could explain the bell-shaped dose-response curves observed in this and other studies using CCK compounds (Dourish *et al.*, 1988; Hendrie *et al.*, 1989). Moreover, the present study supports the existence of regulatory mechanisms between CCK and enkephalin systems. These CCK/opioid interactions may be

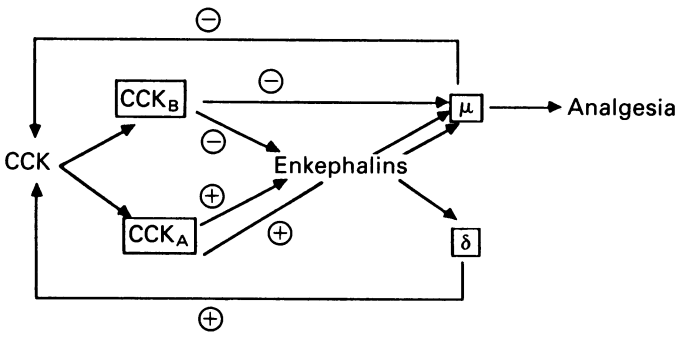


Figure 7 Schematic representation of the proposed regulation loops between the CCK and the opioid systems. The CCK agonists, endogenous and/or exogenous, stimulate the CCK_B and/or the CCK_A receptors which can modulate the opioidergic systems either directly (via binding of opioid agonists or via the C-fibre evoked activity) or indirectly (via the release of endogenous enkephalins). In addition, activation of μ-opioid receptors, which leads to antinociceptive responses, could negatively modulate the release of endogenous CCK, while δ-opioid receptors may enhance it.

mediated supraspinally. Indeed, the responses observed in the hot plate test are believed to be supraspinally organized responses (Schmauss & Yaksh, 1984). However, the results obtained could still be interpreted as spinally mediated effects, since drugs given i.c.v. very rapidly spread into the spinal subarachnoid space.

Schematically, activation of CCK_B receptors could negatively modulate the opioid system either directly (via binding of opioid agonists or C-fibre evoked activity), or indirectly (via release of endogenous enkephalins), while stimulation of CCK_A receptors would enhance opioid release. In addition, it appears that the opioid system can regulate the release of CCK peptides: activation of δ-opioid receptors could increase the synaptic concentration of endogenous CCK, while μ-opioid receptors could decrease it (Figure 7). The existence of these regulation loops between both systems, could mean that the doses of opiates needed to produce analgesia might be reduced by co-administration of CCK agonists. This may reduce the side-effects observed with opiates, although studies are needed to confirm this proposal.

The authors wish to thank C. Dupuis for the preparation of the manuscript. We gratefully acknowledge Ann Beaumont for stylistic revision of the paper.

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(Received January 4, 1993
Revised March 5, 1993
Accepted March 22, 1993)

Br. J. Pharmacol. (1993), **109**, 1071–1078 © Macmillan Press Ltd, 1993

Incomplete reversal of β-adrenoceptor desensitization in human and guinea-pig cardiomyocytes by cyclic nucleotide phosphodiesterase inhibitors

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1 The decreased response to β-adrenoceptor stimulation seen in heart failure may be related to a defect in cyclic AMP production. The inotropic effects of the selective phosphodiesterase (PDE) III inhibitors, SK&F 94120 and SK&F 94836, and the non-selective PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX), alone and when combined synergistically with isoprenaline, were studied in control and β-adrenoceptor-desensitized ventricular myocytes.

2 Myocytes isolated from noradrenaline-treated guinea-pigs had a reduced maximum response to isoprenaline compared with control animals ($60.0 \pm 2.5\%$, $n = 42$ vs $79.5 \pm 1.7\%$ maximum calcium: $n = 46$, $P < 0.001$). Together with an approximately 20 fold increase in the isoprenaline EC_{50}, this is indicative of β-adrenoceptor desensitization as a result with chronic infusion with noradrenaline.

3 The maximum inotropic response of IBMX was depressed following noradrenaline treatment, from $74.9 \pm 4.6\%$ ($n = 7$) in control, to $61.7 \pm 2.70\%$ ($n = 6$), as a percentage of maximum calcium in noradrenaline-treated guinea-pig ventricular myocytes ($P < 0.02$). The pD_2 value for IBMX was also reduced ($P < 0.02$). No significant differences in the inotropic effects of SK&F 94120 and SK&F 94836 were seen between control and β-adrenoceptor desensitized myocytes.

4 Threshold inotropic concentrations of SK&F 94120 and SK&F 94836 caused a five fold decrease in the EC_{50} of control myocytes for isoprenaline, and an 11 fold decrease in the noradrenaline-treated guinea-pig ventricular myocytes.

5 The maximum response to isoprenaline in myocytes isolated from normal guinea-pigs was unaffected by PDE inhibition; either at threshold or maximum inotropic concentrations, or by CPT cyclic AMP, an analogue of cyclic AMP.

6 A significant potentiation of the maximum isoprenaline response by threshold inotropic concentrations was observed with SK&F 94120 ($P < 0.05$), but not with IBMX or SK&F 94836, in myocytes isolated from noradrenaline-treated guinea-pig hearts. This potentiation, however, did not completely restore the response to levels seen in control myocytes.

7 The extent of potentiation of the maximum isoprenaline response by maximum inotropic concentrations of either IBMX or CPT cyclic AMP, was no greater than that by threshold concentrations of IBMX, in myocytes isolated from noradrenaline-treated guinea-pig hearts.

8 In cardiac myocytes isolated from the explanted hearts of 16 patients with heart failure, threshold concentrations of IBMX and SK&F 94120 decreased the isoprenaline EC_{50} by a factor of four and six, respectively, but potentiation of the maximum isoprenaline response occurred only with SK&F 94120. The attenuated isoprenaline response was increased from $60.3 \pm 4.5\%$ to $74.3 \pm 4.2\%$ as a % maximum calcium ($P < 0.05$, $n = 6$), but remained substantially lower than the $116 \pm 7\%$ ($P < 0.001$, $n = 6$) seen in myocytes isolated from non-failing hearts.

9 We conclude that the reduced maximum contraction amplitude with isoprenaline in cardiac myocytes from either patients in end-stage failure, or noradrenaline-treated guinea-pigs, is partly but not solely due to insufficient cyclic AMP levels, since inhibition of cyclic AMP degradation does not result in complete reversal of the β-adrenoceptor desensitization.

Keywords: Cardiac myocyte; phosphodiesterase inhibitor; heart failure; β-adrenoceptor desensitization; cyclic AMP

Introduction

Cyclic nucleotide phosphodiesterase (PDE) enzymes regulate myocardial contraction. They terminate the action of adenosine 3':5'-cyclic monophosphate (cyclic AMP) by catalysing its hydrolysis to 5' adenosine monophosphate (Reeves *et al.*, 1987). Inhibition of PDE results in an increase in cyclic AMP, similar to β-adrenoceptor agonist stimulation of adenylate cyclase. The resulting increased activation of cyclic AMP-dependent protein kinase elicits increased phosphorylation of several regulatory proteins in the heart, with subsequent increased contractility (Feldman *et al.*, 1987; Karczewski *et al.*, 1990; Bristow *et al.*, 1990). The initial aim of this study was to characterize the inotropic effects of PDE inhibition in ventricular myocytes isolated from both human and guinea-pig hearts. Previous work on PDE inhibition in cardiac muscle has been carried out on whole muscle preparations (Feldman *et al.*, 1987; Gristwood *et al.*, 1987; Weishaar *et al.*, 1987; Bohm *et al.*, 1988a,d; Schmitz *et al.*, 1989; Bethke *et al.*, 1991; von der Leyen *et al.*, 1991). The advantages of myocyte over multicellular preparations are numerous. They include the lack of scar tissue or fibrosis, and the avoidance of external neural and humoral influences (Harding *et al.*, 1991). We have previously shown that selected isolated myocytes retain the characteristics of whole heart and isolated papillary muscle in terms of response to isoprenaline and increased extracellular calcium (Harding *et al.*, 1988).

Five isoenzymes of PDE have been defined; PDE I–V, and their inhibitors are classified according to their selectivity (Beavo & Reifsnyder, 1990). PDE III inhibition appears to be necessary for eliciting a positive inotropic effect, irrespective of whether the substance is a selective PDE III inhibitor

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or inhibits cell isoenzymes with similar potencies (Schmitz *et al.*, 1989). In this study, we therefore used the selective PDE III inhibitors (4-acetamido)phenylpyrazin-2(1H)-one (SK&F 94120) (Reeves *et al.*, 1987; Gristwood *et al.*, 1987) and 2-cyano-1-methyl-3- [4-(4-methyl-6-oxo-1,4,5,6-tetrahydropyridazin-3-yl)phenyl]guanidine (SK&F 94836) (Beavo, 1988). Also studied was 3-isobutyl-1-methylxanthine (IBMX), a non-selective PDE inhibitor (Schmitz *et al.*, 1989; Nicholson *et al.*, 1991).

Attenuated contractile response to β-adrenoceptor stimulation, as a consequence of a decrease in both the receptor number (Bristow *et al.*, 1982) and cyclic AMP production (Feldman *et al.*, 1987; Danielsen *et al.*, 1989), is well documented in heart failure (Bohm *et al.*, 1988a,c; Bristow *et al.*, 1990). This is believed to be a result of chronic exposure to the observed high plasma noradrenaline levels of patients with heart failure (Thomas & Marks, 1978). Therefore, our second aim in this study was to investigate the synergistic effects of PDE inhibitors and β-adrenoceptor stimulation by isoprenaline in myocytes isolated from both failing human hearts and the hearts of noradrenaline-treated guinea-pigs. Such analysis will determine to what degree reversal of β-adrenoceptor desensitization is possible in these tissues.

Methods

Male Dunkin-Hartley guinea-pigs of weight 300–700 g were anaesthetized with 2% Hypnorm (0.5 ml kg^{-1}) and a local injection of lignocaine hydrochloride (2 ml kg^{-1}). Osmotic minipumps (model 2001, Alzet, U.S.A.) containing (−)-noradrenaline hydrochloride dissolved in saline and 1 mM ascorbate were incubated in saline for 4 h at 37°C and implanted sub-cutaneously in the neck. The mean pumping rate of the pumps was 0.97 μl h^{-1} with a concentration of noradrenaline such that the guinea-pigs received, over a period of 7 days, 900 μg kg^{-1} h^{-1}.

Human ventricular tissue was obtained from explanted hearts at the time of transplant. All patients were in end stage heart failure; nine due to ischaemic cardiomyopathy, five due to dilated cardiomyopathy, one hypertrophic cardiomyopathy and one primary pulmonary hypertension. Two non-failing hearts from normal donors were also obtained, the circumstances being patient incompatibility and excessive arteriosclerosis.

Isolation of the ventricular myocytes was as previously described (Harding *et al.*, 1990), via enzymatic digestion procedures, involving a Langendorff perfusion method for guinea-pig hearts and a modified chunk isolation for human myocardium. The isolated myocytes were chosen for contraction experiments via strict selection criteria. These were; a sarcomere length greater than 1.60 μm (human) and 1.70 μm (guinea-pig), spontaneous contractions of a rate less than 2 min^{-1} when unstimulated, stable contraction amplitude and diastolic length at a stimulation rate of 0.2 Hz (human) and 0.5 Hz (guinea-pig), and a complete recovery of contraction amplitude after challenge with maximally stimulating concentrations of calcium. Only those cells which contracted in line with their long axis could be studied. Contractility experiments were carried out as previously described (Harding *et al.*, 1988), and continuously monitored with a video camera/edge detection system.

Concentrations of inotropes were increased cumulatively until no further increase in contraction amplitude was shown over one log unit, or until arrhythmic contractions were obtained. The EC_{50}, i.e. concentration at which half maximal response is obtained, values for isoprenaline and the isoprenaline/calcium ratio (the maximum response to isoprenaline relative to that produced by maximally stimulating concentrations of calcium) for ventricular myocytes have been determined to be an indication of the severity of heart failure in cells isolated from failing human hearts (Harding *et al.*, 1992a), and an indication of the degree of desensitization in myocytes from noradrenaline-treated animal hearts (Jones *et al.*, 1990; Brown & Harding, 1992).

The effectiveness of the PDE inhibitors was tested by their ability to potentiate the positive inotropic effect of isoprenaline. Following initial maximum response to calcium and isoprenaline, a threshold inotropic concentration of PDE inhibitor was determined and the concentration-response curve to isoprenaline repeated in its presence. For concentration-response curves, responses were normalised to their own maxima with basal and, when present, minimal PDE responses, subtracted. In the graphs showing % cell shortening and maximum contraction as a percentage of maximum calcium, unless otherwise indicated, no basal subtractions were made. In experiments where the effect of maximum inotropic concentrations of IBMX or 8-(4-chlorophenylthio)-adenosine 3':5'-cyclic monophosphate (CPT cyclic AMP) were investigated, the compounds were added at the point of maximum response to isoprenaline.

Previous results have shown a decline in responsiveness of guinea-pig ventricular myocytes to successive isoprenaline concentration-response curves (a decrease of 9.3 ± 2.35% s.e.mean, $n = 6$, in the maximum contraction amplitude). Subtracting the preceding basal contraction away from each maximum isoprenaline response did not abolish the effect. Thus it was necessary to carry out second control calcium and isoprenaline concentration-response curves following PDE inhibitor washout so that the stability of myocytes could be evaluated. The maximum inotropic effect of isoprenaline in the presence of PDE inhibition was compared to the mean of the two control isoprenaline maxima.

A second control curve to isoprenaline was not routinely carried out in human myocytes. Previous experiments have indicated that there is little decrease in the maximum response to β-adrenoceptor stimulation with time in human cells. Three consecutive challenges with β-agonist were performed on the same cell, 1 h apart. There was no significant change in maximum shortening (5.4 ± 0.5%, 5.5 ± 0.4% and 5.3 ± 0.5% respectively, $n = 7$) or pD_2 (7.55 ± 0.28, 7.32 ± 0.22 and 7.22 ± 0.30, $n = 5$). Thus, comparisons were made only with the initial isoprenaline maximum.

Statistical significance was assessed by Student's t tests (for paired data where possible) and P values quoted. Values quoted throughout are mean ± standard error and n numbers represent the numbers of animals or explanted hearts. Results obtained from several cells for one heart preparation were pooled. For EC_{50}s, t tests were performed on the log transformed variable (the pD_2).

Materials

(−)-Noradrenaline hydrochloride, (−)-isoprenaline hydrochloride and CPT cyclic AMP were obtained from Sigma. SK&F 94120 and SK&F 94836 were from Smithkline and Beecham, the kind gift of Brian Warrington, and were diluted from stock solutions of 10^{-2} M dissolved in 0.1 M NaOH solution in dimethyl sulphoxide (DMSO), IBMX, was obtained from Aldridge Chemical Group, Dorset, and dissolved to 10^{-3} M stock solution in Krebs Henseleit solution.

Results

Guinea-pig ventricular myocytes

Characterization of myocytes isolated from noradrenaline-treated guinea-pigs The maximum percentage cell shortening in the presence of high extracellular calcium was similar in myocytes isolated from control and noradrenaline-treated guinea-pigs. The responses were 10.8 ± 1.8% ($n = 46$) and 10.8 ± 0.6% ($n = 42$) respectively, with a maximal concentration range of 8 to 20 mM Ca^{2+}. Recovery from a challenge with maximum calcium was rapid for both groups. This suggests that those myocytes subjected to high concentrations

of noradrenaline for 7 days were structurally intact. No major cellular defect of excitation-contraction coupling or in the contractile apparatus was detected.

The maximum isoprenaline/calcium ratio for the pooled left ventricular myocytes isolated from the noradrenaline-treated guinea-pigs was $60.0 \pm 2.5\%$ ($n = 42$) which is significantly less ($P < 0.001$) than that of the myocytes isolated from the control guinea-pigs ($79.5 \pm 1.7\%$, $n = 46$). Such a depression is an indication of β-adrenoceptor desensitization as a result of the noradrenaline treatment. This is also shown by the approximately 20 fold increase in the EC_{50} of the isoprenaline concentration-response curves; pD_2 values being 8.72 ± 0.07 μM ($n = 40$) for the control myocytes and 7.47 ± 0.09 μM ($n = 38$) for the noradrenaline treated group ($P < 0.001$).

The isoprenaline/calcium ratio and the pD_2 value for isoprenaline in left ventricular myocytes isolated from sham-operated guinea-pigs were not significantly different from ventricular myocytes isolated from normal/untreated guinea-pig hearts (data not shown). This is consistent with a previous study where we used an animal model of β-agonist induced desensitization (Jones *et al.*, 1990). Thus data obtained from myocytes isolated from sham-operated and untreated guinea-pigs were pooled together and considered control data.

Increase in contraction amplitude with PDE inhibitors Figure 1 shows the concentration-response curves to IBMX in both control and noradrenaline-treated guinea-pig ventricular myocytes. There was a significant rightward shift in the noradrenaline-treated guinea-pig ventricular myocytes compared to control, pD_2 values being 4.37 ± 0.20 μM ($n = 6$) and 5.36 ± 0.15 μM ($n = 7$), respectively ($P < 0.005$). The maximum contraction amplitudes compared to calcium were depressed following noradrenaline treatment, from $74.9 \pm 4.6\%$ ($n = 7$) to $61.7 \pm 2.7\%$ ($n = 6$) ($P < 0.02$).

The necessary amount of solvent used to dissolve the compounds SK&F 94120 and SK&F 94836 limited the maximum concentration which could be used. In the case of SK&F 94120, concentrations of 3 mM or greater had a detrimental effect on the cells due to the negative inotropic effect of the DMSO concentration (a decline in contraction amplitude of $12.7 \pm 4.5\%$, $n = 5$, for dilutions of 3:1000, and 19.8 ± 3.8 μM, $n = 4$, for 1:100 dilutions). For this reason it was not always possible to obtain true EC_{50} values for SK&F 94120 and SK&F 94836, particularly in the noradrenaline-treated animals.

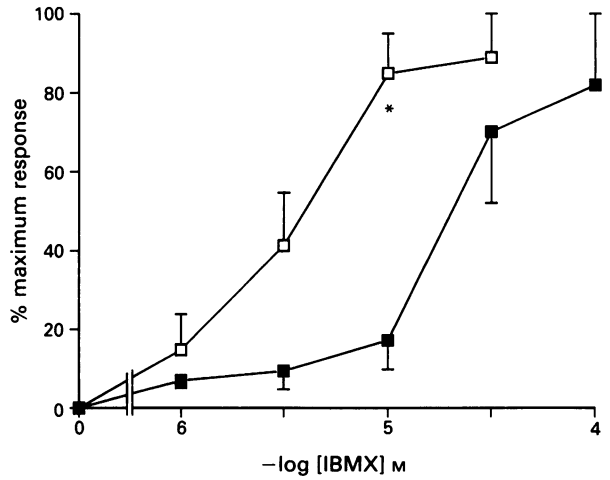


Figure 1 Concentration-response curves to 3-isobutyl-1-methylxanthine (IBMX) in ventricular myocytes isolated from control (□, $n = 7$) and noradrenaline-treated guinea-pig hearts (■, $n = 6$). Values are expressed as a percentage of the maximum isoprenaline response with basal contraction amplitudes subtracted, and are mean ± s.e.mean. *$P < 0.001$ statistically significantly different response at the corresponding concentration of isoprenaline.

Those experiments where maximal inotropic concentrations of SK&F PDE inhibitors were possible, showed a slight shift to the right in the noradrenaline-treated guinea-pig ventricular myocyte-response curve, compared to control responses. These shifts were smaller than those seen with IBMX, and were not statistically significant (pD_2 shifts being 5.01 ± 0.24 μM to 4.48 ± 0.13 μM, $n = 6$, for SK&F 94120 and 4.60 ± 0.33 μM, $n = 8$, to 4.36 ± 0.30 μM, $n = 6$, for SK&F 94836). Noradrenaline treatment did not cause a significant depression of the maximum obtainable response by PDE inhibition with SK&F 94120 or SK&F 94836. However, the changes seen are underestimated due to the necessary omission of cells from the most severely desensitized animals.

In Table 1, myocyte contraction amplitude was normalized to the maximum calcium response. Mean response values to a common PDE inhibitor concentration: 10 μM, were calculated from all the experiments on myocytes isolated from control and noradrenaline-treated guinea-pigs. A significant depression of the response was seen for IBMX in noradrenaline-treated animals, but not for SK&F 94120 or SK&F 94836.

Synergism of PDE inhibition and β-adrenoceptor stimulation Due to the varying degree of desensitization, we did not use a standard concentration of PDE inhibition in the investigation of its synergistic effects with isoprenaline. Instead a minimum inotropic effect was determined for each cell. In the case of SK&F 94120, for the control myocytes the mean threshold concentration used was 1.99 ± 1.08 μM which gave a mean increase in cell shortening over basal, as a percentage of maximum calcium, of $10.1 \pm 1.9\%$ ($n = 9$). These values are not significantly different from those of the noradrenaline-treated guinea-pigs ventricular myocytes, where a concentration of 7.33 ± 1.71 μM was used, giving a response of $8.43 \pm 4.11\%$ ($n = 6$). Similarly, the threshold inotropic response of IBMX was $20.6 \pm 5.1\%$ at a mean concentration of 3.66 ± 0.99 μM ($n = 11$) in control myocytes, not significantly different from $11.7 \pm 5.6\%$ at a mean concentration of 5.67 ± 1.39 μM ($n = 9$) in the noradrenaline-treated guinea-pig ventricular myocytes. Mean threshold concentrations of SK&F 94836 were significantly different for the two groups: 2.79 ± 1.10 μM for the control myocytes and a higher $15.5 \pm 47.$ μM for the myocytes isolated from noradrenaline-treated guinea-pigs ($P < 0.05$). Despite this, the increase over basal was significantly lower in the noradrenaline group as compared to controls: $3.25 \pm 1.77\%$ ($n = 6$) and $22.2 \pm 4.6\%$ ($n = 8$) ($P < 0.01$), respectively.

Table 2 gives the % maximum cell shortening data for isoprenaline concentration-response curves, both alone and in combination with the minimum inotropic concentration of the named PDE inhibitors. In the case of SK&F 94120, these values are expressed graphically in Figure 2a,b; for myocytes isolated from control and noradrenaline-treated guinea-pigs. For every case of PDE inhibition, apart from IBMX in control myocytes, the isoprenaline concentration-response curve was significantly shifted to the left in the presence of a

Table 1 Contraction amplitude of control and noradrenaline-treated guinea-pig ventricular myocytes with 10 μM SK&F 94120, SK&F 94835 and 3-isobutyl-1-methylxanthine (IBMX)

	SK&F 94120	*SK&F 94836*	*IBMX*
Control	25.7 ± 10.2 (6)	22.8 ± 6.6 (9)	61.4 ± 9.1 (7)
Noradrenaline-treated	11.0 ± 4.2 (9)	11.7 ± 4.5 (8)	10.4 ± 4.9 (6)*

Values expressed as % maximum calcium response, mean ± s.e.mean. Number of hearts in parentheses.
*$P < 0.001$ significantly different to corresponding control.

Table 2 Maximum contraction amplitudes and pD_2 values of control and noradrenaline-treated guinea-pig ventricular myocytes to isoprenaline and isoprenaline in the presence of threshold inotropic concentrations of phosphodiesterase (PDE) inhibitors

	SK&F 94120		*SK&F 94836*		*IBMX*	
	Control	*NA-treated*	*Control*	*NA-treated*	*Control*	*NA-treated*
Isoprenaline						
Contraction amplitude (% maximum calcium response)	79.0 ± 3.8 (7)	50.5 ± 7.3 (7)*	68.1 ± 4.2 (6)	45.1 ± 5.0 (5)*	68.5 ± 3.7 (9)	50.3 ± 5.9 (6)*
pD_2 (nM)	8.67 ± 0.08 (6)	7.08 ± 0.23 (6)**	8.81 ± 0.09 (7)	7.17 ± 0.15 (6)**	8.72 ± 0.23 (6)	7.15 ± 0.15 (7)**
Isoprenaline & PDEI						
Contraction amplitude (% maximum calcium response)	83.0 ± 6.8 (6)	70.0 ± 2.0 (6)†	65.4 ± 5.3 (8)	57.3 ± 7.6 (6)	71.1 ± 4.3 (11)	61.9 ± 4.7 (9)
pD_2 (nM)	9.28 ± 0.10 (7)††	8.11 ± 0.20 (6)**††	9.48 ± 0.08 (6)††	8.23 ± 0.24 (5)**††	9.06 ± 0.26 (6)	8.26 ± 0.24 (7)*††

Mean values ± s.e.mean, number of hearts in parentheses. PDEI: PDE inhibition.
*$P<0.01$, **$P<0.001$ significantly different from corresponding control.
†$P<0.05$, ††$P<0.01$ significantly different from initial isoprenaline in corresponding group.

PDE inhibitor. There was a tendency for a greater shift in the noradrenaline-treated guinea-pig ventricular myocytes than in the control myocytes. Figure 2b shows an approximate 12 fold shift in the isoprenaline EC_{50} caused by SK&F 94120 in the noradrenaline-treated guinea-pig ventricular myocytes, comparable to only 4 fold in the control myocytes (Figure 2a).

Figure 3 represents the maximum contractile responses of both myocyte groups to mean control isoprenaline (pre and post-SK&F 94120 treatment) and to isoprenaline in the presence of the threshold inotropic concentration of SK&F 94120. The values are expressed as a percentage of maximum calcium response. Such analysis showed a significant potentiation of the inotropic response of isoprenaline by SK&F 94120 only in the myocytes isolated from the noradrenaline-treated guinea-pigs. An increase in the % cell shortening from 6.51 ± 0.97% ($n = 5$) to 9.45 ± 0.64% ($n = 6$) was found ($P<0.05$). In contrast, SK&F 94120 did not cause a potentiation of the mean isoprenaline response in control myocytes.

IBMX and SK&F 94836 showed the same tendency to increase the maximum contraction in cells from noradrenaline-treated, but not control, animals (Table 2) although the effect escaped statistical significance. To examine the quantitative nature of the effect of PDE inhibition more closely, the data for all PDE inhibitors were pooled (Figure 4). A potentiation for the isoprenaline response was only noticed in the noradrenaline-treated guinea-pig ventricular myocytes, with a significance level of $P<0.01$. However, the initial depressed isoprenaline maximum was not completely reversed to control levels by PDE inhibition at minimal inotropic concentrations.

The effect of the minimum inotropic concentration of IBMX on the initial isoprenaline maximum was compared to that of maximum inotropic concentrations in both control myocytes (30 μM IBMX) and in those from noradrenaline-treated guinea-pig hearts (100 μM IBMX), as shown in Figure 5. The synergistic response of isoprenaline plus PDE inhibition is expressed as a percentage of the initial isoprenaline % cell shortening. There was no significant difference in maximum isoprenaline responses in the presence of threshold inotropic and maximally stimulating concentrations of IBMX, % maximum isoprenaline values being 108 ± 3% ($n = 8$) and 102 ± 2% ($n = 6$), respectively. Similarly, synergistic responses of noradrenaline-treated guinea-pig myocytes were comparable for threshold (125 ± 6%, $n = 6$), and maximum concentrations of IBMX (108 ± 3%, $n = 6$).

The inotropic responses of PDE inhibition and β-adrenoceptor stimulation were further compared to that of maximum concentrations of CPT cyclic AMP, an analogue of cyclic AMP (Collis *et al.*, 1989). Again there was no significant difference, from that of IBMX, in the extent of potentiation over maximum isoprenaline in either group of guinea-pig ventricular myocytes (Figure 5).

Human ventricular myocytes

Results were obtained on isolated ventricular myocytes from 16 failing and two non-failing explanted human hearts. The maximum isoprenaline/calcium ratio for the human failing

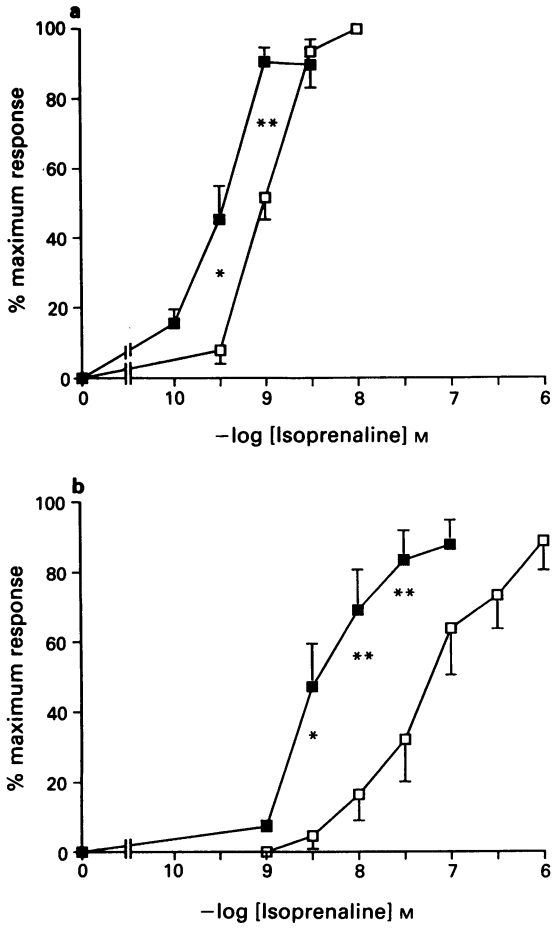


Figure 2 Concentration-response curves to isoprenaline (□) and isoprenaline in the presence of threshold inotropic concentrations of SK&F 94120 (■) of guinea-pig ventricular myocytes isolated from (a) control ($n = 9$), and (b) noradrenaline-treated hearts ($n = 6$). Values are expressed as a percentage of the maximum isoprenaline response with basal contraction amplitudes subtracted, and are mean ± s.e.mean. *$P<0.01$; **$P<0.001$: statistically significantly different response at the corresponding concentration of isoprenaline.

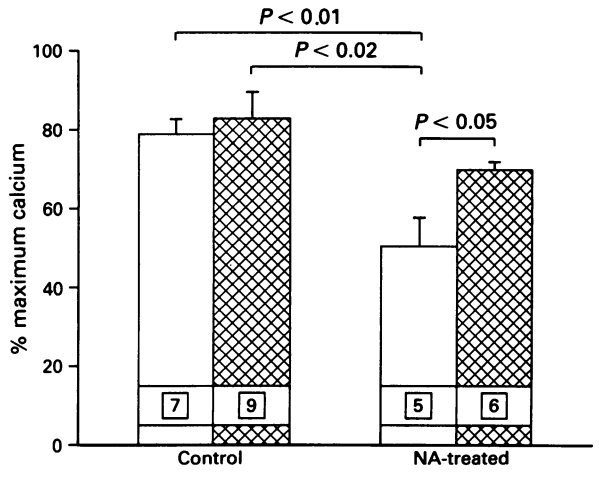


Figure 3 Maximum isoprenaline responses calculated from ventricular myocytes isolated from control and noradrenaline (NA)-treated guinea-pig hearts. Open columns are pooled mean isoprenaline responses before and after PDE inhibition, and the cross hatched columns are isoprenaline responses during exposure to a threshold inotropic concentration of SK&F 94120. Values are expressed as a percentage of the maximum calcium response with no subtraction of basal amplitude, and are mean ± s.e.mean. Number of hearts indicated in boxes for each column.

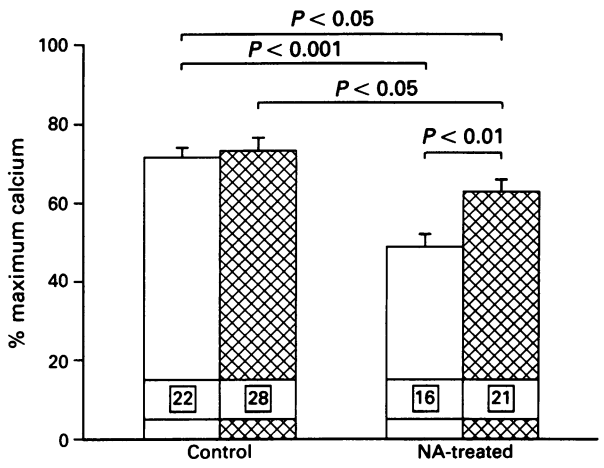


Figure 4 Maximum isoprenaline responses calculated from ventricular myocytes isolated from control and noradrenaline (NA)-treated guinea-pig hearts. Open columns are pooled mean isoprenaline responses before and after PDE inhibition, and the cross hatched columns are pooled values for isoprenaline plus a threshold concentration of SK&F 94120, SK&F 94836 or 3-isobutyl-1-methylxanthine (IBMX). Values are expressed as a percentage of the maximum calcium response with no subtraction of basal amplitude, and are mean ± s.e.mean. Number of hearts indicated in boxes for each column.

group was 56.4 ± 4.1%, $n = 16$. This is significantly less than the maximum response to isoprenaline previously shown in myocytes from non-failing hearts which is close to, or higher than the maximum calcium (116 ± 7%, $n = 6$, $P < 0.001$) (Brown & Harding, 1992). The β-adrenoceptor desensitization was further shown in these failing myocytes by their significantly lower isoprenaline pD_2 values compared to non-failing, 8.06 ± 0.19 ($n = 16$) and 8.89 ± 0.30 ($n = 6$) respectively, ($P < 0.05$).

Twelve myocytes from 10 failing human hearts were challenged with isoprenaline in the presence and absence of IBMX. The mean concentration of IBMX was 40.3 ± 13.4 μM, which gave a minimal inotropic response of 25.0 ± 6.19% ($n = 10$). Figure 6 shows the potentiation of low

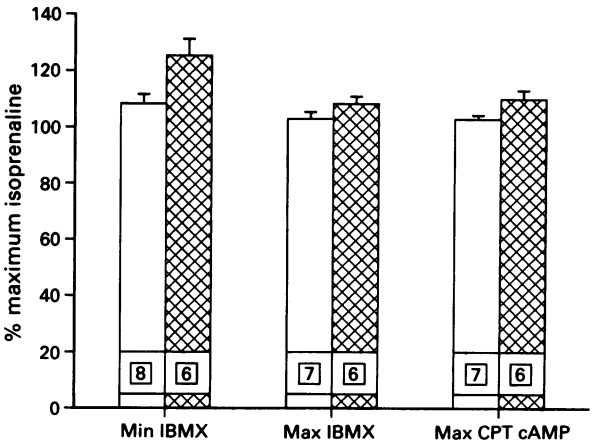


Figure 5 Potentiation of the maximum isoprenaline response by threshold (min) and maximum inotropic (max) concentrations of 3-isobutyl-1-methylxanthine (IBMX), and by maximum inotropic concentrations of CPT cyclic AMP, of ventricular myocytes isolated from control (open columns) and noradrenaline-treated (cross hatched columns) guinea-pig hearts. Concentrations were, respectively, 3.6 ± 1.0 μM and 5.7 ± 1.4 μM for threshold IBMX, 30 μM and 100 μM for maximum IBMX, 100 μM and 1 mM for CPT cyclic AMP. Values are expressed as a percentage of the maximum isoprenaline response, and are mean ± s.e.mean. Number of hearts indicated in boxes for each column.

isoprenaline concentrations by IBMX. There was no significant potentiation of the maximum effect of isoprenaline by IBMX in these 12 failing myocytes, see Figure 7. In the two non-failing myocytes used for this study, threshold inotropic concentrations of IBMX again potentiated the low concentrations of isoprenaline, but failed to potentiate the maximum. Figure 8 of a trace recording shows the actual responses of one of these myocytes.

SK&F 94120 at a mean threshold inotropic concentration of 100 ± 45 μM gave a response of 9.02 ± 4.13% in six failing human myocytes. Such concentrations caused an approximate nine fold decrease in the isoprenaline EC_{50}, pD_2 values being 8.05 ± 0.49 for isoprenaline alone and 8.84 ± 0.23 ($n = 3$), when in the presence of SK&F 94120. SK&F 94120 was shown to cause a significant potentiation of the isoprenaline response in myocytes isolated from failing human myocardium (see Figure 7). The mean % cell shortening

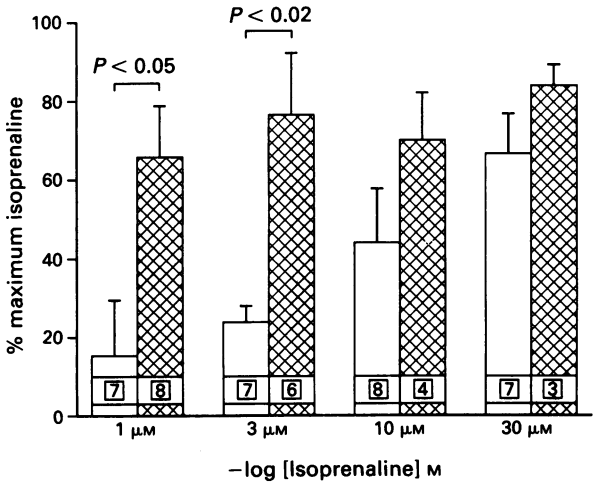


Figure 6 Contractile responses of failing human ventricular myocytes to four concentrations of isoprenaline, both alone (open columns) and in the presence of a threshold inotropic concentration of 3-isobutyl-1-methylxanthine (cross hatched columns). Values are expressed as a percentage of the maximum isoprenaline response with basal contraction amplitudes subtracted, and are mean ± s.e.mean. Number of patients indicated in boxes for each column.

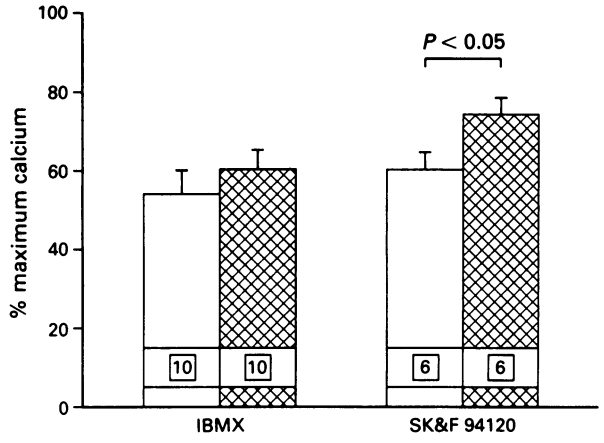


Figure 7 Maximum contractile responses of failing human ventricular myocytes to isoprenaline (open columns) and isoprenaline in the presence of threshold concentrations of 3-isobutyl-1-methylxanthine (IBMX) or SK&F 94120 (cross hatched columns). Values are expressed as a percentage of the maximum calcium response, and are mean ± s.e.mean. Number of patients indicated in boxes for each column.

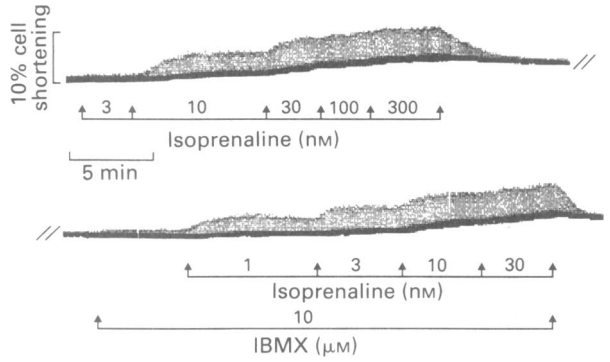


Figure 8 A typical trace showing the contraction amplitude of a human ventricular myocyte isolated from a non-failing donor heart responding to 1 mM Ca^{2+} and to cumulatively increasing concentrations of isoprenaline in the absence and presence of 10 μM 3-isobutyl-1-methylxanthine (IBMX). An upward deflection represents cell shortening.

increased from 6.18 ± 0.59% ($n = 6$) to 7.54 ± 0.40% ($n = 6$) when in the presence of SK&F 94120. The maximum response was significantly ($P < 0.05$, $n = 6$), increased from 60.3 ± 4.5% to 74.3 ± 4.2% maximum calcium. This is substantially lower than the 116 ± 7% ($P < 0.001$, $n = 6$) seen in myocytes isolated from donor hearts.

Discussion

Cyclic nucleotide phosphodiesterase inhibition was shown to elicit a positive inotropic response in guinea-pig ventricular myocytes. At concentrations of 10 μM, the non-specific PDE inhibitor IBMX was shown to be more effective than SK&F 94120 and SK&F 94836 which are PDE III inhibitors. The maximum mean response of IBMX was 60% of that caused by maximally stimulating concentrations of calcium, with an EC_{50} value of 4.4 μM. An EC_{50} value of 70 μM has been reported by Scholz & Meyer (1986), using guinea-pig ventricular papillary muscle preparations. Maximum response values for the SK&F compounds were limited by solubility restrictions, however the mean threshold inotropic concentration of 2 μM for SK&F 94120 is consistent with that of previous reports (Gristwood *et al.*, 1987), which gave a value of 1 μM for guinea-pig papillary muscle preparations.

PDE III is a major component of the pool of cardiac cyclic nucleotide phosphodiesterases (Beavo & Reifsnyder, 1990) and exerts a significant influence on myocardial contraction (Weishaar *et al.*, 1987). Therefore, specific PDE III inhibitors would be expected to be potent in the isolated myocyte preparation. The surprisingly large responses of IBMX may be due to its additional effects, all of which may increase contractility. These include its competitive reversible effect on inhibitory adenosine (Bohm *et al.*, 1988b), its possible activation of the sarcoplasmic reticulum calcium release channels and sensitization of the myofilaments to calcium (Scholz & Meyer, 1986), reduction of the activity of inhibitory guanine nucleotide protein (Gi) function (Parsons *et al.*, 1988) or additional effects on other PDE isoenzymes, see Shahid & Nicholson (1990).

We have previously demonstrated that in myocytes isolated from failing human hearts, the positive inotropic effect via β-adrenoceptor activation is diminished, the degree of which correlates well with the severity of disease (Harding *et al.*, 1992a). Such agonist subsensitivity was also shown in this study where myocytes isolated from failing human ventricle had a lower isoprenaline to calcium ratio and a higher EC_{50} to isoprenaline compared to previous data from non-failing myocytes. Groups working on whole muscle cardiac preparations have also reported a depression in the isoprenaline response in human failing hearts compared to non-failing hearts (Brown *et al.*, 1986; Bohm *et al.*, 1988d; Nabauer *et al.*, 1988; von der Leyen *et al.*, 1991). In this study, myocytes isolated from noradrenaline treated guinea-pigs showed similar diminished isoprenaline responses compared with control myocytes. This is analogous to the β-adrenoceptor desensitization which is seen in patients with heart failure. Our present results are consistent with previous work on noradrenaline-induced desensitization in guinea-pig (Wynne *et al.*, 1991; Brown & Harding, 1992) and rat (Jones *et al.*, 1990) myocytes.

The inotropic responses of noradrenaline treated guinea-pig ventricular myocytes to the PDE inhibitor IBMX, were depressed in comparison to control myocytes, the maximum response being significantly decreased. These results are similar to those of other groups who have reported a diminished response with PDE inhibition in isometrically contracting trabeculae from failing human heart as compared to those from non-failing hearts (Bohm *et al.*, 1988d; von der Leyen *et al.*, 1991; Steinfath *et al.*, 1992). The same studies show no differences in the response to high calcium, or in the basal activity of the PDE enzymes, between failing and non-failing human papillary muscle preparations. Similarly, in this study the responsiveness of the noradrenaline treated guinea-pig ventricular myocytes to calcium was unaltered compared to control myocytes. Therefore, it is unlikely that the lack of efficiency of the PDE inhibitors in the myocytes isolated from noradrenaline-treated guinea-pig hearts was due to an impairment in the contractile apparatus, or due to an enhanced breakdown of cyclic AMP. The concentration of IBMX giving half maximal inhibition of cardiac PDE is reported as 10 μM (Galvan & Schudt, 1990) in guinea-pig myocardium. It has been shown that prenalterol-treated (Bobik & Little, 1984) and noradrenaline-treated (Reithmann & Werdan, 1988) chick embryo cells have depressed levels of cyclic AMP under β-adrenoceptor stimulation. Depressed cyclic AMP levels have also been reported for human failing myocardium compared to non-failing (Danielson *et al.*, 1989). Therefore, the reduced inotropic responses of PDE inhibition seen in myocytes isolated from our animal model of β-adrenoceptor desensitization may be attributable to impaired cyclic AMP synthesis. The second part of this study was aimed at investigating the synergistic effects of PDE inhibition and β-adrenoceptor stimulation. Such stimulation would determine whether elevated cyclic AMP levels alone would be sufficient to restore the diminished isoprenaline responses of myocytes isolated from failing human hearts and noradrenaline-treated guinea-pig hearts, to non-failing and control levels, respectively.

In the presence of PDE inhibitors the concentration-response curves to isoprenaline were shifted to the left. This

was the case for myocytes from human and guinea-pig, with a more pronounced shift seen in the β-adrenoceptor desensitized myocytes. This was also the case in the extent of potentiation of the maximum isoprenaline response. No potentiation of the maximum isoprenaline response by PDE inhibition was seen in control guinea-pig myocytes and myocytes isolated from non-failing human hearts. However in the β-adrenoceptor densensitized myocytes isolated from human failing and noradrenaline-treated guinea-pig hearts, SK&F 94120 potentiated the maximum isoprenaline effect. No significant effect was seen with IBMX or SK&F 94836. In these respects the selective PDE III inhibitor was more potent than the non-selective IBMX. This is consistent with results of Gristwood *et al.* (1987) who, with concentrations within our range, showed potentiation of the maximum isoprenaline response by PDE III inhibition with SK&F 94120 in failing human myocardium, but not in control guinea-pig muscle.

In control, non-β-adrenoceptor desensitized myocytes, β-adrenoceptor agonist-stimulated cyclic AMP is likely to be at levels adequate to generate a maximum inotropic response. Attempting to increase levels further through PDE inhibition would cause no additional increase in inotropic effect. However, in myocytes isolated from failing human and noradrenaline-treated guinea-pig hearts, it is possible for PDE inhibition to increase the reported depressed levels of cyclic AMP (Danielsen *et al.*, 1989; von der Leyen *et al.*, 1991) and hence potentiate the maximum inotropic effect of isoprenaline.

Our results are in agreement with the hypothesis that deficient β-adrenoceptor-stimulated cyclic AMP synthesis may be an underlying defect in hearts of patients with end-stage heart failure, probably due to a defect in the receptor-adenylate cyclase coupling. This may be a result of β-adrenoceptor down regulation in addition to increased levels of Gi in both noradrenaline-treated guinea-pig ventricular myocytes (Reithmann *et al.*, 1989) and in human failing myocardium (Feldman *et al.*, 1988; Neumann *et al.*, 1988).

Pooling of all PDE inhibitor results showed incomplete reversal of the noradrenaline-induced β-adrenoceptor desensitization in the myocytes isolated from the model. There still remained a significant depression of the isoprenaline maximum response, despite the fact that the effects of lower concentrations of isoprenaline were increased more than four fold with these concentrations. Similar results were obtained on human myocytes. The small potentiation observed in the maximum effect of isoprenaline in myocytes from failing human hearts did not restore responses to levels seen in cells from normal hearts, where the isoprenaline response was 116% of the maximum calcium. This is substantiated by additional results which show that there was no significant difference in the degree of potentiation of the maximum isoprenaline response with threshold IBMX concentrations as compared to maximal inotropic concentrations (100 μM) of IBMX. The latter concentration of IBMX was quoted by Scholz & Meyer (1986) as inhibiting almost 100% of cardiac PDE III in guinea-pig papillary muscle preparations. Furthermore no increased potentiation was shown when PDE inhibition was substituted by maximum inotropic concentrations of the analogue of cyclic AMP, CPT cyclic AMP. The results are in accord with our previous findings in myocytes from noradrenaline-treated rats (Jones *et al.*, 1990) and failing human heart (Harding *et al.*, 1992b), where the effects of N^6,$O^{2'}$-dibutyryl-cyclic AMP (db-cyclic AMP, which penetrates intracellularly to mimic cyclic AMP) were reduced compared to control.

These results provide evidence for the existence of a reduction in the effectiveness, as well as in the production, of cyclic AMP in failing human hearts and noradrenaline-treated guinea-pig myocardium. Evidence from studies on intact myocardium indicates that cyclic AMP-dependent phosphorylation of regulatory proteins such as phospholamban in the sarcoplasmic reticulum, troponin I in the myofibrils and a 15 kDa protein in the sarcolemma, may be responsible for at least some of the β-adrenergic effect on the mammalian myocardium (Karczewski *et al.*, 1990). Therefore, a possible lesion at one or more of these pathways could explain the observed post cyclic AMP abnormalities in these tissues.

Our results differ from those of Bohm and co-workers (1988d) who studied the inotropic-effects of β-adrenoceptor agonists, in combination with PDE inhibitors, on the contractility of whole muscle preparations. Bohm *et al.* (1988d) obtained a near maximal response with both β-adrenoceptor agonists and PDE inhibitors together (90% of that obtained with maximum concentrations of calcium). In the human myocytes treated with SK&F 94120, we obtained a maximum response to isoprenaline that was only 74% of maximum with calcium. Various other authors have also reported complete restoration of the diminished positive inotropic response of isoprenaline in failing human myocardium by PDE inhibition (Bethke *et al.*, 1991; von der Leyen *et al.*, 1991; Bartel *et al.*, 1992).

However, all the above studies were carried out on whole muscle preparations. Current studies are in progress in our laboratories to assess whether there is a true difference between myocytes and papillary muscle preparations from the same heart in the maximum attainable with cyclic AMP-raising agents. Our previous work has shown that there can be some quantitative differences in the expression of β-adrenoceptor desensitization between intact muscle strips and isolated cells from the same failing human heart (Harding *et al.*, 1991). Notably, in guinea-pig papillary muscle, unlike myocytes, there is an accumulation of endogenous adenosine which mediates an anti-adrenergic effect upon β-adrenoceptor responses (Hopwood *et al.*, 1987). Alternatively, it may be that patient populations were different between the present study and those of Bohm & Scholz.

To conclude, our findings indicate that reduced cyclic AMP levels, both basal and β-adrenoceptor stimulated, are a contributing factor towards the reduced inotropic responsiveness of β-agonists in myocytes isolated from both noradrenaline-treated guinea-pigs and failing human hearts. Incomplete restoration of the β-adrenoceptor response with PDE inhibition indicates that there exists an abnormality in the effect of cyclic AMP distal to the adenylate cyclase system.

We are grateful for the technical assistance of Dr Federica Del Monte, Mr Peter O'Gara and Mr Graham Storey. This study was supported by the British Heart Foundation.

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(Received December 10, 1992
Revised March 10, 1993
Accepted March 23, 1993)

Br. J. Pharmacol. (1993), **109**, 1079–1084 © Macmillan Press Ltd, 1993

α_2-Adrenoceptor-mediated modulation of the nitrergic innervation of the canine isolated ileocolonic junction

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1 The effects of specific α-adrenoceptor agonists and antagonists on electrically-evoked non-adrenergic non-cholinergic (NANC) relaxations, previously demonstrated as nitrergic, were investigated in isolated circular muscle strips of the canine ileocolonic junction.

2 During a substance P-induced contraction and in the presence of atropine and guanethidine, the specific α_1-adrenoceptor agonist, phenylephrine and antagonist, prazosin, as well as the specific α_2-adrenoceptor antagonist, yohimbine, had no effect on the NANC relaxations evoked by electrical field stimulation. In contrast, clonidine and the more specific α_2-adrenoceptor agonist, UK-14,304, significantly reduced the electrically-induced relaxations, preferentially those in response to low frequency stimulation. The inhibitory effect of UK-14,304 on these relaxations was antagonized by yohimbine.

3 During a noradrenaline-induced contraction, clonidine, but not UK-14,304 significantly augmented the relaxations to electrical stimulation.

4 The adrenoceptor agonists and antagonists used had no effect on concentration-response curves to NO or on the relaxation induced by nitroglycerin.

5 These results indicate that stimulation of prejunctional α_2-adrenoceptors inhibits the nitrergic NANC relaxations induced by field stimulation and thus suggest prejunctional regulation of nitric oxide release via α_2-adrenoceptors in the canine ileocolonic junction.

Keywords: Canine ileocolonic junction; NANC neurotransmission; nitric oxide; prejunctional α_2-adrenoceptors

Introduction

Over the last decade, a great deal of attention has been focused on the concept that neurotransmitter release can be modulated via prejunctional receptors located on, in or near the axon terminals or varicosities. Activation of these receptors can be achieved by specific molecules released from adjacent neurones or cells (heteroregulation) or from the same neurone (autoregulation), resulting in a change in the amount of neurotransmitter that is released. Hence, this process is of key importance in the determination of the synaptic efficiency and the coordination of neuronal communication. Although most of the present knowledge concerning prejunctional modulation is derived from noradrenergic neurones, prejunctional receptors have also been demonstrated on cholinergic neurones as well as on central 5-hydroxytryptamine (5-HT), dopamine and γ-aminobutyric acid (GABA) neurones (Westfall & Martin, 1991). However, evidence suggesting this concept in the peripheral non-adrenergic non-cholinergic (NANC) nervous system, and especially the nitrergic innervation, is rather scarce. In the gastrointestinal tract, prejunctional inhibition of NANC neurotransmission has only been demonstrated in the rat gastric fundus (MacDonald *et al.*, 1990; Lefebvre & Smits, 1992), the guinea-pig proximal colon (Kojima *et al.*, 1988), the rat anococcygeus muscle (Li & Rand, 1989), and the canine lower oesophageal sphincter (Barnette *et al.*, 1990).

Recently, we provided evidence that nitric oxide (NO) or a NO-releasing substance mediates the NANC nerve-induced relaxations in the canine ileocolonic junction (Boeckxstaens *et al.*, 1990a) and, using a bioassay, we actually demonstrated the release of a substance which pharmacologically behaves like NO in response to NANC nerve stimulation (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1991b). Based on these results, we suggested NO as an inhibitory NANC neurotransmitter in this tissue. Interestingly, the NANC relaxations in the canine ileocolonic junction evoked by field stimulation up to 16 Hz, 1 ms are entirely mediated by NO (Boeckxstaens *et al.*, 1990a) and thus nitrergic. Furthermore, the involvement of other putative NANC neurotransmitters such as adenosine 5'-triphosphate, vasoactive intestinal polypeptide, GABA and 5-HT had been excluded earlier (Boeckxstaens *et al.*, 1990b–e). Recently, electrophysiological evidence was also provided confirming the role of NO in the ileocolonic junction (Ward *et al.*, 1992). This knowledge, together with morphological evidence demonstrating the presence of NO synthase in its myenteric neurones (unpublished observations), make the ileocolonic junction an interesting model to study possible prejunctional regulation of NO release. Since the ileocolonic junction receives a prominent adrenergic innervation and since prejunctional modulation of NANC neurotransmission via adrenoceptors has been described (Kojima *et al.*, 1988; MacDonald *et al.*, 1990; Lefebvre & Smits, 1992), we suggest a possible prejunctional adrenergic regulation of the NO release at the canine ileocolonic junction.

Methods

Tissue preparation

Mongrel dogs of either sex (body weight 10–30 kg) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.) and a laparotomy was performed. The ileum and colon were resected 5 cm above and 3 cm below the ileocolonic junction respectively. After the resected specimen was cleaned and rinsed, the mucosa was removed from the ileum and ileocolonic junction by means of sharp dissection. Circular muscle strips of the ileocolonic junction were cut, approximately 1.5 cm long and 0.3 cm wide and mounted in organ

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baths (25 ml) (Pelckmans et al., 1989) filled with a modified Krebs-Ringer solution (mM: NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, CaEDTA 0.026 and glucose 11.1). The solution was maintained at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2.

Isometric tension recording

One end of each muscle strip was connected to a metal rod while the other end was attached to a strain gauge transducer (Statham UC2) for continuous recording of isometric tension. After the muscle strips were brought to the optimal point of their length-tension relationship (Pelckmans et al., 1989), they were washed three times and then allowed to equilibrate for at least 45 min before experimentation.

Experimental protocols

All experiments were performed in the presence of atropine (0.3 μM) and guanethidine (3 μM) and during a contraction induced by substance P (0.1 μM) or noradrenaline (30 μM). After each contraction, the muscle strips were washed at least 3 times with an interval of 5 min resulting in a cycle time of at least 20 min.

In a first series of experiments, the muscle strips were contracted with substance P (0.1 μM). Substance P was chosen as contractile agent to avoid any interference with the adrenoceptor agents used. In a first protocol, the effects of the specific adrenoceptor agonists and antagonists clonidine (1 μM), UK-14,304 (0.1–3 μM), prazosin (1–3 μM), phenylephrine (3–10 μM) and yohimbine (0.1–1 μM) were investigated on relaxations induced by electrical impulses (2–16 Hz, 0.5–2 ms, pulse trains lasting 10 s), NO (1 μM) and nitroglycerin (1 μM).

In another protocol, the effects of these agents were also studied on concentration-response curves to NO (0.3–30 μM). Finally, the effect of UK-14,304 (0.1 μM) on the electrically-induced relaxations and on those to NO (1 μM) and nitroglycerin (1 μM) was reinvestigated in the presence of yohimbine (0.1 μM).

In a second series of experiments, the effects of clonidine (1 μM) and UK-14,304 (1 μM) on the relaxations to electrical stimulation (2–16 Hz, 1–2 ms), NO (0.3–30 μM) and nitroglycerin (1 μM) were re-evaluated during a noradrenaline (30 μM)-induced contraction.

Electrical impulses (rectangular waves, 100 mA, 9 V) were delivered by a GRASS stimulator and a direct current (d.c.) amplifier in trains of stimuli of 10 s. Pharmacological agents were added at least 10 min before stimulation of the tissue.

Drugs used

The following drugs were used: atropine sulphate (Federa, Brussels, Belgium), clonidine (Boehringer Ingelheim, Germany), guanethidine (Ciba-Geigy, Switzerland), noradrenaline hydrogentartrate (Fluka AG, Buchs SG, Switzerland), phenylephrine (Sigma Chemical Co, St. Louis, MO, U.S.A.), prazosin (Pfizer, Sandwich, England), substance P, yohimbine hydrochloride (Sigma Chemical Co, St. Louis, MO, U.S.A.). UK-14,304 tartrate (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline) was a gift of Pfizer Central Research, Sandwich, England.

All drugs were administered as aqueous solutions. Ascorbic acid (57 mM) was added to the stock solution of noradrenaline and phenylephrine. The solutions were prepared on the day of experimentation and administered in volumes not exceeding 0.5% of the bath volume. NO solutions were prepared as described by Kelm et al. (1988).

Presentation of results and statistical analysis

Results are expressed as g contraction or as percentage decrease of the noradrenaline or substance P-induced contraction and are shown as mean ± s.e.mean for the number of dogs indicated.

For statistical analysis, a two tailed Student's t test for paired observations was used. P values of less than 0.05 were considered to be significant.

Results

In the presence of atropine (0.3 μM) and guanethidine (3 μM), and during a substance P (0.1 μM)-induced contraction, the specific α_1-adrenoceptor antagonist prazosin (1–3 μM), and the specific α_2-adrenoceptor antagonist yohimbine (0.1–1 μM) had no effect on the spontaneous activity of the canine ileocolonic junction (n = at least 6). In contrast, the specific α_1-adrenoceptor agonist, phenylephrine (3 μM: 0.4 ± 0.2 g; 10 μM: 0.4 ± 0.2 g, n = 8), and the specific α_2-adrenoceptor agonists, clonidine (1 μM; 0.4 ± 0.2 g, n = at least 6) and UK-14,304 (0.1 μM; 0.7 ± 0.5 g; 1 μM: 1.1 ± 0.2 g; 3 μM: 0.9 ± 0.1 g, n = at least 6), induced a tonic contraction

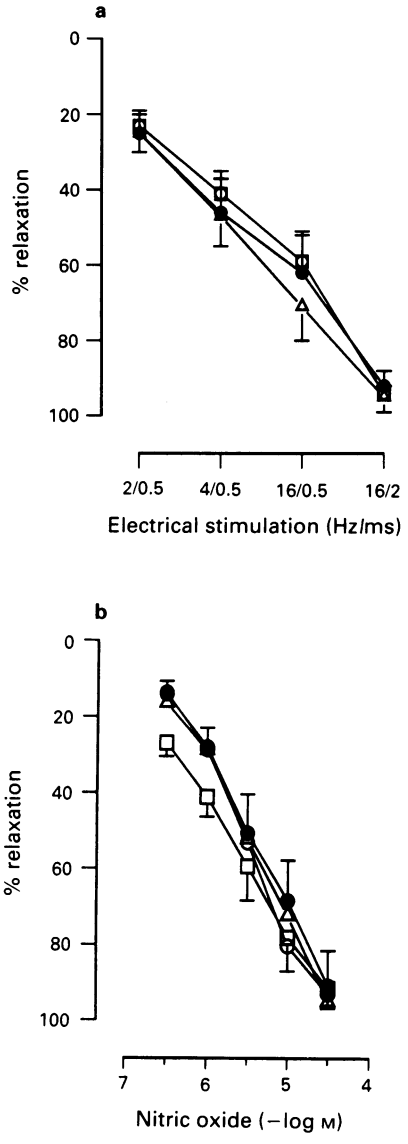


Figure 1 Effect of yohimbine (△, 1 μM), phenylephrine (□, 10 μM) and prazosin (●, 3 μM) on (a) electrically-evoked NANC relaxations (□, 2–16 Hz, 0.5–2 ms) and (b) the concentration-response curve to NO (○, 0.3–3 μM) in the canine ileocolonic junction during a substance P (0.1 μM)-induced contraction. Results are shown as mean ± s.e.mean for 5–8 experiments and are expressed as percentage decrease of the substance P-induced contraction.

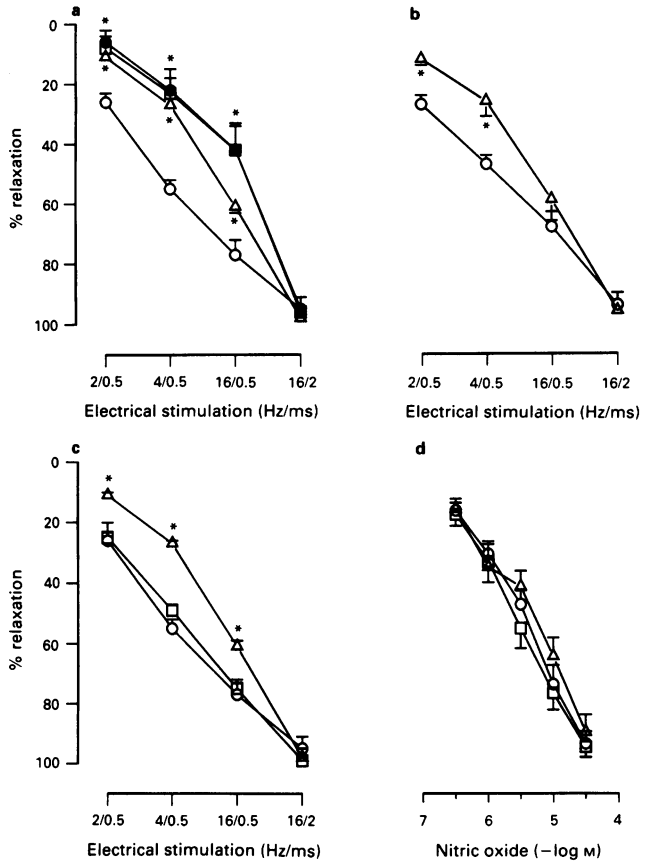


Figure 2 Effect on electrically-evoked NANC relaxations (2–16 Hz, 0.5–2 ms) (○) of (a) UK-14,304 (△, 0.1 μM; □, 1 μM; ●, 3 μM), of (b) clonidine (△, 1 μM) and of (c) UK-14,304 (0.1 μM) in the absence (△) and in the presence of (□) of yohimbine (0.1 μM). Effect of UK-14,304 (△, 3 μM) and clonidine (□, 1 μM) on the concentration-response curve to NO (○, 0.3–3 μM) in the canine ileocolonic junction (d). All experiments were performed during a substance P (0.1 μM)-induced contraction. Results are shown as mean ± s.e.mean for 4–6 experiments and are expressed as percentage decrease of the substance P-induced contraction. $^*P<0.05$, significantly different from control, Student's t test for paired observations.

confirming our previous results (Pelckmans *et al.*, 1990). None of the adrenoceptor agents affected the substance P-induced contraction (3.2 ± 0.5 g, $n = 7$).

Phenylephrine, prazosin, and yohimbine had no effect on the nitrergic relaxations evoked by electrical impulses (2–16 Hz, 0.5–2 ms), on the concentration-response curve to NO (0.3–30 μM) (Figure 1) or on the relaxations to nitroglycerin (1 μM) (results not shown). However, the α_2-adrenoceptor partial agonist clonidine (1 μM) and the more specific α_2-adrenoceptor agonist UK-14,304 (0.1–3 μM) significantly reduced the electrically-evoked NANC relaxations, preferentially those in response to low frequency stimulation (2 and 4 Hz, 0.5 ms) (Figures 2a, b and 3). The inhibitory effect of UK-14,304 was concentration-dependent (Figure 2a) and was antagonized by the specific α_2-adrenoceptor antagonist, yohimbine (0.1 μM) (Figures 2c and 3). Yohimbine also inhibited the contraction to UK-14,304 (0.1 μM) (Figure 3). Neither clonidine nor UK-14,304 had an effect on the concentration-response curve to NO (Figure 2d) or on the relaxation to nitroglycerin (1 μM, Figure 3).

Since in previous studies all our experiments were performed during a noradrenaline (30 μM)-induced contraction, a second series of experiments was designed in which we re-evaluated the effect of UK-14,304 (1 μM) and clonidine (1 μM) under such experimental conditions. To obtain relaxations of similar amplitude as during a substance P-induced contraction, the pulse duration of electrical impulses had to

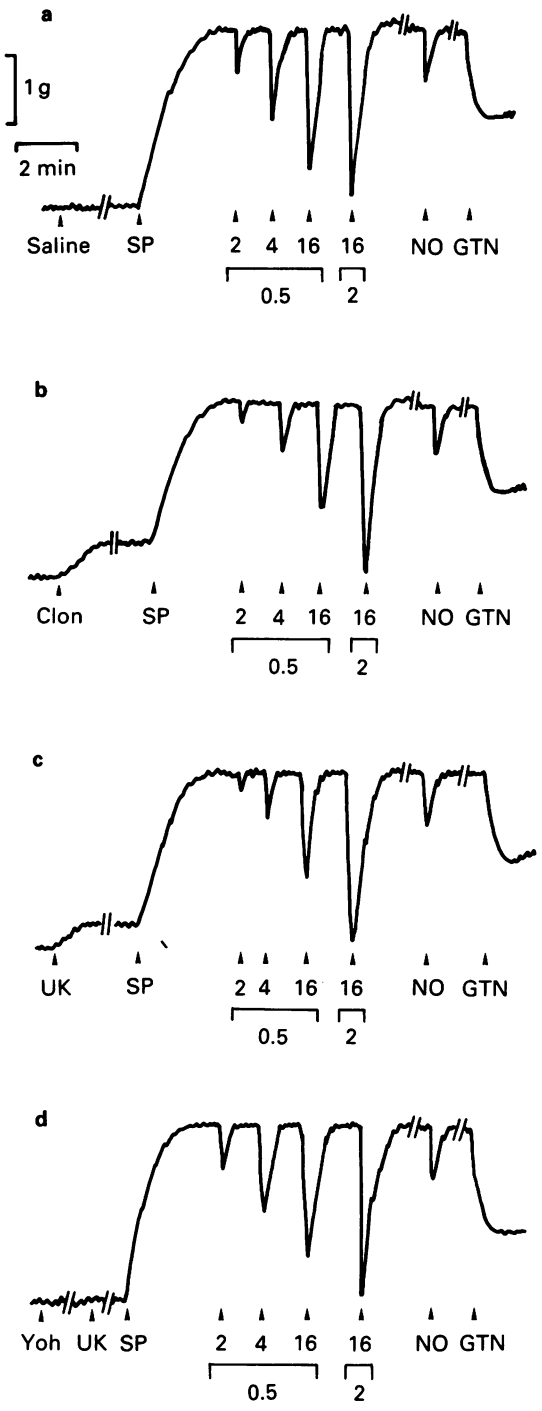


Figure 3 Isometric tension recordings showing (a) the control and the effect of (b) clonidine (Clon, 1 μM) and UK-14,304 (UK, 0.1 μM) in (c) in the absence and (d) the presence of yohimbine (Yoh, 0.1 μM) on NANC relaxations induced by electrical stimulation (2–16 Hz, 0.5–2 ms), NO (1 μM) and nitroglycerin (GTN, 1 μM) in a circular muscle strip of the ileocolonic junction. All experiments were performed during a substance P (SP, 0.1 μM)-induced contraction. Breaks in the tracings represent periods of tissue equilibration. Similar results were obtained in tissues from 3–5 other dogs.

be raised from 0.5 ms to 1 ms. In contrast to the results described above, UK-14,304 had no effect on the electrically-evoked (2–16 Hz, 1–2 ms) relaxations. On the other hand, clonidine significantly enhanced these NANC relaxations (Figures 4 and 5), preferentially those in response to low frequency stimulation (2–4 Hz, 1 ms).

Neither UK-14,304 nor clonidine had an effect on the concentration-response curve to NO, on the relaxation to

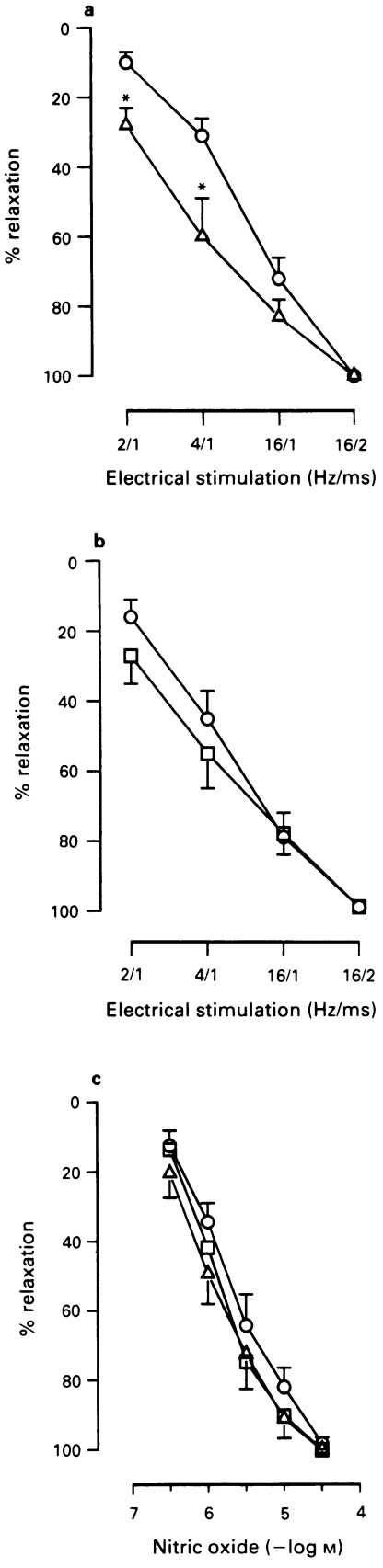


Figure 4 Effect of (a) clonidine (Δ, 1 μM) and (b) UK-14,304 (□, 1 μM) on electrically (2–16 Hz, 1–2 ms) evoked NANC relaxations (○) and (c) the concentration-response curve to NO (○, 0.3–30 μM) during a noradrenaline (30 μM)-induced contraction in the canine ileocolonic junction. Results are shown as mean ± s.e.mean for 6–7 experiments and expressed as percentage decrease of the noradrenaline contraction. $^*P<0.05$, significantly different from control, Student's t test for paired observations.

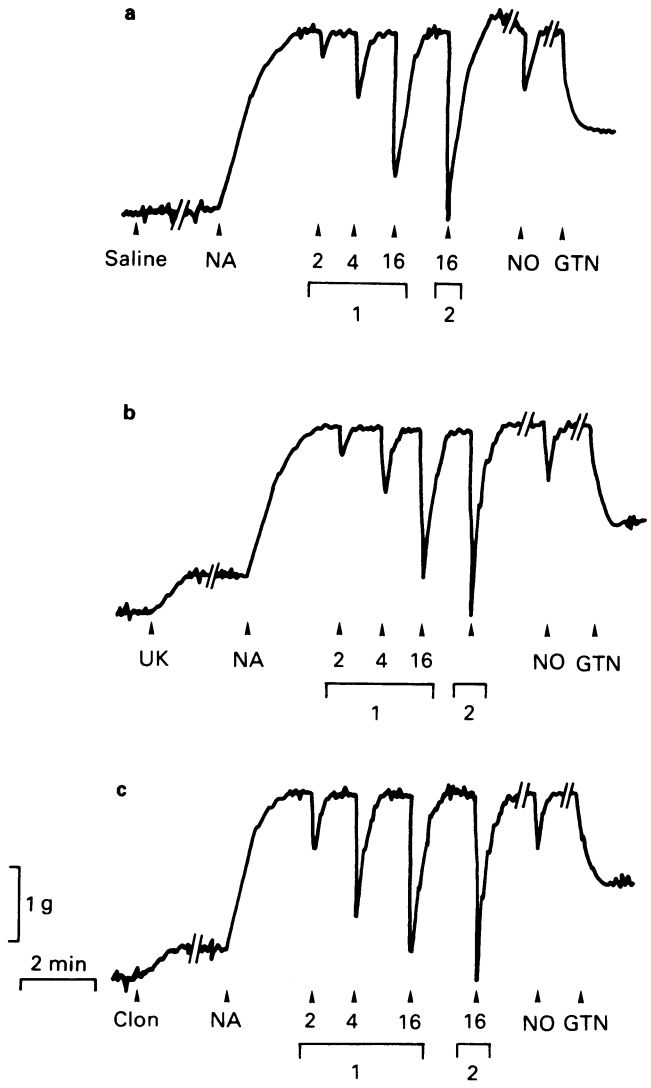


Figure 5 Isometric tension recordings showing (a) the control and the effect of (b) UK-14,304 (UK, 1 μM) and (c) clonidine (Clon, 1 μM) on NANC relaxations induced by electrical stimulation (2–16 Hz, 1–2 ms), NO (1 μM) and nitroglycerin (GTN, 1 μM) in a circular muscle strip of the canine ileocolonic junction. All experiments were performed during a noradrenaline (NA, 30 μM)-induced contraction. Breaks in the tracings represent periods of tissue equilibration. Similar results were obtained in tissues from 5–6 other dogs.

nitroglycerin (Figures 4 and 5) or on the contraction to noradrenaline.

Discussion

It is now well established that adrenergic neurones possess α_2-autoreceptors localized on the axon terminals. These prejunctional α_2-adrenoceptors are involved in the modulation of the calcium-dependent release of noradrenaline through a negative feedback mechanism mediated by the neurotransmitter itself (Langer, 1981). α_2-Adrenoceptors however subserve many more functions than autoinhibition of noradrenaline release and have also been found localized on non-adrenergic nerve terminals. Activation of such prejunctional α_2-adrenoceptors inhibits the calcium-dependent release of neurotransmitters such as acetylcholine, 5-HT and dopamine (Vizi, 1979; Gothert & Hunt, 1980; Dubocovich, 1984). The present study provides evidence indicating that these receptors are also involved in the prejunctional modulation of nitric oxide

release from NANC nerves in the canine ileocolonic junction.

In previous studies (see Introduction), we demonstrated that the inhibitory NANC innervation of the canine ileocolonic junction is mainly nitrergic; the NANC nerve-mediated relaxations induced by electrical impulses up to 16 Hz, 1 ms are completely blocked by inhibitors of the L-arginine:NO pathway, and in addition, the involvement of other putative NANC neurotransmitters, such as adenosine 5'-triphosphate (ATP) and vasoactive intestinal polypeptide (VIP), in the electrically-evoked NANC relaxations has been excluded. As such, the canine ileocolonic junction is a suitable model to study the nitrergic neurotransmission. Here, we demonstrate that in the absence of noradrenaline (muscle strips contracted with substance P), only the α_2-adrenoceptor agonists, clonidine and UK-14,304, significantly reduced the electrically-evoked NANC relaxations, especially those to low frequency stimulation. This effect was antagonized by the specific α_2-adrenoceptor antagonist, yohimbine, indicating that this effect was mediated by α_2-adrenoceptors. As previously reported (Pelckmans *et al.*, 1990), the canine ileocolonic junction possesses postjunctional excitatory α_2-adrenoceptors, explaining the contractile response to clonidine and UK-14,304. It seems rather unlikely however that these postjunctional receptors are involved in the inhibitory effect of clonidine and UK-14,304 on the electrically-evoked NANC relaxations, especially because these adrenoceptor agonists had no effect on the responses to exogenous NO. Furthermore, although clonidine did not affect the tone of the tissue in 3 out of 6 experiments, it did however inhibit the NANC relaxations in response to electrical stimulation in these experiments. Therefore, our results suggest the presence of prejunctional α_2-adrenoceptors which upon activation inhibit NO release. This is further confirmed by the observation that, compared to data obtained with α_2-adrenoceptor agonists on adrenergic neurones, the prejunctional receptor on the nitrergic NANC neurones has many features in common with the adrenergic autoreceptor. As demonstrated in numerous peripheral preparations (for review, see Gillespie, 1980), the inhibitory effect of α_2-adrenoceptor agonists on noradrenaline release is inversely proportional to the frequency of stimulation. In our study, both UK-14,304 and clonidine were indeed most effective on the relaxations induced by low frequency stimulation, but ineffective on those in response to high frequency stimulation (16 Hz). Furthermore, when there is much noradrenaline in the vicinity of the prejunctional α_2-adrenoceptor, it is well established that α_2-adrenoceptor agonists are least effective in inhibiting neurotransmitter release. This explains why UK-14,304 failed to affect nitrergic relaxations when the muscle strips were precontracted with high concentrations of noradrenaline. In contrast, however, it has been reported that partial agonists such as clonidine might even increase neurotransmitter release under such conditions (Medgett *et al.*, 1978; Gillespie, 1980), as also indicated in our experiments (muscle strips contracted with noradrenaline). Indeed, if the maximal inhibitory effect is already being produced by noradrenaline itself, it will be impossible to produce further inhibition by another agonist. If the agonist however has a lower efficacy than noradrenaline, its effect may be manifested as an antagonism of the effect of noradrenaline. This mechanism explains the potentiating action of clonidine on the NANC nerve-mediated relaxations observed in the canine ileocolonic junction during a noradrenaline-induced contraction. Finally, the prejunctional α_2-adrenoceptor activation by exogenous noradrenaline may also be responsible for the finding that the duration of the electrical stimuli applied during a noradrenaline-induced contraction had to be raised from 0.5 to 1 ms to obtain relaxations of the same amplitude as during a substance P-induced contraction. Similarly, endogenous noradrenaline released upon electrical stimulation can act on these α_2-adrenoceptors as well to inhibit NO release, an effect which should be blocked by yohimbine, thereby enhancing the NANC relaxations. However, as all experiments were performed in the presence of guanethidine to block the adrenergic neurotransmission, yohimbine had no such effect. Finally, circulating catecholamines released *in vivo* during stress situations can also influence NO release via activation of these prejunctional α_2-adrenoceptors, possibly playing a role in the motility disturbances observed under such conditions. Whether the prejunctional α_2-adrenoceptor is localized on the nerve terminals or varicosities or on the somatodendritic region of the nitrergic NANC neurones cannot be determined from our experiments.

Prejunctional modulation of NANC neurotransmission in the gastrointestinal tract was previously decribed in only a few tissues (see Introduction); in the rat gastric fundus (MacDonald *et al.*, 1990) and the guinea-pig proximal colon (Kojima *et al.*, 1988), the prejunctional inhibition of the NANC relaxations was reported to be mediated by α_2-adrenoceptors. Recently Lefebvre & Smits (1992) showed in the rat gastric fundus that UK-14,304 blocked the NANC relaxations induced by short train stimulation, previously demonstrated to be mediated by NO (Li & Rand, 1990; Boeckxstaens *et al.*, 1991a), suggesting prejunctional inhibition of NO release via α_2-adrenoceptor activation. In contrast to our results, clonidine did not affect the NANC relaxations in the rat gastric fundus. It should also be emphasized that in contrast to the canine ileocolonic junction, VIP is released by the NANC nerves of the rat gastric fundus as well as NO (De Beurme & Lefebvre, 1988), complicating the study of prejunctional regulation of NO release.

In conclusion, our results provide evidence indicating the presence of prejunctional α_2-adrenoceptors localized on nitrergic neurones in the canine ileocolonic junction. Activation of these receptors inhibits the nitrergic relaxations evoked by NANC nerve stimulation, suggesting that nitric oxide release is modulated via prejunctional α_2-adrenoceptors. This finding might suggest an interaction between the adrenergic and nitrergic innervation in the canine ileocolonic junction. Stimulation of the splanchnic nerves *in vivo* results in contraction of the ileocolonic junction via stimulation of α-adrenoceptors on the smooth muscle (for review, see Ouang, 1992). As adrenergic stimulation is excitatory, one might speculate on the presence of prejunctional inhibition of the release of NO, the main inhibitory neurotransmitter in this tissue, by adrenergic fibres favouring the contractile response. As mentioned in the Introduction, such prejunctional mechanisms are of key importance for the coordination of neuronal communication. The exact physiological importance of the described α_2-adrenoceptors however still needs to be determined. Also whether autoregulatory mechanisms exist in nitrergic neurotranmission or prejunctional regulation of the adrenergic and/or cholinergic innervation by NO occurs in the canine ileocolonic junction certainly needs further study.

This work was supported by the Belgian Fund for Medical Research (Grant 3.0014.90). The authors gratefully acknowledge F.H. Jordaens for technical assistance and Mrs L. Van de Noort for typing the manuscript.

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(Received February 2, 1993
Revised March 18, 1993
Accepted March 24, 1993)

Br. J. Pharmacol. (1993), **109**, 1085–1090 © Macmillan Press Ltd, 1993

Signal transduction pathways involved in the acute potentiation of NMDA responses by 1S,3R-ACPD in rat hippocampal slices

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1 A grease-gap recording technique has been used to investigate the mechanisms underlying the acute potentiation of N-methyl-D-aspartate (NMDA) responses by aminocyclopentane-1S,3R-dicarboxylic acid (1S,3R-ACPD) in area CA1 of rat hippocampal slices.

2 1S,3R-ACPD (10 μM), but not 1R,3S- ACPD (10 μM), potentiated submaximal responses to NMDA (dose-ratio of 0.81 ± 0.02 (mean ± s.e.mean); $n = 55$), but not to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), in a readily reversible manner. Potentiation also occurred in slices treated with 0.2 μM tetrodotoxin, and in slices perfused with Mg^{2+}-free medium.

3 1S,3R-ACPD-induced potentiation was unaffected by the protein kinase inhibitors K-252b (0.1 μM) and staurosporine (1 μM) and the intracellular Ca^{2+} store depletor, thapsigargin (10 μM). Coapplication of staurosporine and thapsigargin was also without effect.

4 1S,3R-ACPD-induced potentiation was unaffected by inhibitors of arachidonic acid formation, bromophenacyl bromide (50 μM) and RG80267 (100 μM). Arachidonic acid (10–50 μM) depressed reversibly NMDA-induced responses. The potentiation was unaffected by 8-bromo cyclic AMP (500 μM) or the PKA inhibitor Rp-adenosine 3,5-cyclic monophosphothioate triethylamine (Rp-cAMPS; 50 μM).

5 1S,3R-ACPD-induced potentiation was abolished in slices perfused with Ca^{2+}-free medium. The potentiation was also blocked by phorbol-12,13-diacetate (1 μM), in a staurosporine-sensitive manner.

6 It is concluded that the potentiation of NMDA responses by 1S,3R-ACPD is not mediated by protein kinase A or C, by release of Ca^{2+} from intracellular stores or by arachidonic acid. It involves a Ca^{2+}-sensitive process and is negatively regulated by protein kinase C.

Keywords: Metabotropic receptor; mGluR; hippocampus; protein kinase; long-term potentiation; LTP

Introduction

The activation of metabotropic glutamate receptors (mGluRs) can result in a variety of physiological effects, including the modulation of voltage-dependent conductances (Stratton *et al.*, 1989; Charpak *et al.*, 1990) and inhibition of neurotransmitter release (Baskys & Malenka, 1991). There is currently considerable interest in determining the signal transduction pathways that may mediate these effects. In this respect, activation of mGluRs can increase phosphoinositide (PI) turnover (Sladeczek *et al.*, 1986; Nicoletti *et al.*, 1986) and release Ca^{2+} from intracellular stores (Murphy & Miller, 1988; Irving *et al.*, 1990), alter levels of adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Tanable *et al.*, 1992), elevate arachidonic acid (Aramori & Nakanishi, 1992) and may couple to ion channels directly (Lester & Jahr, 1990).

We have reported previously that activation of mGluRs by the specific agonist, aminocyclopentane-1S,3R-dicarboxylate (1S,3R-ACPD), can potentiate selectively responses induced by N-methyl-D-aspartate (NMDA) in the CA1 region of the hippocampus (Harvey *et al.*, 1991). In the present study, we have explored the signal transduction processes that may mediate this effect.

Methods

Experiments were performed on 400 μm transverse hippocampal slices, obtained from adult female rats (33 ± 7 days old) by use of a grease-gap method, as described previously (Blake *et al.*, 1988). In brief, following removal of the CA3 region, the alveus was partially dissected from the CA1 region and placed under a Ag/AgCl wick electrode. The alveus and wick electrode assembly were covered by an insulating layer of grease (80:20 petroleum jelly:liquid paraffin) and d.c. potentials were recorded between this electrode and a similar one placed in the perfusing medium. This solution, which was perfused at a rate of 1.3 ml min^{-1}, and was maintained at 28°C, comprised (mM): NaCl 124, $NaHCO_3$ 26, KCl 3, $CaCl_2$ 2, $MgSO_4$ 1, and D-glucose 10 (bubbled with 95% O_2/5% CO_2:pH 7.4). Drugs were added to the perfusate; NMDA and AMPA were applied as 2 ml aliquots at regular intervals of between 15 and 20 min whereas the other compounds were applied continuously. The peak amplitude was used as a quantitative measure of responses throughout. The Schaffer collateral-commissural pathway was stimulated with a bipolar metal electrode, delivering 0.1 ms pulses at 0.033 Hz, positioned in stratum radiatum.

Drugs

N-methyl-D-aspartate, thapsigargin, bromophenacyl bromide, 8-bromo cyclic AMP, phorbol-12,13-diacetate, arachidonic acid and tetrodotoxin were obtained from Sigma. Rp-adenosine 3,5-cyclic monophosphothioate triethylamine was obtained from Research Biochemicals Incorporated. Staurosporine and (8R*,9S*,11S*)-(−)-9-hydroxy-9-methyoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11a-triazadibenzo (a,g) cycloocta (c,d,e) trinden-1-one-9-carboxylic acid (K-252b) were gifts from Dr Kase (Kyowa Hakko, Kogyo Co Ltd, Tokyo, Japan), RG-80267 was a gift from Rhone-Poulenc Rorer, USA, and α-amino-3-hydroxy-5-methyl-4-isooxazolepropionate, aminocyclopentane-1S,3R-dicarboxylate and aminocyclopentane-1R,3S-dicarboxylic acid were gifts from Professor J.C. Watkins (Department of Pharmacology, University of Bristol). Thapsigargin, staurosporine, K252b, arachidonic acid and RG-80267 (1,6-bis(cyclohexyloximinocarbonylamino)hexane) were dissolved in dimethyl sulphoxide (DMSO) (final concentration < 1% DMSO) and bromophenacyl bromide was dissolved in ethanol (final concentration 0.5% ethanol). At these concentrations both

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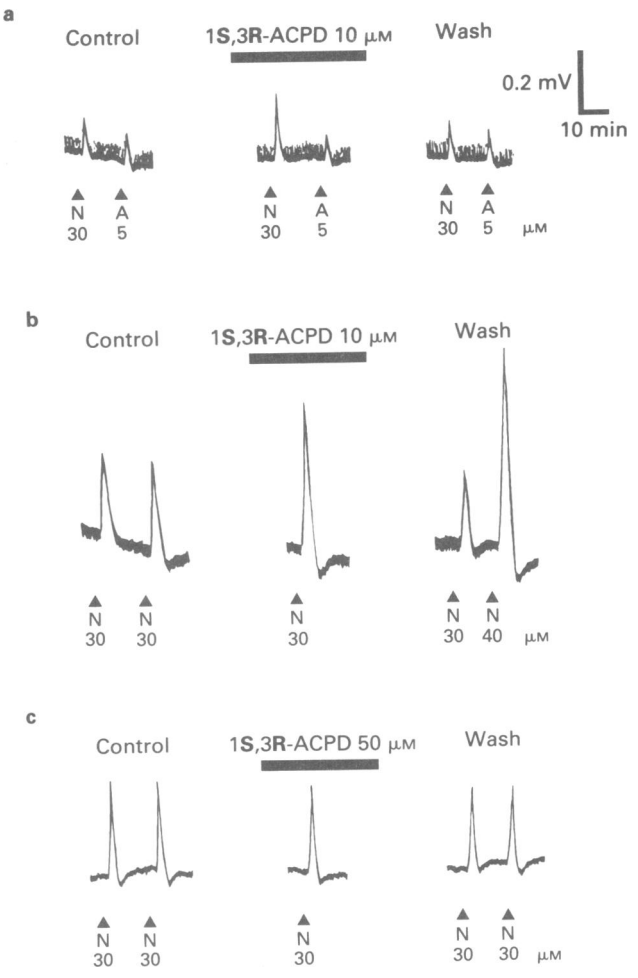


Figure 1 1S,3R-ACPD potentiates responses induced by NMDA: (a) shows that the application of 1S,3R-ACPD potentiates responses induced by NMDA but not AMPA, (b) illustrates the standard protocol to obtain an approximate dose-ratio. Following washout of 1S,3R-ACPD a higher concentration of NMDA was applied to enable the potentiated response to be bracketed between two control responses on the linear region of the log dose response curve. (c) The enantiomer 1R,3S-ACPD did not affect responses to NMDA. In this and subsequent figures the traces are computer scanned excerpts of chart-recordings of d.c. potential versus time. NMDA (and in (a) also AMPA) were applied as 2 ml aliquots at the times indicated by arrowheads below the traces. The numbers refer to the concentration in μM. All other drugs were applied continuously as indicated by bars above the traces. The regular upward deflections visible in some traces are filtered versions of synaptic responses. For abbreviations in this and subsequent figure legends, see text.

DMSO and ethanol had no effects. All other drugs were directly dissolved in artifical CSF (aCSF) or equimolar base.

Results

Dose-dependency and stereoselectivity of ACPD-induced potentiation of NMDA responses

1S,3R-ACPD (10 μM) potentiated submaximal responses to NMDA (10–40 μM) in 101 of 106 slices tested. In contrast, submaximal responses to AMPA (5–15 μM) were not appreciably affected ($n = 4$; Figure 1a). The effect developed fully by the time of the first application of NMDA in the presence of 1S,3R-ACPD (10–15 min after the addition of 1S,3R-ACPD). The potentiation was sustained for the duration of the application of 1S,3R-ACPD (up to 40 min) and reversed by the time of the first application of NMDA in the absence of 1S,3R-ACPD (5–10 min after the start of the washout of 1S,3R-ACPD). The effect was reproduced with subsequent applications of 1S,3R-ACPD to the same slice. The size of the potentiation, expressed in terms of % change, varied between 19 and 650%. This marked variability is due to the position of the control NMDA response on the dose-response curve. Therefore, to provide a more accurate quantification of the effect, an approximate dose-ratio was calculated for each slice (Childs *et al.*, 1988), by applying a higher dose of NMDA after washout of 1S,3R-ACPD (Figure 1b). Under control conditions, 10 μM 1S,3R-ACPD yielded a dose-ratio of 0.81 ± 0.02 ($n = 55$). At 1 μM, 1S,3R-ACPD had no descernible effect on responses to NMDA ($n = 4$). At high concentrations (50–400 μM) 1S,3R-ACPD affected the d.c. potential and depressed synaptic responses (Harvey *et al.*, 1991). Therefore, 10 μM 1S,3R-ACPD was used as the standard dose throughout. The effects of ACPD were stereoselective since 1R,3S-ACPD (1–100 μM) did not affect responses to NMDA ($n = 5$; Figure 1c).

1S,3R-ACPD-induced potentiation is not affected by tetrodotoxin or by perfusion of slices with Mg^{2+}-free medium

To determine whether 1S,3R-ACPD-induced potentiation of NMDA responses requires synaptic activity, experiments were performed in tetrodotoxin-containing medium. Tetrodotoxin (0.2 μM) abolished synaptic responses but did not affect the ability of 1S,3R-ACPD to augment NMDA-induced responses (dose-ratio, 0.83 ± 0.01; $n = 4$) (not illustrated).

Since 1S,3R-ACPD (at 10 μM) did not affect either the base-line potential or the size of excitatory postsynaptic potentials (e.p.s.ps) it is unlikely that the potentiation of NMDA responses is due indirectly to depolarization of the membrane (which would lead to a reduction of the level of Mg^{2+} block of NMDA channels). If it were, then 1S,3R-ACPD would be expected to reduce NMDA-induced responses in Mg^{2+}-free medium. However, in slices perfused with Mg^{2+}-free medium (and tetrodotoxin), 1S,3R-ACPD potentiated NMDA responses to a similar extent as under

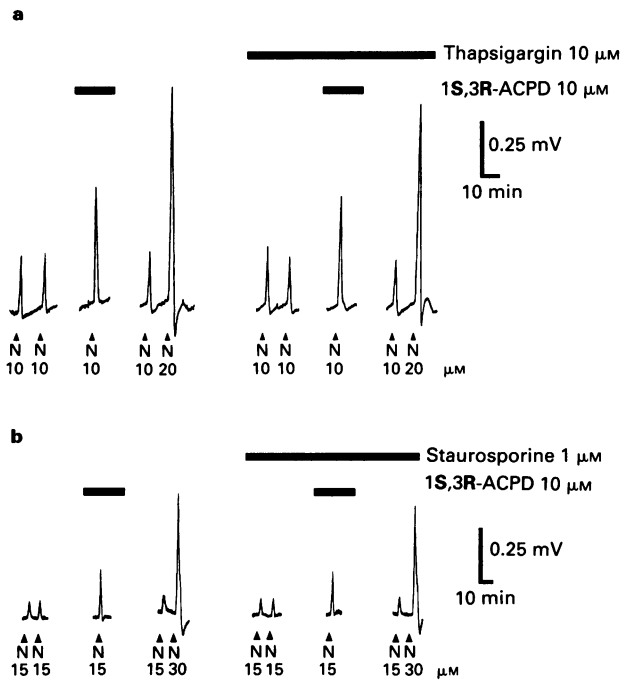


Figure 2 1S,3R-ACPD-induced potentiation is not affected by thapsigargin or staurosporine: (a) shows similar potentiations under control conditions and following perfusion of thapsigargin (10 μM) for 30 min; (b) shows similar potentiations under control conditions and following perfusion of staurosporine (1 μM) for 30 min. Neither thapsigargin nor staurosporine had effects on responses to NMDA *per se*.

Table 1 Effects of various treatments on the 1S,3R-ACPD-induced potentiation of NMDA responses

Treatment	*Dose*	*Control*	*Treated*	n
Thapsigargin	(10 μM)	0.78 ± 0.04	0.78 ± 0.03	3
Staurosporine	(1 μM)	0.78 ± 0.03	0.78 ± 0.04	4
K-252b	(0.1 μM)	0.83 ± 0.03	0.83 ± 0.02	3
Staurosporine + thapsigargin		0.76 ± 0.02	0.77 ± 0.04	3
8-bromo cyclic AMP	(500 μM)	0.80 ± 0.03	0.79 ± 0.01	4
RpcAMPS	(50 μM)	0.81 ± 0.02	0.81 ± 0.02	3
Bromophenacyl bromide	(50 μM)	0.79 ± 0.02	0.78 ± 0.04	3
RG 80267	(100 μM)	0.79 ± 0.04	0.83 ± 0.05	5
Ca^{2+}-free		0.77 ± 0.02	1.00 ± 0.01	5*
Phorbol-12,13-diacetate	(1 μM)	0.81 ± 0.02	1.00 ± 0.02	5*

The actions of each drug were assessed by comparing the dose-ratio of the 1S,3R-ACPD-induced potentiation of NMDA responses, under control conditions, with the dose-ratio for potentiation obtained in the presence of the drug.
*$P < 0.05$ (paired t test).

control conditions (dose-ratio, 0.83 ± 0.04; $n = 9$) (not illustrated).

1S,3R-ACPD-induced potentiation does not require either activation of protein kinase C or release of Ca^{2+} from intracellular stores

Two important consequences of mGluR activation are the activation of protein kinase C (PKC) and the release of Ca^{2+} from intracellular stores. To investigate whether the release of Ca^{2+} from intracellular stores is required for potentiation we applied thapsigargin, which depletes most intracellular

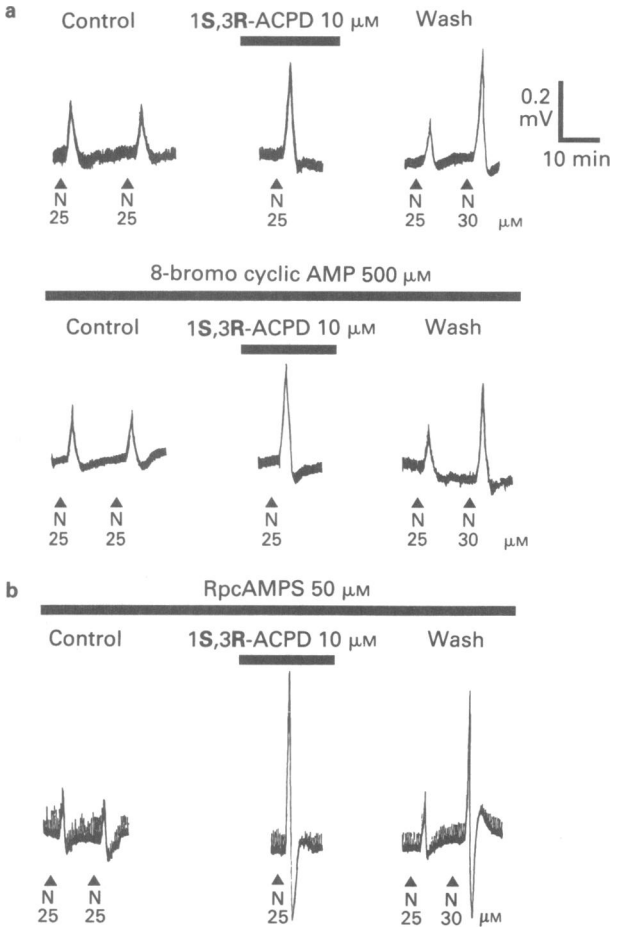


Figure 3 1S,3R-ACPD-induced potentiation is not mediated by cyclic AMP: (a) shows similar potentiations under control conditions (upper traces) and following the addition of 8-bromo-cyclic AMP (500 μM) for 30 min (lower traces); (b) illustrates that treatment with Rp-cAMPS (50 μM) for 30 min had no effect on the ability of 1S,3R-ACPD to potentiate NMDA responses.

Ca^{2+} stores by blocking ATP-dependent Ca^{2+} uptake into intracellular compartments (Thastrup *et al.*, 1990; Irving *et al.*, 1992). Thapsigargin (10 μM) had no effect on 1S,3R-ACPD-induced potentiation ($n = 3$; Figure 2a; Table 1). The drug was active in the system, since in parallel experiments it prevented tetanus-induced long-term potentiation (LTP) (not illustrated), as described previously (Harvey & Collingridge, 1992). To test the involvement of PKC directly we used two potent inhibitors, staurosporine (1 μM) (Tamaoki *et al.*, 1986) and K-252b (0.1 μM) (Kase *et al.*, 1987). Neither compound had any effect on the ability of 1S,3R-ACPD to augment NMDA-induced responses (Figure 2b; Table 1) (K-252b not illustrated). Both compounds were active since, in parallel experiments, they prevented the induction of LTP but not short-term potentiation (STP) (not illustrated), as described previously (Reymann *et al.*, 1990). In addition, staurosporine (K-252b not tested) prevented PDA from facilitating synaptic transmission (not illustrated). It is conceivable that either activation of PKC or release of Ca^{2+} from intracellular stores is able to mediate the potentiation such that blockade of one system alone has no effect. We therefore applied staurosporine (1 μM) and thapsigargin (10 μM) together (not illustrated); this treatment also failed to affect 1S,3R-ACPD-induced potentiation ($n = 3$; Table 1).

1S,3R-ACPD-induced potentiation is not mediated by cyclic AMP

In addition to coupling to phospholipase C (PLC), activation of mGluR can increase or decrease (Aramori & Nakanishi, 1992) levels of cyclic AMP. However, treatment with 8-bromo cyclic AMP (500 μM), which elevates cyclic AMP to activate protein kinase A (PKA) (Chneiweiss, 1991), had no effect on responses to NMDA or on the ability of 1S,3R-ACPD to augment these responses ($n = 4$; Figure 3a; Table 1). Similarly, Rp-adenosine 3,5-cyclic monophosphothioate triethylamine, (RpcAMPS), a substance which competitively inhibits the activation of PKA by cyclic AMP (Van Hasstert *et al.*, 1984), was without affect on 1S,3R-ACPD-induced potentiation ($n = 3$; Figure 3b; Table 1).

1S,3R-ACPD-induced potentiation is not mediated by arachidonic acid

It has been reported previously that, in addition to coupling to PLC, mGluRs also couple to PLA_2 (Aramori & Nakanishi, 1992) and that arachidonic acid potentiates responses to NMDA (Miller *et al.*, 1992). Thus another possible mediator of 1S,3R-ACPD-induced potentiation is arachidonic acid. Bromophenacyl bromide (50 μM), an inhibitor of phospholipase A_2 (Hofmann *et al.*, 1982) did not affect 1S,3R-ACPD-induced potentiation ($n = 3$, Figure 4a; Table 1). Bromophenacyl bromide was active since it blocked the generation of tetanus-induced LTP (but not STP; data not shown), as reported previously (Okada *et al.*, 1989). Ara-

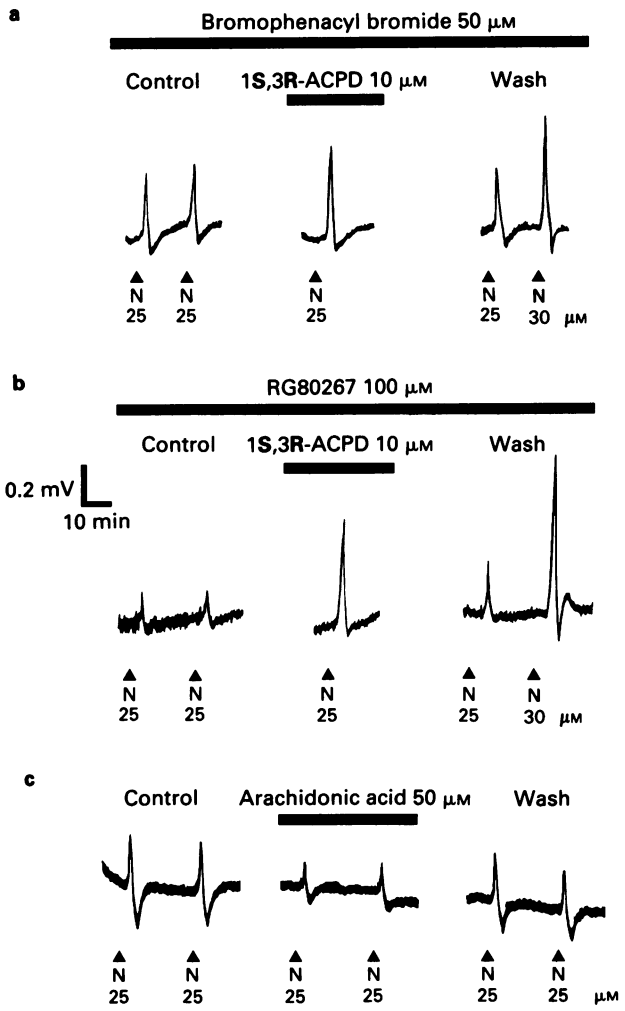


Figure 4 1S,3R-ACPD-induced potentiation is not mediated by arachidonic acid. The upper two sets of records show that 1S,3R-ACPD potentiates responses to NMDA in the presence of the inhibitors of arachidonic acid formation, (a) bromophenacyl bromide (50 μM) and (b) RG80267 (100 μM). Similar potentiations were obtained prior to addition of the inhibitors and neither compound affected responses to NMDA *per se*. (c) Illustrates that arachidonic acid (50 μM) depresses reversibly responses to NMDA.

chidonic acid can also be formed by the action of diacylglycerol lipase. However, RG80267 (100 μM), a substance which has been reported to inhibit this enzyme (Schimmel, 1989), was also without consistent effect on 1S,3R-ACPD-induced potentiation (Figure 4b, Table 1). Arachidonic acid (10–50 μM), itself, did not potentiate responses to NMDA: rather it depressed reversibly responses to NMDA ($n = 4$; Figure 4c).

1S,3R-ACPD-induced potentiation involves a Ca^{2+}-dependent process

The next series of experiments were aimed at determining whether a Ca^{2+}-dependent process is involved in the potentiation of NMDA responses. This was achieved by comparing the ability of 1S,3R-ACPD to potentiate NMDA responses in the presence ($[Ca^{2+}] = 2$ mM) and absence of added $CaCl_2$ in the perfusing medium. Under control conditions, reversible potentiation of NMDA responses was obtained and then Ca^{2+}-free medium was perfused until e.p.s.ps, evoked at supra-maximal stimulus strengths, were abolished. Under these conditions, 1S,3R-ACPD no longer potentiated responses to submaximal doses of NMDA ($n = 5$; Figure 5; Table 1). Re-introduction of Ca^{2+}-containing medium restored synaptic transmission and the ability of 1S,3R-ACPD to augment responses to NMDA (not illustrated).

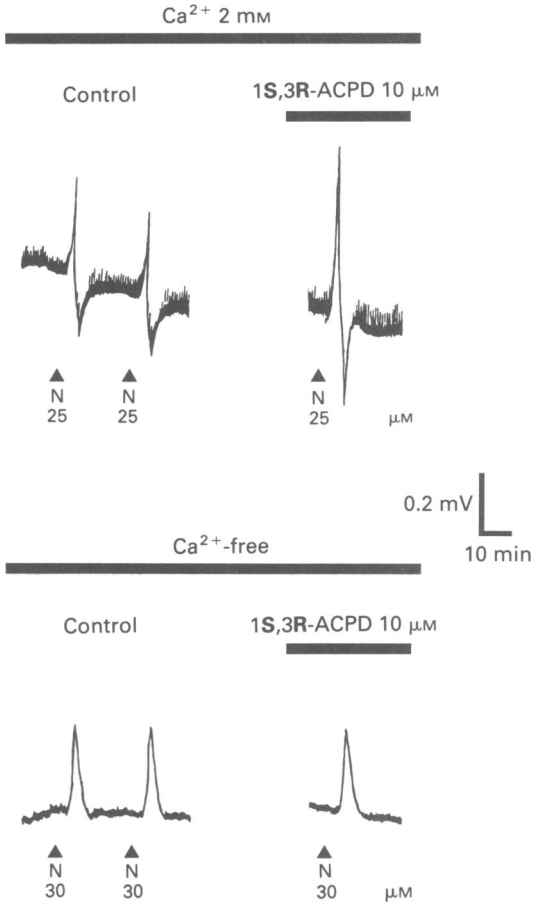


Figure 5 1S,3R-ACPD-induced potentiation is blocked by perfusion with Ca^{2+}-free medium. The upper traces show potentiation of NMDA responses in the presence of 2 mM Ca^{2+}. The lower traces show the lack of potentiation after perfusion with a Ca^{2+}-free medium for 60 min. These conditions abolished synaptic transmission and blocked the afterhyperpolarization, associated with responses to NMDA.

1S,3R-ACPD-induced potentiation is negatively regulated by PKC

To determine whether PLC is involved in the acute potentiating action of 1S,3R-ACPD we applied phorbol-12,13-diacetate (PDA) which, through activation of PKC, inhibits phosphoinositide (PI) hydrolysis (Nishizuka, 1986; Schoepp & Johnson, 1988), via a selective interaction with PLC (Aramori & Nakanishi, 1992). PDA (1 μM) enhanced synaptic transmission whilst having no effect on responses to NMDA. It blocked 1S,3R-ACPD-induced potentiation in a reversible manner ($n = 5$; Figure 6a; Table 1). This action of PDA was probably via the activation of PKC since staurosporine inhibited the effect ($n = 3$; Figure 6b).

Discussion

The present observation that 1S,3R-ACPD potentiates acutely responses mediated by NMDA extends our earlier report (Harvey *et al.*, 1991), which has since been confirmed by other groups (Aniksztejn *et al.*, 1992; Bleakman *et al.*, 1992). Independently, a similar potentiation of NMDA responses by ACPD was described in mRNA-injected oocytes (Kelso *et al.*, 1992). 1S,3R-ACPD has also been shown to potentiate NMDA receptor-mediated synaptic responses (Kinney & Slater, 1992; Collins, 1993). In the CA1 region of the hippocampus, the effect is selective for NMDA since responses to AMPA are not altered. However, in dorsal horn neurones

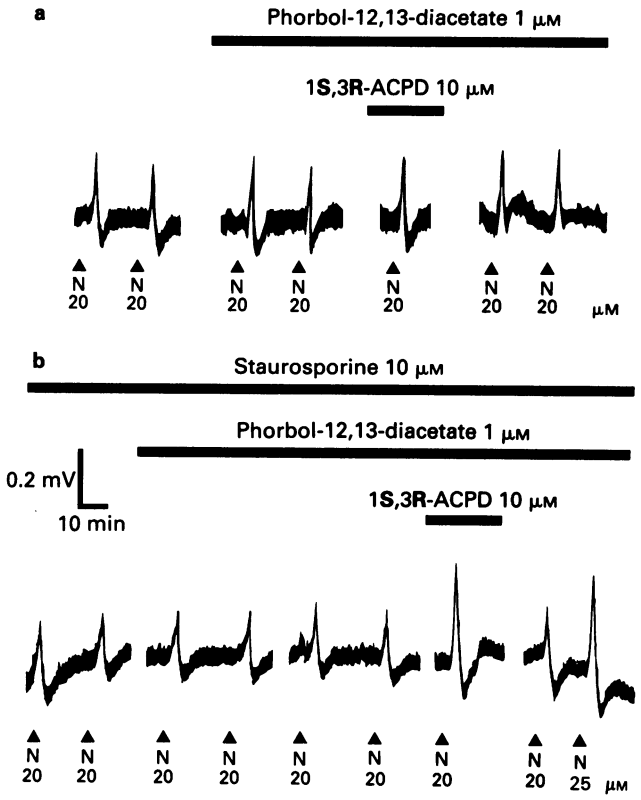


Figure 6 1S,3R-ACPD-induced potentiation is blocked by a phorbol ester, in a staurosporine-sensitive manner: (a) shows an example of the inhibition by phorbol-12,13-diacetate (1 μM) of 1S,3R-ACPD-induced potentiation. Note that the phorbol ester had no effect on responses to NMDA *per se*. (b) Shows that staurosporine prevents phorbol-12,13-diacetate from inhibiting 1S,3R-ACPD-induced potentiation.

and cerebellar Purkinje neurones 1S,3R-ACPD has also been shown to potentiate responses to AMPA and kainate (Bleakman *et al.*, 1992; Cerne & Randic, 1992; Glaum *et al.*, 1992). Potentiation of NMDA-induced responses has also been described following the activation of certain other G-protein coupled receptors, such as muscarinic (Markram & Segal, 1992), 5-hydroxytryptamine (5-HT) (Blake & Long, 1991) and opioid (Chen & Huang, 1991) receptors. It is likely, but by no means certain, that these effects will be mediated through a common intracellular pathway.

In mRNA-injected oocytes, 4-β-phorbol-12,13-dibutyrate mimics and occludes the potentiation of NMDA responses by the activation of mGluRs, implicating a role for PKC (Kelso *et al.*, 1992). Similarly, PKC has been proposed to mediate the potentiation of NMDA responses by ACPD in the CA1 region of the hippocampus, since the effect is blocked by sphingosine and the intracellular injection of a peptide PKC inhibitor (Aniksztejn *et al.*, 1992). In contrast, our data do not support a role of PKC in the potentiation of NMDA responses by 1S,3R-ACPD. Thus, PDA did not potentiate responses induced by NMDA and two potent PKC inhibitors, which were active in our preparation, were without effect on the potentiation of these responses by 1S,3R-ACPD. Rather, our data suggests that PKC could be involved in negative regulation of the potentiation mechanism. In this respect, the mechanism may be similar to that whereby the activation of mGluRs by quisqualate inhibits certain voltage-gated Ca^{2+} channels (Lester & Jahr, 1990). The inhibition of K^+ conductances by ACPD has also been shown to be independent of PKC (Gerber *et al.*, 1992).

The present data do not support a role for cyclic AMP/PKA in 1S,3R-ACPD-induced potentiation, since the potentiation was neither mimicked nor blocked by 8-bromo cyclic AMP, a treatment which would be expected to elevate cyclic AMP. In addition Rp-cAMPS did not affect the potentiation. Furthermore, both staurosporine and K-252b, which would be expected to block PKA in the concentrations used, were without effect. Recent experiments with 8-bromo cyclic AMP and staurosporine have also excluded a role for PKA in the ACPD-mediated reduction of K^+ conductances (Gerber *et al.*, 1992). Similarly, arachidonic acid does not appear to mediate the potentiation. Thus, unlike in cultured cerebellar granule neurones and acutely dissociated hippocampal neurones (Miller *et al.*, 1992), it did not potentiate NMDA responses. In addition, inhibitors of arachidonic acid formation did not prevent 1S,3R-ACPD-induced potentiation of NMDA responses. However, these data do not preclude a non-exclusive role for arachidonic acid formed by lipases insensitive to the inhibitors tested in the present investigation.

It has been reported that the potentiation of NMDA responses by acetylcholine (ACh) involves the formation of IP_3 and the liberation of Ca^{2+} from intracellular stores (Markram & Segal, 1992). In the present study, however, thapsigargin did not affect 1S,3R-ACPD-induced potentiation of NMDA responses. In other studies we have found that thapsigargin completely blocks both ACh and 1S,3R-ACPD-induced Ca^{2+} mobilization from intracellular stores in cerebellar granule cells (Irving *et al.*, 1992) and is able to block the induction of LTP in hippocampal slices (Harvey & Collingridge, 1992). Thus, although we cannot eliminate the possibility that thapsigargin failed to deplete a small but critical pool of Ca^{2+} which is involved in the potentiation effect, we believe it is more likely that release of Ca^{2+} from intracellular stores is not required. These data, however, do not entirely preclude a different role of inositolphosphates in the potentiation response. The potentiation of NMDA responses by 1S,3R-ACPD is, like that induced by ACh (Markram & Segal, 1992), a Ca^{2+}-dependent process. Ca^{2+} may be needed to facilitate the coupling of mGluRs to PLC (Irving *et al.*, 1992) or for processes downstream of this. The observation that PDA blocks 1S,3R-ACPD-induced potentiation suggest that PLC is involved. Phorbol esters selectively block this effector pathway of mGluRs (Aramori & Nakanishi, 1992), presumably by activating PKC which results in feedback inhibition of the transduction process (Iino *et al.*, 1988; Schoepp & Johnson, 1988). Consistent with this idea, staurosporine prevented the PDA block of 1S,3R-ACPD-induced potentiation. These data do not preclude an alternative mechanism which is also subject to inhibition following the activation of PKC.

In conclusion, the acute potentiation of NMDA responses by 1S,3R-ACPD does not involve either the activation of PKC or the release of Ca^{2+} from intracellular stores. The inhibition of the potentiation by the phorbol ester suggests the involvement of either a PLC-coupled mechanism independent of these processes or a different effector system which can also be negatively regulated by PKC. In all respects the mechanism is similar to that by which activation of mGluRs inhibits Ca^{2+} currents (Lester & Jahr, 1990). This potentiation may have a physiological role in forms of synaptic plasticity which are dependent on the activation of NMDA receptors, such LTP in the Schaffer collateral commissural pathway (Collingridge *et al.*, 1983). In particular, it can explain how a mixture of 1S,3R-ACPD and 1R,3S-ACPD rapidly enhances STP (the initial phase of LTP) (McGuinness *et al.*, 1991). In addition, however, 1S,3R-ACPD can induce a long lasting enhancement of synaptic transmission in the CA1 region of the hippocampus. This latter effect shows a pharmacological profile that is distinct from 1S,3R-ACPD-induced potentiation of NMDA responses, as the effect is eliminated by staurosporine, K-252b and thapsigargin (Bortolotto & Collingridge, 1993). The time course of this enhancement of synaptic transmission is consistent with a role in the later phases of LTP. Thus, activation of mGluRs by 1S,3R-ACPD may modulate synaptic plasticity through two distinct mechanisms which have different temporal profiles and utilize different transduction mechanisms.

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(Received December 21, 1992
Revised March 19, 1993
Accepted March 24, 1993)

Br. J. Pharmacol. (1993), **109**, 1091–1096 © Macmillan Press Ltd, 1993

Influence of epithelium on the inhibition of melittin-induced contraction of guinea-pig isolated trachea by the potassium channel opener NIP-121

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1 We have investigated the effect of the potassium channel opener, NIP-121, on contraction elicited by melittin (a phospholipase A_2 activator) in epithelium-intact and epithelium-denuded trachea isolated from guinea-pigs. The effects of NIP-121 were compared with those of isoprenaline, aminophylline and hydrocortisone.

2 In the presence of the cyclo-oxygenase inhibitor, indomethacin (5 μM), melittin (3 μg ml^{-1}) caused time-dependent contraction. The melittin-induced contractile response was significantly augmented by removal of the epithelium and was concentration-dependently and completely inhibited by the phospholipase A_2 (PLA_2) inhibitor, *p*-bromophenacyl bromide (BPB 10–100 μM), and the lipoxygenase inhibitor, phenidone (10–300 μM). Similar inhibition of the melittin response by BPB (10 μM) and phenidone (10 μM) was observed in the epithelium-denuded trachea.

3 Concentration-dependent inhibition of the melittin response was induced by preincubation with NIP-121 (0.03 and 0.1 μM), isoprenaline (0.001 and 0.01 μM), aminophylline (30 and 100 μM) and hydrocortisone (100 and 300 μM). The effect of NIP-121 was abolished by glibenclamide (1 μM).

4 The inhibitory effect of NIP-121 on the melittin response was greatly reduced by removing the epithelium while that of the isoprenaline, aminophylline or hydrocortisone was not changed.

5 The inhibition of the melittin response by these drugs was similar to their inhibition of the contraction elicited by a low concentration (3 nM) of leukotriene D_4 (LTD_4) in the epithelium-intact trachea. Inhibition of the LTD_4 response by NIP-121 was not observed in the epithelium-denuded trachea. However, higher concentrations of NIP-121 (0.3 and 1 μM) did inhibit LTD_4-induced contractions of epithelium-denuded trachea.

6 These findings suggest that melittin causes epithelium-dependent contraction of the guinea-pig isolated trachea which is mediated by products of lipoxygenase activity. NIP-121 may inhibit the melittin response by activating glibenclamide-sensitive potassium channels, which appear to be epithelium-dependent (an indirect effect of NIP-121 apart from its direct effect on the airway smooth muscle) while isoprenaline, aminophylline and hydrocortisone act directly to relax the trachealis smooth muscle.

Keywords: NIP-121; melittin; leukotriene D_4; isoprenaline; aminophylline; hydrocortisone; airway epithelium; guinea-pig trachea

Introduction

Airway hyperresponsiveness is a characteristic feature of bronchial asthma and is associated with damage to, or loss of, the epithelium lining the airways (Laitinen *et al.*, 1985; Seltzer *et al.*, 1986). Airway responsiveness to bronchoactive substances is augmented by mechanically removing the epithelium in airway preparations isolated from several animal species including man (Barnes *et al.*, 1985; Holroyde, 1986; Aizawa *et al.*, 1988; Sparrow & Mitchell, 1991). Barnes (1986) also proposed the hypothesis that the lack of epithelium sensitizes the C-fibre afferent nerve thereby promoting the release of bronchoconstrictor neuropeptides, such as substance P. The epithelium, therefore, may play an important role in the regulation of airway smooth muscle responsiveness.

The potassium channel opener, cromakalim, induces not only relaxation of guinea-pig (Arch *et al.*, 1988) and human (Black *et al.*, 1990) airway smooth muscle but also prevention of bronchoconstriction mediated by cholinergic (McCaig & De Jonckheere, 1989) and non-adrenergic non-cholinergic (Ichinose & Barnes, 1990) nerve stimulation. These data suggest that a potassium channel opener may be a useful drug for the airway hyperreactivity evoked by neurogenic inflammation mediated by activation of non-adrenergic, non-cholinergic nerves. NIP-121, a potassium channel opener (Masuda *et al.*, 1991), has potent bronchodilator activity in the guinea-pig isolated trachea (Shikada *et al.*, 1991). We reported recently that NIP-121, as well as cromakalim, inhibited neurally-mediated contraction (Yamamoto *et al.*, 1992) and epithelium-dependent potentiation of sodium nitroprusside-induced relaxation in the guinea-pig isolated trachea (Shikada & Tanaka, 1992). Wessler *et al.* (1992) found that the inhibitory effect of cromakalin on parasympathetic nerve-induced acetylcholine release is epithelium-dependent. These data postulate that a potassium channel opener may act on the epithelium in airway tissues. Since epithelium releases putative broncho-relaxing substances that regulate the airway smooth muscle responsiveness (Hay *et al.*, 1987; Fernandes & Goldie, 1990), it is important to study the interaction between a potassium channel opener and airway epithelium.

In the present study we have further investigated the mode of action of NIP-121 on airway tissues, by examining the influence of the epithelium on the inhibition by NIP-121 of contractions induced by a phospholipase A_2 (PLA_2) activator, melittin, in the guinea-pig isolated trachea. The effects of NIP-121 have been compared with those of the anti-asthma drugs isoprenaline, aminophylline and hydrocortisone. We have previously reported that melittin caused contraction of the isolated guinea-pig trachea and that the response was similar to that of exogenously applied arachidonic acid (AA) (Yamamoto *et al.*, 1990).

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Methods

Tracheae were removed from male Hartley guinea-pigs (280–350 g) which had been killed by stunning and bleeding. Each trachea was cut spirally and divided into two or three segments (one was used as a control). In some experiments, the epithelium was removed mechanically by gently rubbing the luminal surface with a cotton-tipped applicator. Individual tissues were suspended under an applied load of 1 g in a 10 ml organ bath containing 8 ml of modified Tyrode solution at 37°C and gassed continuously with 95% O_2 + 5% CO_2. The composition of the modified Tyrode solution was (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHPO_4$ 0.3, $NaHCO_3$ 20 and dextrose 11. Trachealis length changes were measured isotonically (transducer type TD-112S, Nihon Koden). After the tissues had equilibrated for 50–60 min, the maximal response to histamine (100 μM) was evoked. Subsequent contractile responses were expressed as percentages of the maximal response to histamine. The tissues were washed several times for 30 min to re-establish the baseline and were then incubated for an additional 30 min with the test drug or vehicle in the presence of 5 μM indomethacin. Melittin at a concentration of 3 μg ml^{-1} and leukotriene D_4 (LTD_4) at concentrations from 0.1–30 nM were singly and cumulatively added to the organ bath respectively. The area under the time contraction curve (AUC) in the melittin response was calculated by a computer programme and compared with that of the paired control.

In a separate experiment, the potencies of the test drugs in suppressing spontaneous tone of the guinea-pig isolated trachea were measured. Cumulative concentration-relaxation curves for test drugs were generated by successive increases in the organ bath concentration of these drugs. These experiments were performed on epithelium-intact trachea in the absence of indomethacin. The relaxant response was expressed as a percentage of the maximum relaxation obtained with 1 mM aminophylline added to the organ bath at the end of the experiment.

In all experiments, only one concentration-response curve for a relaxant drug was constructed in a given tissue. All results are expressed as means ± s.e.mean, and the statistical significance ($P<0.05$) was determined by Student's one-tailed t test for paired observations.

Drugs

The following agents were used: histamine hydrochloride (Wako Pure Chemical Industries, Japan); LTD_4 (Ultrafine Chemicals, England); indomethacin, melittin (bee venom, purity 85%), (−)-isoprenaline hydrochloride, aminophylline hydrate, hydrocortisone, 1-phenyl-1-pyrazolidone (phenidone), glibenclamide, pyrilamine maleate (Sigma Chemical Co., USA); atropine sulphate (E. Merck, Germany); *p*-bromophenacyl bromide (BPB, Nacalai tesque, Japan); NIP-121 ((+)-7,8-dihydro-6,6-dimethyl-7-hydroxy-8-(2-oxo-piperidin-1-yl)6H-pyrano[2,3-f]benz-2,1,3-oxadiazole) and cromakalim were synthesized by Nissan Chemical Industries Ltd., Central Research Laboratory, Japan. Indomethacin was dissolved in 100% ethanol, and hydrocortisone, BPB, phenidone, glibenclamide, NIP-121 and cromakalim were dissolved in 100% dimethylsulphoxide. Other drugs were dissolved in distilled water. At the final bath concentrations used, the drug vehicles did not cause any trachealis length changes.

Results

Melittin-induced contraction of guinea-pig isolated trachea and influence of epithelium removal on the effect of inhibitors of the arachidonic acid (AA) lipoxygenase pathway on the melittin response

In the presence of indomethacin (5 μM), melittin at a concentration of 3 μg ml^{-1} induced a slowly developing contraction of the guinea-pig tracheal strip. The peak response usually occurred within 10–15 min and the magnitude of the contraction was about 50% of the histamine maximum (Figure 1). When the tracheal luminal surface was gently rubbed with cotton to remove the epithelium, the melittin-induced contractile response was significantly enhanced (Figure 1). The extent of the contraction judged by the AUC was also signficantly increased (Table 1).

The PLA_2 inhibitor, BPB and the lipoxygenase inhibitor, phenidone concentration-dependently and completely, inhibited the melittin response observed in epithelium-intact trachea (Table 1). Similar inhibition of the melittin response by these inhibitors was also observed in the epithelium-denuded trachea. Pyrilamine (1 μM) and atropine (1 μM) each had no inhibitory effect on the melittin response in intact tissue.

Effect of NIP-121, isoprenaline, aminophylline and hydrocortisone on melittin response in epithelium-intact and epithelium-denuded trachea

To investigate the effect of the test drugs on the melittin response, we determined concentrations of the test drugs that caused equivalent suppression of the spontaneous tone of the

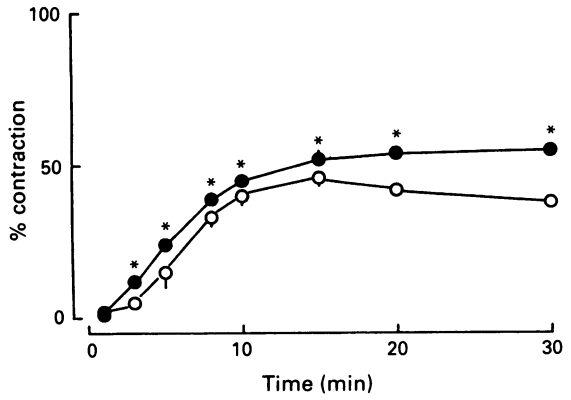


Figure 1 Effect of epithelium removal on melittin (3 μg ml^{-1})-induced contraction of the guinea-pig isolated trachea. Each point represents the mean ± s.e.mean of 5 preparations: (○) epithelium-intact tissues and (●) epithelium-denuded tissue. *Indicates that the data points from the epithelium-denuded tissues were significantly greater ($P<0.05$) than the corresponding data points from the epithelium-intact tissues.

Table 1 Effect of *p*-bromophenacyl bromide (BPB), phenidone, pyrilamine and atropine on melittin-induced contraction of epithelium-intact and epithelium-denuded trachea isolated from guinea-pigs

Inhibitor	*concentration (μM)*	*Epithelium*	*% of paired control AUC*
None		denuded	129 ± 5
BPB	10	intact	41 ± 4
	100	intact	0
	10	denuded	38 ± 7
Phenidone	10	intact	50 ± 7
	100	intact	23 ± 4
	300	intact	5 ± 4
	10	denuded	62 ± 5
Pyrilamine	1	intact	100 ± 9
Atropine	1	intact	109 ± 14

Values are expressed as the mean ± s.e.mean of 4–6 preparations. The AUC was calculated by a computer programme and is expressed as % of the paired control AUC.

trachea. All test drugs concentration-dependently suppressed the spontaneous tone of the epithelium-intact trachea in the absence of indomethacin. In these experiments we determined two concentrations of each drug that caused about 30–40% and 80–90% decreases in spontaneous tone, respectively. Table 2 shows the effect of two concentrations of the test compounds against the spontaneous tone of the trachea. We used 300 μM hydrocortisone as the upper tested concentration of hydrocortisone. Further increase in the tested concentration of hydrocortisone was limited by its solubility in dimethylsulphoxide.

Table 2 Effect of NIP-121 and reference drugs on spontaneous tone in the epithelium-intact trachea isolated from guinea-pig

Drug	*Concentration (μM)*	*% relaxation*
NIP-121	0.03	31 ± 13
	0.1	81 ± 7
Isoprenaline	0.001	36 ± 8
	0.01	93 ± 1
Aminophylline	30	42 ± 3
	100	88 ± 3
Hydrocortisone	100	33 ± 6
	300	48 ± 6

% relaxation is expressed as the maximum response induced by 1 mM aminophylline added at the end of the experiment. Values are expressed as the mean ± s.e.mean of 4–7 preparations.

In the epithelium-intact trachea, preincubation with the low concentration of NIP-121 (0.03 μM) markedly suppressed the melittin response while low concentrations of isoprenaline, aminophylline and hydrocortisone had no significant effect (Figure 2). At the higher concentration, isoprenaline completely blocked the melittin response while NIP-121, aminophylline and hydrocortisone moderately inhibited it to about the same extent (Figures 2 and 3a). Reduction of the NIP-121 concentration to 0.01 μM, which did not suppress the spontaneous tone, had little effect on the melittin response (116 ± 7% of the paired control AUC, $n = 4$). The combination of the ATP-sensitive potassium channel blocker

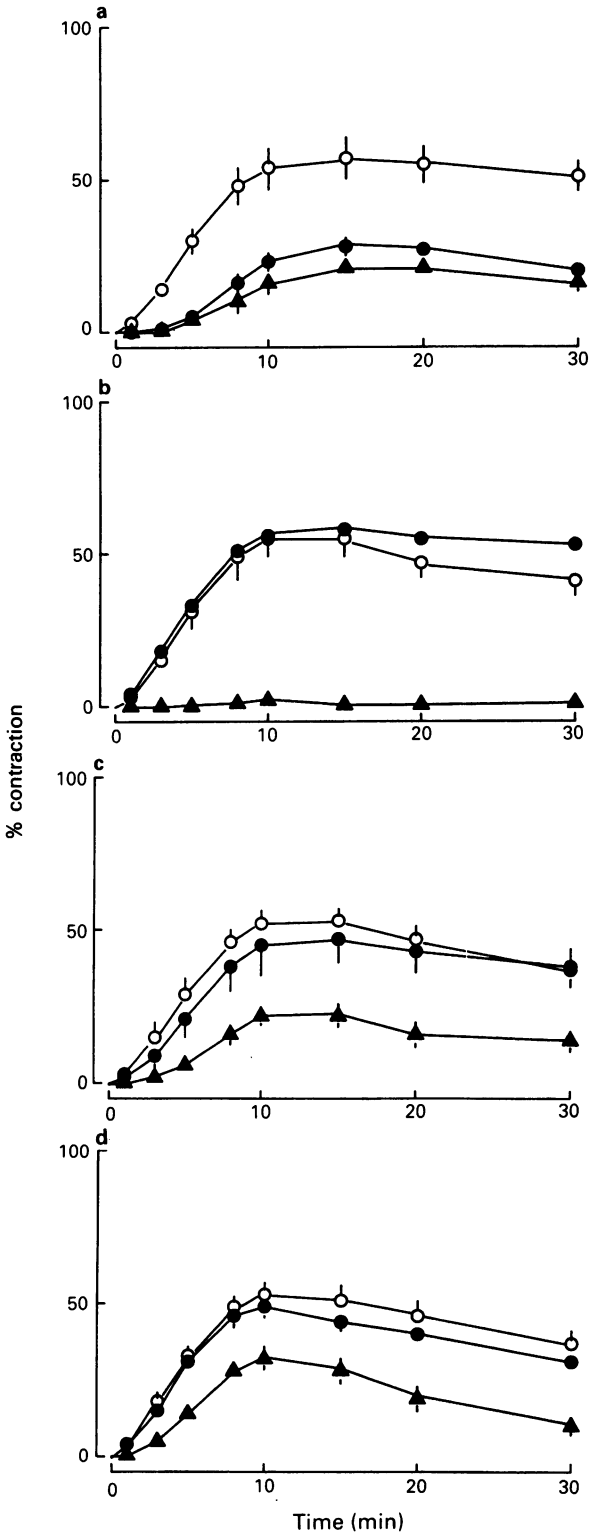


Figure 2 Effect of NIP-121 (a (●) 0.03 μM; (▲) 0.1 μM), isoprenaline (b (●) 0.001 μM; (▲) 0.01 μM), aminophylline (c (●) 30 μM; (▲) 100 μM) and hydrocortisone (d (●) 100 μM; (▲) 300 μM) on melittin-induced contraction of the epithelium-intact guinea-pig isolated trachea. Each point represents the mean ± s.e.mean of 4–5 preparations in paired control (○) and in test tissues.

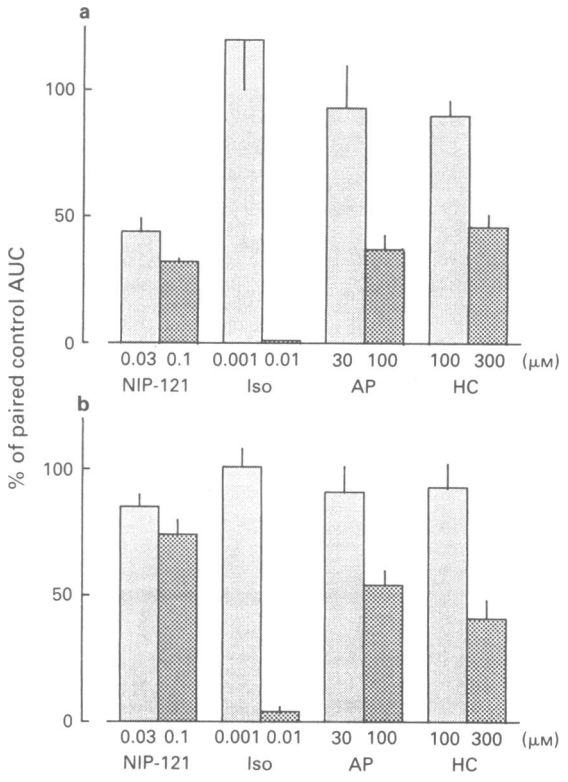
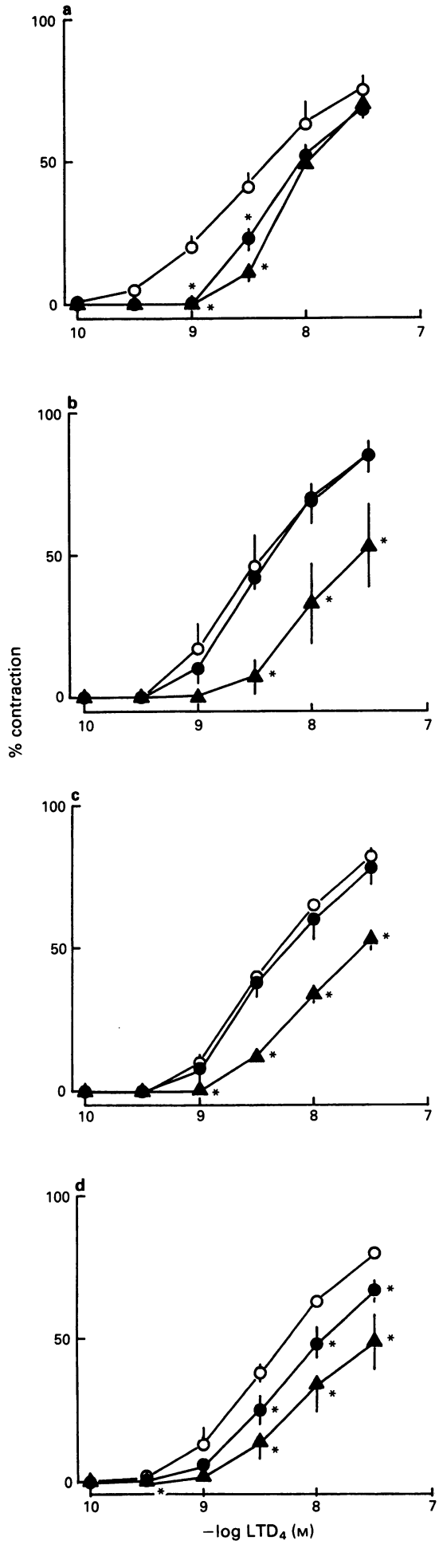


Figure 3 Effect of NIP-121, isoprenaline (Iso), aminophylline (AP) and hydrocortisone (HC) on melittin-induced contraction of the epithelium-intact (a) and the epithelium-denuded (b) guinea-pig isolated trachea. The AUC in the test tissue is expressed as % of the paired control AUC. Each value shows the mean ± s.e.mean of 4–5 preparations.



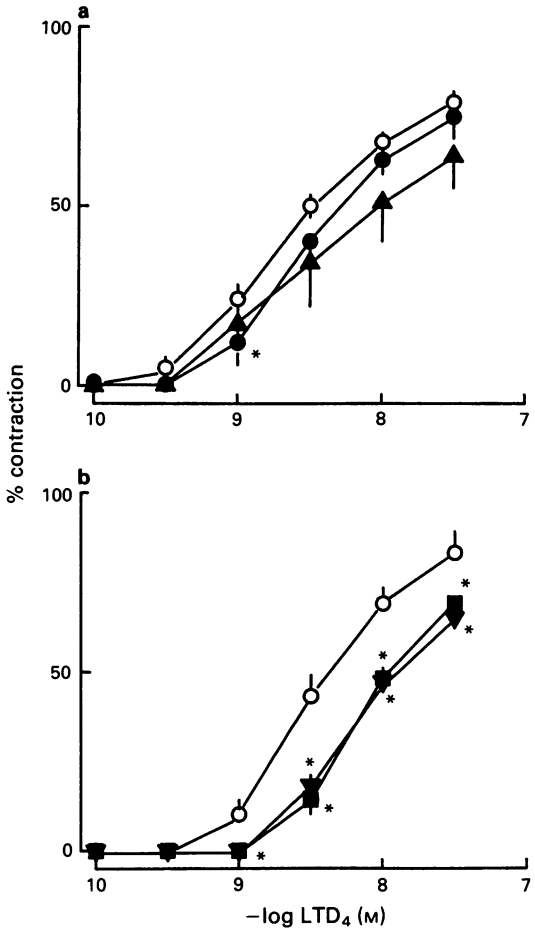


Figure 5 Effect of NIP-121 (a (●) 0.03 μM; (▲) 0.1 μM), (b (■) 0.3 μM; (▼) 1 μM) on leukotriene D_4 (LTD_4)-induced contraction of the epithelium-denuded guinea-pig isolated trachea. Each point represents the mean ± s.e.mean of 5 preparations in paired control (○) and in test tissues. *Indicates that the data points of the test tissues were significantly different ($P<0.05$) from the corresponding data points of the control tissues.

glibenclamide (1 μM) with 0.1 μM NIP-121 failed to inhibit the melittin response (102 ± 14% of the paired control AUC). This finding suggests that the inhibition of the melittin response by NIP-121 is dependent upon the activation of ATP-sensitive potassium channels.

In the epithelium-denuded trachea, the inhibition of the melittin response by NIP-121 was greatly reduced but inhibition by isoprenaline, aminophylline and hydrocortisone was not different from that in the epithelium-intact trachea (Figure 3b). A similar reduction of the inhibition of the melittin response was also observed in the case of cromakalim. Cromakalim at a concentration (0.1 μM) which caused appreciable suppression of spontaneous tracheal tone (68 ± 12%, $n = 5$) inhibited the melittin response in the epithelium-intact trachea (70 ± 23% of the paired control AUC, $n = 5$) but the inhibition disappeared in the epithelium-denuded trachea (106 ± 8% of the paired control AUC, $n = 5$).

Figure 4 Effect of NIP-121 (a (●) 0.03 μM; (▲) 0.1 μM), isoprenaline (b (●) 0.001 μM; (▲) 0.01 μM), aminophylline (c (●) 30 μM; (▲) 100 μM) and hydrocortisone (d (●) 100 μM; (▲) 300 μM) on the leukotriene D_4 (LTD_4) log concentration-contraction curve in the epithelium-intact, guinea-pig isolated trachea. Each point represents the mean ± s.e.mean of 4 preparations in paired control (○) and in test tissues. *Indicates that the data points of the test tissues were significantly different ($P<0.05$) from the corresponding data points of the control tissues.

Effect of NIP-121 and reference drugs on the LTD_4 concentration-response curve in epithelium-intact and epithelium-denuded tracheae

In the epithelium-intact trachea, the low concentration of NIP-121 (0.03 μM) caused significant inhibition of the contractions induced by low concentrations of LTD_4 (1 and 3 nM). The low concentration (100 μM) of hydrocortisone also had a slight but significant inhibitory effect on LTD_4 responses. The low concentrations of isoprenaline and aminophylline did not have any significant effect on the LTD_4 response (Figure 4). The high concentrations of these drugs induced marked inhibition of the LTD_4 response. The magnitude of inhibition by these drugs of contraction induced by 3 nM LTD_4 (approximately 40–50% of the histamine maximum response) was similar to that observed against the melittin response in the epithelium-intact trachea, except that the low dose of hydrocortisone had a slight but significant effect on the LTD_4 response.

We have previously reported that epithelium removal causes a significant leftward shift of the LTD_4 concentration-response curve without affecting its maximal response (Shikada & Tanaka, 1992). In the epithelium-denuded trachea, the inhibitory effect of NIP-121 on the LTD_4 contraction was greatly reduced (Figure 5). The reduction of the effect of NIP-121 on the LTD_4 response by removing the epithelium was similar to that obtained with the melittin response. However, higher concentrations of NIP-121 (0.3 and 1 μM) did inhibit LTD_4-induced contractions of epithelium-denuded trachea (Figure 5).

Discussion

Melittin caused a contraction that was slow in onset of the tracheal strip isolated from a guinea-pig in the presence of the cyclo-oxygenase inhibitor, indomethacin. Our previous study showed that the melittin-induced contractile response was significantly inhibited by both the peptide-leukotriene receptor antagonist, FPL55712, and the lipoxygenase inhibitor, nordihydroguaiaretic acid; similar results were obtained for AA-induced contraction (Yamamoto *et al.*, 1990). We therefore suggested that melittin could contract the guinea-pig isolated trachea by promoting the formation of AA lipoxygenase products. The present data support this hypothesis since not only the PLA_2 inhibitor BPB but also another lipoxygenase inhibitor, phenidone, concentration-dependently and completely, inhibited the melittin response. The inhibitory activity of BPB and phenidone against the melittin response in the epithelium-intact trachea is about the same as that obtained in the epithelium-denuded trachea. This result suggests that when melittin is applied to the trachea, lipoxygenase products are generated in trachealis smooth muscle and thereby induce contraction. It is conceivable that peptide leukotrienes may mediate the melittin response since they are more potent bronchoconstrictors in man (Dahlen *et al.*, 1980; Jones *et al.*, 1982) and guinea-pigs (Drazen *et al.*, 1980; Weichman *et al.*, 1982) than other lipoxygenase products such as LTB_4, which induce cyclo-oxygenase-dependent contraction (Lawson *et al.*, 1986) and lipoxins, which are inactive in tracheal preparations (Jacques *et al.*, 1988).

When the epithelium was removed by rubbing the tracheal luminal surface with cotton, the melittin response was significantly increased. The enhancement of the melittin response by removal of epithelium may be due to the epithelium being a metabolic site of loss for melittin. This interpretation is supported by reports that the epithelium is a site of metabolism for some agents including catecholamines (Farmer *et al.*, 1986) and neurokinins (Devillier *et al.*, 1988). Some inhibitory substances released from epithelium have been reported to suppress tracheal responsiveness against bronchoactive substances (Hay *et al.*, 1987; Fernandes & Goldie, 1990). It is possible that such epithelium-derived putative inhibitory substances may also modulate the melittin-induced contraction. Strek *et al.* (1992) reported that PLA_2- induced, epithelium-dependent substances increased muscarinic responsiveness and the effect was attenuated by eicosanoid blockage. If melittin-induced epithelium-dependent substances increase airway hyperresponsiveness, the present study further supports the theory that the activation of endogenous PLA_2 is an important step in the development of airway hyperresponsiveness.

Two concentrations of NIP-121, isoprenaline, aminophylline and hydrocortisone which have the ability to suppress the spontaneous tone of the epithelium-intact trachea by about 30–40% and 80–90% respectively, were used to investigate the effect on melittin-induced contraction. Although the low concentration of NIP-121 greatly inhibited the melittin response as compared to the reference drugs, lowering the dose of NIP-121 to 0.01 μM affected neither the spontaneous tone nor the melittin response. These results suggest that the effects of concentrations of NIP-121 that reduce the melittin contraction are related to the direct inhibitory effects of the drug on the tracheal responses. Furthermore, of the various agents tested, isoprenaline was the most potent inhibitor of the melittin response. The reason for the difference in the potency among these test drugs in inhibiting the melittin response may be the ability of these drugs to inhibit LTD_4-induced contraction. The extent of inhibition of 3 nM-LTD_4 induced contraction (about 40–50% of the histamine maximum) by these drugs was very similar to that of inhibition of the melittin response. These results suggest that the effect of NIP-121 as well as reference drugs on the melittin response may be closely associated with the inhibition of LTD_4-induced contraction by these drugs, and also strongly support the hypothesis that peptide-leukotrienes contribute to the melittin-induced tracheal contraction. The finding that the inhibition of the melittin response by NIP-121 was completely inhibited by the presence of the ATP-sensitive potassium channel blocker, glibenclamide, suggests that the effect of NIP-121 on the melittin response is dependent on activation of the glibenclamide-sensitive potassium channels.

The inhibitory effect of NIP-121 on the melittin response was greatly reduced in the epithelium-denuded trachea while that of the reference drugs was not changed. A similar reduction was also observed in the effect of the standard potassium channel opener, cromakalim, on the melittin response in the epithelium-denuded trachea. We reported recently that NIP-121 and cromakalim augmented the relaxation induced by sodium nitroprusside and that the effect was epithelium-dependent in the guinea-pig isolated trachea (Shikada & Tanaka, 1992). Moreover, Wessler *et al.* (1992) found that potassium channel openers inhibit acetylcholine release in the airway via an indirect, epithelium-dependent pathway. We therefore propose that the potassium channel openers NIP-121 and cromakalim may inhibit the melittin response via an indirect, epithelium-dependent mechanism. This interpretation is supported by reduction of the effect of NIP-121 on the LTD_4 response in the epithelium-denuded trachea. However, since higher concentrations of NIP-121 inhibited LTD_4-induced contractions of epithelium-denuded trachea, we feel that NIP-121 may have at least two mechanisms in the guinea-pig tracheal preparation; a direct relaxation of smooth muscle and an indirect effect via an epithelium-dependent pathway. The mechanism of the action of NIP-121 on the tracheal epithelium is not clear but may be related to an increase in either the release of epithelium-dependent inhibitory substances or their activity.

In conclusion, melittin causes epithelium-dependent contraction of the guinea-pig isolated trachea which is mediated by products of lipoxygenase activity. NIP-121 may inhibit the melittin response by activating glibenclamide-sensitive potassium channels, which appears to be an epithelium-dependent, indirect effect of NIP-121 apart from its direct effect on the airway smooth muscle, while isoprenaline, aminophylline and hydrocortisone primarily act directly to relax the trachealis smooth muscle.

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(Received January 20, 1993
Revised March 22, 1993
Accepted March 24, 1993)

Br. J. Pharmacol. (1993), **109**, 1097–1100 © Macmillan Press Ltd, 1993

Effects of cromakalim on the electrical slow wave in the circular muscle of guinea-pig gastric antrum

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1 In circular muscle strips of the antrum of guinea-pig stomach, the effects of cromakalim were studied on mechanical activity and intracellular membrane potential.

2 Cromakalim inhibited mechanical activity at concentrations higher than 1 μM, accompanied by membrane hyperpolarization and a decrease in membrane resistance. The hyperpolarization was markedly potentiated in K^+-free solution and was still observed in the absence of Na^+.

3 Slow wave electrical activity was relatively resistant to cromakalim. Changes in its amplitude and frequency were not consistent but blockade of slow waves was never observed. In many preparations cromakalim induced spike-like potentials at the top of slow waves, or when spike-like potentials already existed they were potentiated. However, mechanical activity was always inhibited.

4 Inhibition by cromakalim of the phasic contractions associated with the slow waves, could not be reversed by increasing the external K^+ concentration (12–30 mM).

5 The results suggest that in guinea-pig stomach muscle mechanical suppression by cromakalim does not simply result from membrane hyperpolarization or from inhibition of slow waves. A clear dissociation was found between the mechanical and electrical activities. Slow waves, particularly their frequency, are relatively insensitive to membrane hyperpolarization.

Keywords: Cromakalim; slow wave; gastric muscle

Introduction

Cromakalim is considered to activate potassium (K^+) channels which are sensitive to intracellular ATP and which can be blocked by sulphonylureas, such as glibenclamide (Cook, 1988; Weston, 1989; Weston & Edwards, 1992). Cromakalim relaxes several types of smooth muscle including that from the gastrointestinal tract and this effect is also antagonized by glibenclamide (Cavero *et al.*, 1989; McPherson & Angus, 1990; Quast, 1992). Such relaxation is likely to be mainly due to a decrease in calcium (Ca^{2+}) influx resulting from membrane hyperpolarization (Weston, 1989), but in addition to this mechanism, the direct inhibition of Ca^{2+} influx (Nakao *et al.*, 1988; Okabe *et al.*, 1990; Bray *et al.*, 1991; Post *et al.*, 1991) and intracellular Ca^{2+} release (Bray *et al.*, 1991) may also be involved.

Spontaneous electrical activity, which takes the form of slow waves in the antrum region of the guinea-pig stomach, is known to be relatively insensitive to Ca^{2+} channel blockers, such as verapamil (Golenhofen & Lammel, 1972). The frequency and amplitude of the slow wave are also only very weakly voltage-dependent, when studied by the sucrose-gap method (Tomita, 1981). In the canine colon, the main effect of the racemate cromakalim or its (−)-enantiomeric component, levcromakalim was to decrease slow waves duration. The frequency of slow waves was found to be either decreased (Post *et al.*, 1991) or remained constant (Farraway & Huizinga, 1991), during hyperpolarization caused by these K^+ channel openers. On the other hand, a marked decrease of amplitude in the slow wave of guinea-pig gastric muscle, was observed in the presence of 1 μM cromakalim, although the frequency was not significantly altered (Ito *et al.*, 1992). In the present experiments, we studied the effects of cromakalim on the electrical and mechanical activities in the circular muscle of guinea-pig gastric antrum to determine any correlation between mechanical inhibition and the amplitude of the slow wave. Some of these results have been reported in a preliminary form (Tomita & Brading, 1990).

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Methods

The methods used were essentially the same as those described previously by Chihara & Tomita (1987). Hartley guinea-pigs (250–350 g) of either sex were killed by stunning and bleeding and the stomach was removed. After careful removal of the mucosa, muscle strips (1 mm × 5 mm) were dissected from the antral region in the direction of the circular muscle fibres. A strip was mounted horizontally in a small chamber (0.3 ml) by fixing one end with small pins and connecting the other end to a strain gauge with fine thread for recording tension development. The membrane potential was measured with a conventional intracellular microelectrode technique. In some experiments, a partition method (Abe & Tomita, 1968) was used to measure changes in electrotonic potentials by applying 1–2 s pulses of a constant potential field to the tissue between slow waves.

The chamber was perfused with a physiological solution (prewarmed to 34°C) at a rate of 2 ml min^{-1}. The normal solution contained (mM): NaCl 127, $KHCO_3$ 6, $CaCl_2$ 2.4, $MgCl_2$ 1.2, glucose 12, Tris-HCl 10, the pH being adjusted to 7.4 at 34°C. Meclofenamate (0.3 μM) was added to the perfusate throughout the experiments to eliminate any contribution of endogenous prostaglandins (Syed *et al.*, 1990). Numerical values are expressed as the mean ± s.e.mean with the number of observations (*n*).

In some experiments, only mechanical responses were recorded. For these experiments, preparations of a size similar to those used for electrical recordings were held vertically in a small tube (1 ml) and superfused with the physiological solution prewarmed to 34°C at a rate of 2 ml min^{-1}.

Drugs used were cromakalim (a gift from SmithKline Beecham Laboratories), meclofenamate monohydrate (Parke-Davis) and glibenclamide (Sigma). The stock (10 mM) solutions of cromakalim and glibenclamide were prepared in ethanol and dimethylsulphoxide, respectively.

Results

Effects of spontaneous electrical and mechanical activity

The circular muscle of guinea-pig gastric antrum produced rhythmic electrical slow waves, each accompanied by a contraction. The configuration of the slow wave varied to some extent in different preparations and even in different regions of the same tissue. A notch was often noticeable in the falling phase of the wave, suggesting the presence of two components (cf. Tomita, 1981). Cromakalim at a concentration higher than 1 μM reduced muscle tone and inhibited phasic contractions, accompanied with membrane hyperpolarization, as shown in Figure 1. On average, the hyperpolarization was 7.4 ± 1.5 mV ($n = 14$) with 10 μM cromakalim. Effects on amplitude and frequency were not consistent, but cessation of the slow wave was never observed. In many preparations, the lower slow part of the slow wave became smaller, whereas the spike-like potential when present at the upper part became larger, or started to appear during hyperpolarization, as shown in Figure 1a. The extremes in change in amplitude of slow waves were a decrease to 30% and an increase to 145% in 12 preparations, but in most preparations the change was less than 20% with 10 μM cromakalim. The slow wave frequency was not clearly modified in 4 preparations, it slightly (3–6%) increased in 3, or slightly (5–15%) decreased in 7 out of 14 preparations with 10 μM cromakalim.

Changes in electrotonic potential were examined in 3 preparations. An example of the result is shown in Figure 1b. The amplitude of electrotonic potentials was clearly reduced to 73 ± 6% ($n = 3$) during hyperpolarization produced by 10 μM cromakalim.

Figure 2a shows an atypical effect of cromakalim. In this preparation, 3 μM cromakalim increased the slow wave amplitude markedly (40%) and the slow wave frequency slightly (4%) during hyperpolarization. Mechanical activity was strongly inhibited in spite of the significant increase in amplitude of the slow wave and the appearance of a spike-like potential. The cromakalim-induced increase in amplitude and frequency was even greater when the temperature of the organ bath was lowered from 34°C to 24°C (Figure 2b), but the mechanical inhibition was still very clear.

Antagonism by glibenclamide

The effects of cromakalim were strongly antagonized by glibenclamide, as previously reported (Ito *et al.*, 1992). Glibenclamide itself did not show any noticeable effect on its own up to 3 μM. However, as shown in Figure 3, cromakalim

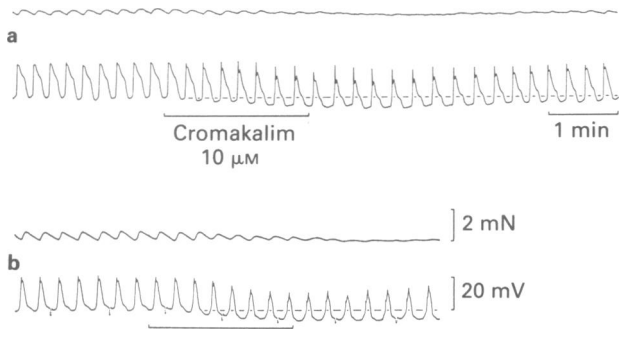


Figure 1 Effects of cromakalim (10 μM: at horizontal bars) on mechanical (upper) and electrical activity (slow waves, lower trace) in the circular muscle of guinea-pig gastric antrum. Examples obtained from two different preparations (a and b) are shown. In (b), electrotonic potentials were produced by applying a constant potential field (2 s in duration) after every three slow waves using the partition methods.

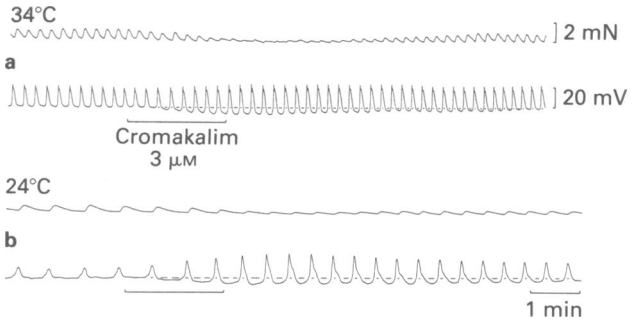


Figure 2 Effects of cromakalim (3 μM: at horizontal bars) at different temperatures (34°C and 24°C). Records (a) and (b) were obtained from the same preparation, but from different cells.

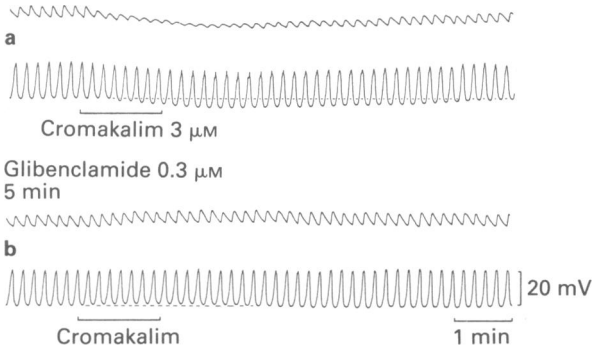


Figure 3 Inhibition of cromakalim effect with glibenclaimide. (a) Control, and (b) cromakalim (3 μM) was applied 5 min after application of glibenclamide (0.3 μM). Both recordings were obtained from the same cell.

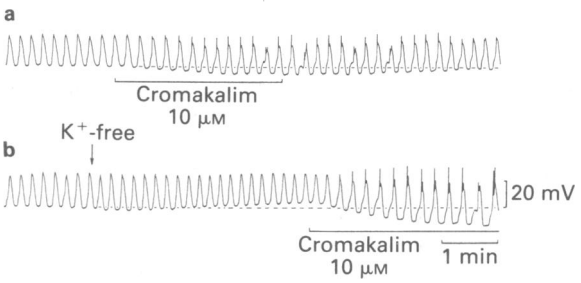


Figure 4 Effects of cromakalim (10 μM: at horiztonal bars) in the presence of normal K^+ (6 mM) (a) and nominally K^+-free solution (b), obtained from different cells in the same preparation.

(3 μM) did not produce hyperpolarization and inhibition of mechanical activity in the presence of glibenclamide (0.3 μM). In 2 out of 4 preparations, there was a small cromakalim-induced depolarization and an increase in muscle tone. A glibenclamide-resistant inhibitory effect of cromakalim has been reported (Ito *et al.*, 1992), but we failed to observe this phenomenon using 1 μM glibenclamide and 10 μM cromakalim.

Effects of changing $[K^+]_o$

Hyperpolarization produced by cromakalim was increased significantly in K^+-free solution (Figure 4). When K^+ was removed, the membrane was slowly depolarized, as previously observed with the sucrose-gap method (Ohba *et al.*, 1977). Cromakalim (10 μM), applied 4 min after K^+ removal, produced 13 mV hyperpolarization from the control level (b), compared with 5 mV in normal solution (a).

The relaxant effect of cromakalim was reduced by increas-

ing the external K^+ concentration, but a clear mechanical inhibition was still observed when cromakalim (3 μM) was applied during a steady contracture in 40 mM K^+ solution, in which rhythmic contractions were blocked. Figure 5 shows the effects of excess K^+ (12–30 mM) on mechanical activity in the presence and absence of cromakalim (3 μM). Excess K^+ raised muscle tone concentration-dependently. Phasic contractions were increased with 12 mM K^+ (Figure 5a), but reduced with K^+ higher than 24 mM (Figure 5 e.g). Cromakalim abolished phasic contractions and reduced the K^+-contracture slightly. In the presence of cromakalim, no recovery of phasic contractions was observed by increasing K^+ concentrations, although a tonic contraction was produced concentration-dependently, which was smaller than the tonic component in the absence of cromakalim. The same results were confirmed in two other preparations.

Effects of cromakalim were also studied in the absence of Na^+ (Figure 6). In this experiment, 127 mM NaCl was replaced with 63.5 mM $MgCl_2$ plus 63.5 mM sucrose, keeping the Cl^- concentration constant (134 mM).

In Na^+-free solution, the membrane was depolarized and the slow waves were reduced in amplitude. Mechanical activity was strongly inhibited in excess Mg^{2+} (Na^+-free) solution. When N-methyl-D-glucamine was used as a Na^+ substitute, the change in membrane potential was essentially the same, but a greater tension developed, making stable intracellular recording difficult. Cromakalim repolarized the membrane and restored the amplitude of the electrical rhythmic activity (Figure 6b). The frequency of this electrical activity was higher than that of the slow waves in normal solution and a hump could not be observed in the falling phase. In two preparations, cromakalim also repolarized the membrane in the presence of ouabain (5 μM) which slowly depolarized the membrane by 5–10 mV over a period of 10 min (not shown).

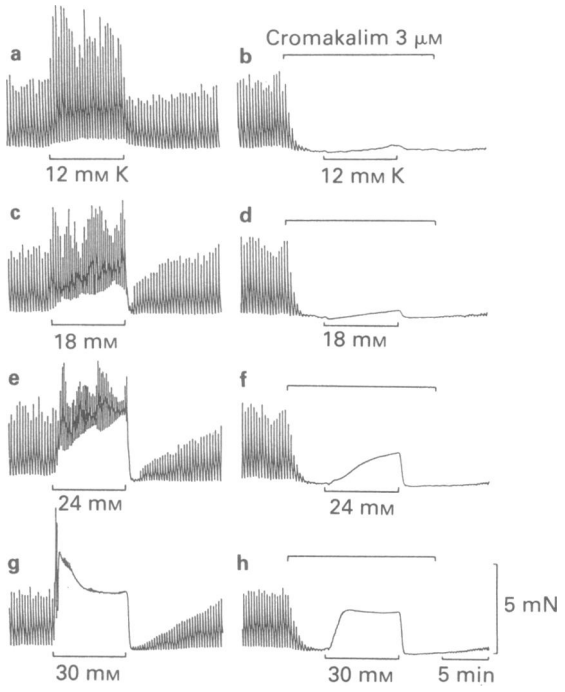


Figure 5 Mechanical activity in response to excess K^+ in the absence (left) and in the presence of cromakalim (right). Excess K^+ (12–30 mM) was applied every 30 min, and cromakalim (3 μM) was applied 4 min before the second application of a given concentration of K^+, as indicated above the recordings.

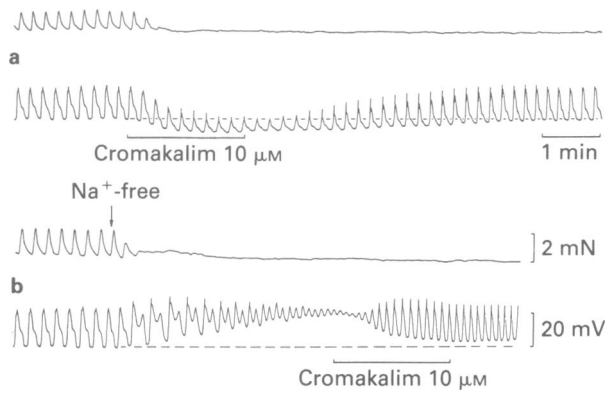


Figure 6 Effects of cromakalim in normal Na^+ and 0 mM Na^+ solution. After observing the control response to cromakalim (10 μM) (a), 127 mM Na^+ was replaced with 63.5 mM $MgCl_2$ and 63.5 mM sucrose (Na^+-free) and 4 min later cromakalim was applied (b). (a) and (b) are from different cells in the same preparation.

Discussion

In the circular muscle of guinea-pig gastric antrum, cromakalim (3–10 μM) hyperpolarized the membrane and this was accompanied by a decrease in membrane resistance, as judged by the decrease in the amplitude of the electrotonic potentials. The hyperpolarization could be readily blocked by glibenclamide, confirming the previous report by Ito *et al.* (1992). In contrast, the hyperpolarization was markedly potentiated in K^+-free medium. Furthermore, cromakalim could still produce hyperpolarization in the absence of Na^+ or in the presence of ouabain. These results strongly suggest that cromakalim increases the K^+ conductance of the plasma membrane, through activation of glibenclamide-sensitive K^+ channels, as reported for many other smooth muscles (Weston & Edwards, 1992).

Mechanical inhibition by cromakalim is probably due in part to hyperpolarization and a decrease in slow wave amplitude. These factors should reduce Ca^{2+} influx, the basic mechanism believed to underlie cromakalim-induced relaxation in other smooth muscles (Weston, 1989; Quast, 1992). However, some additional factor seems to be involved in the cromakalim-induced changes in gastric circular muscle. In some preparations, mechanical activity was strongly inhibited by cromakalim even when the slow wave amplitude was not reduced or even increased. In gastric antral muscle, the spike-like component at the top of the slow wave is considered the main determinant of the magnitude of the phasic contraction (Golenhofen & Lammel, 1972). Verapamil inhibits the spike-like component with little effect on slow waves and suppresses mechanical activity. Since cromakalim often increased the spike-like component, one might expect that this change would be accompanied by an increase in contraction amplitude, such as occurs on exposure of the guinea-pig gastric muscle to adrenaline (Chihara & Tomita, 1987) or with tetraethylammonium in the canine gastric muscle (Szurszewski, 1978). However, no such mechanical potentiation was actually observed in the presence of cromakalim.

If the inhibition of mechanical activity by cromakalim were simply due to membrane hyperpolarization, contractions would be expected to recover when the membrane is depolarized by excess K^+. However, in the presence of 10 μM cromakalim, no such recovery was observed when $[K^+]_o$ was between 12 and 30 mM. Since cromakalim had no effect on Ca^{2+}-induced contraction in chemically skinned muscle strips (guinea-pig trachea: Allen *et al.*, 1986; rat myometrium: Hollingworth *et al.*, 1987), it is very likely that cromakalim exerts some inhibitory action on excitation-contraction coupling processes.

There is some evidence that cromakalim may inhibit Ca^{2+} influx directly and that this mechanism may be involved in relaxation, in addition to the inhibition of Ca^{2+} influx which occurs as a consequence of membrane hyperpolarization (rat

portal vein: Hamilton *et al.*, 1986; guinea-pig mesenteric artery and vein: Nakao *et al.*, 1988). In single muscle cells of rat portal vein (Okabe *et al.*, 1990) and in canine colon (Post *et al.*, 1991), inhibition of inward Ca^{2+} current by cromakalim has been demonstrated with patch-clamp experiments. It is therefore possible that the mechanical inhibition of cromakalim in gastric muscle observed in the present study is partly due to this mechanism, but enhancement of the spike-like potential by cromakalim and the accompanying inhibition of mechanical activity rather contradict this possibility. The ionic mechanisms underlying these paradoxical effects need further investigation. Modification of the function of intracellular Ca^{2+} stores might also be responsible for the cromakalim-induced mechanical inhibition, since some evidence has been presented in rabbit aorta that cromakalim interferes with the refilling of intracellular Ca^{2+} stores and impairs the ability of noradrenaline to release Ca^{2+} from the stores (Bray *et al.*, 1991).

If spontaneous activity in guinea-pig stomach is generated by a voltage-dependent ionic mechanism, its frequency should be decreased, or even completely inhibited, as the membrane becomes hyperpolarized. It has been reported that cromakalim blocks spontaneous spike activity during hyperpolarization in guinea-pig taenia caeci (Weir & Weston, 1986; Den Hertog *et al.*, 1989) and rat portal vein (Hamilton *et al.*, 1986) or slow rhythmic electrical activity in guinea-pig trachealis (Allen *et al.*, 1986). On the other hand, the frequency of slow waves in the guinea-pig gastric antrum is not significantly affected by the hyperpolarization caused by cromakalim (Ito *et al.*, 1992; present study). Such results are consistent with those of previous studies obtained with the sucrose-gap method that slow wave frequency is only weakly voltage-dependent (Tomita, 1981). In the canine colon (circular muscle), it has been reported that cromakalim (10 μM) has either no consistent effect on the slow wave frequency (Farraway & Huizinga, 1991) or that it reduces the frequency by 30% with a hyperpolarization of 7 mV (Post *et al.*, 1991). It may be that the slow wave in guinea-pig stomach is less dependent on membrane potential than that in the canine colon. Nevertheless, in general, the pacemaker activity of slow wave in gastrointestinal muscles seems much more stable in response to changes in membrane potential than that of slow wave in guinea-pig airways muscle (Allen *et al.*, 1986) and spike-like activity in other smooth muscles (Hamilton *et al.*, 1986; Weir & Weston, 1986; Den Hertog *et al.*, 1989) suggesting that different mechanisms underlie the pacemaker processes which trigger these electrical activities.

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(Received February 22, 1993
Revised March 22, 1993
Accepted March 24, 1993)

Br. J. Pharmacol. (1993), **109**, 1101–1109 © Macmillan Press Ltd, 1993

Demonstration of the suitability of CGP 12177 for *in vivo* studies of β-adrenoceptors

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1 Positron emission tomography (PET) with appropriate radioligands offers the possibility of studying receptors non-invasively in man. The suitability of CGP 12177, a hydrophilic non-selective β-adrenoceptor antagonist which can be labelled with the positron emitter ^{11}C, as a ligand for *in vivo* studies of β-adrenoceptors was assessed in rats.

2 [^{3}H]-CGP 12177 was injected into the tail veins of restrained conscious rats. Serial blood samples were taken from tail arteries to determine clearance from plasma. Rats were killed and tissues removed to determine tissue uptake. Radioactivity was assessed by liquid scintillation counting.

3 The uptake of (−)-[^{3}H]-CGP 12177 in various tissues was compared to that of (±)-[^{3}H]-CGP 12177. Maximum tissue:plasma ratios obtained for the (−)-enantiomer in lung, heart and liver were 170, 42 and 13 compared with 60, 15 and 12 for the racemate. Prior injection of excess unlabelled (±)-CGP 12177 blocked the uptake of both (−)- and (±)-[^{3}H]-CGP 12177 in lung and heart but not liver, tissue:plasma ratios for both tracers being reduced to 7, 3 and 7 respectively.

4 Clearance of (−)-[^{3}H]-CGP 12177 from plasma was rapid during the first 5 min but showed only small changes during 5 to 90 min. Uptake in lung and heart reached a maximum at 1 to 5 min and showed a slow decrease during 5–90 min. Prior injection of unlabelled (±)-CGP 12177 reduced uptake in lung and heart to 10% and 20% respectively. Injection of unlabelled (±)-CGP 12177 at 15 min displaced ~75% of the radioactivity by 90 min.

5 (±)-Propranolol had a similar effect to that of unlabelled (±)-CGP 12177. Prior injection reduced uptake of radioactivity in lung and heart to 15% and 20% respectively. Injection of (±)-propranolol at 15 min displaced ~60% of the radioactivity by 90 min indicating that the tracer binds to β-adrenoceptor sites *in vivo*.

6 *In vivo* saturation curves, obtained by injection of (−)-[^{3}H]-CGP 12177 with increasing amounts of unlabelled (−)-CGP 12177, gave values of B_{max} for lung of ~45 pmol per g wet weight of tissue and for heart of ~6 pmol per g wet weight of tissue. K_D could only be expressed as nmol injected per kg body weight, that for lung (2.5 nmol kg^{-1}) being greater than that for heart (1.3 nmol kg^{-1}).

7 Competition studies carried out by co-injecting (−)-[^{3}H]-CGP 12177 with unlabelled (±)-CGP 12177 or (−)-propranolol gave similar values for B_{max} (lung 44 pmol g^{-1}, heart 6 pmol g^{-1},). Values of K_D for (±)-CGP 12177 (lung 4.7 pmol kg^{-1}, heart 2.6 pmol kg^{-1}) were approximately twice those for (−)-CGP 12177. Values of K_D for (−)-propranolol (lung 38 nmol kg^{-1}, heart 104 nmol kg^{-1}) were greater.

8 The results show that (−)-[^{3}H]-CGP 12177 is a suitable ligand for assessing β-adrenoceptors *in vivo*.

Keywords: Adrenoceptors in rat; β-adrenoceptor antagonists; CGP 12177; heart, rat; lung, rat; propranolol; B_{max}; β-adrenoceptors; *in vivo* ligand binding

Introduction

The use of radiolabelled ligands has given considerable insight into the role of receptors in normal physiology and disease. The methods used, however, involve preparation of membrane fractions or tissue sections and incubation of these preparations with appropriate ligands *in vitro*. Such methods may only be used to investigate receptors in man if it is possible to take biopsies at operation, usually of advanced disease, or to obtain tissues at *post mortem*. Information about receptors *in situ* has only been argued indirectly by assessing physiological response to receptor stimulation.

The development of positron emission tomography (PET) has made it possible to measure regional distribution and kinetics of radiolabelled tracers *in vivo* (Phelps *et al.*, 1986; Schuster, 1989). Used with appropriate ligands labelled with positron emitting radioisotopes such as carbon-11 (^{11}C) or fluorine-18 (^{18}F), it offers the possibility of assessing receptor distribution and function non-invasively in man (Syrota *et al.*, 1985; Farde *et al.*, 1986). Two areas in which PET would be of particular value is in assessing the role of β-adrenoceptors in diseases of the heart (e.g. congestive heart failure and the cardiomyopathies; Jones *et al.*, 1989) and lung (e.g. asthma; Davis, 1988). In addition, PET could be used to measure changes in β-adrenoceptors during and following the administration of the β-antagonist or agonist drugs used to ameliorate the symptoms of these diseases.

The non-selective β-adrenoceptor antagonist, CGP 12177 (4-(3-*t*-butylamino-2-hydroxypropoxy)-benzimadazol-2-one), has a number of properties which suggest that it is an appropriate ligand for use in PET. It has a high affinity for β-adrenoceptors and, as a consequence of its low lipophilicity, it shows low non-specific binding *in vitro* (Staehelin *et al.*, 1983). It is not thought to accumulate inside cells and has therefore been proposed as a ligand for surface β-adrenoceptors (Staehelin *et al.*, 1983). It has been used *in vitro* to assess the cellular distribution of β-adrenoceptors (Hertel *et al.*, 1983) and the competitive binding of other β-antagonists (Lacasa *et al.*, 1985). Both the racemate and (−)-enantiomer of CGP 12177 have been labelled with ^{11}C (Boullais *et al.*, 1986; Brady *et al.*, 1991) but the difference in the affinities of the (−) and (+)-enantiomers (Affolter *et al.*, 1985) indicate that (−)-[^{11}C]-CGP 12177 would be preferable to the racemate for PET.

The present study was initiated to assess the suitability of CGP 12177 for PET. Initially the (−)-enantiomer was com-

pared with the racemate. The results confirmed the superiority of the (−)-enantiomer and detailed studies of (−)-[^{3}H]-CGP 12177 was then carried out.

Methods

Animals

Adult male Sprague Dawley rats (220–300 g) were anaesthetized by isoflurane/N_2O/O_2 and catheters (o.d. 0.96 mm) were inserted into a tail artery and vein. Animals were allowed to recover from the anaesthesia for 2–3 h in Bollman's restraining cages.

[^{3}H]-CGP 12177 (74–740 MBq) in 200–300 μl of saline was injected as a bolus via the tail vein. Aliquots of the injectate were diluted in water to determine the radioactivity injected into each animal. Six arterial blood samples (~200 μl) were taken from each animal at selected times after injection of the tracer. An aliquot of whole blood was taken and the remainder centrifuged (2 min at 20000 *g*) to separate the plasma. Animals were killed by intravenous injection of sodium pentobarbitone (Euthatal) at 200 mg kg^{-1} and tissues rapidly removed.

The thorax was opened by an incision on each side of the sternum and the heart and lungs were removed together. The individual lobes of the lung were separated and a sample (~20 mg), distal to the bronchus, was taken from each lobe. The heart was dissected into 5 regions, left and right atrial walls, left and right ventricular walls and interventricular septum, excluding the heart valves. The ventricular walls and septum were divided to give samples less than 100 mg. A length of aorta (10 mm), mainly the aortic arch, and trachea (5–10 mm) were cleared of connective tissue. Samples (100–200 mg) of liver, kidney (cortex and medulla), fat from the lower abdomen and striated muscle (gastrocnemius) were also removed.

Preparation of samples

Tissue samples were blotted, weighed and dissolved in 1 ml of Soluene at 37°C overnight. Blood (15 μl) and plasma (30 μl) were dissolved in 200 μl 1 N NaOH overnight and acidified with 200 μl 4 N HCl. Ten millilitres of scintillant (Hiionic fluor, Packard, acidified by the addition of 1% glacial acetic acid) were added to each dissolved sample for the determination of radioactivity by scintillation counting (Beckman LS 6800). Radioactivity was expressed as becquerels per gram of wet tissue (Bq g^{-1}) or becquerels per millilitre of blood or plasma (Bq ml^{-1}).

Normalization of results

Preliminary experiments showed that the uptake of radioactivity in heart and lung following injection of (−)-[^{3}H]-CGP 12177 was rapid, reaching a plateau at 1–2 min which was maintained until 20 min. Radioactivity in the plasma decreased rapidly during the first 5 min and showed little change during the next 15 min. Before studying tissue uptake and plasma clearance in detail (see Figures 2, 3 and 6 below), the relationship between uptake and amount of (−)-[^{3}H]-CGP 12177 injected was assessed. Animals were killed at 10–20 min after injection of 0.4 to 5 kBq g^{-1} (0.2 to 2.8 nmol kg^{-1}). Figure 1 shows radioactivity in lung, heart (left ventricular wall) and liver as a function of (−)-[^{3}H]-CGP 12177 injected either alone or 5 min after unlabelled (±)-CGP 12177 (7 μmol kg^{-1}). Prior injection of unlabelled (±)-CGP 12177 reduced the uptake of radioactivity, consistent with β-adrenoceptor blockade, in lung and heart but not in liver. Below 2 kBq g^{-1} (1.5 nmol kg^{-1}) uptake in both lung and heart was proportional to the amount injected (correlation coefficients >0.88, $P<0.001$) and the small differences in the specific activities of the batches of (−)-[^{3}H]-CGP

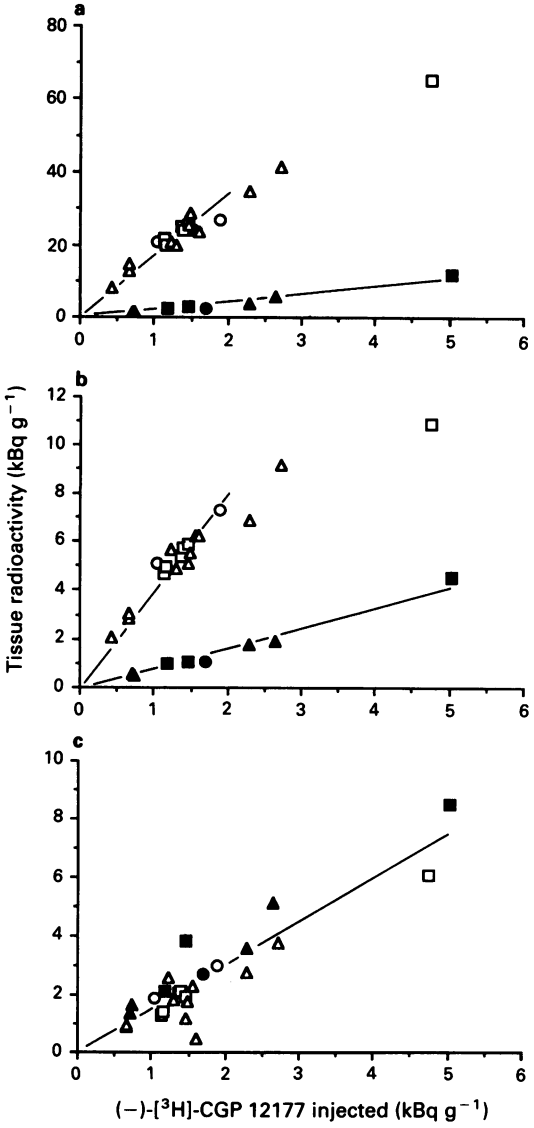


Figure 1 Radioactivity in lung (a), left ventricular wall (b) and liver (c) at 10 to 20 min after intravenous injection of (−)-[^{3}H]-CGP 12177 plotted as a function of radioactivity injected per body weight. (−)-[^{3}H]-CGP 12177 was injected alone (open symbols) or 5 min after unlabelled (±)-CGP 12177 at 7 μmol kg^{-1} (closed symbols). The specific activity of the (−)-[^{3}H]-CGP 12177 was 1.6 (○ ●), 1.8 (□ ■) or 2.0 (△ ▲) TBq mmol^{-1}.

12177 used had no significant effect on the relationship (Figure 1). In subsequent experiments, therefore, the amount injected was always less than 2 kBq g^{-1}. To correct for differences in animal body weight and injected dose, results were expressed as an index of radioactivity defined as:-

$$\text{Index of radioactivity} = \frac{\text{tissue radioactivity (Bq)/tissue wet weight (g)}}{\text{radioactivity injected (Bq)/body weight (g)}}$$

The preliminary experiments also showed that prior injection of unlabelled (±)-CGP 12177 reduced the rate of clearance of (−)-[^{3}H]-CGP 12177 from the plasma. Using the index of radioactivity to compare the tissue uptakes observed with or without prior injection of (±)-CGP 12177 to block receptors does not take into account the increase in plasma levels of (−)-[^{3}H]-CGP 12177 observed after blocking. Therefore, an additional method for comparing individual rats was used. Tissue uptakes were expressed as tissue:plasma ratios, defined as:-

$$\text{Tissue:plasma ratio} = \frac{\text{tissue radioactivity (Bq)/tissue wet weight (g)}}{\text{plasma radioactivity (Bq)/plasma weight (g)}}$$

Plan of experiments

Clearance of [^{3}H]-CGP 12177 from plasma and uptake in tissues were measured under various conditions: (i) [^{3}H]-CGP 12177 was injected alone; (ii) unlabelled (±)-CGP 12177 (7 μmol kg^{-1}) or (±)-propranolol (7 μmol kg^{-1}) was injected as a bolus (200–300 μl) either 5 min before the tracer to block receptors or 15 min after the tracer to displace any radioactivity bound to receptors; (iii) increasing amounts (0.4–1000 nmol kg^{-1}) of unlabelled (−)-CGP 12177, (±)-CGP 12177 or (−)-propranolol were co-injected with a constant amount of tracer to assess saturation and competition with [^{3}H]-CGP 12177 binding.

Materials

(−)-[^{3}H]-CGP 12177 (1.61–2.03 TBq mmol^{-1}) and (±)-[^{3}H]-CGP 12177 (1.13 TBq mmol^{-1}) were obtained from Amersham International plc (Amersham, Bucks). Unlabelled (−)-CGP 12177 and (±)-CGP 12177 were kindly supplied by Dr K.A. Jaeggi, Ciba Geigy (Basel, Switzerland). (−)-Propranolol hydrochloride and (±)-propranolol hydrochloride were obtained at the highest purity from Sigma Chemical Co. (Poole, Dorset).

Data analysis

For each animal, the radioactivity in each sample of lung, septum, left ventricular wall and right ventricular wall was determined and the mean value for each tissue calculated. In the majority of cases the standard errors on these means were smaller than the symbols used in the figures and in all cases they were less than the variation between the means for individual rats.

The significance of differences between groups of animals was tested using the *t* test or the Behren's test, a modification of the *t* test used when the variances of the means were significantly different ($P<0.05$).

The results of competition experiments were analysed using the curve fitting routines of the Ligand programme, originally written by Munson & Rodbard (1980) and modified for microcomputers by G.A. McPherson. This programme uses non-linear curve fitting techniques to obtain estimates of K_D and B_{max} and generates an approximate standard error which gives an indication of the reliability of the estimates.

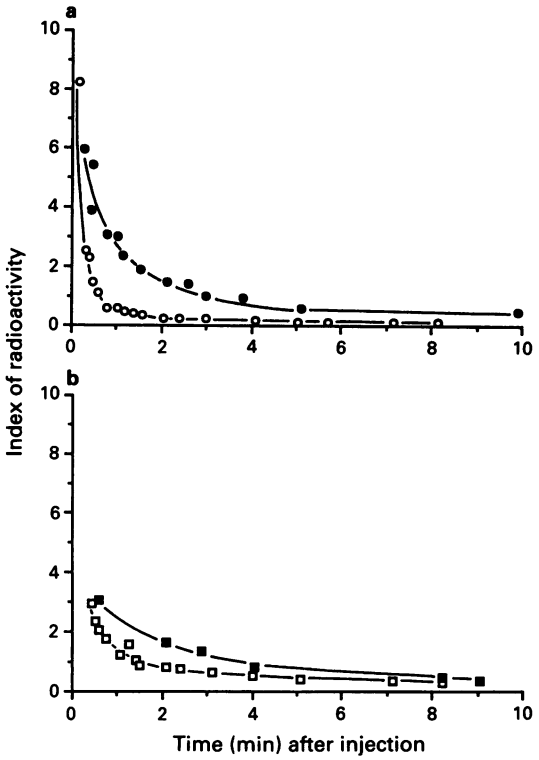


Figure 2 Radioactivity in plasma during the first 10 min after intravenous injection of [^{3}H]-CGP 12177. (−)-[^{3}H]-CGP 12177 (a) or (±)-[^{3}H]-CGP 12177 (b) was injected alone (○ □) or 5 min after unlabelled (±)-CGP 12177 at 7 μmol kg^{-1} (● ■).

Results

Clearance of [^{3}H]-CGP 12177 from plasma

Clearance of radioactivity from plasma after injection of [^{3}H]-CGP 12177 is illustrated in Figures 2 and 3. Data for all rats (6 time points for each rat) have been pooled and for clarity of presentation mean values for selected time intervals plotted. Initial clearance of (−)-[^{3}H]-CGP 12177 was rapid (Figure 2), radioactivity falling to 1–2% during the first 5 min. Prior injection of unlabelled (±)-CGP 12177 (Figure 2) or (±)-propranolol (data not shown) reduced the rate of clearance such that the radioactivity in the plasma at 10 min was about 4 times that observed after (−)-[^{3}H]-CGP 12177 alone. Clearance of (±)-[^{3}H]-CGP 12177 was intermediate between that of the (−)-enantiomer injected alone and after blocking. When receptors were saturated by prior injection of unlabelled (±)-CGP 12177, clearance of (±)-[^{3}H]-CGP 12177 was the same as that of (−)-[^{3}H]-CGP 12177 after blocking (Figure 2).

After the first 10 min clearance of (−)-[^{3}H]-CGP 12177, injected alone or after (±)-CGP 12177, was slow compared with that observed at early times and the difference between tracer injected alone and after unlabelled drug was maintained for the duration (90 min) of the study (Figure 3). Injection of (±)-CGP 12177 at 15 min after the tracer increased the radioactivity in the plasma to a level which, for a given time after injection of the tracer, was about 1.5 times that observed when receptors were blocked by prior injection of unlabelled drug (Figure 3). This increased level was maintained for the duration of the study.

(±)-Propranolol had the same effects on plasma radioactivity as had unlabelled (±)-CGP 12177 (data not shown).

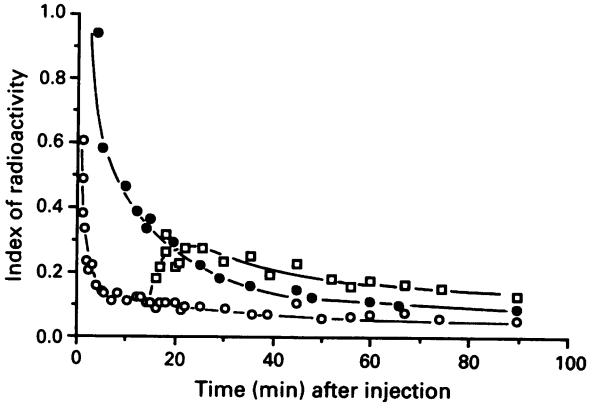


Figure 3 Radioactivity in plasma during 1 to 90 min after intravenous injection of (−)-[^{3}H]-CGP 12177. (−)-[^{3}H]-CGP 12177 was injected alone (○) or with unlabelled (±)-[^{3}H]-CGP 12177 given 5 min before (●) or 15 min after (□) the tracer. Data for 1 to 10 min are also shown in Figure 2. Note the difference in the scales used in Figures 2 and 3.

Distribution of [^{3}H]-CGP 12177 in tissues

The uptake of (−)-[^{3}H]-CGP 12177 in various tissues is illustrated in Figure 4. There was little difference between uptake in the various regions of the heart (Figure 4 insert). The mean value for all regions is shown in the main panel of Figure 4 for comparison with other tissues. Prior injection of either unlabelled (±)-CGP 12177 or (±)-propranolol to saturate receptors reduced the uptake of radioactivity in lung, heart and, to a lesser extent, aorta and trachea ($P<0.05$) but not in the other tissues shown in Figure 4. Uptake in the brain was low (data not shown). After injection of (−)-[^{3}H]-CGP 12177, indices of radioactivity for various regions of the brain ranged from 0.1 to 0.3, with the exception of the pineal body for which values were 4. Prior injection of unlabelled (±)-CGP 12177 reduced the uptake to 0.05–0.08 and 2 respectively.

A large dose of (−)-isoprenaline (7 μmol kg^{-1}) given either as a bolus injection or as an infusion reduced the uptake of (−)-[^{3}H]-CGP 12177 in lung and heart, but not liver, the indices of radioactivity being reduced to 6, 2 and 3 respectively.

Uptake of (±)-[^{3}H]-CGP 12177 in the various tissues is compared with that of (−)-[^{3}H]-CGP 12177 in Figure 5. Uptake of the racemate in lung and heart was approximately 60% that of the (−)-enantiomer. Prior injection of unlabelled (±)-CGP 12177 reduced the uptake to that observed for the (−)-enantiomer after blocking.

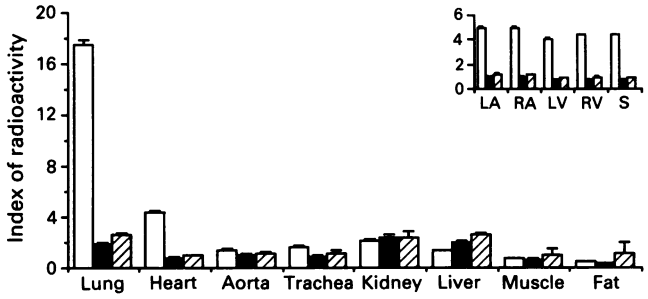


Figure 4 Distribution of (−)-[^{3}H]-CGP 12177 in tissues of the rat at 10 to 20 min after intravenous injection. (−)-[^{3}H]-CGP 12177 was given alone (□) or 5 min after intravenous injection of unlabelled (±)-CGP 12177 (■) or (±)-propanolol (▨), both unlabelled drugs at 7 μmol kg^{-1}. The insert shows uptake in various regions of the heart, namely, left atrial wall (LA), right atrial wall (RA), left ventricular wall (LV), right ventricular wall (RV) and interventricular septum (S). The mean value for all regions (Heart) is shown in the main panel for comparison with other tissues. Bars indicate standard errors.

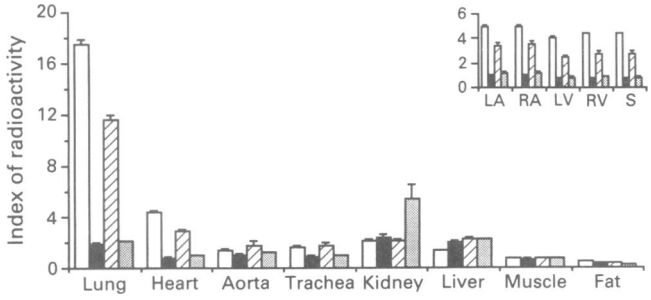


Figure 5 Distribution of (±)-[^{3}H]-CGP 12177 (▨ ▩) in tissues of the rat at 10 to 20 min after intravenous injection compared with that of (−)-[^{3}H]-CGP 12177 (□ ■). Each tracer was injected alone (□ ▨) or 5 min after intravenous injection of unlabelled (±)-CGP 12177 at 7 μmol kg^{-1} (■ ▩). The insert shows uptake in various regions of the heart, namely left atrial wall (LA), right atrial wall (RA), left ventricular wall (LV), right ventricular wall (RV) and interventricular septum (S). The mean value for all regions (Heart) is shown in the main panel for comparison with other tissues. Bars indicate standard errors.

The tissue uptake after intravenous injection of [^{3}H]-CGP 12177 includes radioactivity in the vascular and interstitial spaces as well as that bound to receptor and non-specific sites. Fractional blood volumes, measured in a few animals by injecting [^{99m}Tc]-labelled albumin 10 min before the rats were killed and assuming tissue haematocrit to be similar to large vessel haematocrit, were 0.15 for lung and 0.11 for heart. Thus at 1 min after injection of (−)-[^{3}H]-CGP 12177 alone, when blood levels of (−)-[^{3}H]-CGP 12177 were high, radioactivity in the whole blood was 1.3% of the total radioactivity in the lung and 3.4% of that in the heart. By 15 min, however, when plasma levels had decreased, these percentages had fallen to 0.8% and 2.3% respectively. Prior injection of unlabelled (±)-CGP 12177 increased these values to 4.3% in lung and 7.1% in heart at 15 min after injection of tracer.

Tissue:plasma ratios corresponding to the results shown in Figures 4 and 5 are given in Table 1. Prior injection of unlabelled (±)-CGP 12177 or (±)-propranolol significantly ($P<0.05$) reduced the tissue:plasma ratios for all tissues, although the effects in lung and heart were an order of magnitude greater. This is explained, in part by the increased amount of (−)-[^{3}H]-CGP 12177 in the plasma after receptors had been blocked by an excess of antagonists.

Time course of uptake and displacement

Figure 6 shows the radioactivity in lung and left ventricular wall following injection of (−)-[^{3}H]-CGP 12177 (1–2 MBq kg^{-1}, 0.6–1.1 nmol kg^{-1}). Although the total uptake in the lung was 3.5 times that in the heart the time courses were similar but with a slightly faster clearance from the heart. In each tissue, maximum uptake was reached at 2–5 min after injection of tracer alone and there was a small decrease in radioactivity during the next 85 min. Prior injection of unlabelled (±)-CGP 12177 reduced radioactivity to about 10% in lung and 20% in heart but there was little change during 1 to 90 min. Injection of unlabelled (±)-CGP 12177 at 15 min caused a gradual loss of radioactivity, approximately 75% of the radioactivity being displaced at 90 min (Figure 6). (±)-Propranolol had similar effects to those of unlabelled (±)-CGP 12177. Injection of (±)-propranolol 5 min before (−)-[^{3}H]-CGP 12177 reduced the uptake of radioactivity in the lung and heart to 15% and 20% respectively. Injection of (±)-propranolol at 15 min displaced ~60% of the radioactivity.

Uptake in trachea, aorta, fat and muscle, although less than in heart (Figure 4), reached a maximum at 1–2 min and showed little change during the next 90 min. Prior injection of unlabelled (±)-CGP 12177 or (±)-propranolol had a small effect on radioactivity in the trachea and aorta, reducing it at 20 to 90 min, whereas injection of the unlabelled drugs at 15 min had no significant effect.

Radioactivity in the liver and kidney decreased, after the initial uptake, more rapidly than in the other tissues. The rapid loss from the kidney during the first 5 min after injection followed that from the plasma. That this corresponded to excretion of radioactivity was confirmed in 4 rats from which urine was collected with a catheter inserted directly into the bladder via a small suprapubic incision. Ten to twenty percent of the injected dose was excreted during the first 5 min whereas only 5–10% was excreted during the next 40 min.

Uptake in blood cells was calculated from the radioactivity in whole blood and plasma using the measured peripheral haematocrit. Data for all rats (6 time points for each rat) were pooled and for clarity mean values for selected time intervals are shown in Figure 7. Prior injection of unlabelled (±)-CGP 12177 (Figure 7) or (±)-propranolol (data not shown) blocked the uptake of (−)-[^{3}H]-CGP 12177 to the same extent but, in contrast to the results for lung and heart, injection of the unlabelled drugs at 15 min had little effect on the gradual loss of radioactivity from these cells. It is possi-

Table 1 Uptake of [^{3}H]-CGP 12177 in tissues of the rat

	Tissue:plasma ratio				
	(−)-[^{3}H]-CGP 12177			*(±)-[^{3}H]-CGP 12177*	
Tissue	*Alone*	*After (±)-CGP 12177*	*After (±)-propranolol*	*Alone*	*After (±)-CGP 12177*
Lung	170 ± 8 (20)	6.34 ± 0.42 (7)	9.58 ± 2.22 (2)	60.1 ± 6.2 (5)	7.25 ± 1.20 (4)
Heart: left atrial wall	47.4 ± 1.8 (20)	3.41 ± 0.21 (7)	4.24 ± 1.18 (2)	17.6 ± 2.0 (5)	4.23 ± 0.80 (4)
right atrial wall	47.6 ± 2.0 (20)	3.33 ± 0.16 (7)	4.27 ± 0.99 (2)	18.3 ± 2.2 (5)	4.19 ± 0.39 (4)
left ventricular wall	39.1 ± 1.8 (20)	2.51 ± 0.13 (7)	3.21 ± 0.67 (2)	12.8 ± 1.3 (5)	2.90 ± 0.46 (4)
right ventricular wall	42.3 ± 1.8 (20)	2.81 ± 0.15 (7)	3.45 ± 0.85 (2)	14.3 ± 1.4 (5)	3.29 ± 0.50 (4)
interventricular septum	42.3 ± 1.8 (20)	2.59 ± 0.13 (7)	3.20 ± 0.68 (2)	14.1 ± 1.4 (5)	2.95 ± 0.41 (4)
Aorta	13.9 ± 1.3 (18)	3.44 ± 0.17 (7)	4.29 ± 1.02 (2)	9.35 ± 2.95 (5)	4.28 ± 0.74 (4)
Trachea	16.1 ± 1.0 (18)	3.13 ± 0.17 (7)	4.36 ± 1.63 (2)	9.11 ± 2.32 (5)	3.61 ± 0.49 (4)
Kidney	21.1 ± 0.9 (18)	8.26 ± 1.09 (7)	9.01 ± 3.47 (2)	10.9 ± 1.5 (5)	18.3 ± 0.80 (3)
Liver	13.0 ± 0.8 (20)	6.61 ± 0.33 (7)	9.35 ± 1.29 (2)	11.7 ± 1.0 (5)	7.55 ± 0.71 (4)
Muscle	7.09 ± 0.42 (19)	2.31 ± 0.24 (7)	4.06 ± 2.52 (2)	3.67 ± 0.21 (5)	2.68 ± 0.56 (3)
Fat	4.57 ± 0.83 (19)	1.23 ± 0.22 (7)	4.60 ± 4.02 (2)	1.65 ± 0.29 (5)	0.888 ± 0.099 (3)

Table gives means ± standard errors and number of animals in parentheses, with the exception of column 4 ((±)-propranolol) where means and ranges are given.

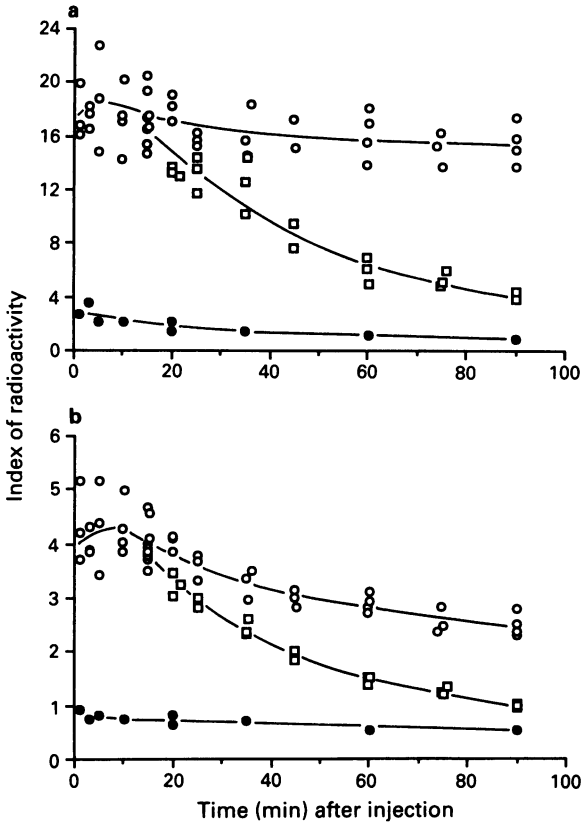


Figure 6 Radioactivity in lung (a) and left ventricular wall (b) after intravenous injection of (−)-[^{3}H]-CGP 12177 alone (○) or with unlabelled (±)-CGP 12177 at 7 μmol kg^{-1} given 5 min before (●) or 15 after (□) the tracer. Each symbol represents the mean of estimates for an individual rat. Lines through the data were drawn by eye.

ble, however, that displacement of bound activity from blood cells was not observed because there was a substantial increase in free (−)-[^{3}H]-CGP 12177 in the plasma which would result in an increase in both non-specific and specific binding to these cells.

Competition studies

A constant amount of (−)-[^{3}H]-CGP 12177 (0.7 nmol kg^{-1}) was co-injected with increasing amounts of unlabelled (−)-CGP 12177, unlabelled (±)-CGP 12177 or unlabelled (−)-propranolol. The rate of loss of radioactivity from the plasma decreased as the amount of unlabelled drug was increased. Radioactivity in lung and left ventricular wall at 15 min after co-injection is plotted against the amount of unlabelled drug injected (nmol kg^{-1}) in Figure 8.

Saturation curves for (−)-CGP 12177 were constructed with data from Figure 8. Specific binding was calculated as the difference between the tissue radioactivity observed after (−)-[^{3}H]-CGP 12177 alone and that after pre-injection of (±)-CGP 12177 at 7 μmol kg^{-1} and was expressed as pmol g^{-1} tissue using the specific activity of the injected (−)-[^{3}H]-CGP 12177. (The specific activity decreased as the amount of unlabelled (−)-CGP 12177, co-injected with the labelled (−)-[^{3}H]-CGP 12177, increased). Results were plotted as a function of the total amount of (−)-CGP 12177 (i.e. labelled plus unlabelled) injected (Figure 9). To combine results obtained with the (−)-enantiomer and the racemate, it was assumed that 50% of the racemate was the (−)-enantiomer and that the binding of the (+)-enantiomer to β-adrenoceptors was insignificant (Affolter *et al.*, 1985).

The data were analysed by the curve fitting routines of the Ligand programme (Munson & Rodbard, 1980). Values for B_{max} were computed as pmol g^{-1} tissue wet weight. Values for K_D could only be expressed in terms of drug injected per body weight (nmol kg^{-1}) and therefore were not directly comparable to those obtained by *in vitro* techniques. When the results obtained when (−)-[^{3}H]-CGP 12177 was co-in-

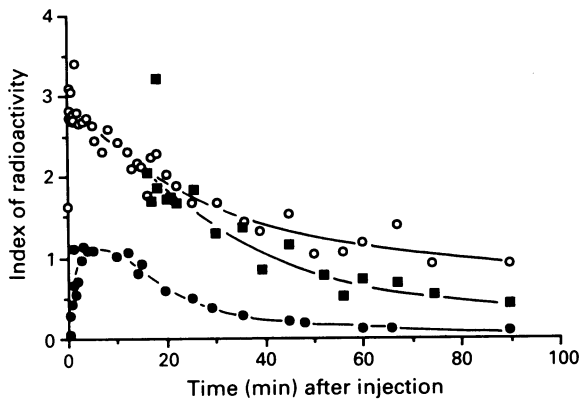


Figure 7 Radioactivity in blood cells after intravenous injection of (−)-[^{3}H]-CGP 12177 alone (○) or with unlabelled (±)-CGP 12177 at 7 μmol kg^{-1} given 5 min before (●) or 15 min after (■) the tracer. Data for all rats (6 time points for each rat) were pooled and mean values for each time interval plotted.

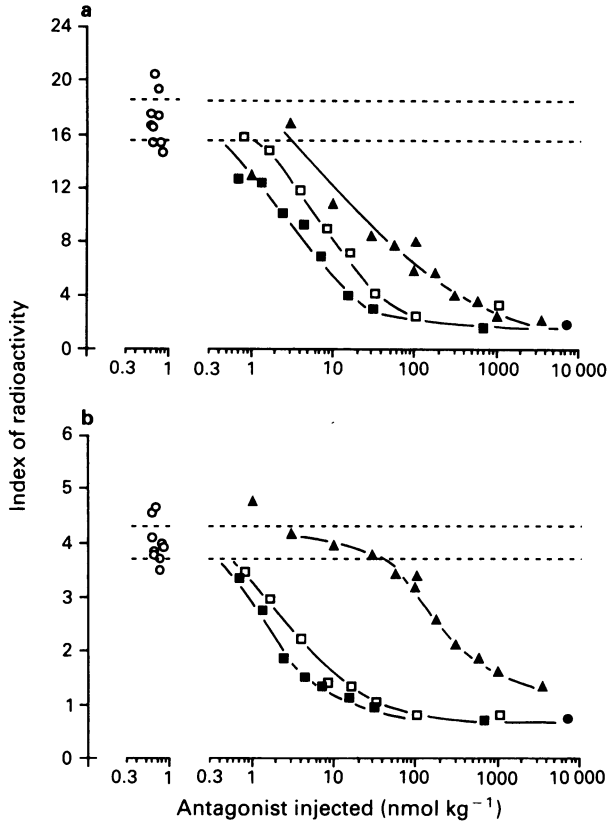


Figure 8 Competition between (−)-[^{3}H]-CGP 12177 and unlabelled antagonists in lung (a) and left ventricular wall (b). Radioactivity at 15 min after intravenous co-injection of (−)-[^{3}H]-CGP 12177 (~0.7 nmol kg^{-1}) with (−)-[^{3}H]-CGP 12177 (■), (±)-CGP 12177 (□) or (−)-propranolol (▲) is plotted as a function of the unlabelled antagonist injected (nmol kg^{-1}). Radioactivity after injection of the tracer alone (○) or 5 min after (±)-CGP 12177 (●) is also shown. The dotted lines indicate ±95% confidence limits on the mean of the values for (−)-[^{3}H]-CGP 12177 alone. Each symbol represents radioactivity for an individual rat.

jected with (−)-CGP 12177 were analysed, the best fits were obtained with a single receptor site. Values for both B_{max} and K_D were greater in the lung than in the heart (Table 2). The values computed for K_D in Table 2 were used to analyse the

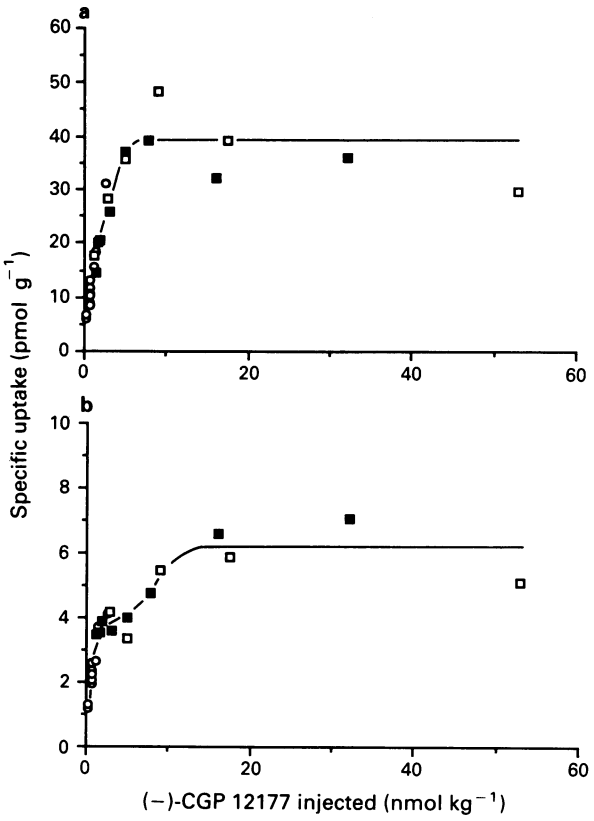


Figure 9 *In vivo* 'saturation' curves for (−)-CGP 12177 in lung (a) and left ventricular wall (b). (−)-[^{3}H]-CGP 12177 was injected alone (○) or with unlabelled (−)-CGP 12177 (■) or (±)-CGP 12177 (□). Uptake of (−)-CGP 12177 was expressed as pmol kg^{-1} wet tissue and plotted against the total amount (labelled and unlabelled) (−)-CGP 12177 injected (see text).

data obtained when (−)-[^{3}H]-CGP 12177 was co-injected with (±)-CGP 12177 or (−)-propranolol (Table 3). Values obtained for B_{max} were similar to those obtained using (−)-CGP 12177 (Table 2). Values for K_D for (±)-CGP 12177 were approximately twice those for (−)-CGP 12177 in both lung and heart. Those for (−)-propranolol were an order of magnitude greater in the lung than for (−)-CGP 12177 and two orders of magnitude greater in the heart.

Discussion

Specificity of tissue binding

The observation that radioactivity in lung and heart reached a plateau which was maintained despite a rapid loss from plasma is consistent with binding of [^{3}H]-CGP 12177 to tissue. That the major part of this binding was to β-adrenoceptors was shown by the inhibition of uptake by prior injection of the unlabelled β-antagonist, (±)-propranolol, or the agonist, (−)-isoprenaline, and by the displacement initiated by injecting the antagonist at 15 min after injection of ligand. Prior injection of an excess of unlabelled (±)-CGP 12177 before (−)-[^{3}H]-CGP 12177 indicated that the non specific binding was ~10% of the total binding in lung and 20% that in heart. Similar percentages have been reported for myocytes (Limas & Limas, 1984) and cardiac tissue preparations *in vitro* (Endoh *et al.*, 1989) although higher values (30%) have been observed in myocardial tissue slices (Watson-Wright *et al.*, 1989).

Recent studies in anaesthetized rats have given results for uptake of (−)-[^{3}H]-CGP 12177 in lung and heart and for

Table 2 *In vivo* binding of (−)-CGP 12177 in lung and heart of rat

Tissue	K_{11} (kg mol^{-1} × 10^8)	K_D (nmol kg^{-1})	B_{max} (pmol g^{-1})
Lung	4.02 ± 0.69	2.49 ± 0.43	44.2 ± 4.1
Heart: left atrial wall	8.27 ± 2.01	1.21 ± 0.29	7.13 ± 0.77
right atrial wall	6.44 ± 0.92	1.55 ± 0.22	7.70 ± 0.50
left ventricular wall	7.35 ± 1.57	1.36 ± 0.29	6.22 ± 0.58
right ventricular wall	8.63 ± 1.54	1.16 ± 0.21	6.01 ± 0.44
interventicular septum	7.80 ± 1.60	1.28 ± 0.26	6.50 ± 0.58

Binding constants were determined by injecting (−)-[^{3}H]-CGP 12177 with unlabelled (−)-CGP 12177 and assessing radioactivity in each tissue. Computer analysis by curve fitting procedures (Ligand programme) was used to estimate binding constants K_{11} (association constant) and K_D (reciprocal of K_{11}) in terms of drug injected per body weight and B_{max} (maximum density of binding sites) in terms of drug per tissue wet weight.

Table 3 *In vivo* binding of β-adrenoceptor antagonists in lung and heart of rat

	(±)-CGP 12177			*(−)-Propranolol*		
Tissue	K_{21} (kg mol^{-1} × 10^8)	K_D (nmol kg^{-1})	B_{max} (pmol g^{-1})	K_{21} (kg mol^{-1} × 10^6)	K_D (nmol kg^{-1})	B_{max} (pmol^{-1})
Lung	2.12 ± 0.67	4.71 ± 1.49	44.6 ± 3.6	26.1 ± 6.3	38.3 ± 9.2	43.7 ± 3.6
Heart: left atrial wall	3.23 ± 0.53	3.10 ± 0.51	6.73 ± 0.32	8.08 ± 2.08	124 ± 32	5.00 ± 0.30
right atrial wall	2.99 ± 0.52	3.35 ± 0.58	7.48 ± 0.38	13.6 ± 3.71	73.3 ± 19.9	7.15 ± 0.51
left ventricular wall	4.28 ± 0.63	2.33 ± 0.34	5.77 ± 0.25	8.91 ± 1.48	112 ± 19	5.95 ± 0.25
right ventricular wall	4.54 ± 0.80	2.20 ± 0.39	5.55 ± 0.29	9.40 ± 1.93	106 ± 22	5.85 ± 0.30
interventricular septum	4.84 ± 0.66	2.07 ± 0.28	6.22 ± 0.26	9.52 ± 1.84	105 ± 20	6.18 ± 0.30

Binding constants were determined by injection of (−)-[^{3}H]-CGP 12177 with unlabelled (±)-CGP 12177 or (−)-propranolol and assessing radioactivity in each tissue. Computer analysis by curve fitting procedures (Ligand programme), using the values for K_{11} (association constant for (−)-[^{3}H]-CGP 12177) shown in Table 2, was used to estimate binding constants K_{21} (association constant for unlabelled antagonist to (−)-[^{3}H]-CGP 12177 binding sites) and K_D (reciprocal of K_{21}) in terms of drug injected per body weight and B_{max} in terms of drug per tissue wet weight.

clearance from plasma which are in agreement with those reported here. As in the present study, prior injection of (±)-propranolol decreased the tissue uptake of (−)-[^{3}H]-CGP 12177 but its effect on the time course of plasma clearance was not reported (van Waarde *et al.*, 1992a). That (−)-[^{3}H]-CGP 12177 binds to β-adrenoceptors *in vivo* was confirmed by predosing the rats with various α- or β-adrenoceptor agonists or antagonists (van Waarde *et al.*, 1992b).

Uptake of (−)-[^{3}H]-CGP 12177 in brain was low as might be expected for a hydrophilic ligand which would be excluded from the tissues by the blood brain barrier. Prior injection of (±)-CGP 12177 blocked the uptake of tracer indicating there was specific binding to β-adrenoceptors as had been demonstrated in brain slices *in vitro* (de Paermentier *et al.*, 1989) where the problem of delivery of the tracer to the tissue does not arise.

Injection of unlabelled (±)-CGP 12177 or (±)-propranolol before (−)-[^{3}H]-CGP 12177, caused small but significant reductions in the uptake of radiolabelled ligand, expressed as the index of radioactivity, in trachea and aorta but not in muscle, fat, liver or kidney (Figure 4). In contrast, prior injection of antagonists significantly reduced tissue:plasma ratios in all the tissues (Table 1). This discrepancy is probably explained by the fact that a large dose of unlabelled antagonist increased the amount of (−)-[^{3}H]-CGP 12177 in the plasma. Non-specific tissue binding measured as the index of radioactivity would be increased relative to that which would have been observed if plasma levels of labelled ligand were unchanged. On the other hand, tissue:plasma ratios include changes in plasma levels of (−)-[^{3}H]-CGP 12177. Predosing with antagonists reduced these ratios to 20–50% of those observed after (−)-[^{3}H]-CGP 12177 alone, indicating specific binding in agreement with *in vitro* studies using [^{3}H]-CGP 12177 which have demonstrated β-adrenoceptors in liver (Kawai *et al.*, 1986), muscle (Watson-Wright & Wilkinson, 1986), fat (Lacasa *et al.*, 1985) and proximal tubules in kidney (Morgunov & Hirsch, 1990). Other radiolabelled ligands have shown the presence of β-adrenoceptors in the aorta (Molenaar *et al.*, 1987) and trachea (Barnes *et al.*, 1982).

Another factor which may complicate the delivery of the ligand to tissues is that there are reports that CGP 12177 has a partial agonist effect *in vitro* (Staehelin *et al.*, 1983; Becker & Porzig, 1984). The tracer doses of (−)-[^{3}H]-CGP 12177 (0.7 nmol kg^{-1}) used in the present experiments had no effect on heart rate and blood pressure, measured via an indwelling tail artery catheter, although the dose used to determine non-specific uptake (7 μmol kg^{-1}) caused a 30% increase in heart rate which persisted for 2 h after intravenous injection but no consistent changes in blood pressure.

A potential disadvantage of using radiolabelled ligands *in vivo* is that the radioactivity in plasma or tissues represents both the ligand and any labelled metabolites. There is limited information about the metabolism of CGP 12177 in the dog, the rat and man. Rapid metabolism of (±)-[^{11}C]-CGP 12177 in dogs has been reported by Delforge *et al.* (1991). At 5 min after intravenous injection, less than 12% of the total radioactivity in the blood was unchanged (±)-[^{11}C]-CGP 12177. In contrast, a detailed study of (−)-[^{3}H]-CGP 12177 in dogs failed to identify metabolites with high performance liquid chromatography (h.p.l.c.), although a small percentage

(<5%) of polar metabolites was detected in the plasma of rats at 15 min after injection of ligand (Jones *et al.*, 1991). Similar results for (−)-[^{3}H]-CGP 12177 in rats have been reported with thin layer chromatography (van Waarde *et al.*, 1992a). At 20 min after injection, 92% of the plasma radioactivity co-eluted with unchanged (−)-CGP 12177, although by 80 min this had fallen to 40%. Recent studies have confirmed that (−)-[^{11}C]-CGP 12177 remains unchanged in canine and human plasma up to 80 min after injection although it was partially metabolized in the rat, the percentage of unchanged (−)-[^{11}C]-CGP 12177 being 92% at 5 min and 74% at 80 min (Luthra *et al.*, 1992).

Estimation of B_{max} *and* K_D

Injection of a tracer amount of (−)-[^{3}H]-CGP 12177 with increasing amounts of unlabelled (−)-CGP 12177 decreased the uptake of radioactivity in lung and heart (Figure 8). The data were best fitted to a single receptor site in both lung and heart. Different values for K_D, however, were calculated for lung and heart (Table 2). These values are not directly comparable with the dissociation constant K_D determined *in vitro*. Although the uptake of (−)-[^{3}H]-CGP 12177 in heart and lung is clearly related to the amount of drug injected (Figures 1, 8 and 9), the tissue environment is one of changing levels of ligand and never reaches an equilibrium as assumed when using the Ligand computer programme to analyse *in vitro* binding data. In addition, results can only be expressed as nmol injected per kg body weight which does not take into account differences in the availability of free antagonist, related to blood flow and tissue extraction, to β-adrenoceptors in various tissues. The differences in K_D for (−)-[^{3}H]-CGP 12177 observed for lung and heart, therefore, could be related to microscopic differences in tissue concentrations of free ligand.

Alternatively, the difference in the values of K_D for lung and heart could reflect a difference in the affinity of (−)-[^{3}H]-CGP 12177 to the different β-adrenoceptor sub-types. Studies of binding of (−)-[^{3}H]-CGP 12177 to membrane or cell preparations from tissues with predominantly β_1- or β_2-adrenoceptors give a wide range of values for K_D with no clear correlation with β-adrenoceptor sub-type. Two studies using selective antagonists, however, indicate that the affinity of (−)-[^{3}H]-CGP 12177 to β_1-adrenoceptors in rat heart membranes is two to three times greater than that to β_2-adrenoceptors (Nanoff *et al.*, 1987; Tsuchihashi *et al.*, 1989). This small difference is not detectable by non linear curve fitting procedures using (−)-[^{3}H]-CGP 12177 alone or with non-selective antagonists. As β-adrenoceptors in lung are predominantly of the β_2 sub-type and those in the heart are predominantly β_1 (Minneman *et al.*, 1979), this difference might be expected to result in the K_D for lung being greater than that for heart. That this was observed in the present study (Table 2) supports a selectivity of (−)-[^{3}H]-CGP 12177 for β-adrenoceptors sub-types.

Table 3 indicates that the K_D for (±)-[^{3}H]-CGP 12177 is approximately twice that for (−)-[^{3}H]-CGP 12177. A similar relationship has been observed between the K_D's for the racemate and (−)-enantiomer in glioma cells *in vitro* (Affolter *et al.*, 1985). *In vitro* binding studies also show that the affinity of CGP 12177 for β-adrenoceptors is about 2.5 times that of propranolol (Tsuchihahsi *et al.*, 1989). The fact that the differences indicated in Table 3 are considerably greater could be due to the high lipophilicity of propranolol resulting in high non specific binding *in vivo*. The difference between values for heart and lung, however, was not predicted from the literature. This may be due to the distribution of propranolol to the tissues. The lung has a high affinity for propranolol (Dollery & Junod, 1976) and may therefore remove a significant amount from the circulation on first pass after an intravenous injection thus reducing the amount reaching the heart (Syrota, 1988). This effect would be most apparent at low ligand doses and could explain the difference in the relationship between injected dose and radioactivity shown in Figure 9.

The estimates of B_{max} given in Tables 2 and 3 are expressed as pmol g^{-1} wet tissue. If it is assumed that there are 100 mg protein per gram of wet tissue, values for B_{max} become 440 fmol mg^{-1} protein for lung and ~60 fmol mg^{-1} for heart. The B_{max} of 60 fmol mg^{-1} is within the range of values (12–70 fmol mg^{-1}) determined with [^{3}H]-CGP 12177 in rat cardiac membranes or myocytes *in vitro* (Limas & Limas, 1987; Shepherd *et al.*, 1987; Lauk *et al.*, 1989; Steinkraus *et al.*, 1989; Tsuchihahsi *et al.*, 1989; Mauz & Pelzer, 1990; Reithmann *et al.*, 1990; Rocha-Singh *et al.*, 1991). Although the regional variations were not significant the results are in agreement with autoradiographic studies using lipophilic ligands such as [^{125}I]-iodocyanopindolol which indicate that the number of β-adrenoceptors is greatest in the atrial walls (Summers *et al.*, 1987). Values of B_{max} have not been published for (−)-[^{3}H]-CGP 12177 in rat lung. The value of 440 fmol g^{-1} reported here, however, is similar to that obtained in rat lung (406 fmol g^{-1}) with [^{125}I]-iodocyanopindolol (Winter *et al.*, 1986).

Conclusion

Following intravenous injection, specific uptake, determined by prior injection of an excess of antagonist, was 80–90% total binding and was 75% displaceable. Values of β_{max} determined using (−)-[^{3}H]-CGP 12177 in combination with unlabelled CGP 12177 or propranolol were comparable to values reported for *in vitro* assays in the literature. Although it was not possible to measure values of K_D in terms of the true concentration of ligand in equilibrium conditions, comparison of apparent K_D's could be made. The results confirm that (−)-CGP 12177 is a suitable ligand for assessing β-adrenoceptors *in vivo*. Labelled with ^{11}C, it is now being used for assessing β-adrenoceptors in man with positron emission tomography (de Silva *et al.*, 1992; Ueki *et al.*, 1992).

I would like to thank Dr K.A. Jaegii (Ciba-Geigy) for the gift of unlabelled (±)-CGP 12177 and (−)-CGP 12177, Janice Burgin for excellent technical assistance and Dr T. Jones, Dr J.M.B. Hughes, C.G. Rhodes and other colleagues at the Cyclotron Unit for their advice and encouragement.

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(Received December 18, 1992
Revised February 18, 1993
Accepted March 24, 1993)

Br. J. Pharmacol. (1993), **109**, 1110–1119 © Macmillan Press Ltd, 1993

Estimation of competitive antagonist affinity from functional inhibition curves using the Gaddum, Schild and Cheng-Prusoff equations

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1 The estimation of antagonist affinity from functional experiments in which the effect of a fixed agonist concentration is reduced by a range of antagonist concentrations ('functional inhibition curves') has been considered from both a theoretical and experimental viewpoint.

2 Theoretical predictions are compared with results obtained from the stimulation of [^{35}S]-GTPγS binding by acetylcholine to membranes of Chinese hamster ovary (CHO) cells stably transfected with human m1–m4 muscarinic receptors, and inhibition of the stimulated binding by pirenzepine and AQ-RA 741.

3 The usual procedure of applying the Cheng-Prusoff correction is shown to be theoretically invalid, and predictions are made of the size and distribution of errors associated with this procedure.

4 A different procedure for estimating antagonist affinity, using the principles of dose-ratio analysis and analogous to use of the Gaddum equation, is found to be accurate and theoretically valid.

5 A novel method of analysis allows accurate estimation of both antagonist affinity and Schild slope, by fitting the combined data from an antagonist inhibition curve and an agonist activation curve directly to a form of the Schild equation (derived by Waud) using non-linear regression analysis.

6 It is shown that the conventional Schild analysis can be enhanced by treating part of the data as a family of inhibition curves and including in the Schild plot dose-ratios estimated from the inhibition curves.

Keywords: Muscarinic receptor subtypes; GTPγS binding; antagonist affinity, dose-ratio analysis; receptor theory

Introduction

Antagonist affinity is frequently estimated in binding studies by measuring the inhibition of binding of a fixed concentration of radioligand and converting the observed IC_{50} (concentration of antagonist inhibiting binding by 50%) to a K_i (estimate of antagonist dissociation constant) with the equation of Cheng & Prusoff (1973). This equation assumes that both agents interact in a reversible competitive fashion at a single site according to the law of mass action, that the reaction is at equilibrium, and that the free concentrations of the agents are known and constant throughout the experiment. The K_d of the radioligand must also be known.

In functional studies the dissociation constant of the agent providing the signal, the agonist, is not known, and the relationship between observed response and agonist occupancy is also not known. In order to overcome these limitations, null methods have been devised which involve the same assumptions as in the binding experiments and, in addition, the assumption that a particular response reflects a certain level of agonist occupancy regardless of the level of antagonist occupancy. The central feature of these methods is the measurement of 'dose ratios', ratios of agonist concentrations in the presence and absence of antagonist which produce the same response. The simplest such method uses the Gaddum equation (Gaddum, 1957) and involves the measurement of agonist effect over a range of concentrations in the absence and presence of a fixed concentration of antagonist. The method of Arunlakshana & Schild (Schild, 1957) uses dose-ratios obtained in the presence of a number of antagonist concentrations.

The experimental designs which are used in Gaddum and Schild analyses typically involve the construction of two or more agonist concentration-effect curves in the absence and presence of fixed antagonist concentrations, for the practical reason that most whole tissues produce a rapid response to agonist but do not maintain a stable response to a fixed agonist concentration for the often long periods of time needed for equilibration of antagonist. Some functional responses, however, notably those measured biochemically, readily lend themselves to designs involving an antagonist titration in the presence of a fixed concentration of agonist, an 'inhibition curve' design.

Although the application of inhibition curve design to Gaddum analysis (Lazareno & Roberts, 1987) and, recently, to Schild analysis (Pöch *et al.*, 1992) have been described, most authors have been content to analyse functional inhibition curves with the Cheng-Prusoff equation (e.g. Ford *et al.*, 1992), despite the fact that the parameters and assumptions of this analysis are not applicable to functional experiments. The Cheng-Prusoff equation, however, is virtually identical to the null-method equation of Lazareno & Roberts (1987) at high agonist concentrations, so under this condition the Cheng-Prusoff equation should yield the correct result. Eglen & Whiting (1989) have explicitly investigated the applicability of the Cheng-Prusoff equation to functional data and have reached the opposite conclusion: they claimed to demonstrate, both experimentally and theoretically, that the equation may, by chance, yield the correct result with low fixed agonist concentrations, but results in serious errors when used with high agonist concentrations. If this result were correct, it would invalidate the null-method inhibition-curve type of design of Lazareno & Roberts (1987) and Pöch *et al.* (1992).

Here we demonstrate (i) the validity of Gaddum analysis as applied to inhibition curves, (ii) the direct fitting of the Schild equation (Waud, 1975; 1976) to single inhibition curves to yield estimates of both Schild slope and pK_b, and (iii) the possibility of enhancing the power of the normal

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Schild analysis by extracting dose-ratio values from inhibition curves as well as activation curves. We also explore the validity of the Cheng-Prusoff equation in functional experiments using a theoretical model of agonism, the Operational Model of Black & Leff (1983). The functional measure we have used is the agonist stimulated binding of [^{35}S]-GTPγS to membranes from Chinese hamster ovary (CHO) cells stably transfected with the m1–m4 human muscarinic receptors.

Some of these data have been presented in preliminary form (Lazareno *et al.*, 1993).

Methods

Cell culture and membrane preparation

CHO cells stably expressing human m1–m4 muscarinic receptors (Buckley *et al.*, 1989) were generously provided by Dr N. Buckley (N.I.M.R., London). These were grown in MEM-alpha medium (GIBCO) containing 10% (v/v) new born calf serum and harvested by scraping the cells in a hypotonic medium (20 mM HEPES + 10 mM EDTA, pH 7.4). Membranes were prepared at 0°C by homogenization with a Polytron followed by centrifugation (40,000 *g*, 15 min), were washed once in 20 mM HEPES + 0.1 mM EDTA, pH 7.4, and were stored at − 70°C in the same buffer. The yields of receptor were 3, 1, 3 and 4 pmol mg^{-1} of total membrane protein at m1, m2 m3 and m4 subtypes respectively.

[^{35}S]-GTPγS binding

Membranes were suspended in a buffer containing 20 mM HEPES, 100 mM NaCl and 10 mM $MgCl_2$, pH 7.4 at a protein concentration of 25–50 μg ml^{-1}. Polystyrene tubes (5 ml) containing 1 ml of membrane suspension were incubated with GDP, ACh and antagonist at 30°C for 20 min and then transferred to ice for 15 min. [^{35}S]-GTPγS was added to a final concentration of 100 pM and the samples incubated for 30 min at 30°C. The samples were filtered over glass fibre filters (Whatman GF/B) using a Brandel cell harvester and washed with 5 ml water. The filter discs were extracted overnight in 3 ml scintillant and counted by liquid scintillation spectrometry at an efficiency of about 97%. Assays were conducted in duplicate, with each set of replicates filtered together.

Reagents

[^{35}S]-GTPγS was purchased from Du Pont; acetylcholine (ACh), pirenzepine (PRZ) and GDP were from Sigma, and AQ-RA 741 (11-({4-[4-(diethylamino)-butyl]-1-piperidinyl} acetyl))-5,11-dihydro-6H-pyrido(2,3-b)(1,4)-benzodiazepine-6-one) was a gift from Dr Karl Thomae GmbH.

Data analysis

In Experiment 1 the d.p.m. were not transformed. In Experiment 2 there were small but consistent differences between the 1st and 2nd replicates of the m3 and m4 assays, caused perhaps by small differences in the incubation time or washing procedure, so for all subtypes each set of replicates was expressed as a % of basal activity: this transformation resulted in a small improvement in the coefficient of variation.

The data were analysed by non-linear regression analysis with two programmes. The direct fits to the Schild equation and simulations were conducted with SigmaPlot (Jandel Scientific, Germany), which was also used to produce all the graphs. The logistic fitting utilised Allfit (De Lean *et al.*, 1978, a gift from Dr Munson, NIH); SigmaPlot could have been used, but Allfit was more convenient as it is designed specifically for the analysis of families of logistic curves, with the option of sharing or fixing some or all of the fitted parameters between curves, and testing the statistical validity of such constraints. In all cases the statistical validity of sharing parmeters, where appropriate, was assessed by the 'extra sum of squares' test (Munson & Rodbard, 1980) and a significance level of $P<0.05$.

Cheng-Prusoff analysis

Agonist activation curves and antagonist inhibition curves in the presence of a fixed agonist concentration [A] were fitted to logistic functions to yield the parameters EC_{50} and IC_{50}. The Cheng-Prusoff estimate of dissociation constant (K_{CP}) was obtained with the formula.

$$K_{CP} = IC_{50}/([A]/EC_{50} + 1).$$

A theoretical consideration of the validity of this analysis in functional experiments is contained in the Appendix.

Analysis of inhibition curves using Gaddum analysis

The procedure has been described by Lazareno & Roberts (1987). Gaddum analysis involves the estimation of equiactive agonist concentrations of agonist in the presence and absence of a certain antagonist concentration, and insertion of the ratio of the former to the latter agonist concentration (the 'dose-ratio') in the equation

$$K_b = [B]/(\text{dose-ratio} - 1),$$

where K_b is the estimate of antagonist K_d and [B] is the concentration of antagonist.

Both the traditional and inhibition curve designs contain a titration of agonist alone, to which a second titration is related. In the traditional design the antagonist concentration is fixed by the experimenter, a second agonist titration is performed in its presence, and analysis of the two curves allows an estimation of the equieffective agonist concentration in the presence of antagonist (measured typically at the 50% response level). In the inhibition curve design the equieffective agonist concentration is fixed by the experimenter, the antagonist concentration reducing its effect by 50% is estimated from an antagonist titration in the presence of the fixed agonist concentration, and the agonist concentration causing the same effect (50% that of the fixed concentration) is estimated from the agonist titration. As in the conventional design, there are two titrations which provide estimates for the analysis.

The antagonist inhibition curve and the agonist activation curve containing agonist concentrations *up to the fixed concentration used in the inhibition curve*, were fitted simultaneously to a logistic model with the maxima and minima either defined (Experiment 1) or shared (Experiment 2) (the pK_b estimates were almost identical with either type of constraint) to yield an IC_{50}* for the antagonist and an EC_{50}* for the agonist. The IC_{50}* is the concentration of antagonist which produces, in the presence of the fixed agonist concentration, a response level of 50% of the response obtained with the fixed agonist concentration alone. The EC_{50}* is the agonist concentration which, by itself, produces a response level of 50% of the response obtained with the fixed agonist concentration alone. Note that the EC_{50}* will not be the same as the true EC_{50} unless the fixed agonist concentration itself produces the E_{max} response, and the IC_{50}* is not necessarily identical to the true IC_{50} estimated without constraints. The necessary condition of the analysis is that the antagonist IC_{50}* in the presence of the fixed agonist concentration and the agonist EC_{50}* correspond to the same response level. (The analysis was done here with logistic curve fitting, but Hill analysis, using a logit-log transformation and defined maxima and minima, would have done just as well). The Gaddum equation can now be expressed as

$$K_b = IC_{50}^*/([A]/EC_{50}^* - 1),$$

where [A] is the fixed agonist concentration. Note that at

high agonist concentrations, ($EC_{50}^* \approx EC_{50} << [A]$) this equation is almost identical to the Cheng-Prusoff equation.

Schild analysis

Schild plots were constructed by plotting log (dose-ratio − 1) agonist log([B]), where 'dose-ratio' is the ratio of equiactive agonist concentrations in the presence and absence of antagonist concentration [B]. The plots were analysed by linear regression and, when it was established that the plots were linear with slopes not significantly different from unity, the intercept from the regression analysis with slope constrained to unity was taken as the pK_b estimate.

Direct fitting of data to the Schild model

It is worthwhile reviewing this powerful and underused equation which was first derived by Waud (1975, 1976). If an agonist causes a 50% effect at a concentration of $[EC_{50}^0]$ in the absence of antagonist, and at a concentration of $[EC_{50}]$ in the presence of a fixed concentration [B] of antagonist, then the dose ratio (dr) is $[EC_{50}]/[EC_{50}^0]$, and

$$[EC_{50}] = dr.[EC_{50}^0]. \quad (1)$$

The Schild model relates the dose-ratio to [B] according to the equation $\log(dr-1) = s.\log[B] - \log(K_b)$, where K_b is the antagonist dissociation constant and s is the Schild slope, so

$$dr = ([B]^s/K_b) + 1. \quad (2)$$

If the agonist curves can be described with a logistic function, then effect = (E_{max}-basal) / $(1 + ([EC_{50}]/[A])^b)$ + basal, where b is the slope factor of the agonist curve and [A] is the agonist concentration. Substituting (1) and (2) we obtain the Waud equation

$$\text{Effect} = (E_{max}\text{-basal})/\left(1 + \left\{\frac{[EC_{50}^0] \cdot \left(\frac{[B]^s}{K_B} + 1\right)}{[A]}\right\}^b\right) + \text{basal} \quad (3)$$

A slightly different equation has been used by Waud *et al.* (1978)

$$\text{Effect} = (E_{max}\text{-basal})/\left(1 + \left\{\frac{[EC_{50}^0] \cdot \left(\left(\frac{[B]}{K_B}\right)^s + 1\right)}{[A]}\right\}^b\right) + \text{basal} \quad (4)$$

Here the slope s represents a logistic slope factor of the antagonist occupancy function rather than the molecularity of the antagonist-receptor interaction. Both equations yield identical parameter estimates when fitted to experimental data, except for the estimate of K_b. Small variations in s can lead to quite large variations in K_b when equation (3) is used, but minor variations when (4) is used, and K_b is estimated with greater precision (and less correlation with s) by (4). The Schild slope can only be assigned molecular significance if it is an integer, otherwise it indicates some deviation from the underlying assumptions of the model, and the K_b estimate can only be considered as an estimate of antagonist potency. If the Schild slope is not significantly different from 1 then the best estimate of K_b is obtained by constraining the Schild slope to 1 (as with all the data reported here), but if this is not valid then equation (4) will yield a better estimate of antagonist potency.

Results

Theoretical

Errors caused by the use of the Cheng-Prusoff equation Figure 1 shows the Cheng-Prusoff estimate of dissociation constant divided by the true dissociation constant (K_{CP}/K_i) as a function of log(fixed agonist concentraton/EC_{50}) for various

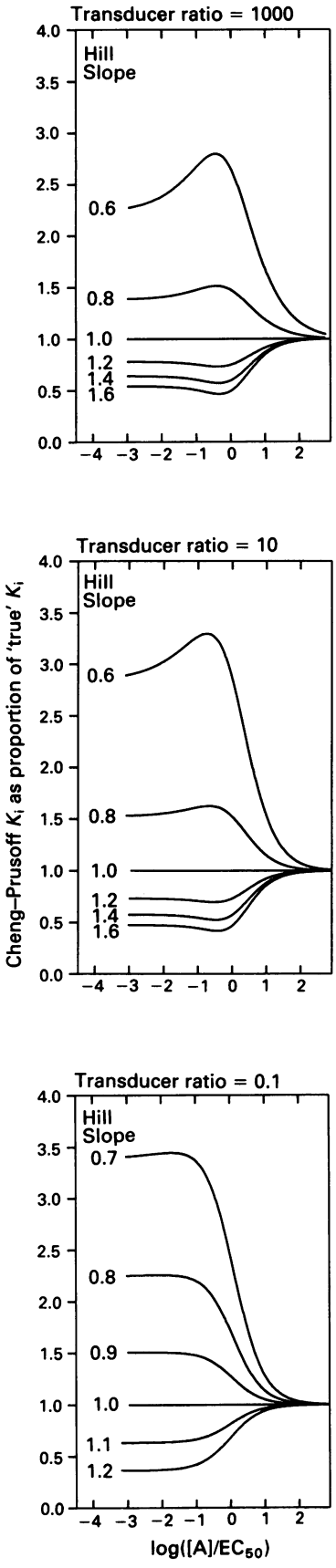


Figure 1 Theoretical error in the use of the Cheng-Prusoff equation to estimate antagonist affinity from IC_{50} values obtained from functional inhibition experiments. 'Cheng-Prusoff' K_i estimates were derived from an 'operational model' of agonist effect as described in the Appendix. Errors (K_{CP}/K_i) are shown as a function of log(fixed agonist concentration/EC_{50}), the Hill slope factor of the logistic function relating [agonist] to effect (see Appendix), and the transducer ratio, τ, which corresponds roughly to receptor reserve.

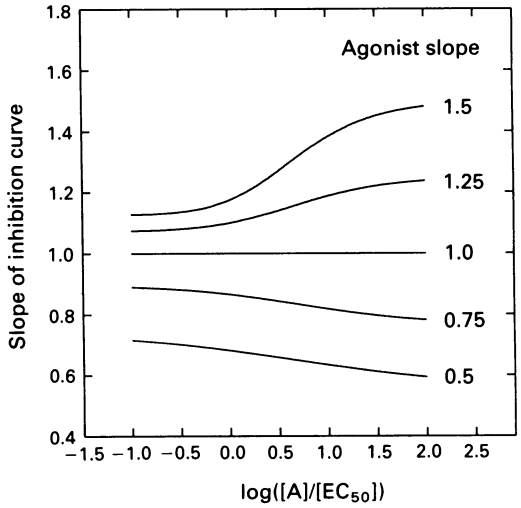


Figure 2 Logistic slope factors of theoretical antagonist functional inhibition curves derived from simulations with the Waud equation using various agonist slope factors. The antagonist Schild slopes were fixed at 1.

values of Hill (logistic) slope of the agonist curve and τ (transducer ratio, related to 'receptor reserve'). When the Hill slope is 1, or when the fixed agonist concentration is 100 fold or more greater than the EC_{50}, the Cheng-Prusoff equation provides a good estimate of the 'true' antagonist K_d. Errors arise when lower agonist concentrations are used and the Hill slope deviates from 1. The magnitude of the error increases as the Hill slope deviates from 1, and for a particular Hill slope, increases with decreasing receptor reserve. The limiting cases, with very large or very small [A], are as described in the Appendix. In addition, a range of fixed agonist concentrations give larger deviations than when [A] is very small, the largest deviation occurring with ($EC_{50}/10 < [A] < EC_{50}$).

Slopes of antagonist inhibition curves If the agonist curve is truly a logistic function with slope $\neq 1$, then the antagonist inhibition curve cannot be a logistic function, though inaccuracies in fitting such inhibition curves to a logistic function are likely to be trivial. Simulated inhibition curves with Schild slopes of 1 were generated using the Waud equation at various levels of $[A]/[EC_{50}]$ and agonist slope. The results in Figure 2 demonstrate that inhibition curves only have slope factors of 1 if the agonist curve is itself rectangular hyper-

Table 1 Parameters for acetylcholine (ACh) and pirenzepine (PRZ) from analysis of Experiment 1

	m1	*m2*	*m3*	*m4*
Fixed [PRZ] concentration (M)	3×10^{-8}	3×10^{-7}	3×10^{-7}	10^{-7}
	10^{-7}	3×10^{-6}	10^{-6}	10^{-6}
	3×10^{-7}	3×10^{-5}	3×10^{-6}	10^{-5}
Analysis				
1 ACh alone pEC_{50} (− log M)	6.10	7.39	5.59	6.91
4 ACh alone pEC_{50} (− log M)	6.10	7.41	5.57	6.91
1 ACh logistic slope	0.53	0.73	0.97	0.75
4 ACh logistic slope	0.55	0.72	0.97	0.75
1 Schild slope	1.14	1.03	0.84	1.03
2 Schild slope	1.12	0.96	0.83	0.90
3 Schild slope	1.13	1.01	0.84	0.97
4 Schild slope	1.15	1.00	0.84	1.03
1 pK_b	8.37	6.60	6.77	7.65
2 pK_b	8.37	6.64	6.85	7.73
3 pK_b	8.38	6.62	6.81	7.69
4 pK_b	8.39	6.66	6.74	7.66

Analysis: (1) activation curves fitted to logistic function, Schild plots from dose-ratios; (2) inhibition curves and corresponding activation curve fitted to logistic with fixed limit parameters, Schild plots from dose-ratios; (3) dose-ratios from (1) and (2) combined in Schild plots; (4) direct fit of dataset to Waud equation; Schild slope estimated from unconstrained analysis, pK_b (and control ACh slope and pEC_{50}) estimated from analysis with Schild slope set to 1.

Table 2 Parameters for acetylcholine (ACh), pirenzepine (PRZ) and AQ-RA 741 from analysis of Experiment 2

	m1	*m2*	*m3*	*m4*
Fixed [ACh] (M)	3×10^{-5}	3×10^{-6}	10^{-4}	3×10^{-6}
Analysis				
1 ACh slope	0.50	0.64	0.84	0.68
3 ACh slope	0.47	0.65	0.85	0.70
1 ACh pEC_{50} (− log(M)	5.69	7.31	5.43	6.97
3 ACh pEC_{50} (− log(M)	5.66	7.31	5.43	6.97
1 PRZ logistic slope	0.71	0.72	0.84	0.74
3 PRZ Schild slope	1.08	0.96	0.95	1.00
1 PRZ pIC_{50}	6.78	4.70	5.30	6.11
2 PRZ pK_b	8.46	6.66	6.81	7.76
3 PRZ pK_b	8.37	6.65	6.79	7.74
4 PRZ pK_{CP}	7.97	6.50	6.75	7.58
1 AQ-RA logistic slope	0.56	0.78	0.86	0.77
3 AQ-RA Schild slope	0.93	1.10	1.03	1.07
1 AQ-RA pIC_{50}	6.56	7.12	5.86	7.01
2 AQ-RA pK_b	8.28	9.09	7.38	8.65
3 AQ-RA pK_b	8.21	9.09	7.34	8.63
4 AQ-RA pK_{CP}	7.76	8.92	7.31	8.47

Analysis: (1) full activation and inhibition curves fitted to logistic function; (2) inhibition curve and corresponding activation curve up to the fixed concentration fitted to logistic with shared limit parameters, IC_{50}* and EC_{50}* values applied to the Gaddum equation; (3) direct fit of inhibition curve and corresponding activation curve to the Waud equation; Schild slope estimated from unconstrained analysis, pK_b (and control ACh slope and pEC_{50}) estimated from analysis with Schild slope set to 1; (4) parameters from analysis (1) applied to Cheng-Prusoff equation.

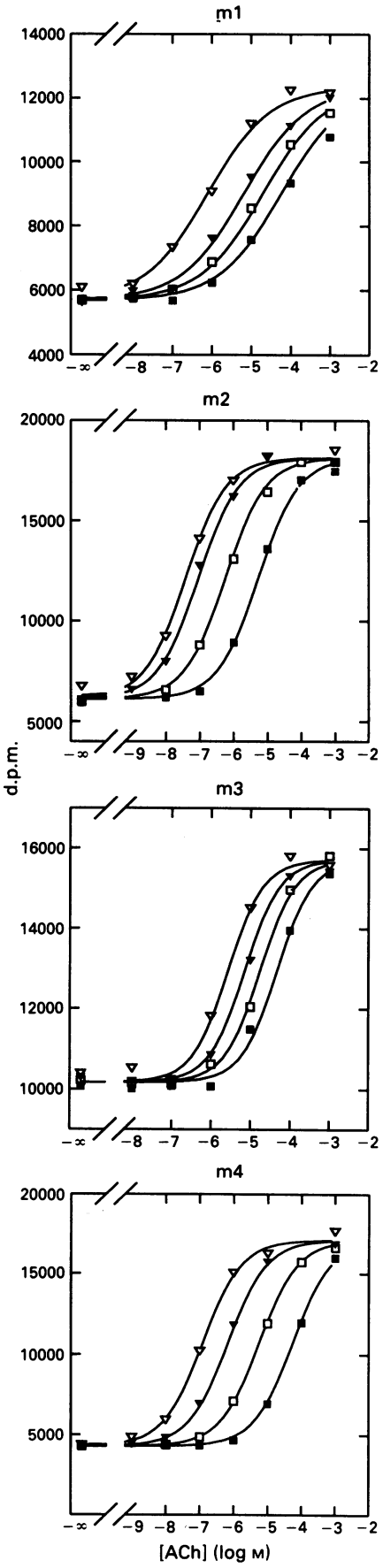


Figure 3 Experiment 1: effect of acetylcholine (ACh) in the presence of various concentrations of pirenzepine on [^{35}S]-GTPγS binding to membranes from CHO cells transfected with m1–m4 muscarinic receptors. The lines show the fit to the Waud equation with Schild slope fixed at 1. Each point is the mean of duplicate observations. The following pirenzepine concentrations were used (M):

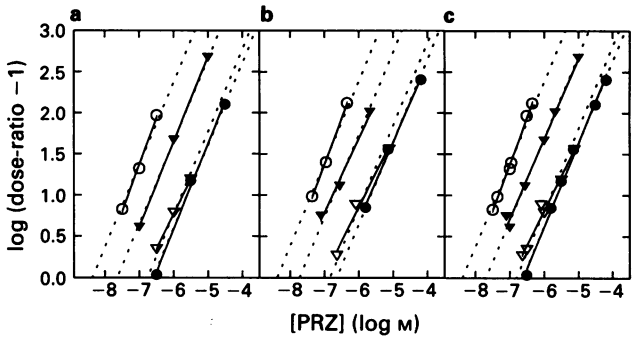


Figure 4 Schild plots of the effect of pirenzepine (PRZ) on acetylcholine potency in stimulating GTPγS binding via m1 (○), m2 (●), m3 (▽) and m4 (▼) receptors (Experiment 1). (a) Dose-ratios derived from EC_{50} values obtained from logistic analysis of the curves shown in Figure 3; (b) Dose-ratios derived from Gaddum analysis of EC_{50}* and IC_{50}* values obtained from the curves shown in Figure 5, as described in Methods; (c) Combination of (a) and (b). The lines show linear regression, with Schild slopes unconstrained (solid) or fixed at 1 (dotted).

bolic, otherwise the antagonist slope will be similar to the agonist slope but will also depend on the fixed agonist concentration. The logistic slopes of functional inhibition curves by themselves therefore provide no information as to e.g. the presence of multiple receptors, unless the agonist curve has a slope of 1: the Schild slope factor obtained by direct fitting to the Waud equation may be more informative.

Experimental

The presence of GDP was required to reveal an agonist induced increase in [^{35}S]-GPTγS binding mediated by m2 and m4 receptors, and improved the signal mediated by m1 and m3 receptors (Hilf *et al.*, 1989; Farries, unpublished observations). The optimal GDP concentration differed at different subtypes (Lazareno *et al.*, 1993; Farries, unpublished), here, 10^{-7} M (Experiment 1) and 3×10^{-7} (Experiment 2) was used with m1 and m3 receptors, and 10^{-6} M was used with m2 and m4 receptors.

It was found necessary to pre-equilibrate the membranes with both agonist and antagonist before initiating the [^{35}S]-GPTγS binding, since preincubation with pirenzepine alone led to concentration-dependent decreases in the agonist E_{max} at m1–m4 receptor subtypes (data not shown). Preincubation with ACh at m1 or m4 receptors did not reduce either the potency of ACh or its E_{max} (data not shown).

Experiment 1 ACh activation curves were constructed at m1–m4 receptors alone and in the presence of three concentrations of pirenzepine. The greater potency of ACh at m2 and m4 receptors allowed a wider range of pirenzepine concentrations than at m1 and m3 receptors.

Pirenzepine did not significantly affect basal activity, E_{max} or slope. Direct fitting of the data to the Waud equation revealed that the Schild slopes were not significantly different from 1, and Figure 3 shows the data with fits constrained to Schild slopes of 1. The parameter estimates are shown in Table 1.

Figure 4a shows conventional Schild plots derived from EC_{50} values obtained by logistic analyses in which the basal, E_{max} and slope parameter estimates were shared between the

Subtype/ Symbol	(▽)	(▼)	(□)	(■)
m1	0	3×10^{-8}	10^{-7}	3×10^{-7}
m2	0	3×10^{-7}	3×10^{-6}	3×10^{-5}
m3	0	3×10^{-7}	10^{-6}	3×10^{-6}
m4	0	10^{-7}	10^{-6}	10^{-5}

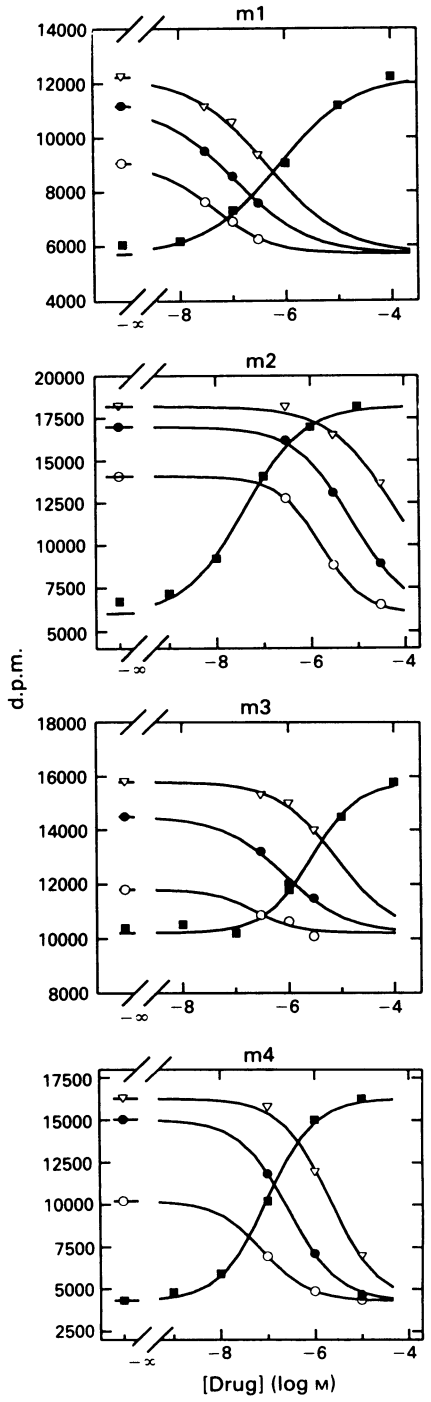


Figure 5 Inhibition curves of pirenzepine in the presence of different fixed acetylcholine (ACh) concentrations (○, ● and ▽), constructed from part of the data shown in Figure 3, together with control acetylcholine curves (■).

curves. The Schild slopes were not significantly different from 1 and the pK_b values were very similar to those obtained by direct fitting to the Waud equation (Table 1).

Figure 5 shows inhibition curves constructed from parts of the complete data set. Each inhibition curve was analysed together with the portion of the control activation curve up to the fixed agonist concentration [A], with common defined basal and maximum parameters, to yield EC_{50}* and IC_{50}* values. The dose ratios ([A]/EC_{50}*) and corresponding IC_{50}* values are shown as Schild plots in Figure 4b, and Schild plots combining the data from the activation and inhibition curves are shown in Figure 4c. The results (Figures 4b and 4c, Table 1) show that in all cases the Schild plots were linear with slopes close to 1. This reflects the fact that the pK_b estimates from the inhibition curves were close to the 'true'

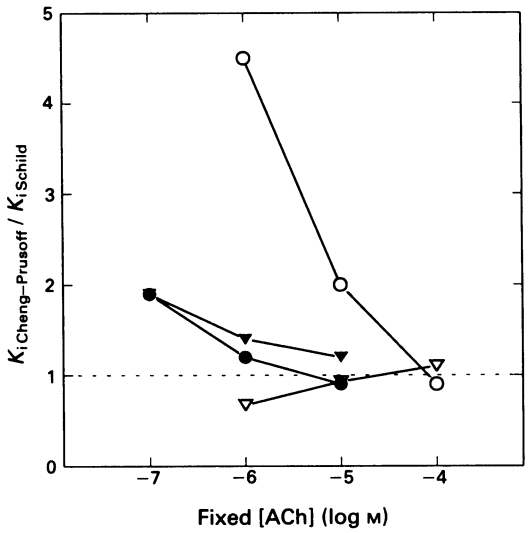


Figure 6 Error caused by using the Cheng-Prusoff equation to derive pirenzepine affinity estimates from the inhibition curves shown in Figure 5, at m1 (○), m2 (●), m3 (▽) and m4 (▼) receptors. The 'true' affinity was taken to be that derived from the direct fit to the Waud equation with Schild slope fixed at 1.

pK_b and were not correlated with the fixed agonist concentrations (data not shown). These results also show that the dose-ratios derived from the inhibition curves complement those obtained from activation curves to provide, in the case of m2 and m4 receptors, more detailed Schild plots over a wider range of antagonist concentrations (both those chosen by the experimenter and those estimated as IC_{50} values from inhibition curves).

Figure 6 shows the deviation of the Cheng-Prusoff estimates of pirenzepine dissociation constant from the 'true'

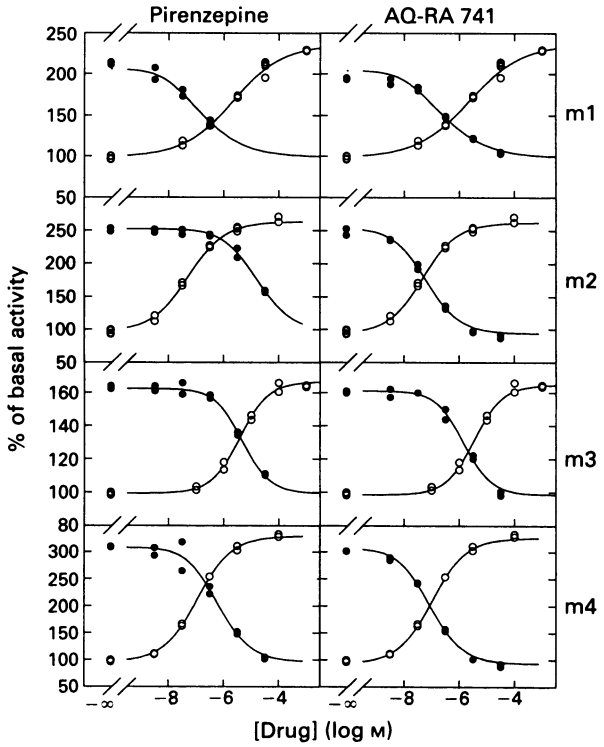


Figure 7 Experiment 2: acetylcholne activation curve for stimulation of GTPγS binding via m1–m4 muscarinic receptors (○) and inhibition curves for pirenzepine and AQ-RA 741 in the presence of a fixed acetylcholine concentration (●). The data are individual replicates expressed as % of mean basal activity. The lines show the direct fit of each activation + inhibition curve data set to the Waud equation with Schild slope fixed at 1.

values obtained from direct fitting to the Waud equation. The deviations are quite consistent with those derived theoretically (see Appendix and Figure 1) being minimal at the highest fixed agonist concentrations and at the m3 receptor, where the ACh curves had slopes close to 1.

Experiment 2 At each muscarinic receptor subtype, a full ACh activation curve was constructed, together with titrations of pirenzepine and AQ-RA 741 alone and in the presence of a fixed submaximal ACh concentration. In some cases the antagonists alone seemed slightly to inhibit basal activity (data not shown) with the largest apparent effect, 5–10% inhibition, seen with AQ-RA 741 at m4 receptors. These effects were too small and inconsistent to quantify and were not considered in subsequent analyses.

Figure 7 shows each inhibition curve and its corresponding activation curve fitted directly to the Waud equation with Schild slopes constrainted to 1. The unconstrained Schild slopes were not significantly different from 1 (Table 2), in marked contrast to the flat logistic slopes of the inhibition curves which are completely in agreement with the theoretical predictions shown in Figure 2.

Each inhibition curve was analysed together with the portion of the corresponding activation curve up to the fixed agonist concentration with basal and 'maximum' parameters constrained to be shared, to yield EC_{50}* and IC_{50}* values. pK_b estimates derived with the equation $pK_b = \log(IC_{50}^*/([A]/[EC_{50}^*]-1))$ were very close to the estimates obtained from the direct fit (Table 2).

The values obtained for pirenzepine in this experiment using single inhibition curves were almost identical to those obtained in Experiment 1 from full Schild analysis, and the affinity estimates for both antagonists are consistent with previously reported values (Lazareno *et al.*, 1990; Dorje *et al.*, 1991).

Discussion

Schild analysis is among the most robust and theoretically valid techniques in pharmacology (Colquhoun, 1987). Its robustness derives, in part, from the fact that the analysis is in two parts. The first part considers a family of two or more agonist titrations and allows the assumptions of parallel concentration-effect curves and constant E_{max} (both necessary if the antagonist is acting as a simple competitive inhibitor) to be assessed empirically. The second part considers dose-ratios derived from the first part, and tests the assumption that log(dose-ratio − 1) is a linear function of log antagonist concentration with slope of unity, again necessary if the antagonist is acting as a simple competitive inhibitor. As well as testing some of the underlying assumptions of the analysis, the two-stage nature of conventional Schild analysis allows the estimation of antagonist affinities from responses with which, for practical reasons, only a single dose-ratio estimate can be made in each experiment.

Conventional Schild analysis does have drawbacks, however, the most obvious of which is inefficiency: the analyses in Experiment 1 above each contained 56 data points and yet the conventional pA_2 estimate was derived from only three points. The range of antagonist concentrations which can be studied may also be limited by the potency of the agonist and hence the largest dose-ratio which can be measured without encountering nonspecific effects of high agonist concentrations.

In biochemical, and some whole-tissue, experiments it is often practically feasible to assess antagonist affinity by titrating the antagonist against a fixed concentration of agonist. Such a design allows efficient study of a large range of antagonist concentrations and is therefore widely used. The correct design and analysis of such experiments has, however, received little attention.

Binding experiments often use an inhibition curve design, and in principle the Cheng-Prusoff correction is a valid method for obtaining affinity estimates (though there are pitfalls in such experiments, and enhanced methods to account for them, see e.g. Munson & Rodbard, 1988). McKinney *et al.* (1991) have shown that the Cheng-Prusoff correction is valid in functional experiments where the agonist curve has a slope of 1, a conclusion with which we agree. In contrast, Eglen & Whiting (1989) claim to have demonstrated, both empirically and theoretically, that application of the Cheng-Prusoff equation is invalid at high agonist concentrations. This conclusion is surprising, since at high agonist concentrations the Cheng-Prusoff equation is almost identical to the null method equation of Lazareno & Roberts (1987, see Methods above). In fact, Eglen & Whiting showed only that application of the equation to their data yielded discrepant results, they did not demonstrate that the error lay in the application of the equation rather than in the data: in fact, application of a null method of analysis to their inhibition data would have given the same discrepant results. Leff & Dougall (1993) have recently derived a theoretically valid form of the Cheng-Prusoff equation which takes account of the slope of the agonist curve.

From our theoretical consideration, the following conclusions can be made regarding the applicability of the Cheng-Prusoff equation to those functional preparations which can be described by the Operational Model of Black & Leff (1983): (1) the equation is valid for rectangular hyperbolic E/[A] functions; (2) the equation is not valid for other E/[A] functions except when high fixed agonist concentrations are used (at least 10 fold greater than EC_{50}); (3) the equation gives the greatest error when concentrations between $EC_{50}/10$ and EC_{50} are used as fixed agonist concentrations; (4) the Hill slope of the E/[A] function is the major determinant of the size of the error – the equation gives increasing error as the slope deviates from 1, but for slopes between 0.7 and 1.2 the error will not exceed about 4 fold; (5) for a particular Hill slope, the error will increase with decreasing receptor reserve. The empirical results shown in Figure 6 are consistent with these conclusions, as are results obtained from a larger population of data (Lazareno & Birdsall, 1993).

Although use of the Cheng-Prusoff correction is invalid, there is a valid method for analysing a single inhibition curve, together with an activation curve, to provide a dose-ratio from which a valid pK_b can be estimated (Lazareno & Roberts, 1987, see Methods above). We found that these pK_b estimates were in excellent agreement with pA_2 values measured with full Schild analysis. The logistic slope of the inhibition curve, however, cannot be interpreted directly since it is strongly determined by the slope of the agonist curve. It is, nevertheless, possible to estimate the Schild slope from an inhibition curve. In principle, each point on an inhibition curve could be converted to a dose-ratio and the data could be treated as a Schild plot. We have found, however, that this treatment magnifies small errors at the top and bottom of the curve and does not provide reliable data. The solution is to use non-linear regression analysis to fit the combined inhibition and activation curves directly to the Schild model using the Waud equation. This provides reliable estimates of both the K_b and the Schild slope, and thus this design and analysis allows a test of one of the underlying assumptions of Schild analysis, namely that competitive antagonists have Schild slopes of 1. It is important to note that the slope of the inhibition curve can *only* be interpreted by using this analysis in conjunction with the activation curve.

Since a single inhibition curve can be used to provide an accurate estimate of K_b, it follows that a family of inhibition curves, with different fixed agonist concentrations, can be used to construct a Schild plot. Pöch *et al.* (1992) constructed families of activation curves in the normal Schild design and then extracted inhibition curves from their data, as we did in Experiment 1 above. They also showed that their 'alternative Schild plots', using dose-ratios derived from the inhibition curves, gave essentially the same results as the 'conventional'

Schild plots. Their analysis differs from our form of Gaddum analysis only in that they used the parameters of the logistic fit to the control activation curve to estimate the concentration of agonist alone giving the effect of 50% the fixed agonist concentration. Our results are completely consistent with those of Pöch *et al.* (1992), and demonstrate that dose-ratios obtained from inhibition curves have equal status with those obtained from activation curves. It therefore makes sense, if the design of the experiment allows, to consider the data from a family of activation curves also as a family of inhibition curves and to combine the dose ratios from both types of curves into a single Schild plot. As shown above, this can double the number of points on the Schild plot and provide more precision and a wider range of antagonist concentrations. Much attention has been given to the significance and analysis of Schild plots which are not linear or have slopes different from 1 (Kenakin, 1982). The use of inhibition curves to obtain more detailed and extensive Schild plots may therefore be of practical value.

A way to enhance further the power and efficiency of the full Schild analysis is to fit the entire data set directly to the Schild model with the Waud (1975, 1976) equation using non-linear regression analysis. This procedure uses all the data, rather than just a few dose-ratio values, to estimate the pA_2 and Schild slope, and it therefore provides better estimates of these parameters. This in turn means that fewer data points are required for reliable results.

In conclusion, antagonist affinities can be estimated from functional inhibition curves either by a null method of dose-ratio analysis or by a direct fit to the Schild model with the Waud equation. Use of the Cheng-Prusoff equation is usually invalid and inaccurate, and always unnecessary.

We gratefully acknowledge financial support from Sankyo Co. Ltd., Tokyo.

Appendix

Theoretical assessment of the validity of application of the Cheng-Prusoff equation to functional experiments, using the Operational Model of agonism (Black & Leff, 1983)

The operational model starts with the same basic assumptions as the traditional null methods, that agonists and competitive antagonists interact at the receptor according to the Law of Mass Action and that the response is a function of agonist receptor occupancy at equilibrium. In addition, the operational model assumes explicitly that the function relating occupancy to response (the 'transducer function') is a logistic function. While such a model cannot be universally true, it is sufficiently general to be of interest, and has been found to generate consistent results when applied to pharmacological data (Leff, 1988).

Receptor occupancy by agonist alone is given by

$$[AR] = \frac{R_0 \cdot [A]}{[A] + K_A} \quad (1)$$

where [AR] = concentration of agonist-occupied receptors, R_0 = total receptor concentration, [A] = agonist concentration, K_A = agonist dissociation constant.

Receptor occupancy by agonist in the presence of competitive antagonist is given by

$$[AR] = \frac{R_0 \cdot [A]}{[A] + K_A \cdot \left(1 + \frac{[I]}{K_I}\right)} \quad (2)$$

where [I] = antagonist concentration, K_i = antagonist dissociation constant.

Response is assumed to be a logistic function of agonist occupancy

$$E = \frac{E_m \cdot [AR]^n}{K_E{}^n + [AR]^n} \quad (3)$$

where E = the response, E_{max} = the maximum possible response of the tissue, K_E = the concentration of agonist-occupied receptors required to produce a response of $E_m/2$, n = the slope factor of the logistic transducer function.

A useful quantity, τ (the 'transducer ratio'), is defined as R_0/K_E, and is a function of the efficacy of the agonist.

Substituting (1) into (3) we get

$$E = \frac{E_m \cdot \tau^n \cdot [A]^n}{([A] + K_A)^n + \tau^n \cdot [A]^n} \quad (4)$$

and

$$[A_{50}] = \frac{K_A}{(2 + \tau^n)^{\frac{1}{n}} - 1} \quad (5)$$

where $[A_{50}]$ = the agonist concentration giving half its own maximum response.

When the concentration-effect curve is rectangular hyperbolic, the transducer function has a slope of 1

$$E = \frac{E_m \cdot \tau \cdot [A]}{K_A + (1 + \tau) \cdot [A]} \quad (6)$$

and

$$[A_{50}] = \frac{K_A}{1 + \tau} \quad (7)$$

In the presence of competitive antagonist we substitute (2) into (3) and obtain

$$E = \frac{E_m \cdot \tau^n \cdot [A]^n}{([A] + K_A \cdot (1 + \frac{[I]}{K_I}))^n + \tau^n \cdot [A]^n} \quad (8)$$

and, if the transducer function has a slope of 1,

$$E = \frac{E_m \cdot \tau \cdot [A]}{[A] \cdot (1 + \tau) + K_A \cdot (1 + \frac{[I]}{K_I})} \quad (9)$$

Consider first the simple case, where the slope, n, of the transducer function is 1 (which will result in the concentration-effect curve also having a slope of 1).

If $[I_{50}]$ is the antagonist concentration which inhibits the effect of a particular agonist concentration by 50%, then, from (6) and (9),

$$\frac{2 \cdot E_m \cdot \tau \cdot [A]}{[A] \cdot (1 + \tau) + K_A \cdot (1 + \frac{[I_{50}]}{K_I})} = \frac{E_m \cdot \tau \cdot [A]}{[A] \cdot (1 + \tau) + K_A}$$

and

$$K_I = \frac{[I_{50}]}{[A] \cdot \frac{1 + \tau}{K_A} + 1} \quad (10)$$

Substituting the equation for $[A_{50}]$ (7)

$$K_I = \frac{[I_{50}]}{\frac{[A]}{[A_{50}]} + 1} \quad (11)$$

which is the Cheng-Prusoff equation. So, if the concentration-effect curve has a slope of 1 the Cheng-Prusoff equation gives the correct answer for any concentration of agonist (this conclusion has recently been derived in a different way by KcKinney *et al.* (1991)).

Consider now the more general case. From (4) and (8)

$$\frac{2 \cdot E_m \cdot \tau^n \cdot [A]^n}{([A] + K_A.(1 + \frac{[I_{50}]}{K_I}))^n + \tau^n \cdot [A]^n} = \frac{E_m \cdot \tau^n \cdot [A]^n}{(K_A + [A])^n + \tau^n.[A]^n}$$

$$([A] + K_A + K_A.\frac{[I_{50}]}{K_I})^n = 2.(K_A + [A])^n + \tau^n.[A]^n \quad (12)$$

With very large [A], ($[A] >> K_A$)

$$[A] + K_A.\frac{[I_{50}]}{K_I} = [A].\ (2 + \tau^n)^{\frac{1}{n}} \quad (13)$$

Substituting for K_A in (5) gives

$$K_I = \frac{[I_{50}]}{\frac{[A]}{[A_{50}]}} \quad (14)$$

which is the same as the Cheng-Prusoff equation at large [A], so with high agonist concentrations the Cheng-Prusoff equation gives the correct result.

With very small [A], ([A] → 0)

$$(K_A.\ (1 + \frac{[I_{50}]}{K_I}))^n = 2.K_A^n \quad (15)$$

$$K_I = \frac{[I_{50}]}{2^{\frac{1}{n}} - 1} \quad (16)$$

If K_{CP} is the estimate of K_i obtained with the Cheng-Prusoff equation, then as [A] → 0, $K_{CP} = [I_{50}]$, and

$$\frac{K_{CP}}{K_I} = 2^{\frac{1}{n}} - 1 \quad (17)$$

i.e. at very low agonist concentrations K_{CP} will overestimate K_i if $n < 1$, and will underestimate K_i if $n > 1$.

In general, from (12), the antagonist $[I_{50}]$ is related to the fixed agonist [A] by

$$[I_{50}] = \frac{K_I}{K_A} \cdot ((2.(K_A + [A])^n + \tau^n.[A]^n)^{\frac{1}{n}} - [A] - K_A) \quad (18)$$

The Cheng-Prusoff estimate of K_i (K_{CP}) can then be calculated using the formula for $[A_{50}]$ (5) and $[I_{50}]$ (18).

Hill slopes of agonist E/[A] curves

The 'Hill slopes' quoted in Figure 1 are necessarily approximate because, for n = 1, the E/[A] curve is not a logistic function (Black *et al.*, 1985). The deviation from a logistic function is small, however, so it may reasonably be assumed that the gradient of the midpoint on the E/[A] curve is equal to the midpoint gradient of the logistic function best describing the E/[A] curve. The midpoint gradient of a normalised E/log[A] curve generated by the operational model is (Black *et al.*, 1985)

$$G_{\frac{1}{2}(operational)} = \frac{0.576.n.(2 + \tau^n).((2 + \tau^n)^{1/n} - 1}{(2 + \tau^n)^{1/n}.(1 + \tau^n)} \quad (19)$$

and the midpoint gradient of a logistic function is

$$G_{\frac{1}{2}(logistic)} = 0.576 \cdot \text{Hill slope} \quad (20)$$

so the Hill slope is estimated as

$$\text{Hill slope} = \frac{n.(2 + \tau^n).((2 + \tau^n)^{1/n} - 1)}{(2 + \tau^n)^{1/n}.(1 + \tau^n)} \quad (21)$$

Within the range of values used in Figure 1 the deviation between these Hill slope estimates and slope factors obtained from logistic analysis of simulated E/[A] curves was about 0.03 or less. The value of n corresponding to a given Hill slope was estimated from (21) with an iterative procedure.

When τ is large, Hill slope $\approx$ n, but when τ is small the Hill slope tends towards 1 (Black *et al.*, 1985) so, for a given Hill slope, n $\approx$ Hill slope when τ is big, and n increasingly deviates from 1 as τ becomes smaller. The size of the deviation of the Cheng-Prusoff estimate as [A] → 0 depends only on n and is independent of τ (17), and it increases as n deviates from 1. For a given Hill slope, however, the deviation of the Cheng-Prusoff estimate is inversely related to τ, i.e. the deviation is large when receptor reserve is small and Hill slope $\neq$ 1.

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(Received February 16, 1993
Revised March 22, 1993
Accepted March 26, 1993)

Br. J. Pharmacol. (1993), **109**, 1120–1127 © Macmillan Press Ltd, 1993

Pharmacological characterization of acetylcholine-stimulated [^{35}S]-GTPγS binding mediated by human muscarinic m1–m4 receptors: antagonist studies

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1 We have used dose-ratio analysis to estimate functionally the affinity constants (pK_b) and Schild slope factors of a range of selective or atypical antagonists at human muscarinic m1–m4 receptors.

2 The functional response was the stimulation by acetylcholine of [^{35}S]-GTPγS binding to membranes from Chinese hamster ovary (CHO) cells stably expressing individual receptor subtypes.

3 A novel experimental design and analysis was used which allowed the estimation of affinity and Schild slope factor from a single antagonist inhibition curve, and the results were compared with other methods of analysis, both theoretically valid and invalid.

4 In general, the affinity estimates were very similar to previously reported values obtained in binding studies with animal tissues and cloned human receptors and the Schild slope factors were close to unity.

5 These results demonstrate the validity of the assay and provide no evidence for species differences in antagonist affinity for muscarinic receptor subtypes.

6 The results confirm both the utility of himbacine in distinguishing between m1 and m4 receptors and a previously reported modest m4-selectivity for tropicamide and secoverine.

7 The cholinesterase inhibitor, tacrine (THA), had a potency profile similar to that of gallamine but with less selectivity. Its affinity could not be determined since it had Schild slope factors of about 2 at all subtypes.

8 *o*-Methoxy-sila-hexocyclium had only a modest selectivity for the m1 subtype.

Keywords: Muscarinic receptor subtype; cloned human receptors; guanine nucleotide exchange; GTPγS binding; competitive antagonist; allosteric effect; null method; affinity estimation; tacrine

Introduction

Muscarinic cholinoceptors belong to the superfamily of G-protein coupled receptors. Five subtypes have been identified by molecular biological techniques and are referred to as m1, m2, m3, m4 and m5 receptors (Hulme *et al.*, 1990). Before this family was discovered by cloning techniques, three subtypes were identified pharmacologically and referred to as M_1, M_2 and M_3 receptors. These are now known to correspond to the m1, m2 and m3 receptors. The pharmacological M_4 receptor, corresponding to the m4 gene product, has recently been characterized in animal tissues in binding (Lazareno *et al.*, 1990; Waelbroeck *et al.*, 1990) and functional (Caulfield & Brown, 1991; Bernheim *et al.*, 1992) studies. The cloned m5 receptor has been characterized by binding studies (Buckley *et al.*, 1989; Dörje *et al.*, 1991b) but there have been no studies of the native m5 receptor.

The muscarinic receptor subtypes preferentially couple to different effector systems. The clearest distinction is that m1, m3 and m5 receptors preferentially couple to phospholipase-C (PLC)-mediated phosphoinositide hydrolysis (PI), while m2 and m4 receptors preferentially couple to inhibition of adenylyl cyclase. A single type of receptor can, however, mediate many independent responses: for example, m2 receptors can inhibit cyclase, open K^+ channels and stimulate phosphoinositide hydrolysis (Pfaffinger *et al.*, 1985; Ashkenazi *et al.*, 1987; Nathanson, 1987), suggesting that a single type of receptor may activate more than one type of G-protein.

Although a receptor may mediate more than one independent response, this does not prove that the receptors act via different G-proteins. Katz *et al.* (1992) have recently demonstrated that an m2 receptor can stimulate PLC-β2, presumably by causing dissociation of G-protein α and βγ subunits and thus increasing the free concentration of the βγ subunit which is the stimulus for this isoenzyme of PLC. These authors suggest that this mechanism may explain the stimulation of PI breakdown which is sometimes seen in cells expressing high levels of m2 or m4 receptors (e.g. Ashkenazi *et al.*, 1987; Tietje *et al.*, 1990), since both the cyclase and PI responses are sensitive to pertussis toxin (PTX). PTX causes the ADP ribosylation of certain G-proteins (Gi_1, Gi_2, Gi_3 and Go), and this prevents the G-protein from being activated by the receptor. The fact that two responses are sensitive to PTX does not prove that they are mediated by the same G-protein, but if only one response is PTX-sensitive it is likely that different G-proteins are involved. This is the case in m4-transfected CHO cells where PTX blocks the inhibitory effect on cyclic AMP levels but not the stimulatory effect seen with high agonist concentrations (Jones *et al.*, 1991). Conversely, if a response is only partially sensitive to PTX this implies that different G-proteins are involved, and this is the case with phosphoinositide hydrolysis stimulated by transfected m1 receptors in Chinese hamster ovary (CHO) cells (Ashkenazi *et al.*, 1989).

Even though the different muscarinic receptor subtypes generate different second messenger responses, they have in common the effect of activating G-proteins by stimulating guanine nucleotide exchange. The details of the mechanism of G-protein activation are still being revealed (Cerione, 1991; Bourne & Stryer, 1992), but it seems that the major consequence of the interaction of agonist-occupied receptor and G-protein is that a molecule of GDP dissociates from the α subunit of G-protein and is replaced by a molecule of

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GTP. This activates the α subunit, which may dissociate from the receptor and from the βγ subunit. The α-GTP subunit interacts with and modulates its effector until the GTP is hydrolysed to GDP by a GTPase intrinsic to the α subunit. The receptor does not affect this intrinsic GTPase, but it has recently been shown that the effector may itself act on the α-GTP subunit to increase the rate of GTP hydrolysis and hence inactivation of the G-protein (Berstein *et al.*, 1992). The α-GDP subunit remains inactive until the GDP dissociates and is replaced by GTP, the process which is stimulated by the receptor.

There are many ways of measuring guanine nucleotide exchange. One method uses [^{35}S]-GTPγS. This GTP analogue is relatively resistant to hydrolysis and dissociates slowly from the G-protein, so it accumulates in membranes or vesicles containing G-proteins. Activation of muscarinic receptors increases the binding of [^{35}S]-GTPγS in reconstituted systems (Kurose *et al.*, 1986; Florio & Sternweis, 1989) and in membranes from heart (Hilf *et al.*, 1989; 1992; Shiozaki & Haga, 1992; Chidiac & Wells, 1992) and smooth muscle (Liebmann *et al.*, 1992). We have measured the stimulation by acetylcholine (ACh) of [^{35}S]-GTPγS binding to membranes from CHO cells stably transfected with single human muscarinic m1–m4 receptors (Farries, unpublished observations), and here we have characterized the response pharmacologically with a number of selective or atypical antagonists.

One aim of this study was to assess the validity of the assay as a pharmacological response by comparing the antagonist affinity estimates of a range of known selective antagonists, measured using this functional response at human receptors, with affinity estimates measured in binding studies with human, rat or rabbit receptors. Some atypical antagonists were also studied.

A second aim was to assess a novel experimental design and method of analysis described in the accompanying paper (Lazareno & Birdsall, 1993). This is an 'inhibition curve' design which uses a single agonist titration and a titration of antagonist in the presence of a fixed concentration of agonist. The data from both titrations are fitted simultaneously to the Schild equation (Waud, 1975; 1976) using non-linear regression analysis. We compare this method of analysis with other methods of analysis, both theoretically valid and invalid, which do not require non-linear regression analysis. The large data set (112 experiments) of this study provides an opportunity to compare the distribution of errors incurred by the (invalid) use of the Cheng-Prusoff (1973) correction with the distribution predicted by a theoretical model, and also to compare two slightly different (valid) procedures which are based on the null method of dose-ratio analysis.

Some of the results described in this paper have been presented in preliminary form (Lazareno *et al.*, 1993).

Methods

Cell culture and membrane preparation

CHO cells stably expressing cDNA encoding human muscarinic receptors m1–m4 (Buckley *et al.*, 1989) were generously provided by Dr N.J. Buckley (N.I.M.R., London). These were grown in MEM-alpha medium (GIBCO) containing 10% (v/v) new born calf serum and harvested by scraping the cells off the surface in a hypoosmotic medium (20 mM HEPES + 10 mM EDTA, pH 7.4). Membranes were prepared at 0°C by homogenization with a Polytron followed by centrifugation (40,000 *g*, 15 min), were washed once in 20 mM HEPES + 0.1 mM EDTA pH 7.4, and were stored at − 70°C in the same buffer. The receptor densities (pmol receptor mg^{-1} membrane protein) were 4, 1, 5 and 3 for m1–m4 receptors respectively.

[^{35}S]-GTPγS binding assay

Membranes were suspended in a buffer containing 20 mM HEPES, 100 mM NaCl and 10 mM $MgCl_2$ pH 7.4 at a protein concentration of 25–50 μg ml^{-1}. Polystyrene tubes (5 ml) containing 1 ml of membrane suspension were incubated with GDP (0.1 μM for m1 and m3 receptors, 1 μM for m2 and m4 receptors), ACh and antagonist at 30°C for 20 min and then transferred to ice for 15 min. [^{35}S]-GTPγS was added to a final concentration of 100 pM and the samples incubated for 30 min at 30°C. The preincubation ensured that agonist and antagonist were at equilibrium throughout the labelling period. It was found necessary to pre-equilibrate the membranes with both agonist and antagonist before initiating the [^{35}S]-GTPγS binding, since preincubation with pirenzepine alone led to concentration-dependent decreases in the agonist E_{max} at m1–m4 receptor subtypes (data not shown). Preincubation with ACh did not reduce either the potency of ACh or its E_{max} (data not shown). The samples were filtered over glass fibre filters (Whatman GF/B) using a Brandel cell harvester and washed with 5 ml water. The filter discs were extracted overnight in 3 ml scintillant and counted by liquid scintillation spectrometry at an efficiency of about 97%. There was no detectable cholinesterase activity in CHO cells membranes, so there was no need to include a cholinesterase inhibitor in these assays.

Materials

[^{35}S]-GTPγS was purchased from Du Pont; AQ-RA 741 (11-({4-[4-(diethylamino)-butyl]-1-piperidinyl}acetyl)-5,11-dihydro-6H-pyrido(2,3-b)(1,4)-benzodiazepine-6-one) was a gift from Dr Karl Thomae GmbH; *o*-methoxy-sila-hexocyclium (OMSH) was a gift from Dr G. Lambrecht (Frankfurt), secoverine was a gift from Dr J. Barnes (Glaxo); Himbacine was a gift from Prof. W. Taylor (Sydney); hexahydrosiladiphenidol (HHSiD) and methoctramine were from Research Biochemicals Inc. (Semat, U.K.); acetylcholine (ACh), pirenzepine (PRZ), tacrine (THA), tropicamide and GDP were from Sigma (U.K.). Benzyl-4-DAPine (benzyl-4-diphenyl acetoxy piperidine) was a gift from Dr R.B. Barlow; its results will be presented in detail elsewhere, but they have been included in the population of data used to compare methods of analysis (Figure 3).

Experimental design and analysis

Each assay contained a titration of ACh alone, and titrations of 4 or 5 antagonists in the presence of a fixed concentration of ACh. Assays were conducted in duplicate, with each set of replicates filtered together. In separate assays the effect of the antagonist was studied in the absence of ACh.

For each set of replicates the data were expressed as a % of basal activity. This transformation sometimes reduced the coefficient of variation of the data, and never significantly increased it. The data were analysed in four ways, which have been described in more detail by Lazareno & Birdsall (1993). The first method is the best, but requires a non-linear curve fitting programme which can handle user-defined equations with two independent variables. The other methods do not require non-linear regression. The second method is invalid, but the third and fourth methods are theoretically valid and are equivalent to using the Gaddum equation (Gaddum, 1957) to analyse agonist titrations in the absence and presence of a fixed concentration of antagonist.

(1) For a single antagonist, the combined ACh and antagonist titration data were fitted to the Schild equation (Waud, 1975; 1976)

$$Effect = (E_{max}\text{-}basal)/(1 + \{\frac{[EC_{50}^0] \cdot (\frac{[B]^s}{K_b} + 1)}{[A]}\}^b) + basal$$

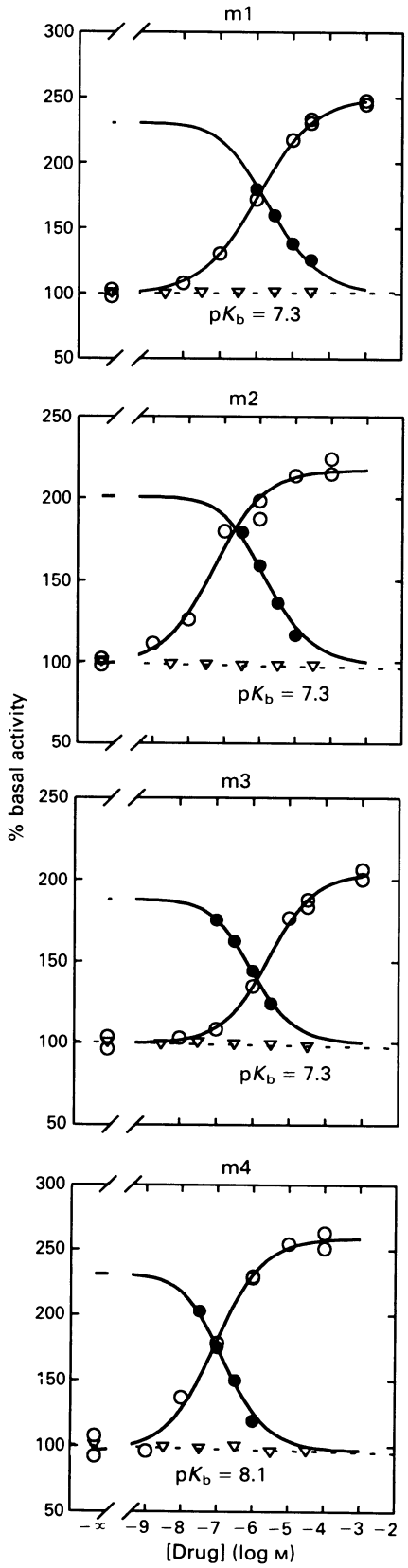


Figure 1 Stimulation of [^{35}S]-GTPγS binding to CHO cell membranes containing Hm1–Hm4 muscarinic receptors by acetylcholine (ACh) and inhibition of the effect of a fixed ACh concentration by tropicamide. The data are expressed as a % of basal activity, and each point represents the mean of duplicate observations. (○) ACh alone; (●) tropicamide in the presence of a submaximal concentration of ACh; solid lines show the fit of the combined data to the Schild equation with Schild slope constrained to unity; (▽) effect of tropicamide on basal activity, the dotted line shows the linear regression; these data were obtained in a separate experiment.

where [*A*] and [*B*] are the agonist and antagonist concentrations respectively, EC_{50}^0 is the EC_{50} of the agonist alone, *b* is the logistic slope factor of the agonist effect. K_b is the antagonist dissociation constant and *s* is the Schild slope factor. Lazareno & Birdsall (1993) have discussed the derivation and utility of this equation and have validated its use in both the traditional Schild-plot type of design and in the inhibition-curve design used here. The non-linear regression analysis was performed with SigmaPlot (Jandel). The data were analysed twice. Initially, the Schild slope factor was unconstrained, and the estimates of this parameter are those reported here. The data were reanalysed with the Schild slope factor constrained to be 1, and the pK_b estimates from these analyses are reported here. When terms such as 'affinity' or 'pK_b' are used they refer to the estimates from this second, constrained, analysis. The results of the two analyses were also used to assess whether the Schild slope factor was significantly different from unity, using the 'extra sum of squares' F-test (Munson & Rodbard, 1980). A pK_b value can only be considered as a true estimate of affinity if the corresponding Schild slope factor is not different from 1.

(2) The data from each assay (titrations of ACh alone and with a fixed concentration in the presence of titrations of 4 or 5 antagonists separately) were simultaneously fitted to logistic functions with the programme Allfit (De Lean *et al.*, 1978, gift of Dr P. Munson, NIH). The 'basal' parameter was constrained to be shared between all the curves (this was always statistically valid), and the 'maximum' parameter was constrained to be shared among the antagonist curves, while the 'maximum' parameter of the ACh curve was unconstrained. The EC_{50} and IC_{50} estimates were used to calculate the $K_{b\text{-}CP}$ using a version of the Cheng-Prusoff equation (Cheng & Prusoff, 1973)

$$K_{b\text{-}CP} = IC_{50}/(A/EC_{50} + 1)$$

where A is the fixed agonist concentration. It is theoretically invalid to use this equation to estimate antagonist affinities from functional inhibition curve data, and Lazareno & Birdsall (1993) have derived some theoretical predictions about the size and distribution of the errors incurred by the use of this form of analysis.

(3) The logistic parameter estimates for the ACh titration obtained in the preceding analysis were used with the IC_{50} estimates to calculate the $K_{b\text{-}P}$ estimate (Pöch *et al.*, 1992)

$$EC_{50}^* = (A^b/((A/EC_{50})^b + 2))^{(1/b)}$$

$$K_{b\text{-}P} = IC_{50}/(A/EC_{50}^* - 1)$$

where *b* is the slope factor of the ACh curve and EC_{50}^* is the ACh concentration causing 50% of the effect caused by the fixed concentration. This form of analysis is valid (Pöch *et al.*, 1992; Lazareno & Birdsall (1993).

(4) The data from each assay (titrations of 4 or 5 antagonists and of ACh alone *up to the fixed agonist concentration*) were simultaneously fitted to logistic functions with the programme Allfit. Both the 'basal' and 'maximum' parameters were constrained to be shared between all the curves. This analysis yielded EC_{50}^* and IC_{50}^* estimates which were used to calculate the $K_{b\text{-}LR}$ (Lazareno & Roberts, 1987)

$$K_{b\text{-}LR} = IC_{50}/(A/EC_{50} - 1)$$

These EC_{50}^* and IC_{50}^* estimates were usually slightly different from the EC_{50}^* and IC_{50} values used to calculate the $K_{b\text{-}P}$. This form of analysis is valid and is equivalent to defining the effect of the fixed agonist concentration as E_{max} and estimating EC_{50}^* and IC_{50}^* with Hill (logit-log) analysis (Lazareno & Roberts, 1987).

Results

ACh caused a dose-dependent increase in [^{35}S]-GTPγS binding (Figure 1, Table 1). The rank order of ACh potency

Table 1 Logistic parameters of acetylcholine (ACh) stimulation of [^{35}S]-GTPγS binding to CHO cell membranes containing single subtypes of human m1–m4 muscarinic receptors

Subtype	*N*	*pEC$_{50}$* (log M)	*Slope*	*E$_{max}$* (% basal)
m1	8	5.91 ± 0.11	0.56 ± 0.02	232 ± 5
m2	9	7.40 ± 0.06	0.69 ± 0.02	217 ± 13
m3	9	5.34 ± 0.08	0.81 ± 0.03	189 ± 11
m4	7	6.79 ± 0.09	0.67 ± 0.02	336 ± 18

Values are mean ± s.e.mean.

between the subtypes was m2 > m4 > m1 > m3. This differed from the rank order of receptor density, which was m3 > = m1 > m4 > m2, and maximal effect, which was m4 > m1 > = m2 > m3. The concentration-effect curves of ACh had logistic slope factors less than 1 with a rank order m3 > m4 = m2 > m1. It is difficult to interpret differences between EC_{50} or E_{max} values because both parameters are modified by GDP in a subtype-dependent manner (Farries, unpublished; Lazareno *et al.*, 1993).

The antagonists caused a dose-dependent inhibition of [^{35}S]-GTPγS binding stimulated by a fixed concentration of ACh (Figure 1, Table 2). They had little effect on basal activity (Figure 1 and data not shown). Often the higher concentrations seemed to inhibit basal activity by 5–10%, but the effect was too small and variable to quantify. We saw a similar lack of effect when a sodium-free Tris-HEPES-Mg assay buffer was used (data not shown).

The Schild slope factors were generally not different from 1 (Table 2). The single exception was THA which had slopes of about 2 at all subtypes, and the slope factors in each experiment with THA were significantly different from unity. In general, the logistic slope factors of the antagonist inhibition curves were always less than the corresponding Schild slope factor.

The antagonist affinity estimates were generally similar to previously reported values (Lazareno *et al.*, 1990; Michel *et al.*, 1990; Dörje *et al.*, 1991b; see Mitchelson, 1988 and Caulfield, 1993 for reviews). Pirenzepine were most potent at m1 receptors, but also had high affinity at m4 receptors. Himbacine had log affinities at about 8 at m2 and m4 receptors and about 7 at m1 and m3 receptors; it was the best drug for distinguishing between m1 and m4 receptors. Gallamine, methoctramine and AQ-RA 741 had different

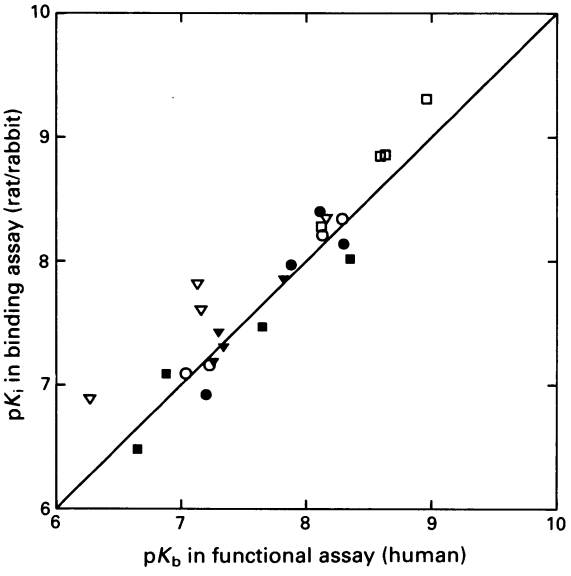


Figure 2 Comparison of log affinity estimates of six antagonists at m1–m4 receptors measured in two studies: the current study using a functional response and cloned human receptors, and the binding study of Lazareno *et al.* (1990) using native rat and rabbit receptors. Each drug is represented by a different symbol and the four instances of each symbol correspond to the drug's affinity at each of the four receptor subtypes. The drugs are: himbacine (○); HHSiD (●); methoctramine (▽); tropicamide (▼); secoverine (□); and pirenzepine (■). The line has a slope of 1 and intercept of 0.

Table 2 Estimates of pK_b and Schild slope factors of antagonists inhibiting acetylcholine (ACh)-stimulated [^{35}S]-GTPγS binding to CHO cell membranes containing single human m1–m4 muscarinic receptors

	pK_b (− log M) and Schild slope factor			
Drug/subtype	*m1*	*m2*	*m3*	*m4*
Pirenzepine	8.35 ± 0.03	6.65 ± 0.01	6.88 ± 0.12	7.65 ± 0.04
	1.05 ± 0.06	1.02 ± 0.04	1.06 ± 0.17	1.07 ± 0.03
Gallamine	5.35 ± 0.17*	6.24 ± 0.13	4.60 ± 0.16	5.42 ± 0.09
	0.75 ± 0.22*	1.07 ± 0.08	1.04 ± 0.18	0.90 ± 0.08
Himbacine	7.24 ± 0.06*	8.29 ± 0.05	7.04 ± 0.12	8.13 ± 0.11
	0.98 ± 0.07*	1.24 ± 0.12	1.23 ± 0.06	1.20 ± 0.01
Methoctramine	7.16 ± 0.04	8.16 ± 0.06	6.27 ± 0.06	7.13 ± 0.10
	0.98 ± 0.16	1.08 ± 1.12	1.10 ± 0.10	1.29 ± 0.16
AQ-RA 741	8.00 ± 0.22*	8.99 ± 0.06	7.19 ± 0.08	8.56 ± 0.07
	0.89 ± 0.04*	1.25 ± 0.09	1.09 ± 0.03	1.11 ± 0.02
HHSiD	8.30 ± 0.07	7.20 ± 0.08	8.10 ± 0.07	7.88 ± 0.09
	0.93 ± 0.08	1.14 ± 0.07	1.13 ± 0.05	1.17 ± 0.10
Tropicamide	7.27 ± 0.03*	7.34 ± 0.04	7.30 ± 0.05	7.82 ± 0.10
	0.98 ± 0.12*	1.27 ± 0.11	1.04 ± 0.01	1.09 ± 0.04
Secoverine	8.59 ± 0.18*	8.63 ± 0.14	8.12 ± 0.06	8.96 ± 0.16
	0.94 ± 0.08*	1.32 ± 0.16	1.15 ± 0.10	1.19 ± 0.15
o-Me-Si-HexC	7.44 ± 0.11	6.36 ± 0.10	6.68 ± 0.12	7.18 ± 0.08
	0.94 ± 0.06	1.52 ± 0.33	1.32 ± 0.05	1.12 ± 0.03
THA[1]	6.18 ± 0.09*	6.30 ± 0.13*	5.46 ± 0.11*	5.75 ± 0.11
	2.01 ± 0.08*	1.98 ± 1.20*	2.33 ± 0.48*	1.75 ± 0.08

The parameters were estimated by fitting data directly to the Schild model using non-linear regression analysis. pK_b values were estimated with Schild slope factor set to 1. Results are mean ± s.e.mean $n \geq 3$ except * $n = 2$. [1]pK_b values are not affinity constants since Schild slopes are steep.

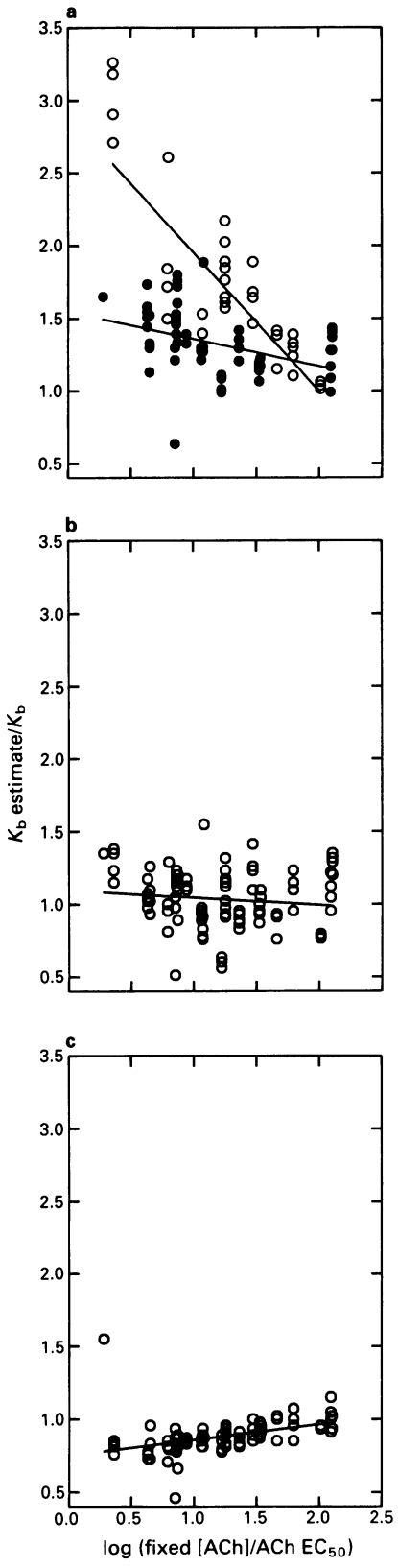


Figure 3 Deviations of antagonist K_b estimates at Hm1–Hm4 receptors derived from different analyses of the same experimental data, expressed as a ratio of the corresponding K_b obtained from a direct fit to the Schild equation with Schild slope constrained to unity. The estimates (see methods) used (a) the Cheng-Prusoff equation (analysis 2), (b) the method of Pöch *et al.* (1992) (analysis 3), (c) the method of Lazareno & Roberts (1987) (analysis 4). In (a) (○) correspond to experiments in which the agonist titration had a logistic slope factor of <0.65; (●) experiments with agonist slopes $>=0.65$. The lines indicate the linear regression, and are included as a guide to the eye.

potencies but similar profiles; they were most potent at m2 receptors, about 50 fold weaker at m3 receptors, and with intermediate potency at m1 and m4; gallamine and methoctramine had similar affinities at m1 and m4, while AQ-RA 741 was more potent at m4 than at m1 receptors. HHSiD is known as an m3-selective drug, but it did not distinguish between m1, m3 and m4 receptors. In confirmation of a previous binding study (Lazareno *et al.*, 1990) tropicamide and secoverine showed small degrees of m4-selectivity (3 and 2 fold respectively).

OMSH had a similar selectivity pattern to pirenzepine (m1 > m4 > m3 > m2) but with only limited m1 selectivity.

THA had a pattern of selectivity (m2 > m1 > m4 > m3) similar to gallamine, though it was much less selective. Its rank order of potency was similar to that found in binding studies of M_1–M_3 receptors (Kiefer-Day *et al.*, 1991). The values quoted in Table 2, however, may not correspond to affinity estimates since THA had Schild slopes greater than 1.

It is of interest to compare the results obtained in this study, using a functional response and cloned human receptors, with those obtained in a binding study which used rat and rabbit tissues (Lazareno *et al.*, 1990). Six drugs were common to both studies. There was a correlation of 0.93 between the affinity estimates of the six drugs at the four receptors in the two studies, the slope of the regression line was not different from 1 and the intercept was not different from 0 (Figure 2). The largest discrepancy (up to 5 fold) was with methoctramine at m1, m3 and m4 receptors, and this is explained by the lack of Mg^{2+} in the binding assays with these receptors.

The pK_b data shown in Table 2 were obtained by fitting the agonist and antagonist titrations directly to the Schild equation, as described in Methods. The fact that the Schild slope factors were generally about 1 (as expected for competitive antagonists) supports the validity of this form of analysis. Other ways of analysing the data, which may be more convenient, were described in Methods. The ratios of the K_b estimates obtained with these methods to those obtained from fitting to the Schild equation are shown in Figure 3.

The pattern of deviations (-fold difference in K_b) obtained from using Cheng-Prusoff analysis is consistent with the theoretical predictions of Lazareno & Birdsall (1993). The antagonist affinity was underestimated, the error was less at high fixed agonist concentrations, increased as the fixed agonist concentration approached the agonist EC_{50}, and was more marked when the agonist curve had a low slope. The estimates obtained by the method of Pöch *et al.* (1992) were similar to those obtained from fitting to the Schild equation and were unrelated to subtype, fixed agonist concentration or the slope of the agonist curve. Overall, the mean and s.e.mean of the deviations obtained with this analysis was 1.04 ± 0.02 ($n = 112$). The method of Lazareno & Roberts (1987) tended to yield small overestimates of antagonist affinity; the mean and s.e.mean of the deviations was 0.86 ± 0.01 ($n = 112$). There was, however, a strong correlation between the size of the deviation and the ratio of fixed agonist concentration to EC_{50} and when this effect was removed by linear regression there was a much smaller residual variance in the deviations of the data derived by the method of Lazareno & Roberts (1987) than in the deviations of the data derived by the method of Pöch *et al.* (1992).

The correlation between the deviations of the Lazareno & Roberts (1987)-derived estimates and the ratio of fixed agonist concentration to EC_{50} is presumably caused by the small but consistent errors caused by forcing a truncated agonist titration into a logistic function. The increased variability of the deviations of the data obtained with the method of Pöch *et al.* (1992) compared with the deviations associated with the method of Lazareno & Roberts (1987) presumably reflect the fact that the parameter estimates were less accurate when the fitting procedure allowed the agonist E_{max} to float than when the E_{max} was constrained. If we

consider data where the fixed agonist concentration was at least 10 fold greater than the agonist EC_{50}, then these K_d estimates, as a ratio of the estimated value derived from the Schild equation, were 1.01 ± 0.03 and 0.92 ± 0.01 using the method of Pöch *et al.* (1992) and Lazareno & Roberts (1987) respectively.

Discussion

In this study we have characterized a range of selective or atypical antagonists at Hm1–Hm4 muscarinic receptors using as the functional response the stimulation by ACh of GTPγS binding. In general, the antagonist affinity estimates obtained in this study with selective antagonists are in excellent agreement with previously reported values. Many of the drugs used in this study were also studied in binding studies using rat and rabbit tissues (Lazareno *et al.*, 1990) or cloned human receptors (Dörje *et al.*, 1991b) and the affinity estimates in the three studies were very similar (see also Mitchelson, 1988 and Caulfield, 1993). Our current results with secoverine and tropicamide confirm the earlier finding in binding studies (Lazareno *et al.*, 1990) that these antagonists have 2 to 3 fold overall selectivity for m4 receptors.

OMSH was reported to have similar affinities to pirenzepine at M_1–M_3 receptors in functional studies, with a pA_2 of 8.3 at rat superior cervical ganglion and 8.4 at rabbit vas deferens (two putative functional M_1 preparations), but in binding studies at M_1 sites its log affinity constant was only 7 (Lambrecht *et al.*, 1988; Feifel *et al.*, 1990). Our results at m1–m3 receptors are almost identical to the binding results at M_1 and functional results at M_2 and M_3 receptors. OMSH had a similar selectivity pattern to pirenzepine (m1 > m4 > m3 > m2) but with only limited m1-selectivity.

These results are mainly confirmatory, but have two important implications. Firstly, they provide no evidence for mammalian species differences in the antagonist profile of muscarinic receptor subtypes. The potential for species differences in muscarinic subtype pharmacology must, however, always be considered. Secondly, the results demonstrate that both the functional GTPγS binding assay with cloned human receptors and the radioligand binding assay with rat/rabbit tissues yield valid data for selective antagonists, despite the fact that most whole tissues contain more than one muscarinic receptor subtype (Dörje *et al.*, 1991a).

The logistic slope factors of the antagonist titrations were usually less than 1, but these slope factors cannot be interpreted in isolation from the slope factor of the agonist titration (Lazareno & Birdsall, 1993). By fitting the combined data of the antagonist and agonist titrations to the Schild equation it was possible to estimate the Schild slope of the antagonist. The slope factors were generally not different from unity, as expected for competitive antagonists. This result also supports the validity of this experimental design and form of analysis.

Two drugs, gallamine and THA, are thought to act, at least in part, through an allosteric mechanism. An apparent allosteric action of gallamine has been detected in functional studies utilising full Schild analysis (Clarke & Mitchelson, 1976; Ehlert, 1988; Kenakin & Boselli, 1989; Pöch *et al.*, 1992), but we saw no evidence for noncompetitive action. We might have seen signs of allosterism if higher fixed agonist concentrations had been used, but the single inhibition curve design is less sensitive than the full Schild analysis for detecting negative allosteric effects. THA has been found to inhibit the binding of antagonist radioligands with logistic slope factors of about 1.5–2 at M_1, M_2 and M_3 sites (Potter *et al.*, 1989; Kiefer-Day *et al.*, 1991), and it also has steep slopes for the allosteric effect of inhibiting antagonist radioligand dissociation (Potter *et al.*, 1989). Interestingly Potter *et al.* (1989) reported that THA inhibited agonist radioligand binding with slopes of about 1 and did not affect agonist ligand dissociation. Here we have found THA to inhibit ACh-stimulated GTPγS binding with similar potency and subtype specificity as has been reported from binding studies, and with Schild slopes of about 2 at all subtypes. This is the first report that THA inhibits muscarinic receptor activation with a steep concentration-response function.

Hilf & Jakobs (1992) found that antagonists inhibit basal GTPγS binding to cardiac membranes by up to 25% with potencies corresponding to their affinities at the m2 receptor. This phenomenon has been interpreted as evidence for 'negative intrinsic efficacy' of the antagonists at receptors precoupled to G-proteins (Hilf & Jakobs, 1992; Costa *et al.*, 1992). We saw little if any inhibition by antagonists of basal response using the standard assay conditions, but that was expected since the phenomenon is strongly inhibited by sodium ions (although the specificity of sodium, as opposed to a general monovalent cation effect, has not been explored). We also failed to see the inhibitory effect, however, when we used a sodium-free buffer. We have no explanation for this discrepancy.

This study demonstrates that accurate estimates of antagonist affinity and Schild slope can be obtained from functional assays with an inhibition-curve design as long as the data are analysed by a valid method. This design is often used with biochemical functional measures, but is more usually analysed with the Cheng-Prusoff equation (e.g. McKinney *et al.*, 1991; Ford *et al.*, 1992). As well as being theoretically invalid, this analysis will often underestimate (or overestimate) the antagonist affinity by up to 3 fold or more (Lazareno & Birdsall, 1993, this study). The best method of analysing such data is to fit them to the Schild equation, since this makes use of all the data without transformation and provides an estimate of Schild slope. If powerful curve-fitting facilities are unavailable, and the Schild slope can be assumed to be 1, then it is still possible to obtain accurate K_b estimates by logistic curve fitting or Hill analysis, or even measurement off a graph. The essence of this method is to estimate the concentration of agonist alone causing half the effect of the fixed concentration, i.e. the same effect as the fixed agonist concentration in the presence of the IC_{50} concentration of antagonist. We have assessed two slightly different methods of making this estimation. Both methods have strengths and weaknesses, but the errors associated with them are small and either method may be used with confidence, especially if the fixed agonist concentration is at least 10 fold greater than the EC_{50}.

The advantage of the single inhibition curve design in assessing antagonist affinity from functional experiments is that it requires less data than full Schild analysis containing a number of agonist (or antagonist) titrations in the presence of different fixed concentrations of antagonist (or agonist). The disadvantage, of course, is that it does not test the assumptions which underlie the analysis i.e. parallel displacement of agonist curves, and lack of effect on basal or maximal activity. In many cases the inhibition curve design will be quite adequate for the rapid assessment of antagonist affinity and Schild slope, and the effect of the antagonist on basal activity can be assessed in separate experiments if required. The full Schild design and analysis, however, preferably using non-linear regression, remains essential for the most thorough characterization of an antagonist.

In conclusion, the stimulation of [^{35}S]-GTPγS binding by muscarinic agonists provides a quick and convenient assay which allows accurate estimation of antagonist affinities, and estimates of antagonist Schild slopes can be obtained from single inhibition curves.

We gratefully acknowledge financial support from Sankyo Co. Ltd., Tokyo.

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(Received February 16, 1993
Accepted March 26, 1993)

Br. J. Pharmacol. (1993), **109**, 1128–1132 © Macmillan Press Ltd, 1993

The effect of endothelins on nitric oxide and prostacyclin production from human umbilical vein, porcine aorta and bovine carotid artery endothelial cells in culture

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1 This study has investigated the effects of the endothelin isopeptides, endothelin-1 (ET-1), ET-2 and ET-3 on the production of the endothelium-derived relaxing factors, nitric oxide (NO) and prostacyclin (PGI_2) from primary cultures of endothelial cells obtained from human umbilical vein (HUVECS), porcine aorta (PAECS) and bovine carotid artery (BCAECS).

2 NO generation was assessed indirectly by measuring production of cyclic GMP and PGI_2 formation was measured by radioimmunoassay of 6-keto $PGF_{1\alpha}$.

3 In HUVECS, histamine (1 μM) increased cyclic GMP and 6-keto $PGF_{1\alpha}$ production by 12.6 ± 2.0 and 4.9 ± 0.7 fold respectively over the corresponding basal values. Haemoglobin (10 μM) and the NO synthase inhibitor N^G-monomethyl-L-arginine (10 μM) significantly inhibited the increase in cyclic GMP formation in response to histamine but had no effect on 6-keto $PGF_{1\alpha}$ production. In contrast to histamine, the endothelin isopeptides (ET-1, ET-2 and ET-3; 0.01–1000 nM) produced no significant change in either cyclic GMP or 6-keto $PGF_{1\alpha}$ production in HUVECS.

4 In a separate series of experiments, ET-3 (0.01–1000 nM) also failed to produce any significant change in cyclic GMP or 6-keto $PGF_{1\alpha}$ production from primary cultures of PAECS and BCAECS. In contrast, bradykinin (0.1 μM) and sodium nitroprusside (1 mM) were used as positive control agents and increased cyclic GMP production in these cells.

5 In conclusion, the endothelin isopeptides do not release NO and PGI_2 from primary cultures of HUVECS, PAECS and BCAECS. This suggests that endothelin receptors are either absent from these cells or are not coupled to NO or PGI_2 production.

Keywords: Bovine carotid artery endothelial cells; cyclic GMP; EDRF; endothelin isopeptides; human umbilical vein endothelial cells; nitric oxide; porcine aortic endothelial cells; prostacyclin

Introduction

At present, differences in the pharmacological potency exerted by the endothelins (ET) suggest the existence of an ET-1- and ET-2-selective vasoconstrictor receptor, located on vascular smooth muscle and a non-selective endothelin receptor, which mediates vasodilatation, located on the vascular endothelium (Yanagisawa & Masaki, 1991). These observations are supported by DNA cloning studies from bovine and rat lung which have confirmed the structure of two distinct endothelin receptor subtypes (Arai *et al.*, 1990; Sakurai *et al.*, 1990). This has led to the ET-1/ET-2-selective vasoconstrictor receptor being termed ET_A and the non-selective vasodilator receptor, ET_B (Vane, 1990).

Experiments in rat isolated mesentery and lung suggest that the ET-induced vasodilatation responses may be mediated indirectly by release of nitric oxide (NO) and prostacyclin (PGI_2) from the endothelium (De Nucci *et al.*, 1988; Whittle *et al.*, 1989). Endothelins have also been reported to release NO and PGI_2 from bovine cultured endothelial cells (Emori *et al.*, 1991a,b; Filep *et al.*, 1991). Our aim, therefore, was to establish whether the endothelins could release NO and PGI_2 from primary cultures of human umbilical vein endothelial cells (HUVECS), thus providing a simple *in vitro* system in which to examine ET_B-mediated responses. We have also investigated the effect of the endothelins on NO and PGI_2 production from primary cultures of porcine aorta (PAECS) and bovine carotid artery endothelial cells (BCAECS). In our studies, endothelin-induced changes in guanosine 3':5'-cyclic monophosphate (cyclic GMP) (a marker of NO formation) and 6-keto $PGF_{1\alpha}$ production (the stable metabolite of PGI_2) were measured and compared with responses to histamine and bradykinin, known releasers of NO and PGI_2 (Adams-Brotherton, 1986; White & Martin, 1989; Chaudhuri *et al.*, 1991).

A preliminary account of our findings has been presented to the British Pharmacological Society (White *et al.*, 1992).

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Methods

Isolation of human umbilical vein endothelial cells (HUVECS)

HUVECS were isolated by a modification of the method of Jaffe *et al.* (1973b). Umbilical cords were collected into Hanks buffered salt solution (Ca^{2+} and Mg^{2+}-free) supplemented with gentamycin (250 μg ml^{-1}) and BM-cyclin 1 (10 μg ml^{-1}), and stored at 4°C. Typically, cords were stored for $\leq$ 48 h before use. Occasionally cords stored for longer periods (up to 5 days) were also used, the cells from which were pooled with those from the fresher cords. Veins were then cannulated, rinsed with phosphate-buffered saline (PBS) and refilled with collagenase (0.5 mg ml^{-1}, Boehringer, collagenase A). After a 10 min incubation at 37°C, endothelial cells were harvested from the vessels by flushing with Eagle's medium (E199) supplemented with: L-glutamine (100 μg ml^{-1}), penicillin (25 units ml^{-1}), streptomycin (10 μg ml^{-1}), BM-cyclin 2 (5 μg ml^{-1}), and sodium bicarbonate (25 mM). Cells were then collected by centrifugation (1,000 g, 5 min), and resuspended in supplemented E199 containing 20% v/v human serum. Cells from 2–3 veins were pooled, seeded

onto 24 multiwell plates, at a density of $\sim 10^5$ cells per well, and grown in culture at 37°C in an atmosphere of 95% humidity, 5% CO_2 in air. Approximately 24 h after isolation, cells were washed in E199 (1 ml) before adding a fresh aliquot (0.5 ml) of supplemented E199 containing human serum. The cells were then grown to confluence over 3–4 days.

Isolation of pig aortic endothelial cells (PAECS) and bovine carotid artery endothelial cells (BCAECS)

PAECS and BCAECS were isolated essentially as described for HUVECS. Briefly, pig aortae and internal or external bovine carotid arteries were flushed through with PBS to remove any blood. Vessels were tied at one end and cannulated at the other with a 60 ml syringe and cleaned of adhering fat and connective tissue. All branching arteries were then ligated and the vessels filled with collagenase A (0.5 mg ml^{-1}) and incubated at 37°C for 10 min. Following incubation, endothelial cells were loosened off the vessels by massaging between the thumb and forefinger. Cells were then harvested, centrifuged (1,000 *g*, 5 min) and resuspended in supplemented E199 (50 ml) containing human serum (20% v/v) for PAECS and foetal calf serum (20% v/v) for BCAECS. Cells from each vessel were then seeded onto a 24 multiwell plate and kept in culture, as described for HUVECS, until they reached confluence over approximately 2 days.

Endothelial cells from each species were characterized histologically, by their monolayer growth of cobblestone appearance and by their ability to release NO and PGI_2. In the case of HUVECS, the cells stained positively for von Willebrand Factor (Jaffe *et al.*, 1973a).

Measurement of cyclic GMP and 6-keto PGF$_{1\alpha}$

Following removal of the tissue culture medium, the cells were washed in warmed (37°C) Krebs solution (1 ml) of the following ionic composition (mM): Na^+ 143, K^+ 5.9, Mg^{2+} 0.6, Ca^{2+} 1.3, Cl^- 124, $H_2PO_4^-$ 1.2, SO_4^- 0.6, HCO_3^- 25, which also contained glucose (11 mM) and the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX; 0.1 or 1 mM). In experiments where 6-keto PGF$_{1\alpha}$ production was not measured, indomethacin (2.8 μM) was also included to inhibit prostanoid formation. After washing the cells, they were then incubated in the Krebs solution (1 ml) under an atmosphere of 95% humidity, 5% CO_2 in air for a period of at least 30 min.

Following incubation, test compounds were added to the cells at the concentrations and times indicated (see Results). The Krebs solution was then quickly removed for radioimmunoassay of 6-keto PGF$_{1\alpha}$ (Metachem, Antisera; Amersham TRK618 tracer), and the cells were extracted with 3% v/v perchloric acid in 20% v/v ethanol (0.5 ml). The cells were then scraped off the multiwell plate with a rubber policeman and harvested. Residual cells on the plate were recovered by a second extraction (0.5 ml). The extracts were then centrifuged (11500 *g*, 3 min) to obtain a supernatant and cell pellet. The supernatant was neutralized (pH 7.0–8.0) with a saturated solution of potassium hydrogen carbonate (140–160 μl) before measurement of cyclic GMP by scintillation proximity assay (Amersham, RPA 540 kit). The protein content of the cell pellet was extracted with NaOH (0.5 ml of 1 M) and determined by the method of Bradford, (1976).

Drugs and reagents

Culture medium E199, gentamycin, Hanks solution, human serum, foetal calf serum, L-glutamine, penicillin, phosphate-buffered saline and streptomycin were all obtained from Flow Laboratories. BM cyclin 1 and 2 and collagenase A were obtained from Boehringer Mannheim. L-Arginine, bradykinin triacetate, indomethacin, isobutylmethylxanthine (IBMX), haemoglobin (porcine), histamine hydrochloride, sodium nitroprusside and superoxide dismutase (bovine erythrocyte) were all obtained from Sigma. Phosphoramidon was obtained from Sigma and BDH Chemicals Ltd. N^G-monomethyl-L-arginine (L-NMMA) was obtained from Ultrafine Chemicals (Salford). Mepyramine was purchased from Rhone Pôulenc, and ranitidine was supplied by Glaxo Group Research Ltd. Endothelin-1, endothelin-2, and endothelin-3 were all obtained from Peninsula Laboratories.

All drugs were prepared freshly on a daily basis in Krebs solution, except for haemoglobin, which was prepared and stored as described by Martin *et al.* (1985). The endothelin isopeptides were made up as stock solutions in 0.1 M acetic acid and stored (− 20°C) as aliquots. Aliquots of the endothelins were then thawed and diluted with Krebs solution as required to provide working solutions which were kept on ice.

Expression of results

Basal levels of cyclic GMP and 6-keto PGF$_{1\alpha}$ are expressed in relation to the protein content (mg) of each well. Basal levels of cyclic GMP and 6-keto PGF$_{1\alpha}$ varied between batches of cells, therefore drug-induced changes in cyclic GMP and 6-keto PGF$_{1\alpha}$ are expressed as a ratio of their corresponding basal control value. All data are expressed as the mean ± s.e.mean, where n represents the number of replicate multiwell plates from different cell sources. All comparisons were made by a Student's paired t test. A probability value of $P = 0.05$ or less was taken to reflect a significant difference.

Results

Basal levels of cyclic GMP and 6-keto PGF$_{1\alpha}$ in HUVECS: effect of histamine

The mean basal levels of cyclic GMP and 6-keto PGF$_{1\alpha}$ measured in primary cultures of HUVECS are shown in Table 1.

Histamine (0.01–100 μM, 3 min) produced a concentration-dependent (pEC_{50} 6.69 ± 0.07) increase in cyclic GMP content in HUVECS that was abolished by pretreatment for 30 min with mepyramine (1 μM) but unaffected by ranitidine (1 μM, data not shown). A submaximal concentration of histamine (1 μM, 3 min) produced a mean 12.6 ± 2.0 and 4.9 ± 0.7 fold ($n = 19$) increase in cyclic GMP and 6-keto PGF$_{1\alpha}$ production, respectively.

The effect of haemoglobin, L-NMMA and L-arginine on the cyclic GMP content of HUVECS

Treatment of HUVECS with either haemoglobin (10 μM) or the NO synthase inhibitor L-NMMA (10 μM) for 30 min, produced approximately a 50% reduction of the basal content of cyclic GMP in the cells (Figure 1a). In contrast, L-arginine (1 mM, 30 min) alone, or in combination with L-NMMA (10 μM), produced a significant 2–3 fold rise in the basal cyclic GMP content of HUVECS (Figure 1a). Addition of histamine (1 μM, 3 min), to HUVECS, produced

Table 1 Basal levels of cyclic GMP and 6-keto PGF$_{1\alpha}$ measured over 3 min in human umbilical vein (HUVECS), porcine aorta (PAECS) and bovine carotid artery endothelial cells (BCAECS)

Cells	*Cyclic GMP* (pmol mg^{-1} protein)	*6-keto PGF$_{1\alpha}$* (ng mg^{-1} protein)
HUVECS	6.2 ± 1.0	95.0 ± 13.5
PAECS	8.6 ± 1.0	61.0 ± 12.0
BCAECS	13.3 ± 6.4	17.2 ± 4.8

Values are mean ± s.e.mean of 5–19 observations.

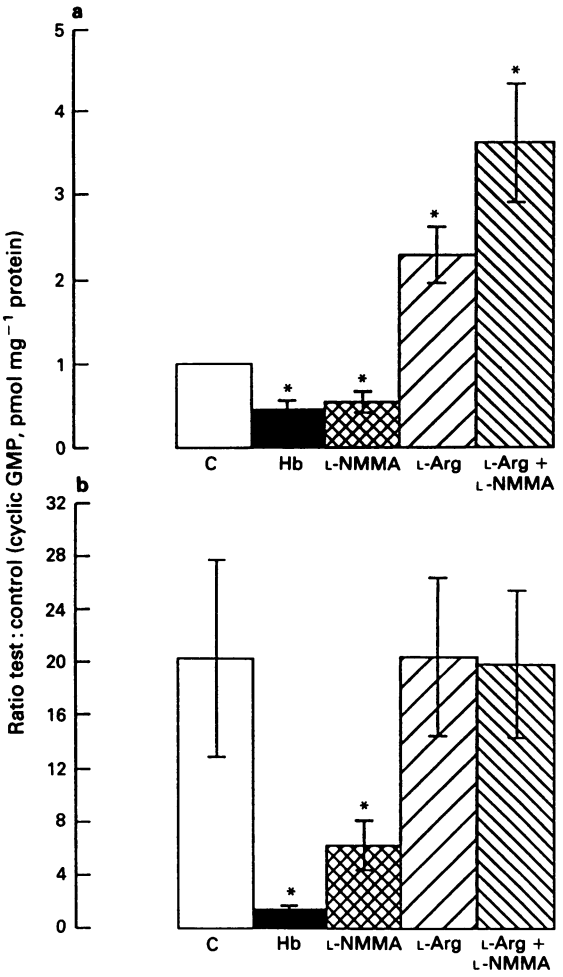


Figure 1 The effects of pretreatment (30 min) of human umbilical vein endothelial cells in culture with haemoglobin (Hb, 10 μM), N^G-monomethyl-L-arginine (L-NMMA, 10 μM), L-arginine (L-Arg, 1 mM) or a combination of L-NMMA + L-arginine on (a) basal and (b) histamine (1 μM, 3 min)-stimulated cyclic GMP content of cells. Data are mean ± s.e.mean of 4 observations. $*P<0.05$ denotes a significant difference from control (C).

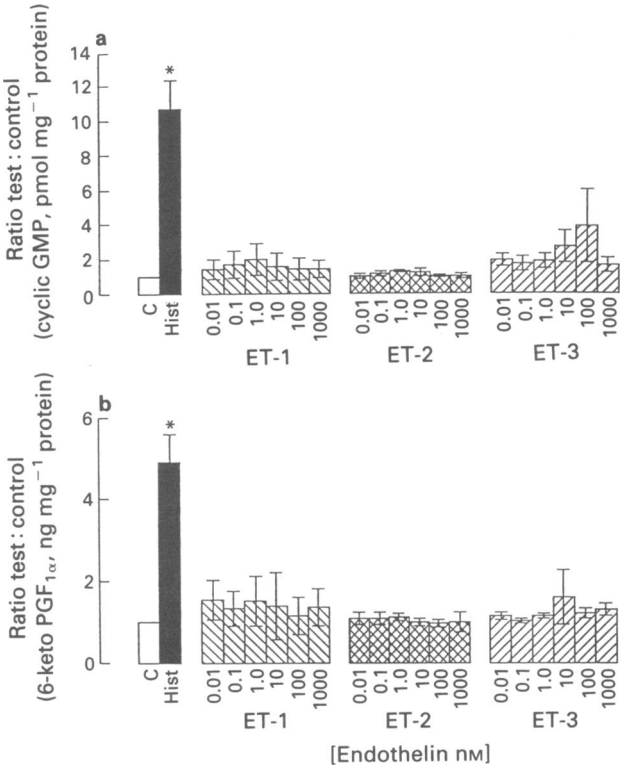


Figure 2 Comparison of the effect of histamine (Hist, 1 μM, 3 min) and the endothelin isopeptides ET-1, ET-2 and ET-3 (0.01–1000 nM, 3 min) on (a) the cyclic GMP content and (b) 6-keto $PGF_{1\alpha}$ productin from human umbilical vein endothelial cells in culture. Data are mean ± s.e.mean of 4–15 observations. $*P<0.05$ denotes a significant difference from control (C).

a 20.3 ± 7.4 fold ($n = 4$) increase in cyclic GMP, that was substantially inhibited by pretreatment with haemoglobin or L-NMMA (10 μM, 30 min; Figure 1b). L-Arginine (1 mM, 30 min) alone had no effect on the histamine-induced increase in cyclic GMP but reversed the inhibitory effects of L-NMMA on histamine-induced increases in this nucleotide (Figure 1b).

The effect of the endothelin isopeptides on cyclic GMP and 6-keto $PGF_{1\alpha}$ formation in HUVECS

Treatment of HUVECS with either ET-1, ET-2 or ET-3 (0.01–1000 nM, 3 min) did not alter cyclic GMP or 6-keto $PGF_{1\alpha}$ production in HUVECS (Figure 2). In contrast, histamine (1 μM, 3 min, $n = 15$), in the same cells, produced a 10.7 ± 1.7 fold increase in cyclic GMP, and a 4.9 ± 0.7 fold increase in 6-keto $PGF_{1\alpha}$ production (Figure 2).

Increasing the concentration of IBMX (1 mM) in combination with superoxide dismutase (SOD, 30 units ml^{-1}, to retard NO breakdown), and at the same time increasing the incubation time of ET-3 (100 nM) to 10 min also failed to uncover any change in the cyclic GMP or 6-keto $PGF_{1\alpha}$ levels. In contrast, under the same conditions, histamine (1 μM, 10 min) produced 34.0 ± 12.3 and 6.2 ± 1.8 fold ($n = 6$) increases in cyclic GMP and 6-keto $PGF_{1\alpha}$ production, respectively. Pretreatment of HUVECS with the endothelin convertase inhibitor, phosphoramidon (60 μM, 24 h) also failed to reveal any ET-3-induced cyclic GMP production in the cells (data not shown).

The effect of histamine, bradykinin and the endothelin isopeptides, on cyclic GMP and 6-keto $PGF_{1\alpha}$ production in PAECS and BCAECS

The basal levels of cyclic GMP in PAECS and BCAECS were comparable to those measured in HUVECS (Table 1). In contrast, BCAECS produced substantially less 6-keto $PGF_{1\alpha}$ than HUVECS or PAECS (Table 1). In PAECS, ET-3, (0.01–1000 nM, 10 min) was without effect on cyclic GMP or 6-keto $PGF_{1\alpha}$ production. In contrast, bradykinin (0.1 μM, 10 min) produced 11.5 ± 0.9 and 3.3 ± 0.7 fold increases in cyclic GMP and 6-keto $PGF_{1\alpha}$ production, respectively. In BCAECS, neither ET-3 (0.1–1000 nM, 10 min) nor histamine (1.0 μM, 10 min) altered cyclic GMP or 6-keto $PGF_{1\alpha}$ production although sodium nitroprusside (1 mM, 10 min) produced a 4.0 ± 0.8 fold increase in cyclic GMP, without affecting 6-keto $PGF_{1\alpha}$ production.

Discussion

The aim of the present investigation was to examine whether the endothelin isopeptides elicit the release of NO and PGI_2 from primary cultures of HUVECS, thus providing a simple *in vitro* system in which to examine ET_B-mediated responses. Our study has shown, however, that in contrast to histamine, ET-1, ET-2 or ET-3 do not alter either cyclic GMP or PGI_2 (6-keto $PGF_{1\alpha}$) formation in HUVECS.

Changes in cyclic GMP have been used previously as a marker for NO (EDRF) formation in cultured endothelial cells (Martin *et al.*, 1988). Although NO-releasing agents

produce increases in cyclic GMP in HUVECS they have not been shown, to our knowledge, to be NO-mediated (Adams-Brotherton, 1986). In this study, histamine-induced increases in cyclic GMP were inhibited by mepyramine but not ranitidine, suggesting that the responses were H_1 receptor-mediated. Haemoglobin, which binds and inactivates NO, also inhibited the histamine-induced increases in cyclic GMP. Furthermore, the NO synthase inhibitor, L-NMMA (Moncada *et al.*, 1989) inhibited histamine-induced rises in cyclic GMP, an effect which was reversed by L-arginine, a precursor amino acid for NO. Together, these observations indicate that in HUVECS, histamine-induced increases in cyclic GMP formation reflect NO-mediated responses. Therefore, the inability of the endothelins to increase cyclic GMP in HUVECS suggests that these peptides do not release NO from these cells.

Several explanations for the endothelin isopeptides failing to release NO and PGI_2 from HUVECS can be considered. The simplest explanation is that endothelin receptors are either absent from or not coupled to NO and PGI_2 production in HUVECS. An alternative explanation is that the endothelins may release NO and PGI_2, but in amounts so small as to be undetectable by the assay methods used. The latter explanation seems unlikely, as haemoglobin and L-NMMA both significantly reduced basal levels of cyclic GMP, indicating that the nucleotide assay was sufficiently sensitive to detect even basal NO release. Furthermore, treatment of HUVECS with superoxide dismutase to remove superoxide anions, which inactivate NO (Rubanyi & Vanhoutte, 1986, Gryglewski *et al.*, 1986), in combination with increasing the ET-3 incubation time and the IBMX concentration, did not unmask any increases in the cyclic GMP signal. Finally, it is recognised that HUVECS in culture can secrete ET-1 (Saijonmaa *et al.*, 1990; Kohno *et al.*, 1990). Consequently, endothelin receptor densensitization to endogenously formed endothelins could account for the inability of exogenous endothelins to release NO and PGI_2 from HUVECS. This explanation can probably be excluded, however, since ET-3 failed to increase cyclic GMP or PGI_2 formation in HUVECS, even following pretreatment with the endothelin convertase inhibitor, phosphoramidon (60 μM) which has been previously shown to inhibit ET-1 production from bovine endothelial cells (Sawamura *et al.*, 1991).

Interestingly, L-arginine treatment enhanced basal levels of cyclic GMP in HUVECS and thus its availability may represent a rate-limiting step and a key determinant of basal NO synthesis. However, the NO synthase inhibitor L-NMMA failed to block this L-arginine-induced enhancement of basal NO production in HUVECS. An explanation for this result probably resides in the 100 fold molar excess of L-arginine compared to L-NMMA (10 μM) used to compete for NO synthase.

The failure of the endothelin isopeptides to release NO and PGI_2 from HUVECS is at variance with similar measurements made in bovine cultured aortic and carotid artery endothelial cells (Emori *et al.*, 1991a,b; Filep *et al.*, 1991). Therefore, in a separate series of experiments, we examined the effects of ET-3 on cyclic GMP and PGI_2 production in PAECS and BCAECS. In these cells, as in HUVECS, ET-3 failed to increase cyclic GMP (NO) and PGI_2 production. In contrast, in PAECS, bradykinin increased production of both cyclic GMP (NO) and PGI_2, consistent with previous reports that in response to agonist stimulation these cells release both mediators (White & Martin, 1989). In BCAECS, histamine failed to increase either cyclic GMP or PGI_2 production. However, since sodium nitroprusside significantly increased cyclic GMP, the lack of effect of histamine is not due to the absence of a soluble guanylate cyclase in BCAECS. It is more likely that histamine receptors, like endothelin receptors, are either absent or not coupled to NO and PGI_2 production in these cells.

Our findings in HUVECS, PAECS and BCAECS are consistent with those previously reported in cultured PAECS, where bradykinin but not ET-1 caused NO release (measured by cyclic GMP; Vanhoutte *et al.*, 1988). These same investigators also reported that in endothelium-intact canine isolated femoral arteries and veins, ET-1 does not alter basal cyclic GMP content, consistent with ET-1 failing to release NO from these preparations. As discussed, however, our results differ from observations in bovine cultured aortic and carotid artery endothelial cells (Emori *et al.*, 1991a,b; Filep *et al.*, 1991) and from those in rat isolated mesentery and lung (De Nucci *et al.*, 1988; Warner *et al.*, 1989), where ET-1 and ET-3 are reported to release both NO and PGI_2. The reason(s) for these differences is unclear but possibly species differences in the distribution and/or coupling of endothelin receptors may occur. Such a view is consistent with other pharmacological receptor types on the vascular endothelium, where agents can elicit endothelium-dependent vasodilatation in one tissue but not in another tissue from the same species, or in the same tissue-type from a different species (Cherry *et al.*, 1982).

It is unclear why our findings differ from those of Emori *et al.* (1991a,b) who demonstrated that ET-3 induced the release of NO and PGI_2 from BCAECS. One key difference is that primary cultured endothelial cells were used in the present study, whilst Emori and colleagues used cells subcultured between passages 9–14. It is interesting to speculate that an up-regulation in the number of endothelin receptors may occur upon subculturing of endothelial cells. In this regard Eguchi *et al.* (1991) have shown, using affinity labelling of ET-1 and ET-3, that specific endothelin receptor binding occurs on subcultured BCAECS. Generally, however, the responsiveness of endothelial cells to agonists which release NO and PGI_2 is recognised to decline with time in culture (Ager & Martin, 1983).

In conclusion, endothelin isopeptides are unable to release NO and PGI_2 from primary cultures of HUVECS, PAECS and BCAECS, suggesting that endothelin receptors are either absent or not coupled to NO or PGI_2 production in these cells and excluding their use as a simple *in vitro* assay system for ET_B-receptor activity.

Note added in proof

Further experiments using both intact confluent HUVEC cells and a membrane preparation have revealed no specific binding of [^{125}I]-ET-3. These data therefore consistent with the premise that ET_B-like receptors are absent from primary cultures of HUVECS.

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(Received November 10, 1992
Revised March 22, 1993
Accepted March 29, 1993)

Br. J. Pharmacol. (1993), **109**, 1133–1139 © Macmillan Press Ltd, 1993

Comparison of the airways relaxant and hypotensive potencies of the potassium channel activators BRL 55834 and levcromakalim (BRL 38227) *in vivo* in guinea-pigs and rats

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1 BRL 55834, a novel potassium channel activator, has been compared with levcromakalim (BRL 38227) for its relaxant effects *in vivo* on the airways and vasculature of the guinea-pig and rat.

2 When administered intravenously 2 min prior to challenge, BRL 55834 and levcromakalim each inhibited histamine-induced increases in airways resistance (R_{aw}) in the anaesthetized guinea-pig, with BRL 55834 showing a 4.5 fold greater potency than levcromakalim ($ED_{25} = 2.5\ \mu g\ kg^{-1}$ and $11.3\ \mu g\ kg^{-1}$ respectively). By contrast, both compounds had similar hypotensive potencies ($ED_{18} = 8.5\ \mu g\ kg^{-1}$ and $6.5\ \mu g\ kg^{-1}$ respectively).

3 In the same guinea-pig model, intraduodenally administered BRL 55834 (100 and $250\ \mu g\ kg^{-1}$) and levcromakalim ($500\ \mu g\ kg^{-1}$) each protected against histamine-induced changes in R_{aw} and dynamic lung compliance (C_{dyn}), both compounds showing a rapid onset of action that persisted for more than 50 min. The lower dose of BRL 55834 had a similar bronchodilator effect to that of levcromakalim, yet both doses of BRL 55834 elicited substantially smaller effects than levcromakalim on mean arterial blood pressure.

4 In the anaesthetized rat, BRL 55834 and levcromakalim each evoked a dose-related inhibition of inhaled methacholine-induced changes in R_{aw} and C_{dyn} when given i.v., with BRL 55834 showing some four fold greater potency than levcromakalim (BRL 55834: R_{aw} $ED_{35} = 3.7\ \mu g\ kg^{-1}$, C_{dyn} $ED_{35} = 5.9\ \mu g\ kg^{-1}$; levcromakalim: R_{aw} $ED_{35} = 16\ \mu g\ kg^{-1}$, C_{dyn} $ED_{35} = 23.5\ \mu g\ kg^{-1}$). As in the guinea-pig, BRL 55834 had a reduced propensity to lower mean arterial blood pressure ($ED_{11} = 8\ \mu g\ kg^{-1}$ for BRL 55834, $11 \pm 3\%$ being its maximum effect; $ED_{11} = 16\ \mu g\ kg^{-1}$, maximum effect $= 34 \pm 6\%$ for levcromakalim.

5 When administered intraduodenally to anaesthetized rats, BRL 55834 (10, 20 and $100\ \mu g\ kg^{-1}$) evoked rapid and dose-related inhibitions of methacholine-induced R_{aw} and C_{dyn} changes which persisted for over 30 min. At the lower and middle dose there was little effect on mean arterial blood pressure (<10% fall). Levcromakalim ($500\ \mu g\ kg^{-1}$) by contrast elicited transient airways responses that diminished rapidly after 5 min, while the effects on blood pressure were well maintained (>20% at 65 min). Levcromakalim ($100\ \mu g\ kg^{-1}$) did not affect airways responses but also evoked a marked and sustained fall in blood pressure.

6 BRL 55834, administered *per os*, prolonged the time to histamine-induced dyspnoea in conscious guinea-pigs. The greatest effect of BRL 55834 was observed when it was administered 60 min prior to challenge, a dose of $0.20\ mg\ kg^{-1}$ doubling the mean time to collapse. A similar level of protection was afforded by levcromakalim ($1.25\ mg\ kg^{-1}$), with maximal activity occurring between 30 and 60 min.

7 The present studies in guinea-pigs and rats indicate that BRL 55834 is the first potassium channel activator to exhibit greater bronchodilator potency than levcromakalim but reduced tendency to lower arterial blood pressure. It is suggested that BRL 55834 may have greater potential than levcromakalim as a bronchodilator for therapeutic use in man.

Keywords: BRL 55834; levcromakalim; potassium channel activator; bronchodilator; airways; respiratory dynamics; blood pressure

Introduction

The potassium channel activators are potent and efficacious relaxants of spontaneous and induced tone in the airways of animals and man (Arch *et al.*, 1992). Studies *in vivo* have confirmed that when administered by a variety of different routes such compounds protect animals from the bronchospastic effects of a variety of stimuli (Arch *et al.*, 1988; Paciorek *et al.*, 1990a; Bowring *et al.*, 1991; Raeburn & Karlsson, 1991; Englert *et al.*, 1992). Furthermore, work with cromakalim has demonstrated that a single oral dose not only protects human volunteers against the effects of inhaled histamine (Baird *et al.*, 1988), but also, when given just prior to retiring, attenuates the morning dip in lung function in patients suffering from nocturnal asthma (Williams *et al.*, 1990).

In addition to bronchodilatation via smooth muscle relaxation, there is mounting evidence that the potassium channel activators will attenuate the release of neuropeptides (such as substance P and neurokinin A) from excitatory non-adrenergic, non-cholinergic (eNANC) unmyelinated nerves (C fibres) (Ichinose & Barnes, 1990; Lewis & Raeburn, 1990; Burka *et al.*, 1991; Good *et al.*, 1992). In particular, inhibition of the release of substance P, which is a potent inflammatory agent in human airways (Rogers *et al.*, 1989), may represent an additional beneficial effect of the potassium channel activators in asthma.

First generation potassium channel activators such as cromakalim discriminate little between smooth muscle types and significantly lower blood pressure in anaesthetized animals as a consequence of vasodilatation (Arch *et al.*, 1988; Bowring *et al.*, 1991). As a result of reflex tachycardia such effects are less prominent in conscious animals including man

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(Baird *et al.*, 1988), but cardiovascular side-effects are nevertheless dose-limiting (Arch *et al.*, 1992). A potassium channel activator with an enhanced selectivity for the airways relative to the vasculature may offer considerable advantages over existing compounds, although no such agent has yet been described.

In this paper we describe the *in vivo* effects of BRL 55834 [(3S,4R)-3,4-dihydro-2,2-dimethyl-4-(2-oxopiperidin-1-yl)-6-pentafluoroethyl-2H-1-benzopyran-3-ol] in the guinea-pig and rat following intravenous, oral and intraduodenal administration, and contrast the results with those found for levcromakalim (BRL 38227), the active enantiomer of cromakalim. Some of these results have been reported previously in preliminary form (Bowring *et al.*, 1992).

Methods

Anaesthetized guinea-pigs and rats

Guinea-pigs (male Dunkin-Hartley, 500–800 g) or rats (male Spraque-Dawley, 250–400 g) were anaesthetized with urethane (1.25 g kg^{-1}, i.p.). A jugular vein and carotid artery were cannulated for the administration of compounds and measurement of blood pressure respectively. The trachea was cannulated for the measurement of tidal airflow via a heated Fleisch pneumotachograph. The oesophagus was ligated above the gastro-oesophageal junction and cannulated to measure intrathoracic pressure. Airway resistance (R_{aw}) and dynamic lung compliance (C_{dyn}) were derived by use of a computer and software developed within our laboratories.

Guinea-pigs were challenged with histamine (2–4 $\mu g\ kg^{-1}$ histamine diphosphate, i.v.) every 10 min. The dose was adjusted so that R_{aw} increased by about 200% and C_{dyn} decreased by about 30%. Rats were challenged every 15 min with methacholine by inhalation according to the following procedure. A T-piece was attached to the pneumotachograph, and methacholine aerosol, generated using an ultrasonic nebulizer, was passed across the airway for 2 min. The concentration of methacholine solution was varied (0.5 to 2.5 mM) to produce an approximate increase in R_{aw} of 100% and a decrease in C_{dyn} of 40%. When stable responses were established in guinea-pigs or rats, BRL 55834 or levcromakalim was injected intravenously 2 min before each bronchoconstrictor challenge. The change in mean arterial blood pressure evoked by a given dose of BRL 55834 or levcromakalim was measured with respect to the mean pressure recorded immediately before the administration of that dose of the potassium channel activator. In general the maximal hypotensive effect of these agents occurred within 2 min of their intravenous injection. BRL 55834 (2–8 $\mu g\ kg^{-1}$) was given in increasing doses to guinea-pigs, each increasing dose being given when airways responses and blood pressure had returned to baseline. The 16 $\mu g\ kg^{-1}$ dose in guinea-pigs and each dose of BRL 55834 in rats were given to animals that received no other dose. Levcromakalim was given in increasing doses to guinea-pigs, but in rats the 3.1 and 200 $\mu g\ kg^{-1}$ doses were given to different animals from those receiving the 12.5 and 50 $\mu g\ kg^{-1}$ doses. When given intraduodenally, single doses of either BRL 55834 or levcromakalim were administered to each animal.

Conscious guinea-pigs

Guinea-pigs were placed individually in a perspex chamber of approximately 8 litres capacity at various times following the oral administration of BRL 55834 or levcromakalim and exposed for up to 240 s to an aerosol generated for 20 s from a 5 mM solution of histamine diphosphate. Those animals that collapsed were immediately removed from the chamber. They were allowed to recover and were not used for further experiments. The time from the introduction of the aerosol to collapse was recorded, with those animals not responding within the 4 min observation time being considered to be fully protected.

Drugs and solutions

BRL 55834 (Buckle *et al.*, 1992) and levcromakalim (BRL 38227) were synthesized in the laboratories of SmithKline Beecham Pharmaceuticals. Both compounds were administered i.v. in 0.9% NaCl and a maximum of 10% (w/v) dimethylsulphoxide and intraduodenally in a maximum of 1% (w/v) dimethylsulphoxide. The dosage volume was 0.1 ml 100 g^{-1} bodyweight for the i.v. route and 5 ml for the intraduodenal route.

Analysis of results

For experiments in anaesthetized animals, the response to the last bronchoconstrictor challenge prior to the administration of BRL 55834 or levcromakalim was used for calculation of the percentage inhibition of the bronchoconstrictor response. Statistical analysis was by single-sample *t* test. Potencies of BRL 55834 and levcromakalim have been compared for regions of the respective dose-response curves that overlap along the ordinate. Hence x in ED_x is not always a conventional number. Since ED_x values could in general not be estimated for individual animals, confidence intervals have been estimated by analysis of the linear part of the meaned dose-response curves using Fieller's theorem (Finney, 1980) and employing a pooled estimate of the residual variance from the entire curve. ED_{25} values for R_{aw} could be estimated for each guinea-pig given i.v. BRL 55834 or levcromakalim, however, and geometric means with 95% confidence intervals have in this instance been calculated in the conventional manner. For experiments in conscious animals, times of 240 s were allocated to animals that were fully protected for calculation of mean collapse times. Statistical analysis in these experiments was by one-tailed Mann-Whitney U-test.

Results

Baseline values

Mean resting R_{aw}, C_{dyn} and arterial blood pressure in guinea-pigs and rats are shown in Table 1. Administration of vehicle had no significant effect on baseline R_{aw}, C_{dyn} or arterial blood pressure or on the response of R_{aw} and C_{dyn} to histamine or methacholine.

Bronchoconstriction in anaesthetized guinea-pigs

Intravenous route When administered by the i.v. route to freely respiring anaesthetized guinea-pigs, BRL 55834 (2 to 16 $\mu g\ kg^{-1}$) elicited a dose-dependent inhibition of the histamine-induced increase in R_{aw}, providing 66% inhibition at the highest dose tested (Figure 1a). The dose of BRL 55834 providing a 25% inhibition (ED_{25}) of the histamine response was 2.5 (2.1–3.9) $\mu g\ kg^{-1}$ (geometric mean and 95% confidence interval). Inhibition of the histamine-induced change in R_{aw} was smaller 12 min after giving BRL 55834 than after 2 min in most animals (4 $\mu g\ kg^{-1}$: 56 ± 6%

Table 1 Mean resting values for R_{aw}, C_{dyn} and mean blood pressure

	R_{aw} ($cmH_2O\ ml^{-1}\ s^{-1}$)	C_{dyn} ($ml\ cmH_2O^{-1}$)	*Mean blood pressure* (mmHg)
Guinea-pigs	0.049 ± 0.022	1.50 ± 0.05	51.7 ± 8.0
Rats	0.017 ± 0.003	0.60 ± 0.13	81.1 ± 8.6

Results are shown as mean ± s.d. for at least 30 values.

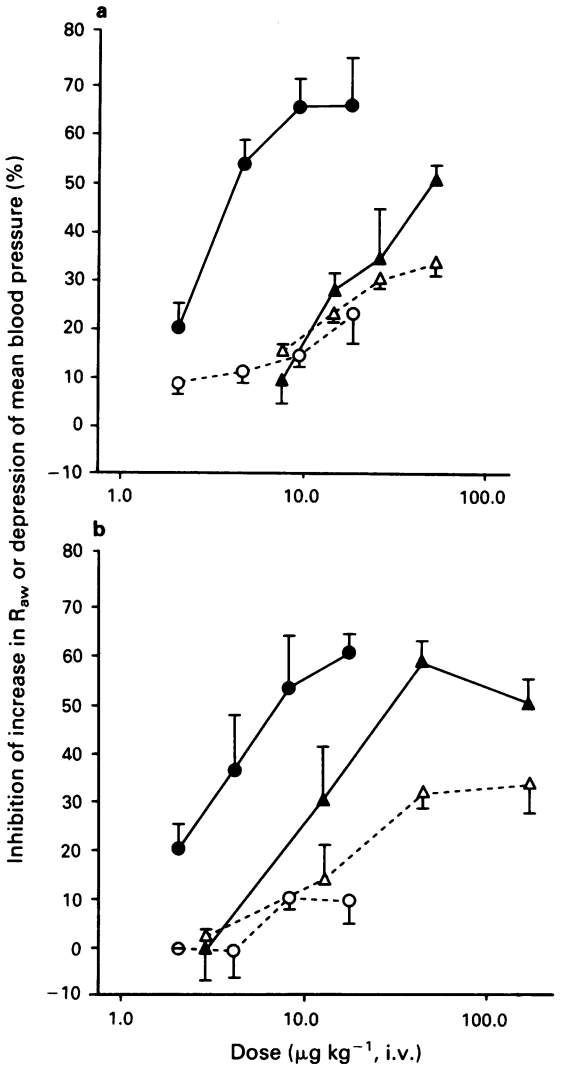


Figure 1 The effects of i.v. BRL 55834 and levcromakalim on baseline mean blood pressure and histamine-induced increases in R_{aw} in anaesthetized guinea-pigs (a) or methacholine-induced increases in R_{aw} in anaesthetized rats (b). Effects of BRL 55834 (●) and levcromakalim (▲) on airways; effects of BRL 55834 (○) and levcromakalim (△) on blood pressure. Responses were measured 2 min after the administration of BRL 55834 or levcromakalim. Details of whether cumulative dose-response curves were constructed or whether animals were given single doses of BRL 55834 or levcromakalim are given in the Methods section.

(mean ± s.e.mean) at 2 min, 29 ± 15% at 12 min, $n = 8$; 8 μg kg^{-1}: 67 ± 7% at 2 min, 46 ± 11% at 12 min, $n = 8$; 16 μg kg^{-1}: 66 ± 10% at 2 min, 63 ± 11% at 12 min). At 16 μg kg^{-1} the protective effect of BRL 55834 persisted for at least 22 min (17 ± 4%, $n = 5$). BRL 55834 evoked only a small, albeit significant ($P < 0.01$ at all dose levels), reduction of mean arterial blood pressure (Figure 1a), the dose inducing an 18% fall (ED_{18}) being 8.5 (5.1–15.3) μg kg^{-1}. To provide assurance that no tachyphylaxis occurred in response to BRL 55834, a single dose of 4 μg kg^{-1} was administered to a separate set of animals. In this instance the protection provided (54 ± 5%, $n = 11$) was the same as that achieved by the cumulative dosage regimen shown in Figure 1a.

Levcromakalim (6.25 to 50 μg kg^{-1}) also caused a dose-related inhibition of the histamine-induced changes in R_{aw} in this model (Figure 1a), with ED_{25} = 11.3 (8.7–14.6) μg kg^{-1}. Like BRL 55834, levcromakalim lowered mean arterial blood pressure ($P < 0.001$ at each dose level), but, its ED_{18} value [6.5 (4.7–9.1) μg kg^{-1}] was lower than its ED_{25} value for inhibiting changes in R_{aw} (Figure 1a).

Essentially similar results were obtained for the inhibitory effects of both BRL 55834 and levcromakalim on the histamine-induced decrease in C_{dyn}, although the data (not shown) were generally more variable than those with R_{aw} changes.

Intraduodenal route BRL 55834 and levcromakalim were also compared in this model when given by the intraduodenal route. Doses of BRL 55834 (100 μg kg^{-1}) and levcromakalim (500 μg kg^{-1}) were chosen to provide similar levels of protection based on intravenous potencies, and the fact that earlier studies (not published) had shown little protective effect of levcromakalim at a dose of 150 μg kg^{-1}. A higher dose of BRL 55834 (250 μg kg^{-1}) was included to ensure that the response to the lower dose was not supramaximal.

Both compounds showed a rapid onset of anti-bronchoconstrictor activity, with the lower dose of BRL 55834 providing similar protection to levcromakalim against the histamine-induced changes in R_{aw} and the protective effect of both compounds persisting over the 50 min observation period (Figure 2a). The higher dose of BRL 55834 was substantially more effective than the lower dose over the early time period, although by 50 min the two doses had similar effects. The onset of hypotensive activity with levcromakalim was also rapid and prolonged (Figure 2b). BRL 55834, by contrast, lowered arterial blood pressure at a slower rate than levcromakalim, even at the higher 0.25 mg kg^{-1} dose (Figure 2b). Furthermore, at least at the lower dose, the extent of blood pressure lowering was less with BRL 55834 than levcromakalim, since an experiment over a 2 h time course showed that the peak effect of BRL 55834 was achieved by 60 min. The effects of BRL 55834 on C_{dyn} (not shown) followed a similar trend to those on R_{aw} but were more variable.

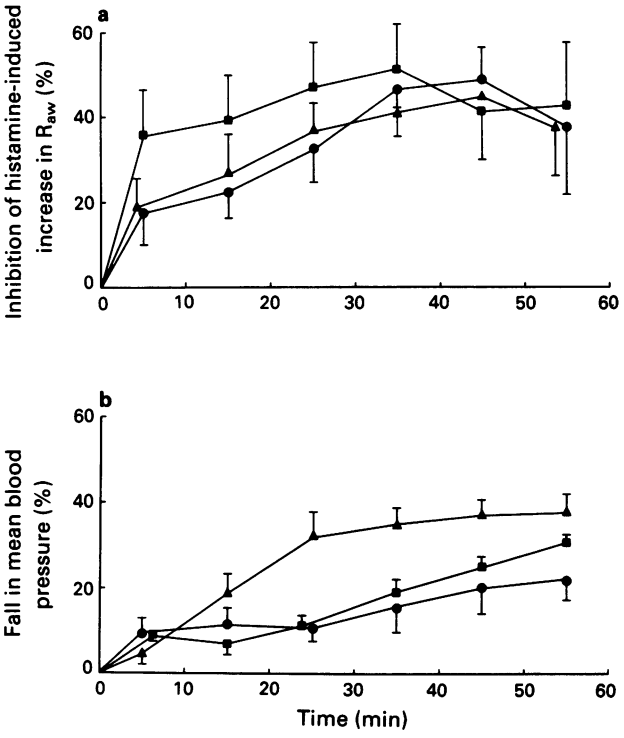


Figure 2 The effects of intraduodenal BRL 55834 (●, 100 μg kg^{-1}; ■, 250 μg kg^{-1}) and levcromakalim (▲, 500 μg kg^{-1}) on (a) histamine-induced increases in R_{aw} and (b) baseline mean blood pressure in anaesthetized guinea-pigs. The points are means of 6–8 values, ± s.e. All the points represent statistically significant effects, except for the effect of BRL 55834 (100 μg kg^{-1}) on blood pressure at 5 min.

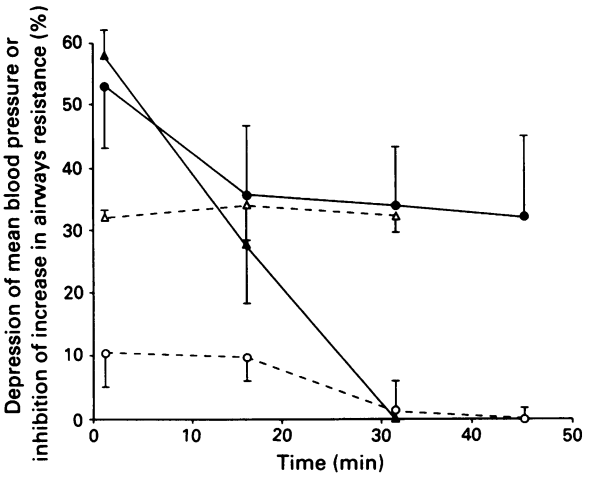


Figure 3 Time course of the effects of i.v. BRL 55834 (8 μg kg^{-1}) and levcromakalim (50 μg kg^{-1}) on methacholine-induced increases in R_{aw} and mean baseline blood pressure in anaesthetized rats. BRL 55834 (●) and levcromakalim (▲) on airways; BRL 55834 (○) and levcromakalim (△) on blood pressure. The points are means of 5 values, ± s.e.

Bronchoconstriction in the anaesthetized rat

Intravenous route Peak effects of BRL 55834 and levcromakalim on both the airways and the vasculature were observed 2 min after their i.v. administration to freely respiring, anaesthetized rats. BRL 55834 (2 to 16 μg kg^{-1}) evoked a dose-related inhibition of the increase in R_{aw} in response to inhaled methacholine (ED_{35} = 3.7 [1.5–6.7] μg kg^{-1}), providing a 60% inhibition at the highest dose (Figure 1b). Similarly, between 2 and 8 μg kg^{-1}, it evoked a dose-related inhibition of the decrease in C_{dyn} (ED_{35} = 5.9 [4.0–10.9] μg kg^{-1}), the maximum inhibition being 44% (data not shown). BRL 55834 was without effect on arterial blood pressure at the lower doses and its maximum effect was only 11 ± 3% (at 8 μg kg^{-1}, Figure 1b). In a similar experiment, levcromakalim showed a lower potency (ED_{35} = 16 [10–26] μg kg^{-1} resistance; ED_{35} = 23.5 [10.9–43.4] μg kg^{-1} compliance) on the airways, causing dose-related inhibitions over the range 3.1 to 50 μg kg^{-1}. Unlike BRL 55834, however, levcromakalim (ED_{11} = 8 [3–18] μg kg^{-1}) had a significant effect on arterial blood pressure at all doses which showed a respiratory protective effect (Figure 1b).

Evaluation of the time courses of the effects of BRL 55834 and levcromakalim in the rat demonstrates an even clearer distinction between their selectivities. BRL 55834 (8 μg kg^{-1}) provided a prolonged airways protectant effect, lasting over the duration of the experiment (>40 min) (Figure 3). Its effects on arterial blood pressure, however, had returned to control levels at 30 min and were minimal at all time points. The airways protectant effects of an equieffective dose of levcromakalim (50 μg kg^{-1}) by contrast were short-lived, returning to baseline values at 30 min, whereas the effects of this dose of levcromakalim on arterial blood pressure were well maintained (Figure 3).

Intraduodenal route BRL 55834 was a potent inhibitor of inhaled methacholine-induced R_{aw} and C_{dyn} changes in the anaesthetized rat when given by the intraduodenal route. BRL 55834 (10, 20 and 100 μg kg^{-1}) caused a dose-related inhibitory effect which persisted for over 30 min at the lower and middle doses, and for the duration of the experiment (65 min) at the higher dose (Figure 4a and b). Arterial blood pressure was unaffected by BRL 55834 at the lower and middle doses (the transient effect at the middle dose was not statistically significant), but was reduced by approximately 15% at the high dose (Figure 4c). Levcromakalim (100 μg kg^{-1}) afforded no protective effects against R_{aw} and C_{dyn} changes at any time point, whereas a 500 μg kg^{-1} dose resulted in a transient response which diminished rapidly after 5 min (Figure 4a and b). Consistent with the intravenous data, both doses of levcromakalim evoked a pronounced and prolonged reduction in arterial blood pressure which persisted for the duration of the experiment (65 min) (Figure 4c).

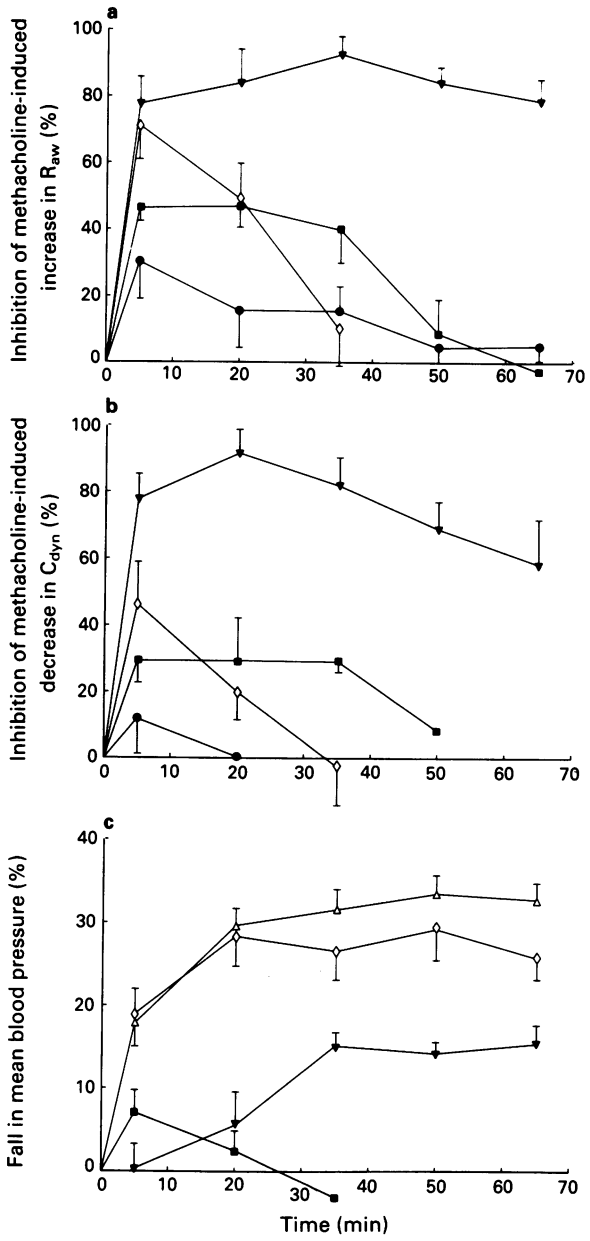


Figure 4 The effects of intraduodenal BRL 55834 (●, 10 μg kg^{-1}; ■, 20 μg kg^{-1}; ▼, 100 μg kg^{-1}) and levcromakalim (△, 100 μg kg^{-1}; ◇, 500 μg kg^{-1}) on (a) methacholine-induced increases in R_{aw}, (b) methacholine-induced decreases in C_{dyn} and (c) baseline mean blood pressure in anaesthetized rats. Levcromakalim, 100 μg kg^{-1}, had no effect on R_{aw} or C_{dyn} and BRL 55834, 10 μg kg^{-1}, had no effect on blood pressure. The points are means of 5 values with s.e. Statistically significant effects ($P<0.05$) were for BRL 55834 (100 μg kg^{-1}): all times for R_{aw} and C_{dyn} and from 35 min for blood pressure; BRL 55834 (20 μg kg^{-1}): to 35 min for R_{aw} and C_{dyn}; levcromakalim (100 μg kg^{-1}): all times for blood pressure; levcromakalim (500 μg kg^{-1}): 10 and 20 min for R_{aw}, 10 min only for C_{dyn}, all times for blood pressure.

Bronchoconstriction in the conscious guinea-pig

Orally administered BRL 55834 (0.5 mg kg^{-1}) and levcromakalim (2.5 mg kg^{-1}) protected conscious guinea-pigs from

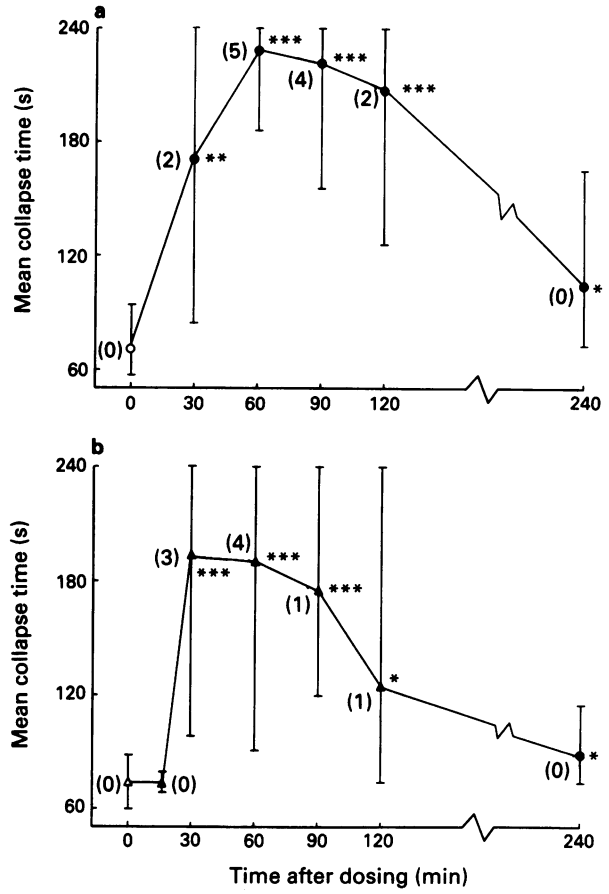


Figure 5 Time course of the effects of (a) BRL 55834 (0.5 mg kg^{-1}, p.o.) and (b) levcromakalim (2.5 mg kg^{-1}, p.o.) on the time to collapse following administration of an histamine aerosol to conscious guinea-pigs. Mean collapse times for controls are shown as the time 0 points. The points are means of 6 values and the vertical lines show the range of collapse times, with 240 s indicating full protection. The numbers in parentheses indicate the number of animals that were protected for 240 s out of the 6 challenged at each time. $*P<0.05$; $**P<0.01$; $***P<0.001$ compared to collapse times for controls.

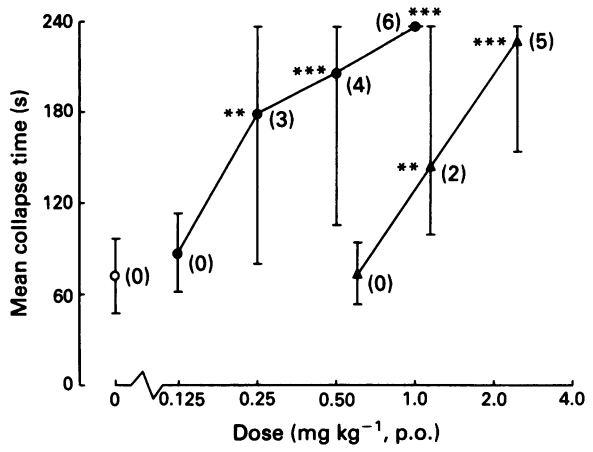


Figure 6 Dose-response relationships for the protection of conscious guinea-pigs from histamine aerosol by BRL 55834 (●) and levcromakalim (▲) given 60 min before challenge. The points are means of 6 values (12 for controls) and the vertical lines show the range of collapse times, with 240 s indicating full protection. The numbers in parentheses indicate the number of animals that were protected for 240 s. $**P<0.01$; $***P<0.001$ compared to collapse times for controls.

dyspnoea in response to a subsequent histamine aerosol challenge, with maximum protection occurring 60 min after dosing with BRL 55834 and between 30 and 60 min after levcromakalim (Figure 5). From subsequent dose-response studies carried out at the 60 min time point both BRL 55834 (0.125 to 1.00 mg kg^{-1}) and levcromakalim (0.625 to 2.5 mg kg^{-1}) inhibited dyspnoea in a dose-dependent fashion. ED_{100} doses (doubling of mean time to collapse) were 0.20 mg kg^{-1} and 1.25 mg kg^{-1} for BRL 55834 and levcromakalim respectively (Figure 6).

Discussion

The smooth muscle relaxant activity of the potassium channel activators has been extensively demonstrated in a wide range of tissues, suggesting that such compounds may have a potential utility in many diseases (Longman & Hamilton, 1992). It is this same diversity, however, that may restrict their clinical usefulness in certain disorders, particularly where competing smooth muscle events may be dose-limiting. This situation is especially evident with first generation potassium channel activators in which potent vasodilator activity is manifested as marked antihypertensive activity in animal models (Arch *et al.*, 1988; Paciorek *et al.*, 1990a; Mondot *et al.*, 1988). Cromakalim is relatively free of blood pressure lowering activity in normotensive human volunteers and in asthmatic patients (Baird *et al.*, 1988; Williams *et al.*, 1990), but cerebral vasodilatation, resulting in headache, is dose-limiting (Arch *et al.*, 1992).

Despite the structural diversity of the many known potassium channel activators, BRL 55834, a compound developed from the benzopyran series, is the first compound for which airway selectivity *in vivo* has been described. In contrast, a preliminary report from our laboratory (Taylor *et al.*, 1992c) has failed to demonstrate that BRL 55834 is airways-selective *in vitro*, in that it has similar IC_{50} values for inhibition of 30 mM KCl-induced tone in guinea-pig portal vein and for inhibition of spontaneous tone and tone induced by various spasmogens in guinea-pig tracheal spirals. However, levcromakalim differs from BRL 55834 in being less potent in tracheal spirals than in portal vein. It is for this reason that BRL 55834 has been extensively evaluated for its bronchodilator activity in animal models. It is quite possible, however, that the differing *in vitro* profiles of BRL 55834 and levcromakalim are of no relevance to the *in vivo* selectivity of BRL 55834.

In order to compare the anti-bronchoconstrictor and vasodilator potencies of BRL 55834 *in vivo* with those of the reference compound levcromakalim, experiments have been carried out in freely respiring anaesthetized guinea-pigs and rats. The guinea-pig, in particular, can be used as a model of human asthma because of the similarities that exist between the two in their response to various agonists and to known relaxants (Kallos & Kallos, 1984). A second species, the rat, was chosen to confirm and support the selectivity that was subsequently established in the guinea-pig. In both species, urethane anaesthesia largely abolishes the reflex tachycardia evoked in response to vasodilatation and lowered blood pressure. Consequently, the fall in blood pressure in these models provides a sensitive index of vasodilator activity. Anti-bronchoconstrictor activity was assessed in terms of agonist-induced changes in both R_{aw} and C_{dyn}, which are measures of large and small airway changes, respectively.

Largely in accord with its *in vitro* potencies relative to levcromakalim (Taylor *et al.*, 1992c),BRL 55834 (i.v.) was 4.5 times more potent than levcromakalim as an inhibitor of histamine-induced increases in R_{aw}, yet had similar potency as a blood pressure lowering agent in the anaesthetized guinea-pig (Figure 1a). Essentially similar effects were seen on both R_{aw} and C_{dyn}, a result which parallels those found earlier for levcromakalim (Bowring *et al.*, 1991; Paciorek *et al.*, 1991; Buckle *et al.*, 1993), cromakalim (de Souza *et al.*,

1989) and Ro 31-6930 (Paciorek *et al.*, 1991). In contrast, the β_2-adrenoceptor agonist, salbutamol, showed greater potency on the larger relative to the smaller airways in this model (Bowring *et al.*, 1991), although no difference has been shown for salmeterol on these parameters in either the guinea-pig or the cat (Paciorek *et al.*, 1991). Thus, BRL 55834 shows similar potency on both the large and small airways in the guinea-pig, a result which parallels that found earlier with levcromakalim. Whether these differences seen between the potassium channel activators and salbutamol have any clinical relevance remains to be established.

In order to demonstrate that BRL 55834 had the potential to protect against provoked bronchospasm when administered orally, experiments were carried out in which the compound was introduced directly into the duodenum via an indwelling cannula. In this study BRL 55834 showed a rapid onset of protective action, comparable to that of levcromakalim, and was again some five fold more potent than levcromakalim administered in the same manner (Figure 2a). That selectivity for the airways was retained was evident from the finding that an equi-bronchoprotective dose of BRL 55834 showed a significantly reduced effect on arterial blood pressure (Figure 2b). Whether an intraduodenal dose of BRL 55834 that does not affect blood pressure would elicit a useful bronchodilator effect was not determined in the guinea-pig. Studies in conscious guinea-pigs confirmed the ability of BRL 55834 to protect against a histamine challenge when given orally (Figures 5 and 6). In comparative experiments, BRL 55834 was six fold more potent than levcromakalim. In the guinea-pig therefore, BRL 55834 retains selectivity as an airways relaxant relative to levcromakalim, whether administered intravenously or intraduodenally.

In the anaesthetized rat, methacholine was chosen as the respiratory spasmogen since preliminary studies showed that the rat was insensitive to histamine challenges at sub-toxic doses. In this model i.v. BRL 55834 was found to be at least four fold more potent than BRL 38227 at protecting rats from the ventilatory effects of inhaled methacholine, yet elicited similar hypotensive activity (Figures 1b and 4). It is particularly significant that by both i.v. and intraduodenal routes BRL 55834 shows effects on airways parameters at doses that do not affect blood pressure and that those hypotensive effects that do occur are less than those observed with doses of levcromakalim required to provide an equal degree of respiratory protection. In addition to its greater potency, BRL 55834 showed a prolonged effect on the airways of rats when administered either intravenously or intraduodenally, whereas the effects of levcromakalim were relatively short (Figures 3 and 4). Since the duration of the effects of BRL 55834 and levcromakalim on arterial blood pressure were reversed (Figures 3 and 4), it was evident that BRL 55834 showed a preferential and more beneficial action on the airways of both the guinea-pig and rat than levcromakalim.

Few published data exist on previously described potassium channel activators which would allow a definitive assessment of the intrinsic airway selectivity of these agents. From our own experience, however, most compounds show either a profile similar to that of levcromakalim *in vitro* or exhibit a greater effect on the vasculature (unpublished results). BRL 55834 is to our knowledge the first potassium channel activator for which a selectivity for the airways has been unequivocally demonstrated *in vivo*.

If the *in vivo* selectivity of BRL 55834 is related to it having a different *in vitro* profile from levcromakalim (see above), it is appropriate to search for an explanation for this selectivity at a molecular level. Available physiological evidence supports the notion that BRL 55834 exerts its relaxant activity through potassium channel activation (Taylor *et al.*, 1992b). Furthermore, preliminary electrophysiological studies with BRL 55834 in bovine isolated trachealis have shown that in addition to the low conductance, ATP-inhibited and glibenclamide-sensitive potassium channel (30 pS) opened by levcromakalim, BRL 55834 also opens a high conductance potassium channel (247 pS) having characteristics similar to those of the calcium-activated, glibenclamide-insensitive channel (Ward *et al.*, 1992). Some workers have claimed that levcromakalim also opens the high conductance potassium channel, but others have failed to reproduce these findings (see Noack *et al.*, 1992). The fact that one group (Ward *et al.*, 1992) has found a difference in the abilities of levcromakalim and BRL 55834 to open this channel serves to highlight a difference between the compounds at the molecular level. Whether this difference is responsible for the airway selectivity of BRL 55834 *in vivo* remains to be established.

Based on the concentrations of cromakalim required to relax tone in human isolated bronchi (Taylor *et al.*, 1992a), it has been argued that the clinical effects of this agent cannot be due to direct smooth muscle relaxation since insufficient plasma concentrations are achieved (Small *et al.*, 1992). One suggestion is that cromakalim inhibits neural mechanisms underlying airways hyper-responsiveness, since available data suggest that cromakalim and other potassium channel activators affect hyper-responsiveness at doses that have no bronchoprotective effect in normal airways (Chapman *et al.*, 1991; 1992; Burka *et al.*, 1991). However, irrespective of the effects of the potassium channel activators on airways hyper-responsiveness, they are unequivocally relaxants of normal airways smooth muscle. In addition to its high potency on the isolated trachealis of the guinea-pig (Taylor *et al.*, 1992c), BRL 55834 is a potent relaxant of tone in human isolated bronchi (unpublished). Taken together with the selectivity found *in vivo* in the guinea-pig and rat, these data suggest that BRL 55834 may provide useful bronchodilatation in man.

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(Received January 20, 1993
Revised March 19, 1993
Accepted March 29, 1993)

Br. J. Pharmacol. (1993), **109**, 1140–1148 © Macmillan Press Ltd, 1993

β-Adrenoceptor subtypes and the opening of plasmalemmal K^+-channels in trachealis muscle: electrophysiological and mechanical studies in guinea-pig tissue

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1 Mechanical and electrophysiological studies of guinea-pig isolated trachealis have been made with the objectives of (a) identifying which of the β-adrenoceptor subtypes mediates the opening of plasmalemmal K^+-channels, (b) gaining further insight into the properties of the novel, long-acting β-adrenoceptor agonist, salmeterol and (c) clarifying the role of K^+-channel opening in mediating the relaxant actions of agonists at β-adrenoceptors.

2 Noradrenaline (10 nM–100 μM) caused a concentration-dependent increase in the rate of beating of guinea-pig isolated atria. The selective β_1-adrenoceptor blocking drug, CGP 20712A (100 nM–10 μM) caused concentration-dependent antagonism of noradrenaline. The selective β_2-adrenoceptor blocking drug, ICI 118551, also produced concentration-dependent antagonism of noradrenaline, but only when used in concentrations greater than 300 nM.

3 Cromakalim (100 nM–10 μM), isoprenaline (1–100 nM), procaterol (0.1–30 nM), salbutamol (1 nM–1 μM), salmeterol (1–100 nM) and theophylline (1 μM–1 mM) each caused concentration-dependent suppression of the spontaneous tone of guinea-pig isolated trachealis.

4 ICI 118551 (10 nM–1 μM) antagonized isoprenaline, procaterol and salmeterol in suppressing the spontaneous tone of the isolated trachea. The antagonism was concentration-dependent. In contrast, ICI 118551 (1 μM) antagonized neither cromakalim nor theophylline. CGP 20712A (up to 1 μM) failed to antagonize cromakalim, isoprenaline, procaterol, salmeterol or theophylline. In trachea treated with indomethacin (2.8 μM) and carbachol (10 μM), salmeterol (1 μM) antagonized the effects of isoprenaline but not aminophylline.

5 Intracellular electrophysiological recording from guinea-pig isolated trachealis showed that the relaxant effects of cromakalim (10 μM), isoprenaline (100 nM), procaterol (10 nM) and salbutamol (10 nM–1 μM) were accompanied by the suppression of spontaneous electrical slow waves and by cellular hyperpolarization. In contrast, the relaxant effects of salmeterol (10 nM–1 μM) were not accompanied by significant cellular hyperpolarization.

6 CGP 20712A (1 μM) inhibited the hyperpolarization but not the relaxation induced by isoprenaline (100 nM). In contrast ICI 118551 (100 nM) inhibited both the hyperpolarization and the relaxation induced by isoprenaline (100 nM). Neither CGP 20712A (1 μM) nor ICI 118551 (100 nM) inhibited the hyperpolarization induced by cromakalim (10 μM). Salmeterol (1 μM) inhibited the hyperpolarization induced by isoprenaline (100 nM) but not that induced by cromakalim (10 μM).

7 It is concluded that activation of either β_1- or β_2-adrenoceptors can promote the opening of K^+-channels in the trachealis plasmalemma. The poor ability of salmeterol to hyperpolarize trachealis muscle reflects neither its selectivity in activating β_2-adrenoceptors as opposed to β_1-adrenoceptors nor a non-specific action in stabilizing the cell membrane. Instead, it may reflect low intrinsic efficacy of the drug at β_2-adrenoceptors. The opening of plasmalemmal K^+-channels plays a supportive rather than a crucial role in mediating the tracheal relaxant actions of agonists at β-adrenoceptors.

Keywords: Trachealis muscle; electrophysiology; β-adrenoceptor subtypes; K^+-channels; isoprenaline; procaterol; salbutamol; salmeterol; CGP 20712A; ICI 118551

Introduction

In bovine and canine trachealis the mechano-inhibitory effects of agonists at β-adrenoceptors (adrenaline, isoprenaline and procaterol) are associated with hyperpolarization of the muscle cells (Suzuki *et al.*, 1976; Kirkpatrick, 1981; Ito & Tajima, 1982; Cameron *et al.*, 1983; Fujiwara *et al.*, 1988). In human and guinea-pig trachealis, agonists at β-adrenoceptors, such as isoprenaline and terbutaline, suppress spontaneous mechanical tone, suppress electrical slow waves and evoke cellular hyperpolarization (Allen *et al.*, 1985; Honda *et al.*, 1986; Honda & Tomita, 1987). Since the slow wave suppression and the hyperpolarization are inhibited by propranolol (Kirkpatrick, 1981; Ito & Tajima, 1982; Allen *et al.*, 1985; Honda *et al.*, 1986) it seems clear that these electrophysiological changes are mediated by the activation of β-adrenoceptors. However, the adrenoceptor subtype (β_1- or β_2-) responsible for mediating these electrophysiological changes has yet to be determined.

Electrotonic potentials induced by transmembrane current pulses are reduced in amplitude during the hyperpolarization of airways smooth muscle induced by agonists at β-adrenoceptors (Ito & Tajima, 1982; Cameron *et al.*, 1983; Fujiwara *et al.*, 1988). This may suggest that the hyperpolarization is a consequence of an increase in membrane conductance. Furthermore, since isoprenaline-induced hyperpolarization approaches the predicted K^+ equilibrium potential for trachealis muscle (Kirkpatrick, 1981; Allen *et al.*, 1985) it is likely that this conductance change involves the opening of membrane K^+-channels. Direct support for this suggestion comes from the patch clamp studies of Kume *et al.* (1989).

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Using cells freshly dispersed from rabbit trachealis smooth muscle, and recording from cell-attached patches, these authors showed that the addition of isoprenaline (0.2 μM) to the bathing medium stimulated the opening of plasmalemmal K^+-channels of large conductance.

The ability of charybdotoxin, in guinea-pig trachealis and human bronchus, to antagonize agonists at β-adrenoceptors such as isoprenaline and salbutamol (Jones *et al.*, 1990; Murray *et al.*, 1991; Miura *et al.*, 1992) suggests that the opening of the large conductance, Ca^{2+}-dependent K^+-channel (BK_{Ca}) is involved in the relaxant activity of the agonists at β-adrenoceptors. However, whether BK_{Ca} channel opening plays a crucial or merely a supportive role in mediating the mechano-inhibitory effects of the agonists at β-adrenoceptors is currently a matter of active debate (Jones *et al.*, 1990; Murray *et al.*, 1991; Cook & Small, 1991; 1992).

In the present study we have employed agonists (e.g. procaterol and salmeterol) and antagonists (e.g. CGP 20712A and ICI 118551) showing high selectivity for β_1- or β_2-adrenoceptors in experiments designed to determine which of the β-adrenoceptor subtypes mediates plasmalemmal K^+-channel opening in trachealis muscle. Other objectives of the present experiments were to shed more light on the role of K^+-channel opening in mediating the mechano-inhibitory effects of the agonists at β-adrenoceptors and to characterize further the pharmacological properties of the novel, long-acting β_2-adrenoceptor agonist, salmeterol (Ball *et al.*, 1991; Dougall *et al.*, 1991). The present electrophysiological and mechanical studies of guinea-pig trachealis were carried out in parallel with studies of mechanical activity and $^{86}Rb^+$ efflux in bovine trachealis (Chiu *et al.*, 1993). Some of our findings have been the subject of communications to the British Pharmacological Society (Chiu *et al.*, 1992; Cook *et al.*, 1992; Cook & Small, 1992).

Methods

Preparation of isolated tissues

Guinea-pigs of either sex (weight 300–800 g) were killed by stunning and bleeding. The heart was quickly excised from each animal and immersed in Krebs solution. Following removal of the pericardium, ventricular tissue was dissected from the atria taking care to preserve the integrity of the atrioventricular septum. Tracheae were excised from the animals, cleaned of adhering adipose and connective tissue and opened by cutting longitudinally, diametrically opposite the trachealis.

Tissue bath studies with isolated atria

Isolated, spontaneously-beating atria were set up in Krebs solution (37.5°C) under an initial resting tension of 1 g. Atrial tension changes were recorded with a force displacement transducer coupled to a Grass 7P1 preamplifier mounted in a Grass model 79D polygraph. The tension change signal was used to trigger a Grass model 7P44 tachograph so that recordings of atrial tension and rate of beating were made simultaneously. Once atrial rate had stabilized, test tissues were treated with CGP 20712A (100 nM, 1 μM or 10 μM) or ICI 118551 (100 nM, 300 nM, 1 μM or 10 μM) for 20 min prior to constructing a cumulative log concentration-chronotropic effect curve for noradrenaline (10 nM–100 μM) in the presence of one of the antagonists at β-adrenoceptors. Concentration increments for noradrenaline were made at 3 min intervals. Time-matched control tissues were treated identically to test tissues except that they were not exposed to CGP 20712A or ICI 118551.

Tissue bath studies with isolated trachea

Small segments of trachea were set up for the isometric recording of tension changes essentially as described by Foster *et al.* (1983). At the onset of each experiment, tissues were subjected to an imposed tension of 1.5 g. Approximately 20 min later, aminophylline (1 mM) was added in order to determine the recorder pen position at zero tissue tone. The aminophylline was removed from the tissue (initial wash followed by two further washes at 10 min intervals) and when spontaneous tone subsequently became maximal (40 min after initial wash), study of bronchodilator drugs commenced. A cumulative log concentration-effect curve against spontaneous tone was constructed for one of the following: cromakalim (100 nM–10 μM), isoprenaline (1–100 nM), procaterol (0.1–30 nM), salbutamol (1 nM–1 μM) or theophylline (1 μM–1 mM) in each test tissue. The concentration of isoprenaline was increased at 4 min intervals, that of procaterol, salbutamol or theophylline at 5 min intervals and that of cromakalim at 8 min intervals. Once the response to the highest concentration of the relaxant agonist had equilibrated, aminophylline was added to determine the recorder pen position at zero tissue tone. All relaxant responses were measured in terms of the maximal response to aminophylline. Following washout of the relaxant agonist, CGP 20712A (100 nM, 1 μM or 10 μM) or ICI 118551 (10 nM, 100 nM or 1 μM) was incubated with the tissue for a period of 40 min. The log concentration-effect curve for the relaxant agonist was then reconstructed in the presence of the antagonist. Time-matched control tissues were treated identically to test tissues but were not exposed to CGP 20712A or ICI 118551.

Responses of the isolated trachea to salmeterol required a protracted period (>30 min) to equilibrate and such responses proved very difficult to reverse by repeated changes of the bath fluid. Accordingly, salmeterol was studied by a modified protocol. Test tissues were preincubated (40 min) with CGP 20712A (100 nM, 1 μM or 10 μM) or ICI 118551 (10 nM, 100 nM or 1 μM) for 40 min prior to the application of a single concentration of salmeterol (1 nM, 10 nM, 100 nM, 1 μM, 10 μM or 100 μM). The relaxant response to salmeterol was allowed to develop for a period of 45 min before the addition of aminophylline (1 mM) in order to determine the pen position corresponding to zero tissue tone. Time-matched control tissues were treated similarly but were not exposed to CGP 20712A or ICI 118551.

A further group of experiments was performed in order to determine whether salmeterol might (as reported by Dougall *et al.*, 1991) act as a partial agonist at β_2-adrenoceptors. In these experiments, indomethacin (2.8 μM) was added to the Krebs solution 20 min after the initial setting up of the tracheal segments. Following indomethacin-induced suppression of spontaneous tissue tone (60 min), carbachol (10 μM) was added to the bath fluid. Once the spasmogenic response to carbachol had equilibrated (40 min), a cumulative log concentration-relaxation curve for isoprenaline (1 nM–10 μM) was constructed, essentially as described above. Following isoprenaline washout (40 min), test tissues were incubated with salmeterol (1 μM) for 45 min before reconstructing the log concentration-relaxation curve for isoprenaline in the presence of the salmeterol. Time-matched control tissues were treated similarly but were not exposed to salmeterol. Similar experiments were performed using aminophylline (1 μM–30 mM) instead of isoprenaline.

Intracellular electrophysiological recording from trachealis

Simultaneous recording of intracellular electrical activity and mechanical changes of a contiguous segment of trachea was performed with the technique and tissue holder described by Dixon & Small (1983). In brief, part of the trachealis was immobilized to permit long-term electrical recording while mechanical activity of contiguous muscle bundles was measured under an initial, imposed tension of 1.5 g. The recording microelectrodes were filled with 3 M KCl and were of resistance greater than 40MΩ.

After impalement of a trachealis cell, several minutes were allowed to elapse to check the stability of the record of electrical activity. Tracheal relaxant drugs (cromakalim, 10 μM; isoprenaline, 100 nM; procaterol, 10 nM; salbutamol, 10 nM, 100 nM or 1 μM; salmeterol, 10 nM, 100 nM or 1 μM) were then studied by their addition to the Krebs solution superfusing the tissue. Whenever possible, the effects of relaxant drugs on the electrical activity of the impaled cell were monitored for periods of 4 (isoprenaline), 5 (salbutamol), 6 (procaterol), 8 (cromakalim) or 14 min (salmeterol). When the effects of the tracheal relaxant drugs were examined in the presence of a modifying agent (CGP 20712A, ICI 118551 or salmeterol), the modifying agent was allowed to pre-equilibrate with the tissue for at least 20 min.

Drugs and solutions/statistical analysis of results

Drug concentrations are expressed in terms of the molar concentration of the active species. The following substances were used: aminophylline (BDH), CGP 20712A (2-hydroxy-5-(2-((2-hydroxy-3-(4-((1-methyl-4-trifluoro-methyl)-1H-imidazole-2-yl)-phenoxy)propyl)amino)ethoxy)-benzamide monomethanesulphonate; Ciba-Geigy Pharmaceuticals), cromakalim (SmithKline Beecham Pharmaceuticals), ICI 118551 (erythro-DL-1-(7-methylindan-4-yloxyl)-3-(isopropylamino-butan-2-ol hydrochloride); ICI Pharmaceuticals), (−)-isoprenaline hydrochloride (Sigma), (−)-noradrenaline bitartrate (Sigma), procaterol (Sigma), salbutamol (Glaxo Group Research), salmeterol (Glaxo Group Research), theophylline (Sigma). Stock solutions of most drugs were prepared in twice-distilled water. That of cromakalim was prepared in 70% ethanol. Isoprenaline, noradrenaline, salbutamol and salmeterol stock solutions were prepared in 0.1 M HCl. Dilutions of isoprenaline and noradrenaline were made with distilled water containing 0.57 mM ascorbic acid.

The Krebs solution used in the tissue bath experiments and for the microelectrode recording of membrane potential changes had the following composition (mM): Na^+ 143.5, K^+ 5.9, Ca^{2+} 2.6, Mg^{2+} 1.2, Cl^- 127.6, HCO_3^- 25, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2 and glucose 11.1 This solution (pH 7.4) was maintained at 37°C and was gassed with a mixture of 95% O_2:5% CO_2.

The significance of differences between means was assessed by use of a two-tailed, unpaired t test. The null hypothesis was rejected when $P < 0.05$.

Results

Studies with isolated atria

Noradrenaline (10 nM−100 μM) caused a concentration-dependent increase in the rate of atrial beating. CGP 20712A (100 nM−10 μM) did not significantly alter the resting atrial rate but antagonized noradrenaline in a concentration-dependent fashion. At a concentration of 1 μM, CGP 20712A caused a rightward shift of the noradrenaline log concentration-effect curve which was greater than 100 fold (Figure 1a). ICI 118551 (100 nM−10 μM) caused no change in the resting rate of atrial beating. At concentrations in the range 100−300 nM, ICI 118551 failed to modify the chronotropic action of noradrenaline. However, at concentrations in the range 1−10 μM, ICI 118551 antagonized noradrenaline in a concentration-dependent manner (Figure 1b).

Studies of the mechanical activity of isolated trachealis

Isoprenaline (1−100 nM; Figure 2), procaterol (0.1−30 nM; Figure 2), salbutamol (1 nM−1 μM) and salmeterol (1−100 nM; Figure 3) each caused concentration-dependent suppression of the spontaneous tone of guinea-pig isolated trachealis. Each of these agents was able to cause full suppression of the spontaneous tone of the trachea. The rank

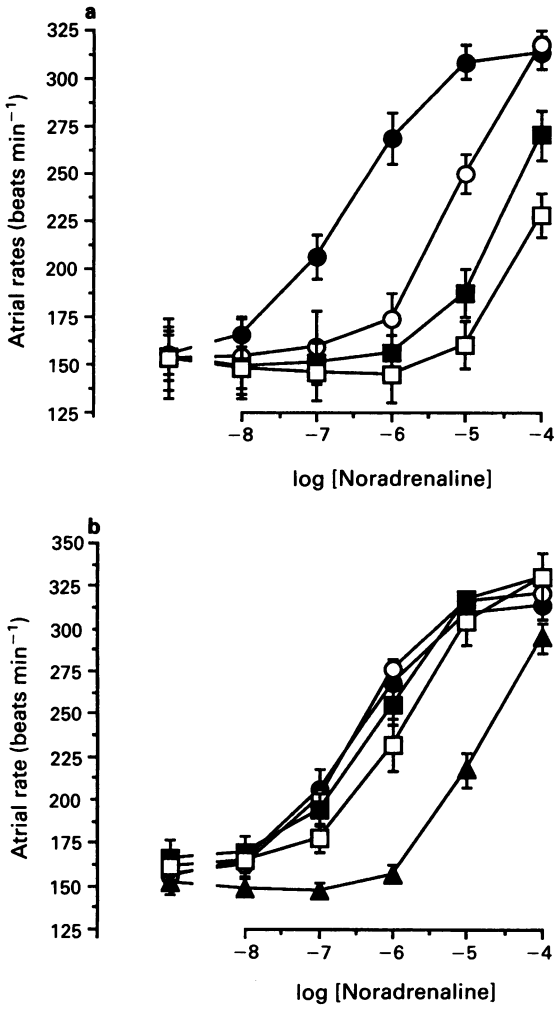


Figure 1 The effects of (a) CGP 20712A and (b) ICI 118551 against the chronotropic action of noradrenaline acting on guinea-pig isolated atria. In each panel the abscissa scale indicates the molar concentration of noradrenaline on a $\log_{10}$ scale while the ordinate scale indicates atrial rate in beats min^{-1}. In both panels (●) indicates the time-matched control log concentration-effect curve for noradrenaline. (a) Shows log concentration-effect curves for noradrenaline obtained in the presence of 100 nM (○), 1 μM (■) and 10 μM (□) CGP 20712A; (b) shows log concentration-effect curves for noradrenaline obtained in the presence of 100 nM (○), 300 nM (■), 1 μM (□) and 10 μM (▲) ICI 118551. All data points indicate the mean (±s.e.mean) of values obtained from at least 6 tissues. The data points shown on the extreme left in each panel indicate the resting atrial rate prior to the administration of noradrenaline.

order of relaxant potency for the agonists at β-adrenoceptors was procaterol > isoprenaline = salmeterol > salbutamol (Table 1). Cromakalim (100 nM−10 μM) and theophylline (1 μM−1 mM) also produced concentration-dependent suppression of tracheal tone. The maximal relaxant effect of theophylline was equivalent to that of aminophylline, whereas the maximal effect of cromakalim was 80−90% of that of aminophylline (data not shown).

CGP 20712A (in concentration up to 1 μM) did not alter the spontaneous tone of the trachea, nor did it antagonize isoprenaline, procaterol, salbutamol or salmeterol. However, at a concentration of 10 μM, CGP 20712A caused minor (< four fold) antagonism of procaterol and salbutamol (Figures 2 and 3; Table 1). CGP 20712A (1 μM) did not modify the relaxant actions of either cromakalim or theophylline (Table 1). Like CGP 20712A, ICI 118551 (10 nM−1 μM) did not alter the spontaneous tone of the trachea, nor did it (at a concentration of 100 nM) antagonize either cromakalim or theophylline (Table 1). However, ICI

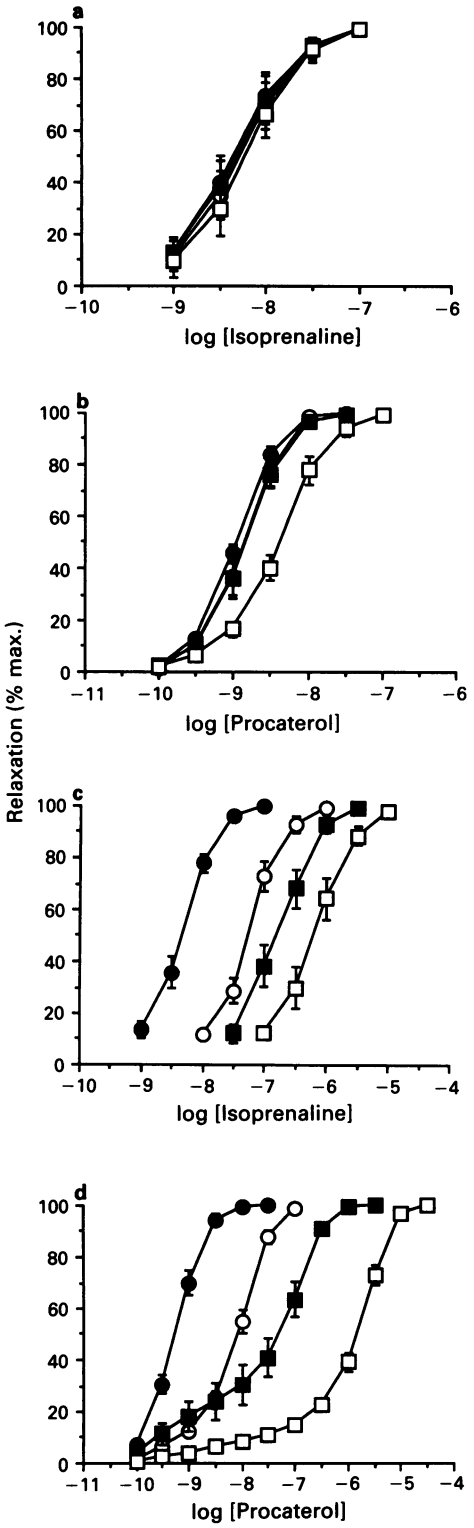


Figure 2 The effects of CGP 20712A and ICI 118551 against the actions of isoprenaline and procaterol in suppressing the spontaneous tone of guinea-pig isolated trachealis muscle. In each case the abscissa scale indicates the $\log_{10}$ molar concentration of isoprenaline or procaterol while the ordinate scale indicates relaxation expressed as a percentage of the maximal relaxation induced by aminophylline. In all panels (●) indicates the log concentration-effect curve of the agonist at β-adrenoceptors as observed in the absence of any antagonist. (a) and (b) show log concentration-effect curves for the agonists at β-adrenoceptors as observed in the presence of 100 nM (○), 1 μM (■) or 10 μM (□) CGP 20712A; (c) and (d) show log concentration-effect curves for the agonist at β-adrenoceptors as observed in the presence of 10 nM (○), 100 nM (■) or 1 μM (□) ICI 118551. In each panel all data points indicate the means of values from at least 6 issues ± s.e.mean.

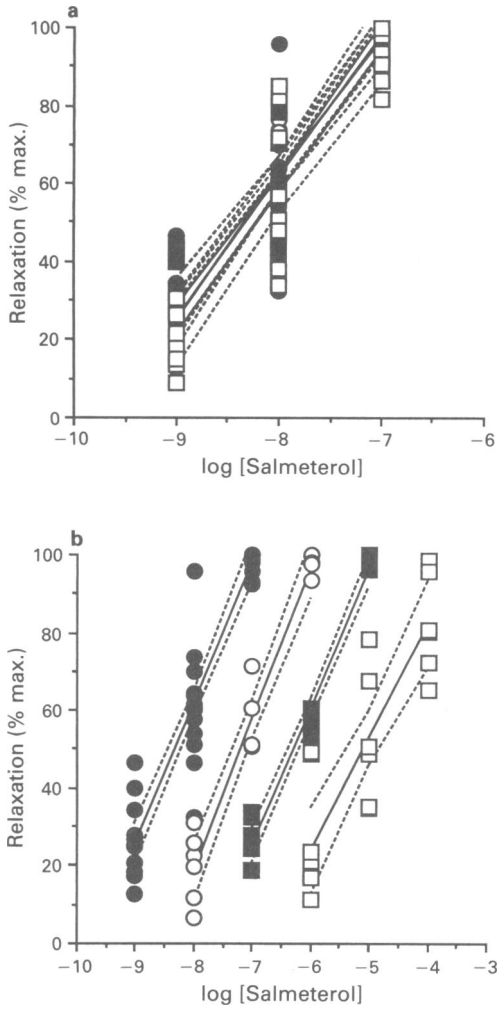


Figure 3 The effects of (a) CGP 20712A and (b) ICI 118551 against the action of salmeterol in suppressing the spontaneous tone of guinea-pig isolated trachealis muscle. In each case the abscissa scale indicates the $\log_{10}$ molar concentration of salmeterol and the ordinate scale indicates relaxation expressed as a percentage of the maximal relaxation induced by aminophylline. In both panels the solid lines indicate the linear regression of response versus the log molar concentration of salmeterol while the broken lines indicate the upper and lower 95% confidence limits of the regression lines. In (a) responses to salmeterol were obtained in the absence (●) and presence of 100 nM (○), 1 μM (■) and 10 μM (□) CGP 20712A. In (b) responses to salmeterol were obtained in the absence (●) and presence of 10 nM (○), 100 nM (■) and 1 μM (□) ICI 118551.

118551 antagonized isoprenaline, procaterol, salbutamol and salmeterol in a concentration-dependent manner (Figures 2 and 3, Table 1).

In tracheal segments treated with indomethacin (2.8 μM) and carbachol (10 μM), isoprenaline (1 nM–10 μM) and aminophylline (1 μM–30 mM) each evoked concentration-dependent relaxation. The incubation of test tissues with salmeterol (1 μM) caused some reduction (approximately 20%) in the carbachol-induced tone. This reduction in tone did not render the tissues more sensitive to isoprenaline. On the contrary, the log concentration-effect curve for isoprenaline seen in the salmeterol-treated tissues was shifted to the right ($-\log EC_{50}$ values for control and test tissues were 6.62 ± 0.06 and 5.59 ± 0.11 respectively; mean ± s.e.mean, $n = 6$) compared with that seen in the time-matched control tissues. Furthermore, the maximal response to isoprenaline was slightly reduced in the test tissues (data not shown). Salmeterol (1 μM) did not, however, antagonize aminophylline ($-\log EC_{50}$ values for control and test tissues 3.33 ± 0.15 and 3.64 ± 0.10 respectively) in suppressing carbachol-induced contraction of the trachea.

Table 1 The effects of CGP 20712A and ICI 118551 against the actions of cromakalim, theophylline and some agonists at β-adrenoceptors in suppressing the spontaneous tone of guinea-pig isolated trachealis

	Time-matched control	*CGP 20712A*		
Agonist	(no antagonist)	100 nM	1 μM	10 μM
Cromakalim	6.50 ± 0.10	†	6.53 ± 0.10	†
Theophylline	4.32 ± 0.06	†	4.29 ± 0.05	†
Isoprenaline	8.35 ± 0.10	8.27 ± 0.15	8.32 ± 0.15	8.23 ± 0.15
Procaterol	8.93 ± 0.05	8.85 ± 0.09	8.87 ± 0.08	8.36 ± 0.08*
Salbutamol	7.70 ± 0.10	7.63 ± 0.11	7.59 ± 0.11	7.26 ± 0.09*
Salmeterol	8.34 (8.21–8.50)	8.33 (8.21–8.45)	8.36 (8.22–8.52)	8.20 (8.06–8.36)
	Time-matched control	*ICI 118551*		
Agonist	(no antagonist)	10 nM	100 nM	1 μM
Cromakalim	6.09 ± 0.10	†	5.79 ± 0.10	†
Theophylline	4.13 ± 0.05	†	3.97 ± 0.04	†
Isoprenaline	8.39 ± 0.05	7.27 ± 0.07*	6.78 ± 0.12*	6.18 ± 0.12*
Procaterol	9.23 ± 0.06	8.07 ± 0.07*	7.47 ± 0.25*	5.84 ± 0.05*
Salbutamol	7.86 ± 0.11	6.54 ± 0.22*	5.50 ± 0.32*	4.36 ± 0.15*
Salmeterol	8.29 (8.14–8.45)	7.18* (7.06–7.32)	6.29* (6.21–6.37)	5.09* (4.84–5.36)

For most agents the data indicate mean (± s.e.mean) values of $-\log_{10} EC_{50}$. For salmeterol the mean $-\log_{10} EC_{50}$ and its lower and upper 95% confidence limits are shown. For all agents, data from at least 6 tissues contribute to the mean.
†indicates a parameter not measured.
*indicates a value significantly ($P < 0.05$) different from that observed in the absence of the relevant antagonist.

Intracellular electrophysiological recording from trachealis

The addition of either cromakalim (10 μM) or isoprenaline (100 nM; Figures 4 and 6) to the Krebs solution superfusing the trachealis muscle reduced the mechanical tone of the tissue, suppressed spontaneous electrical slow waves and caused hyperpolarization of the impaled cell. In this respect we have been able to confirm the results of our earlier studies (Allen *et al.*, 1986). Procaterol (10 nM) and salbutamol (10 nM–1 μM) also produced relaxation accompanied by slow wave suppression and by cellular hyperpolarization (Figure 4 and Table 2).

As anticipated from the results of the tissue bath studies, salmeterol (10 nM–1 μM) produced relaxant effects, but these were very slow to develop compared with those of the other agonists at β-adrenoceptors. Salmeterol (10 nM–1 μM) failed to evoke significant hyperpolarization of the trachealis cells (Figure 5 and Table 2). At concentrations of 100 nM and 1 μM this agent did, however cause some reduction in slow wave amplitude or frequency (Figure 5b,c). Pretreatment of the trachealis with salmeterol (1 μM) abolished the mechanical tone of the tissue, failed to modify the hyperpolarization induced by cromakalim (10 μM) but reduced the ability of isoprenaline (100 nM) to cause hyperpolarization of the impaled cell (Table 2).

The addition of CGP 20712A (1 μM) to the Krebs solution superfusing the trachealis muscle modified neither the mechanical tone of the tissue nor the electrical behaviour of the impaled cell. CGP 20712A (1 μM) did not modify the relaxant action of cromakalim (10 μM) nor the ability of cromakalim to hyperpolarize the trachealis cells (Table 2). CGP 20712A (1 μM) did not reduce the relaxant action of isoprenaline (100 nM) but significantly reduced the hyperpolarization induced by isoprenaline (Figure 6 and Table 2).

ICI 118551 (100 nM) did not itself modify either the mechanical tone of the tissue or the ongoing electrical behaviour of the impaled cell. ICI 118551 (100 nM) also failed to modify electrical and mechanical responses of the trachea to cromakalim (Table 2). In contrast ICI 118551 (100 nM) significantly reduced both the relaxation induced by isoprenaline and the ability of isoprenaline to hyperpolarize the trachealis cells (Figure 6 and Table 2).

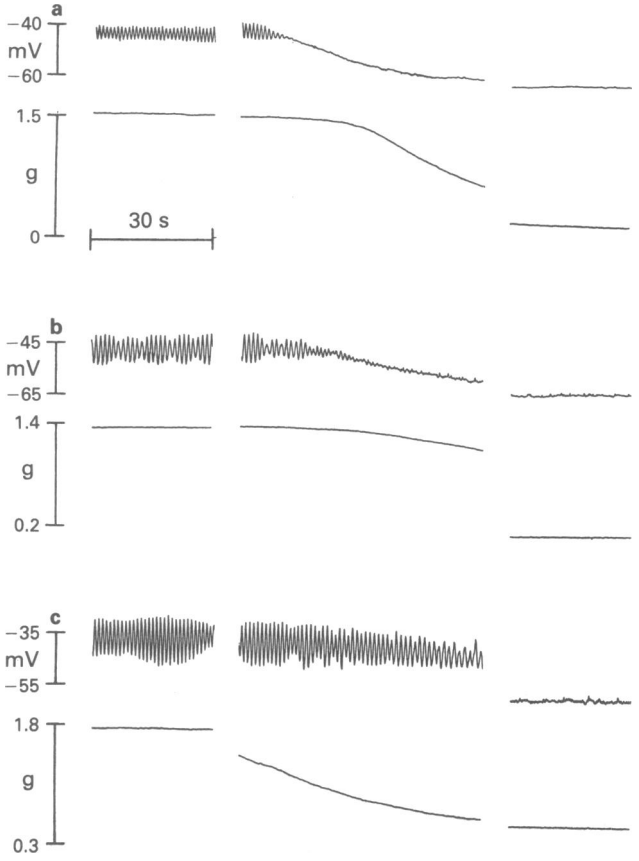


Figure 4 The effects of (a) isoprenaline (100 nM) (b) salbutamol (1 μM) and (c) procaterol (10 nM) on the electrical and mechanical activity of guinea-pig isolated trachealis. In each row of recordings the upper trace represents membrane potential changes recorded from a single cell while the lower trace represents tension changes recorded from a contiguous segment of trachea. In each row the left hand panel represents control activity recorded immediately prior to the administration of the agonist at β-adrenoceptors. The centre panels show activity recorded 45 s after the administration of the β-adrenoceptor agonist. The right hand panels indicate activity recorded 4 (isoprenaline), 5 (salbutamol) or 6 min (procaterol) after the administration of the β-adrenoceptor agonist.

Table 2 Some drug effects on the electrical and mechanical activity of guinea-pig isolated trachealis muscle

Agent (s)	*Hyperpolarization* (mV)	*Relaxation* (g)	(*n*)
Isoprenaline (100 nM)	13.6 ± 1.3*	1.20 ± 0.10	9
Isoprenaline (100 nM) + CGP 20712A (1 μM)	5.8 ± 1.4*†	1.10 ± 0.20	11
Isoprenaline (100 nM) + ICI 118551 (100 nM)	−0.4 ± 0.8†	0.14 ± 0.06	7
Isoprenaline (100 nM) + salmeterol (1 μM)	−4.1 ± 1.4*†	–	8
Procaterol (10 nM)	6.8 ± 2.4*	1.30 ± 0.10	9
Salbutamol (10 nM)	−0.3 ± 1.7	0.50 ± 0.10	8
Salbutamol (100 nM)	7.7 ± 1.5*	1.50 ± 0.20	9
Salbutamol (1 μM)	9.7 ± 1.6*	1.10 ± 0.10	6
Salmeterol (10 nM)	0.3 ± 1.4	0.38 ± 0.10	4
Salmeterol (100 nM)	3.4 ± 2.1	0.76 ± 0.11	12
Salmeterol (1 μM)	2.2 ± 1.1	0.89 ± 0.10	12
Cromakalim (10 μM)	23.7 ± 3.7*	0.90 ± 0.20	4
Cromakalim (10 μM) + CGP 20712A (1 μM)	21.0 ± 2.6*	0.70 ± 0.10	4
Cromakalim (10 μM) + ICI 118551 (100 nM)	22.3 ± 0.9*	0.91 ± 0.18	4
Cromakalim (10 μM) + salmeterol (1 μM)	23.0 ± 2.7*	–	4

Data indicate the means (± s.e.mean) of values from the indicated (*n*) number of tissues.
Measurements of membrane potential and tension changes were made 4, 5, 8, 6 and 14 min respectively after the administration of isoprenaline, salbutamol, cromakalim, procaterol and salmeterol.
*indicates a mean value of membrane potential change significantly ($P < 0.05$) different from zero.
†indicates a significant ($P < 0.05$) difference from the corresponding value for the agonist measured in the absence of any antagonist.

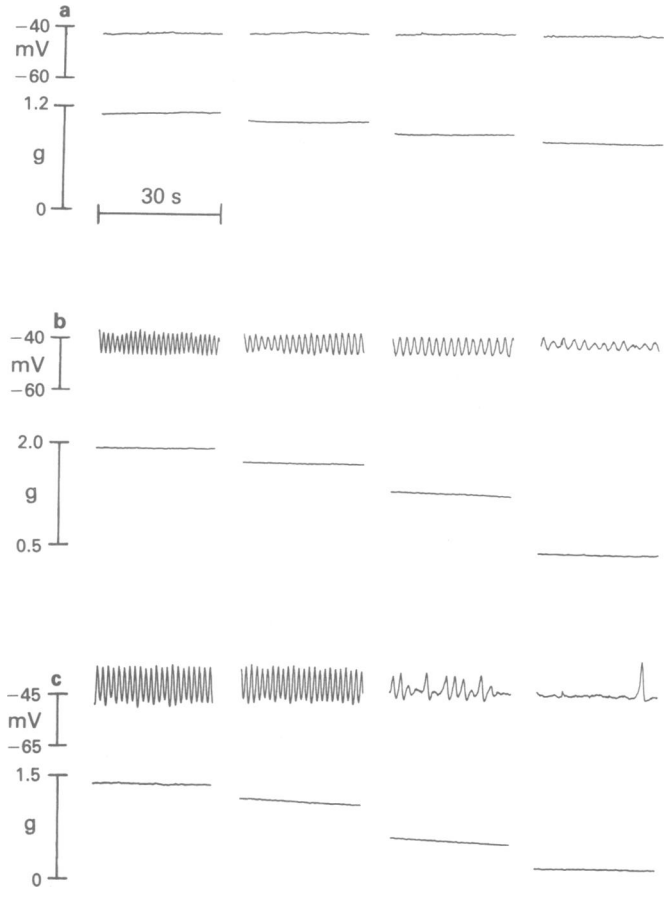


Figure 5 The effects of salmeterol 10 nM (a), 100 nM (b) and 1 μM (c) on the electrical and mechanical activity of guinea-pig isolated trachealis. In each row of recordings the upper trace represents membrane potential changes recorded from a single cell while the lower trace represents tension changes recorded from a contiguous segment of trachea. In each row the left hand panel represents control activity recorded immediately prior to the administration of salmeterol. The subsequent panels show activity recorded 1, 4 and 14 min respectively after the administration of salmeterol. Note that, at the outset, the cell illustrated in (a) lacked spontaneous electrical slow wave activity. Note also the failure of salmeterol (10 nM–1 μM) to cause cellular hyperpolarization.

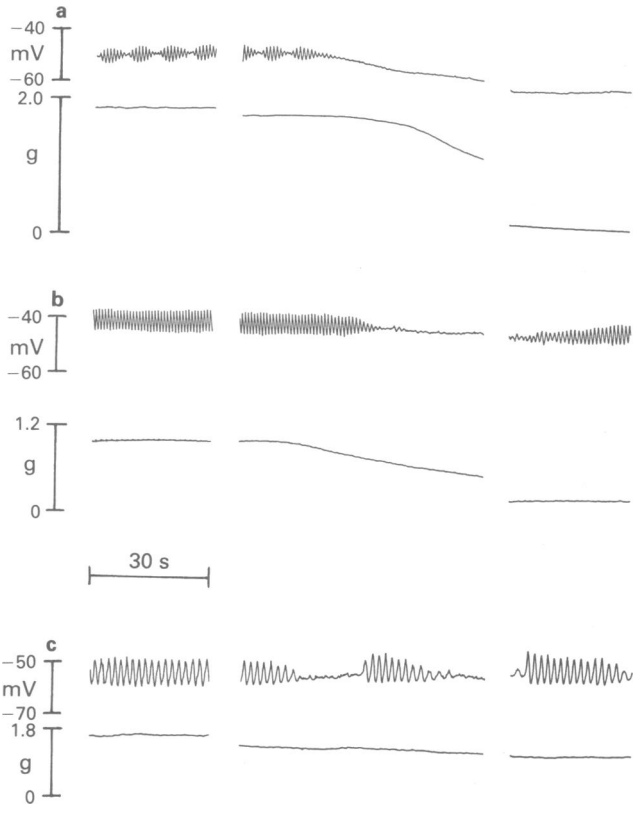


Figure 6 The effects of CGP 20712A and ICI 118551 on the electrical and mechanical responses of guinea-pig isolated trachealis to isoprenaline (100 nM). In each row of recordings the upper trace represents membrane potential changes recorded from a single cell while the lower trace represents tension changes recorded from a contiguous segment of trachea. In each row the left hand panel represents control activity recorded immediately prior to the addition of isoprenaline (100 nM). The subsequent panels show activity recorded 45 s and 4 min, respectively, after the addition of isoprenaline. (a) Shows the response to isoprenaline recorded in the absence of any modifying agent; (b) and (c) show responses to isoprenaline (100 nM) recorded from tissues equilibrated with CGP 20712A (1 μM) and ICI 118551 (100 nM) respectively. Note that CGP 20712A inhibited the hyperpolarization but not the relaxation induced by isoprenaline. Note also that ICI 118551 attenuated both the hyperpolarization and the relaxation induced by isoprenaline.

Discussion

Properties of CGP 20712A and ICI 118551

In the present study, CGP 20712A (100 nM–10 μM) failed to modify the spontaneous tone or spontaneous electrical activity of guinea-pig trachealis muscle. CGP 20712A (1 μM) failed to inhibit the tracheal relaxant action of theophylline (Table 1). Furthermore, the same concentration of CGP 20712A failed to inhibit the tracheal relaxation or the cellular hyperpolarization induced by the potassium channel opener, cromakalim (Tables 1 and 2). Collectively, these findings suggest that CGP 20712A (100 nM–10 μM) does not directly inhibit the plasmalemmal K^+-channels controlling the excitability of the trachealis cells nor the K^+-channel (K_{KCO}) involved in the action of cromakalim. The fact that CGP 20712A (100 nM–1 μM) antagonized noradrenaline in increasing atrial rate (Figure 1) without antagonizing isoprenaline, procaterol, salbutamol or salmeterol in suppressing tracheal tone (Figures 2 and 3; Table 1) is entirely consistent with reports (Dooley & Bittinger, 1984; Lemoine *et al.*, 1985) that CGP 20712A is an antagonist having very much higher (at least 10,000 times) affinity for β_1- than for β_2-adrenoceptors.

ICI 118551 (10 nM–100 nM) failed to modify the spontaneous tone or the spontaneous electrical activity of guinea-pig trachealis muscle. ICI 118551 (100 nM) failed to inhibit the tracheal relaxant action of theophylline (Table 1). Furthermore, the same concentration of ICI 118551 failed to inhibit the tracheal relaxation or the cellular hyperpolarization induced by cromakalim (Tables 1 and 2). Collectively, these observations suggest that, like CGP 20712A, ICI 118551 does not directly inhibit the plasmalemmal K^+-channels controlling the excitability of the trachealis cells nor does it directly inhibit K_{KCO}. Our observation that ICI 118551 (10–100 nM) antagonized isoprenaline, procaterol, salbutamol and salmeterol in suppressing tracheal tone without antagonizing noradrenaline in increasing atrial rate (Figures 1, 2 and 3; Table 1) is entirely consistent with reports (O'Donnell & Wanstall, 1980; Bilski *et al.*, 1983; Rimele *et al.*, 1988) that ICI 118551 is an antagonist having much higher (50–125 times) affinity for β_2-adrenoceptors than for β_1-adrenoceptors.

The results of our mechanical studies in the atria and trachea suggest that CGP 20712A (1 μM) produces marked antagonism at β_1-adrenoceptors with negligible concurrent antagonism at β_2-adrenoceptors. Similarly, it may be suggested that ICI 118551 (100 nM) produces marked antagonism at β_2-adrenoceptors with negligible concurrent antagonism at β_1-adrenoceptors.

Effects of salmeterol on the electrical and mechanical activity of trachealis muscle

Earlier reports (Ball *et al.*, 1991; Dougall *et al.*, 1991) that salmeterol exerts relaxant effects in trachealis muscle, that such effects require a protracted (>20 min) period to reach equilibrium and are poorly reversible by removal of the drug from the bathing medium have been confirmed in the present study. The claim (Ball *et al.*, 1991) that salmeterol is an agonist that is highly selective for β_2- as opposed to β_1-adrenoceptors is supported by the present findings that the tracheal relaxant action of salmeterol was antagonized by ICI 118551 but not by CGP 20712A (Figure 3 and Table 1).

Other agonists (i.e. salbutamol and procaterol) with reported selectivity for β_2- as opposed to β_1-adrenoceptors had relaxant effects in guinea-pig trachealis that were associated with hyperpolarization of the trachealis cells (Figure 4 and Table 2). In view of this, we were surprised to observe (Figure 5 and Table 2) that relaxant concentrations of salmeterol, while causing some inhibition of slow wave activity, failed significantly to modify the membrane potential of the trachealis cells.

We considered the possibility that salmeterol might, in addition to acting as an agonist at β_2-adrenoceptors, stabilize the trachealis cell membrane in a non-specific manner. A secondary action of this kind might prevent expression of any membrane hyperpolarization induced by the activation of β_2-adrenoceptors. However, salmeterol failed to reduce cromakalim-induced hyperpolarization (Table 2) suggesting that, at concentrations causing suppression of the spontaneous tone of guinea-pig trachea, salmeterol does not stabilize the plasmalemma by a non-specific mechanism.

Several groups of investigators (e.g. Ball *et al.*, 1991; Dougall *et al.*, 1991; Waldeck & Kallstrom, 1991) have reported that salmeterol is a partial rather than a full agonist at β_2-adrenoceptors. Our finding that salmeterol antagonized the relaxant action of isoprenaline (but not that of aminophylline) in carbachol-treated trachea supports this idea. It may also explain our observation that salmeterol could inhibit the hyperpolarization of trachealis cells induced by isoprenaline (Table 2). We therefore propose that the failure of salmeterol to induce significant hyperpolarization of the trachealis cells is a reflection of its low intrinsic efficacy (Dougall *et al.*, 1991) at β_2-adrenoceptors.

Subtypes of β-adrenoceptor and the activation of plasmalemmal K^+-channels

Electrophysiological studies of guinea-pig trachealis have shown that the relaxant action of isoprenaline is accompanied by cellular hyperpolarization (Allen *et al.*, 1985; Honda *et al.*, 1986) whereas no such membrane potential change accompanies the relaxant action of salmeterol (Cook & Small, 1992; Figure 5). In view of the high selectivity of salmeterol as an agonist at β_2- rather than β_1-adrenoceptors (Ball *et al.*, 1991) and the failure of salmeterol to evoke hyperpolarization of trachealis muscle, Cook & Small (1992) proposed that activation of β_1- but not β_2-adrenoceptors might promote the opening of plasmalemmal K^+-channels. However, several observations now suggest that this hypothesis is untenable.

Procaterol, salbutamol and terbutaline are all agonists selective for β_2- as opposed to β_1-adrenoceptors. Procaterol causes hyperpolarization of trachealis muscle from the guinea-pig (Figure 4) and the dog (Fujiwara *et al.*, 1988). Terbutaline (Honda *et al.*, 1986) and salbutamol (Figure 4) both induce hyperpolarization of guinea-pig trachealis muscle. Furthermore, as discussed above, the failure of salmeterol significantly to hyperpolarize guinea-pig trachealis may be attributed not to its great selectivity in activating β_2- as opposed to β_1-adrenoceptors, but instead to its low intrinsic efficacy at β_2-adrenoceptors. Therefore, although salmeterol fails to hyperpolarize trachealis cells, the ability of procaterol, salbutamol and terbutaline to induce cellular hyperpolarization suggests that the activation of β_2-adrenoceptors can promote plasmalemmal K^+-channel opening. This suggestion receives strong support from our findings (Figure 6, Table 2) that ICI 118551 (100 nM) ablated the tracheal hyperpolarization induced by isoprenaline.

Isoprenaline-induced hyperpolarization of guinea-pig trachealis was also significantly attenuated by CGP 20712A (1 μM; Figure 6; Table 2) suggesting that the activation of β_1-adrenoceptors can also promote K^+-channel opening in the plasmalemma. We thus conclude that the activation of either β_1- or β_2-adrenoceptors can promote K^+-channel opening in trachealis muscle. This conclusion receives strong support from the results of our studies of $^{86}Rb^+$ efflux from bovine trachealis (Chiu *et al.*, 1993).

Role of plasmalemmal K^+-channel opening in mediating the tracheal relaxation induced by agonists at β-adrenoceptors

In guinea-pig trachealis (Jones *et al.*, 1990; Murray *et al.*, 1991) and human bronchus (Miura *et al.*, 1992) charybdotoxin inhibits the relaxant effects of agonists at β-adreno-

ceptors such as isoprenaline and salbutamol. Since charybdotoxin has been reported to inhibit BK_{Ca} channels in airways smooth muscle (Green *et al.*, 1991; Murray *et al.*, 1991), these findings suggest that the opening of BK_{Ca} channels contributes to the relaxant activity of the agonists at β-adrenoceptors. It is commonly assumed that BK_{Ca} channel opening causes plasmalemmal hyperpolarization and therefore causes relaxation by inhibiting Ca^{2+} influx through voltage-operated channels. However, a number of findings now suggest that such a mechanism cannot play a crucial role in mediating the mechano-inhibition induced by the agonists at β-adrenoceptors.

In guinea-pig trachealis, for example, low concentrations of isoprenaline evoke relaxation without causing hyperpolarization (Allen *et al.*, 1985). In the same tissue, salmeterol is capable of fully suppressing spontaneous tone without causing significant change in membrane potential (Cook & Small, 1992; Figure 5 and Table 2). The K^+-channel inhibitor, procaine, can abolish the trachealis muscle hyperpolarization induced by isoprenaline without markedly affecting its relaxant activity (Allen *et al.*, 1985). Furthermore, CGP 20712A, an antagonist selective for β_1-adrenoceptors, can markedly attenuate the hyperpolarization but not the relaxation of guinea-pig trachealis induced by isoprenaline (Cook *et al.*, 1992; Figures 2 and 6 and Table 2). The relaxant activity of isoprenaline and salbutamol in trachealis is also largely retained when the tissue is bathed by a K^+-rich (80–120 mM) medium of osmolality similar to that of Krebs solution (Kumar, 1978; Allen *et al.*, 1985; Small *et al.*, 1989; Cook & Small, 1991). In this situation the K^+ equilibrium potential and the resting membrane potential are so closely apposed that K^+-channel opening can cause very little hyperpolarization. Accordingly, agonists at β-adrenoceptors cannot be expected to cause hyperpolarization sufficient to ensure the closure of voltage-operated Ca^{2+}-channels and hence the inhibition of Ca^{2+} influx. Unless K^+-channel opening can evoke relaxation by mechanisms not depending on hyperpolarization of the plasmalemma, we can conclude that K^+-channel opening induced by the agonists at β-adrenoceptors plays merely a supportive and not a crucial role in mediating their mechano-inhibitory effects.

S.J.C. was supported by Glaxo Group Research and P.C. by the Medical Research Council, R.C.S., R.W.F. and S.J.D. were supported by the National Asthma Campaign. CGP 20712A, cromakalim, ICI 118551, salbutamol and salmeterol were gifts from their respective manufacturers.

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(Received December 7, 1992
Revised March 4, 1993
Accepted March 30, 1993)

Br. J. Pharmacol. (1993), **109**, 1149–1156 © Macmillan Press Ltd, 1993

β-Adrenoceptor subtypes and the opening of plasmalemmal K^+-channels in bovine trachealis muscle: studies of mechanical activity and ion fluxes

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1 Studies of mechanical activity and $^{86}Rb^+$ efflux have been made in bovine isolated trachealis with the objectives of (a) identifying which of the β-adrenoceptor subtypes mediates the opening of plasmalemmal K^+-channels, (b) gaining further insight into the properties of the novel, long-acting β_2-adrenoceptor agonist, salmeterol and (c) clarifying the role of K^+-channel opening in mediating the mechano-inhibitory actions of agonists at β-adrenoceptors.

2 In bovine trachealis muscle strips precontracted with histamine (460 μM), isoprenaline (0.1 nM–1 μM), procaterol (0.1–10 nM) and salmeterol (0.1–10 nM) each caused concentration-dependent relaxation.

3 ICI 118551 (10 nM–1 μM) antagonized isoprenaline, procaterol and salmeterol in suppressing histamine-induced tone of the isolated trachealis muscle. The antagonism was concentration-dependent. In contrast, CGP 20712A (10 nM–1 μM) failed to antagonize isoprenaline, procaterol or salmeterol.

4 Salmeterol (1– 10 μM) antagonized isoprenaline in relaxing strips of bovine trachea which had been precontracted with carbachol (1 μM).

5 Cromakalim (10 μM), isoprenaline (100 nM–10 μM), procaterol (10 nM–1 μM) and salbutamol (100 nM–10 μM) each promoted the efflux of $^{86}Rb^+$ from strips of bovine trachealis muscle preloaded with the radiotracer. In contrast, salmeterol (100 nM–10 μM) failed to promote $^{86}Rb^+$ efflux.

6 CGP 201712A (1 μM), ICI 118551 (100 nM) and salmeterol (1 μM) did not themselves modify $^{86}Rb^+$ efflux from trachealis muscle strips, nor did they affect the promotion of $^{86}Rb^+$ efflux induced by cromakalim (10 μM). In contrast, CGP 20712A (1 μM) and ICI 118551 (100 nM) were each able to inhibit the promotion of $^{86}Rb^+$ efflux induced by isoprenaline (1 μM) or procaterol (100 nM). Furthermore, salmeterol (10 μM) inhibited isoprenaline (1 μM)-induced promotion of $^{86}Rb^+$ efflux.

7 It is concluded that, in bovine trachealis, activation of either β_1- or β_2-adrenoceptors can promote the opening of $^{86}Rb^+$-permeable K^+-channels in the plasmalemma. The failure of salmeterol to promote plasmalemmal K^+-channel opening may reflect, not its selectivity in activating β_2- as opposed to β_1-adrenoceptors, but rather its low intrinsic efficacy at β_2-adrenoceptors. The opening of plasmalemmal K^+-channels plays a supportive rather than a crucial role in mediating the mechano-inhibitory effects of agonists at β-adrenoceptors acting on trachealis muscle.

Keywords: Trachealis muscle; $^{86}Rb^+$ efflux; β-adrenoceptor subtypes; K^+-channels; isoprenaline; procaterol; salbutamol; salmeterol; CGP 20712A; ICI 118551

Introduction

As background to the experiments described in the preceding paper (Cook *et al.*, 1993) we reviewed the substantial evidence that suggests that agonists at β-adrenoceptors relax airways smooth muscle by mechanisms associated with the opening of plasmalemmal K^+-channels. That such K^+-channel opening is mediated by the activation of β-adrenoceptors is indicated by the ability of propranolol to inhibit the hyperpolarization of airways smooth muscle induced by isoprenaline (Ito & Tajima, 1982; Allen *et al.*, 1985; Honda *et al.*, 1986). However, since propranolol has approximately equal affinity for β_1- and β_2-adrenoceptors, this agent does not help in the identification of the β-adrenoceptor subtype responsible for promoting the K^+-channel opening.

In order to shed more light on (a) the identity of the β-adrenoceptor subtype mediating K^+-channel opening, (b) the importance of K^+-channel opening to the mechano-inhibitory effects of agonists at β-adrenoceptors and (c) the pharmacological properties of salmeterol (Ball *et al.*, 1991; Dougall *et al.*, 1991), we studied mechanical and electrophysiological changes in guinea-pig isolated trachealis (Cook *et al.*, 1993). In these studies of guinea-pig trachealis, the membrane hyperpolarization induced by agonists at β-adrenoceptors was used as an index of plasmalemmal K^+-channel opening. The present work with bovine trachealis muscle was carried out in parallel with the studies of guinea-pig tissue and had identical objectives. In the case of bovine trachealis, we have used drug-induced promotion of $^{86}Rb^+$ efflux as an index of the ability of the agonists at β-adrenoceptors to promote K^+-channel opening. The results of our electrophysiological (Cook *et al.*, 1993) and ion flux measurements (present study) both suggest that (a) activation of either β_1- or β_2-adrenoceptors can promote K^+-channel opening, (b) that K^+-channel opening and the consequential cellular hyperpolarization do not play a crucial role in the process by which agonists at β-adrenoceptors induce relaxation and (c) that salmeterol fails to promote K^+-channel opening by virtue of its low intrinsic efficacy at β_2-adrenoceptors. Some of our findings have been the subject of preliminary reports (Chiu *et al.*, 1992; Cook *et al.*, 1992; Cook & Small, 1992).

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Methods

Tissue preparation

Bovine tracheae were collected from the local abattoir and transported to the laboratory immersed in cold Krebs solution. The trachea was opened by cutting longitudinally, diametrically opposite the trachealis muscle. A segment of tracheal wall containing the trachealis muscle was then dissected from the opened organ and pinned out under Krebs solution with the adventitial surface uppermost. Adventitial tissue was carefully dissected from the preparation to expose the dorsal surface of the trachealis muscle. The preparation was then pinned out mucosal surface uppermost and the mucosal and submucosal tissue were carefully removed in order to expose the ventral surface of the trachealis muscle. Strips (approximately 1.25 cm in length) of the cleaned trachealis muscle were prepared by cutting in the longitudinal axis of the muscle bundles.

Mechanical studies of bovine trachealis muscle

Strips of bovine trachealis were set up in Krebs solution containing indomethacin (5.6 μM). Isometric recording of tension changes was performed with an initial, imposed tension of 2 g. Following the initial setting up of each tissue, three exchanges of bath fluid were performed at 15 min intervals. Following the first two exchanges of bath fluid, the imposed tension was readjusted to 2 g. One hour after the initial setting up of each tissue, histamine (460 μM) was added to the bath fluid in order to induce tissue tone. When the contractile response to histamine had equilibrated (20 min) study of a relaxant drug commenced.

The effects of isoprenaline (0.1 nM–1 μM) or procaterol (0.1 nM–10 nM) were studied by constructing, within a single tissue, a cumulative log concentration-effect curve for the relaxant. In both cases, increments in drug concentration were made at 4 min intervals. When the relaxant effect of the highest tested concentration of the agonist at β-adrenoceptors had reached a plateau, aminophylline (1 mM) was added to determine zero tissue tone. All relaxant responses to the agonists at β-adrenoceptors were measured in terms of the maximal response to aminophylline.

In the case of isoprenaline, construction of the initial log concentration-effect curve was followed by washout of the isoprenaline, aminophylline and histamine. An antagonist at β-adrenoceptors (test tissues) or vehicle (time-matched control tissues) was then added to the bath fluid; 45 min later, histamine (460 μM) was again added to the bath fluid and, after a further 20 min, the log concentration-effect curve for isoprenaline was reconstructed in the presence of the antagonist (test tissues) or its vehicle (control tissues). In the case of procaterol only one log concentration-effect curve was constructed in a single tissue. Such tissues were pre-incubated (65 min) with vehicle or an antagonist at β-adrenoceptors before the log concentration-effect curve for procaterol was constructed in the presence of the antagonist or its vehicle. The relaxant effects of salmeterol (0.01–100 nM) were studied in a fashion similar to that described for procaterol. However, only one concentration of salmeterol (tissue contact time 90 min) was tested on each tissue. All relaxant responses to salmeterol were corrected for the tone loss observed over the same period of time in the vehicle-treated control tissues. A further series of experiments was performed in order to assess whether salmeterol acts as a partial agonist at β_2-adrenoceptors. In these experiments salmeterol (1 or 10 μM) was added to the Krebs solution bathing test tissues; 60 min later, carbachol (1 μM) was added in order to induce tissue tone. Once the spasmogenic response to carbachol had reached a plateau (30 min), a cumulative log concentration-effect curve for isoprenaline was constructed (in the presence of salmeterol), essentially as

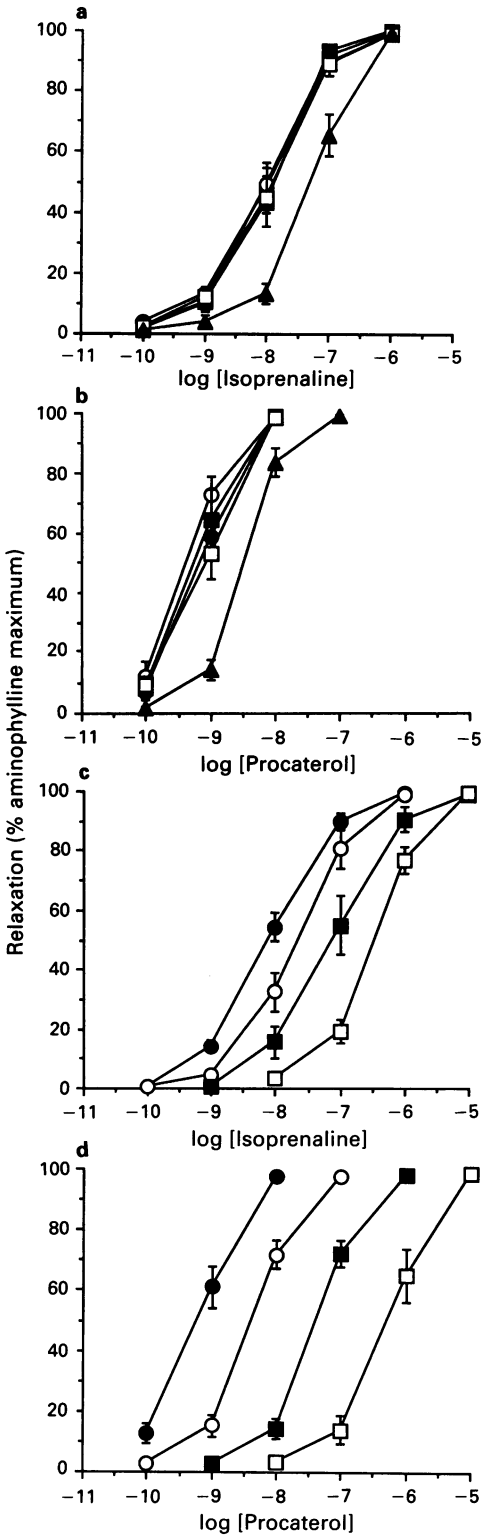


Figure 1 Bovine trachealis pre-contracted with histamine (460 μM): the effects of CGP 20712A and ICI 118551 against the relaxant actions of isoprenaline and procaterol. The abscissae indicate the molar concentration of isoprenaline (panels a and c) or procaterol (panels b and d) on a log scale. The ordinates indicate relaxation expressed as a percentage of the maximal relaxation induced by aminophylline. In each panel, (●) indicates the log concentration-effect curve of the agonist at β-adrenoceptors as observed in the absence of antagonist; (a) and (b) show the effects of isoprenaline and procaterol, respectively, as observed in the presence of CGP 20712A 10 nM (○), 100 nM (■), 1 μM (□) and 10 μM (▲); (c) and (d) show the effects of isoprenaline and procaterol, respectively as observed in the presence of ICI 118551 10 nM (○), 100 nM (■) and 1 μM (□). Data points indicate the means of values from tissue strips from at least 6 tracheae. Where not contained within the plot symbol, vertical bars indicate s.e.mean.

described above. Time-matched control tissues were treated similarly to test tissues but were not exposed to salmeterol.

Studies of $^{86}Rb^+$ efflux from bovine trachealis

The efflux of $^{86}Rb^+$ from bovine trachealis was studied essentially as described by Longmore *et al.* (1991). In brief, strips of bovine trachealis were impaled on hypodermic needles and loaded with $^{86}Rb^+$ by incubation for 90 min in Krebs solution maintained at 37°C and gassed with a mixture of 95% O_2 and 5% CO_2. This loading medium contained 185 kBq ml^{-1} $^{86}Rb^+$ and the Rb concentration was less than 50 μM. Following loading with the radiotracer, each tissue strip was transferred to the first of a series of efflux tubes containing 3 ml of Krebs solution gassed with a mixture of 95% O_2 and 5% CO_2 and maintained at 37°C. Each tissue strip was transferred to the next tube in the series at 4 or 2 min intervals. At the end of the efflux period the contents of each efflux tube was assayed for radioactivity in a gamma counter. Each tissue strip was blotted and similarly assayed for radioactivity. In the case of test tissues, cromakalim (10 μM) or an agonist at β-adrenoceptors was present in the efflux medium for a period of 20 (50 in the case of salmeterol) min. This period of tissue exposure to cromakalim or an agonist at β-adrenoceptors started at least 28 (and generally 40) min after the beginning of the efflux period, at which time the efflux rate coefficient had assumed a relatively low and slowly-changing value. The β-adrenoceptor agonists tested were isoprenaline (100 nM, 1 μM or 10 μM), procaterol (10 nM, 100 nM or 1 μM), salbutamol (100 nM, 1 μM or 10 μM) and salmeterol (100 nM, 1 μM or 10 μM). In the case of time-matched control tissues, the efflux medium, over the same time period, contained the appropriate vehicle for the agonist at β-adrenoceptors. In experiments where CGP 20712A (1 μM), ICI 118551 (100 nM) or salmeterol (1 or 10 μM) were tested as potential inhibitors of the $^{86}Rb^+$ efflux induced by cromakalim or one of the tested agonists at β-adrenoceptors, the inhibitor under test (test tissues) or its vehicle (control tissues) was present in the efflux medium for the whole of the efflux period. For both test and control tissues, the inducer of $^{86}Rb^+$ efflux was added to the bathing medium for a period of 20 min commencing 40 min after the start of the efflux period.

Drugs and solutions/statistical analysis of results

Drug concentrations are expressed in terms of the molar concentration of the active species. The following substances were used: aminophylline (BDH), CGP 20712A (2-hydroxy-5-(2-((2-hydroxy-3-(4-((1-methyl-4-trifluoro-methyl)-1H-imidazole-2-yl)-phenoxy)propyl)amino)ethoxy)-benzamide monomethane sulphonate; Ciba-Geigy Pharmaceuticals), carbachol chloride (Sigma), cromakalim (SmithKline Beecham Laboratories), histamine hydrochloride (Sigma), ICI 118551 (erytho-DL-1-(7-methylindan-4-yloxyl)-3-isopropylaminobutan-2-ol hydrochloride; ICI Pharmaceuticals), indomethacin (Sigma), (−)-isoprenaline hydrochloride (Sigma), procaterol (Sigma), salbutamol (Glaxo Group Research), salmeterol (Glaxo Group Research).

Stock solutions of most drugs were prepared in twice-distilled water. A stock solution of cromakalim was prepared in 70% ethanol. Isoprenaline, procaterol, salbutamol and salmeterol stock solutions were prepared in 0.1 M HCl. Dilutions of the isoprenaline stock solution were made in distilled water containing 0.57 mM ascorbic acid. The Krebs solution used in the tissue bath experiments and in the studies of $^{86}Rb^+$ efflux had the following composition (mM): Na^+ 143.5, K^+ 5.9, Ca^{2+} 2.6, Mg^{2+} 1.2, Cl^- 127.6, HCO_3^- 25, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2 and glucose 11.1. This solution (pH 7.4) was maintained at 37°C and was gassed with a mixture of 95% O_2:5% CO_2. The significance of differences between means was assessed by use of a two-tailed, unpaired t test. The null hypothesis was rejected when $P<0.05$.

Results

Mechanical studies of bovine trachealis

The addition of histamine (460 μM) to the indomethacin (5.6 μM)-containing Krebs solution evoked tension development which reached its peak value approximately 15 min after histamine addition. Thereafter the developed tension fell slightly to a plateau, which was either well-sustained or which exhibited a slow decline.

Isoprenaline (0.1 nM–1 μM), procaterol (0.1 nM–10 nM) and salmeterol (0.01 nM–100 nM) each produced concentration-dependent suppression of histamine-induced tone in the trachealis strips. The maximal effects of each of the three agonists at β-adrenoceptors were equivalent to those of aminophylline (Figures 1 and 2). CGP 20712A (10 nM–1 μM) did not itself induce tension development, nor did it modify histamine-induced tone. Furthermore, CGP 20712A (10 nM–1 μM) did not antagonize isoprenaline, procaterol or salmeterol in suppressing histamine-induced tone (Table 1 and Figures 1 and 2). When the concentration of CGP 20712A was raised to 10 μM, it caused minor (five fold, three fold and seven fold, respectively) antagonism of isoprenaline, procaterol and salmeterol (Table 1).

Like CGP 20712A, ICI 118551 (1 nM–1 μM) did not itself induce tension development by the trachealis muscle, nor did it modify histamine-induced tone. However, ICI 118551 produced concentration-dependent antagonism of isoprenaline,

Table 1 The effects of CGP 20712A and ICI 118551 against the actions of some agonists at β-adrenoceptors in suppressing histamine (460 μM)-induced tone of bovine isolated trachealis

	Time-matched control		*CGP 20712A*		
Agonist	(no antagonist)	10 nM	100 nM	1 μM	10 μM
Isoprenaline	7.95 ± 0.07	7.98 ± 0.14	7.88 ± 0.16	7.90 ± 0.16	7.26 ± 0.11*
Procaterol	9.01 ± 0.04	9.28 ± 0.05	9.20 ± 0.13	9.03 ± 0.12	8.48 ± 0.05*
Salmeterol	9.28 (9.13–9.44)	–	–	9.29 (9.04–9.61)	8.42* (8.27 ± 8.59)
	Time-matched control		*ICI 118551*		
Agonist	(no antagonist)	1 nM	10 nM	100 nM	1 μM
Isoprenaline	8.08 ± 0.10	8.19 ± 0.12	7.64 ± 0.14*	7.10 ± 0.19*	6.46 ± 0.07*
Procaterol	9.19 ± 0.11	9.15 ± 0.11	8.37 ± 0.08*	7.37 ± 0.07*	6.33 ± 0.11*
Salmeterol	9.28 (9.13–9.44)	9.23 (9.01–9.48)	8.19* (8.07–8.32)	7.39* (7.24–7.57)	6.34* (6.08–6.68)

For isoprenaline and procaterol the data indicate mean (± s.e.mean) values of $-\log_{10} EC_{50}$. For salmeterol the mean $-\log_{10} EC_{50}$ and its lower and upper 95% confidence limits are shown. For all agents, data from at least 6 tissues contribute to the mean. A dash indicates a measurement not performed.
*indicates a value significantly ($P<0.05$) smaller than that observed in the absence of the relevant antagonist.

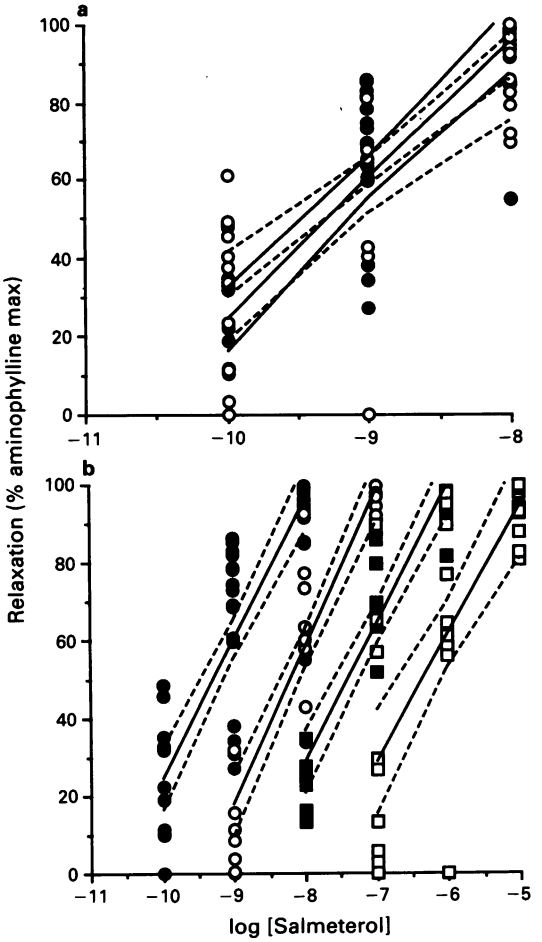


Figure 2 Bovine trachealis pre-contracted with histamine (460 μM): the effects of CGP 20712A and ICI 118551 against the relaxant action of salmeterol. The abscissae indicate the molar concentration of salmeterol on a log scale. The ordinates indicate relaxation expressed as a percentage of the maximal relaxation induced by aminophylline. In (a) the data points indicate responses to salmeterol as observed in the absence (●) and presence (○) of CGP 20712A (1 μM). The linear regression of response upon log molar concentration of salmeterol as observed in the absence of CGP 20712A (1 μM), and its 95% confidence limits are represented by the solid lines. The equivalent regression line and 95% confidence limits observed in the presence of CGP 20712A (1 μM) are represented by the broken lines. In (b) the data points indicate responses to salmeterol as observed in the absence of (●) and presence of ICI 118551 10 nM (○), 100 nM (■), 1 μM (□). In (b) the solid lines indicate the linear regression of response against log molar concentration of salmeterol as observed in the absence and presence of the various concentrations of ICI 118551 tested. The broken lines indicate the upper and lower 95% confidence limits of each regression line.

procaterol and salmeterol (Table 1 and Figures 1 and 2).

The application of carbachol (1 μM) to indomethacin (5.6 μM)-treated strips of bovine trachealis evoked spasm which required 30 min to approach its peak value. In contrast to our observations in equivalent experiments on guinea-pig trachealis (Cook *et al.*, 1993), pretreatment of test strips of bovine trachealis with salmeterol (1 or 10 μM) did not significantly reduce the spasm evoked by carbachol. In control tissues, isoprenaline (1 nM–1 mM) caused concentration-dependent suppression of the spasm evoked by carbachol. Isoprenaline produced similar effects in the salmeterol-treated test tissues. In the presence of salmeterol, however, the log concentration-effect curve for isoprenaline was shifted rightward without change in the maximal response. The mean (± s.e.mean; $n = 6$) $\log_{10}$ concentration ratios for isoprenaline (presence of salmeterol: absence of salmeterol) measured at the level of the half-maximal response were 0.416 ± 0.12 (salmeterol 1 μM) and 0.670 ± 0.15 (salmeterol 10 μM).

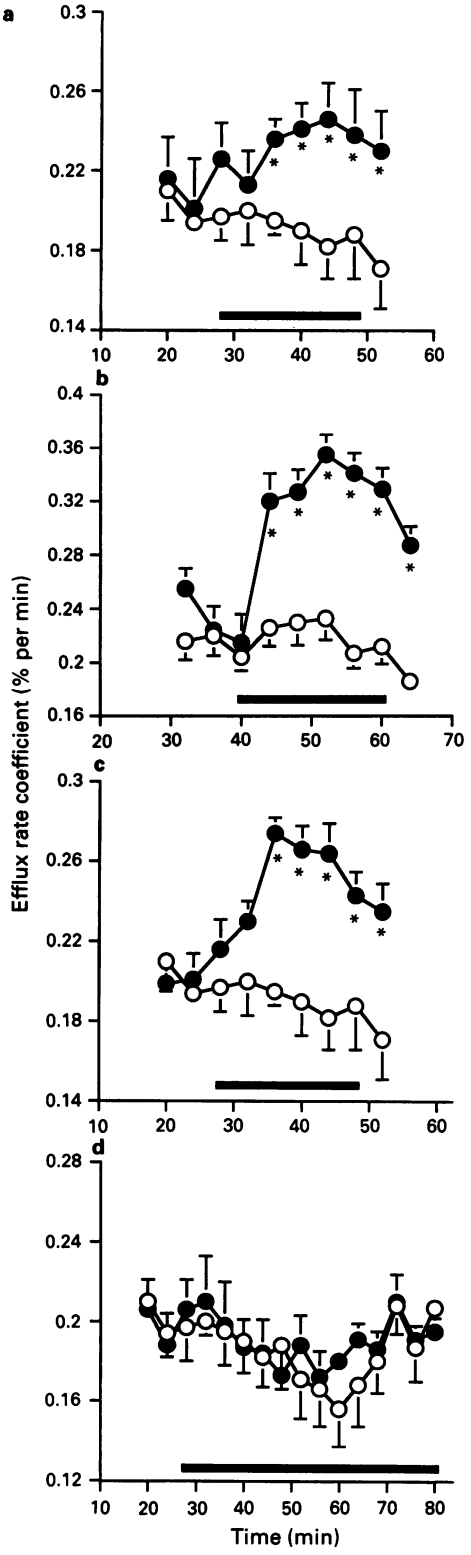


Figure 3 The effects of isoprenaline, procaterol, salbutamol and salmeterol on the efflux of $^{86}Rb^+$ from strips of bovine trachealis muscle preloaded with the radiotracer. In each panel the abscissa scale indicates the time (min) measured from the start of the efflux period. The ordinate scale indicates the efflux rate coefficient (% per min). In each panel the horizontal bar indicates the period for which an agonist at β-adrenoceptors or its vehicle was present in the efflux medium. In each panel (○) indicates time-matched, vehicle-treated control tissues while (●) indicates test tissues treated with 1 μM isoprenaline (a), 100 nM procaterol (b), 10 μM salbutamol (c) or 10 μM salmeterol (d). Data points indicate the mean ± s.e.mean of values from tissue strips from at least 6 tracheae. *indicates a significant ($P < 0.05$) difference from the corresponding data point in the vehicle-treated control tissues. Note that, in contrast to the other agonists at β-adrenoceptors, salmeterol did not modify the efflux of the radiotracer from the trachealis muscle.

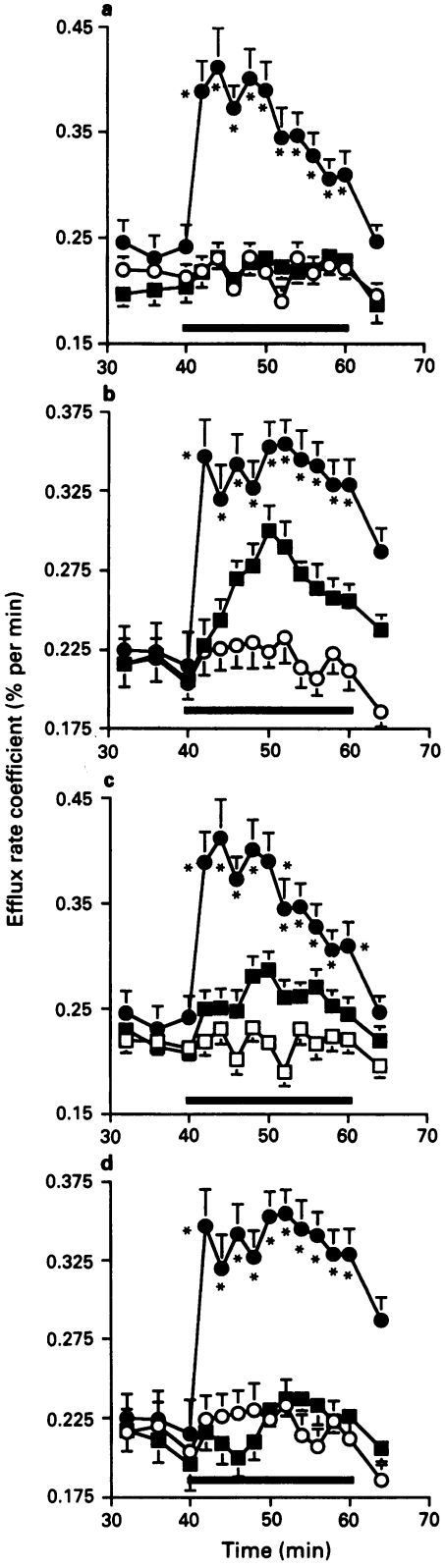


Figure 4 The effects of CGP 20712A (1 μM; a and b) and ICI 118551 (100 nM; c and d) on isoprenaline- or procaterol-induced promotion of $^{86}Rb^+$ efflux from strips of bovine trachealis muscle. In each panel the abscissa scale indicates the time (min) measured from the start of the efflux period. The ordinate scale indicates the efflux rate coefficient (% per min). Time-matched control tissues (○) were not exposed to an agonist at β-adrenoceptors. All other tissues were exposed to either isoprenaline (1 μM; a and c) or procaterol (100 nM; b and d) for the period indicated by the horizontal bar. (●) Indicates tissues exposed to an agonist, but not to an antagonist, at β-adrenoceptors; (■) indicates tissues exposed both to an agonist and to an antagonist at β-adrenoceptors. Where used, the antagonist at β-adrenoceptors was present throughout the efflux period. Data points indicate the mean of values ± s.e.mean from tissue strips from at least 6 tracheae. *indicates a significant ($P<0.05$) difference from the corresponding data point for tissues treated with both the agonist and the antagonist at β-adrenoceptors.

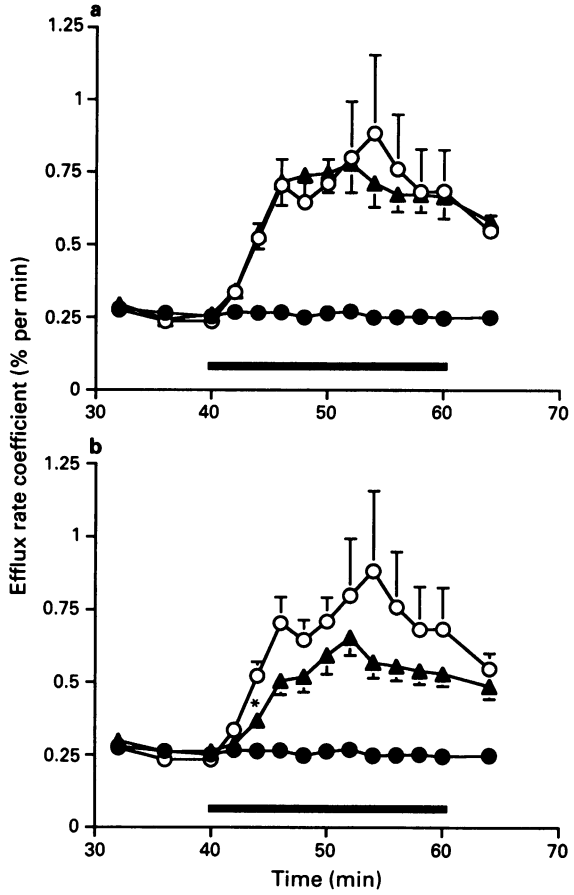


Figure 5 The effects of CGP 20712A (1 μM) and ICI 118551 (100 nM) on cromakalim-induced promotion of $^{86}Rb^+$ efflux from strips of bovine trachealis muscle. In each panel the abscissa scale indicates the time (min) measured from the start of the efflux period. The ordinate scale indicates the efflux rate coefficient (% per min). The horizontal bar indicates the period during which cromakalim (10 μM) was present in the efflux medium. In the case of test tissues (▲), CGP 20712A (1 μM; a) or ICI 118551 (100 nM; b) was present throughout the efflux period. Time-matched control tissues (○) were challenged with cromakalim but were exposed neither to CGP 20712A nor to ICI 118551. A further group of control tissues (●) were not challenged with cromakalim and were exposed neither to CGP 20712A nor to ICI 118551. In the case of this latter group of control tissues, the vehicle for the antagonist at β-adrenoceptors was present throughout the efflux period and the vehicle for cromakalim was present for the period indicated by the horizontal bar. Data points indicate the mean of values ± s.e.mean from tissue strips from at least 6 tracheae. *indicates a significant ($P<0.05$) difference between the mean efflux rate coefficient for tissues treated with a combination of cromakalim and ICI 118551 and that for tissues treated with cromakalim alone.

Studies of $^{86}Rb^+$ efflux from bovine trachealis

Fifteen min after the start of the efflux period, the $^{86}Rb^+$ efflux rate coefficient had declined to a relatively low (0.2–0.3% min^{-1}) value, suggesting that the rapidly-exchanging compartment for $^{86}Rb^+$ had cleared. This was true for both test and control tissues. In the case of the control tissues, the $^{86}Rb^+$ efflux rate coefficient thereafter fell slowly throughout the remainder of the efflux period.

Isoprenaline (100 nM, 1 μM and 10 μM), procaterol (10 nM, 100 nM and 1 μM) and salbutamol (100 nM, 1 μM and 10 μM)

each increased the $^{86}Rb^+$ efflux rate coefficient. In the case of procaterol and salbutamol, concentration-dependency of this effect was suggested by the peak amplitude of the increase in $^{86}Rb^+$ efflux rate coefficient evoked by each of the tested concentrations. In contrast to isoprenaline, procaterol and salbutamol, salmeterol (100 nM, 1 μM and 10 μM) failed to increase the $^{86}Rb^+$ efflux rate coefficient significantly (Figure 3).

CGP 20712A (1 μM) did not itself modify the $^{86}Rb^+$ efflux rate coefficient. However, CGP 20712A (1 μM) ablated the increase in $^{86}Rb^+$ efflux rate coefficient induced by isoprenaline (1 μM). It also attenuated the increase in $^{86}Rb^+$ efflux rate coefficient induced by procaterol (100 nM) (Figure 4). Like CGP 20712A, ICI 118551 (100 nM) did not itself modify the $^{86}Rb^+$ efflux rate coefficient. However, ICI 118551 (100 nM) ablated the increase in $^{86}Rb^+$ efflux rate coefficient induced by procaterol (100 nM) and markedly attenuated that induced by isoprenaline (1 μM) (Figure 4).

Cromakalim (10 μM) induced an increase in the $^{86}Rb^+$ efflux rate coefficient, thereby supporting our earlier findings with the active enantiomer of this agent, BRL 38227 (Berry *et al.*, 1992). The cromakalim-induced increase in the $^{86}Rb^+$ efflux rate coefficient was greater than that evoked by isoprenaline, procaterol or salbutamol (compare Figure 5 with Figures 3 and 4). CGP 20712A (1 μM) did not modify the promotion of $^{86}Rb^+$ efflux induced by cromakalim (10 μM) (Figure 5). In the presence of ICI 118551 (100 nM) the increase in $^{86}Rb^+$ efflux rate coefficient induced by cromakalim (10 μM) appeared slightly smaller than that evoked by cromakalim in the absence of antagonist. However, a significant difference in the $^{86}Rb^+$ efflux rate coefficient was only observed for one of the collection periods (42–44 min from the start of efflux) (Figure 5).

Pretreatment of strips of bovine trachealis muscle with salmeterol (1 μM) did not modify cromakalim (10 μM)-induced promotion of $^{86}Rb^+$ efflux (data not shown). Salmeterol (1 μM) caused an apparent reduction in the promotion of $^{86}Rb^+$ efflux induced by isoprenaline (1 μM). However, no significant differences were observed between mean values of efflux rate coefficient for tissues treated with isoprenaline alone compared with tissues treated with a combination of isoprenaline and salmeterol (Figure 6a). When the concentration of salmeterol was raised to 10 μM, it reduced the ability of isoprenaline to promote $^{86}Rb^+$ efflux (Figure 6b).

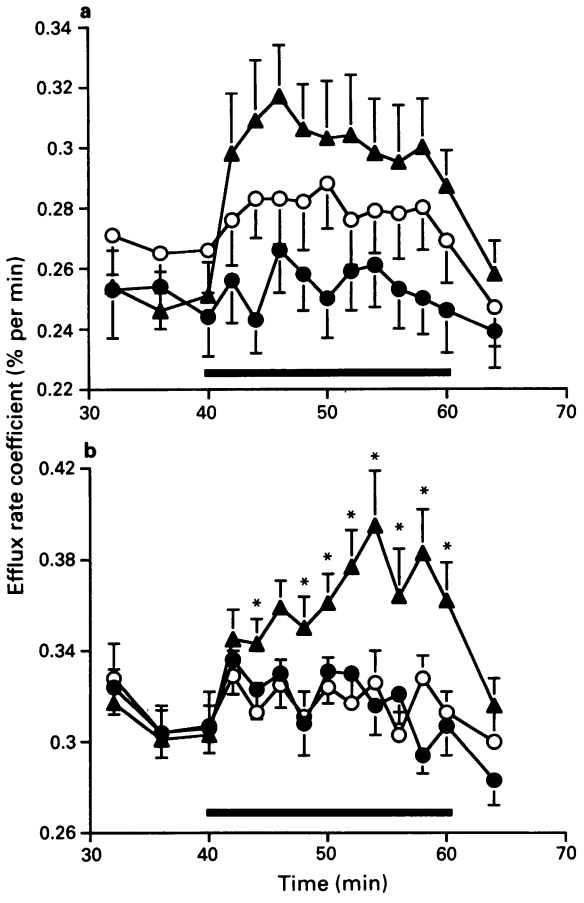


Figure 6 The effects of salmeterol (1 and 10 μM) on isoprenaline (1 μM)-induced promotion of $^{86}Rb^+$ efflux from strips of bovine trachealis muscle. In each panel the abscissa scale indicates the time (min) measured from the start of the efflux period. The ordinate scale indicates the efflux rate coefficient (% per min). The horizontal bar indicates the period during which isoprenaline (1 μM) was present in the efflux medium. Where appropriate, salmeterol 1 μM (a) or 10 μM (b) was present throughout the efflux period; (▲) indicates tissues treated with isoprenaline alone, (○) tissues treated with isoprenaline in combination with salmeterol and (●) tissues treated with salmeterol alone. Each data point indicates the mean of values ± s.e.mean from at least 6 tissues. *indicates a significant ($P<0.05$) difference from the corresponding data point for tissues treated with a combination of salmeterol and isoprenaline. Note the ability of salmeterol (10 μM) to reduce the efflux of $^{86}Rb^+$ promoted by isoprenaline.

Discussion

Properties of CGP 20712A and ICI 118551

In guinea-pig trachealis muscle, CGP 20712A (100 nM–10 μM) did not modify the spontaneous mechanical tone or electrical activity of the tissue. Furthermore, CGP 20712A (1 μM) did not inhibit the tracheal relaxation induced by theophylline or cromakalim or the hyperpolarization of guinea-pig trachealis cells induced by cromakalim (10 μM) (Cook *et al.*, 1993). In the present study, CGP 20712A (100 nM–1 μM) did not induce tension development by bovine trachealis muscle nor did it, *per se*, modify the efflux of $^{86}Rb^+$ from tissue pre-loaded with the radiotracer. CGP 20712A (1 μM) also failed to attenuate the promotion of $^{86}Rb^+$ efflux induced by cromakalim (10 μM) (Figure 5). These findings all support the assertion (Cook *et al.*, 1993) that CGP 20712A does not directly inhibit the plasmalemmal K^+-channels that regulate the excitability of the trachealis cells and does not directly inhibit the K^+-channel (K_{KCO}) involved in the action of cromakalim. Furthermore, the results of the present mechanical studies of bovine trachealis and of studies (Cook *et al.*, 1993) of guinea-pig atria and trachealis are consistent with reports (Dooley & Bittinger, 1984; Lemoine *et al.*, 1985) that CGP 20712A is an antagonist having much greater affinity for β_1- than β_2-adrenoceptors.

In guinea-pig trachealis, ICI 118551 (10–100 nM) did not modify the spontaneous mechanical tone or electrical activity of the muscle cells. ICI 118551 (100 nM) did not inhibit the tracheal relaxant actions of theophylline or cromakalim, or the cellular hyperpolarization induced by cromakalim (10 μM) (Cook *et al.*, 1993). In the present study, ICI 118551 (10 nM–1 μM) did not induce tension development by bovine trachealis muscle not did it, *per se*, modify the efflux of $^{86}Rb^+$ from tissue preloaded with the radiotracer. ICI 118551 (100 nM) also had little or no effect against the promotion of $^{86}Rb^+$ efflux induced by cromakalim (10 μM) (Figure 5). These findings all support the assertion (Cook *et al.*, 1993) that, like CGP 20712A, ICI 118551 does not directly inhibit the plasmalemmal K^+-channels that control the excitability of the trachealis cells not does it inhibit K_{KCO}. Furthermore, reports (O'Donnell & Wanstall, 1980; Bilski *et al.*, 1983; Rimele *et al.*, 1988) that ICI 118551 is an antagonist having greater affinity for β_2- than for β_1-adrenoceptors, have been substantiated by the present mechanical studies of bovine trachealis and by studies (Cook *et al.*, 1993) of guinea-pig atria and trachealis.

In view of these considerations we propose that CGP

20712A (1 μM) and ICI 118551 (100 nM) produce selective antagonism at β_1- and β_2-adrenoceptors respectively and that their blockade of the relevant β-adrenoceptor is not complicated by their acting directly to inhibit the opening of plasmalemmal K^+-channels.

Agonists at β-adrenoceptors and the opening of plasmalemmal K^+-channels in trachealis muscle

In guinea-pig trachealis, isoprenaline, procaterol and salbutamol each cause hyperpolarization (Allen *et al.*, 1985; Honda *et al.*, 1986; Cook *et al.*, 1993). The present observations (Figures 3 and 4) that isoprenaline, procaterol and salbutamol each promote the efflux of $^{86}Rb^+$ from bovine trachealis preloaded with the radiotracer, provides supportive and more direct evidence that these three agonists at β-adrenoceptors open plasmalemmal K^+-channels. The failure of salmeterol to evoke significant hyperpolarization of guinea-pig trachealis muscle (Cook *et al.*, 1993) was paralleled in the present study by its failure to promote $^{86}Rb^+$ efflux from bovine trachealis (Figure 3). These observations provide very strong evidence to suggest that, in contrast to isoprenaline, procaterol and salbutamol, salmeterol is ineffective in provoking the opening of plasmalemmal K^+-channels in trachealis muscle. Studies of guinea-pig trachealis pre-contracted with carbachol (Ball *et al.*, 1991; Dougall *et al.*, 1991; Waldeck & Kallstrom, 1991; Cook *et al.*, 1993) have indicated that salmeterol is a partial rather than a full agonist at β_2-adrenoceptors. The partial agonist activity of salmeterol was not revealed by comparing the maximal relaxant effects of isoprenaline and salmeterol in histamine-precontracted bovine trachealis (Figure 1 and 2). However, the present observation that salmeterol can antagonise isoprenaline in relaxing bovine trachealis precontracted with carbachol lends support to the results of the earlier studies of guinea-pig tissue. In the present experiments, salmeterol (100 nM–10 μM) itself failed to promote the efflux of $^{86}Rb^+$ from bovine trachealis muscle (Figure 3) yet, in a concentration of 10 μM, it attenuated the increase in $^{86}Rb^+$ efflux induced by isoprenaline. That salmeterol (1 μM) did not exert a non-specific effect in inhibiting plasmalemmal K^+-channels is suggested by its failure to modify $^{86}Rb^+$ efflux induced by cromakalim (10 μM). Equivalent experiments with salmeterol (10 μM) were not performed. However, that salmeterol (10 μM) does not act as a non-specific inhibitor of plasmalemmal K^+-channels is suggested by its failure to evoke tracheal spasm. That salmeterol (10 μM) does not exert a non-specific action in airways smooth muscle is also suggested by the susceptibility of its relaxant effect to ICI 118551 (1 μM) (Cook *et al.*, 1993; present study).

The present findings concerning the effects of salmeterol on $^{86}Rb^+$ efflux, parallel the electrophysiological observations (Cook *et al.*, 1993) in guinea-pig trachealis that salmeterol itself fails to cause cellular hyperpolarization but can inhibit the hyperpolarization induced by isoprenaline. We suggest that the weakness of the effects of salmeterol on $^{86}Rb^+$ efflux from trachealis muscle and on membrane potential, combined with its ability to antagonize a full agonist, may be explicable in terms of its reported (Dougall *et al.*, 1991) low intrinsic efficacy at β_2-adrenoceptors.

Subtypes of β-adrenoceptor and the activation of plasmalemmal K^+-channels

The reported high selectivity of salmeterol as an agonist at β_2-adrenoceptors (Ball *et al.*, 1991) and the failure of salmeterol to evoke hyperpolarization of guinea-pig trachealis prompted Cook & Small (1992) to propose that activation of β_1- but not β_2-adrenoceptors might promote the opening of plasmalemmal K^+-channels. However, Cook *et al.* (1993) subsequently showed that (a) isoprenaline-induced hyperpolarization of guinea-pig trachealis could be inhibited either by CGP 20712A (1 μM) or by ICI 118551 (100 nM), (b) that other agonists (salbutamol and procaterol) that selectively activate β_2-adrenoceptors could evoke tracheal hyperpolarization and (c) that the failure of salmeterol to induce hyperpolarization was attributable not to its selectivity for β_2- as opposed to β_1-adrenoceptors but, instead to its low intrinsic efficacy at β_2-adrenoceptors. In view of these findings, Cook *et al.* (1993) rejected the hypothesis that activation of β_1- but not β_2-adrenoceptors could cause K^+-channel opening. Instead, they proposed that activation of either β-adrenoceptor subtype could lead to K^+-channel opening. This new proposal receives support from the present findings that β_2-selective agonists such as procaterol and salbutamol were able to promote the efflux of $^{86}Rb^+$ from bovine trachealis muscle (Figure 3) and that CGP 20712A (1 μM) and ICI 118551 (100 nM) were each able to inhibit the efflux of $^{86}Rb^+$ induced by isoprenaline.

Since procaterol is an agonist exhibiting selectivity for β_2- as opposed to β_1-adrenoceptors (Yabuuchi, 1977; O'Donnell & Wanstall, 1985), we were surprised to observe (Figure 4) that CGP 20712A could significantly attenuate the $^{86}Rb^+$ efflux induced by this agent. It is difficult to decide whether this reflects a limitation of the β_2:β_1 selectivity of procaterol or the β_1:β_2 selectivity of CGP 20712A. However, CGP 20712A is reported (Dooley & Bittinger, 1984; Lemoine *et al.*, 1985) to have a very much greater (at least 10,000 times) affinity for β_1- than β_2-adrenoceptors and, at a concentration of 1 μM, CGP 20712A failed to antagonize isoprenaline, procaterol, salbutamol or salmeterol in relaxing either guinea-pig (Cook *et al.*, 1993) or bovine (present study) trachealis. Procaterol is reported to have 600–850 fold greater affinity for β_2-adrenoceptors than for β_1-adrenoceptors (Yabuuchi, 1977; O'Donnell & Wanstall, 1985). In view of the poorer selectivity ratio of procaterol between the two β-adrenoceptor subtypes and the fact that the concentration of procaterol chosen for use in the $^{86}Rb^+$ efflux studies was more than 100 times greater than its EC_{50} in suppressing histamine-induced tone of bovine trachealis, we ascribe the effectiveness of CGP 20712A against procaterol-induced $^{86}Rb^+$ efflux to this concentration (100 nM) of procaterol being high enough to cause some activation of β_1-adrenoceptors.

Role of plasmalemmal K^+-channel opening in mediating the relaxation of airways smooth muscle induced by agonists at β-adrenoceptors

Opening of K^+-channels could cause plasmalemmal hyperpolarization and hence a voltage-dependent inhibition of Ca^{2+} influx into trachealis cells. However, in the preceding paper (Cook *et al.*, 1993) we have reviewed the substantial evidence that now suggests that such a mechanism does not play a crucial role in mediating the mechano-inhibitory effects of the agonists at β-adrenoceptors. The ability of salmeterol to relax airways smooth muscle without hyperpolarizing the tissue (Cook & Small, 1992; Cook *et al.*, 1993) forms a novel and important part of this evidence. The present observation that salmeterol, in contrast to other agonists at β-adrenoceptors, fails to promote $^{86}Rb^+$ efflux from bovine trachealis is consistent with its lack of effect on membrane potential and provides a strong indication that the relaxant action of salmeterol does not involve the opening of plasmalemmal K^+-channels. Of course, measurements of membrane potential and $^{86}Rb^+$ efflux are somewhat indirect indices of K^+-channel activity and the outcome of patch-clamp studies with salmeterol is awaited with interest.

In conclusion we suggest that, in bovine trachealis, activation of either β_1- or β_2-adrenoceptors can promote the opening of $^{86}Rb^+$-permeable K^+-channels in the plasmalemma. The failure of salmeterol to promote plasmalemmal K^+-channel opening reflects not its selectivity in activating β_2- as opposed to β_1-adrenoceptors, but rather its low intrinsic efficacy at β_2-adrenoceptors. The opening of plasmalemmal K^+-channels plays, if anything, a supportive rather than a

crucial role in mediating the mechano-inhibitory effects of agonists at β-adrenoceptors.

S.J.C. was supported by Glaxo Group Research and P.C. by the Medical Research Council. R.C.S., R.W.F. and S.J.D. were supported by the National Asthma Campaign. CGP 20712A, ICI 118551, salbutamol and salmeterol were gifts from their respective manufacturers.

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(Received December 7, 1992
Revised March 4, 1993
Accepted March 30, 1993)

Br. J. Pharmacol. (1993), **109**, 1157–1163 © Macmillan Press Ltd, 1993

Ligand binding properties of putative β_3-adrenoceptors compared in brown adipose tissue and in skeletal muscle membranes

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1 The β-adrenoceptor population was characterized in membrane preparations from rat brown adipose tissue (BAT) and from soleus muscle by use of the radioligand [^{125}I]-iodocyanopindolol ([^{125}I]-ICYP). In addition, atypical binding sites for [^{125}I]-ICYP found in both tissues were examined, and the relationship between these sites and the putative rat β_3-adrenoceptor is discussed.

2 It was established that BAT membranes host a mixed population of β_1- and β_2-adrenoceptors. Of these two sites, 55% showed a high affinity for the β_1-selective ligand CGP 20712A (pK 8.5), and 45% showed a high affinity for the β_2-selective antagonist ICI 118551 (pK 8.6). Soleus muscle membranes were found to host a population of β_2-adrenoceptors, characterized by a high affinity for ICI 118551 (pK 9.1), but β_1-adrenoceptors could not be detected in this preparation. 5-Hydroxytryptamine receptors were not detected in either preparation.

3 In addition to β_1- and β_2-adrenoceptors, atypical binding sites were identified in both tissues using high concentrations of radioligand (0.5–0.6 nM) and in the presence of 1 μM (−)-propranolol. The atypical sites were abundant, representing 80 and 81% of the total [^{125}I]-ICYP binding sites in BAT and soleus muscle respectively. When the pK values for 11 ligands were compared, the correlation coefficient for atypical sites in BAT and soleus muscle was 0.94.

4 The atypical binding sites showed a moderate affinity for (±)-cyanopindolol (pK 7.3–7.7), poor stereoselectivity for the (+)- and (−)-enantiomers of alprenolol (<10 fold), and a low affinity for β-adrenoceptor antagonists and partial agonists in the order: (±)-cyanopindolol > (−)-alprenolol > (−)-propranolol = (±)-ICI 118551 >> (±)-CGP20712A. The affinity of these ligands for the atypical sites reflects their behaviour in functional studies of putative β_3-adrenoceptors in rat BAT, white adipose tissue, intestine and colon.

5 The atypical sites labelled by [^{125}I]-ICYP were resistant to agonist binding, and while the order of affinity of the agonists BRL 37344 > isoprenaline > noradrenaline matches their order of potency at putative β_3-adrenoceptors, none of these compounds caused displacement of the radioligand at concentrations below 10 μM.

6 It is concluded that the atypical binding sites for [^{125}I]-ICYP found in rat BAT and soleus muscle membranes are the same, and that these sites show some relationship to the putative rat β_3-adrenoceptor identified in functional studies using antagonists. However, under the conditions used in the present study, pK values obtained for β_3-agonist binding are not useful.

Keywords: Brown adipose tissue; skeletal muscle; putative β_3-adrenoceptors

Introduction

In addition to standard β_1- and β_2-adrenoceptors, there is strong evidence for the existence of a third β-adrenoceptor in brown and white adipose tissue (BAT and WAT) (Arch *et al.*, 1984; Hollenga & Zaagsma, 1989). This discovery was based initially on the observation that functional responses of rat BAT and WAT were not typical of those seen by the activation of standard β-adrenoceptors, and was later supported by the identification of human and rat β_3-adrenoceptor genes (Emorine *et al.*, 1989; Granneman *et al.*, 1991). As a result of this work, novel, selective β_3-agonists have been developed, and these compounds offer a new therapeutic opportunity for the treatment of obesity (Arch *et al.*, 1991; Holloway *et al.*, 1991). However, while β_3-agonists may become useful medicinal agents, their success or failure will depend in part on whether they cause any adverse or desirable effects in tissues other than fat. Thus, it would be useful to know the distribution of putative β_3-adrenoceptors in organs and tissues throughout the body.

There is evidence from studies involving a number of animal species that β_3-adrenoceptors may not be found exclusively in adipose tissue. In the rat, the strongest evidence emanates from studies of atypical β-adrenoceptors in smooth muscle of the small intestine (Van Der Vliet *et al.*, 1990), colon (Bianchetti & Manara, 1990; McLaughlin & MacDonald, 1990; Kirkham & Kelly, 1992), gastric fundus (McLaughlin & MacDonald, 1991) and oesophagus (Buckner & Christopherson, 1974). Skeletal muscle is a tissue which may host β_3-adrenoceptors also, and this could be important for two reasons. First, muscle tremor is a common response to β_2-agonist treatment and is a factor which may limit the usefulness of certain β_3-agonists as anti-obesity agents (Holloway *et al.*, 1991). This problem has been overcome partly by the development of agents with increased β_3-/β_2-adrenoceptor selectivity (Holloway *et al.*, 1991). However, the identification of β_3-adrenoceptors in skeletal muscle would suggest that alternative strategies might be needed to limit this unwanted side-effect. The second reason for determining whether skeletal muscle hosts β_3-adrenoceptors, is to predict whether β_3-agonists are likely to increase muscle growth. Because of the large amount of water which accompanies each gram of muscle protein deposited, such an effect would have a con-

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siderable impact on the pattern of weight change in an obese individual being treated with these agents.

Compared with other tissues hosting β-adrenoceptors, skeletal muscle has not been studied intensively. Challiss et al. (1988) proposed that an atypical β-adrenoceptor may mediate the metabolic responses to a novel β-agonist in rat soleus muscle, while others have identified an atypical binding site in this muscle using autoradiography (Molenaar et al., 1991). Recently it was proposed that this atypical site could be characterized by competitive ligand binding experiments using [^{125}I]-iodocyanopindolol ([^{125}I]-ICYP) as the radioligand (Roberts et al., 1991).

The radioligand binding technique offers the ability to screen rapidly many tissues for a given receptor subtype. Studies can be performed on small biopsy samples, and by this method receptors may be quantified and temporal changes in their density can be examined. Unfortunately, previous attempts to characterize the putative β_3-adrenoceptor using the ligand binding technique have not been as successful as functional experiments. Although [^{125}I]-ICYP is the radioligand with highest affinity for standard β-adrenoceptors, some workers have suggested that this compound is unsuitable for labelling atypical β-adrenoceptors (Van Der Vliet et al., 1990), while other workers (Ahmad et al., 1990) have described an atypical binding site which we believe could be an enantiomeric artefact caused by the unusually high affinity of (+)[^{125}I]-ICYP for binding to β_2-adrenoceptors (Moore et al., 1991). A number of different factors which have led to the incomplete characterization of the β-adrenoceptor population in adipose tissue by ligand binding techniques have been outlined by Arch (1989) and by Zaagsma & Nahorski (1990).

There were three aims of the present study: first, to characterize the β-adrenoceptor populations in rat soleus muscle and in BAT using the ligand binding technique; second, to compare the binding properties of atypical sites found in these two tissues; and third to determine how drug affinity constants obtained for the atypical sites compare with published values for putative rat β_3-adrenoceptors obtained from functional experiments.

Methods

Preparation of tissue homogenates

Female Wistar rats (200–250 g) were killed by cervical dislocation. Interscapular BAT was removed and the soleus muscles were dissected from each hind limb. Tissue from batches of ten rats was pooled and minced with scissors. The BAT and soleus muscles were homogenized separately; each in 10 ml of ice-cold buffer (50 mM Tris 7.0, 0.25 M sucrose, 1 mM EGTA; pH 7.4 at 4°C) using an Ultra-Turrax tissue homogenizer set at half-maximum speed and run for 10–20 s.

All centrifugations were performed at 4°C. The homogenates were first centrifuged for 10 min at 1,000 *g*. The pellets were discarded and each supernatant was filtered through a triple layer of surgical gauze. The gauze was washed with a further 2 ml of buffer and the filtrates and washings were centrifugated for 15 min at 10,000 *g*. Next, the supernatants were transfered to ultracentrifuge tubes and centrifuged for a further 30 min at 100,000 *g*. The supernatants were discarded and the pellets were each resuspended in 3 ml of ice-cold buffer (50 mM Tris 7.0, 5 mM $MgCl_2$, 1 mM EGTA; pH 7.4 at 4°C). After a further centrifugation again at 100,000 *g* for 30 min, the supernatants were discarded and the pellets were each resuspended in 1 ml of ice-cold Tris assay buffer (50 mM Tris 7.7, 10 mM $MgCl_2$, 0.15 M NaCl; pH 7.4 at 37°C) and stored at −80°C. Membrane protein concentration was determined by the Bradford Protein Assay (Bio-Rad, Richmond, U.S.A.) with bovine serum albumin standards.

Radioligand binding assays and data analysis

The same procedure was applied to BAT and soleus muscle membranes. Assays were performed in triplicate by incubating 100 μl of cell membrane suspension (incubation concentration 0.1–0.2 mg protein ml^{-1}) with 50 μl [^{125}I]-ICYP, 50 μl of GppNHp (incubation concentration 100 μM) and 50 μl of various drugs, in polystyrene tubes (12 mm × 75 mm). Assays were initiated by the addition of cell membranes, and tubes were incubated for 90 min in a shaking water bath set at 37°C and 120 cycles min^{-1}. All components of the assay were in Tris assay buffer. Ascorbic acid (2.8 mM) was added to the Tris buffer when catecholamines were used in the assay to reduce the rate of oxidation of these ligands. Separation of bound from free radioligand was achieved by filtering through Whatman GF-C glass fibre filter papers using a Brandel cell harvester (Beckman Instruments, Brisbane, Australia). Each tube was rinsed 5 times with 5 ml ice-cold Tris assay buffer. Radioactivity remaining on the filters was determined with a Gammamaster gamma counter (Linbrook, Brisbane, Australia) at a counting efficiency of 68.3%.

For saturation studies, tubes were incubated with [^{125}I]-ICYP at increasing concentrations. A range of concentrations from 1–200 pM was used determine the K_D value for binding to standard β_1- and β_2-adrenoceptors, with nonspecific binding fixed at the level defined by 1 μM (−)-propranolol. The K_D value for the binding of [^{125}I]-ICYP to atypical sites was determined using a radioligand concentration range of 5–2500 pM, with nonspecific binding defined by 0.5 mM (−)-propranolol. Specific binding to both standard and atypical sites increased linearly over the protein concentration range 0.04–0.3 mg protein ml^{-1}. Results were analysed using the Ligand computer programme (Munson & Rodbard, 1980).

Competition studies were performed with 0.5–0.6 nM [^{125}I]-ICYP. From the results of a preliminary competition study (Figure 1a), 0.5 mM (−)-propranolol was selected for use in obtaining an initial estimate of non-specific binding, while 1 μM (−)-propanolol was used to estimate binding not accounted for by β_1- or β_2-adrenoceptors. Final estimates for non-specific binding, for binding site density (B_{max}) and pK values, were obtained using the Ligand programme, allowing each parameter to float. A comparison of pK values obtained for the atypical binding sites in BAT and soleus muscle was made with the Spearman Rank Correlation Test.

Drugs

(−)-Isoprenaline, (±)-pindolol, (−)-propranolol hydrochloride, 5-hydroxytryptamine hydrochloride and all buffer salts were purchased from Sigma Chemical Co (St. Louis, U.S.A.). Bovine serum albumin was from Armour (Kanakee, U.S.A.). (−)-[^{125}I]-iodocyanopindolol was from Dupont (North Ryde, Australia). Guanylylimidodiphosphate (GppNHp) was from Boehringer Mannheim (New Farm, Australia). Alprenolol hydrogen tartrate ((+) and (−)-enantiomers) was purchased from Labkemi (Tyresol, Sweden). The following drugs were gratefully received as gifts: (±)-cyanopindolol (Sandoz, Basle, Switzerland); CGP20712A (1-[2-((3-carbamoyl-4-hydroxy)-phenoxy) ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)-phenoxy]-propan-2-ol) Ciba Geigy, Basel, Switzerland); ICI 118551 (erythro-1-(7-methylindan-4-yloxy)-3-(isopropylamino)-butan-2-ol) (ICI, Macclesfield, England); BRL 37344 (4-[2-[(2-hydroxy-2-(3-chlorophenyl)ethyl)amino]-propyl]-phenoxyacetic acid) (SmithKline Beecham, Epsom, England); (−)-noradrenaline bitartrate and (−)-adrenaline bitartrate (Sterling Winthrop, Rensselaer, U.S.A.).

Results

Brown adipose tissue membranes

Saturation studies using BAT cell membranes revealed the presence of two binding sites for [^{125}I]-ICYP. A high-affinity/

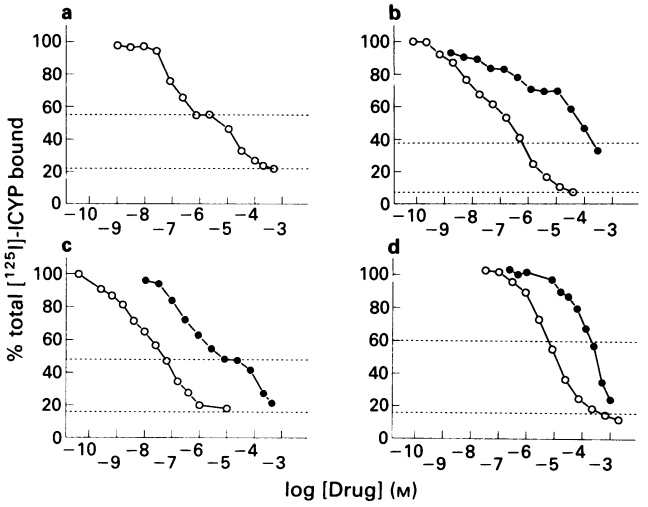


Figure 1 Representative curves showing the displacement of [^{125}I]-iodocyanopindolol ([^{125}I]-ICYP) (0.5–0.6 nM) from brown adipose tissue membranes by various competing ligands. Upper and lower horizontal lines show [^{125}I]-ICYP binding in the presence of 1 μM and 0.5 mM (−)-propranolol respectively. Competing ligands were: (a) (−)-propranolol; (b) ICI 118551 (○) and CGP20712A (●); (c) cyanopindolol (○) and pindolol (●); (d) (+)-alprenolol (○) and BRL 37344 (●).

low-density site was observed (pK 10.9 ± 0.04, B_{max} 109 ± 12 fmol mg^{-1} protein) as well as a lower-affinity/high-density site (pK 8.77 ± 0.11, B_{max} 430 ± 61 fmol mg^{-1} protein) (Figure 2).

When competition studies were performed, the high affinity/low-density [^{125}I]ICYP binding site in BAT behaved in a manner characteristic of a mixed population of β_1- and β_2-adrenoceptors. The ligands (−)-propanolol (pK 8.69 ± 0.03), cyanopindolol (pK 10.14 ± 0.20), and pindolol (pK 8.41 ± 0.07) could not distinguish between the two adrenoceptors, but their presence was revealed with the ligands CGP 20712A and ICI 118551 (Figure 1b) which are selective for β_1- and β_2-adrenoceptors respectively. CGP 20712A bound to the larger population of β_1-adrenoceptors with a high affinity (pK 8.47 ± 0.18) and to the smaller poulation of β_2-adrenoceptors with a lower affinity (pK 5.45 ± 0.12). Conversely, ICI 118551 bound to β_2-adrenoceptors with a high affinity (pK 8.58 ± 0.09) and to β_1-adrenoceptors with a lower affinity (pK 5.79 ± 0.23). ICI 118551 was among the compounds which revealed the presence of a third binding site in BAT, and because of the dominance of this third site it was difficult to assess accurately the relative proportions of β_1- and β_2-adrenoceptors present. Nevertheless, the mean proportion estimated from six competition studies using ICI 118551 and CGP 20712A was 55% β_1-adrenoceptors, 45% β_2-adrenoceptors. Competition experiments using 5-hydroxytryptamine (5-HT) failed to reveal any significant displacement of radioligand at concentrations up to 12 μM, indicating the absence of 5-HT binding sites.

In addition to standard β-adrenoceptors, the presence of a high density atypical binding site in BAT was apparent when (−)-propranolol and pindolol were used as the competing ligands. Displacement curves for these compounds were clearly bi-phasic (Figure 1a,c) and were best described by models having two binding sites ($P<0.01$). Cyanopindolol and ICI 118551 produced shallow displacement curves (Figure 1b,c) which, although not distinctly bi-phasic, were best described by models of two binding sites also ($P<0.001$). Other ligands such as (+)-alprenolol and BRL 37344 showed little selectivity between the standard β-adrenoceptors and the atypical binding site, and produced displacement curves which were steep and monophasic (Figure 1d). However, when these data are modelled, the Ligand programme calculated a level of non-specific binding which was much lower than that defined by 1 μM (−)-propranolol, but similar to that defined by 0.5 mM (−)-propranolol. In order to determine the affinity constants of (+)-alprenolol, BRL 37344, and other ligands not selective against the atypical site, displacement experiments were performed in the presence of 1 μM (−)-propranolol to block [^{125}I]-ICYP binding to β_1- and β_2-adrenoceptors. The pK values for the binding of all ligands tested to the high density atypical site are presented in Table 1.

Skeletal muscle membranes

Saturation studies using soleus muscle membranes produced results very similar to those described above for BAT. Binding parameters for [^{125}I]-ICYP to the high affinity/low density site, and to the low affinity/high density atypical site were pK 11.01 ± 0.02, B_{max} 48 ± 11 fmol mg^{-1} protein and pK 9.16 ± 0.30, B_{max} 203 ± 70 fmol mg^{-1} protein respectively (Figure 2).

In contrast to BAT, the high affinity/low density in soleus muscle behaved in a manner consistent with a homogeneous population of β_2-adrenoceptors. Binding sites which could be saturated with 1 μM (−)-propranolol (pK 8.12 ± 0.10) were occupied by the β_2-adrenoceptor-selective ligand ICI 118551 with similarly high affinity (pK 9.09 ± 0.25). Displacement studies using the β_1-selective antagonist, CGP20712A, failed to reveal any binding sites with high affinity for this ligand, consistent with the absence of β_1-adrenoceptors from the preparation. No displacement of the radioligand was observed by 5-HT at concentrations up to 1 mM, consistent with the absence of labelled 5-HT binding sites.

As with BAT membranes, the presence of high-density atypical binding site in soleus muscle was identified with (−)-propranolol, and was confirmed using the ligands cyanopindolol, pindolol and ICI 118551. All of these ligands yielded displacement curves which were best described by models having two binding sites ($P<0.05$). The pK values for other ligands were determined in the presence of 1 μM (−)-propranolol, and the results for all ligands tested are presented in Table 1.

Correlations

When the pK values for all ligands were compared, results obtained for the atypical binding site in soleus muscle correlated well with those obtained in BAT ($r = 0.944$, $P<0.01$, $n = 11$). A plot of these data is shown in Figure 3. Because the true affinity of β_3-adrenoceptor agonists might not be represented under the experimental conditions used, the data were also analysed after excluding the pK values for the compounds BRL 37344, isoprenaline, adrenaline and noradrenaline. A significant correlation between BAT and soleus muscle pK values was still observed ($r = 0.905$, $P<0.05$, $n = 8$).

Table 1 Dissociation constants for atypical ligand binding sites in brown adipose tissue (BAT) and soleus muscle membranes

Ligand	pK[a] *BAT*	*Soleus muscle*
(−)-[^{125}I]-iodocyanopindolol	8.77 ± 0.11	9.16 ± 0.30
(±)-Cyanopindolol	7.66 ± 0.19	7.33 ± 0.04
(±)-Pindolol	3.94 ± 0.09	4.03 ± 0.22
(−)-Propranolol	4.93 ± 0.10	5.00 ± 0.18
(±)-ICI 118551	5.00 ± 0.10	5.05 ± 0.09
(±)-GCGP 20712A[b]	<4.00	<4.00
(−)-Alprenolol[b]	5.37 ± 0.21	5.83 ± 0.04
(+)-Alprenolol[b]	5.02 ± 0.19	5.45 ± 0.21
(±)-BRL 37344[b]	3.87 ± 0.03	4.19 ± 0.09
(−)-Isoprenaline[b]	3.14 ± 0.16	2.32 ± 0.37
(−)-Adrenaline[b]	2.20 ± 0.07	2.61 ± 0.37
(−)-Noradrenaline[b]	1.99 ± 0.03	2.09 ± 0.07

[a]Negative log of the dissociation constant.
[b]Ligands tested in the presence of 1 μM (−)-propranolol. Values are means ± s.e.mean ($n = 3$).

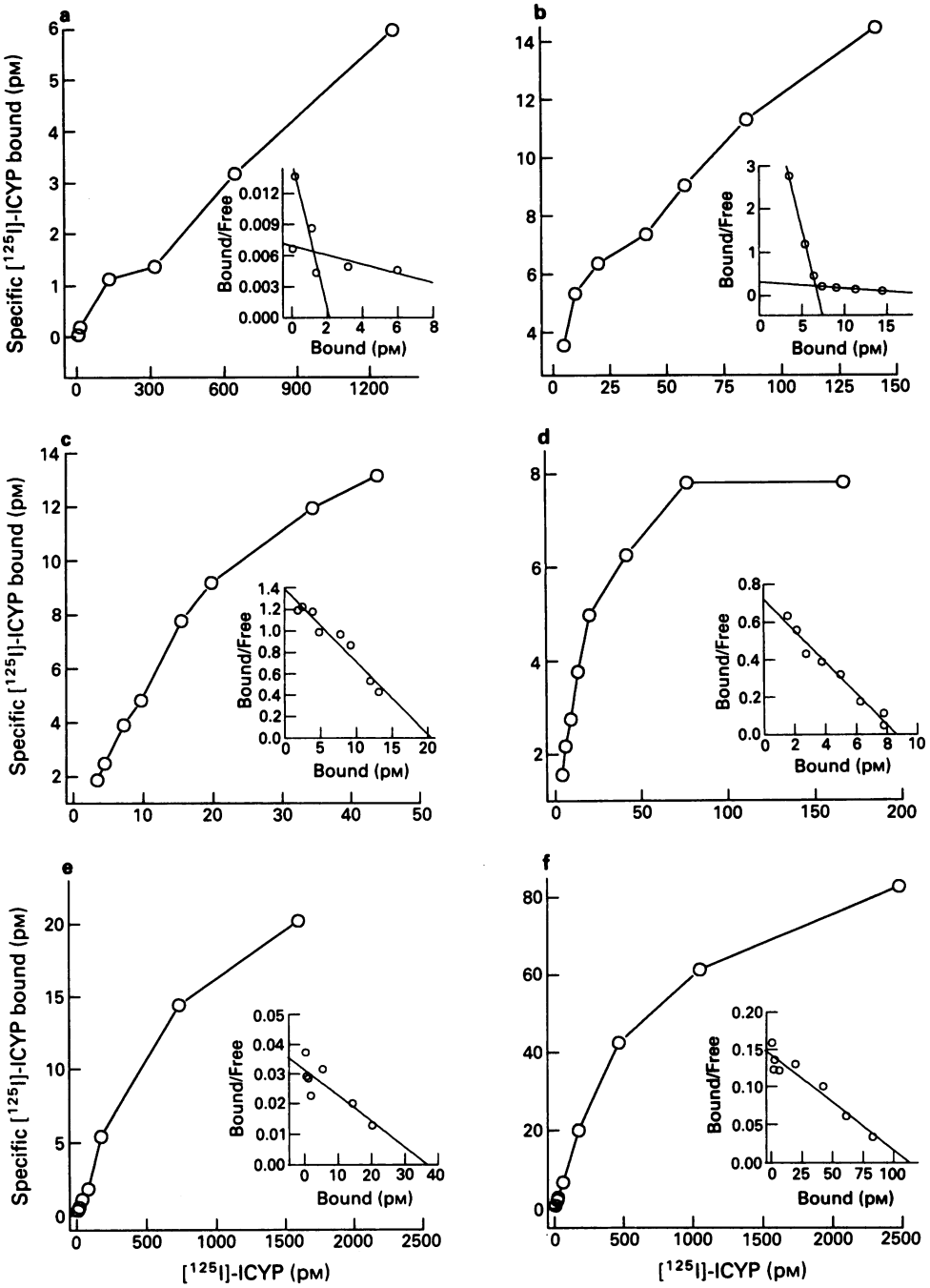


Figure 2 Specific binding of [^{125}I]-iodocyanopindolol ([^{125}I]-ICYP) to brown adipose tissue (a,c,e) and soleus muscle (b,d,f) membranes studied under different experimental conditions. (a,b) Biphasic saturation curves from preliminary experiments using low concentrations of radioligand with non-specific binding defined by use of 0.5 mM (−)-propranolol. Inset shows Scatchard analysis of these data with regression lines fitted to the two distinct binding sites. (c,d) Typical saturation studies used to determine the affinity of [^{125}I]-ICYP for β_1/β_2-adrenoceptors using low concentrations of radioligand with non-specific binding defined by use of 1 μM (−)-propranolol. Inset shows a single binding site by Scatchard analysis. (e,f) Typical saturation studies used to determine the affinity of [^{125}I]-ICYP for atypical binding sites using high concentrations of radioligand with non-specific binding defined by use of 0.5 mM (−)-propranolol. Inset shows Scatchard analysis.

Discussion

The results obtained in the present study for the identification of standard β-adrenoceptors in soleus muscle and BAT agree with those of earlier reports. Rat skeletal muscle is a well-known source of β_2-adrenoceptors (Williams *et al.*, 1984; Rothwell *et al.*, 1987; Kim *et al.*, 1991), and some workers have suggested that soleus muscle hosts a minor population of β_1-adrenoceptors (Rothwell *et al.*, 1987; Kim *et al.*, 1991). However, as in a previous study (Sillence *et al.*, 1991), we failed to detect any β_1-adrenoceptors in this tissue. By using the highly sensitive technique of autoradiography. Molenaar *et al.* (1991) found that [^{125}I]-ICYP labels a small number of 5-HT receptors in soleus muscle; however, these workers were unable to detect labelled 5-HT binding sites in competitive ligand binding experiments (R.J. Summers, personal communication) and the results of the present study confirm this latter observation. The present observation that BAT hosts a mixed population of β_1- or β_2-adrenoceptors also agrees with a number of earlier findings (for review see Arch, 1989), and the proportion of these two sites observed in the present study is in particularly close agreement with

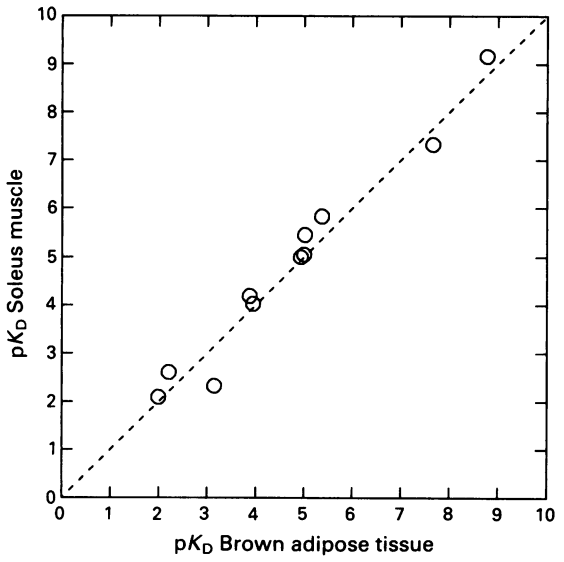


Figure 3 The relationship between atypical [^{125}I]-iodocyanpindolol binding sites in soleus muscle and brown adipose tissue. Values for pK (negative log dissociation constant) were obtained for eleven compounds from competitive ligand binding experiments.

the proportion of 59% β_1- and 41% β_2-adrenoceptors reported by Rothwell *et al.* (1985).

Previous reports of atypical binding sites being present in rat skeletal muscle are relatively few (Moore *et al.*, 1990; 1991; Kim *et al.*, 1991; Roberts *et al.*, 1991). Furthermore, when Molenaar *et al.* (1991) reported the localization of a propranolol-resistant [^{125}I]-ICYP binding site in soleus muscle, they found certain discrepancies between their autoradiography data and pA_2 values reported in the literature for the atypical receptor in rat adipocytes. This prompted Molenaar *et al.* (1991) to suggest that a family of atypical receptors may exist rather than a single β_3-adrenoceptor. To address this question we have made a direct comparison between the atypical [^{125}I]-ICYP binding sites present in soleus muscle and those in BAT. The strong correlation of binding affinities observed for the 11 ligands tested supports the conclusion that the atypical binding sites in these two tissues are the same. Furthermore, the high density atypical binding sites in both tissues (~80% of the total β-adrenoceptor population) agrees with the results of binding studies conducted recently by Fève *et al.* (1991) who reported that β_3-adrenoceptors form about 90% of the total β-adrenoceptor population in cultured 3T3-F442A adipocytes.

The final aim of this study was to determine the relationship between the atypical binding sites observed in BAT and soleus muscle and those putative β_3-adrenoceptors thought to be present in rat adipose tissue and gut from the results of functional studies. When comparing ligand binding data with the results of functional experiments, comparisons made using antagonists provide the most useful information, and this has been noted particularly for the β_3-adrenoceptor (Arch, 1989). The results published previously for antagonists and for partial agonists of the rat β_3-adrenoceptor show some relationship to those obtained in the present study in terms of stereoselectivity, order of potency and in absolute pK values.

The low stereoselectivity observed for alprenolol binding in the present study is consistent with the suggestion of Harms *et al.* (1977) that low stereoselectivity for standard β-adrenoceptor antagonists is a characteristic of atypical rat β-adrenoceptors. While this feature is useful to distinguish atypical β-adrenoceptors from standard β-adrenoceptors, it also increases the difficulty for workers using ligand binding techniques to discriminate between authentic β_3-adrenoceptors and nonspecific lipophilic binding sites. Indeed, when two independent groups observed similar atypical binding sites in smooth muscle, the observed lack of stereoselectivity for propranolol caused these workers to reject the hypothesis that these sites were related to β-adrenoceptors (Blue *et al.*, 1990; Van Der Vliet *et al.*, 1990).

If differences in pK values of less than 0.1 log units are ignored, then the order of affinity observed in the present study from the binding of partial agonists and antagonists to atypical sites is: cyanopindolol>(−)-alprenolol>(−)-propranolol = ICI 118551>>CGP20712A. This order is similar to that obtained in functional studies of the β_3-adrenoceptor when different combinations of these drugs were tested in rat WAT (Hollenga & Zaagsma, 1989; Kirkham & Kelly, 1992), colon (Kirkham & Kelly, 1992) and BAT (Arch *et al.*, 1984), guinea-pig ileum (Blue *et al.*, 1990) and cultured adipocytes (Fève *et al.*, 1991).

When comparing ligand binding data with pK values obtained from functional studies it is important to consider the experimental conditions used, and in particular to be aware of the possible contribution of multiple receptors to the response being studied. For example, the pK value observed for (−)-propranolol in the present work agrees with the value of 5.0 reported by Roberts *et al.* (1991) using ligand binding techniques. However, this value is lower than published values from functional studies against isoprenaline, which range from 6.1 in rat small intestine (Van Der Vliet *et al.*, 1990) to 7.3 for BAT lipolysis (Arch *et al.*, 1984). We suggest that the latter pK values are over-estimates which reflect the contribution of both β_1- and β_3-adrenoceptors to the functional response. This hypothesis is supported by the finding that when isoprenaline is replaced by an agonist more selective for β_3-adrenoceptors, a fall in the pK value for propranolol of between 0.4 and 0.5 log units is seen in WAT (Wilson *et al.*, 1984) and BAT (Arch *et al.*, 1984).

In contrast to the results obtained with propranolol, studies in adipose tissue which have employed ICI 118551 against isoprenaline probably give a more accurate estimate of the antagonist pK value because β_2-adrenoceptors do not contribute to the functional response, and because ICI 118551 has a relatively low affinity for β_1-adrenoceptors. This was illustrated in WAT when a difference in the pK values for ICI 118551 of less than 0.2 log units was observed when isoprenaline was replaced by the β_3-selective agonist BRL 37344 (Hollenga & Zaagsma, 1989). The pK values observed for ICI 118551 in the latter study (5.33), and in a similar study using BAT (5.7) (Arch *et al.*, 1984) are acceptably close to those of the present work. The third antagonist for which there are published data from functional studies is (±)-cyanopindolol. In 1992, Kirkham & Kelly reported the results of a study of both WAT lipolysis and colon relaxation. These workers used isoprenaline as the agonist, but carried out their experiments in the presence of 0.1 μM propranolol to attenuate the contribution of β_1- and β_2-adrenoceptors to the response. The pK values for cyanopindolol obtained by Kirkham & Kelly (1992) for lipolysis (7.5) and colon relaxation (7.0) agree well with the results of the present study.

Data for agonist binding obtained in the present study are difficult to interpret. It is a concern that extremely high drug concentrations were required to displace the radioligand in the case of all agonists tested, and these data suggest the possibility that the atypical binding sites observed in the present study are purely lipophilic sites, unrelated to atypical β-adrenoceptors. An alternative explanation is that antagonists and agonists may bind differently to the β_3-adrenoceptor, and that the active site for agonists was not occupied by the radioligand under the conditions we employed. It has been argued that the only meaningful pK values for agonists are those obtained for an induced high-affinity state, whereas under binding conditions which employ GppNHp and high concentrations of Na^+ ions, low affinity states of the receptor are strongly favoured (Molenaar *et al.*, 1991). In this regard it is noteworthy that Fève *et al.* (1991), who conducted

binding studies under slightly different conditions, using 3T3-F422A adipocytes and Chinese hamster ovary (CHO) cells transfected with the human β_3-adrenoceptor gene, observed pK values for the agonists BRL 37344, isoprenaline and noradrenaline in the same rank order as that seen in the present study, but which were higher by two orders of magnitude. In contrast, the pK values observed by Fève *et al.* (1991) for the binding of [^{125}I]-ICYP to adipocytes (8.82) and β_3-CHO cells (8.72) are strikingly similar to the pK values for this antagonist seen in the present study. It is also of interest that Fève *et al.* (1991) observed binding of similarly high affinity to both tissues by the hydrophilic radioligand (−)-[^{3}H]-CGP12177. Thus, although agonist pK values seen in the present work do not support the hypothesis that the atypical binding site is the putative β_3-adrenoceptor, it would seem unwise to reject this possibility based on agonist data alone, when the data obtained with a range of antagonists lend the hypothesis such strong support.

In summary, ligand binding studies have shown that membranes from rat BAT and soleus muscle host identical binding sites which are present in abundance in each tissue. From antagonist and partial agonist binding data these sites show a resemblance to the rat β_3-adrenoceptor described by previous workers. If it can be confirmed that these sites are indeed β_3-adrenoceptors, then other tissues could be screened for these receptors using the binding conditions described, to help predict the effects of β_3-agonists *in vivo*. In addition, rat cell membranes might provide a useful primary screen for new β_3-adrenoceptor antagonist drugs. However, we suggest that this system is not useful to obtain valid data for the binding of β_3-agonists.

The authors thank M.J. Stock for supplying the method for the membrane preparation, and are grateful to the pharmaceutical companies who kindly supplied ligands for this work. This study was supported by grants from the Australian Meat Research Corporation and the Australian Research Council. N.G.M. is a recipient of an Australian Postgraduate Research Award.

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(Received June 9, 1992
Revised September 10, 1992
Accepted March 30, 1993)

Br. J. Pharmacol. (1993), **109**, 1164–1168 © Macmillan Press Ltd, 1993

Bicuculline-resistant paired-pulse inhibition in the rat hippocampal slice

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1 An initial observation that paired-pulse inhibition in hippocampal slices was increased rather than decreased by bicuculline prompted the present study to explore the mechanism underlying bicuculline-resistant inhibition.

2 In the presence of bicuculline, paired-pulse interactions were dependent on the interpulse interval (i.p.i.) but a medium-latency inhibition was consistently observed at an i.p.i. of 300 to 500 ms.

3 The medium-latency (300 ms) bicuculline-resistant inhibition produced by paired orthodromic stimuli was substantially reduced by 2-hydroxysaclofen and was probably largely mediated by $GABA_B$-receptor activation. Paired-pulse inhibition produced by an orthodromic/antidromic stimulation sequence was not affected by 2-hydroxysaclofen suggesting the possibility that the $GABA_B$-receptors involved in orthodromic inhibition may be located presynaptically on the Schaffer collateral terminals rather than on the postsynaptic surface. The medium latency inhibition was also reduced by baclofen and under some conditions, by adenosine.

4 In addition to the $GABA_B$-component, a hydroxysaclofen-resistant depression of postsynaptic excitability contributed to bicuculline-resistant paired-pulse inhibition at the 300 ms latency.

Keywords: Paired-pulse facilitation; paired-pulse inhibition; bicuculline; hippocampus; adenosine; 2-hydroxysaclofen; baclofen

Introduction

Paired-pulse effects in the hippocampus (facilitation and inhibition) between extracellularly-recorded field potentials (single population spikes or the corresponding field e.p.s.ps) have been well characterized both *in vivo* and *in vitro*. In the CA1 region, in the hippocampal slice preparation facilitation may be seen when both conditioning and test stimuli activate the same Schaffer collateral/CA1 dendritic synapses and the inter-pulse interval (i.p.i.) between conditioning and test stimuli is greater than about 40 ms. Paired-pulse facilitation can sometimes be seen up to an i.p.i. of several seconds (Creager *et al.*, 1980; Dunwiddie *et al.*, 1980). Conversely, inhibition is most easily demonstrated at i.p.is less than 40 ms (Creager *et al.*, 1980; Dunwiddie *et al.*, 1980; Lynch *et al.*, 1981).

Paired-pulse inhibition is thought to be mainly or completely mediated by γ-aminobutyric acid (GABA)-releasing feedback and feedforward interneurones and it was therefore surprising to find, as part of another investigation, that the $GABA_A$ antagonist, bicuculline, increased paired-pulse inhibition in the CA1 area at moderately long (> 300 ms) i.p.is. In the presence of bicuculline, repetitive firing of pyramidal cells occurs following a single stimulation to the Schaffer collateral fibres. However, the prolonged population potential that reflects this multiple firing is still only about 30 ms in duration and seems unlikely to account for increased inhibition at a latency of 300 ms. Although there is a good deal of relevant information on inhibitory potentials, both in the normal and the disinhibited hippocampus, from work using intracellular recording techniques, paired-pulse interactions between extracellularly recorded potentials in the disinhibited hippocampus do not seem to have been studied systematically. The present study was therefore designed to characterize the action of bicuculline on paired-pulse interactions between these extracellular potentials in more detail and in particular to investigate the mechanisms of this medium-latency inhibition.

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Methods

Male Wistar rats (170 to 220 g) were anaesthetized with urethane and cooled on ice whilst breathing oxygen enriched air (Newman *et al.*, 1992). When their rectal temperatures reached 30°C (about 15 min) they were killed by cervical dislocation.

Hippocampal slices were prepared 450 μm thick in artificial CSF (ACSF) of composition (mmol l^{-1}): KH_2PO_4 2.2, KCl 2, $NaHCO_3$ 25, NaCl 115, $CaCl_2$ 2.5, $MgSO_4$ 1.2, glucose 10, saturated with 95% O_2:5% CO_2.

For recording, individual slices were submerged and superfused at 30°C. Drugs were added to the superfusing ACSF. Extracellular population potentials were recorded by single glass microelectrodes in the CA1 pyramidal cell layer. Concentric bipolar stimulating electrodes were placed in the stratum radiatum around the CA1/CA2 junction for orthodromic activation of pyramidal cells and in the alveus for antidromic activation. When the paired stimuli were both orthodromic they were delivered through the same electrode. Evoked responses were amplified, displayed on digital storage oscilloscopes and averaged (8 sweeps) before being plotted onto a chart recorder.

In general, single and double stimuli were alternated: test stimuli, orthodromic or antidromic, adjusted to evoke a population spike between 45% and 75% of maximum, were delivered at 0.1 Hz or 0.05 Hz and conditioning stimuli were delivered before alternate test stimuli. Responses to test stimuli which were preceded by a conditioning stimulus are designated T_{pair}. Responses to unpaired test stimuli are designated T_{single} and responses to conditioning stimuli are designated S_{cond}.

A preliminary series of experiments was carried out to examine the effects of bicuculline on paired-pulse interactions at various interpulse intervals (i.p.is) using orthodromic stimuli only.

A second series of experiments, all at an i.p.i. of 300 ms, was then carried out to examine the effect of agents on the paired-pulse inhibition seen at this latency in the presence of bicuculline. In an attempt (see Discussion) to separate pre- and postsynaptic effects (i.e. on the pyramidal cells or on the Schaffer collateral terminals), for these second experiments, we used two different stimulation paradigms, the standard

(ortho/ortho) paradigm with both conditioning and test stimuli orthodromic and a second arrangement (ortho/anti) in which the conditioning stimulus was orthodromic and the test stimulus antidromic. When assessing the action of agents on paired-pulse inhibition at this latency stimuli were adjusted in the presence of the agent, if necessary, to return T_{single} and S_{cond} to their control size (all test stimuli were still submaximal). To be certain that changes in inhibition were not, nevertheless, due to small changes in the excitatory conditioning potential, inhibition was measured with two strengths of conditioning stimulus, supramaximal (1 mA) and submaximal – producing in bicuculline a potential approximately 50 to 70% of maximal size.

In all experiments facilitation or inhibition was expressed as the percentage change in T_{pair} compared with T_{single}. Response size was taken to be the size of the population spike measured from the peak positivity to the peak negativity. In the presence of bicuculline, response size was taken to be the peak to peak size of the first population spike in each burst. Unless stated otherwise statistical analysis was by paired t test with $P<0.05$ taken to indicate statistical significance.

Slices were only accepted for experiment if the evoked maximum orthodromic population spike in ACSF after 15 min in the recording chamber was at least 4 mV and free from secondary spikes.

Baclofen, and adenosine hemisulphate were obtained from Sigma Biochemicals Ltd., (−)-bicuculline methobromide, and 2-hydroxysaclofen from Research Biochemicals Inc.

Results

Effect of bicuculline on paired-pulse interactions

In the absence of bicuculline, orthodromic stimuli normally exhibited paired-pulse inhibition at short i.p.is (less than 50 ms) and facilitation at longer i.p.is. The i.p.i. at which inhibition changed to facilitation was quite variable between slices ranging from less than 30 ms to over 300 ms (Figure 1).

As expected, in the presence of bicuculline methobromide (10 μmol l^{-1} or greater) a short burst of several population spikes replaced the single population spike seen in response to a stimulus in the absence of drugs. Orthodromic paired-pulse interactions in bicuculline also depended on the interpulse interval, both facilitation and inhibition being demonstrable. With a supramaximal conditioning stimulus two temporally distinct phases of inhibition were seen (Figure 1, solid squares). At i.p.is less than 50 ms and between 300 and 900 ms bicuculline 10 μmol l^{-1} significantly increased inhibition or decreased facilitation compared with drug-free control in the same slices. However, when the i.p.i. was 100 ms the slices showed significantly increased facilitation in the presence of bicuculline (Figure 1). At i.p.is longer than 900 ms there was a second phase of facilitation.

Effect of 2-hydroxysaclofen, baclofen and adenosine on inhibition in the presence of bicuculline

Control values All slices which showed ortho/ortho inhibition at an i.p.i. of 300 ms with supramaximal conditioning stimuli in the presence of 10 μmol l^{-1} bicuculline also showed inhibition with submaximal conditioning stimuli and with all stimuli in the ortho/anti paradigm. Inhibition in the ortho/anti paradigm was generally much less than inhibition in the ortho/ortho paradigm at either submaximal or supramaximal conditioning stimulus strength (see Figures 3, 4 and 5). In the ortho/ortho sequence inhibition was significantly less when the conditioning stimulus was supramaximal rather than submaximal ($P<0.05$, $n=22$ slices).

2-Hydroxysaclofen Superfusion with the $GABA_B$-antagonist 2-hydroxysaclofen 200 μmol l^{-1} (in the presence of bicuculline at i.p.i. = 300 ms) significantly reduced ortho/ortho

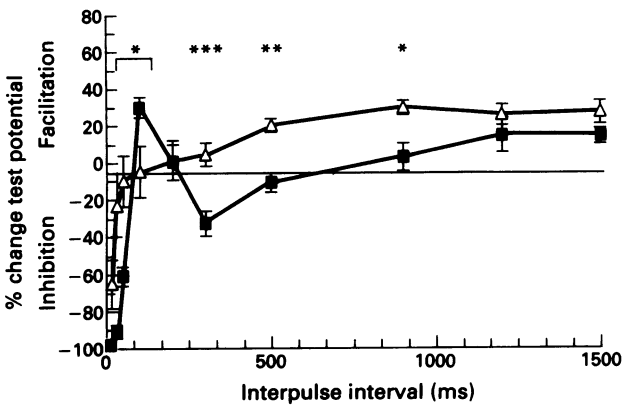


Figure 1 Influence of bicuculline methobromide 10 μmol l^{-1} on paired-pulse interactions at i.p.is up to 1500 ms, $n=9$ slices with each point being the mean of between 4 and 9 values. Paired pulse changes were recorded in the same slices in the absence (△) and presence (■) of bicuculline. The conditioning stimulus was supramaximal at 1 mA. The unpaired test stimulus was readjusted to give a response between roughly 50 and 70% of maximum (constant within each experiment) in the presence of bicuculline. All stimuli were orthodromic. Values are mean ± s.e.mean. $*P<0.05$; $**P<0.01$; $***P<0.001$ for difference between ACSF and bicuculline.

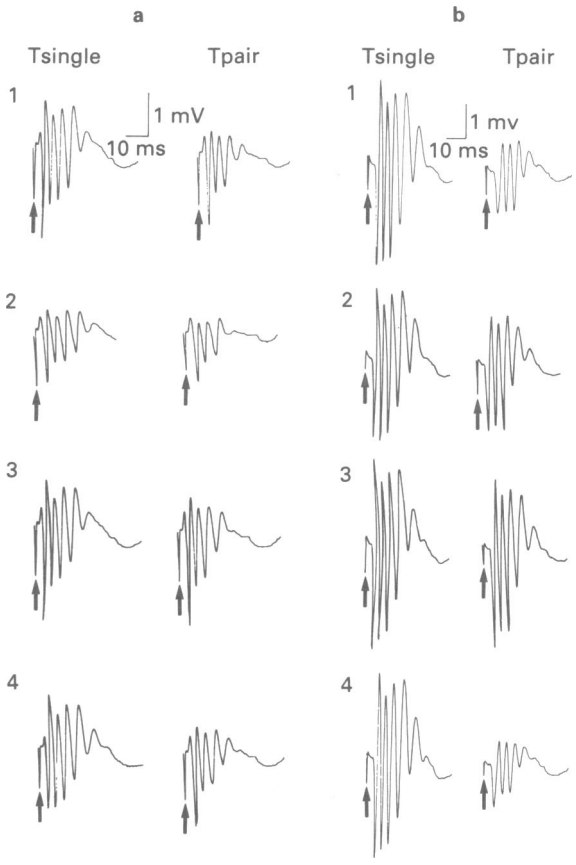


Figure 2 Paired-pulse inhibition between evoked potentials in bicuculline 10 μmol l^{-1}, i.p.i. = 300 ms. All stimuli are orthodromic. Conditioning stimuli and unpaired test stimuli are submaximal. Each potential is the average of eight responses. (a) Effect of baclofen 1 μmol l^{-1}. (b) Effect of 2-hydroxysaclofen 200 μmol l^{-1}. Sequence shows: (1) control values; (2) effect of drug with stimuli unadjusted from control; (3) effect of drug with stimuli adjusted to return S_{cond} and T_{single} to control size; (4) wash with stimulus strength readjusted again in (a). Arrows mark stimulus artifacts. Calibration bars 1 mV and 10 ms.

inhibition both when the conditioning stimulus was supramaximal ($P<0.05$) and submaximal ($P<0.01$, $n=6$ slices) (Figures 2 and 3). In some slices inhibition was reversed to facilitation in the presence of 2-hydroxysaclofen. The effect of 2-hydroxysaclofen partially reversed on washing the drug from the recording chamber.

By contrast, 2-hydroxysaclofen had no significant effect on postsynaptic ortho/anti inhibition in the same slices under the same conditions (Figure 3). As control inhibition in the two paradigms was different, the proportional change in inhibition caused by 2-hydroxysaclofen relative to the respective control was compared by the Wilcoxon matched pairs test between the two paradigms. The difference between the effect of 2-hydroxysaclofen on ortho/ortho and ortho/anti inhibition was significant ($P<0.05$) for both supramaximal and submaximal conditioning stimuli.

Baclofen Baclofen 1 μmol l^{-1}, a $GABA_B$-agonist, also markedly and significantly reduced inhibition in the ortho/ortho paradigm both when the conditioning stimulus was supramaximal ($P<0.01$) and submaximal ($P<0.001$, $n=7$ slices) (Figures 2 and 4). The effect of baclofen partially reversed when the drug was washed from the recording chamber.

Baclofen at this concentration had no significant effect on the ortho/anti inhibition. The difference between the effect of baclofen on ortho/ortho and ortho/anti inhibition was significant ($P<0.05$ Wilcoxon matched pairs test) for both maximal and submaximal conditioning stimuli when the proportional change in inhibition caused by baclofen relative to the respective control was compared between the two paradigms.

Although the effects of baclofen and 2-hydroxysaclofen were similar in some respects, baclofen initially decreased both T_{single} and T_{pair} (but T_{single} more than T_{pair}) before the stimulation strength was adjusted, whereas 2-hydroxysaclofen increased the size of T_{pair} (Figure 2).

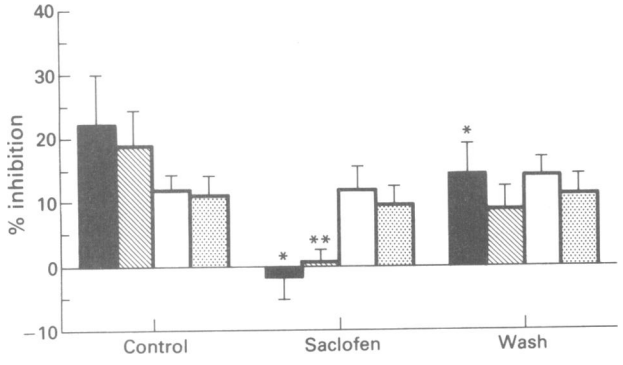


Figure 3 Effect of 2-hydroxysaclofen 200 μmol l^{-1} on paired-pulse inhibition at an interpulse interval of 300 ms in the presence of bicuculline methobromide 10 μmol l^{-1}. Both ortho/ortho and ortho/anti stimulation paradigms were used (see text) and each was repeated with a supramaximal (1 mA) conditioning stimulus and a submaximal conditioning stimulus set to give a response between 50 and 70% of maximum (constant within each experiment). The unpaired test stimuli were also set to give a response between 50 and 70% maximum. The unpaired test stimuli and the submaximal conditioning stimuli were re-adjusted if necessary in the presence of 2-hydroxysaclofen and after washout to keep them as constant as possible. Solid columns show ortho/ortho responses with conditioning stimulus supramaximal; cross hatched columns show ortho/ortho responses with conditioning stimulus submaximal; open columns show ortho/anti responses with conditioning stimulus supramaximal; stippled columns show ortho/anti responses with conditioning stimulus submaximal. $n=6$ slices. Values are mean ± s.e.mean. $*P<0.05$; $**P<0.01$ for difference between drug and control or wash and drug.

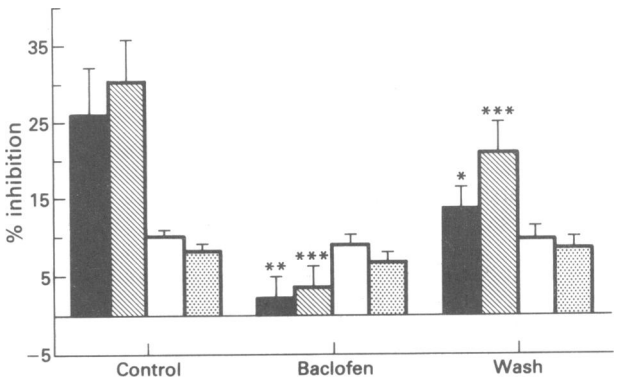


Figure 4 Effect of baclofen 1 μmol l^{-1} on paired-pulse inhibition at an interpulse interval of 300 ms in the presence of bicuculline methobromide 10 μmol l^{-1}, $n=7$ slices. See Figure 3 for details. Values are mean ± s.e.mean. $*P<0.05$; $**P<0.01$; $***P<0.001$ for difference between drug and control or wash and drug.

Adenosine The effect of adenosine 20 μmol l^{-1} on paired-pulse inhibition was examined at an i.p.i. of 300 ms in the presence of bicuculline 10 μmol l^{-1}. Adenosine (Figure 5) had no significant effect on ortho/anti inhibition with maximal or submaximal conditioning stimuli or on ortho/ortho inhibition when the conditioning stimulus was maximal (Figure 5).

With a submaximal conditioning stimulus adenosine significantly ($P<0.01$, $n=9$ slices) and reversibly reduced inhibition in the ortho/ortho paradigm (Figure 5). Adenosine, like baclofen, markedly reduced the size of both T_{single} and T_{pair} before the stimuli were readjusted to their control amplitude.

Discussion

In the presence of bicuculline, paired-pulse inhibition was markedly increased at short and intermediate i.p.is compared with the bicuculline-free control slices. In bicuculline, evoked potentials are prolonged to 30 ms or more as a result of repetitive firing of pyramidal cells (see Figure 2). However, the later inhibition cannot be explained by refractoriness of the postsynaptic membrane since it follows a period of facilitation at 100 ms.

In CA1 area of the normal hippocampus, pyramidal cell inhibitory postsynaptic potentials (i.p.s.ps) consist of two components, a short-latency $GABA_A$-mediated component

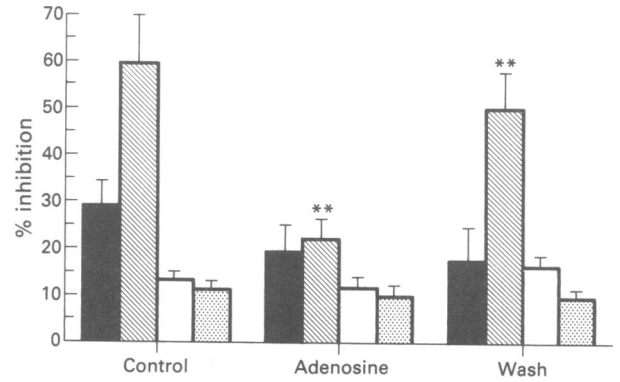


Figure 5 Effect of adenosine 20 μmol l^{-1} on paired-pulse inhibition at an interpulse interval of 300 ms in the presence of bicuculline methobromide 10 μmol l^{-1}, $n=9$ slices. See Figure 3 for details. Values are mean ± s.e.mean. $**P<0.01$ for difference between drug and control or wash and drug.

and a longer latency, longer duration $GABA_B$ component (Alger & Nicoll, 1982a,b; Newberry & Nicoll, 1984; 1985; Peet & McLennan, 1986; Dutar & Nicoll, 1988; Davies *et al.*, 1990; Lambert *et al.*, 1991a). Also, under certain conditions, a long-lasting $GABA_A$-mediated depolarizing inhibitory potential may be seen (Alger & Nicoll, 1982a,b; Newberry & Nicoll, 1985; Avoli & Perreault, 1987; Lambert *et al.*, 1991a). In the presence of $GABA_A$ antagonists $GABA_B$-i.p.s.ps recorded in the pyramidal cells are enhanced and merge with a prolonged K_{Ca}-mediated membrane after-hyperpolarization (Schwartzkroin & Stafstrom, 1980; Newberry & Nicoll, 1984). This is probably the result of increased activation of recurrent interneurones secondary to repetitive pyramidal cell firing as well as increased activation of feedforward interneurones which will be subject to decreased $GABA_A$-mediated inhibition (Lacaille, 1991).

The fact that 2-hydroxysaclofen caused a marked reduction in ortho/ortho inhibition in the present work would support the view that it was at least partially mediated by $GABA_B$ receptors. The time course of the inhibition is also consistent with $GABA_B$ activation. Davies *et al.* (1990) measured a latency to onset of 29 ms, with a peak at 135 ms and duration of 723 ± 135 ms for pure isolated $GABA_B$ i.p.s.ps on CA1 pyramidal cells.

The ortho/anti paradigm was used to examine pyramidal cell excitability 300 ms after an orthodromic conditioning stimulus. A small but consistent inhibition was demonstrated. Since this was not significantly reduced by 2-hydroxysaclofen (or by baclofen or adenosine which also reduced ortho/ortho inhibition), this postsynaptic decrease in excitability may be non-GABA-mediated. Furthermore this leaves unresolved the question of the location of the $GABA_B$ receptors which are involved in paired-pulse inhibition at this latency. In addition to $GABA_B$ receptors located postsynaptically on the CA1 pyramidal cells, there are known to be presynaptic $GABA_B$ receptors whose activation inhibits excitatory transmission at the Schaffer collateral terminals (Blaxter & Carlen, 1985; Harrison *et al.*, 1990). It is possible these mediate at least part of the 2-hydroxy-saclofen sensitive component.

The reduction in ortho/ortho inhibition produced by baclofen, a $GABA_B$ agonist also suggests that this inhibition is substantially mediated by GABAergic interneurones. In the absence of other drugs, baclofen is known to decrease paired-pulse inhibition of single population spikes in the CA1 area (Steffensen & Henriksen, 1991; Ault & Nadler, 1983). It acts at receptors on interneurone terminals to reduce GABA release (Davies *et al.*, 1990; Lambert *et al.*, 1991b; Nathan & Lambert, 1991; Thompson & Gahwiler, 1992) and depresses interneurone excitability by inducing hyperpolarization (Madison & Nicoll, 1988; Misgeld *et al.*, 1989). The receptors mediating this effect are also involved in synaptic transmission to the interneurones since slow, $GABA_B$-mediated i.p.s.ps have been recorded from hippocampal interneurones (Segal, 1990; Lacaille, 1991).

Adenosine, like baclofen, decreases excitatory synaptic transmission and hence the evoked orthodromically evoked population spike in the hippocampus, at least in part by a presynaptic mechanism (Okada & Ozawa, 1980; Corradetti *et al.*, 1984; Burke & Nadler, 1988; Yoon & Rothman, 1991; Thompson *et al.*, 1992). Adenosine, however, has virtually no presynaptic action to reduce GABA release from interneurone terminals: it does not affect monosynaptic i.p.s.ps resulting from the direct stimulation of interneurones (Lambert & Teyler, 1991; Yoon & Rothman, 1991; Thompson *et al.*, 1992) or the potassium-evoked release of GABA from cortical slices except at millimolar concentration (Hollins & Stone, 1980). In the absence of other drugs adenosine has been reported to increase paired-pulse facilitation of the field excitatory postsynaptic potential by a presynaptic action at the Schaffer collateral terminals (Dunwiddie & Haas, 1985). We were thus interested in the effect of adenosine on paired-pulse inhibition in the presence of bicuculline.

The effect of adenosine was less clear-cut than that of baclofen. Although it reversibly reduced ortho/ortho inhibition when the conditioning stimulus was submaximal it was apparently not effective when the conditioning stimulus was supramaximal (Figure 5). It is possible that adenosine is working through a different mechanism from baclofen.

In summary, following an observation in rat hippocampal slices that paired pulse inhibition at moderately long i.p.is was increased by the $GABA_A$ antagonist bicuculline, the mechanism of the inhibition has been examined in more detail. The medium latency (300–500 ms) inhibition was found to be substantially mediated by 2-hydroxysaclofen-sensitive $GABA_B$ receptors which did not influence antidromic excitability and may not therefore be located postsynaptically. A decrease in postsynaptic excitability which was resistant to both bicuculline and 2-hydroxysaclofen contributed to paired-pulse inhibition at this latency.

M.J.H. is supported by a Medical Graduate Fellowship from the Wellcome Trust.

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(Received February 10, 1993
Revised March 18, 1993
Accepted March 30, 1993)

Br. J. Pharmacol. (1993), **109**, 1169–1177 © Macmillan Press Ltd, 1993

A novel metabotropic glutamate receptor agonist: marked depression of monosynaptic excitation in the newborn rat isolated spinal cord

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1 Neuropharmacological actions of a novel metabotropic glutamate receptor agonist, (2S,1'R,2'R,3'R)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV), were examined in the isolated spinal cord of the newborn rat, and compared with those of the established agonists of (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine (L-CCG-I) or (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid ((1S,3R)-ACPD).

2 At concentrations higher than 10 μM, DCG-IV caused a depolarization which was completely blocked by selective N-methyl-D-aspartate (NMDA) antagonists. The depolarization was pharmacologically quite different from that caused by L-CCG-I and (1S,3R)-ACPD.

3 DCG-IV reduced the monosynaptic excitation of motoneurones rather than polysynaptic discharges in the nanomolar range without causing postsynaptic depolarization of motoneurones. DCG-IV was more effective than L-CCG-I, (1S,3R)-ACPD or L-2-amino-4-phosphonobutanoic acid (L-AP4) in reducing the monosynaptic excitation of motoneurones.

4 DCG-IV (30 nM–1 μM) did not depress the depolarization induced by known excitatory amino acids in the newborn rat motoneurones, but depressed the baseline fluctuation of the potential derived from ventral roots. Therefore, DCG-IV seems to reduce preferentially transmitter release from primary afferent nerve terminals.

5 Depression of monosynaptic excitation caused by DCG-IV was not affected by any known pharmacological agents, including 2-amino-3-phosphonopropanoic acid (AP3), diazepam, 2-hydroxysaclofen, picrotoxin and strychnine.

6 DCG-IV has the potential of providing further useful information on the physiological function of metabotropic glutamate receptors.

Keywords: Excitatory amino acid; metabotropic glutamate receptor; presynaptic inhibition; spinal reflex; monosynaptic excitation; newborn rat spinal cord

Introduction

A metabotropic glutamate receptor, that is linked to the stimulation of phosphoinositide hydrolysis or the reduction of adenylcyclase activity, has recently been described (Sladeczek *et al.*, 1985; Nicoletti *et al.*, 1986; Sugiyama *et al.*, 1987; 1989; Palmer *et al.*, 1989; Tanabe *et al.*, 1992; Nakanishi, 1992). This glutamate receptor subtype in the mammalian central nervous system is activated by some kinds of glutamate analogues such as quisqualate, ibotenate, 1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) and the (2S,1'S,2'S)-isomer (L-CCG-I, previously reported as (2S,3S,-4S)-CCG) of 2-(carboxycyclopropyl)glycine (CCG) (Monaghan *et al.*, 1989; Sugiyama *et al.*, 1989; Schoepp *et al.*, 1990; Nakagawa *et al.*, 1990; Ishida *et al.*, 1990b; Shinozaki & Ishida, 1992; Porter *et al.*, 1992). (1S,3R)-ACPD and L-CCG-I appeared to be selective agonists for this receptor (Irving *et al.*, 1990; Shinozaki *et al.*, 1989b; Ishida *et al.*, 1990b) and others are non-selective agonists. The physiological effects of activation of metabotropic glutamate receptors have been examined in a number of brain regions (Schoepp *et al.*, 1990; Desai & Conn, 1991; Miller, 1991; Boss *et al.*, 1992). However, much less is known of the pharmacology of metabotropic glutamate receptors than is the case for ionotropic glutamate receptors which are characterized by their unique sensitivity to low concentrations of N-methyl-D-aspartic acid (NMDA), kainic acid and (RS)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propanoic acid (AMPA) (Monaghan *et al.*, 1989; Simon, 1992; Krogsgaard-Larsen & Hansen, 1992). A major contributory factor has been lack of potent and selective agonists and antagonists of this class of L-glutamate receptors.

CCG is a conformationally restricted glutamate analogue (Shinozaki *et al.*, 1989b; Shinozaki & Ishida, 1991), and L-CCG-I is almost equal to (1S,3R)-ACPD and more potent than L-glutamate on a molar basis in causing a temperature-dependent depolarization of the newborn rat spinal motoneurone (Ishida *et al.*, 1990b). The depolarizing responses to L-CCG-I and (1S,3R)-ACPD were neither depressed by selective NMDA antagonists nor by a non-NMDA blocker, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), demonstrating a clear preference for non-NMDA, non-kainate and non-AMPA receptors (Shinozaki *et al.*, 1989b). L-CCG-I stimulates phosphoinositide hydrolysis in a concentration-dependent manner in rat hippocampal synaptoneurosomes (Nakagawa *et al.*, 1990), and it induces chloride-mediated oscillatory responses in *Xenopus* oocytes injected with rat brain mRNA in a manner quite similar to quisqualate or *trans*-ACPD (Ishida *et al.*, 1990b). Thus, L-CCG-I proved to be a potent agonist for metabotropic glutamate receptors. Recently, L-CCG-I was demonstrated to be a potent agonist for cloned 'mGluR2' glutamate receptors which are negatively coupled to adenylcyclase, although it stimulated cloned 'mGluR1' and 'mGluR4' glutamate receptors as well (Hayashi *et al.*, 1992).

It is known that (1S,3R)-ACPD depresses monosynaptic excitation of motoneurones in the newborn rat spinal cord (Sunter *et al.*, 1991). L-CCG-I also depresses it markedly rather than polysynaptic discharges in the low micromolar range (Ishida *et al.*, 1990a; Shinozaki & Ishida, 1992; 1993).

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These effects have been considered to be due to their presynaptic actions. In addition to L-CCG-I, we recently found that another L-CCG-I analogue, (2S,1'R,2'R,3'R)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV), depressed monosynaptic excitation at lower concentrations than those required by L-CCG-I (Ohfune *et al.*, 1993; Shinozaki & Ishida, 1993). DCG-IV possesses the glutamate skeleton of both L-CCG-I and the (2S,1'R,2'S)-isomer (L-CCG-IV) of CCG (Figure 1). L-CCG-IV is a potent agonist for NMDA receptors (Shinozaki *et al.*, 1989a,b; Kawai *et al.*, 1992) and is about 300 times more potent than NMDA in increasing the intracellular Ca^{2+} concentration in rat cultured hippocampal neurones (Kudo *et al.*, 1991; Shinozaki *et al.*, 1992), while L-CCG-IV is several times more potent than NMDA in causing a depolarization of spinal motoneurones of newborn rats. The structural similarity of DCG-IV to both L-CCG-I and L-CCG-IV might lead to the assumption that DCG-IV would show similar pharmacological properties to them, that is, DCG-IV would be expected to activate both NMDA receptors and metabotropic glutamate receptors simultaneously. In fact, DCG-IV reduced forskolin-induced adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation in the rat hippocampal neurones about 10–15 times more effectively than (1S,3R)-ACPD, but did not stimulate phosphoinositide hydrolysis (Maruyama *et al.*, unpublished observation). DCG-IV definitely caused an NMDA-like depolarization but was much less active than NMDA or L-CCG-IV, and its pharmacological actions are not always similar to those of L-CCG-I or (1S,3R)-ACPD. Therefore, DCG-IV would be expected to be a metabotropic glutamate receptor agonist of the other type. In the present paper we describe neuropharmacological actions of DCG-IV in the isolated spinal cord of the newborn rat and compare them with those of L-CCG-I or (1S,3R)-ACPD.

Methods

The methods used for the electrophysiological experiments in the newborn rat isolated spinal cord were essentially similar to those described previously (Shinozaki *et al.*, 1989b). The spinal cords of 1–7 day-old Wistar rats were used for the experiments. Under ether anaesthesia, the spinal cord below the thoracic region was isolated, hemisected sagitally and placed in a 0.15 ml bath perfused at a fixed flow rate of 5–6 ml min^{-1} with artificial cerebrospinal fluid (ACSF) (in mM: NaCl 138.6, KCl 3.4, $CaCl_2$ 1.3, $MgCl_2$ 1.2, $NaHCO_3$ 21.0, NaH_2PO_4 0.58, glucose 10.0, pH 7.4) which was oxygenated with a gas mixture of 95% O_2 and 5% CO_2. In some experiments, isolated spinal cords were used without hemisection in order to record the contralateral ventral root potential. Tetrodotoxin (TTX, 0.5 μM) was sometimes added to ACSF in order to block spontaneous depolarization and indirect drug effects, and in some cases Mg^{2+}-free solution was used to test the depolarizing activity of glutamate analogues including NMDA-type agonists. The potential changes generated in the motoneurones were recorded extracellularly from the L4 or L5 ventral root with a suction electrode, and in some experiments the evoked reflex responses were recorded from the L4 or L5 ventral roots with suction electrodes when the ipsi- or contralateral root of the corresponding segment was stimulated with a suction electrode. Dorsal root potential (DRP) was recorded from a dorsal root (L4 or L5) adjacent to the stimulated root. All test compounds were applied to the preparation either by perfusion or by the brief-pulse injection into the perfusion system at a constant duration. The temperature of the perfusing fluid was kept at 27 ± 0.2°C.

Drugs

The following compounds were used: acetylsalicylate (Sigma), (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid ((1S,3R)-ACPD, Tocris), (RS)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propanoic acid (AMPA, Tocris), L-2-amino-4-phosphonobutanoic acid (L-AP4, Tocris), DL-2-amino-3-phosphonopropanoic acid (AP3, Tocris), baclofen (Sigma), bicuculline (Sigma), (RS)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP, Tocris), (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine (L-CCG-I), (2S,1'R,2'S)-2-(carboxycyclopropyl)glycine (L-CCG-IV), (RS)-4-carboxy-3-hydroxyphenylglycine (4C3H-PG, Tocris), 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, Tocris), chlorzoxazone (Sigma), diazepam (Horizon inj, Yamanouchi), (2S,1'R,2'R,3'R)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV), furosemide (Sigma), 2-hydroxysaclofen (Tocris), matrine (a gift from Dr M. Terada, Hamamatsu Univ. Shizuoka, Japan), N-methyl-D-aspartic acid (NMDA, Sigma), picrotoxin (Tokyo Kasei), piperazine hydrochloride (Sigma), pertussis toxin (List Biol. Lab.), sodium L-glutamate mono (Wako), strychnine hemisulphate (Sigma), tetrodotoxin (TTX, Seikagakukogyo) and theanine (a gift from Dr M. Nakagawa, National Research Institute of Tea, Shizuoka, Japan). The carbon numbers of CCG derivatives previously reported were renumbered according to the IUPAC nomenclature in the present paper.

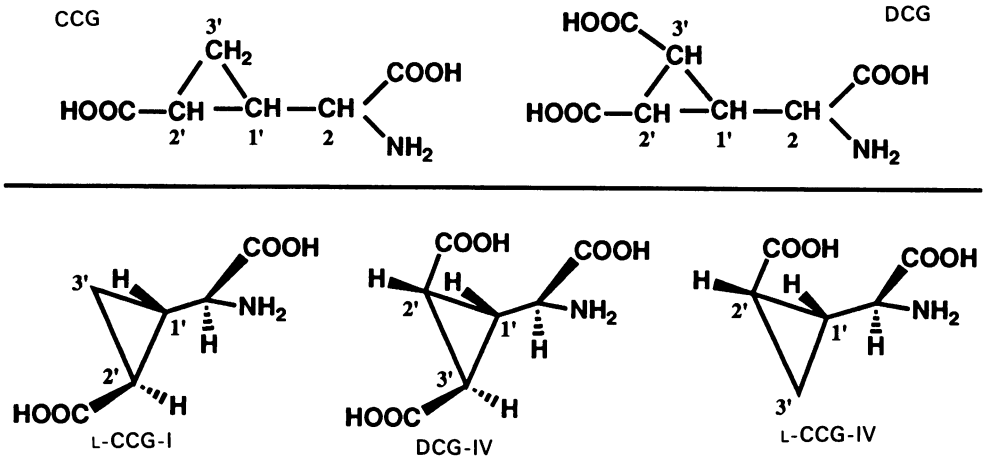


Figure 1 Plane chemical structure of 2-(carboxycyclopropyl)glycine (CCG) and 2-(2,3-dicarboxycylcopropyl)glycine (DCG), and stereochemical display of (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine (L-CCG-I), (2S,1'R,2'R,3'R)-2-(2,3-dicarboxycyclopropyl)-glycine (DCG-IV) and (2S,1'R,2'S)-2-(carboxycyclopropyl)glycine (L-CCG-IV). The carbons 1', 2' and 3', previously numbered as 3, 4 and 6, respectively, were renumbered according to the IUPAC nomenclature.

Results

Depolarization of motoneurones induced by DCG-IV

The potential changes generated in spinal motoneurones of newborn rats were recorded extracellularly from the L4 or L5 ventral root of the hemisected spinal cord. When L-CCG-I, (1S,3R)-ACPD and DCG-IV were added to the TTX (0.5 μM) containing and Mg^{2+}-free bathing solution for a period of 30 s, they caused a depolarization of spinal motoneurones in a concentration-dependent manner. Their threshold concentrations for depolarization were less than 10 μM. L-CCG-I and (1S,3R)-ACPD showed slightly slower responses (the prolonged time course of action) than those to L-glutamate. The depolarizing activity of DCG-IV was about 20 times higher than that of L-glutamate on a molar basis, but lower than that of NMDA or L-CCG-IV. Figure 2 shows the depolarizing responses to L-glutamate, NMDA, AMPA, DCG-IV, L-CCG-I and (1S,3R)-ACPD in the presence and absence of (RS)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) and CNQX. The depolarization induced by L-CCG-I or (1S,3R)-ACPD was only slightly reduced by CPP, and the pharmacological properties of the depolarization seemed quite similar (Shinozaki *et al.*, 1989b; Ishida *et al.*, 1990b). On the other hand, the depolarizaiton induced by DCG-IV (20 μM) was completely blocked by CPP (10–50 μM), suggesting that DCG-IV activated NMDA receptors, as might be anticipated from the structural similarity between DCG-IV and L-CCG-IV (a potent NMDA agonist). It seems likely that the glutamate folded skeleton in the molecule of DCG-IV, which is quite similar to that of L-CCG-IV, contributes directly to the activation of NMDA receptors.

When the potential changes were recorded from the L4 or L5 dorsal root with a suction electrode, L-CCG-I, (1S,3R)-ACPD and DCG-IV caused depolarizations that were markedly smaller than those of ventral roots. Their threshold concentrations for depolarization were about 10 μM (TTX 0.5 μM containing, Mg^{2+}-free solution), demonstrating no significant difference in their threshold concentrations between the potentials from ventral and dorsal roots. The depolarization induced by DCG-IV completely disappeared in the presence of CPP (50 μM), but others were not reduced even in the combined treatment with CPP (50 μM) and CNQX (50 μM).

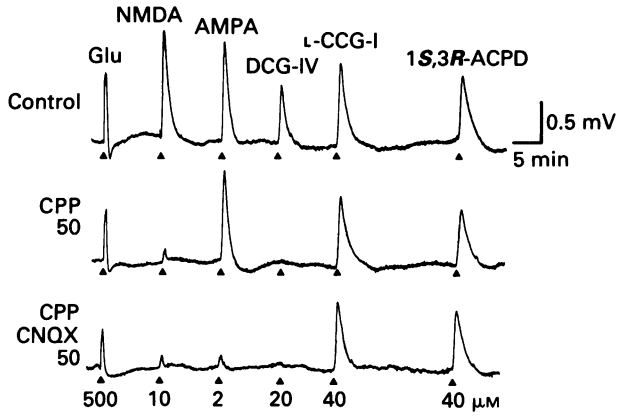


Figure 2 Sample records of the depolarizing responses to (2S,1'R,-2'R,3'R)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV), (2S,1'S,2'S)-2-(carbodycyclopropyl)glycine (L-CCG-I), (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid ((1S,3R)-ACPD) and representative excitatory amino acids in the newborn rat spinal motoneurone. Test samples were added to the tetrodotoxin (0.5 μM) containing and Mg^{2+}-free solution at triangles for a period of 30 s in the absence (control) and presence of (RS)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) and/or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). Numbers show the concentration (μM) of the test samples. Glu: L-glutamate, NMDA: N-methyl-D-aspartate, AMPA: (RS)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propanoic acid.

One of the pronounced features of the depolarization induced by the prolonged application of L-CCG-I and (1S,3R)-ACPD was a desensitization-like phenomenon, which was not observed in the case of DCG-IV. When L-CCG-I or (1S,3R)-ACPD was added to the bathing fluid (TTX 0.5 μM containing, Mg^{2+}-free) for 2 min in various concentrations, the depolarization induced by L-CCG-I or (1S,3R)-ACPD was not maintained but decreased during its application in a concentration-dependent manner (Figure 3a). On the other hand, DCG-IV and representative ionotropic glutamate receptor agonists, such as kainic acid, NMDA and AMPA, induced slight desensitization only when amplitudes of depolarizing responses were significantly large, in contrast to L-CCG-I and (1S,3R)-ACPD (data were not shown). The desensitization-like phenomenon observed here was also demonstrated when L-CCG-I and (1S,3R)-ACPD were repeatedly or alternately applied at short intervals. This desensitization-like action crossed between L-CCG-I and (1S,3R)-ACPD (Figure 3b). These time-dependent actions were not observed with repetitive application of NMDA, kainate and AMPA.

It has been reported that DL-2-amino-3-phosphonopropanoic acid (AP3), L-2-amino-4-phosphonobutanoic acid (L-AP4) and (S)-4-carboxy-3-hydroxyphenyl-glycine ((S)-4C3H-PG) inhibit depolarizing responses to *trans*- or (1S,3R)-ACPD in various preparations (Schoepp *et al.*, 1990; Irving *et al.*, 1990; Jones *et al.*, 1992). AP3 and L-AP4 induced slight depolarization of the newborn rat spinal motoneurone at a concentration greater than 0.3 mM, but neither AP3 nor L-AP4 reduced depolarizing responses to L-glutamate (1 mM, 10 s application), L-CCG-I (0.1 mM, 10 s), quisqualate (2 μM, 10 s), (1S,3R)-ACPD (0.1 mM, 10 s) and DCG-IV (50 μM, 10 s) even at higher concentrations than the threshold for depolarization of motoneurones. 4C3H-PG reduced the amplitude of depolarizing responses to L-CCG-I (40 μM, 30 s) and (1S,3R)-ACPD (40 μM, 30 s) to about half at a high concentration (300 μM) without causing depolarization of motoneurones in the TTX-containing solution.

Suppression of fluctuation of ventral root potentials

When the spinal preparation was perfused with the TTX-free ACSF, the base line of the potential derived from the L4 or L5 ventral root fluctuated in a range of about 0.5 mV, probably due to spontaneous discharges of motoneurones through the release of neurotransmitters. Low concentrations of L-CCG-I (0.1–1 μM, 10 min application) or DCG-IV (30–100 nM, 10 min application) almost completely reduced the baseline fluctuation without causing detectable depolarization of spinal motoneurones (Figure 4a), demonstrating the block of transmitter release from primary afferent nerve terminals. The recovery from the action of L-CCG-I was relatively rapid

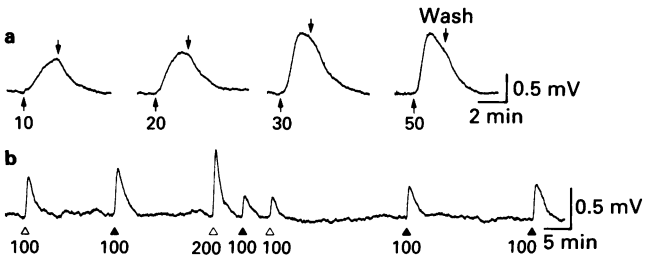


Figure 3 The desensitization-like responses revealed by (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine (L-CCG-I) and (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid ((1S,3R)-ACPD) in the newborn rat motoneurone. Test samples were added to the tetrodotoxin (0.5 μM)-containing and Mg^{2+}-free solution for a period indicated by arrows. Numbers show the concentration (μM) of the sample. (a) Depolarizing responses to L-CCG-I (2 min application, at intervals of 30 min); (b) 10 s application. (△) L-CCG-I; (▲) (1S,3R)-ACPD.

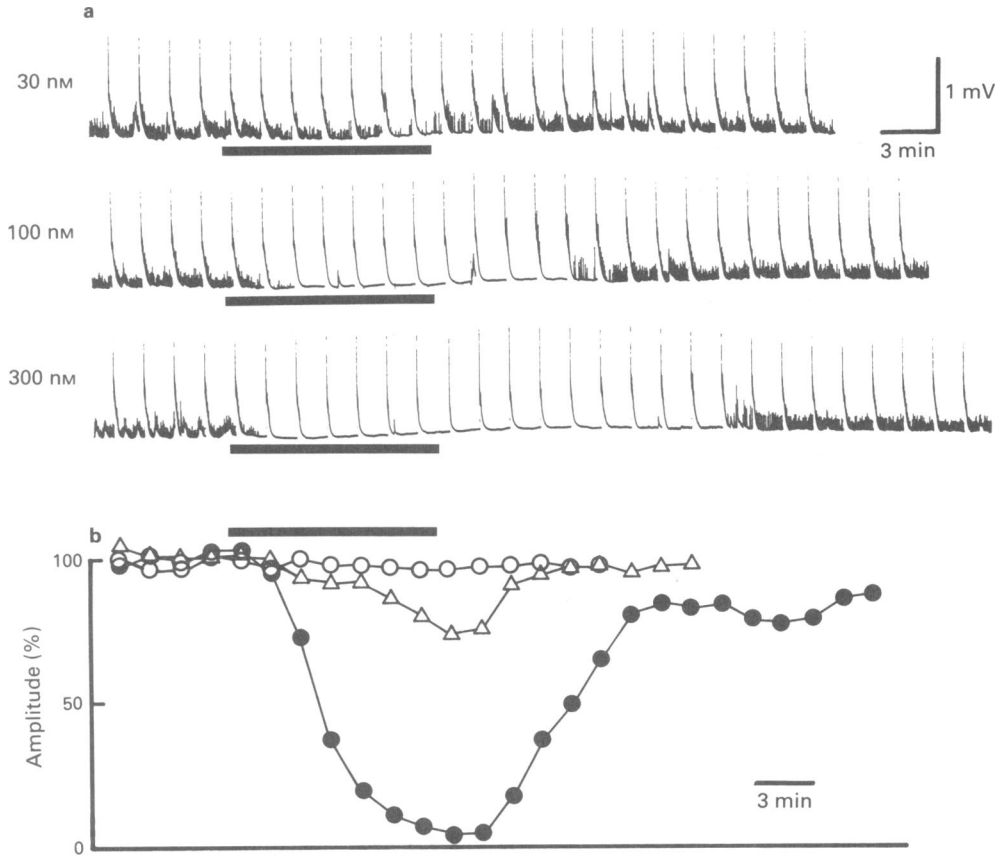


Figure 4 (a) Sample records of spinal reflexes evoked by electrical stimulation of L5 dorsal root of the isolated spinal cord of newborn rats (7-day-old). The potential changes were recorded from ipsilateral L5 ventral root. (2S,1'R,2'R,3'R)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV) was added to the perfusing fluid at a concentration of 30 (upper trace), 100 (middle trace) and 300 nM (lower trace) for a period indicated by horizontal bars. The fluctuation of the base line reduced in a dose-dependent manner. In these traces, the monosynaptic component of spinal reflexes were not shown because of the frequency response of the pen-writer (see b). The larger deflections represent polysynaptic discharges. (b) Time courses of dose-dependent reduction of the monosynaptic component of spinal reflexes in the presence of DCG-IV. Data were obtained from the same preparation as that in (a). DCG-IV 30 (○), 100 (△) and 300 (●) nM.

after washing the preparation (within about 5 min), but that of DCG-IV was slightly slower than that of L-CCG-I. (1S,3R)-ACPD (0.3–1 μM) did not reduce the fluctuation of the base line at concentrations less than the threshold concentration for depolarization of motoneurones, but rather slightly increased it.

Depression of spinal reflexes

The spinal reflex was repetitively evoked at intervals of 90 s by electrical stimulation of the L4 or L5 dorsal root of the newborn rat. Single shocks were delivered to dorsal root through stimulating electrodes. The potential changes (DR-VRP) were recorded from the ipsilateral ventral roots of the corresponding segment. The DR-VRP was reversibly depressed by DCG-IV at markedly low concentrations in a similar manner to L-CCG-I, and in particular, the DR-VRP monosynaptic component was preferentially decreased by DCG-IV at the nanomolar range, which was much lower than the threshold concentration (about 10 μM in case of TTX-free ACSF) for depolarization of motoneurones without reducing polysynaptic discharges (Figure 4b). In the presence of 0.1–0.3 μM DCG-IV (10 min application), the DR-VRP monosynaptic component almost disappeared with no associated depolarization of the postsynaptic membrane of motoneurones, but polysynaptic discharges were hardly affected. However, polysynaptic components of the DR-VRP were gradually decreased as the concentration was increased and at 5 μM (10 min application), DCG-IV completely reduced the polysynaptic component of the DR-VRP.

In order to test antagonistic actions of DCG-IV against various excitatory amino acids, AMPA (2 μM), NMDA (10 μM), kainic acid (3 μM), (1S,3R)-ACPD (40 μM) and L-CCG-I (40 μM) were added to the TTX-containing and Mg^{2+}-free ACSF. DCG-IV (30 nM–1 μM) did not reduce the depolarization of motoneurones thus induced, suggesting that DCG-IV does not affect depolarizing responses to excitatory amino acids including agonists for metabotropic glutamate receptors. The DR-VRP monosynaptic component is effectively reduced by non-NMDA antagonists, but not by selective NMDA blockers (Jahr & Yoshioka, 1986). As mentioned above, DCG-IV depressed the DR-VRP monosynaptic component at markedly low concentrations which did not cause depolarization of motoneurones. Even in a high concentration (50 μM), CPP did not reduce the DR-VRP monosynaptic component. On the other hand, DCG-IV depressed the DR-VRP monosynaptic component in the presence of high concentrations of CPP, suggesting that the inhibitory action of DCG-IV on spinal reflexes was neither the result of activation of NMDA receptors nor antagonism against neurotransmitters on postsynaptic membranes. It is likely that DCG-IV stimulates presynaptic events to depress the monosynaptic excitation of motoneurones.

L-AP4 has been regarded as one of representative agonists for an ionotropic glutamate receptor (Koerner & Cotman, 1981; Yamamoto *et al.*, 1983; Monaghan *et al.*, 1989), and L-AP4 is known to suppress the DR-VRP monosynaptic component in the spinal cords of both cats (Davies & Watkins, 1982) and immature rats (Evans *et al.*, 1982) at relatively low concentrations. Current information on

presynaptic L-AP4 receptors is also consistent with a second messenger mechanism in which L-AP4 blocks activation of voltage-sensitive Ca^{2+} channels (Koerner & Johnson, 1992). Furthermore, L-AP4 is a potent agonist for the cloned 'mGluR4' glutamate receptor expressed in Chinese hamster ovary cells, which is negatively coupled with adenyl cyclase (Nakanishi, 1992). So, the inhibitory actions of DCG-IV on the DR-VRP were compared with those of L-AP4, L-CCG-I and (1S,3R)-ACPD (Figure 5). L-AP4 (0.3–100 μM, 10 min application) depressed the fluctuation of the base line and the DR-VRP monosynaptic component without causing postsynaptic depolarization of the motoneurone as well as L-CCG-I or DCG-IV, but the actions were slightly less intense than those of L-CCG-I, and markedly less than those of DCG-IV on a molar basis. The threshold concentration of L-AP4 for suppression of the DR-VRP monosynaptic component was about 0.3 μM, which was similar to that of L-CCG-I but considerably higher than that of DCG-IV, but at concentrations of 30–100 μM, both monosynaptic excitation and polysynaptic discharges still remained in lessened amplitudes, suggesting the difference in action between DCG-IV and L-AP4. The threshold concentrations of DCG-IV, L-CCG-I and L-AP4 for decreasing polysynaptic discharges were about 0.3, 1 and 1 μM, respectively. (1S,3R)-ACPD (1–10 μM, 10 min application) depressed both mono- and polysynaptic components of the DR-VRP almost in parallel, with depolarization of motoneurones. Thus, the action of DCG-IV is similar to that of L-CCG-I in that they depress preferentially the monosynaptic component of the DR-VRP at lower concentrations, although there is a large variation of their effective concentration-range.

Effect of some pharmacological agents

For the pharmacological characterization of DCG-IV, the influence of picrotoxin (20 μM), bicuculline (3 μM), 2-hydroxysaclofen (100 μM), strychnine (1 μM), diazepam (10–100 μM), AP3 (100–300 μM), 4C3H-PG (10–300 μM), theanine (1 mM) (Shinozaki & Ishida, 1978), acetylsalicylate (100 μM), piperazine (100 μM), furosemide (100 μM), chlorzoxazone (100 μM) and matrin (0.5 mM) (Ishida & Shinozaki, 1984) were examined on the reduction of the DR-VRP monosynaptic component caused by DCG-IV. At concentrations greater than 30 μM, 4C3H-PG itself depressed both mono- and polysynaptic components of spinal reflexes (see Jones *et al.*, 1992); therefore, the action was examined at a concentration of 10 μM. Any tested compounds in the present experiments (10 min application) had no influence on the action of DCG-

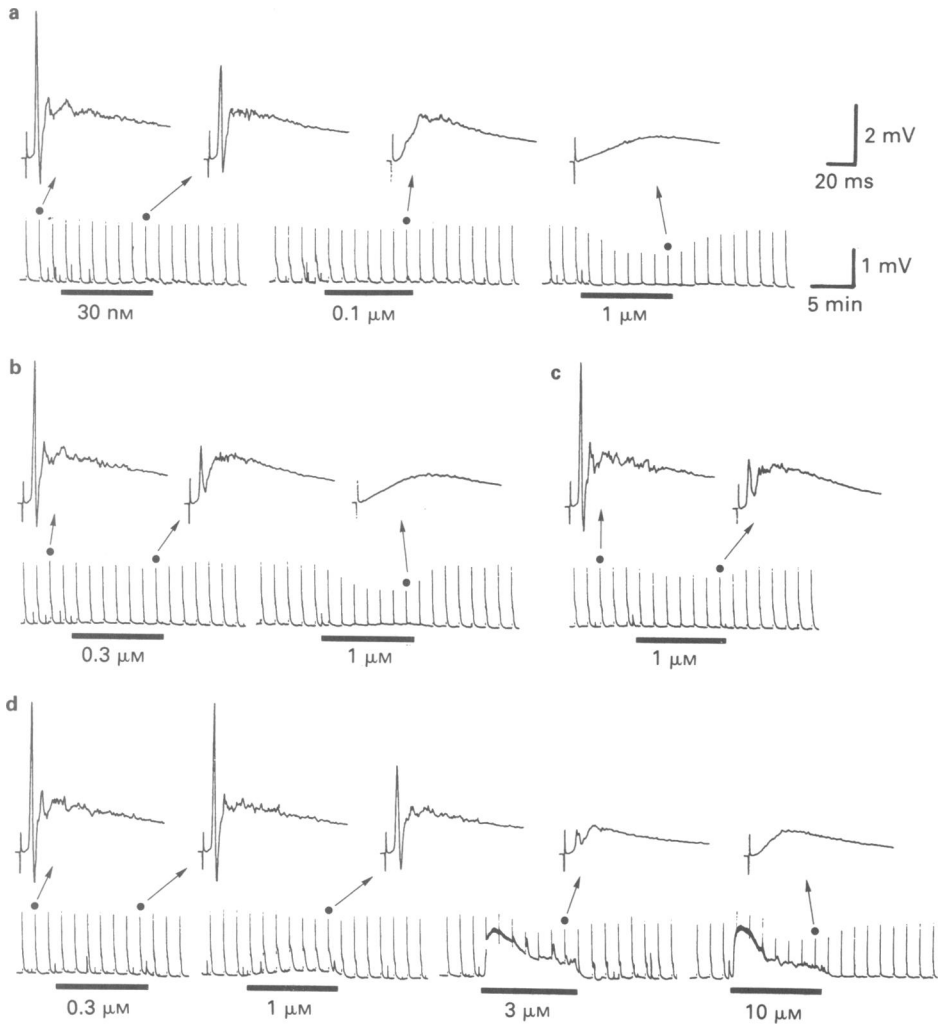


Figure 5 Sample records of spinal reflexes repetitively evoked at intervals of 90 s by electrical stimulation of the L5 dorsal root of the isolated spinal cord of newborn rats (1-day-old). The potential changes were recorded from the ipsilateral L5 ventral root. Upper chart: records on an X-Y recorder; Lower chart: records on a pen-writer. Upward fluctuation represents amplitudes of polysynaptic discharges. The following compounds were added to the perfusing fluid (tetrodotoxin-free, Mg^{2+} (1.2 mM) containing) for a period indicated by horizontal lines: (a) (2S,1'R,2'R,3'R)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV); (b) (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine (L-CCG-I); (c) L-2-amino-4-phosphonobutanoic acid (L-AP4); (d) (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid ((1S,3R)-ACPD).

IV. CNQX itself (0.3–10 μM) reduced effectively the DR-VRP monosynaptic component, so the action of CNQX was not examined. In some experiments where the preparation was treated with pertussis toxin (3 μM) for 3 h, the DR-VRP monosynaptic component disappeared but the polysynaptic discharges still remained; therefore, it was impossible to examine the effect of DCG-IV on the DR-VRP monosynaptic component. However, in such cases, L-CCG-I (100 μM, 10 s application) still caused a depolarization, suggesting that the toxin failed to enter into the nerve cell.

DCG-IV and L-CCG-I have a common glutamate skeleton which takes an extended form that is supposed to activate metabotropic glutamate receptors (Ishida *et al.*, 1990b). If DCG-IV and L-CCG-I act on a common receptor, the action of DCG-IV might be expected to be attenuated or modified by L-CCG-I, although their effective concentration-range was different in depressing the monosynaptic component of spinal reflexes. In the presence of DCG-IV (0.3 μM), the DR-VRP monosynaptic component almost disappeared, and L-CCG-I (0.3 μM) reduced its amplitude to about 60% of the control. When both L-CCG-I (0.3 μM) and DCG-IV (0.1 μM) were added to the ACSF, the DR-VRP monosynaptic component disappeared entirely, suggesting that L-CCG-I did not antagonize against DCG-IV on the monosynaptic excitation. Furthermore, during combined treatment with a relatively low concentration of L-CCG-I (0.3 μM) and DCG-IV (0.03 μM), the DR-VRP monosynaptic component was reduced in an almost additive manner.

Comparison with baclofen

The above experiments show that the action of DCG-IV is not mediated by GABAergic systems, because picrotoxin, bicuculline and 2-hydroxysaclofen did not affect the action of DCG-IV. However, it is of great interest to compare the neuropharmacological actions of baclofen and DCG-IV, because both DCG-IV and baclofen reduce forskolin-induced cyclic AMP formation in nerve cells (Hill, 1985; Karbon & Enna, 1985), and baclofen is so far the most potent amino acid in depressing spinal reflexes of the newborn rat spinal preparation (Akagi & Yanagisawa, 1987). The L4 or L5 ventral root potential alternatively evoked by electrical stimulation of the ipsi- and contralateral dorsal root fibre of the corresponding segment (ipsi- and contralateral DR-VRP), and the dorsal root potential (DR-DRP) derived from the dorsal root adjacent to the stimulated root were recorded in the absence and presence of DCG-IV, baclofen and L-AP4. DCG-IV was more potent than baclofen in reducing the monosynaptic component of the ipsilateral DR-VRP (Figure 6a). On the other hand, DCG-IV was less active than baclofen in reducing the DR-DRP, although there was no difference in the effective concentration-range of baclofen between the monosynaptic component of the ipsilateral DR-VRP and the DR-DRP (Figure 6b). When the potential was recorded from the contralateral ventral root of the same segment as that of the stimulated dorsal root, fast and slow components of reflexes were observed. The slow component (contralateral slow DR-VRP) had a time-to-peak of 2–5 s and lasted 20–30 s. Baclofen depressed the contralateral slow DR-VRP at much lower concentrations than those required for depression of the monosynaptic component of the ipsilateral DR-VRP (Figure 7). Similar results were obtained by Akagi & Yanagisawa (1987). DCG-IV also reduced the contralateral slow DR-VRP at slightly higher concentrations than those required for the reduction of the monosynaptic component of the ipsilateral DR-VRP. These results suggest that the distribution of $GABA_B$ receptors in the newborn rat spinal cord is different from that of the DCG-IV-sensitive receptors. L-AP4 also blocks the contralateral slow DR-VRP more effectively than DCG-IV. The action of (1S,3R)-ACPD and L-CCG-I was slightly less potent than that of DCG-IV.

When the ED_{50} value for decreasing the ipsilateral DR-VRP monosynaptic component was compared between

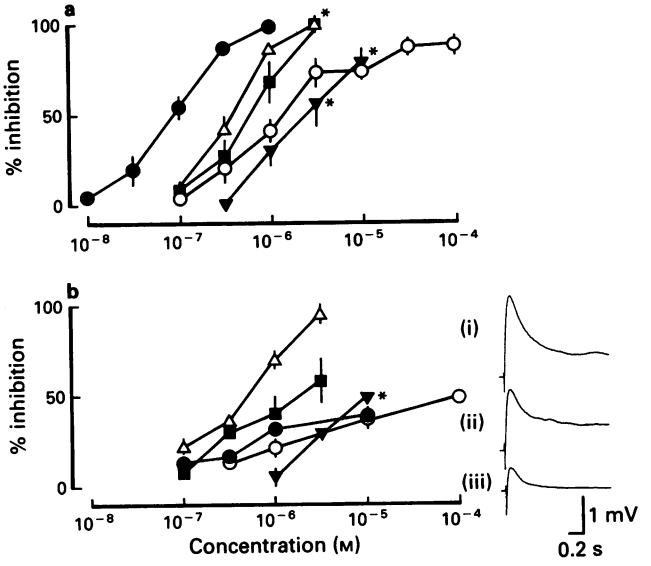


Figure 6 (a) The potential changes generated in the mononeurones were recorded extracellularly from the L4 ventral root with a suction electrode, and an ipsilateral dorsal root was electrically stimulated (10 V, 300 μs duration, 90 s interval) with a suction electrode. Dose-response curves for (2S,1'R,2'R,3'R)-2-(2,3-dicarboxycyclopropyl)-glycine (DCG-IV) (●); baclofen (△); (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine (L-CCG-I) (■); L-2-amino-4-phosphonobutanoic acid (L-AP4) (○) and (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid ((1S,3R)-ACPD) (▼) on the reduction of the monosynaptic component of spinal reflexes of the newborn rat (3 or 4-day-old). Percentage inhibition of amplitudes of monosynaptic components of spinal reflexes was plotted against the concentrations. Vertical bars represent s.e.mean (*n* at least 4). *Concentrations which caused depolarization of motoneurones. (b) Dose-response curves for baclofen (△), L-CGG-I (■), DCG-IV (●), L-AP4 (○) and (1S,3R)-ACPD (▼) on the reduction of dorsal root potentials (DR-DRP). Vertical bars represent s.e.mean (*n* at least 4). The potential changes (DR-DRP) generated in the L4 dorsal root were recorded extracellularly with a suction electrode when the ipsilateral L5 dorsal root or an adjacent segment was electrically stimulated (10 V, 300 μs duration, 90 s interval) with a suction electrode. (i) control, (ii) DCG-IV (1 μM), (iii) baclofen (1 μM). *Concentrations which caused depolarization of dorsal roots.

1- and 7-day-old rats, the value increased to about three times with the lapse of days after birth in the case of DCG-IV, L-CCG-I, (1S,3R)-ACPD and L-AP4, suggesting that receptors sensitive to these amino acids decrease in number or activity immediately after birth by developmental regulation. However, it was not until the rat was at least 7 days old that the sensitivity to baclofen was changed markedly.

Discussion

The interactions of glutamate with its various receptor subtypes are not well understood with respect to their preference for particular conformers of the neurotransmitter molecules. (1S,3R)-ACPD and L-CCG-I, which have been established as potent agonists for metabotropic glutamate receptors (Nakagawa *et al.*, 1990; Ishida *et al.*, 1990b; Shinozaki & Ishida, 1991; Hayashi *et al.*, 1992;Krogsgaard-Larsen & Hansen, 1992; Schoepp *et al.*, 1992), possess a glutamate skeleton in their molecules which takes an extended form. Although the active form of other agonists for metabotropic glutamate receptors is not always known, it seems reasonable to assume that one of the possible conformations for activating metabotropic glutamate receptors is an extended form (Ishida *et al.*, 1990b). Both L-CCG-I and (1S,3R)-ACPD stimulate phosphoinositide hydrolysis, and L-CCG-I reduces the aden-

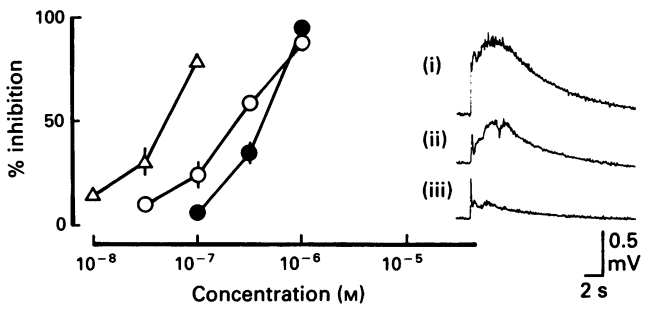


Figure 7 Reduction of the evoked reflex responses derived from L5 ventral roots, which were induced by an electrical stimulation (10 V, 300 μs duration, 180 s intervals) of the contralateral L5 dorsal root (contralateral slow DR-VRP) of the newborn rat (3 or 4-day-old). Left: Dose-response curves for baclofen (Δ), L-2-amino-4-phosphonobutanoic acid (L-AP4) (○) and (2S,1'R,2'R,3'R)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV) (●). Percentage inhibition of amplitudes of the contralateral slow DR-VRP was plotted against the concentrations. (i) Control, contralateral slow DR-VRP; (ii) DCG-IV (0.3 μM); (iii) baclofen (0.1 μM).

ylcyclase activity one order of magnitude more effectively than (1S,3R)-ACPD (Hayashi *et al.*, 1992). DCG-IV possesses the L-glutamate skeleton of an extended form which is quite similar to that of L-CCG-I, and DCG-IV does not stimulate phosphoinositide formation unlike L-CCG-I, but decreases forskolin-induced cyclic AMP formation as well as L-CCG-I (Maruyama *et al.*, unpublished observation). Therefore, DCG-IV is expected to be an agonist for metabotropic glutamate receptors, although there are some differences in the neuropharmacological actions of L-CCG-I and DCG-IV. The pharmacological profile of the recently cloned 'mGluR2' glutamate receptors which is negatively coupled to adenyl cyclase would be expected to be different from that of the metabotropic glutamate receptor coupled to phospholipase C. In the present study, DCG-IV did not cause any depolarization of motoneurones except for that mediated by NMDA receptors, while L-CCG-I and (1S,3R)-ACPD caused a depolarization that was to some extent decreased by 4C3H-PG; but both reduced preferentially the monosynaptic excitation of motoneurones at much lower concentrations than the threshold concentration for depolarization. The quisqualate-induced depolarization also consisted of various components including a metabotropic one, which was quite resistant to CPP and CNQX (Shinozaki *et al.*, 1989b). Quisqualate stimulated the cloned 'mGluR2' glutamate receptor expressed in Chinese hamster ovary cells less effectively than *trans*-ACPD (Nakanishi, 1992). Therefore, it seems probable that the depolarization caused by activating metabotropic glutamate receptors is a consequence of the stimulation of phosphoinositide hydrolysis in newborn rat spinal motoneurones.

At present, there are a few glutamate-related compounds such as DCG-IV that depress the monosynaptic excitation in the nanomolar range which is much lower than the threshold concentration for depolarization of motoneurones without affecting depolarizing responses to excitatory amino acids. Glutamic acid analogues which have been investigated in neural systems share with their parent compound two centres of negatively charged substituents and one with a positive charge. These structural features are believed to be essential for interaction of glutamic acid as an agonist with its neurotransmitter receptors. The structural homology of DCG-IV and L-CCG-I with glutamic acid is particularly close, and DCG-IV differs only by replacement of the hydrogen atom with a carboxylic group moiety on the C3' position of CCG, i.e. DCG-IV is one of the structural isomers of 3'-carboxylated CCG (see Figure 1). Attachment of the (3'R)-methoxymethyl and benzyloxymethyl group to the C3' position of L-CCG-IV (a potent NMDA agonist) converted the NMDA-like property into the non-NMDA type. (3'R)-methoxymethyl and benzyloxymethyl CCG derivatives caused kainate-like depolarization of the dorsal root fibre of newborn rats, while the (3'S)-CCG derivative demonstrated similar properties to parent compound, L-CCG-IV (Ishida *et al.*,1991). It is of great interest that the NMDA-like depolarizing activity of DCG-IV is less than that of L-CCG-IV, and, in addition, that the ability to stimulate phosphoinositide hydrolysis is abolished by 3'R-carboxylation. In passing, a tricarboxylic amino acid, L-4-carboxyglutamic acid (0.1 mM), reduced the monosynaptic component of ipsilateral DR-VRP in a similar manner to DCG-IV without reducing the polysynaptic discharges or causing postsynaptic depolarization, although the threshold concentration was markedly higher than that of DCG-IV (unpublished observation). This does not exclude the possibility that three centres of negatively charged substituents may be effective for activation of metabotropic glutamate receptors. In any case, C3'-substituted CCG compounds would provide useful information about the physiological function of excitatory amino acids.

The structural homology of DCG-IV with L-AP4 is not particularly close, but DCG-IV certainly stimulates L-AP4 receptors; a pharmacology based on antagonists for the L-AP4 receptor does not yet exist, and few known structural analogues of excitatory amino acids mimic the activity and potency of L-AP4, although recently some conformationally restricted cyclic analogues of L-AP4 have been synthesized (Koerner & Johnson, 1992). The L-AP4 receptor, like metabotropic glutamate receptors, operates via a second messenger system, but the most likely second messenger for the ON-bipolar cell L-AP4 receptor is cyclic GMP (Nawy & Jahr, 1990; Schiells & Falk, 1990). This fact, and the circumstance that known L-AP4 receptors and metabotropic glutamate receptors are pharmacologically and functionally distinct, justifies placing them in separate subclasses (Koerner & Johnson, 1992). In the present study, there were differences in the neuropharmacological actions of DCG-IV and L-AP4. DCG-IV completely reduced the DR-VRP monosynaptic component at the nanomolar range, but both monosynaptic excitation and polysynaptic discharges still occurred but at lower amplitudes in the presence of higher concentrations of L-AP4. Furthermore, in the case of the contralateral slow DR-VRP, L-AP4 attenuated it more effectively than DCG-IV, contrary to the case of the monosynaptic component of the ipsilateral DR-VRP, suggesting a difference in the distribution of their receptors.

It has been suggested that monosynaptic transmission of motoneurones may be mediated through non-NMDA receptors (Jahr & Yoshioka, 1986). Since DCG-IV did not affect the depolarizing responses to excitatory agonists for ionotropic glutamate receptors, the reduction of the DR-VRP monosynaptic component caused by DCG-IV would be expected to be a result of activation of presynaptic events, in addition, this is not due to activation of presynaptic ionotropic glutamate receptors, namely AMPA, kainate and NMDA receptors. Metabotropic glutamate receptors seem to regulate transmitter release in the hippocampus and other sites (Baskys & Malenka, 1991; Lovinger, 1991; Sunter *et al.*, 1991). In the present study, it is suggested that DCG-IV and L-CCG-I act on presynaptic terminals of primary afferent fibres directly or through activation of interneurones to decrease transmitter release, although their effective concentrations are considerably different. In the TTX-free ACSF, the baseline of the potential derived from the ventral root fluctuated considerably. The baseline fluctuation is due to spontaneous discharges through the release of neurotransmitters from nerve terminals. The fact that fluctuation of the baseline was abolished by L-CCG-I, L-AP4 and DCG-IV, could be explained by their block of transmitter release. On the other hand, (1S,3R)-ACPD did not reduce the fluctuation but rather enlarged it to increase excitability of motoneurones.

These results may differentiate their pharmacological properties to some extent.

The metabotropic glutamate receptors seem to be decreased in number or sensitivity due to developmental regulation with the lapse of days after birth (Nicolleti *et al.*,1986; Palmer *et al.*, 1990; Baskys & Malenka, 1991); therefore, the question remains as to whether the actions of DCG-IV and L-CCG-I are not common to those of the adult rats. However, when DCG-IV and L-CCG-I were given intraventricularly to the adult rat at a dose of 5–10 nmol or 50–600 nmol, respectively, both compounds caused marked sedation in rats, although there was a difference in the course of the development of sedation between L-CCG-I and DCG-IV. DCG-IV often caused a sedative effect after an excitatory stage in which the rat sometimes jumped abruptly or extended the hind limb convulsively. When 100 nmol of DCG-IV was administered intraventrically to the rat, the rat died from inhibition of respiration within about 10 min, while 600 nmol of L-CCG-I caused only marked reduction of muscle tone in the whole body, and this condition continued for more than 1 h. These results suggest that DCG-IV and L-CCG-I are still active even in adult rats, although receptors for DCG-IV and L-CCG-I may decrease in number or activity after birth. DCG-IV is expected to provide further useful information on the physiological function of metabotropic glutamate receptors.

This work was supported in part by Grant-in-Aids for Scientific Research from the Ministry of Education, Science and Culture of Japan to M.I. and H.S., and a Grant-in-Aid for Scientific Research on Priority Area from the Ministry of Education, Science and Culture of Japan to H.S.

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(Received January 18, 1993
Revised March 8, 1993
Accepted March 30, 1993)

Br. J. Pharmacol. (1993), **109**, 1178–1185 © Macmillan Press Ltd, 1993

Transmitter-mediated local contracture of the endplate region of the focally innervated mouse diaphragm treated with anticholinesterase

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1 Local contraction of the endplate region in response to nerve stimulation was studied in isolated mouse diaphragms. In normal preparations, muscle contractions involved the whole length of the muscle fibre with rise and decay times in the order of tens of ms whether evoked with a single or train of pulses.

2 When acetylcholinesterase was inhibited with neostigmine, tetanic stimulation produced a twitch-like phasic contraction and a delayed tonic contracture. A brief train of pulse (10 ms, 300 Hz) was enough to trigger a full size tonic contracture which reached an amplitude about one tenth that of control tetanus and had a duration of about 4 s.

3 Tetanic stimulation evoked a non-propagating prolonged depolarization at the endplate region lasting for about 1 s following a few muscle action potentials.

4 μ-Conotoxin, a specific inhibitor of muscle Na^+ channel, selectively abolished the phasic contraction and the muscle action potentials leaving the tonic contracture and the prolonged depolarization unaffected.

5 Both the tonic contracture and the prolonged depolarization were highly sensitive to blockade by tubocurarine (IC_{50} 0.05–0.1 μM) and vesamicol (1 μM, an inhibitor of packaging acetylcholine into synaptic vesicles), were attenuated by increasing Ca^{2+} concentration and were prolonged by decreasing Ca^{2+}.

6 The results suggest that prolonged activation of endplate nicotinic receptors by endogenously released transmitter can produce substantial contractions of the endplate region when acetylcholinesterases are inhibited. The source of Ca^{2+} for the contraction seems to come mainly from intracellular stores.

Keywords: Anticholinesterase; muscle contracture; neuromuscular junction

Introduction

The physiological process that triggers excitation-contraction coupling in skeletal muscle is depolarization of the sarcolemma (Ríos & Pizarro, 1991). For the multiply innervated muscle fibres, as found in amphibia, birds, fish and extraocular muscles of mammals, which have high densities of innervations and widespread distribution of acetylcholine (ACh) receptors, the whole muscle membrane can be directly depolarized by the transmitter released from adjacent nerve terminals and the resulting depolarization is sufficient to activate the contractile mechanism (cf. Bowman, 1990). On the other hand, most mammalian muscle fibres are focally innervated and the depolarization of the whole plasma membrane relies on the propagative action potential that is initiated by the junctional endplate potential (e.p.p.) which is confined to the endplate region. The question then arises whether e.p.p. produces a local contraction of the motor endplate region of the focally innervated muscle fibre. Burd & Ferry (1987) reported a contraction of the endplate region after a single indirect stimulation when treated with anticholinesterase. In the present study, we explored the direct effects of e.p.p. on the contraction of the endplate region of mouse diaphragm, a focally innervated striated muscle, by deliberately abolishing the muscle action potential with μ-conotoxin, a specific blocker of the muscle Na^+ channel (Gray & Olivera, 1988; Hong & Chang, 1989b). We investigated in particular the effect of repetitive stimulation on the contraction of the endplate region when the hydrolysis of ACh by acetylcholinesterase is retarded since under such a condition the amplitude and duration of the e.p.ps are increased (Katz & Miledi, 1975) and the regeneratively released neurotransmitter depolarizes enplate region for a prolonged period of time (Chang & Hong, 1986; Hong & Chang, 1989a).

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Methods

Phrenic nerve-hemidiaphragm preparations were isolated from mice (ICR strain, 25–30 g). The organ chamber contained 15 ml Tyrode solution (composition in mM: NaCl 137, KCl 2.8, $CaCl_2$ 1.8, $MgCl_2$ 1.1, $NaHCO_3$ 11.9, NaH_2PO_4 0.33 and dextrose 11.2) unless otherwise indicated. The solution was maintained at 34–36°C and the pH adjusted to 7.2–7.4 by aerating with a gas mixture of 5% CO_2 in O_2. Preparations were loaded with 1–1.5 g resting tension. Indirect contractions were evoked by stimulation of the trunk of the phrenic nerve with supramaximal pulses of 0.05 ms duration and direct contractions by field stimulation with 0.5 ms duration unless otherwise indicated. Tetanic contractions were elicited by a train of pulses consisting of variable number of pulses (2–600) at different frequencies (25–300 Hz). The interval between trains of stimulation was 10–15 min to minimize post-tetanic influences. Mechanical responses were recorded with a sensitive isometric transducer (Gould BG25), which can differentiate a small change of tension (0.03 g) from environmental vibrations. The contraction of the diaphragm was also visualized through a stereomicroscope, equipped with a plan or achromat objective (Zeiss SV 8).

Three procedures were used to paralyze diaphragms for

electrophysiological studies. (1) Preparations were treated with μ-conotoxin (0.75–1.25 μM). (2) Formamide, a chemical that modifies the function of sarcoplasmic reticulum (Del Castillo & Escalona De Motta, 1978) was used. The nerve-muscle preparations were incubated in Tyrode solution containing 2–3 M formamide under direct stimulation at 2 Hz for 25–35 min and thereafter, equilibrated in normal Tyrode solution for 30–60 min. (3) In some experiments, cut muscle preparations were used. Both ends of diaphragm muscles were crushed with forceps and left standing in Tyrode solution for 1.5–2 h until the resting membrane potential declined from about − 80 mV to − 40 to − 46 mV. Immobilizations were considered adequate if the developed tension in response to a test train of direct stimulation (200 Hz, 3 s) was reduced from a control level of 15–20 g to less than 0.3 g. Membrane potentials, e.p.ps and miniature e.p.ps were recorded conventionally with a high impedance amplifier (Dagan 8100). Glass microelectrodes filled with 3 M KCl had resistance of 5–15 MΩ. The signals were filtered at 10 kHz and digitized at 100 kHz and recorded on an electrostatic recorder (Gould ES 2000). Membrane potentials in each preparation from 25–30 muscle fibres were pooled excluding the lowest 10% of the values. The microelectrode was assumed to be targeted at the endplate region when the rise time of the sampled miniature e.p.p. was less than 0.6 ms (without treatment of neostigmine) or 1 ms (after neostigmine). The quantal releases of e.p.ps were estimated by direct comparisons of e.p.p./miniature e.p.p. in preparations paralyzed with μ-conotoxin, in which e.p.ps (evoked at a rate of 0.7 Hz) and miniature e.p.ps were sampled from the same junction for 35 s. Both amplitudes of e.p.ps and miniature e.p.ps were corrected for non-linear summation with a reference membrane potential of − 80 mV as previously described (Chang *et al.*, 1986).

The duration of contraction or endplate depolarization was defined as the time spanning the time course of 30% peak amplitude. The rise and decay times of contraction were defined as the time for 10 to 90% and from 90 to 10% peak amplitude, respectively. All data are presented as means ± standard error of recordings obtained from at least 4 preparations. Differences between means were analyzed by Student's *t* test and a statistical *P* value less than 0.05 was considered significant.

Chemicals used were neostigmine, tetrodotoxin and tubocurarine (Sigma Chem. Co., St. Louis, U.S.A.). μ-Conotoxin was purchased from Peptide Institute (Osaka, Japan). Vesamicol (AH5183) was generously supplied by Glaxo (London, U.K).

Results

Effects of neostigmine on muscle contraction

In control preparations, diaphragms contracted and relaxed rapidly in response to single direct or indirect stimulations with the following parameters: amplitude 1.6 ± 0.2 g, rise time 14 ± 1 ms, decay time 34 ± 3 ms, and duration 36 ± 3 ms ($n = 5$). In order to produce a well-summated tetanic contraction, repetitive pulses greater than 75 Hz were required; otherwise the contractile force was far less than maximum and had a ripple contour (Figure 1). At a stimulation frequency of 200–300 Hz, the amplitude of tetanic contraction approached maximum after about 10 pulses of stimulation and the developed force was nearly 10 fold that of single twitch. On termination of stimuli, the tetanic tension decayed to the pre-stimulation basal level almost as rapidly as a single twitch (Figure 1a).

After treatment with neostigmine at 0.2 μM, a concentration sufficient to provide substantial inhibition of acetylcholinesterase but not to produce side effects on the ACh receptor ion channel (Chang *et al.*, 1987), the direct twitch force was potentiated 4–5 fold (7.8 ± 0.9 g) and the rise time of twitches was approximately doubled (26 ± 2 ms). All of these changes can be ascribed to the repetitive firings of muscle action potentials in response to a single indirect stimulation (cf. Hobbiger, 1976). The decay time of twitches appeared unchanged (32 ± 3 ms). When stimulated with a train of pulses at 40–300 Hz, the contraction of the preparations had two distinct components: an initial twitch-like phasic contraction with a fast relaxation (i.e., a tetanic fade) and a late tonic contracture which reached the peak 400–600 ms after the start of the train of pulses (Figure 1b). The amplitude of the phasic contraction was about 70% that of the control tetanic contraction (11.2 ± 0.8 vs 15.7 ± 1.1 g, $n = 8$), i.e., the tetanic contraction waned before reaching the maximal contractility. The amplitude of the tonic contracture (1.7 ± 0.1 g) was about one tenth that of the control maximal tetanus but its duration was more than a hundred times longer than that of the twitches (3924 ± 210 vs 36 ± 3 ms). This late tonic contracture was characterized by a slow rise and slow decay as compared to those of the phasic contraction. Once triggered, both the magnitude and duration of the tonic contracture remained the same at stimulation frequencies ranging from 50 to 300 Hz. The higher the stimulation frequency the fewer the number of pulses were needed such that, at 300 Hz, a 10 ms short train of stimulation consisting of 4 pulses were sufficient to elicit the fullsize tonic contracture. Additional pulses extending over 1000 ms did not change the profile of contracture but increased slightly the tension at decay phase (Figure 1b4 vs 1b3). The tonic contracture could be repeated and the size maintained for 2 h if the interval of tetanic stimulations was longer than 10 min.

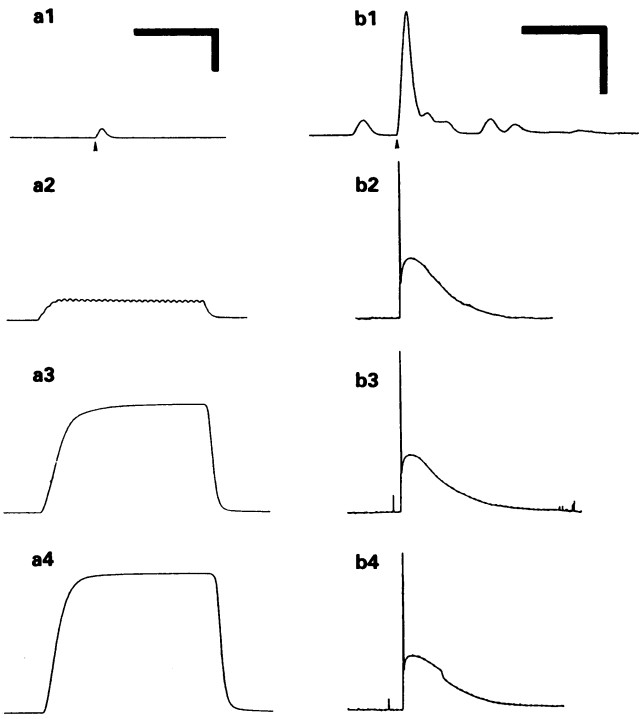


Figure 1 Effects of pulse number and stimulation frequency on indirect contraction of mouse phrenic nerve-diaphragm. (a) Control contractile responses showing single twitch (a1) and tetanic contractions for 0.6 s evoked at 50 Hz (a2), 100 Hz (a3) and 200 Hz (a4). Calibrations: 0.3 s and 5 g. (b) Contractile responses of the same preparation after 30 min incubation with 0.2 μM neostigmine showing the spontaneous fasciculation and the evoked response after a single pulse (arrowhead, b1) and the evoked responses to train of pulses at 50 Hz for 0.6 s (b2) and at 200 Hz for 0.03 s (b3) or for 3 s (b4). Calibrations: 0.3 s (b1), 6 s (b2–b4) and 5 g. Note in (b) the tonic contractures of equal amplitude and duration irrespective of pulse frequency or length of train of pulses.

However, when train of pulses were repeated at intervals shorter than 5 min the size of tonic contracture declined within 1 h.

Effects of μ-conotoxin and formamide on the phasic and tonic contractions

μ-Conotoxin at a concentration of 0.75–1.25 μM abolished the indirect as well as direct twitches and tetanic contractions (Figure 2), confirming the blockade of muscle Na^+ channels (Hong & Chang, 1989b). Moreover, a train of indirect stimulations produced no change of light transmittance at the endplate region, indicating that no local contraction is elicited even in the presence of repetitive e.p.ps when acetylcholinesterase is not inhibited. After further addition of neostigmine, single indirect stimulations evoked small amplitude twitches (the amplitude was 7–15% that of control, Figure 2) with a slow rise time and a prolonged duration (Figure 3a), referred as slow twitches hereafter. A train of stimulation (at 40–300 Hz) now evoked a full-sized tonic contracture without the initial phasic twitch component (Figure 2a4). During optical examination the transmittance of light around the endplate region, but not the rest of the muscle, was reduced at times when indirect stimulations elicited the tonic contracture, indicating that only the endplate region is involved in the tonic contracture. When added to neostigmine-pretreated preparations, μ-conotoxin inhibited the phasic response completely without changing the tonic contracture in response to trains of stimulation (Figure 2b). The profiles and magnitudes of the control tetanus and the tonic contracture are summarized in Figure 3b. When preparations were immobilized with formamide, neither single nor repetitive indirect stimuli produced observable contraction, no matter whether acetylcholinesterase was inhibited or not.

Effect of tubocurarine on the tonic contracture

If the muscle Na^+ channel was not inhibited, tubocurarine (0.03–0.3 μM) partially antagonized the tetanic fade produced by neostigmine when the nerve was stimulated with a train of pulses at 75–100 Hz (Chang *et al.*, 1988; Figure 4a4). By contrast, in preparations paralyzed with μ-conotoxin, tubocurarine dose-dependently inhibited the magnitude of the slow twitches and the tonic contractures and shortened the duration of the contractures (Figure 4 and Table 1). At 0.2 μM, tubocurarine abolished all indirect contractions. In this respect, tubocurarine was 10 fold more potent than in inhibitions of twitches in normal preparations (IC_{50}: 0.07 ± 0.02 vs 0.86 ± 0.10 μM, $n = 5$).

Effects of Ca^{2+} on the tonic contracture

The influence of low (0.45 mM) and high (7.2 mM) Ca^{2+} Tyrode solution on the slow twitch and the tonic contracture was studied. Low and high Ca^{2+} Tyrode solution changed the quantal release to 25 ± 4 and 167 ± 10% of control ($n = 4$), respectively. These changes in Ca^{2+} concentration had negligible effect on direct twitches within 40 min (being 93 ± 3% of control, $n = 4–8$). The small slow twitches evoked after treatment with neostigmine and μ-conotoxin were abolished in low Ca^{2+} Tyrode solution but were potentiated in high Ca^{2+} Tyrode solution (Figure 5). Interestingly, the effects of Ca^{2+} on the tonic contracture were different. In low Ca^{2+} Tyrode solution though more pulses were needed to evoke the tonic contracture, the contracture had a more prolonged duration than that obtained in normal Tyrode solution. While in high Ca^{2+} Tyrode solution both the duration and the magnitude of the tonic contracture were suppressed (Figure 5, Table 1).

Inhibition of tonic contracture by vesamicol

The effects of modifying ACh release on the prolonged responses were tested in another way by the use of vesamicol, which is a specific inhibitor of the vesicular ACh transport system (Marshall & Parsons, 1987). In preparations treated with neostigmine but without indirect stimulation, treatment with vesamicol at 1 μM did not change the phasic contraction nor the tonic contracture tested at 2 h of incubation. How-

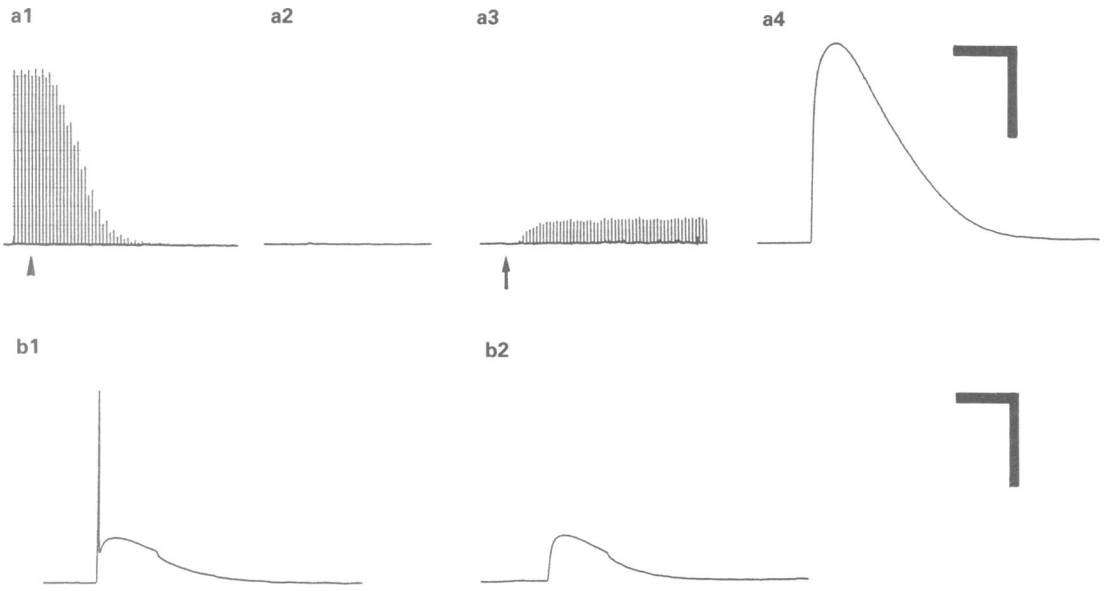


Figure 2 Effects of μ-conotoxin on direct and indirect contractions of mouse phrenic nerve-diaphragm. (a) The preparation was treated, in sequence, with μ-conotoxin (1.25 μM) at ▲ and with neostigmine (0.2 μM) at ↑. The stimulation modes were: (a1) indirect and direct pulses alternatively at 0.1 Hz; (a2) indirect train of pulse at 300 Hz for 3 s; (a3) indirect pulse at 0.1 Hz, and (a4) indirect train of pulse at 300 Hz for 0.01 s. Calibrations: 1 g and 3 s except in a1 and a3 where the intervals between single twitches were 10 s. (b) Another preparation pretreated with 0.2 μM neostigmine and stimulated with train of pulses at 50 Hz for 3 s. (b1) Control, and (b2) 15 min after 1.25 μM μ-conotoxin. Calibrations: 5 g and 3 s. Note that μ-conotoxin isolates the late tonic contracture from the phasic contraction.

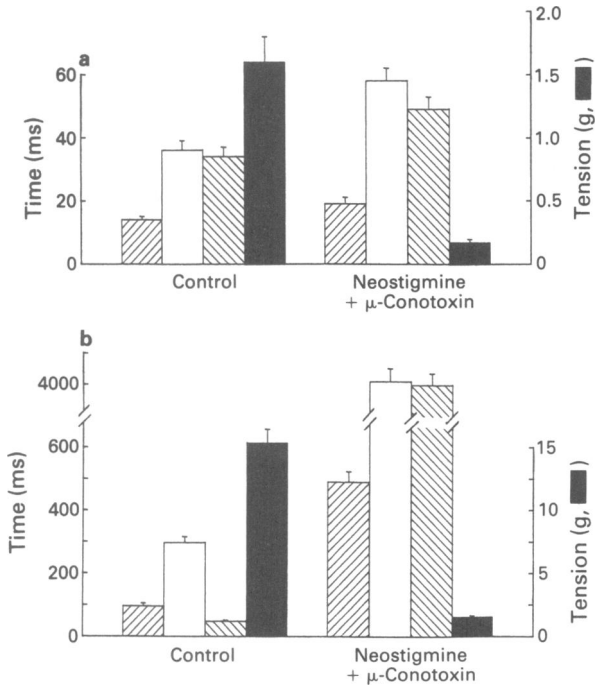


Figure 3 Effects of neostigmine and μ-conotoxin on the time course and amplitude of indirect contraction of mouse phrenic nerve-diaphragm. Phrenic nerves were stimulated either with a single (a) or with a train of pulses at 200 Hz for 0.3 s (b). The contractile responses were evoked in the absence or presence of neostigmine (0.2 μM) and μ-conotoxin (1.25 μM). Four parameters of indirect contraction were measured: ▨ 10–90% rise time; □ 70% duration; ▧ 90–10% decay time, and ■ contractile force. Data were pooled from 5–8 preparations.

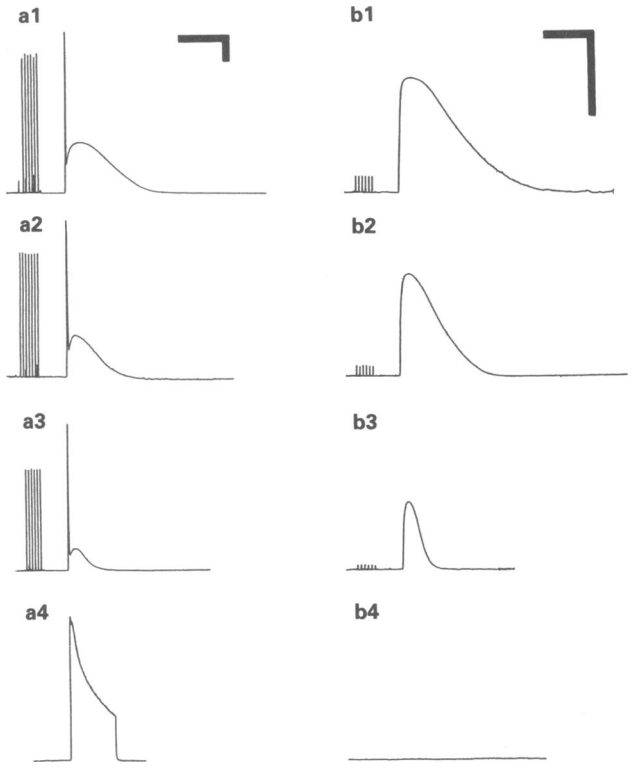


Figure 4 Effects of tubocurarine and μ-conotoxin on tonic contracture of mouse phrenic nerve-diaphragm treated with neostigmine. Preparations were treated with 0.2 μM neostigmine (a) or, in addition, with 1.25 μM μ-conotoxin (b). Tubocurarine was added at concentrations of 0, 0.05, 0.1 and 0.2 μM, respectively, for panels 1, 2, 3 and 4. Phrenic nerves were stimulated first with 6–7 single pulses at 0.1 Hz and then at 200 Hz for 0.1 s, except in panel 4, where stimulation at 75 Hz for 3 s was applied. Calibrations: 1 g and 3 s (for tetanic contraction or tonic contractures). Note that tubocurarine dose-dependently inhibited tonic contractures (a2, a3, b2, b3 and b4) and slow twitches (b2 and b3) while partially restoring the tetanic contraction at 75 Hz (a4).

ever, when the phrenic nerve was periodically stimulated with trains of pulses (100 Hz for 3 s every 10 min for 40 min), vesamicol increased the amplitude of the phasic contraction slightly by 9 ± 2% but inhibited the tonic contracture by 49 ± 5% (estimated from depressions of contracture areas, $n = 4$). The increase of the phasic contraction is a common phenomenon also observed on treatment with low concentrations of tubocurarine or low Ca^{2+} Tyrode solution (data not shown) and seems to be due to an inhibition of the induction of the prolonged endplate depolarization and hence to an increased number of initial muscle action potentials.

Effects of membrane potentials of endplate

In formamide-immobilized preparations treated with neostigmine, trains of indirect stimulation produced initially a few muscle action potentials and then a local prolonged

Table 1 Effects of Ca^{2+}, tubocurarine and μ-conotoxin on the depolarization of endplate and the late tonic contracture of mouse phrenic nerve-diaphragm

Treatment[a]	*Tonic contracture (%)* *Amplitude*	*Duration*	*Endplate depolarization (%)*[b] *Amplitude*	*Duration*
Ca^{2+} (mM)				
0.45	93 ± 4	121 ± 5*	89 ± 9	128 ± 11*
1.8[c]	100	100	100	100
3.6	79 ± 7*	72 ± 8*	105 ± 4	62 ± 10*
7.2	54 ± 9*	41 ± 7*	96 ± 6	30 ± 3*
Tubocurarine (μM)				
0.05	84 ± 3*	70 ± 6*	93 ± 3	49 ± 8*
0.1	49 ± 6*	33 ± 5*	75 ± 7*	22 ± 5*
μ-Conotoxin (μM)				
1.25	96 ± 4	98 ± 3	98 ± 5	103 ± 4

[a]Preparations were incubated in Tyrode solution containing 0.2 μM neostigmine and phrenic nerves were stimulated at 200 Hz for 0.1–0.3 s.
[b]Cut muscle preparations were used and the 70% duration of the prolonged endplate depolarization (6–11 endplates for each preparation) was calculated.
[c]The control values for the amplitude and duration in normal Tyrode solution were 1.6 ± 0.2 g and 3924 ± 210 ms (for the tonic contracture), and 20.1 ± 1.9 mV and 924 ± 67 ms (for the endplate depolarization), respectively.
*$P < 0.05$ vs the values in Tyrode solution containing 1.8 mM Ca^{2+}.
Data were pooled from 4–8 preparations.

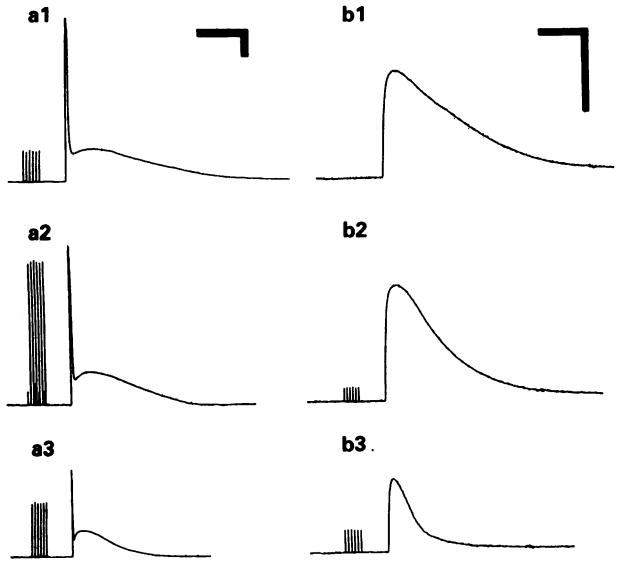


Figure 5 Effects of Ca^{2+} on indirect contraction of mouse phrenic nerve-diaphragm treated with neostigmine. Preparations were pretreated with 0.2 μM neostigmine (a) or, in addition, with 1.25 μM μ-conotoxin (b). Calcium concentrations in Tyrode solution were 0.45, 1.8 and 7.2 mM, respectively, for panels 1, 2 and 3. Phrenic nerves were first stimulated at 0.1 Hz for 6 pulses and then with train of pulse at 200 Hz for 0.1 s. Calibrations: 1 g and 3 s (for tonic contractures). Note changes in amplitude and duration of tonic contractures (a and b) and amplitude of slow twitches (b).

depolarization of the endplate (Figure 6). The action potentials can account for the twitch-like phasic contraction in non-immobilized preparations. The magnitude of depolarization at plateau was about 20–30 mV and the duration lasted up to 1000 ms. Once the prolonged depolarization occurred, continued stimulation of the motor nerve failed to elicit phasic e.p.p, and the stimulations had no apparent effect on the magnitude and duration of the prolonged depolarization as previously reported in cut muscle preparations (Chang & Hong, 1986; Hong & Chang, 1989a). When recorded at a site about 3–4 mm away from the endplate region, only 3–8 muscle action potentials were registered at the beginning of a train of pulse each having a uniform size of amplitude. Thereafter, no residual membrane depolarization was observed. These results demonstrate that the tonic contracture was not associated with muscle action potentials and that the depolarization was confined to the endplate area.

μ-Conotoxin abolished the muscle action potentials but totally preserved the prolonged endplate depolarization (Figure 6). As compared in the figure, the endplate depolarization occurred ahead of the tonic contracture although it was of shorter duration. At a stimulation frequency of 35 Hz, about two thirds of junctions failed to produce the prolonged endplate depolarization. Simultaneously, it was observed that the amplitude as well as the duration of the tonic contracture decreased.

The prolonged depolarization was shortened and inhibited by tubocurarine (Table 1) and by vesamicol (1 μM, data not shown) in parallel with the depression of tonic contracture. Low and high Ca^{2+} Tyrode solution affected the duration of the prolonged depolarization in the same way as they both changed the duration of the tonic contracture (Table 1).

Contraction evoked by direct stimulation of muscle

In view of the relationship between tonic contracture and prolonged depolarization, the effect of a long direct electrotonic depolarization of muscle, bypassing activation of nicotinic receptor and muscle Na^+ channel, was studied in the presence of tubocurarine and tetrodotoxin. An intensive field stimulation of the diaphragm with a rectangular pulse of 10–300 ms duration evoked a prolonged contracture for 2–6 s with a characteristic slow decay (Figure 7). Both the amplitude and duration of the contracture increased as the pulse duration was lengthened. By contrast, the tetanic contractions produced by a train of short pulses (pulse duration 0.5 ms at 300 Hz for 3 s) were of lower amplitude than that induced by a long pulse (100 ms) and relaxed rapidly after termination of stimuli, suggesting that a mechanism for tonic contracture is triggered by a prolonged depolarization but hardly by repetitive short pulses. These contractions were not blocked in Ca^{2+}-free Tyrode solution containing 3 mM EGTA, suggesting that they utilize the Ca^{2+} mobilized from intracellular stores. The changes of muscle membrane potential during such a prolonged contracture were studies in formamide-immobilized diaphragms. The recording microelectrode was inserted into the portion of muscle fibre close to one of the field stimulation electrodes. After the application of a long pulse (100 ms), the membrane potential remained depolarized by 20–40 mV for 0.4–0.8 s and then repolarized to pre-stimulation level. Clearly, the time course of the pulse-induced contracture outlasted that of the pulse-induced membrane depolarization. This temporal relation between contracture and membrane depolarization is similar to that between the tonic contracture and the prolonged endplate depolarization evoked by indirect stimulations in neostigmine-treated preparations.

Discussion

The present results revealed that, unlike the multiply innervated muscle fibres, the endplate region of the focally innervated fibres do not contract in response to single or trains of high frequency e.p.ps in normal condition when the muscle action potential is abolished by μ-conotoxin. This situation, however, is changed and muscles contract in response to indirect stimulation when the hydrolysis of released ACh is retarded. In the presence of neostigmine, single stimuli elicited slow twitches and a train of pulses evoked tonic contractures. Both contractions are not associated with muscle action potential, are resistant to μ-conotoxin but are inhibited by tubocurarine. These results indicate that the contractions are initiated by the endogenous ACh released from the nerve terminal and are confined to the endplate regions of muscle fibres. In the presence of anticholinesterase, the single e.p.p. is not only enhanced but also prolonged (Katz & Miledi, 1975). This may account for the slow twitch responses. Since repetitive stimulation tends to provoke a very marked long-lasting endplate depolarization presumably due to a regenerative release of ACh (Chang & Hong, 1986; Hong & Chang, 1989a), it is likely that the endplate region elicits the tonic contracture in response to the prolonged local depolarization.

The late tonic contracture and the prolonged endplate depolarization observed in neostigmine-treated preparations share several characteristic features. (1) Both phenomena are resistant to μ-conotoxin and are restricted to the endplate area. (2) Both phenomena, once triggered, bear an 'all' property as revealed by the full-size of the tonic contracture and depolarization, irrespective of pulse number and stimulation frequency. (3) The two responses are evoked in parallel by indirect stimulation in terms of stimulation frequency and number of pulses, are modulated in the same way by changes of Ca^{2+} concentration, and show high susceptibilities to blockade by tubocurarine or vesamicol. Moreover, 3,4-diaminopyridine, a K^+ channel blocker also known to produce a prolonged endplate depolarization on indirect stimulation (Hong & Chang, 1990), provoked a similar tonic contracture (unpublished observation), indicating that an inhibition of acetylcholinesterase *per se* is not a prerequisite for such

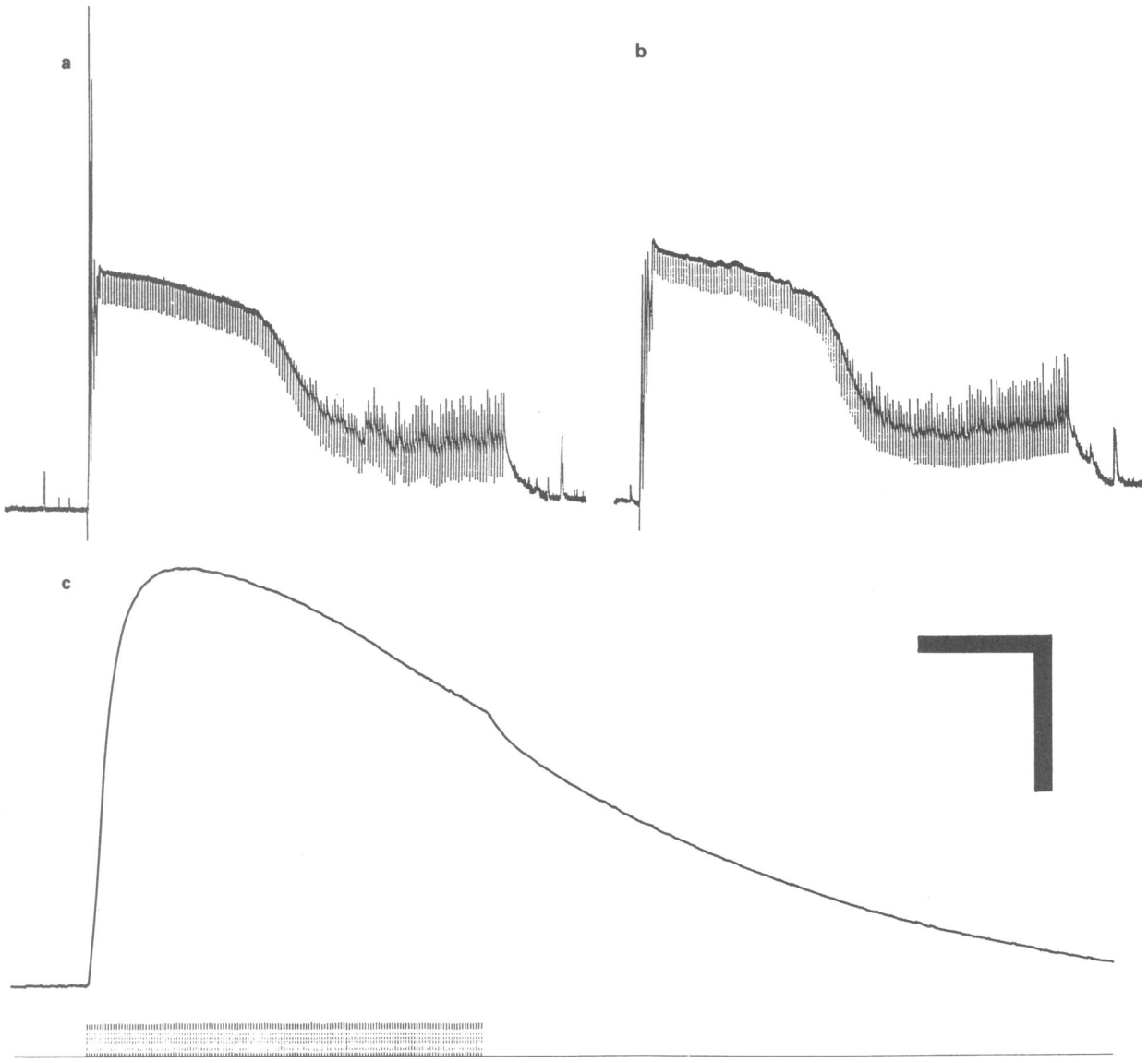


Figure 6 Temporal correlations of the prolonged endplate depolarization and the tonic contracture of mouse phrenic nerve-diaphragm treated with neostigmine. The electrophysiological tracings (a and b) were taken from the same endplate of a preparation immobilized with formamide and treated with 0.2 μM neostigmine before (a) or 30 min after a further treatment with 1.25 μM μ-conotoxin (b). The resting membrane potentials were − 72 mV (a) and − 74 mV (b). The contraction tracing (c) was taken from another preparation treated with neostigmine and μ-conotoxin. Phrenic nerves were stimulated with a train of pulse at 50 Hz for about 3 s (as indicated in the bottom). Calibrations: 1 s and 25 mV (a,b) or 0.5 g (c). Note that the muscle action potentials were observed in (a) but not in (b).

phenomenon. It may be inferred that the long-lasting contracture is caused by the intensive prolonged endplate depolarization, though the duration of the former greatly outlasts that of the latter. The mechanism that provokes the regenerative release of ACh and hence the prolonged endplate depolarization appears to involve activation of presynaptic ACh autoreceptors and Ca^{2+} channels (Hong & Chang, 1989a; Hong *et al.*, 1992). Since vesamicol inhibits ACh mobilization and enhances run-down of endplate current which are composed of quantal releases (Pemberton *et al.*, 1992), whether the mode of the unusual regenerative release of ACh is non-quantal (Zemková *et al.*, 1990) or quantal remains to be elucidated. The inhibitory effects of high Ca^{2+} Tyrode solution on the prolonged depolarization and tonic contracture may be due to activation of the large conductance Ca^{2+}-activated K^+ channels (Anderson *et al.*, 1988a,b) or desensitization of nicotinic receptors (Giniatullin *et al.*, 1989). The results using long pulse stimulation also demonstrate that a mechanism to provoke prolonged contracture upon prolonged depolarization exists along the whole muscle fibre. The mechanism appears not to be triggered by repetitive short pulses. The duration of the tonic contractures, whether induced by direct stimulation or by an indirect train of stimulation in the presence of neostigmine, appears to be related to the length of membrane depolarization. The dose-dependent shortenings of both phenomena by tubocurarine are in support of the idea that the tonic contracture is related to prolonged depolarization.

The tonic contracture is characterized by slow rise and slow fall of tension in comparison with the fast rise and decay of normal tetanic contraction. Moreover, the contracture lasted longer than the prolonged depolarization. It seems that, unlike the brief elevation of intracellular Ca^{2+} concentration triggered by muscle action potential, the prolonged depolarization might increase the cellular Ca^{2+} level continuously for an extended period of time. Since the Ca^{2+}

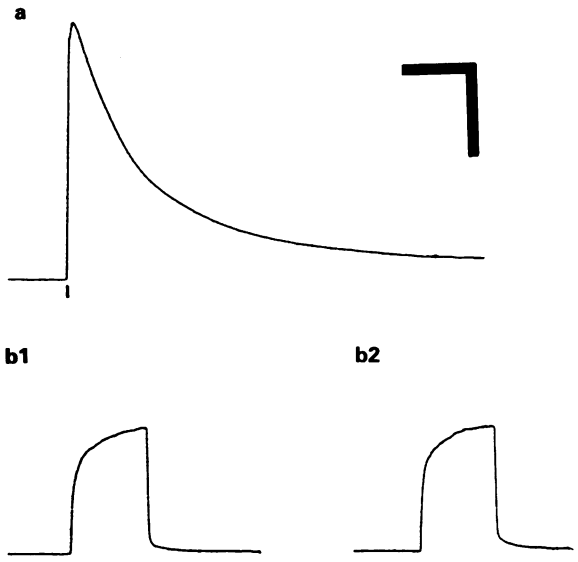


Figure 7 Contractions of mouse diaphragm evoked by direct stimulation. (a) The preparation was incubated in Ca^{2+}-free Tyrode solution containing EGTA (3 mM), tetrodotoxin (10 μM) and tubocurarine (10 μM) for 15 min and then stimulated with a pulse of 100 ms duration (|). (b) The preparation was pretreated with μ-conotoxin (1.25 μM) for 30 min and directly stimulated at 300 Hz for 3 s (pulse width 0.5 ms) before (b1) or 30 min after treatment with tetrodotoxin and tubocurarine (b2). Calibrations: 3 s and 1 g.

release from the sarcoplasmic reticulum is regulated by a Ca^{2+}-dependent inactivation process (Schneider & Simon, 1988), the present observations raise questions whether the prolonged depolarization provokes less inactivation process, desensitizes the inactivation mechanism or retards the Ca^{2+} uptake by sarcoplasmic reticulum.

Since the magnitude of the fullsize tonic contracture was about one tenth that of the maximal normal tetanic contraction, it may be assumed that about 10% of the contractile machinery is involved in the local contraction. This would mean that about 1 mm length of the muscle fibre of mouse diaphragm, the length of which is about 8–12 mm, participates in the contraction. This estimated length of muscle fibres is in accord with the length constant of the endplate depolarization but is more than 10 times larger than the surface dimension of the synaptic endplate (Salpeter & Eldefrawi, 1973; Danielo & Vogel, 1975). The observation that disturbances of the function of sarcoplasmic reticulum by formamide abolished the tonic contracture without impairment of generation of the prolonged endplate depolarization suggests that the majority of Ca^{2+} involved in the tonic contracture comes from the prolonged depolarization-induced Ca^{2+} release from subsynaptic sarcoplasmic reticulum, rather than from the Ca^{2+} influx through the ACh receptor ion channel. However, it cannot be ruled out that the mobilization of Ca^{2+} from intracellular stores by the ACh-dependent second messenger system (Giovannelli *et al.*, 1991) and the translocation of Ca^{2+} via the ACh receptor ion channel which becomes greater at more depolarized potentials (Decker & Dani, 1990) may contribute to some extent.

In mouse diaphragms treated with anticholinesterase, indirect stimulation produces a long-lasting Ca^{2+} signal at endplate region. This Ca^{2+} signal is different from the muscle action potential-induced fast Ca^{2+} transient in that it is slow in onset, enhanced by high Ca^{2+} solution and resistant to μ-conotoxin (Kimura *et al.*, 1991a,b). Although this slow Ca^{2+} signal was described as 'noncontractile', it is probable that the authors missed the low amplitude slow twitches (Figure 3c). Since under physiological conditions, e.p.p. itself produced no discernible local contraction it appears that the transient local depolarization of the endplate and Ca^{2+} influx across the postsynaptic membrane during the normal lifetime of transmitter action is not enough to cause contraction. On the other hand, prolonged and intensified activation of motor endplate nicotinic receptors by endogenous ACh can trigger a mechanism for prolonged local contractions. The possible profound increase in intracellular Ca^{2+} concentration at the nerve terminal and endplate region, as deduced from the intensive release of ACh and the tonic contracture, may bear a relation to the anticholinesterase-induced pathological changes of nerve endings and focal necrosis of muscle endplates (Laskowski *et al.*, 1975; Wecker *et al.*, 1978).

This work was supported by a grant from the National Science Council (NSC82-0412-B002-483).

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(Received February 15, 1993
Revised March 30, 1993
Accepted March 31, 1993)

Br. J. Pharmacol. (1993), **109**, 1186–1191 © Macmillan Press Ltd, 1993

Halothane inhibits the pressor effect of diphenyleneiodonium

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1 We have recently found that diphenyleneiodonium (DPI), a novel inhibitor of nitric oxide (NO) synthase, causes pressor and tachycardic responses in pentobarbitone- but not halothane-anaesthetized rats. The present study investigated the mechanism by which halothane suppresses the pressor response of DPI. The effects of halothane on the pressor response of DPI were also compared with those of other anaesthetic agents.

2 In conscious rats, i.v. bolus injections of DPI (0.025– 1.6 mg kg^{-1}) caused dose-dependent increases in mean arterial pressure (MAP), with ED_{50} of 0.07 ± 0.01 mg kg^{-1} and maximal rise of MAP (E_{max}) of 59 ± 2 mmHg. While ketamine potentiated E_{max} without altering the ED_{50} and pentobarbitone increased the ED_{50} without changing E_{max} of the pressor response to DPI, chloralose, urethane and ethanol displaced the curve to the right and potentiated E_{max}. In contrast, halothane (0.5–1.25%) dose-dependently and non-competitively reduced the pressor responses to DPI.

3 Intravenous bolus injection of a single dose of DPI (1.6 mg kg^{-1}) caused immediate and large increases in plasma noradrenaline and adrenaline, as well as MAP in conscious rats. Halothane (1.25%) almost completely inhibited these increases.

4 The results suggest that DPI causes a pressor response in conscious rats by activating the sympathetic nervous system and halothane abolishes this pressor response by inhibiting activities of the sympathetic nervous system. The results also show that influences of anaesthetics must be taken into consideration when evaluating pressor response of vasoactive agents.

Keywords: Diphenyleneiodonium (DPI); nitric oxide synthase inhibitor; anaesthetics; halothane; pentobarbitone; ketamine; urethane; ethanol; chloralose; pressor; sympathetic nervous system; noradrenaline; adrenaline; endothelium-dependent vasodilatation

Introduction

Diphenyleneiodonium (DPI), a bivalent iodine compound, was first reported to be a potent hypoglycaemic agent (Stewart & Hanly, 1969; Gatley & Martin, 1979). It was later shown to suppress the activities of neutrophil and macrophage NADPH-dependent oxidase (Cross & Jones, 1986), as well as macrophage nitric oxide (NO) synthase (Stuehr *et al.*, 1991), probably via the inhibition of a flavoprotein (Hancock & Jones, 1987; Ellis *et al.*, 1989; Stuehr *et al.*, 1990). DPI was also found to inhibit endothelium-dependent vasodilatation *in vitro* (Stuehr *et al.*, 1991; Poon *et al.*, 1993) and *in vivo* (Poon *et al.*, 1993). Recently, we found that DPI caused pressor and tachycardic effects in pentobarbitone-anaesthetized rats; the cardiovascular effects were due to the modulation of sympathetic nerve activities (Wang & Pang, 1993). In our preliminary experiments, we also found that halothane markedly suppressed the pressor effect of DPI.

Halothane has been shown to inhibit sympathetic nervous transmission at several levels (Seagard *et al.*, 1982; Larach *et al.*, 1987; Rorie *et al.*, 1990). Halothane was also found to alter endothelium-dependent vasodilatation (Muldoon *et al.*, 1988; Blaise, 1991), and to abolish the pressor responses of other NO synthase inhibitors, namely N^G-nitro-L-arginine (L-NNA) and its methyl ester (L-NAME) (Wang *et al.*, 1991a; Pang *et al.*, 1992). The aim of this study was to (1) investigate the characteristics of the inhibitory effect of halothane on the pressor response to DPI; (2) to examine whether halothane suppresses the pressor response of DPI by inhibiting activities of the sympathetic nervous system; (3) to compare the effect of halothane with those of intravenous and inhalation anaesthetic agents on the pressor response of DPI.

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Methods

Surgical preparation

Sprague-Dawley rats (300–360 g) were used in this study. Cannulae (PE50) were inserted into the left iliac vein for the administration of drugs, and into the left iliac artery for the recordings of mean arterial pressure (MAP) by a P23DB pressure transducer (Gould Statham, CA, U.S.A.) and heart rate which was determined electronically from the upstroke of the arterial pulse pressure using a tachograph (Grass, Model 7P4G). In some rats, another PE50 cannula was also inserted into the right iliac artery to collect blood samples. The body temperature of the anaesthetized rats was maintained at 37°C with a heating lamp connected to a thermostat (73A, Yellow Springs Instruments). In halothane-anaesthetized rats in Protocol 2 and 3 (see later), tracheostomy was also performed with a PE160 catheter. All anaesthetized rats were equilibrated for 20 min at the appropriate anaesthetic doses before the commencement of studies. The conscious rats in all the protocols were first anaesthetized with halothane (1.5% in air), to allow surgical preparation, and were allowed to recover for at least 6 h from the effects of anaesthesia before further use. The catheters were tunnelled subcutaneously and exteriorized at the back of the neck.

Measurement of plasma catecholamines

Plasma catecholamines levels were determined by a catecholamine Assay kit (Amersham Canada Ltd., Ont., Canada) (Passon & Peuler, 1974). The radioactivity (^3H) was detected by a 1600 TR liquid scintillation analyzer (Packard Instrument Co., CT, U.S.A.). Blood samples (0.5 ml) were immediately placed in prechilled tubes containing EGTA and reduced glutathione and centrifuged at 1,200 *g* at 4°C. Afterwards, the plasma was removed and stored at – 70°C until assayed within two weeks. Duplicate assays were run for the

standard (in blank plasma) and plasma samples (50 μl each) using distilled water as a blank control for each run. The sensitivity of the catecholamine assay was 0.005 ng ml^{-1} for each catecholamine.

Drugs

Halothane and diphenyleneiodonium (DPI) sulphate were obtained from Ayerst Lab. (Quebec, Canada) and Colour Your Enzyme Ltd. (Ont., Canada), respectively. Urethane ethyl carbamate and ketamine hydrochloride were from Sigma Chemical Co. (MO, U.S.A.). The following anaesthetics were also used: α-chloralose (BDH Chemical Ltd., Poole, England), sodium pentobarbitone (M.T.C. Pharmaceuticals, Cambridge, Ontario, Canada) and anhydrous ethyl alcohol (Stanchem Co., Quebec, Canada). The powder drugs were dissolved in normal saline (0.9% NaCl) except for DPI which was solubilized in 5% glucose solution by 10 min sonication.

Experimental protocol

The rats ($n = 6$ each group) were randomly assigned into groups. MAP and HR were continuously monitored throughout the experiments.

(1) *Dose-MAP response curves to DPI in conscious and anaesthetized rats* Six groups of rats were used: conscious rats and rats anaesthetized with sodium pentobarbitone (65 mg kg^{-1}), ketamine (140 mg kg^{-1}), urethane (2 g kg^{-1}), ethanol (4 g kg^{-1}) and chloralose (150 mg kg^{-1}). Dose-MAP response curves to i.v. bolus injections of DPI (0.025–1.6 mg kg^{-1}) were constructed at dose-intervals of 3–6 min, the time required to recover from the effects of the previous dose.

(2) *Dose-MAP response curves of DPI in conscious and halothane-anaesthetized rats* Five groups of rats were used: conscious rat and rats anaesthetized with different concentrations of halothane (0.5, 0.75, 1 and 1.25% in air) at flow rates of 1500 ml min^{-1}. The dose-MAP response curves of i.v. bolus injections of DPI (0.025–6.4 mg kg^{-1}) were constructed at dose-intervals of 3–6 min as above.

(3) *Effects of DPI on plasma catecholamine levels in conscious and halothane-anaesthetized rats* One group of conscious rats and one group of halothane (1.25%)-anaesthetized rats were injected i.v. with a single bolus dose of DPI (1.6 mg kg^{-1}). Blood samples were collected 20 min prior to and 30 s after the injections of DPI. MAP was continuously monitored.

Calculation and statistical analysis

Maximum effect (E_{max}), half-effective dose (ED_{50}) and Hill coefficient (n), from individual dose-response curves were fitted by a computer programme using non-linear least-squares to the relation $Y = a + bx$, where Y = response and $x = [D]^n/(ED_{50}^n + [D]^n)$ (Quastel & Saint, 1988; Wang & Pang, 1993). All results were expressed as mean ± standard error (s.e.mean) and analyzed by the analysis of variance followed by Duncan's multiple range test with $P < 0.05$ selected as the criterion for statistical significance.

Results

Effects of anaesthetics on DPI-induced MAP response

Baseline MAP in rats anaesthetized with urethane, ethanol and chloralose, but not pentobarbitone or ketamine, was less than that in conscious rats (Table 1).

In conscious rats, DPI caused an immediate (approximately 15 s in onset) and transient (1–2 min in duration) pressor response. The pressure response was dose-dependent (Figure 1) with ED_{50} of 0.07 ± 0.01 mg kg^{-1} and maximal MAP reached at 59 ± 2 mmHg, based on the best-fitted calculations (Table 1). The Hill coefficient n of 3.3 ± 0.5 was significantly different from 1, 2 and 5 but not from 3 or 4. DPI also caused tachycardia at lower doses (0.025–0.1 mg kg^{-1}), bradycardia at higher doses (0.2–1.6 mg kg^{-1}) and movements following the onset of the pressor response (data not shown).

Pentobarbitone, chloralose, urethane and ethanol but not ketamine displaced the dose-MAP curve of DPI to the right (Figure 1) by increasing ED_{50}s (Table 1). On the other hand, ketamine, chloralose, urethane and ethanol but not pentobarbitone potentiated the maximal MAP response to DPI (Figure 1 and Table 2). None of the anaesthetic agents significantly altered the n value of the dose-response curves of DPI (Table 1). Under the influence of all anaesthetics, the hindlimbs and occasionally the forelimbs displayed kicking motion immediately following injections of DPI.

Inhibitory effect of halothane on DPI pressor response

Halothane (0.5–1.25%) reduced baseline MAP in a dose-dependent manner (Table 1). In conscious rats, i.v. bolus injections of DPI also caused similar pressor responses as in protocol (1) (Figure 2 and Table 1). Halothane dose-dependently reduced the maximal effect of DPI and shifted the DPI curve to the right (Figure 2). ED_{50} values were linearly correlated while E_{max} values were inversely correlated with the

Table 1 Values of baseline mean arterial pressure (MAP), as well as parameters (Hill coefficient n, ED_{50} and E_{max}) of the dose-pressor response curves of diphenyleneiodonium (DPI) in conscious rats and rats anaesthetized with sodium pentobarbitone (65 mg kg^{-1}), ketamine (140 mg kg^{-1}), urethane (2 g kg^{-1}), ethanol (4 g kg^{-1}), chloralose (150 mg kg^{-1}) and halothane (0.5–1.25%)

Anaesthetics	*Baseline MAP* mmHg	*Dose-response curve to DPI* *n*	ED_{50} (mg kg^{-1})	E_{max} (mmHg)
Protocol 1				
Conscious	118 ± 2	3.3 ± 0.5	0.07 ± 0.01	59 ± 2
Ketamine	107 ± 7	3.2 ± 0.4	0.08 ± 0.01	72 ± 2*
Pentobarbitone	111 ± 6	3.3 ± 0.7	0.22 ± 0.04*	64 ± 4
Chloralose	89 ± 3*	3.7 ± 0.4	0.12 ± 0.01*	90 ± 4*
Urethane	72 ± 7*	2.9 ± 0.6	0.20 ± 0.03*	71 ± 4*
Ethanol	57 ± 6*	2.5 ± 0.4	0.26 ± 0.03*	78 ± 4*
Protocol 2				
Conscious	113 ± 4	2.9 ± 0.5	0.07 ± 0.01	59 ± 2
0.5% Halothane	96 ± 10	2.6 ± 0.2	0.48 ± 0.09*	62 ± 4
0.75% Halothane	83 ± 4*	2.7 ± 0.5	0.57 ± 0.08*	47 ± 4*
1% Halothane	80 ± 7*	3.8 ± 0.7	0.77 ± 0.15*	34 ± 3*
1.25% Halothane	69 ± 2*	–	–	13 ± 4*,[a]

Values are mean ± s.e.mean $n = 6$ for each group
*denotes significant difference from the conscious rat groups ($P < 0.05$). [a]represents observed data.

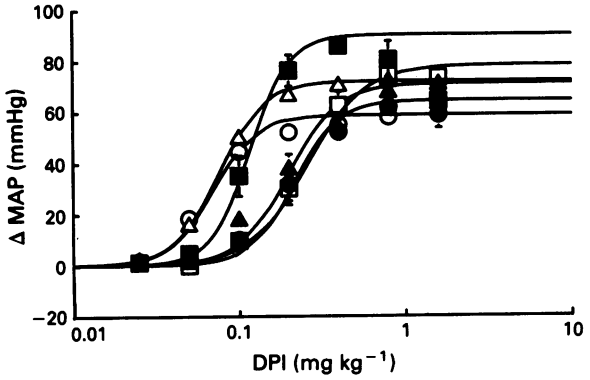


Figure 1 Dose-response (mean ± s.e.mean) curves of i.v. bolus injections of diphenyleneiodonium (DPI) on mean arterial pressure (MAP) in conscious rats (○), and rats anaesthetized with pentobarbitone (65 mg kg^{-1}) (●), ketamine (140 mg kg^{-1}) (△), urethane (2 g kg^{-1}) (▲), ethanol (4 g kg^{-1}) (□) and chloralose (150 mg kg^{-1}) (■). $n = 6$ each group. The lines represent theoretical curves using the formula $Y = E_{max}([D]^n/ED_{50}^n + [D]^n)$ and best-fitted n, ED_{50} and E_{max} shown in Table 1.

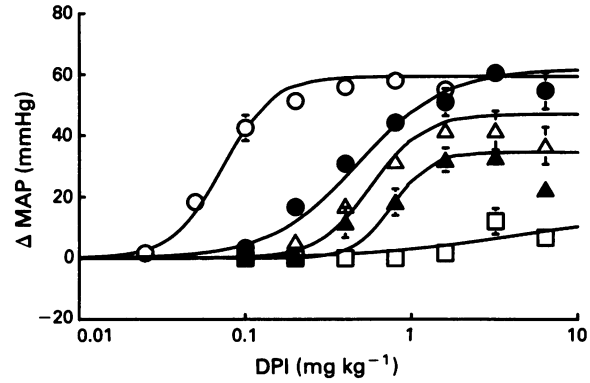


Figure 2 Dose-response (mean ± s.e.mean) curves of i.v. bolus injections of diphenyleneiodonium (DPI) on mean arterial pressure (MAP) in conscious rats and halothane (0.5–1.25%)-anaesthetized rats ($n = 6$ each group). Halothane: 0 (○); 0.5% (●); 0.75% (△); 1% (▲); 1.25% (□). The lines represent theoretical curves using the formula $Y = E_{max}([D]^n/ED_{50}^n + [D]^n)$ and best-fitted n, ED_{50} and E_{max} shown in Table 1.

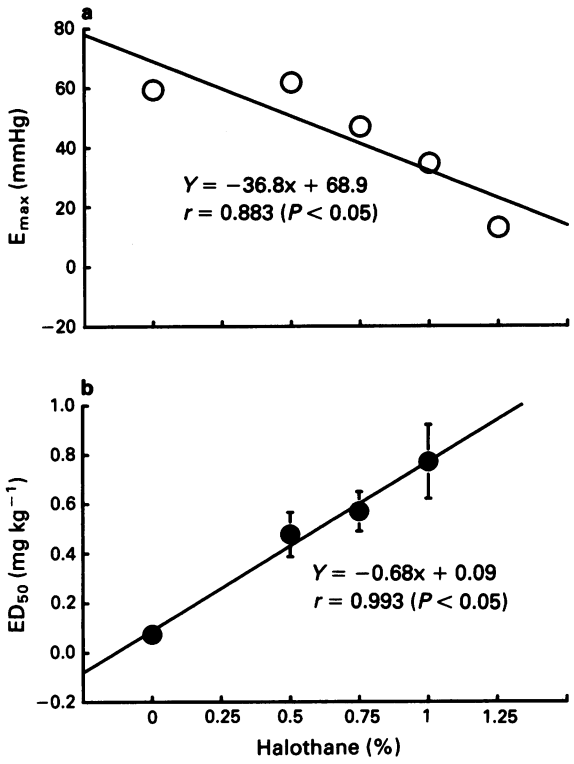


Figure 3 Effects of halothane (0.5–1.25%) on the maximal effect (E_{max}, a) and half-effective dose (ED_{50}, b) of the dose-mean arterial pressure response curves of i.v. bolus injections of diphenyleneiodonium (0.025–6.4 mg kg^{-1}) in rats ($n = 6$ each group).

Table 2 Baseline values of plasma catecholamines and mean arterial pressure (MAP) in conscious and halothane (1.25%)-anaesthetized rats

	Plasma catacholamine (ng ml^{-1})			
	Noradrenaline	*Adrenaline*	*Dopamine*	*MAP*
Conscious	0.26 ± 0.05	0.12 ± 0.01	0.10 ± 0.01	119 ± 4
Halothane	0.25 ± 0.03	0.06 ± 0.01*	0.10 ± 0.01	76 ± 2*

Values are mean ± s.e.mean. $n = 6$ each group.
*denotes significant difference from conscious rats ($P < 0.05$).

concentration of halothane (Figure 3). None of the doses of halothane affected the n value of the curves (Table 1). Halothane also inhibited DPI-induced tachycardia, bradycardia as well as movements (data not shown).

Effect of halothane on DPI-induced catecholamine release

Baseline MAP in halothane (1.25%)-anaesthetized rats was lower than that in conscious rats (Table 2). Baseline plasma level of adrenaline but not noradrenaline or dopamine in halothane-anaesthetized rats was also significantly lower than that in conscious rats (Table 2).

In conscious rats, i.v. bolus injection of DPI (1.6 mg kg^{-1}) caused immediate and large increases (more than 1 ng ml^{-1}) in plasma noradrenaline and adrenaline and a smaller increase (0.1 ng ml^{-1}) in plasma dopamine (Figure 4a), as well as immediate pressor response (Figure 4b). Halothane markedly attenuated DPI-induced increases in plasma catecholamines (Figure 4a) and in MAP (Figure 4b); the reductions of plasma noradrenaline, adrenaline and MAP were 86%, 81% and 95%, respectively.

Discussion

As in previous experiments with pentobarbitone-anaesthetized rats (Wang & Pang, 1993), DPI caused transient and dose-dependent increases in MAP in conscious rats with a Hill coefficient not significantly different from 3 or 4. Moreover, none of the anaesthetics affected the Hill coefficient value. The results suggest that the pressor effects of DPI in conscious and anaesthetized rats are due to the positive cooperation of 3 or 4 molecules of DPI with the corresponding 'receptors' (see Rang, 1971; see discussion of Pennefather & Quastel, 1982; Wang & Pang, 1993).

Although DPI inhibits endothelium-dependent vasodilatation *in vitro* and *in vivo* (Stuehr *et al.*, 1991; Poon *et al.*, 1993), the pressor response to DPI is not a consequence of this inhibition. In a previous study, we found that DPI caused pressor and tachycardic responses by sympathetic stimulation as reflected by concurrent elevations of blood pressure and plasma noradrenaline and adrenaline (Wang & Pang, 1993). Moreover, the pressor response of DPI was attenuated by manoeuvres which impair sympathetic nerve transmission,

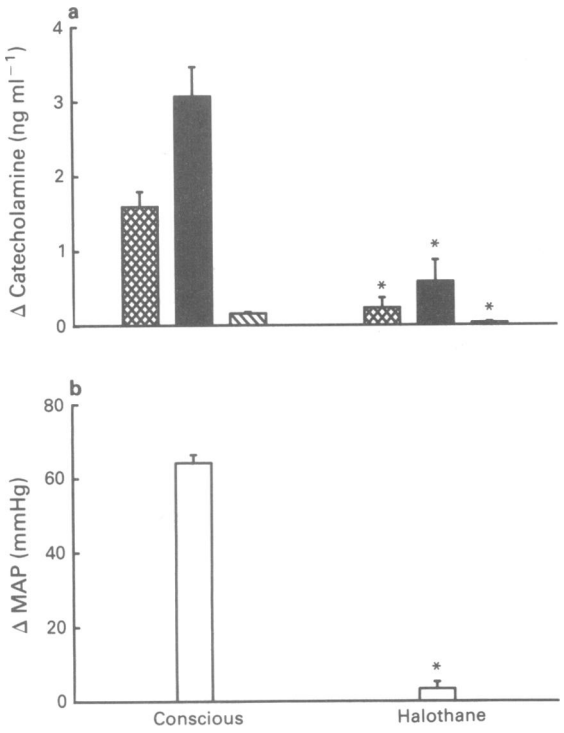


Figure 4 Effects (mean ± s.e.mean) of i.v. bolus injection of diphenyleneiodonium (1.6 mg kg^{-1}) on plasma catecholamines (a) and mean aterial pressure (MAP, b) in conscious and halothane (1.25%)-anaesthetized rats ($n = 6$ each group). In (a), noradrenaline: cross hatched columns; adrenaline: solid columns; dopamine; hatched columns.

namely, pithing, spinal cord transection, tetrodotoxin, mecamylamine, guanethidine, reserpine and phentolamine (Wang & Pang, 1993). In accordance with our previous results, the pressor responses of DPI in conscious rats in the present study were also accompanied by large increases in plasma noradrenaline and adrenaline. Although it is not clear how DPI activates sympathetic nerve activities, the following mechanisms are unlikely to be involved. Firstly, the effect of DPI on sympathetic nerve activity is unrelated to its hypoglycaemic action since the blood pressure and sympathetic stimulatory effects of DPI are immediate and transient (present study) while its hypoglycaemic effect is slow in onset (plateau at 4 h after i.p. administration) and prolonged in action (Gatley & Martin, 1979). Secondly, the pressor response to DPI is unlikely due to the activation of 'pain receptors' as pretreatment with capsaicin (100 mg kg^{-1} s.c. for 2 d) blocked DPI-induced limb kicking but not pressor responses in pentobarbitone-anaesthetized rats ($n = 6$, unpublished observations). Thirdly, the possible inhibitory effect of DPI on brain NO synthesis is unlikely to be responsible for the increase in sympathetic outflow (see discussion of Wang & Pang, 1993).

DPI caused tachycardia in pentobarbitone-anaesthetized rats, with the same time course and pharmacodynamics (ED_{50} and Hill coefficient) as the pressor response (Wang & Pang, 1993). It should be pointed out that the pressor and tachycardic responses of DPI are not interdependent, as propranolol blocked tachycardia but not the pressor response while phentolamine attenuated the pressor but potentiated the tachycardic response (Wang & Pang, 1993). In conscious rats in the present study, DPI produced tachycardia at low doses and bradycardia at high doses. In pentobarbitone-anaesthetized rats, the latter response was absent (Wang & Pang, 1993). The abilities of pentobarbitone anaesthesia to inhibit bradycardic responses to high doses of DPI and phentolamine to potentiate tachycardic responses suggest that bradycardia in response to high doses of DPI in conscious rats was secondary to hypertension-induced baroreflex activation. Different anaesthetic agents have variable influences on baroreflex activity. Due to the difficulty in separating variable direct and indirect effects of DPI on heart rate in different anaesthetic conditions, detailed kinetic analyses of the heart rate effect on DPI were not performed.

Our results also show that halothane dose-dependently and non-competitively inhibited the pressor responses of DPI. It should be emphasized that the inhibitory effect of halothane is not due to its hypotensive action as DPI caused greater pressor responses in rats anaesthetized with chloralose, urethane or ethanol where baseline blood pressures were either similar or lower than those in halothane-anaesthetized rats. Therefore, anaesthetics, at standard anaesthetic doses, have differential effects on the potency and/or efficacy of the pressor response to DPI, and this can be classified as follows: (1) no change in ED_{50} but potentiation of E_{max} of the pressor response to DPI – ketamine; (2) increase in ED_{50} and no change in E_{max}-pentobarbitone; (3) increase in ED_{50} and potentiation of E_{max} - urethane, ethanol and chloralose; (4) increase in ED_{50} and reduction of E_{max} - halothane. The results suggest that the variable influence of anaesthetic agents must be taken into consideration when evaluating the pressor effects of DPI and other pressor agents (Wang *et al.*, 1991a; Abdelrahman *et al.*, 1992).

Halothane has been shown to potentiate (Blaise, 1991) or reduce (Muldoon *et al.*, 1988) endothelium-dependent vasodilatation in different vascular preparations. We have also found that halothane (1.25%) abolishes the pressor effects of L-NNA and L-NAME but not those of noradrenaline or angiotensin II (Wang *et al.*, 1991a; Pang *et al.*, 1992). Although it appears that halothane has a selective inhibitory effect on NO synthase inhibitors, the mechanism by which it suppresses pressor responses to L-NNA and L-NAME is different from that of DPI. L-NNA and L-NAME cause pressor responses by inhibiting NO synthase and subsequent endothelium-dependent relaxation (Wang *et al.*, 1993a; see Moncada *et al.*, 1991). The pressor responses of L-NNA and L-NAME are L-arginine-sensitive (Wang & Pang, 1990; Wang *et al.*, 1991b; 1992; see Moncada *et al.*, 1991) and are independent of the integrity of the central and sympathetic nervous systems (Wang & Pang, 1991; Wang & Pang, 1993). The mechanism by which halothane inhibits pressor responses to L-NNA may involve selective increase in NO synthesis and/or release, or potentiation of the effect of NO (Wang *et al.*, 1991b; Pang *et al.*, 1992) and, this may be analogous to the inhibitory effect of sodium nitroprusside on the pressor effect of L-NNA (Wang *et al.*, 1993b).

Halothane markedly inhibited DPI-induced increases in both plasma catecholamines and MAP. The results suggest that the inhibitory effect of halothane on the pressor response to DPI is primarily due to the suppression of sympathetic activation rather than inhibition of endothelium-dependent vasodilatation. Halothane has been shown to depress activities of the sympathetic nervous system at different levels (1) areas of the central nervous system controlling sympathetic nerve activity (Price *et al.*, 1963; Millar *et al.*, 1969; Larach *et al.*, 1987; Bazil & Minneman, 1989), (2) sympathetic ganglia (Skovsted *et al.*, 1969; Christ, 1977; Bosnjak *et al.*, 1982; Seagard *et al.*, 1982), and (3) sympathetic nerve endings located in the walls of blood vessels (Muldoon *et al.*, 1975; Lunn & Rorie, 1984; Rorie *et al.*, 1990). In addition, a small component of non-specific inhibition by halothane may also be responsible for its effect on the pressor response to DPI. Halothane, at 1.25%, inhibited the maximal pressor response to DPI by 95%, and the increases in plasma noradrenaline and adrenaline by 86% and 81%, respectively. These results are consistent with those of our other study in which the same concentration of halothane reduced the pressor response produced by exogenous noradrenaline or angiotensin II by 18% (Pang *et al.*, 1992). Therefore, the inhibition by halothane of the pressor response to DPI is primarily (approximately 80%)

attributable to the inhibition of sympathetic transmission and secondarily (approximately 20%) due to non-specific inhibition of vascular smooth muscle contraction.

The influence of ketamine and pentobarbitone on sympathetic transmission may also account for their effects on the pressor response to DPI. Ketamine activates the sympathetic nervous system (Traber & Wilson, 1969) by inhibiting neuronal noradrenaline uptake (Nedergaard, 1973; Clanachan & McGrath, 1976). The blockade of uptake$_1$ by ketamine may cause potentiation of the pressor effect of DPI. This interpretation is consisent with our unpublished observation that the uptake$_1$ inhibitor, cocaine also potentiates pressor and tachycardic responses of DPI ($n = 6$). The inhibitory effect of pentobarbitone may involve the suppression of catecholamine release since pentobarbitone has been shown to inhibit the release of noradrenaline from the peripheral sympathetic nerve terminals in rabbit and chicken isolated hearts (Gothert & Rieckesmann, 1978; see Richter & Holtman, 1982), as well as releases of noradrenaline and adrenaline from the perfused cow adrenal glands (see Richter & Holtman, 1982).

In summary, i.v. bolus injections of DPI caused immediate and transient pressor responses in conscious rats; the pressor response was accompanied by large increases in plasma noradrenaline and adrenaline. Among the various anaesthetics (ketamine, urethane, ethanol, chloralose) studied, halothane is the only one which dose-dependently and non-competitively inhibits DPI-induced pressor response and the rises in plasma noradrenaline and adrenaline. The results suggest that (1) DPI causes a pressor response by stimulating sympathetic nerve activities in conscious rats; (2) halothane inhibits the pressor response of DPI by suppressing sympathetic nerve activities rather than interfering with endothelium-dependent vasodilatation; (3) the variable effects of anaesthetics must be taken into consideration when assessing the pressor and heart rate responses of DPI and other vasoactive agents.

This work was supported by a grant from the Medical Research Council of Canada (MRC) and a MRC post-doctoral fellowship to Y.-X. Wang. We would like to express our appreciation to Ms Su Lin Lim for her skilled technical assistance.

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(Received November 26, 1992
Revised February 23, 1993
Accepted April 1, 1993)

Br. J. Pharmacol. (1993), **109**, 1192–1195 © Macmillan Press Ltd, 1993

Characterization of the 5-hydroxytryptamine receptor mediating the positive inotropic response in guinea-pig isolated left atria

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1 5-Hydroxytryptamine (5-HT), in the presence of propranolol (1 μM), atropine (3 μM) and ketanserin (1 μM), induced a positive inotropic response of guinea-pig isolated electrically paced left atria ($pEC_{50} = 7.52$). The positive inotropic response was mimicked by α-methyl-5-HT ($pEC_{50} = 7.26$) and 5-carboxamidotryptamine (5-CT; $pEC_{50} = 6.56$) but not by sumatriptan or 1-(*m*-chlorophenyl) piperazine (m-CPP).

2 The 5-HT induced positive inotropic response was competitively antagonized by both mesulergine ($pA_2 = 7.68$) and methiothepin ($pA_2 = 6.67$). Methysergide was a surmountable antagonist at 3 nM producing a rightward shift in the 5-HT concentration-response curve giving an apparent $pA_2 = 9.2$ with no significant reduction in the maximum. At higher concentrations, methysergide behaved as an insurmountable antagonist, significantly reducing the maximum response to 5-HT as well as producing rightward shifts in the 5-HT concentration-response curves.

3 The 5-HT-induced positive inotropic response was not antagonized by either tropisetron (10 μM) or yohimbine (10 μM).

4 The guinea-pig atrial 5-HT receptor does not satisfy the criteria for any of the currently recognised 5-HT receptor subtypes and appears to have some similarities to the atypical 5-HT receptors previously described in other peripheral tissues.

Keywords: Guinea-pig atria, inotropic response; 5-HT; 5-carboxamidotryptamine (5-CT); α-methyl-5-HT; sumatriptran; 1-(*m*-chlorophenyl) piperazine; 5-HT receptor

Introduction

5-Hydroxytryptamine (5-HT) has marked tachycardiac and inotropic effects in a variety of animal species which are mediated via various 5-HT receptor subtypes. A 5-HT_4 receptor-mediated positive inotropic response has been described in human and porcine isolated left atria (Sanders & Kaumann, 1992; Kaumann, *et al.*, 1991) and 5-HT_1-like receptors have been shown to mediate positive inotropic responses in cat isolated left atria (Kaumann, 1986) and 5-HT-induced tachycardia in the spinal cat (Saxena *et al.*, 1985). In the rabbit heart, the tachycardia and inotropic responses induced by 5-HT are mediated by noradrenaline release from the postganglionic cardiac sympathetic nerves via 5-HT_3 receptor stimulation (Fozard, 1984). In guinea-pig atria, 5-HT can act directly via 5-HT receptors to produce both positive chronotropic and positive inotropic responses (Trendelenburg, 1960; Walter *et al.*, 1984). Studies of the positive chronotropic effect of 5-HT in the guinea-pig have suggested that the response is not mediated by β-adrenoceptors, 5-HT_1-like, 5-HT_2 or 5-HT_3 receptors (Walter *et al.*, 1984; Dhasmana *et al.*, 1988; Eglen & Whiting, 1989) but have failed to identify clearly the receptor involved. There is evidence to suggest that the 5-HT-induced positive inotropic response in guinea-pig atria is due to the release of calcitonin gene-related peptide (Bernoussi & Rioux, 1989; Tramontana *et al.*, 1993) and that this effect is due to the stimulation of 5-HT_3 receptors (Tramontana *et al.*, 1993). Using some 5-HT agonists and antagonists we have attempted to characterize further the 5-HT receptor involved in the 5-HT induced positive inotropic response in guinea-pig isolated left atria.

Methods

Dunkin-Hartley guinea-pigs (male, 300–400 g) were killed by cervical dislocation and the left atria removed and suspended under 1.0 g preload in Krebs solution of the following composition (mM); NaCl 118.0, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.0, $NaHCO_3$ 25.0; maintained at 32°C and gassed with 5% CO_2 in O_2. To eliminate indirect effects of 5-HT, due to release of noradrenaline or acetylcholine, (±)-propranolol (1 μM) and atropine (3 μM) were added to the Krebs solution: ketanserin (1 μM) was also included in the Krebs solution to exclude any action at 5-HT_2 receptors. The atria were in contact with one end of a pair of platinum stimulating electrodes with the other electrode located in the Krebs solution and then paced at 2.2 Hz with a square-wave pulse of 1 ms duration and a current of 10 mA. Tension changes were recorded with Grass FT03 isometric transducers and a Grass polygraph. After a 45 min stabilization period, non-cumulative concentration-response curves to 5-HT (1 nM–100 μM) were constructed (1 min contact time, 5 min between doses) in the absence or presence of antagonists. Antagonists were equilibrated for 60 min before preparing the second concentration-response curves. In some tissues concentration-response curves to α-methyl-5-HT (1 nM–30 μM), 5-carboxamidotryptamine (5-CT, 1 nM–30 μM), sumatriptan (0.1–100 μM) or 1-(*m*-chlorophenyl) piperazine (m-CPP, 0.1–100 μM) were constructed. Responses were expressed as the % increase in force of contraction and concentration-response curves were analysed by the Allfit programme (Delean *et al.*, 1979). Schild analysis (Arunlakshana & Schild, 1959) was carried out by linear regression analysis of pooled data. Estimates of error (95% confidence limits) are indicated in parentheses.

Drugs

The following were used: 5-hydroxytryptamine hydrochloride (Fluorochem), α-methyl-5-HT (Cookson Chemicals), 1-(*m*-

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chlorophenyl)piperazine, yohimbine hydrochloride, (±)-propranolol hydrochloride and atropine sulphate (Sigma), ketanserin tartrate (Janssen), mesulergine and methysergide maleate (gifts from Sandoz Pharma Ltd., Basle, Switzerland), 5-carboxamidotryptamine, sumatriptan, methiothepin maleate and tropisetron hydrochloride were synthesized at Wyeth Research (U.K). Mesulergine and methiothepin were dissolved in dimethylsulphoxide, the other drugs were dissolved in distilled water and then diluted to required concentration in Krebs solution.

Results

The resting mean force of contraction of the paced left atria was 0.79 ± 0.04 g, $n = 30$. 5-HT, α-methyl-5-HT and 5-CT produced concentration-dependent increases in force of contraction with pEC_{50} and maximum % increases in force values respectively of: 5-HT, 7.52 (7.41–7.62) and 84.0 ± 5.7%, $n = 20$; α-methyl-5-HT, 7.26 (7.14–7.38) and 84.7 ± 5.6%, $n = 8$; 5-CT, 6.56 (6.48–6.64) and 87.6 ± 11.6%, $n = 6$ (Figure 1). Sumatriptan or m-CPP up to 100 μM had no positive inotropic effect. In time control preparations, the geometric mean dose-ratio for two consecutive 5-HT concentration-response curves was 1.07 (0.8–1.42), $n = 20$. The positive inotropic response to 5-HT was not antagonized by tropisetron (10 μM); geometric mean dose-ratio = 1.0 (0.6–1.6), $n = 4$, or yohimbine (10 μM); geometric mean dose-ratio = 1.52 (0.7–3.4), $n = 4$. The 5-HT-induced positive inotropic response was antagonized by methiothepin (0.3–3 μM), mesulergine (0.3–3 μM) and methysergide (3–30 nM), (Figures 1 and 2). Both mesulergine and methiothepin behaved as competitive antagonists against 5-HT and Schild analysis gave pA_2 values of 7.68 (7.20– 8.77), slope = 0.93 (0.56–1.30), $n = 16$ and 6.67 (6.46–7.07), slope = 1.28 (0.81–1.74), $n = 15$ respectively. Methysergide was a surmountable antagonist at 3 nM producing a rightward shift in the 5-HT concentration-response curve; geometric mean dose-ratio = 5.99 (1.64–21.8), giving an apparent $pA_2 = 9.2$ (8.2–10.1), $n = 4$ with no significant reduction in the maximum. At higher concentrations, methysergide behaved as an insurmountable antagonist, significantly reducing the maximum response to 5-HT as well as producing rightward shifts in the 5-HT concentration-response curve, with geometric mean dose-ratios at 10 nM and 30 nM of 14.7 (3.5–62.3), $n = 4$, and 132.4 (39.8–440.3), $n = 4$, respectively and reductions in the maximum response to 5-HT by 33.7 ± 14.5% and 47.4 ± 10.7% respectively. None of the antagonists studied had any direct effect on atrial contraction.

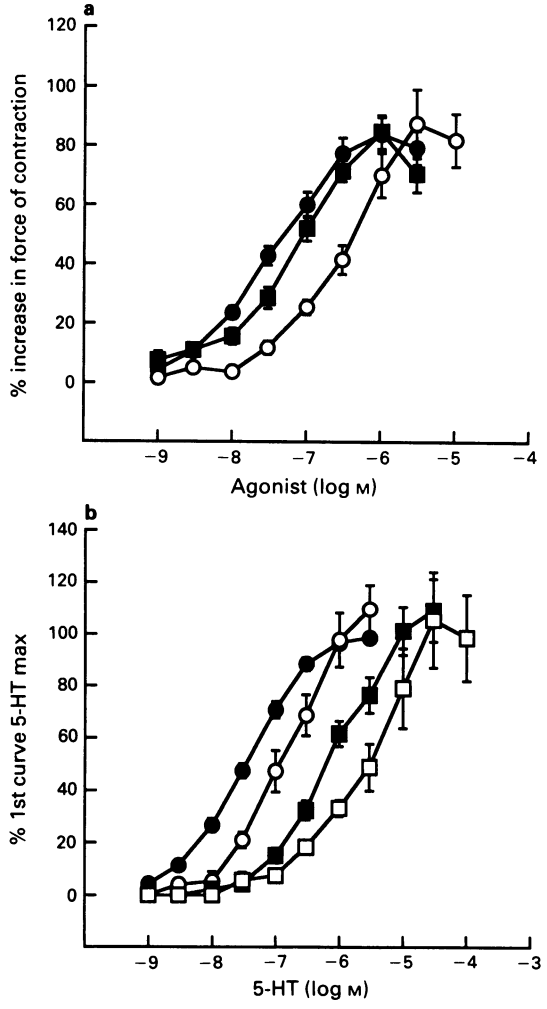


Figure 1 Concentration-response curves for the positive inotropic response of the guinea-pig left atria to (a) 5-hydroxytryptamine (5-HT) ($n = 20$) (●); α-methyl-5-HT ($n = 8$) (■); 5-carboxamidotryptamine (5-CT) ($n = 6$) (○); responses are expressed as % increase force of contraction, (b) 5-HT in the presence of mesulergine; control (●); 0.3 μM (○); 1.0 μM (■) or 3.0 μM (□); responses are expressed as a percentage of the first curve maximum to 5-HT, 4–6 tissues were used in each group. Mean values and s.e.mean are shown.

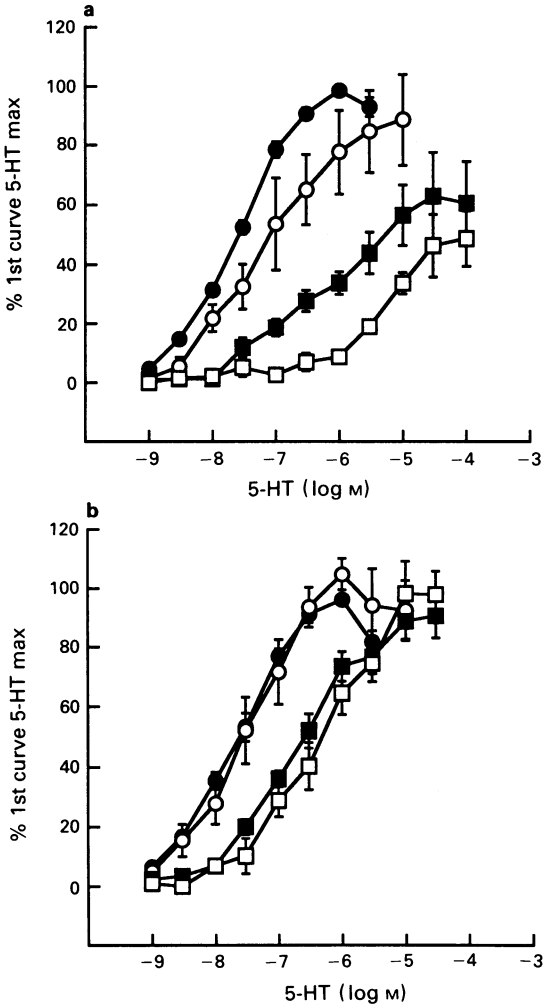


Figure 2 Concentration-response curves for the positive inotropic response of the guinea-pig left atria to 5-hydroxytryptamine (5-HT) in the presence of (a) methysergide; control (●); 3.0 nM (○); 10 nM (■) or 30 nM (□); (b) methiothepin: control (●); 0.3 μM (○); 1.0 μM (■) or 3.0 μM (□); responses are expressed as a percentage of the first curve maximum to 5-HT, 4–6 tissues were used in each group. Mean values and s.e.mean are shown.

Discussion

Previous studies on the chronotropic effects of 5-HT in the guinea-pig atria (Walter *et al.*, 1984; Dhasmana *et al.*, 1988; Eglen & Whiting, 1989), have failed to identify clearly the 5-HT receptor involved but have suggested that the response is not mediated by β-adrenoceptors, 5-HT_1-like, 5-HT_2 or 5-HT_3 receptors. In our study, 5-HT, α-methyl-5-HT and 5-CT produced positive inotropic responses in the guinea-pig isolated left atria with the potency order of 5-HT > α-methyl-5-HT > 5-CT; interestingly the pEC_{50} obtained for the effect of 5-HT on the atria in the study by Walter *et al.* (1984) is not significantly different from the pEC_{50} obtained for 5-HT in our experiments. The lack of any agonism by sumatriptan or antagonism by yohimbine suggests that the positive inotropic response to 5-HT does not involve 5-HT_{1D} receptors or the 5-HT_1-like receptors of the type reported to mediate contraction of dog saphenous veins (Humphrey *et al.*, 1988). The failure of tropisetron to antagonize responses to 5-HT make it unlikely that 5-HT_3 or 5-HT_4 receptor mechanisms contribute to the observed inotropic response. This is in contrast to the results obtained by Tramontana *et al.* (1993) who have shown that the 5-HT-induced positive inotropic responses in guinea-pig atria are antagonized by both ondansetron and tropisetron, evidence to suggest an action at a 5-HT_3 receptor. Tramontana *et al.* (1993) used reserpinized guinea-pigs, administered 5-HT in a cumulative manner and from their control concentration-response curves it can be seen that much higher concentrations of 5-HT were needed to produce positive inotropic responses than observed in our experiments. There is, however, no clear explanation for the discrepancy between the two sets of results. The lack of a positive inotropic effect of sumatriptan in our study is in agreement with the results of Tramontana *et al.* (1993) who also showed that the 5-HT_{1A} agonist, 8-hydroxy-dipropylaminotetralin (8-OH-DPAT), and the 5-HT_{1C}/5-HT_2 agonist, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), were inactive in guinea-pig atria.

The antagonism of the 5-HT-induced positive inotropic response by methysergide, methiothepin and mesulergine indicates that 5-HT may be acting as a 5-HT_1-like receptor. However, the agonist potency order of 5-HT > α-methyl-5-HT > 5-CT does not agree with one of the criteria originally described by Bradley *et al.* (1986) for classification as a 5-HT_1-like receptor, i.e. that 5-CT should be at least equipotent with 5-HT. In guinea-pig isolated left atria, 5-CT is about 10 times less potent as an agonist than 5-HT. Although the agonist potency order at the guinea-pig atrial 5-HT receptor is similar to that described for the 5-HT_{1C} receptor (Hoyer, 1988), the presence of ketanserin in the Krebs solution excludes any action at 5-HT_{1C} or 5-HT_2 receptors. Yohimbine did not antagonize the 5-HT-induced positive inotropic response and m-CPP showed no agonism; 5-HT does not therefore appear to be acting at a 5-HT receptor of the type reported to contract the rat stomach fundus (Clineschmidt *et al.*, 1985). The guinea-pig atrial 5-HT receptor also differs from the non-endothelial 5-HT_1-like receptors responsible for vascular smooth muscle relaxation, since they do not recognise α-methyl-5-HT as an agonist (Martin *et al.*, 1987; Sumner *et al.*, 1989).

α-methyl-5-HT is a potent agonist at the endothelial 5-HT receptors described in the porcine vena cava (Sumner, 1991) and rabbit jugular vein (Leff *et al.*, 1987; Martin *et al.*, 1987) and neither 5-HT receptor easily fits into any currently defined class of 5-HT receptor subtypes. The guinea-pig atrial 5-HT receptor therefore, has some similarities to these receptors, since α-methyl-5-HT is a good agonist in this preparation; however, the lack of agonism by 5-CT or antagonism by mesulergine at the 5-HT endothelial receptor of the porcine vena cava (Summer, 1991) suggest differences between the 5-HT receptors of guinea-pig atria and porcine vena cava. The agonist potency order at the endothelial 5-HT receptor of the rabbit jugular vein is 5-HT ≥ α-methyl-5-HT > 5-CT and methiothepin and methysergide are antagonists at this receptor (Leff *et al.*, 1987; Martin *et al.*, 1987). This is a similar pharmacological profile to that seen for the guinea-pig atrial 5-HT receptor. Mesulergine has been shown to antagonize the endothelial 5-HT receptor of the rabbit jugular vein (apparent $pK_B = 7.3$; G.R. Martin, personal communication) but a more comprehensive comparative analysis is required to determine whether or not the 5-HT receptors in rabbit jugular vein and guinea-pig atrium are the same subtype.

In conclusion, 5-HT induces a positive inotropic response in guinea-pig isolated paced left atria. The 5-HT receptor mediating the 5-HT induced positive inotropic response of guinea-pig left atria cannot as yet be adequately described by any of the currently defined 5-HT receptor subtypes but appears to have some similarities to the atypical 5-HT receptors previously described in other peripheral tissues.

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(Received March 9, 1993
Accepted April 1, 1993)

Br. J. Pharmacol. (1993), **109**, 1196–1205 © Macmillan Press Ltd, 1993

Selective reduction of N-methyl-D-aspartate-evoked responses by 1,3-di(2-tolyl)guanidine in mouse and rat cultured hippocampal pyramidal neurones

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1 The effects of 1,3-di(2-tolyl)guanidine (DTG) were examined on the responses of cultured hippocampal neurones to the excitatory amino acid analogues N-methyl-D-aspartate (NMDA), kainate, quisqualate and (**RS**)-α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA).

2 In rat hippocampal neurones loaded with the Ca^{2+}-sensitive dye Fura-2, DTG (10–100 μM) produced a concentration-dependent depression of the NMDA-evoked rises in intracellular free calcium ($[Ca^{2+}]_i$), an effect that was not modified by changes in the extracellular glycine concentration. DTG (at 50 and 100 μM) also attenuated, although to a lesser extent, the rises in $[Ca^{2+}]_i$ evoked by naturally-derived quisqualate. In contrast, 50 and 100 μM DTG did not depress responses evoked by kainate, AMPA and synthetic, glutamate-free (+)-quisqualate although on occasions DTG enhanced kainate- and AMPA-evoked rises in $[Ca^{2+}]_i$.

3 DTG attenuated NMDA-evoked currents recorded from mouse hippocampal neurones under whole-cell voltage-clamp with an IC_{50} (mean ± s.e.mean) of 37 ± 5 μM at a holding potential of −60 mV. The DTG block of NMDA-evoked responses was not competitive in nature and was not dependent on the extracellular glycine or spermine concentration. The block did, however, exhibit both voltage-, and use-, dependency. The steady-state current evoked by naturally-derived quisqualate was also attenuated by DTG whereas those evoked by kainate and AMPA were not.

4 We conclude that DTG, applied at micromolar concentrations, is a selective NMDA antagonist in cultured hippocampal neurones, the block exhibiting both Mg^{2+}- and phencyclidine-like characteristics. Given the nanomolar affinity of DTG for σ binding sites it is unlikely that the antagonism observed here is mediated by σ-receptors, but the data emphasize the potential danger of ascribing the functional consequences of DTG administration solely to σ receptor-mediated events.

Keywords: Glutamate receptors; N-methyl-D-aspartate (NMDA); kainate; quisqualate; (**RS**)-α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA); 1,3-di(2-tolyl)guanidine (DTG); σ-receptors; cultured hippocampal pyramidal neurones

Introduction

Originally phencyclidine (PCP) and 6,7-benzomorphans such as N-allylnormetazocine, cyclazocine and pentazocine were believed to produce their shared behavioural effects through interaction with a common site, the σ-opiate receptor (Martin *et al.*, 1976; Zukin & Zukin, 1981). Further studies determined, however, that these compounds bind to two non-opioid receptor populations distinct both pharmacologically and autoradiographically (Largent *et al.*, 1986; Sircar *et al.*, 1986; Quirion *et al.*, 1987). PCP, related arylcyclohexylamines such as 1-[1-(2-thienyl)cyclohexyl]piperidine (TCP; Vignon *et al.*, 1983) and the compound, dizocilpine maleate (MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo hepten-5,10-imine-maleate; Wong *et al.*, 1986) bind preferentially to a site, the PCP site, located within the ion channel linked to the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor. Conversely (+)-6,7-benzomorphans bind preferentially to the σ site, but also possess some affinity for the PCP site and attenuate NMDA-evoked responses in central mammalian neurones (Berry *et al.*, 1984; Church & Lodge, 1990).

Only with the identification of the selective σ receptor ligands 1,3-di(2-tolyl)guanidine (DTG; Weber *et al.*, 1986) and (+)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine ((+)3-PPP; Largent *et al.*, 1984) did a more rigorous assessment of the functional correlates of σ receptor occupancy become possible. DTG in particular has been widely employed for this purpose both *in vivo* and *in vitro*, although recent observations have suggested that whilst DTG is a selective σ receptor ligand at low (nM) concentrations (see Quirion *et al.*, 1992) it may, at higher (μM) concentrations, also interact with excitatory amino acid receptors in the mammalian CNS. For example, rats trained to discriminate DTG (3 mg kg^{-1}) from saline generalize to PCP whereas DTG substitutes completely for PCP when the latter compound is used as the training stimulus (Holtzman, 1989), and DTG has been shown to depress NMDA-induced currents in cultured hippocampal neurones (Keana *et al.*, 1989). In contrast, DTG was observed to be inactive as an NMDA antagonist in the cortical slice preparation (Aram *et al.*, 1988) and in *in vivo* recordings from the central tegmental area (French & Ceci, 1990) but, variously, inactive (Malouf *et al.*, 1988) or active (Connick *et al.*, 1992) on CA1 neurones excited by iontophoretic application of NMDA in hippocampal slices. Furthermore the selectivity of DTG as an excitatory amino acid antagonist remains in doubt, as a recent study suggested that DTG non-selectively depresses both NMDA and quisqualate responses (Connick *et al.*, 1992).

The present experiments were undertaken to investigate the selectivity and potency of DTG as an excitatory amino acid antagonist in cultured hippocampal neurones. Initially, we utilized Fura-2-loaded, rat hippocampal neurones to establish the selectivity of DTG as an excitatory amino acid antagonist. Whole-cell, voltage-clamp techniques were then employed in mouse hippocampal neurones to both confirm

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the findings of the fluorescence study and to examine in greater detail the mechanisms whereby DTG depresses NMDA-evoked responses.

Methods

Fluorescent dye studies

Hippocampal neurones were obtained from 18-day-old foetal Wistar rats according to Banker & Cowan (1977) and plated on glass coverslips at a density of $1-3 \times 10^5$ cells cm^{-2}. Cultures were used at 9–28 days after plating. Following loading with Fura-2 (see below), coverslips were placed in a chamber at 20–23°C and continuously superfused at a rate of 1.5 ml min^{-1} with a nominally Mg^{2+}-free solution containing (mM): NaCl 136.5, KCl 3, NaH_2PO_4 1.5, D-glucose 10, $CaCl_2$ 2, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 10. Tetrodotoxin 0.5 μM was added and the pH adjusted to 7.35–7.40 with 10 M NaOH. The amino acid excitants (NMDA, 20 μM; kainate, 80 μM; naturally-derived quisqualate, 40–80 μM; synthetic, glutamate-free (+)-quisqualate, 80 μM; and (**RS**)-α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), 40 μM were administered in 1 ml aliquots into the inflow of the perfusion chamber and allowed to remain in contact with the neurones for 25 s before wash-out. High-K^+ solutions (50 mM, by substitution for NaCl) were introduced in a similar fashion. Once reproducible responses were established, DTG was applied by superfusion. Results were expressed as the percentage change of the peak response to each excitant during the administration of the test compound, in relation to the peaks of the control and recovery responses obtained before and after its administration, respectively.

Cytoplasmic free calcium concentrations ($[Ca^{2+}]_i$) were measured by the dual excitation fluorescence ratio method on an Attofluor Digital Fluorescence Microscopy System (Atto Instruments Inc.; Carl Zeiss Canada Ltd.) employing the calcium sensitive fluorophore, Fura-2 (Grynkiewicz *et al.*, 1985). Neurones were incubated at 33–35°C for 60–75 min in 5–7.5 μM Fura-2 AM (Molecular Probes Inc.) and were then washed and left for 30 min before use to ensure complete hydrolysis of the acetomethoxy form of Fura-2. With excitation wavelengths of 334 and 380 nm, fluorescence intensities (at 510 nm) were obtained from multiple neurone somata simultaneously. Raw intensity data at each excitation wavelength were corrected for background prior to calculation of the ratio. During exposure to excitants, a ratio was acquired every 2 s; much lower rates (e.g. one ratio every 30 s) were employed between excitant application to minimize photobleaching and ultraviolet-mediated cytotoxicity. The *in situ* calibration method was used to convert fluorescence ratios into $[Ca^{2+}]_i$. During exposure to 10 μM Br-A23187, calibration parameters (R_{max}, R_{min} and β) were obtained in the presence (2 mM) and absence (nominally zero-Ca^{2+} solution in the presence of 100 μM ethylene glycol-*bis*(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA)) of Ca^{2+}. The published K_d value for Fura-2 of 135 nM (at 20°C) was employed (Grynkiewicz *et al.*, 1985).

Statistical results are reported as mean ± s.e.mean, where *n* refers to the total number of neurones from which observations were made at each concentration of DTG. The IC_{50} value for DTG (the concentration of DTG resulting in a 50% inhibition of the control NMDA response) was derived by use of the same logistic equation and fitting routine as in the electrophysiological studies (see below). For each concentration of DTG tested, at least three different neuronal cultures were employed. No differences were noted with respect to the extent of the reduction of natural quisqualate-evoked responses by 50 μM DTG when either 40 or 80 μM quisqualate was employed as the excitant and these data have therefore been pooled.

Electrophysiological studies

Dissociated mouse hippocampal neurones grown in culture were used for whole cell recordings with conventional voltage-clamp techniques. The method of culture preparation has been described in detail previously (MacDonald *et al.*, 1989). In brief, hippocampi were dissected from 18-day-old foetal Swiss White mice, mechanically dissociated and neurones plated at densities below 1×10^3 cells cm^{-2} on collagen-coated plates. Cultures were used at 10–21 days following plating.

Dishes were thoroughly rinsed before the experiment was started with an extracellular solution containing (mM): NaCl 140, $CaCl_2$ 1.3, KCl 5.4, HEPES 25, and glucose 33. Tetrodotoxin was added to solutions at 10 nM–1 μM and glycine at 1 or 3 μM before the pH of solution was adjusted to 7.35–7.40 with 1 M NaOH. DTG was made up as a 100 mM stock in HCl and after appropriate dilution the pH adjusted. Agonists and antagonists were superfused over an entire neurone from a multibarrelled perfusion system allowing for rapid (< 50 ms) exchange between compounds (Johnson & Ascher, 1987).

Patch electrodes were constructed from thin-walled borosilicate glass (o.d. 1.5 mm). Electrodes of 1–3 μm tip diameter were pulled with a Narashige PP88 vertical puller and were filled with a solution containing (mM): CsF 110, CsCl 10, HEPES 10 and EGTA 10, pH 7.35–7.40 (adjusted with 2 M CsOH). Occasionally an intracellular solution containing (mM): $CsMeSO_3$ 125, CsCl 15, HEPES 10, EGTA 5, $CaCl_2$ 0.5, $MgCl_2$ 3 with a final pH of 7.2 was employed. Adenosine tri-phosphate (2 mM, Mg^{2+} salt) was added routinely to retard the wash-out of amino acid-evoked currents (MacDonald *et al.*, 1989). Recordings were performed at room temperature (20–24°C). The membrane of the neurone was maintained at −60 mV, unless otherwise indicated, by use of a patch clamp amplifier (Axopatch 1B, Axon Instruments Inc.).

Responses evoked on amino acid application were recorded and averaged with pClamp acquisition and analysis software (Axon Instruments Inc.). Drug effect was expressed as the percentage reduction of the control agonist response at steady state (unless otherwise indicated). Averaged data points were fitted to a standard logistic equation using Sigma Plot (Jandel Scientific) to compute the IC_{50} (the concentration of DTG resulting in a 50% inhibition of the control NMDA response) or the EC_{50} (the concentration of NMDA required for 50% maximum response (Rmax)) where appropriate. Final values indicate mean ± s.e.mean and *n* the number of neurones tested.

Source of compounds

All chemicals were obtained from Sigma Chemical Co., with the exceptions of, in the Fura-2 study, AMPA, synthetic (+)-quisqualate and DTG (Research Biochemicals Inc.) and, in the electrophysiological study, DTG (Research Biochemicals Inc.) and AMPA and a second sample of naturally-derived quisqualate (Tocris Neuramin).

Results

Fluorescent dye studies

DTG (10–100 μM) produced a concentration-dependent reduction of rises in $[Ca^{2+}]_i$ evoked by 20 μM NMDA (Table 1, Figure 1). The IC_{50} value for DTG against NMDA was 33.5 ± 3.3 μM, a value similar to that obtained in the electrophysiological studies (see below). Varying the NMDA concentration from 5 to 20 μM did not alter the degree of inhibition produced by either 50 or 100 μM DTG (not shown). Responses to kainate and AMPA were, in general, attenuated slightly by 50 and 100 μM DTG (Table 1, Figure

Table 1 The effects of 1,3-di(2-tolyl)guanidine (DTG), at the concentrations indicated, on high-$[K^+]_o$-, N-methyl-D-aspartate (NMDA)-, kainate-, natural quisqualate- and (RS)-α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA)-evoked increases in $[Ca^{2+}]_i$

DTG concentration (μM)	*% reduction in responses to* K^+	*NMDA*	*Kainate*	*Quisqualate*	*AMPA*
10	2 ± 2	17 ± 2	NT	NT	NT
	$n = 45$	$n = 45$			
20	7 ± 2	35 ± 3	NT	NT	NT
	$n = 67$	$n = 67$			
50	12 ± 2	62 ± 3	8 ± 2	14 ± 3	9 ± 3
	$n = 19$	$n = 69$	$n = 19$	$n = 26$	$n = 45$
100	43 ± 1	85 ± 2	10 ± 1	28 ± 4	11 ± 4
	$n = 75$	$n = 92$	$n = 38$	$n = 27$	$n = 51$

Data shown are % reductions (mean ± s.e.mean) compared with control values. n = total number of neurones from which observations were made at each concentration of DTG. NT: not tested. Neurones in which DTG produced an increase in kainate- or AMPA-evoked responses (see text) are not included.

2) observations which may reflect, at least in part, the ability of these concentrations of DTG to reduce rises in $[Ca^{2+}]_i$ evoked by high-$[K^+]_o$ medium (Table 1, Figure 1 and 2). In the neurones employed in these experiments, high-$[K^+]_o$-, kainate- and AMPA-mediated responses probably reflect Ca^{2+} influx through voltage-gated Ca^{2+} channels, since they are abolished in the absence of extracellular Ca^{2+} (J.C. and K.A.-H., unpublished observations). The ability of DTG at high concentrations to attenuate Ca^{2+} influx through voltage-gated Ca^{2+} channels is shared by other dextromethorphan/σ receptor ligands (Church *et al.*, 1991). The possibility that the reduction of NMDA-evoked responses by DTG involved an interaction at the glycine binding site on the NMDA receptor-channel complex was investigated in 11 neurones. The addition of 20 μM glycine to the standard, nominally glycine-free, perfusion medium enhanced NMDA-evoked rises in $[Ca^{2+}]_i$ by 11 ± 3% but did not significantly (Student's t test, $P < 0.05$) alter the degree of inhibition associated with the application of 50 μM DTG (60 ± 4% and 55 ± 6% reductions respectively in the absence and presence of 20 μM glycine).

In contrast to the relative lack of antagonism of AMPA-evoked rises in $[Ca^{2+}]_i$, 50 and 100 μM DTG consistently reduced naturally-derived quisqualate-evoked responses. The

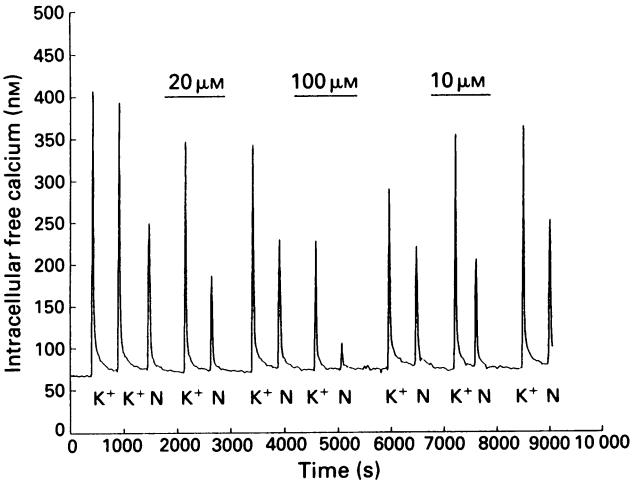


Figure 1 DTG blocks NMDA-evoked increases in $[Ca^{2+}]_i$. The effects of 20, 100 and 10 μM DTG, applied for the periods indicated by the bars above the trace, on high-$[K^+]_o$-(K^+) and NMDA-(N) evoked increases in $[Ca^{2+}]_i$. The record is a mean of measurements obtained from 11 neurones simultaneously. DTG produced a concentration-dependent reduction in NMDA-evoked responses and reduced high-$[K^+]_o$-evoked responses at 100 μM. For abbreviations in this and subsequent figure legends, see text.

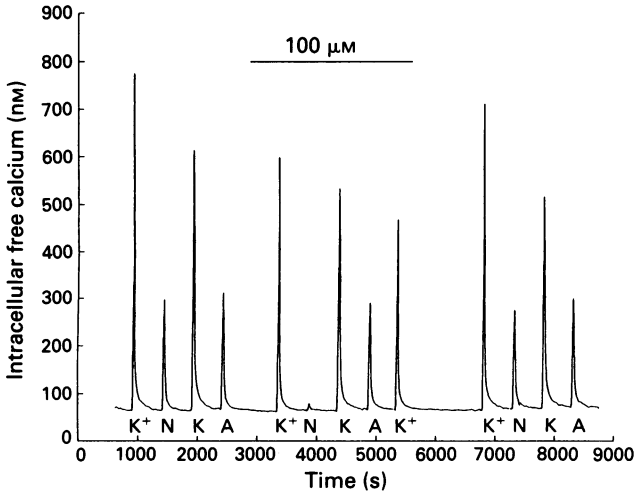


Figure 2 The effect of 100 μM DTG, applied for the period indicated by the bar above the trace, on high-$[K^+]_o$-(K^+), NMDA-(N), kainate-(K) and AMPA-(A) evoked rises in $[Ca^{2+}]_i$. DTG reduced responses evoked by NMDA to a greater extent than those evoked by high-$[K^+]_o$, whereas kainate-and AMPA-evoked responses were little affected. The record is a mean of data obtained from 24 neurones simultaneously.

possible reasons for this finding were examined in greater detail in the electrophysiological studies (see below). However, it is noteworthy that the overall percentage reduction of the response to natural quisqualate (Sigma source; Table 1, Figure 3) was greater than that of the synthetic, glutamate-free (+)-quisqualate (14 ± 3% reduction with 100 μM DTG, $n = 17$). Interestingly, in a subset of the total population of neurones examined, 50 and 100 μM DTG enhanced responses to kainate and/or AMPA, but not to natural or synthetic quisqualate. Thus, 50 μM DTG increased kainate-evoked responses in 2 out of 21 neurones (by 8 and 10%) and AMPA-evoked responses in 6 out of 51 neurones (by 16 ± 6%; range 5–46%) whilst 100 μM DTG increased kainate-evoked responses in 2 out of 40 neurones (by 5 and 15%) and AMPA-evoked responses in 9 out of 60 neurones (by 20 ± 7%; range 6–45%). However, statistical analysis by χ^2 goodness of fit of the potentiated AMPA responses during exposure to DTG failed to demonstrate deviations from the normal distribution and therefore the possibility of the existence of two different neuronal populations could not be substantiated. An example of an enhancement of AMPA-evoked responses, in the presence of a decrease in NMDA- and natural quisqualate-evoked responses, by 100 μM DTG is shown in Figure 3.

Low (nM) concentrations of DTG have been reported to

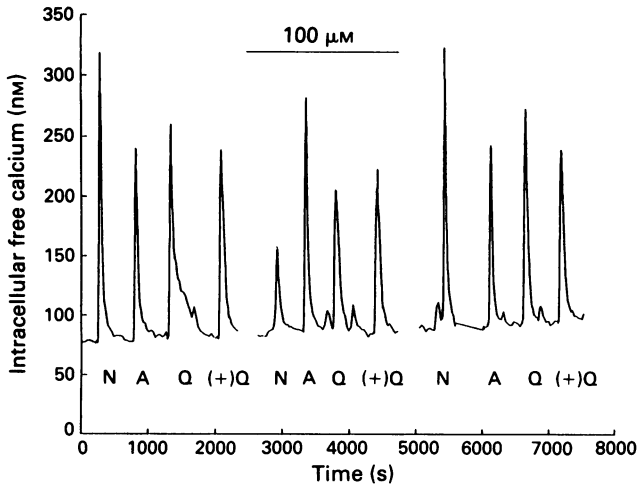


Figure 3 DTG occasionally enhanced AMPA-evoked rises in $[Ca^{2+}]_i$. The record shows the mean response of the 4 neurones, out of a total of 17 recorded simultaneously, which displayed an augmented rise in $[Ca^{2+}]_i$ in response to AMPA (A) in the presence of 100 μM DTG (applied for the period indicated by the bar above the trace). DTG did not affect AMPA-evoked responses in the remaining 13 neurones, but all 17 neurones displayed a reduction in NMDA-(N) evoked rises in $[Ca^{2+}]_i$. Note also that 100 μM DTG reduced the natural quisqualate-(Q) evoked responses to a greater extent than the synthetic, glutamate-free (+)-quisqualate-((+)Q) evoked responses.

potentiate NMDA-evoked responses (Monnet *et al.*, 1990; 1992). The effects of DTG applied at 0.5, 1, 5, 10, 20, and 50 nM were investigated on NMDA-evoked rises in $[Ca^{2+}]_i$ ($n > 15$ at each concentration of DTG tested); in no case was a potentiation of the response observed. At 10, 20 and 50 nM, DTG also failed to affect rises in $[Ca^{2+}]_i$ evoked on application of high-$[K^+]_o$, kainate or AMPA ($n > 6$ at each concentration of DTG tested).

Electrophysiological studies

Selectivity of the DTG block Submaximal, inward currents were evoked in hippocampal neurones by 3 s applications of 60 μM NMDA, 60 μM naturally-derived quisqualate, 10 μM AMPA and 60 μM kainate. Responses to 60 μM NMDA were dose-dependently reduced by DTG. The percentage reduction at each concentration of DTG was estimated only when NMDA responses had reached a stable lower value in the continued presence of DTG, often requiring up to 10 consecutive applications of NMDA (see below). Superfusion with 50 μM DTG depressed both peak and steady-state NMDA-evoked current by 60 ± 4% ($n = 8$) but produced only small reductions of control kainate- or steady-state AMPA-evoked responses (respectively 6 ± 2%, $n = 10$ and 9 ± 2%, $n = 6$). At 300 μM, DTG attenuated the NMDA response by 92 ± 2% ($n = 8$) but with no additional reduction of kainate- or AMPA-evoked responses (respectively 5 ± 1%, $n = 11$ and 9 ± 2%, $n = 11$). Due to the limitations of the perfusion system the rapid peak of the AMPA-evoked responses may be underestimated but there was no apparent inhibition of this particular component on superfusion with 300 μM DTG. The IC_{50} value for DTG against NMDA was 36.6 ± 5.4 μM ($n = 10$) and the slope of the plot was not significantly different from 1 (Student's t test, $P < 0.05$, Figure 4a). An example of the activity of DTG can be readily observed in Figure 4b (using a different protocol from that described above) where DTG was applied during prolonged agonist application to highlight the selectivity and reversibility of the action of DTG on NMDA-evoked events.

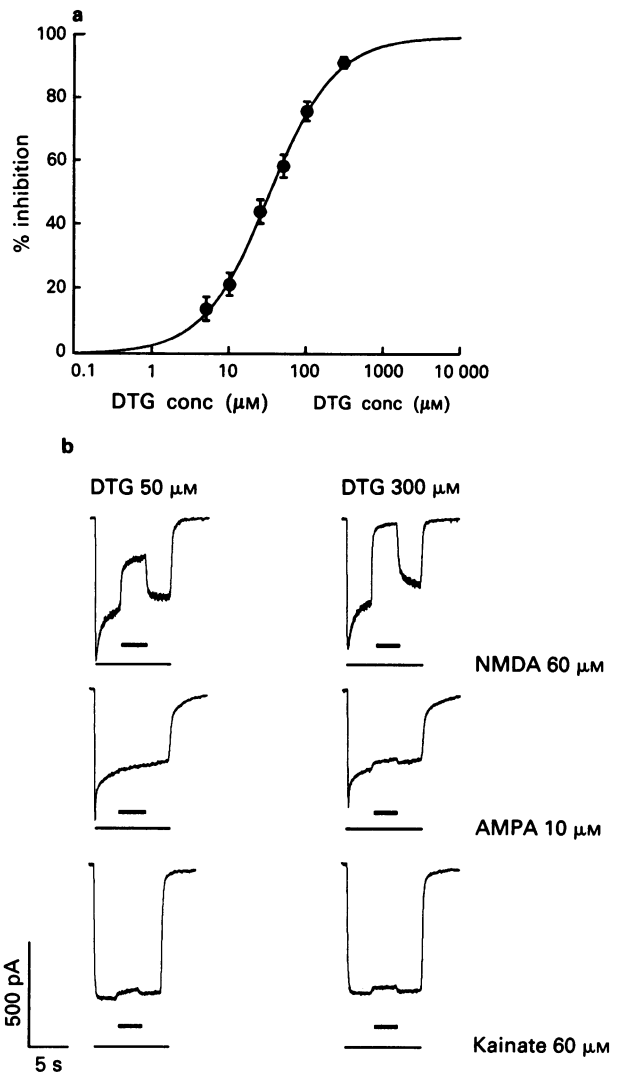


Figure 4 Selectivity of DTG for NMDA-evoked currents. (a) Concentration-inhibition plot for DTG as an NMDA antagonist. NMDA applications (3 s, 60 μM) were repeated in the presence of DTG until steady state block achieved. Data points indicate mean ± s.e.mean and are fitted using a logistic equation. Averaged IC_{50} value for DTG against NMDA was 36.6 ± 5.4 μM with a slope of 1.1 ± 0.2 ($n = 10$). Holding potential of neurone was − 60 mV. Ordinate scale: % inhibition of control response to NMDA. Abscissa scale: DTG concentration in μM. (b) The action of 50 μM DTG (left) and 300 μM DTG (right) on amino acid-evoked currents (applied for period indicated by thin bar below trace) in a single hippocampal neurone. DTG (solid bar) produced a reversible depression of NMDA-evoked currents (53 and 93% at DTG 50 and 300 μM, respectively). Responses to AMPA and kainate were reduced by < 10% at either concentration. Calibration: current amplitude in pA; time in s.

DTG (100 and 300 μM) attenuated the steady-state, but not the peak current, evoked by naturally-derived quisqualate (Figure 5a). At 100 μM, DTG produced a 24 ± 4% reduction of natural quisqualate responses (range 10–50%; $n = 6$), whilst at 300 μM DTG the inhibition increased to 57 ± 10% (range 35–82%; $n = 4$). The degree of inhibition was not dependent on the source of natural quisqualate (Sigma or Tocris) although older stock (stored for more than 12 months at < 4°C) had a greater DTG-sensitive component (at 100 μM DTG, 74 ± 4% reduction; $n = 4$). Superfusion with 10–20 μM DL-2-amino-5-phosphonovalerate (DL-AP5), or 1 mM Mg^{2+}, produced a similar reduction of natural quisqualate responses (for DL-AP5 a 47 ± 5% reduction

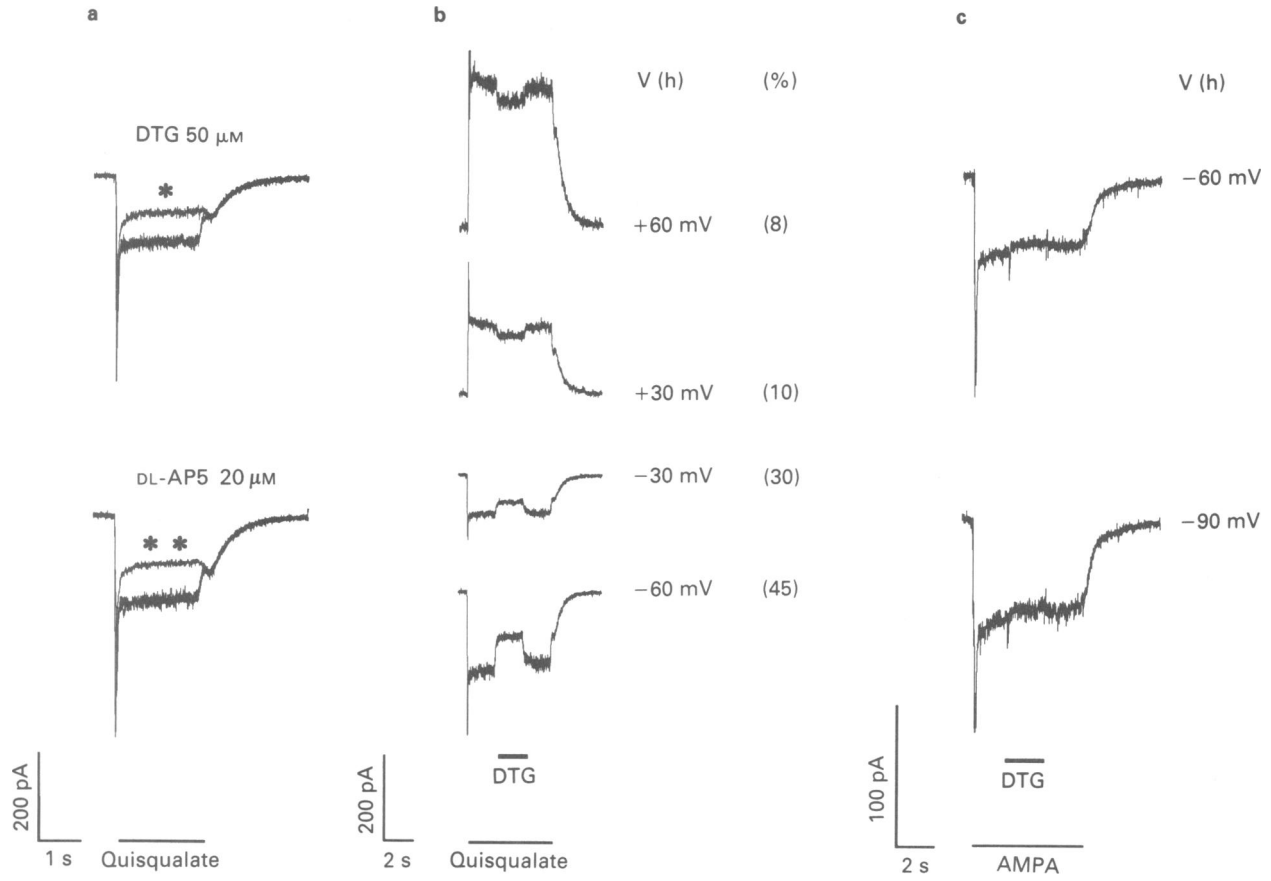


Figure 5 DTG reversibly depressed natural quisqualate-evoked responses. (a) Application of 60 μM natural quisqualate for 2 s evoked a rapidly desensitizing response with a more prolonged plateau. Superfusion with 50 μM DTG (top trace *) or 20 μM DL-AP5 (bottom trace **) depressed the plateau response leaving the rapid peak intact. This effect was fully reversible (wash superimposed on control). (b) In the same neurones as (a), DTG (thick bar) was applied for 2 s during the steady-state phase of the quisqualate response (6 s application, thin bar). The block was examined at a series of holding potentials (V(h)) from − 60 mV to + 60 mV (% inhibition indicated in brackets). (c) On a second neurone, a response to 10 μM AMPA (thin bar) was unaffected by co-application of 50 μM DTG (thick bar). Increasing the holding potential from − 60 mV to − 90 mV did not reveal an inhibition by DTG. Calibration: current amplitude in pA; time in s.

$n = 3$; see Figure 5a) confirming the contribution made by NMDA receptor activation to the natural quisqualate-evoked current. Furthermore, in the presence of 2 mM Mg^{2+} there was no additional reduction of natural quisqualate responses on co-application of 50 or 100 μM DTG ($n = 3$). The reduction of natural quisqualate-evoked currents by DTG appeared to be voltage-dependent (Figure 5b) whilst that of DL-AP5 was not (data not shown).

As hyperpolarization enhanced the DTG inhibition of quisqualate responses (Figure 5b) a similar protocol was applied to reassess the activity of DTG on AMPA-evoked responses. At neither − 60 mV nor − 90 mV did DTG produce an appreciable inhibition of AMPA-evoked responses (Figure 5c), confirming that DTG has low intrinsic activity at the AMPA receptor. In agreement with the fluorescent dye studies, DTG potentiated AMPA-evoked currents in 4 out of 10 neurones tested (range 5–43%), but on no occasion was there potentiation of kainate- or natural quisqualate-evoked currents.

DTG (50 μM) was applied in the presence of haloperidol; on the 3 cells tested 2 μM haloperidol did not reverse the inhibitory action of DTG on NMDA-evoked currents. In addition, DTG applied at concentrations between 0.01 pM and 100 nM, did not potentiate NMDA responses ($n = 8$).

DTG block of NMDA responses was not competitive As DTG has been reported to have non-selective actions at concentrations > 100 μM (Malouf *et al.*, 1988) a concentration of DTG of 50 μM was used in the remaining studies. The latter concentration of DTG produced a 60 ± 5% ($n = 10$) reduction of the maximal response (R_m) evoked on NMDA application with no change in the estimated EC_{50} values for NMDA (respectively 30 ± 6 and 35 ± 5 μM in the absence and presence of DTG, $n = 11$, Figure 6a). The attenuation of NMDA responses by 50 μM DTG was not dependent on agonist concentration; there was no significant difference (Student's t test, $P < 0.05$) in the effect of DTG on responses evoked by 10, 60, 100 or 300 μM NMDA (Figure 6b, top chart).

Increasing the concentration of glycine in the medium from nominally glycine-free to 10 μM glycine increased the amplitude of the NMDA-evoked currents but did not alter the percentage reduction of the control responses by DTG (Figure 6b, middle chart). In the presence of 3 μM glycine, co-application of the polyamine spermine (at 100 μM) produced an additional enhancement of the NMDA-evoked responses (between 10 and 100%, $n = 9$). DTG attenuated both control and polyamine-enhanced currents to a similar degree (Figure 6b, bottom chart) and dose-inhibition plots for DTG were not shifted in the presence of 100 μM spermine ($n = 2$; data not shown).

Voltage-dependent block of NMDA-evoked responses The voltage-dependence of the action of DTG was investigated on 14 neurones. Increasing depolarization of the membrane reduced the percentage inhibition of NMDA-induced cur-

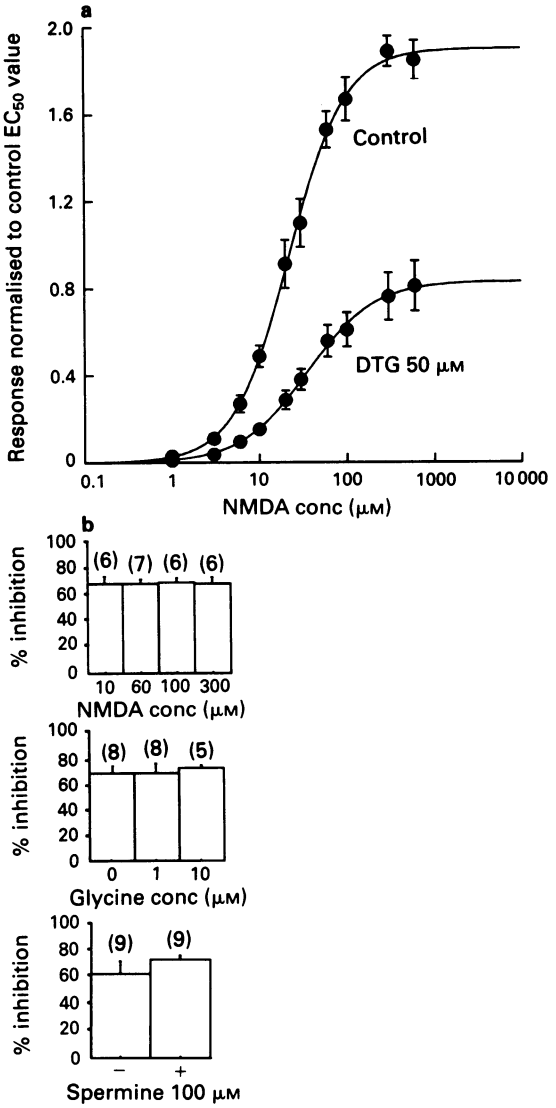


Figure 6 DTG does not interact with agonist-recognition, glycine or polyamine sites. (a) Concentration-response plots for NMDA were constructed in the presence and absence of 50 μM DTG. Responses were normalized to the EC_{50} value for NMDA for each neurone in control solution and data points indicate mean ± s.e.mean ($n = 11$). Control: $EC_{50} = 30 \pm 6$ μM; DTG 50 μM: $EC_{50} = 35 \pm 5$ μM. Ordinate scale: response normalized to the EC_{50} value for NMDA in each neurone. Abscissa scale: NMDA concentration in μM (b) percentage reduction of control response to 60 μM NMDA by 50 μM DTG in the presence of increasing concentrations of NMDA (upper), glycine (middle) and spermine (lower). Under none of the conditions were % inhibitions significantly different from control values (Student's t test, $P < 0.05$). Bars indicate s.e.mean. Number of neurones tested indicated in parentheses above error bars.

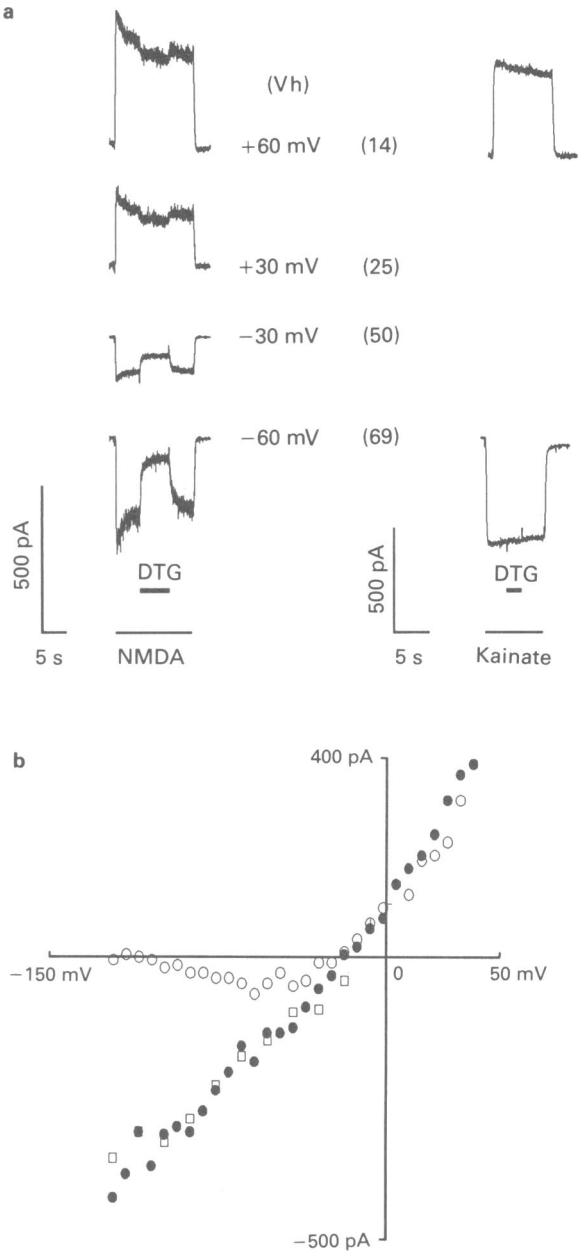


Figure 7 Voltage-dependence of the NMDA block induced by 50 μM DTG. (a) On the left, a 6 s application of DTG (thick bar) reduced steady-state current evoked by NMDA (applied for period indicated by thin bar); those induced by kainate were not affected (right). As the holding potential of the neurone (V(h)) was changed from − 60 mV to + 60 mV, a reduction in the % inhibition (indicated in parentheses) was observed. Calibration: current amplitude in pA; time in s. (b) On a second neurone, superfusion with 50 μM DTG (○) reduced control NMDA-evoked currents (●) in a voltage-dependent manner. The membrane potential was altered in 5 mV increments and allowed to stabilize before NMDA (60 μM) application. Action of DTG was fully reversed on wash in control solution (□). Ordinate scale: current amplitude in pA. Abscissa scale: membrane holding potential in mV.

rents produced by DTG, whilst hyperpolarization increased the inhibition. An example of the voltage-dependency of the block of NMDA-, but not kainate-, evoked currents by DTG can be seen in Figure 7a and b.

Further analysis of the voltage-dependency of the DTG block was carried out using the Woodhull model [1] (Woodhull, 1973) by which one can estimate the fraction (δ) of the transmembrane potential (V) experienced by a channel-blocking ion/molecule on the assumption, made here, that the ion/molecule does not interact with other ions either in the solution or bound to the channel. The equation derived from the model [1] is

$$[1] \quad \frac{I}{Idtg} = 1 + \left(\frac{[dtg]}{Kd(0)} \right) \exp \left(\frac{-zFV\delta}{RT} \right)$$

Where $I/Idtg$ is the ratio of currents in the absence and presence of the antagonist DTG, $Kd(0)$ is the dissociation constant of the antagonist for the binding site at zero transmembrane potential, and R, F and T have their usual values. The equation can be re-arranged and natural logarithms taken to given the linear equation

$$[2] \quad \ln \left(\frac{I}{Idtg - 1} \right) = \ln \left(\frac{[dtg]}{Kd(0)} \right) - \left(\frac{z\delta F}{RT} \right) .V$$

Plotting $\ln(I/I_{dtg}-1)$ vs V gave a straight line for data points which when fitted by least squares gave values for δ and $K_d(0)$ of 0.76 ± 0.08 ($n = 7$) and 163 ± 41 μM, respectively. From the latter value the dissociation constant $K_d(-60\text{ mV})$, the dissociation constant at a transmembrane potential of -60 mV, was calculated to be 27 ± 4 μM which compares well with the IC_{50} value of 37 ± 5 μM obtained from the dose-inhibition plot for DTG at the same holding potential.

Site of action of DTG

A series of combination experiments were carried out with DTG, Mg^{2+} and MK-801 to determine whether the binding sites for these compounds overlap. Superfusion with 300 μM DTG or 10 mM Mg^{2+} resulted in almost complete block of the NMDA-evoked response (Figure 8a and b) with immediate recovery of block by magnesium and slightly slower (within 2 to 3 NMDA applications) recovery from DTG block. By contrast prolonged (~15 s) applications of 3 μM MK-801 were required to block NMDA-evoked responses fully and these were not reversed appreciably within the first 5 min of wash (Figure 8c). Both Mg^{2+} and DTG, when applied prior to and during application of MK-801, prevented the development of the long duration block characteristic of MK-801, suggesting that they both prevent MK-801 from binding within the ion channel.

Use-dependent block of NMDA-evoked responses

Superfusion with DTG resulted in a large reduction of the first NMDA-evoked response in a manner similar to that of DL-AP5 and Mg^{2+}. The block by DTG, but not that of DL-AP5 and Mg^{2+} was, however, further enhanced on subsequent NMDA applications. Approximately $66 \pm 3\%$ ($n = 9$) of the total inhibition was achieved in the presence of DTG on the first NMDA application, implying that the remaining 34% of the block was use-dependent.

The biphasic block of NMDA responses by DTG was further confirmed on estimating the kinetics of the onset and offset of the block with a protocol similar to that in Figure 4b. Onset of the block by DTG could be fitted by two time constants, a fast component of $0.18 \pm 0.02\ 1\ s^{-1}$ ($n = 6$) followed by a slower component of $5.8 \pm 0.7\ 1\ s^{-1}$ ($n = 5$). The recovery from block by DTG was equally well-fitted by two time constants of 0.50 ± 0.07 and $5.55 \pm 1.19\ 1\ s^{-1}$. The fast component contributed to approximately 76% of the final block, in close agreement with the percentage block (66%) of the first NMDA response in the experiments described above.

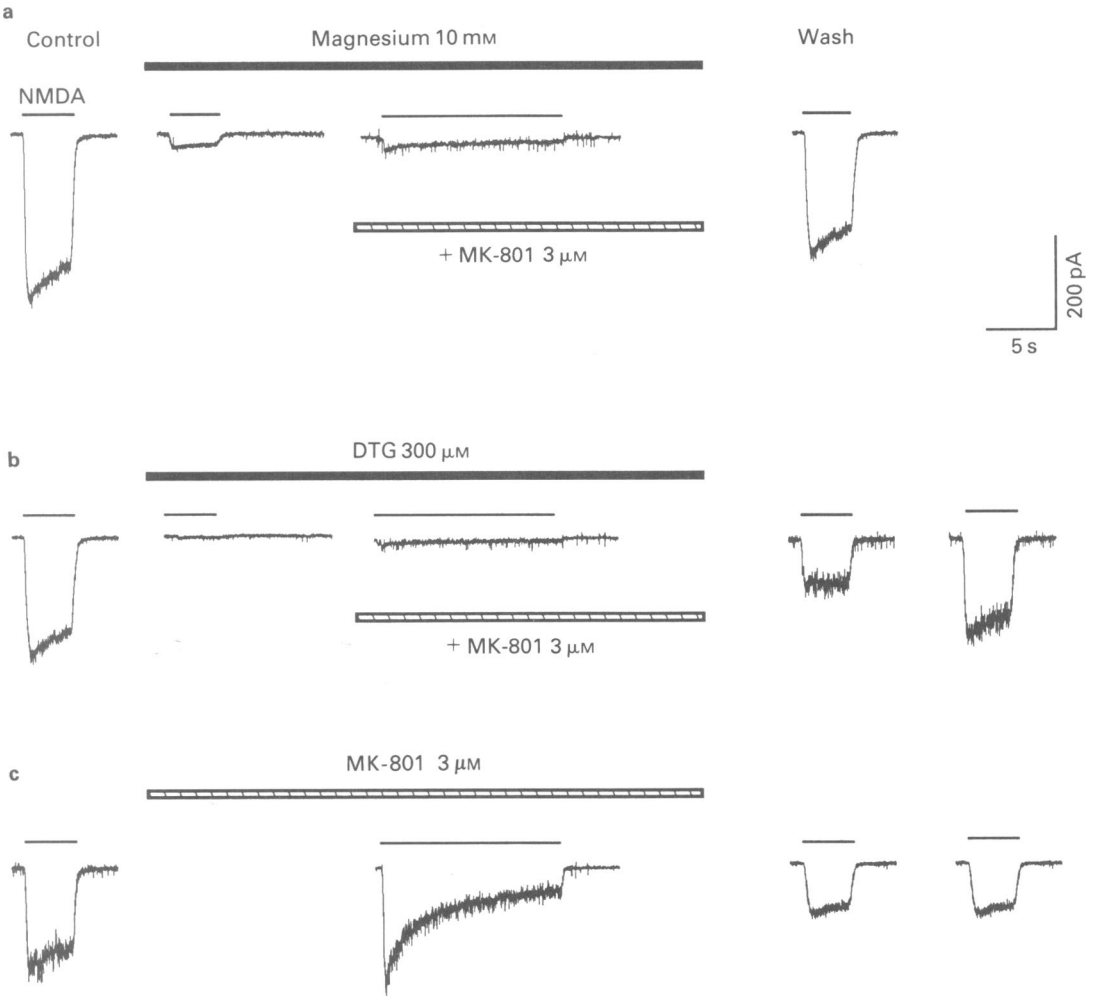


Figure 8 DTG and Mg^{2+} protect against the prolonged block induced by MK-801. The control response to 60 μM NMDA (5 s application, thin bar) was profoundly attenuated by superfusion with (a) 10 mM Mg^{2+} and (b) 300 μM DTG, as indicated by solid bars above traces. MK-801 (3 μM) was then co-applied (hatched bar below traces) in the continued presence of (a) Mg^{2+} or (b) DTG, and NMDA then applied for 12 s. Both antagonists were then washed out for 5 min before NMDA was reapplied (far right). Full recovery from block by Mg^{2+} was achieved on the first application of NMDA (a, trace far right) whilst that from block with DTG required one further NMDA application (b, last 2 traces far right taken at 40 s intervals). In the bottom trace (c) application of MK-801 alone produced a slowly developing block during a 12 s application of NMDA that showed no sign of reversing even with prolonged wash (right). Ordinate scale: 200 pA; Abscissa scale: time in s.

Discussion

DTG is one of the preferred ligands for investigating the functional roles of σ sites. In the present study, concentrations of DTG selective for the latter site (i.e. in the low nanomolar range) had no effect on excitatory amino acid-evoked currents and rises in $[Ca^{2+}]_i$ in cultured mouse and rat hippocampal neurones, respectively. However, higher (μM) concentrations of DTG selectively attenuated NMDA-evoked responses in both assays. The NMDA antagonist action of micromolar concentrations of DTG was haloperidol-insensitive, in agreement with others (Connick *et al.*, 1992), again suggesting a lack of involvement of σ receptors in the NMDA-antagonist properties of micromolar concentrations of DTG. Although high concentrations of DTG (100 μM) have been reported to have non-selective properties (Malouf *et al.*, 1988) the effects observed here cannot be attributed to non-specific effects on membrane excitability. Furthermore as the IC_{50} value for DTG as an antagonist of NMDA-evoked responses in preparations of both rat and mouse hippocampal neurones was similar, one may assume that there is no large species differences in NMDA receptor pharmacology.

The mechanism of the block of NMDA currents by DTG was not competitive but appeared to be both voltage-, and partially use-, dependent in nature. The voltage-dependence of the DTG block is similar to that seen with Mg^{2+} (Nowak *et al.*, 1984) and many other compounds including ketamine (MacDonald *et al.*, 1991), MK-801 (Huettner & Bean, 1988), tricyclic antidepressants (Sernagor *et al.*, 1989), tetrahydroaminoacridine (Hershkowitz & Rogawski, 1991), arcaine (Donevan *et al.*, 1992) and strychnine (Bertolino & Vicini, 1988). Using the Woodhull model (Woodhull, 1973) it was possible to estimate the fraction of the electrostatic field (δ) experienced by DTG at its binding site. In this study δ was 0.76, close to that reported for Mg^{2+} (Ascher & Nowak, 1988). Furthermore, the similarity between the computed affinity of DTG at -60 mV in the kinetic experiments was similar to the IC_{50} value calculated from dose-inhibition plots (also carried out at a holding potential of -60 mV) suggesting that binding at the predicted site within the ion channel can largely account for the inhibitory action of DTG on NMDA-evoked responses. The voltage-dependence of DTG probably stems from a positive charge on the imine group of the molecule at physiological pH. Combination experiments were also suggestive of a Mg^{2+}-type block of NMDA-evoked currents by DTG (Huettner & Bean, 1988; MacDonald *et al.*, 1991). Furthermore, kinetic studies revealed that the rates of onset and offset of DTG block were similar, although not identical, to those of Mg^{2+} and both were significantly faster than those reported for ketamine (MacDonald *et al.*, 1991). In contrast to Mg^{2+}, however, a component of the DTG block was use-dependent, a characteristic of PCP-like ligands. This possibility is supported by binding studies in which DTG displaced [^{3}H]-MK-801 and [^{3}H]-TCP with IC_{50} values of 11 and 8 μM, respectively (Keana *et al.*, 1989). Nevertheless, the present studies are in agreement with those of Keana and colleagues (1989), who proposed that the rapid rate of recovery from the DTG block reflected its low affinity for the NMDA receptor-linked ion channel. Thus the slow rate of recovery from block with MK-801 or ketamine reflects a higher affinity, and thus slower dissociation, of these ligands from the NMDA receptor-channel complex (MacDonald *et al.*, 1991).

Both glycine and polyamines (e.g. spermine) potentiate NMDA receptor-mediated responses through activation of distinct allosteric sites on the NMDA receptor-channel complex (Johnson & Ascher, 1987; Ransom & Stec, 1988). Notably ifenprodil, a non-competitive NMDA antagonist inhibits polyamine-stimulated binding of ^{3}H channel ligands (see Reynolds & Miller, 1989; Beart *et al.*, 1991), and displaces [^{3}H]-(+)-3-PPP binding (Karbon *et al.*, 1990; Benavides *et al.*, 1992) with similar nanomolar affinities, tentatively suggesting an overlap between polyamine and σ binding sites. In the present experiments, DTG did not act as a polyamine or glycine site ligand as the inhibition of NMDA-evoked responses by DTG was not reversed on addition of spermine or glycine. Moreover, DTG did not potentiate NMDA-evoked responses even when an appreciable potentiation on NMDA-evoked responses by glycine and/or spermine could be achieved.

In the absence of an appreciable depression of AMPA-evoked responses by DTG it was surprising that DTG attenuated those evoked by natural quisqualate. As the NMDA receptor antagonists, Mg^{2+} and DL-AP5, produced a similar reduction of quisqualate responses and occluded quisqualate inhibition by DTG, it is likely that natural quisqualate responses are mediated, at least in part, through activation of the NMDA subtype of glutamate receptor. The latter might result from contamination of natural quisqualate with a potent NMDA receptor agonist, most likely to be glutamate and aspartate (Cha *et al.*, 1989), as suggested by our studies on synthetic, glutamate-free (+)-quisqualate. Alternatively, quisqualate may be a partial agonist at the NMDA receptor, a property that AMPA does not, from our studies, appear to possess. In this regard it is noteworthy that quisqualate displaces [^{3}H]-D-AP5 binding from CNS membranes with micromolar affinity (Olverman *et al.*, 1988) and has been reported to evoke responses in *Xenopus* oocytes exclusively expressing an NMDA receptor clone (Moriyoshi *et al.*, 1991). We made no attempt to quantify rigorously the contribution made by each of these factors but our results emphasize once again that quisqualate is not a good choice of ligand for studying drug selectivity at subtypes of glutamate receptor. The reported lack of selectivity of DTG between NMDA- and quisqualate-evoked responses (Connick *et al.*, 1992) may reflect a lack of selectivity of the agonist used rather than that of the antagonist.

Some σ receptor ligands, applied at low concentrations, have been reported to potentiate NMDA receptor-mediated responses in both the hippocampus (Malouf *et al.*, 1988; Monnet *et al.*, 1990; 1992) and elsewhere in the CNS (Iyengar *et al.*, 1990; Rao *et al.*, 1991). We did not observe potentiation of NMDA-evoked responses by nanomolar concentrations of DTG (or (+)-3-PPP; E.J.F., unpublished observations). Although this finding may reflect the fact that our cultured neurones (predominantly derived from the hippocampal CA1 region) do not possess σ binding sites, differences in methodologies may also underlie the discrepancies in the reported NMDA modulatory effects of σ ligands. Under non voltage-clamped conditions (e.g. extracellular recordings *in vivo* or *in vitro* and neurochemical assays) σ ligands may indirectly modulate receptor function through the activation, or inhibition, of other ionic currents. DTG has been reported to block a ligand-gated K^+ current (Bobker *et al.*, 1989) and can reduce ^{86}Rb efflux through K^+ channels (Fletcher *et al.*, 1989). The occasional enhancement of AMPA- and kainate-evoked responses by micromolar concentrations of DTG observed in the present experiments may reflect the interaction of DTG with other ion channels located on, presumably, only a small percentage of our cultured neurones.

In conclusion, we have shown that DTG, at low micromolar concentrations, selectively blocks NMDA receptor-mediated responses in both rat and mouse cultured hippocampal pyramidal neurones. The block of NMDA responses by DTG is not competitive in nature and appears to be both voltage- and use-dependent suggesting that the action of DTG may have characteristics of both Mg^{2+} and PCP-like compounds. The results indicate, however, that DTG, at micromolar concentrations, cannot be employed as a selective probe for investigating the physiological, biochemical, pharmacological or behavioural characteristics of σ receptors. Given the appreciable activity of DTG as an NMDA antagonist, the actions of DTG at these concentrations *in vivo* and *in vitro* may reflect not only the interaction of DTG

with σ receptors but also with the NMDA receptor-channel complex. Indeed, the NMDA antagonist activity associated with micromolar concentrations of DTG and other σ ligands (e.g. caramiphen; see Church *et al.*, 1991) may account in large part for their PCP-like discriminative stimulus properties (Holtzman, 1989; Singh *et al.*, 1990), their neuroprotective properties (Keana *et al.*, 1989; Pontecorvo *et al.*, 1991; Long *et al.*, 1992; Shalaby *et al.*, 1992) and their antiepileptiform activity in studies of Mg^{2+}-free bursting activity (J.C., unpublished observations).

Supported by the Medical Research Council of Canada Grants to J.C. and J.F.M. ('Nerve, Cell and Synapse' Group award) and National Centres of Excellence Grant to J.F.M. E.J.F. is a Medical Research Council of Canada Fellow and J.C. is a B.C. Health Research Foundation Scholar.

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(Received January 8, 1993
Revised March 23, 1993
Accepted April 2, 1993)

Br. J. Pharmacol. (1993), **109**, 1206–1211 © Macmillan Press Ltd, 1993

[^{3}H]-5-carboxamidotryptamine labels 5-HT$_{1D}$ binding sites in bovine substantia nigra

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1 [^{3}H]-5-hydroxytryptamine (5-HT) has been shown to radiolabel at least five types of 5-HT binding sites in mammalian brain tissue, 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1C}$, 5-HT$_{1D}$ and 5-HT$_{1E}$ (Frazer *et al.*, 1990). Selective masking of 5-HT$_{1A}$ and 5-HT$_{1C}$ receptors, has uncovered binding sites which display both high (5-HT$_{1D}$) and low (5-HT$_{1E}$) affinity for 5-carboxamidotryptamine (5-CT). By utilizing [^{3}H]-5-CT we have eliminated a portion of the complex binding (5-HT$_{1E}$) seen when [^{3}H]-5-HT is used as a radioligand.

2 [^{3}H]-5-CT binding to 5-HT$_{1D}$ sites in bovine substantia nigra was rapid, reversible and saturable, displaying high affinity ($K_d = 0.38 \pm 0.04$ nM) and low non-specific binding (>90% specific binding).

3 In bovine substantia nigra, [^{3}H]-5-CT labelled an equivalent number of binding sites to [^{3}H]-5-CT (403 ± 18 and 362 ± 20 fmol mg^{-1} protein, respectively) and binding was sensitive to guanine nucleotides.

4 A linear correlation ($r^2 = 0.99$) existed between the potency of compounds to displace [^{3}H]-5-HT and [^{3}H]-5-CT in bovine substantia nigra.

5 Therefore, [^{3}H]-5-CT is a novel radioligand for the examination of 5-HT$_1$-like binding sites, which under proper experimental conditions can be used to radiolabel selectively 5-HT$_{1D}$-like binding sites.

Keywords: 5-HT$_{1D}$ receptors; 5-carboxamidotryptamine; substantia nigra; [^{3}H]-5-carboxamidotryptamine; bovine brain; 5-hydroxytryptamine

Introduction

Pharmacological and molecular biological techniques have demonstrated the existence of a large family of 5-hydroxytryptamine (5-HT) receptors which have been grouped into several distinct receptor subtypes (5-HT$_1$, 5-HT$_2$, 5-HT$_3$, and 5-HT$_4$, Frazer *et al.*, 1990). While the endogenous ligand 5-HT activates numerous 5-HT receptor types, 5-HT$_1$-like receptors alone are suggested to display an equally high affinity for the agonist, 5-carboxamidotryptamine (5-CT, Bradley *et al.*, 1986). Pharmacological approaches have suggested that 5-HT$_1$-like receptors are a heterogeneous group consisting of 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1C}$, 5-HT$_{1D}$ and 5-HT$_{1E}$ receptors (El Mestikawy *et al.*, 1991; Middlemiss & Hutson, 1990; Hoyer, 1988; Waeber *et al.*, 1990; Leonhardt *et al.*, 1989).

The 5-HT$_{1D}$ receptor has been defined as the [^{3}H]-5-HT binding remaining in the presence of 100 nM (±)-2-dipropylamino-8-hydroxy-1,2,3,4-tetrahydronaphthalene (8-OH-DPAT) and 100 nM mesulergine, which selectively masks the 5-HT$_{1A}$ and 5-HT$_{1C}$ receptors, respectively (Heuring & Peroutka, 1987). However, [^{3}H]-5-HT in the presence of the above masking ligands has been shown to recognize a heterogeneous population of binding sites in porcine caudate (Sumner & Humphrey, 1989) and human cortex (Leonhardt *et al.*, 1989). It has also been demonstrated that in the presence of 100 nM cyanopindolol (to mask 5-HT$_{1A}$ and 5-HT$_{1B}$ sites) and 100 nM mesulergine, 5-CT and sumatriptan produced biphasic displacement of [^{3}H]-5-HT binding in the cortex and/or caudate of dog, guinea-pig, rabbit, pig, man, hamster, and calf (Beer *et al.*, 1992). This binding could be separated into two separate components, displaying high (5-HT$_{1D}$) and low (5-HT$_{1E}$) affinity for 5-CT. Molecular cloning has confirmed the existence of at least two subtypes of the 5-HT$_{1D}$ receptor, each of which display high affinity for 5-CT (Hartig *et al.*, 1992), as well as the 5-HT$_{1E}$ receptor which displays significantly lower affinity for 5-CT (McAllister *et al.*, 1992).

We have taken advantage of the fact that 5-CT can discriminate between 5-HT$_{1D}$ and 5-HT$_{1E}$ receptors and have radiolabelled 5-CT (α-ditritio 5-CT) to obtain a radioligand for investigating the multiplicity in 5-HT$_{1D}$-like binding sites (Mahle *et al.*, 1991). Here we describe the binding characteristics of [^{3}H]-5-CT in comparison with that of the widely used radioligand [^{3}H]-5-HT, in bovine substantia nigra, a region appearing homogeneous with respect to 5-HT$_{1D}$-like binding sites.

Methods

Radioligand binding assay

Bovine brain was obtained from a local slaughterhouse and the substantia nigra dissected, frozen on dry ice and stored at −70°C until assayed, generally less than one week. The tissue was homogenized (1:80 wt/vol) with a polytron (Brinkmann Instruments, Westbury, NY, U.S.A.) in wash buffer (50 mM HEPES, 2.5 mM $MgCl_2$ and 2.0 mM ethylene glycol-*bis* (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), pH adjusted to 7.4 at room temperature, with a saturated Tris base solution). The homogenate was washed twice in wash buffer via centrifugation at 39,000 *g* (10 min, 4°C) with a resuspension between centrifugations, then incubated in wash buffer at 37°C for 10 min to remove endogenous 5-HT (Nelson *et al.*, 1978), and centrifuged as above. The final pellet was resuspended in the initial volume of assay buffer (wash buffer containing 0.1% ascorbic acid and 10 μM pargyline) and stored on ice till assayed.

Radioligand binding assays ([^{3}H]-5-HT, [^{3}H]-5-CT) were carried out in duplicate in a final volume of 1.0 ml consisting of 100 μl radioligand, 50 μl each of 8-OH-DPAT and mesulergine (100 nM final concentration), to inhibit binding to 5-HT$_{1A}$ or 5-HT$_{1C}$ receptors, respectively (Heuring & Peroutka, 1987), 100 μl assay buffer (or guanine nucleotide, competitor, or 5-HT), and 700 μl membrane homogenate (approximately 0.5 mg protein ml^{-1}). Incubations were in-

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itiated by the addition of the membrane preparation and were conducted for 60 min at 25°C. Saturation analyses were performed with 0.05–10 nM radioligand. Nonspecific binding was defined by 10 μM 5-HT. Competition experiments were performed with 0.5 nM [^{3}H]-5-HT and [^{3}H]-5-CT, resulting in approximately 70% and 95% specific binding, respectively. To obtain kinetic rate constants, [^{3}H]-5-CT (total and non-specific binding) was assayed at various time points during association and dissociation. Dissociation was initiated by the addition of 10 μM unlabelled 5-CT.

Bound radioactivity was determined after rapid filtration through Whatman GF/B filter strips, using a Brandel cell harvester (Gaithersburg, MD, U.S.A.), and washing with three washes (5 ml each) of ice-cold wash buffer. Filters were counted by liquid scintillation spectrometry in Atomlight (New England Nuclear) at approximately 47% efficiency. Protein was determined by the method of Bradford (1976), with bovine serum albumin used as the standard.

Chemicals

[^{3}H]-5-HT was purchased from New England Nuclear (Boston, MA, U.S.A.). [^{3}H]-5-CT was developed through custom synthesis by Dr Ron Mattson (Bristol-Myers Squibb) and Dr Stephen Hurt (New England Nuclear, Boston, MA, U.S.A.). Dr John Macor (Pfizer) kindly provided 3-(1,2,5,6-tetrahydropyrid-4-yl)pyrrolo[3,2-b]pyrid-5-one (CP-93,129). Guanyl and adenyl nucleotides were obtained from Sigma Chemical Co (St. Louis, MO, U.S.A.), while other drugs used in competition studies were purchased from Sigma Chemical Co., Research Biochemicals (Natick, MA, U.S.A.), or synthesized locally.

Data analysis

Data were analysed with the non-linear regression analysis programme LIGAND (Munson & Rodbard, 1980), where data were best fitted to a one-site model yielding for saturation studies, the equilibrium dissociation constant (K_d), the maximal number of binding sites (B_{max}), and Hill coefficient (nH). For competition studies, all data were analysed by non-linear regression analysis, LIGAND (Munson & Rodbard, 1980), initially assuming a one-site model of [^{3}H]-5-CT or [^{3}H]-5-HT binding, followed by analysis according to a two-site model. An F test ($P \leqslant 0.01$) was then used to determine whether the data were best described by the two-site model. Kinetic association and dissociation rate constants were obtained with the data analysis programme ENZFITTER (Elsevier Biosoft, Ferguson, MO, U.S.A.), using a first order rate equation or a single exponential decay, respectively. Guanine nucleotide inhibition of ^{3}H-labelled agonist binding was fitted to a four parameter logistic equation to obtain response parameters (IC_{50}, E_{max} and slope index).

Results

As demonstrated in Figure 1, [^{3}H]-5-CT binding, performed under conditions designed to radiolabel 5-HT$_{1D}$ binding sites (Heuring & Peroutka, 1987) rapidly reached equilibrium and was reversible. Association and dissociation rate constants were determined for specific [^{3}H]-5-CT binding (0.5 nM) to bovine substantia nigra membrane homogenates (Figure 1). The association time study yielded a K_{obs} value of 0.17 ± 0.02 min^{-1} which converted to a K_1 value of 1.56×10^8 M^{-1} min^{-1}. The dissociation time study yielded a K_{-1} value of 0.093 ± 0.005 min^{-1}. The resulting K_d value was 0.60 ± 0.07 nM.

[^{3}H]-5-CT binding (under 5-HT$_{1D}$ conditions) in bovine substantia nigra membrane homogenates was homogeneous (nH = 0.99 ± 0.003), saturable and demonstrated high affinity and low non-specific binding. Saturation analysis and the resulting Scatchard transformation (Figure 2) indicated a K_d

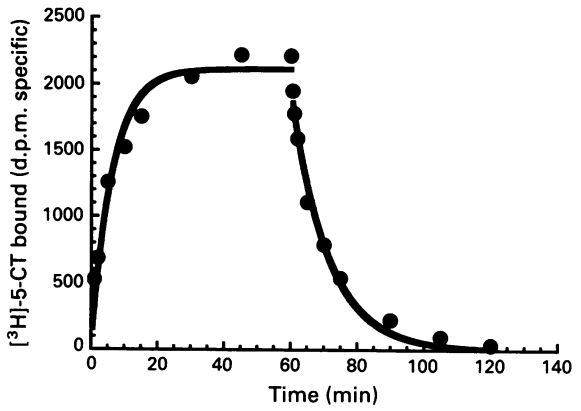


Figure 1 Kinetics of association and dissociation of [^{3}H]-5-carboxamidotryptamine ([^{3}H]-5-CT) binding in bovine substantia nigra membrane homogenates under 5-HT$_{1D}$ conditions. The tissue homogenate (approximately 0.5 mg protein ml^{-1}) was incubated with 0.5 nM [^{3}H]-5-CT and radioactivity measured for 60 min. After that period, an excess of unlabelled 5-CT (1 μM) was added with radioactivity measured for an additional 60 min. Using a first order rate equation (see Methods) and single exponential decay produced a dissociation rate constant of 8.99×10^{-2} and a K_{obs} of 1.58×10^{-1}, which yielded a K_d value of 0.64 nM. Data points shown reflect specific binding. This experiment was performed twice more yielding similar results.

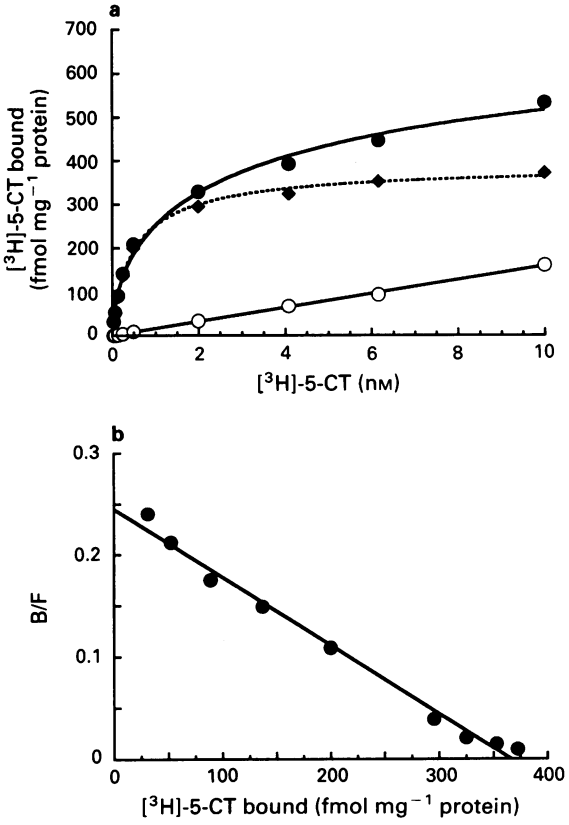


Figure 2 Representative saturation analysis (a, total binding (●); non-specific binding (○) and specific binding (◆)) and Scatchard transformation (b) of [^{3}H]-5-carboxamidotryptamine ([^{3}H]-5-CT) binding in bovine substantia nigra membrane homogenates. Non-specific binding was defined by 10 μM 5-hydroxytryptamine. Data points represent a single experiment performed in duplicate, in the same membrane preparation as used for Figure 3. Values for three separate experiments are described in the text. Non-linear regression analysis of the illustrated experiment yielded the following response parameters: K_d = 0.4 nM, B_{max} = 367 fmol mg^{-1} protein, and nH = 1.00.

value of 0.38 ± 0.04 nM, which was quite similar to that obtained in the kinetic studies (0.60 ± 0.07 nM) and a B_{max} value of 403 ± 32 fmol mg^{-1} protein. In parallel experiments using the same membrane homogenates, [^{3}H]-5-HT saturation analysis (Figure 3, nH = 0.97 ± 0.03, K_d 1.26 ± 0.05 nM), while generating significantly greater non-specific binding, yielded roughly an equivalent number of binding sites (B_{max} = 362 ± 20 fmol mg^{-1} protein) as obtained with [^{3}H]-5-CT.

When unlabelled 5-CT competed for [^{3}H]-5-HT binding in guinea-pig striatal membrane homogenates (Figure 4), a region which possesses both 5-HT$_{1D}$ and 5-HT$_{1E}$ receptors

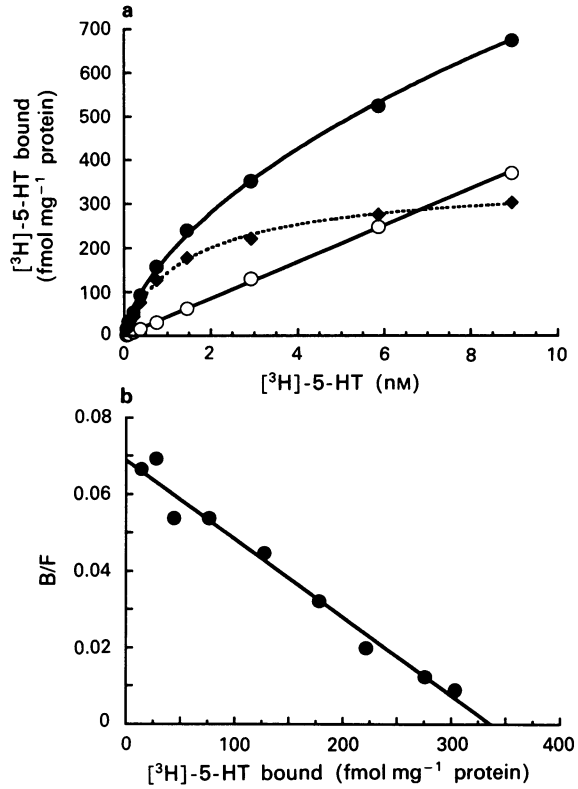


Figure 3 Representative saturation analysis (a, total binding (●); non-specific binding (○) and specific binding (◆)) and Scatchard transformation (b) of [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) binding in bovine substantia nigra membrane homogenates. Non-specific binding was defined by 10 μM 5-HT. Data points represent a single experiment performed in duplicate. Values for three separate experiments are described in the text. Non-linear regression analysis of the illustrated experiment yielded the following response parameters: K_d = 1.29 nM, B_{max} = 338 fmol mg^{-1} protein, and nH = 1.02.

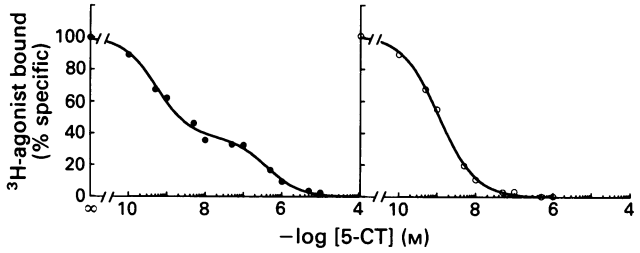


Figure 4 Representative displacement curves of 0.5 nM [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) (●), and [^{3}H]-5-carboxamidotryptamine ([^{3}H]-5-CT) (○) binding by varying concentrations of unlabelled 5-CT in guinea-pig striatal membrane homogenates. Data points represent duplicate determinations with a range of less than 5%. Each experiment was performed an additional two times yielding similar results. Data were analysed by non-linear regression analysis as described in the Methods section, where 5-CT produced a biphasic displacement of [^{3}H]-5-HT: K_H, 0.36 nM (64%) and K_L, 215 nM (36%) and monophasic displacement of [^{3}H]-5-CT: K_i 0.4 nM.

(Beer *et al.*, 1992), a biphasic displacement curve was obtained (slope index 0.36 ± 0.04), which could be separated into a high (K_i = 0.37 ± 0.01 nM, 64%) and a low (K_i = 190 ± 36 nM) affinity component ($P \leq 0.01$). However, unlabelled 5-CT competed for [^{3}H]-5-CT in a monophasic manner (slope index not significantly different from unity) producing a single affinity constant (K_i = 0.68 ± 0.04 nM, slope index 0.91 ± 0.02).

Composite competition curves (n = 3) for both [^{3}H]-5-HT and [^{3}H]-5-CT binding (under 5-HT$_{1D}$ conditions) in bovine substantia nigra membrane homogenates are displayed in Figure 5a and b. With the exception of methiothepin, all compounds produced monophasic displacement ($P \leq 0.01$) of both [^{3}H]-5-HT and [^{3}H]-5-CT binding. Methiothepin inhibition of both [^{3}H]-5-HT and [^{3}H]-5-CT binding produced shallow displacement curves (slope index approximately 0.6), which could be separated into two (nanomolar and micromolar) components. As responses mediated through 5-HT$_1$-like receptors should be antagonized by methiothepin (Bradley *et al.*, 1986), and such responses are antagonized by nanomolar concentrations of methiothepin (Engel *et al.*, 1986), the nanomolar component of agonist displacement was used for the correlation analysis. Table 1 lists the respective K_i values determined by non-linear regression analysis, from both sets of competition curves. A linear correlation (r = 0.99) exists between the affinities of drugs for specific [^{3}H]-5-HT and [^{3}H]-5-CT binding.

A non-hydrolyzable guanine nucleotide (guanosine 5′-β,γ-imidotriphosphate, Gpp(NH)p), caused a concentration dependent attenuation of both [^{3}H]-5-HT and [^{3}H]-5-CT binding in bovine substantia nigra homogenates (Figure 6).

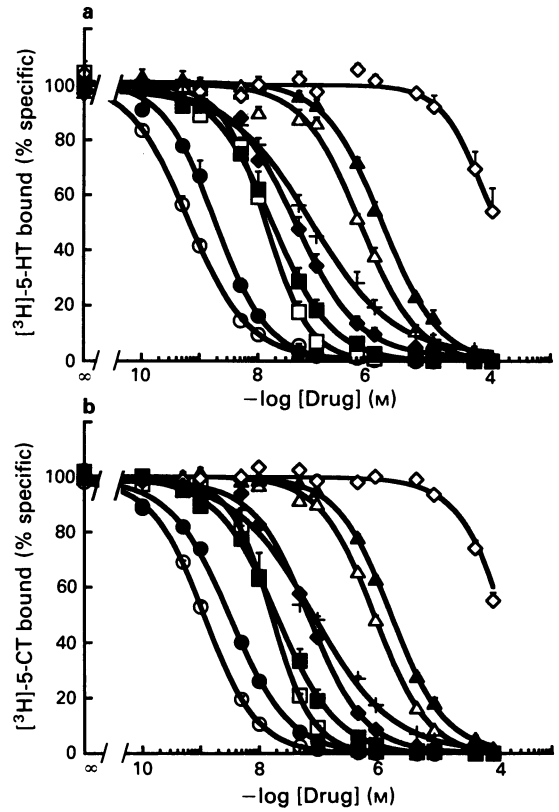


Figure 5 (a) Displacement of 0.5 nM [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) and (b) [^{3}H]-5-carboxamidotryptamine ([^{3}H]-5-CT) binding in bovine substantia nigra membrane homogenates. Competing drugs are denoted by the following symbols: 5-CT (○); 5-HT (●); dihydroergotamine (□); sumatriptan (■); yohimbine (◆); methiothepin (+); DPAT (△); CP-93,129 (▲) and buspirone (◇). Data points are the mean ± s.e. for three separate experiments each performed in duplicate. Data were analysed using non-linear regression analysis as described in the Methods section. Values for the respective concentration-effect curves are illustrated in Table 1.

Table 1 Parameters for the displacement of [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) and [^{3}H]-carboxamidotryptamine ([^{3}H]-5-CT) from 5-HT$_{1D}$ binding sites in bovine substantia nigra membrane homogenates

	[^{3}H]-5-HT		*[^{3}H]-5-CT*	
Drug	K_i (nM)	*Slope index* (nH)	K_i (nM)	*Slope index* (nH)
5-CT	0.59 ± 0.11	0.81 ± 0.06	0.51 ± 0.03	0.97 ± 0.02
5-HT	1.25 ± 0.16	1.04 ± 0.08	1.61 ± 0.11	0.90 ± 0.01
Dihydro-ergotamine	7.56 ± 0.94	1.22 ± 0.05	4.71 ± 0.21	1.17 ± 0.12
Sumatriptan	15 ± 4	0.87 ± 0.02	11 ± 2	0.89 ± 0.04
Yohimbine	34 ± 6	0.85 ± 0.06	31 ± 0.6	0.87 ± 0.02
Methiothepin	22 ± 7 (69%) 1760 ± 1081 (31%)	0.57 ± 0.04	24 ± 4 (78%) 726 ± 29.12 (22%)	0.65 ± 0.02
8-OH-DPAT	406 ± 47	0.92 ± 0.02	401 ± 10	0.98 ± 0.03
CP-93,129	1240 ± 125	0.98 ± 0.14	861 ± 26	0.94 ± 0.09
Buspirone	91649 ± 34436	0.82 ± 0.31	59057 ± 3223	1.17 ± 0.02

Values obtained by non-linear regression analysis (LIGAND).
Mean ± s.e.mean.
$n = 3$ individual repetitions in a common membrane homogenate.

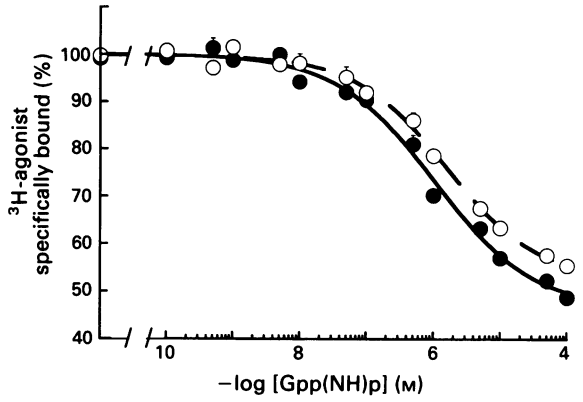


Figure 6 Displacement of 0.5 nM [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) (●) and [^{3}H]-5-carboxamidotryptamine ([^{3}H]-5-CT) (○) binding by varying concentrations of Gpp (NH)p in bovine substantia nigra membrane homogenates. Data points are the mean ± s.e. for three separate experiments each performed in duplicate. Data were fitted by a four parameter logistic equation which generated the following parameters 5-HT and 5-CT, respectively: IC_{50} (nM): 1.02 ± 0.12, 1.40 ± 0.03; E_{max} (%): 53 ± 2, 49 ± 2; and slope index 0.59 ± 0.07, 0.64 ± 0.06.

Gpp(NH)p affected both [^{3}H]-5-HT and [^{3}H]-5-CT binding in a qualitatively and quantitatively similar manner; IC_{50} (nM): 1.02 ± 0.12, 1.40 ± 0.03; E_{max} (%): 53 ± 2, 49 ± 2; and slope index 0.59 ± 0.07, 0.64 ± 0.06, respectively. The adenine nucleotides (adenosine 5′-β,γ-imido)triphosphate, App(NH)p and adenosine 5′-O-(thiotriphosphate, ATPγS) produced a negligible effect on both [^{3}H]-5-HT and [^{3}H]-5-CT binding at a concentration-range from 1 nM to 10 μM (data not shown).

Discussion

Radioligand binding assays have been critical in the elucidation and pharmacological characterization of subtypes of 5-HT receptors in their native tissue. Similar to classical pharmacological studies involving isolated tissue, a prerequisite for the classification of receptor subtypes is selective radiolabelled antagonists. The emerging data suggests that 5-HT receptors can be classified into one of at least four families of receptors, 5-HT$_1$-like, 5-HT$_2$, 5-HT$_3$, or 5-HT$_4$ (Zifa & Fillion, 1992). While the heterogeneity of other 5-HT receptor families is only now being fully appreciated (Kursar *et al.*, 1992), 5-HT$_1$ receptors have long been recognized as a heterogeneous group (Pedigo *et al.*, 1981; Pazos *et al.*, 1985; Heuring & Peroutka, 1987). As there were no selective antagonists and to allow scope for future modification, Bradley *et al.* (1986) deliberately kept the 5-HT$_1$-like receptor classification criteria very broad. 5-HT$_1$-like receptors characteristically possess a high affinity for 5-HT and 5-CT, moderate affinity for methiothepin and methysergide, and low affinity for ketanserin and MDL 72222. However, solely on the basis of radioligand binding techniques in native tissue, with [^{3}H]-5-HT used as the radioligand at least 5 subtypes of 5-HT$_1$ receptors were distinguished: 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1C}$, 5-HT$_{1D}$, and 5-HT$_{1E}$ (Pedigo *et al.*, 1981; Hoyer *et al.*, 1985; Pazos *et al.*, 1985; Heuring & Peroutka, 1987; Leonhardt *et al.*, 1989), which increases the complexity of binding curves and data analysis.

The ability to mask selectively 5-HT$_{1A}$ and 5-HT$_{1C}$ receptors with 8-OH-DPAT and mesulergine, respectively (Heuring & Peroutka, 1987), has decreased the complexity of radioligand binding analysis. The hypothesis that 5-HT$_{1B}$ receptors represent the species homologue of the 5-HT$_{1D}$ receptor (Adham *et al.*, 1992), has also made interpretation of heterogeneous binding curves in porcine and bovine brain considerably less complex. Nonetheless, several compounds, namely 5-CT, ergotamine, and sumatriptan compete for [^{3}H]-5-HT in a biphasic manner generating a high affinity 5-HT$_{1D}$ component and a low affinity 5-HT$_{1E}$ component (Leonhardt *et al.*, 1989; Weisberg & Teitler, 1992; Beer *et al.*, 1992).

Molecular cloning has substantiated in human brain what radioligand binding first predicted in bovine brain, with the cloning and sequencing of genes encoding a 5-HT$_{1D}$ receptor (Hamblin & Metcalf, 1991) and a 5-HT$_{1E}$ receptor (McAllister *et al.*, 1992). Also as predicted on the basis of radioligand binding assays performed in brain membrane homogenates from numerous mammalian species (Leonhardt *et al.*, 1989; Beer *et al.*, 1992), the human 5-HT$_{1E}$ receptor as opposed to the 5-HT$_{1D}$ receptor, has been shown to have a low affinity for 5-CT, ergotamine and sumatriptan. Recently, based upon sequence homology, another new member of the 5-HT$_1$ receptor family has been described (Adham *et al.*, 1993). While this receptor bears the greatest sequence homology with the 5-HT$_{1E}$ receptor, it also shares the pharmacological property of displaying low affinity for 5-CT (approximately 1 μM). Thus this site would not be radiolabelled by low concentrations of [^{3}H]-5-CT. Therefore, 5-CT in the presence of 8-OH-DPAT and mesulergine should selectively bind to 5-HT$_{1D}$ receptor subtypes. Thus in guinea-pig striatum (Figure 4), 5-CT displaces [^{3}H]-5-HT in a biphasic

manner demonstrating a high (5-HT_{1D}) and a low (5-HT_{1E}) affinity component. However, a similar displacement of [^{3}H]-5-CT yielded a monophasic 5-HT_{1D} component.

On this supposition, we have tritiated 5-CT to obtain a high affinity radioligand which would be useful in evaluation of the multiplicity of 5-HT_{1D}-like receptors. We have confirmed our hypothesis (Mahle *et al.*, 1991), in that the radioligand [^{3}H]-5-CT was found to label multiple high affinity 5-HT_{1D}-like binding sites in guinea-pig brain which occurred in a regionally disproportionate manner. However, to validate this compound as a useful radioligand, necessitated conducting parallel studies with [^{3}H]-5-HT in a region where the density of 5-HT_{1E} binding sites would make a negligible contribution. Therefore, bovine substantia nigra was chosen, because in this region 5-CT competed for [^{3}H]-5-HT in a monophasic manner (Figure 5b), suggesting a relative lack of 5-HT_{1E} receptors, as compared to that described in guinea-pig striatum (Figure 4). Another factor supporting the relative homogeneity of bovine substantia nigra is that [^{3}H]-5-HT and [^{3}H]-5-CT also label a nearly equivalent density of binding sites. If an appreciable density of 5-HT_{1E} sites had been present, one would expect the B_{max} observed with [^{3}H]-5-HT to be significantly higher. This was not the case. However, the density observed with [^{3}H]-5-CT was slightly higher (approximately 10–15%), which may indicate the existence of sites which display a greater affinity for 5-CT than for 5-HT (Plassat *et al.*, 1992).

Yet, pharmacologically the binding sites labelled by [^{3}H]-5-HT appeared similar to those labelled by [^{3}H]-5-CT. A series of nine compounds displayed similar affinities regardless of the radioligand used. While typically, antagonists cause monophasic displacement of radiolabelled agonists (De Lean *et al.*, 1980), methiothepin, a 5-HT_1-like antagonist (Bradley *et al.*, 1986), displaced both radioligands in a shallow manner, suggesting a complexity in binding. This however cannot be explained by a heterogeneity of 5-HT_{1D}-like binding sites in bovine substantia nigra, as methiothepin displacement of [^{3}H]-5-CT from the 5-$HT_{1D\alpha}$ receptor expressed in COS cells also generated a shallow slope index (data not shown). It is also highly unlikely, due to the low concentration of agonist radioligand used, that a slight partial agonism of methiothepin is uncovering multiple affinity states of the 5-HT_{1D} receptor. All other agonist compounds competed in a monophasic manner, also suggesting a homogeneity of binding sites and affinity states in bovine substantia nigra.

In conclusion, when 5-CT competed for [^{3}H]-5-HT in guinea-pig striatal membrane homogenates, a biphasic displacement was observed, which consisted of both a 5-HT_{1D} and 5-HT_{1E} component. The low affinity 5-HT_{1E} component was not observed however, when [^{3}H]-5-CT was used as the radioligand, suggesting that [^{3}H]-5-CT (in the presence of 8-OH-DPAT and mesulergine) selectively labels 5-HT_{1D}-like binding sites. Therefore, the high affinity and low non-specific binding coupled with the lack of affinity for 5-HT_{1E} binding sites make [^{3}H]-5-CT a superior radioligand for 5-HT_1-like binding assays. This compound will be useful in examining the heterogeneity of 5-HT_{1D}-like binding sites and will help in the pharmacological evaluation of novel members of the 5-HT receptor family which possess a greater affinity for 5-CT than for the endogenous ligand, 5-HT.

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(Received February 16, 1993
Revised April 2, 1993
Accepted April 5, 1993)

Br. J. Pharmacol. (1993), **109**, 1212–1218 © Macmillan Press Ltd, 1993

Evaluation of ICI D7114, a putative stimulant of brown adipocytes, on histamine-contracted guinea-pig ileum

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1 Experiments were performed to characterize the effects of the novel brown adipocyte stimulant, ICI D7114, in the guinea-pig isolated ileum, right atrium and tracheal chain. In the ileum, agonist-induced inhibition of the contractile response to either histamine or prostaglandin E_2 (PGE_2) was assessed, along with effects on resting rate in the atrium and resting tone in the tracheal chain. In the latter two preparations, antagonism of isoprenaline-induced responses by ICI D7114 was also assessed.

2 Inhibitory responses were obtained in the ileum to ICI D7114, isoprenaline, BRL37344, and noradrenaline. The responses to ICI D7114, isoprenaline and BRL37344 were resistant to blockade with propranolol (5 μM), naloxone (1 μM), methysergide (0.1 μM), cimetidine, indomethacin and 8-phenyltheophylline (all 10 μM). These responses to isoprenaline, in the presence of propranolol (5 μM), were competitively antagonized by alprenolol (1–100 μM) with a pA_2 value of 6.44. The responses to ICI D7114 and BRL37344 were antagonised by single concentrations of alprenolol (1 μM) with apparent pK_B values of 6.53 and 6.57 respectively. These data indicate an effect of ICI D7114 at the atypical β-adrenoceptor in the guinea-pig ileum.

3 The order and relative potency of agonists at the atypical β-adrenoceptor was BRL37344 (4) > isoprenaline (1) = ICI D7114 (1.1) > noradrenaline (0.5).

4 ICI D7114 (1 nM–10 μM) caused no significant change in the rate of beating or the resting tone of the guinea-pig right atrium or tracheal chain respectively. It did, however, cause selective blockade of the responses to isoprenaline in these tissues (apparent pK_B values 7.63 and 5.85 in atrium and tracheal chain respectively). Responses to histamine (atrium) and aminophylline (tracheal chain) were not significantly affected by 10 μM ICI D7114.

5 These results demonstrate that ICI D7114 possesses selective agonist activity at atypical β-adrenoceptors in the guinea-pig ileum and its use as a tool may help to establish a role for the atypical β-adrenoceptor in the control of gastrointestinal motility.

Keywords: Guinea-pig ileum pre-contracted; inhibitory action of ICI D7114; atypical β-adrenoceptors; antagonist action of alprenolol

Introduction

It was suggested by Arch *et al.* (1984) that stimulation of lipolysis following activation of brown adipocytes was the result of an interaction of noradrenaline with an atypical β-adrenoceptor. The authors demonstrated that a series of compounds possessed high potency at this atypical β-adrenoceptor. In particular, BRL37344, (sodium-4-[2-[2-hydroxy-2-(3-chlorophenyl) ethylaminopropyl] phenoxyacetate sesquihydrate) was shown to stimulate lipolysis at much lower concentrations than those required to affect atrial rate or tracheal tone. Since these observations were made, there has been speculation as to the possible existence of a similar atypical β-adrenoceptor that might be involved in the control of gut motility. Bond & Clarke (1987, 1988) identified a propranolol-resistant component to the isoprenaline-induced inhibitory response in electrically stimulated or histamine pre-contracted guinea-pig ileum and further demonstrated that BRL37344 possessed similar potent inhibitory effects. In a more recent study (Blue *et al.*, 1990), the potent non-selective β-adrenoceptor antagonists (−)-alprenolol and (−)-dihydroalprenolol, were shown to produce competitive antagonism of the responses to isoprenaline and BRL37344. However, the affinity constants for these agents were significantly lower than would have been predicted for an interaction at either β_1- or β_2-adrenoceptors, hence it was suggested that an atypical β-adrenoceptor might be responsible for the effects observed. Similar observations have been made in a variety of tissues where responses to catecholamines appear to be resistant to α- or β-adrenoceptor blockade. These tissues include rat stomach fundus (Kelly & MacDonald, 1990), distal colon (McLaughlin & MacDonald, 1990), small intestine (van der Vliet *et al.*, 1990) and rabbit jejunum (Norman & Leathard, 1990). Moreover, Bianchetti & Manara (1990) have recently developed a series of phenylethanolaminotetralines that show a high degree of selectivity for the atypical β-adrenoceptor in rat colon.

It also now appears that atypical β-adrenoceptors may be associated with neuronally-released transmitter. Taneja & Clarke (1991) have recently demonstrated that in electrically stimulated guinea-pig ileum, the neuronally released natural transmitter of the sympathetic nerves, noradrenaline, may, in addition to its actions on classical adrenoceptors, also act at atypical β-adrenoceptors. There is a possibility, therefore, that atypical β-adrenoceptors participate in the physiological mechanisms associated with the control of gut motility.

Hence, there appears to be considerable evidence to support the existence of the atypical β-adrenoceptor in gastrointestinal tissue and that this site appears to bear some resemblance to that already identified in brown adipocytes. ICI D7114 ((S)-4-[2-hydroxy-3-phenoxy-propylamino-ethoxy]-*N*-(2-methoxyethyl) phenoxyacetamide) is a recently developed β-adrenoceptor agonist that stimulates brown adipocytes via an atypical β-adrenoceptor (Holloway *et al.*, 1991a,b). It was of interest, therefore, to evaluate the action of this agent in an isolated tissue system where atypical β-adrenoceptors have been postulated and compare this to any effect at β_1- or β_2-adrenoceptors. The current study describes the pharmacological action of ICI D7114 in the histamine pre-contracted guinea-pig ileum, right atrium and tracheal chain prepara-

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tions. A preliminary account of some of this work was presented to the British Pharmacological Society in Glasgow, July, 1991.

Methods

Tissue preparation

Guinea-pig ileum Guinea-pigs of either sex (Dunkin-Hartley strain, weighing 300–550 g) were killed by cervical dislocation and the distal ileum was removed. After washing out the luminal contents, segments 2–3 cm in length were set up in 10 ml organ baths under an initial resting tension of 500 mg in Krebs solution maintained at 37°C and gassed with 95% O_2, 5% CO_2. The composition (mM) of the Krebs solution was as follows: NaCl 118.9, $NaHCO_3$ 25.0, KCl 4.9, KH_2PO_4 111.2, $MgSO_4$ 1.2, glucose 11.1, $CaCl_2$ 2.5 and ascorbic acid 0.11. Atropine (0.1 μM), phentolamine (3 μM), cocaine (30 μM) and corticosterone (30 μM) were present throughout the experiments to inhibit muscarinic receptors, α-adrenoceptors, neuronal and extraneuronal uptake respectively. The concentrations of phentolamine and atropine were at least 100 times their equilibrium dissociation constants (Bond & Clarke, 1988). In experiments where the effects of compounds at the atypical β-adrenoceptor were assessed, propranolol (5 μM) was present. This concentration of propranolol was used as it had been previously demonstrated (a) to have no effect on smooth muscle tone (Bond & Clarke, 1988) and (b) to 'saturate' β_1- and β_2-adrenoceptors (Bond & Clarke, 1988). Hence, this concentration of propranolol should effectively block any activity of the agonists at classical β-adrenoceptors. In some experiments, the selective β_1- and β_2-adrenoceptor antagonists, CGP 20712A ((±)-1-[2 (3-carbomyl-4-hydroxyphenoxy)-ethylamino]-3-[4-(1-methyl-4-triflouromethyl-2-imidazolyl)-phenoxy]-2-propanol) and ICI 118551 (erythro- (±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol) were used (both 0.1 μM). The antagonism of isoprenaline with these two agents was compared to that obtained with propranolol (5 μM). Tissues were allowed to equilibrate for 30 min before starting experiments. Isotonic recording of tissue length changes were made (Mellor, 1984) and displayed on a Kipp and Zonen BD9 recorder.

Experiments using electrically stimulated guinea-pig ileum were also performed in order to determine the specificity of alprenolol. Ileum was obtained as outlined above and segments 2–3 cm in length were set up between stainless steel ring electrodes in 10 ml organ baths containing Mg^{2+}-free Krebs solution at 37°C and gassed with 95% O_2, 5% CO_2. Tissues were left to equilibrate under 500 mg tension for at least 30 min prior to field-stimulation (supramaximal square wave pulses, 1 ms in duration, delivered at a frequency of 0.1 Hz from a Grass S44 stimulator) to evoke acetylcholine release from enteric neurones. Isotonic recording of tissue length changes were measured as outlined above.

Guinea-pig right atrium Hearts were rapidly removed from the guinea-pigs (sex, strain, weight range as described above) and placed in oxygenated Krebs solution. The right atrium was quickly located, dissected free from the heart and mounted on to a pair of platinum electrodes. Once fixed to the electrodes, the tissue was immersed into a 20 ml organ bath containing Krebs solution (composition as outlined above) at 37°C and gassed with 95% O_2, 5% CO_2. Electrodes were connected to an ICI modular cardiotachometer which counted the number of spontaneous beats in 10 s epochs. A computer (IBM PS200 or equivalent) averaged the epochs in groups of 3 and displayed these on the computer screen at 1 min intervals. Tissues were allowed to equilibrate for 60 min with frequent washings with fresh Krebs solution. During this time, the spontaneous rate fell and became stable.

Guinea-pig tracheal chain Tracheae were removed from guinea-pigs (sex, strain, weight range as described above) and placed into oxygenated Krebs solution. The tissues were then cut into rings. Each preparation consisted of five rings sewn together. These were placed in 10 ml organ baths containing Krebs solution (composition as outlined above) at 37°C and gassed with 95% O_2, 5% CO_2. The tissues were left to equilibrate under 200 mg tension for 60 min during which time frequent rinses with fresh Krebs solution were made. Isotonic recording of tissue length changes were made as outlined above.

Experimental protocols

Guinea-pig ileum Inhibitory actions of the β-adrenoceptor agonists were determined by measuring the reduction in the contractile response to a submaximal concentration of histamine (0.5 μM, see Bond & Clarke, 1988). Thus, responses to histamine were obtained at 5 min intervals with 5 washouts between each application. When a suitable reproducible histamine response was obtained, this was set as 100% and all subsequent responses were compared as a percentage change. Increasing concentrations of β-adrenoceptor agonist were added in a sequential manner 3 min prior to the addition of histamine and retained in the organ bath during the response to histamine. In experiments where antagonist drugs were used, these were added to the reservoir of Krebs and allowed 30 min to equilibrate with the tissue prior to addition of histamine. It was found that when alprenolol was employed as an antagonist, responses to histamine were significantly inhibited by the presence of this agent. In this case, a submaximal concentration of PGE_2 (50 ng ml^{-1}, approx. 0.1 nM) was used instead of histamine.

Concentration-response curves were plotted for the β-adrenoceptor agonists and potency was expressed as an IC_{30} value. This represents the concentration of agonist causing 30% inhibition of the response to histamine. Values are expressed as the mean together with the respective 95% confidence limits. IC_{30} were used to allow direct comparison between this study and those previously performed by other workers using guinea-pig ileum (i.e. Bond & Clarke, 1988).

Some experiments using guinea-pig ileum were performed with electrically-stimulated preparations. These tissues were stimulated to cause release of acetylcholine from enteric neurones, resulting in a 'twitch' response. Following 30 min equilibration, during which time twitch responses became stable, morphine (1 nM–10 μM) was added cumulatively and the inhibition of the twitch response measured. Following a washout period of 30 min stimulation was continued and alprenolol (10 μM) was added to the bath for 10 min after which time, the inhibition-response to morphine was repeated.

Concentration-inhibition curves were plotted for the effects of morphine in the absence and presence of alprenolol. From these curves, IC_{50} values were derived, i.e. the concentration of morphine causing 50% inhibition, and expressed as mean values together with their respective 95% confidence limits.

Guinea-pig right atrium Spontaneous rate of beating of the right atrium was increased by adding either isoprenaline or histamine in a cumulative fashion until no further change in rate was observed. The tissues were then washed several times and left for 30 min for rates to return to normal. ICI D7114 (10 nM–10 μM) was then added to the bathing Krebs solution and left in contact with the tissues for 60 min after which time the concentration-responses to either isoprenaline or histamine were repeated. From the concentration-response curves, EC_{50} values, i.e. the concentration of agonist producing an increase in rate that was 50% of the maximum response, were derived.

Guinea-pig tracheal chain Spontaneous tone of the tracheal chain was reduced by cumulative addition of either iso-

prenaline or aminophylline until no further reduction could be obtained. The tissues were then washed several times until tone had returned; 60 min later, a second concentration response to either isoprenaline or aminophylline was repeated and the tissues were once again washed. When tone had returned, ICI D7114 (1 nM–10 μM) was added to the Krebs bathing medium and left to equilibrate for 60 min after which time, concentration-responses to isoprenaline or aminophylline were repeated. From the concentration-response curves, EC_{50} values were derived.

pA_2 and pK_B values

Agonist concentration-ratios obtained from individual experiments were plotted by the method of Arunlakshana & Schild (1959). Linear regression analysis was used to calculate the pA_2 value and slope of the line. Antagonism was considered to be competitive if the slope of the regression line did not differ significantly from unity. In cases where a single concentration of antagonist was used, 'apparent' pK_B values were derived by the concentration-ratio method of Furchgott (1972), where:

$$K_B = [\text{antagonist}]/\text{agonist concn. ratio} - 1$$

and following on from this:

$$-\log_{10} K_B = pK_B$$

Statistics

Significance levels, where quoted, were determined by Student's paired *t* test after checking the homogeneity of the variances. *P* values of less than 0.05 were considered to indicate a significant difference between the responses being compared.

Drugs

The following drugs were used: histamine dihydrochloride, (−)-isoprenaline hydrochloride, (−)-noradrenaline bitartarate, atropine sulphate, 8-phenyltheophylline, corticosterone-21-acetate, prostaglandin E_2 (all purchased from Sigma Chemical Co.). CGP 20712A, phentolamine mesylate, cocaine hydrochloride (purchased from CIBA-Geigy Pharmaceuticals), methysergide bimaleate (purchased from Sandoz Pharmaceuticals), (±)-propranolol, (±)-alprenolol, (Zeneca Pharmaceuticals), ICI D7114 and BRL37344 (synthesized by B. Rao, Research Function, Zeneca Pharmaceuticals, Alderley Park, Macclesfield). All other chemicals were of analytical grade from either BDH Ltd., Poole, Dorset or Fisons Ltd., Loughborough, Leicester. ICI D7114, BRL37344, methysergide, corticosterone, (±)-alprenolol and 8-phenytheophylline were initially dissolved in dimethyl sulphoxide (DMSO) and subsequently diluted in distilled water. All other agents were dissolved in distilled water.

Results

Guinea-pig ileum

One of the characteristics that helps identify the presence of atypical β-adrenoceptors is the low antagonist potency of propranolol against β-adrenoceptor agonists. In the current studies, the effects of propranolol (5 μM) on inhibitory responses to isoprenaline, BRL37344 and ICI D7114 were evaluated. In the case of isoprenaline, the effects of propranolol were compared with those obtained with CGP 20712A and ICI 118551 (both 0.1 μM), alone or in combination. The effects of propranolol are shown in Figure 1 (a–c). The mean concentration-ratios were 5.7 [2.2–9.0], 12.9 [1.1–32.7] and 1.6 [1.0–2.3] (all $n = 4$) against isoprenaline, BRL37344 and ICI D7114 respectively. These values equate

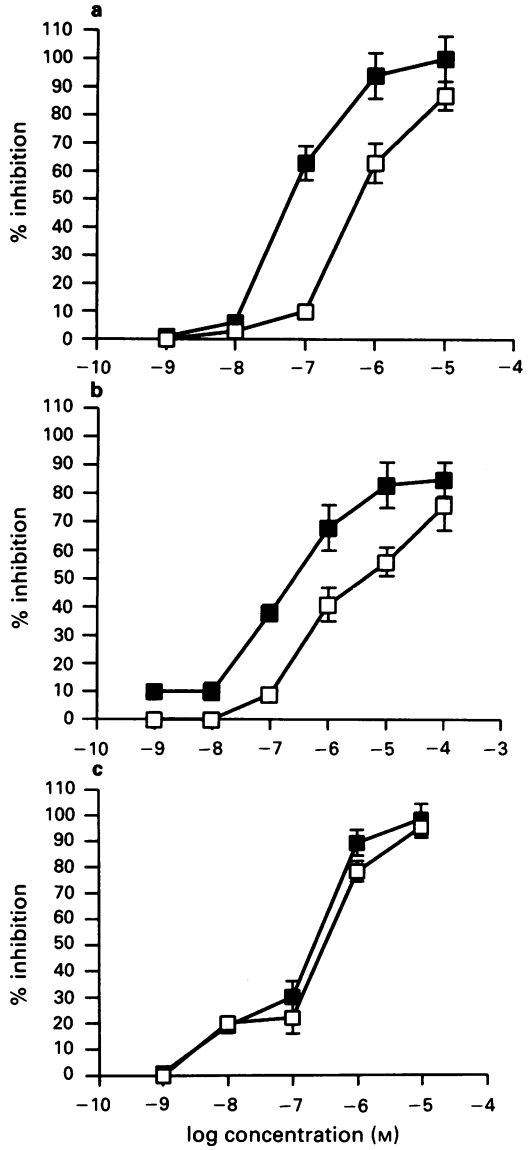


Figure 1 Inhibitory effects of (a) isoprenaline, (b) BRL37344 and (c) ICI D7114 on the contractile responses to histamine (0.5 μM) in guinea-pig isolated ileum: interaction with propranolol (5 μM). (■) Control responses; (□) responses in the presence of propranolol. Each point represents the mean ± s.e.mean percentage (where this exceeds the symbol size) inhibition obtained from tissues from 4 guinea-pigs.

to 'apparent' pK_B values of 5.97 [5.38–6.20], 6.37 [4.30–6.80] and 5.07 [4.3–5.4] respectively. In the presence of CGP 20712A or ICI 118551 alone, small rightward shifts of the isoprenaline concentration-responses were seen (concentration-ratios 2.2 [1.5–3.0] and 4.6 [1.8–11.4] respectively, 'apparent' pK_B values of 7.08 [6.69–7.30] and 7.56 [6.90–8.02], all $n = 4$). When these two antagonists were used in combination, again, small shifts in the concentration-response curves to isoprenaline were observed (concentration-ratio 8.1 [0.7–21.2], 'apparent' pK_B value of 7.85 [6.00–8.3], $n = 4$). The low values obtained with propranolol and either CGP 20712A or ICI 118551 indicate a lack of involvement of either β_1- and β_2-adrenoceptors. In the subsequent studies, propranolol (5 μM) was employed throughout.

Isoprenaline (1 nM–1 mM), BRL37344 (1 nM–1 mM), ICI D7114 (1 nM–1 mM) and noradrenaline (1 nM–1 mM) all produced concentration-related inhibition of the histamine response in the guinea-pig ileum (Figure 2). Isoprenaline and ICI D7114 were approximately equipotent (IC_{30} values 0.20 μM [0.16–0.28 μM] and 0.22 μM [0.04–0.40 μM], $n = 30$

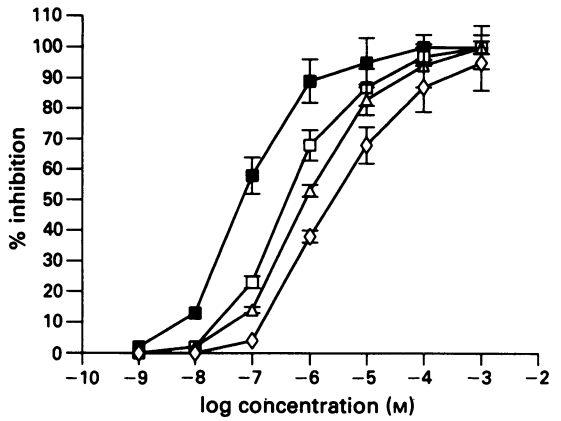


Figure 2 Inhibitory effects of BRL37344 (■, $n = 30$), isoprenaline (□, $n = 30$), ICI D7114 (△, $n = 30$) and noradrenaline (◇, $n = 8$) on the contractile responses to histamine (0.5 μM) in the guinea-pig isolated ileum. Each point represents the mean ± s.e.mean (where this exceeds the symbol size) percentage inhibition and the number of guinea-pigs is given as n.

and 8 respectively). BRL37344 was significantly ($P < 0.01$) more potent than either isoprenaline or ICI D7114 (IC_{30} value 0.052 μM [0.02–0.10 μM], $n = 30$). Noradrenaline (IC_{30} value 0.41 μM [0.16–0.50 μM], $n = 6$) was one-half as potent as isoprenaline. All agonists produced similar maximum responses.

Moreover, the inhibitory responses to isoprenaline, ICI D7114 and BRL37344 were insensitive to the antagonist effects of methysergide (0.1 μM), cimetidine (10 μM), naloxone (1 μM), indomethacin (10 μM) and 8-phenytheophylline (10 μM). However, the inhibitory responses to BRL37344 were significantly ($P < 0.05$) potentiated in the presence of this latter agent (concentration-ratio 0.2 [0.03–0.6]).

The non-selective β-adrenoceptor antagonist (±)-alprenolol, at concentrations above 1 μM, caused a significant inhibition of the histamine-induced contraction of the guinea-pig ileum (data not shown). When PGE_2 was used to contract the tissues, this inhibitory effect was not apparent. Under these latter conditions, alprenolol produced competitive antagonism of the inhibitory responses to isoprenaline (Figure 3a). The Schild plot (Figure 3b) of these data gave a pA_2 value of 6.44 [6.25–6.64, $n = 12$] with a slope (1.10 [0.73–1.45]) not significantly different from unity. Single concentrations of alprenolol (1 μM) also caused antagonism of the inhibitory responses to both BRL37344 (Figure 4a) and ICI D7114 (Figure 4b) in histamine contracted tissues ('apparent' pK_B values 6.57 [6.01–7.42, $n = 4$] and 6.53 [6.26–6.72, $n = 4$] respectively). These values were not significantly different ($P > 0.05$) from those obtained with alprenolol versus isoprenaline in tissues where PGE_2 instead of histamine was used as the contractile agent.

To check on the specificity of alprenolol as a β-adrenoceptor antagonist, its effects were also assessed in the field stimulated guinea-pig ileum against morphine-induced inhibition of twitch. Thus, 10 μM alprenolol caused some inhibition of the twitch response in its own right (40 ± 10%), but was without significant ($P > 0.05$) antagonist effect on the morphine-induced inhibitory effect (Figure 5). In fact, the concentration-response curve to morphine was shifted to the left, although this was not statistically significant ($P > 0.05$, $n = 4$).

Guinea-pig right atrium and tracheal chain

In the guinea-pig right atrium, ICI D7114 (1 nM–10 μM) produced no significant changes in the resting rates of the tissues (0, 13 and 11% increase at 0.001, 0.1 and 10 μM, $n = 4$ in all cases). At 1 nM, no significant antagonist activity was observed, whereas at 0.1 μM and 1 μM, ICI D7114 produced

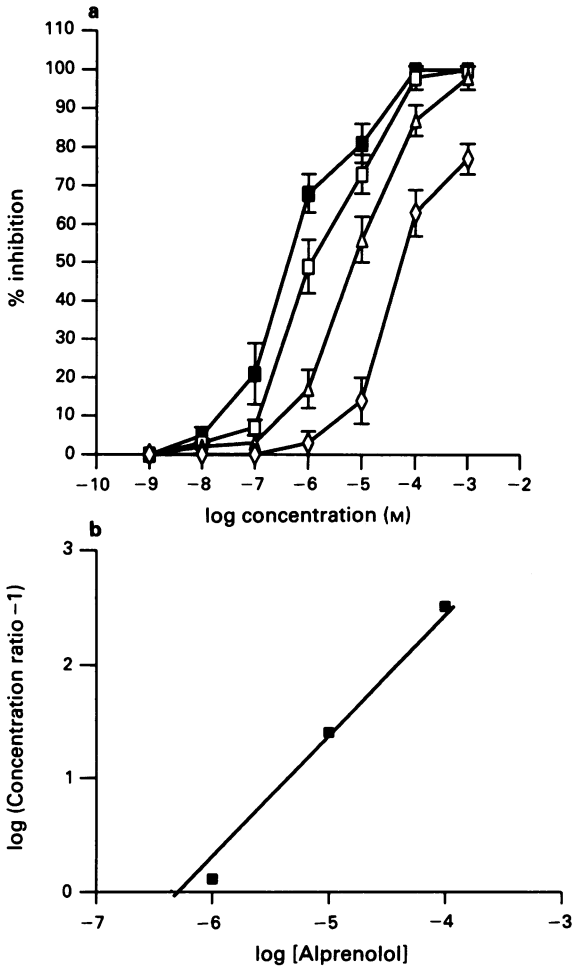


Figure 3 (a) Inhibitory effects of isoprenaline on the contractile response to prostaglandin E_2 (50 ng ml^{-1}) in the guinea-pig isolated ileum: interaction with alprenolol 1 μM–100 μM. (■) Control responses ($n = 12$); responses in the presence of 1 μM (□, $n = 4$), 10 μM (△, $n = 4$) and 100 μM (◇, $n = 4$) alprenolol. Each point represents the mean ± s.e.mean (where this exceeds the symbol size) percentage inhibition and the number of guinea-pigs is given as n. (b) Arunlakshana & Schild (1959) plot for alprenolol versus isoprenaline. Concentration-ratios were measured from the IC_{30} values obtained from the curve shown in (a).

some degree of β_1-adrenoceptor antagonism (concentration-ratios 5.8 [4.0–7.3] and 397.3 [132.0–875.0] respectively). These values equate to 'apparent' pK_B values of 7.68 [7.48–7.79] and 7.59 [7.12–7.94] respectively. Some depression in the maximum response obtained to isprenaline was also seen at these higher concentrations of ICI D7114 (Figure 6a). The effect of ICI D7114 in the guinea-pig right atrium appeared to be specific for β_1-adrenoceptors as high concentrations of the compound (10 μM) failed to antagonize significantly the effects of histamine in this preparation (concentration-ratio 2.28 [1.52–3.75], $n = 4$). In the tracheal chain preparation (Figure 6b), no reduction in resting tone was seen with ICI D7114 at either 1 nM or 0.1 μM. At 10 μM, however, ICI D7114 produced a small and gradual reduction in tone (15% of the isoprenaline maximum response). Moreover, at this high concentration, ICI D7114 produced weak antagonism of the isoprenaline-induced relaxation (concentration ratio 8.07 [6.92–9.29]. This value equates to an 'apparent' pK_B of 5.85 [5.77–5.92]. The weak effect of ICI D7114 in the trachea appeared to be specific for the β-adrenoceptor as the compound (10 μM) failed to antagonize significantly the relaxant responses to aminophylline in this preparation. There was, however, a small but statistically insignificant leftward shift in the aminophylline concentration-response curve (concentration-ratio 0.43 [0.33–0.56]).

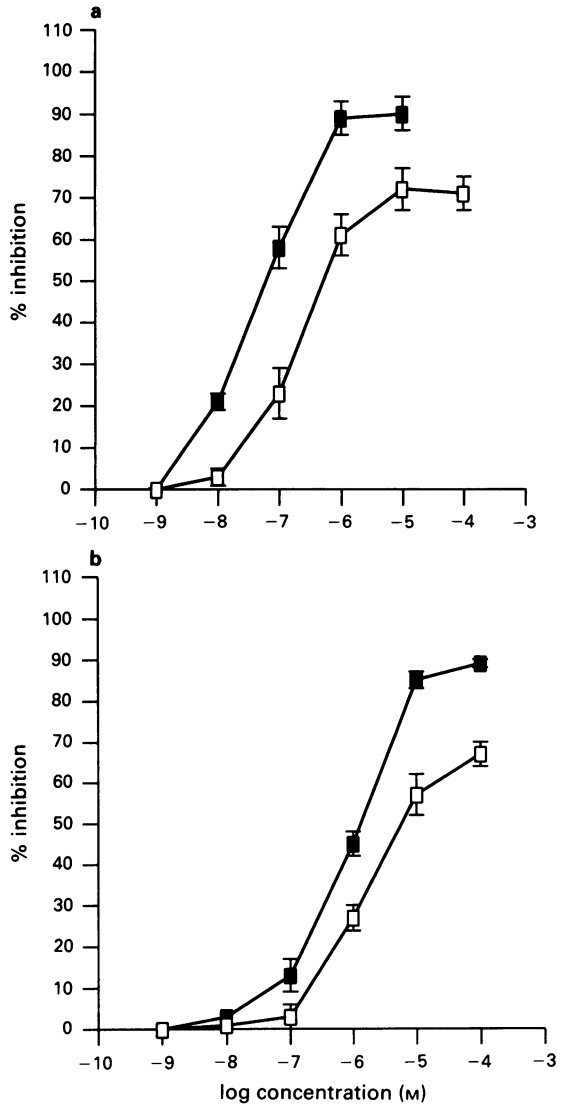


Figure 4 Inhibitory effects of (a) BRL37344 and (b) ICI D7114 on the contractile response to histamine (0.5 μM) in the guinea-pig isolated ileum: interaction with alprenolol (1 μM). (■) Control responses: (□) responses in the presence of alprenolol. Each point represents the mean ± s.e.mean (where this exceeds symbol size) percentage inhibition obtained from tissues from 4 guinea-pigs.

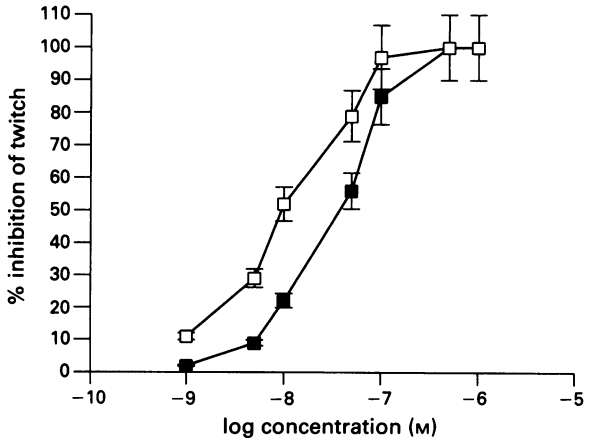


Figure 5 Concentration-responses to morphine in the guinea-pig field-stimulated isolated ileum: interaction with alprenolol. (■) Control concentration-response curve to morphine and (□) concentration-response curve to morphine in the presence of alprenolol (10 μM). Each point represents the mean ± s.e.mean (where this exceeds the symbol size) percentage inhibition obtained in ileum from 4 guinea-pigs.

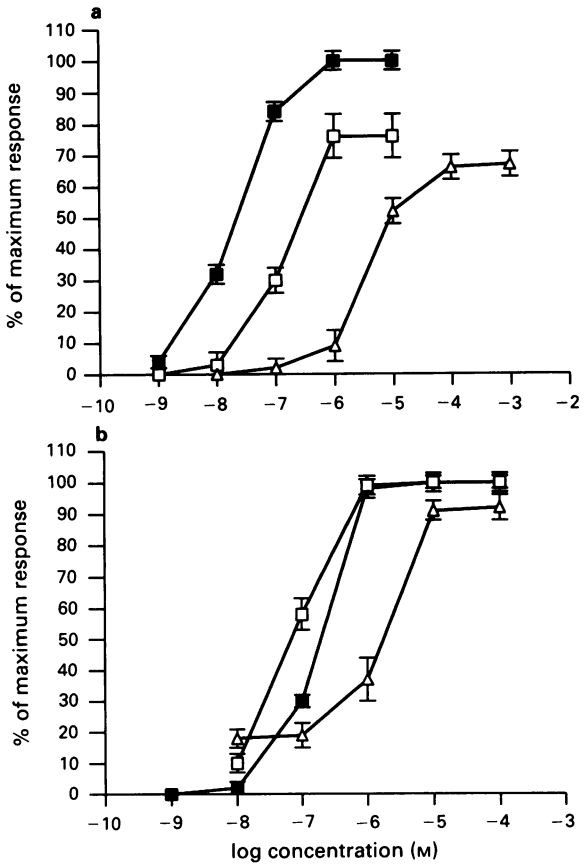


Figure 6 Concentration-responses to isoprenaline in (a) guinea-pig isolated right atria and (b) tracheal chain preparations: interaction with ICI D7114, 0.1 μM and 10 μM. (■) Control responses; responses in the presence of 0.1 μM (□) and 10 μM (△) ICI D7114. Each point represents the mean ± s.e.mean (where this exceeds the symbol size) percentage increase in rate (atria) or decrease in tone (trachea) obtained from 4 guinea-pigs. Effects of ICI D7114 at the lowest concentration used, i.e. 1 nM, have been omitted from the figures for clarity.

Discussion

ICI D7114 has previously been shown to stimulate oxygen consumption potently in conscious rats, cats and dogs and in rat isolated brown adipocytes (Holloway *et al.*, 1991a,b). We have now demonstrated that, in addition to its effect on metabolic parameters, ICI D7114 also causes an inhibition of the response to histamine in the guinea-pig ileum pretreated with a variety of pharmacological agents, including β_1- and β_2-adrenoceptor antagonists. This model has previously been reported to reveal the atypical β-adrenoceptor (Bond & Clarke, 1988). The effect of ICI D7114 in the guinea-pig ileum was similar to that observed with BRL37344, a compound recognised as a potent and selective atypical β-adrenoceptor agonist (Arch *et al.*, 1984), and was only weakly antagonized by (±)-propranolol at concentrations as high as 5 μM. Furthermore, both the isoprenaline and BRL37344-induced inhibition of the histamine responses were also only weakly antagonized by this high concentration of (±)-propranolol. These findings and those that the selective β_1- and β_2-adrenoceptor antagonists (CGP 20712A and ICI 118551) also produced only weak antagonism of the responses to isoprenaline, suggest that conventional β_1- and β_2-adrenoceptors play a minor role in the inhibitory responses produced by isoprenaline, ICI D7114 and BRL37344. These observations are in agreement with the findings of other workers employing the guinea-pig ileum (Bond & Clarke, 1988), rat gastric fundus (McLaughlin & MacDonald, 1991) distal colon (McLaughlin & MacDonald,

1990) and small intestine (van der Vliet *et al.*, 1990), although in this latter preparation, BRL37344 expresses itself as a partial agonist producing a relaxation that was only 60% of that achieved with a maximal concentration of isoprenaline.

In the current study, it was demonstrated that no significant effect of either ICI D7114 or BRL37344 was mediated via α-adrenoceptors, 5-HT_1, 5-HT_2 histamine H_2, opioid, adenosine receptors or via an interaction with prostanoid biosynthesis. It was interesting, however, that the inhibitory responses to BRL37344 were significantly enhanced in the presence of 8-phenyltheophylline. Although the exact reason(s) for this effect are not known, it could be that some degree of phosphodiesterase inhibition was induced by the presence of 8-phenyltheophylline. This, could in turn, lead to an enhancement of relaxation/inhibition. It appears that in the current study BRL37344 was more sensitive to this effect than any of the other β-adrenoceptor agonists employed. In tissues containing β_1- and β_2-adrenoceptors (guinea-pig right atrium and tracheal chain, respectively), ICI D7114 produced only weak agonist activity. BRL37344, on the other hand, has been shown to possess significant agonist activity at both β_1- and β_2-adrenoceptors. Concentrations of 0.68 μM and 0.04 μM caused half-maximal responses in guinea-pig right atrium and trachea respectively (Arch *et al.*, 1984). As ICI D7114 possessed little intrinsic activity in either guinea-pig right atrium (a tissue containing predominantly β-adrenoceptors) or the tracheal chain (a tissue containing predominantly β_2-adrenoceptors), it was checked for its ability to produce antagonism of isoprenaline at these sites. The data revealed β_1- and weak β_2-adrenoceptor antagonist effects to be present and this appeared to be specific for the β-adrenoceptors as no effects were observed with ICI D7114 against either histamine-induced chronotropism or aminophylline-induced relaxation in the atrium and trachea, respectively. Moreover, the effects of ICI D7114 at β_1- and β_2-adrenoceptors have been shown not to be responsible for the action of the compound on brown adipocytes (Holloway *et al.*, 1991a) or its inhibitory effect on the contractile response to histamine in the guinea-pig ileum (current study).

The rank order of agonist potency of isoprenaline, BRL37344 and noradrenaline obtained in the current study is in good agreement with that obtained by other workers (e.g. Bond & Clarke, 1988), from which it may be inferred that similar mechanisms/receptor sites were involved in these determinations in both studies. However, the usefulness of relative agonist potency has it limitations when attempting to classify/categorize the receptor(s) involved. For example, similar (or even identical) receptors can behave quite differently depending on the stimulus-response relationship (Kenakin & Beek, 1980) and if agonists fail to achieve full efficacy, then any comparison is negated. Hence, in tissues where agents behave as partial agonists (BRL37344, rat jejunum, van der Vliet *et al.*, 1990), or have no efficacy whatsoever, (ICI D7114, rat ileum, Growcott *et al.*, 1992; rat colon, MacDonald & Lamont, 1992), then rank orders agonist potency cannot be utilised in the discussion of receptor homogeneity/heterogeneity.

Hence, it is pertinent, whenever possible, to use selective and competitive antagonists to classify receptor sub-types. In an attempt to characterize further the atypical β-adrenoceptor, Blue *et al.* (1990) employed alprenolol or dihydroalprenolol as antagonists. In the current study, alprenolol was used to characterize effects of agonists at the atypical β-adrenoceptor. This agent possessed some degree of 'partial agonist' activity in the guinea-pig ileum, such that at concentrations above 1 μM responses to histamine were almost abolished and the twitch responses in the electrically-stimulated preparation were markedly attenuated. This 'partial agonist' effect has been previously observed in the electrically-stimulated guinea-pig ileum (Blue *et al.*, 1990) and these authors suggest that this latter action of alprenolol might be due to an effect at atypical β-adrenoceptors. These observations are not totally surprising as other pindolol analogues have been demonstrated to possess partial agonist activity in rat adipocytes (Engel *et al.*, 1981). Thus, in the current study, alprenolol produced competitive antagonism of the inhibitory responses to isoprenaline, the Schild analysis revealing a pA_2 value of 6.44. This value is not dissimilar to that reported by Blue *et al.* (1990) where a pA_2 of 6.47 was obtained for (−)-alprenolol against the inhibitory effects of isoprenaline in the electrically stimulated guinea-pig ileum or that obtained by Nials *et al.* (1992) in the guinea-pig gastric fundus ($pA_2 = 6.0$ versus isoprenaline). In the current study, when a single concentration (1 μM) of alprenolol was used to antagonize the inhibitory effects of either ICI D7114 or BRL37344, the calculated 'apparent' pK_B values were 6.53 and 6.57 respectively, which were not significantly ($P > 0.05$) different from the value obtained against isoprenaline. These values are somewhat lower than those obtained for alprenolol at β_1- and β_2-adrenoceptors (pA_2 values = 8.1 and 8.0 respectively, Hoefle *et al.*, 1975) lending support to the notion that a single population of atypical β-adrenoceptors are involved in the inhibitory responses to agents such as ICI D7114. However, there is a possibility that atypical β-adrenoceptors may not be homogeneous. In the rat colon, Bianchetti & Manara (1991) found that alprenolol caused competitive antagonism of the inhibitory responses to isoprenaline (pA_2 7.6) and a novel phenylethanolaminotetraline, SR 5875A (pA_2 7.5). These values are substantially higher than those previously reported for the effect of alprenolol (e.g. Blue *et al.*, 1988) and are closer to the affinity of alprenolol for classical β-adrenoceptors (i.e. pA_2 approx. 8.0). Thus, it could be argued that SR 58375A is acting at both atypical and classical β-adrenoceptors in the study of Bianchetti & Manara (1990) as it is not clear from their findings whether β_1- and β_2-adrenoceptors were blocked when assessment of the antagonist potency of alprenolol was made.

Now that the human atypical β-adrenoceptor has been cloned (Emorine *et al.*, 1989), it was interesting to check for homology between atypical sites identified in animal tissue and those found in human tissue. Already, there appears to be some variance in data. In the human cloned atypical β-adrenoceptor, it has been shown that cyclic AMP production is potently stimulated by BRL37344 (Emorine *et al.*, 1989). However, this same agent has been shown to lack intrinsic activity on human colon (McLaughlin *et al.*, 1991) and adipocytes (Hollenga *et al.*, 1990). It is possible that BRL37344 could therefore act as an antagonist, in the situation where agonist efficacy is low; however, neither McLaughlin *et al.* (1991) nor Hollenga *et al.* (1990) checked for this possibility.

In conclusion, therefore, the present study has indicated that a potent and selective lipolytic agent, ICI D7114, possesses properties similar to BRL37344. The inhibitory effects in the guinea-pig ileum appear to be mediated via an atypical β-adrenoceptor; whether this site is identical to that identified on adipose tissue remains to be resolved. ICI D7114 should provide a valuable tool to probe these sites and help reveal the true nature of the atypical β-adrenoceptor.

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(Received December 9, 1992
Revised March 30, 1993
Accepted April 5, 1993)

Br. J. Pharmacol. (1993), **109**, 1219–1225 © Macmillan Press Ltd, 1993

Effects of N^G-nitro-L-arginine methyl ester on the cardiovascular system of the anaesthetized rabbit and on the cardiovascular response to thyrotropin-releasing hormone

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1 The effects of 300 mg kg^{-1} of the nitric oxide (NO) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) on the regional blood flow, on the flow response to 1 mg kg^{-1} of thyrotropin-releasing hormone (TRH) and on cerebral blood flow autoregulation were studied in urethane anaesthetized rabbits subjected to unilateral sectioning of the cervical sympathetic chain. The blood flow measurements were performed by the tracer microspheres method.

2 The cerebral arteriovenous difference in oxygen saturation (CAVOD) was measured before and after the administration of L-NAME and TRH in order to ascertain whether the effects on cerebral blood flow that were observed were secondary to changes in cerebral metabolism.

3 L-NAME caused a significant decrease in blood flow in several cerebral regions; CBF_{tot} decreased to $72 \pm 4\%$ of control ($P < 0.001$). An increase in blood pressure and a concurrent decrease in heart rate and cardiac output were noted.

4 In the eye, L-NAME caused a reduction in uveal blood flow which was more pronounced on the sympathetically intact side; in the retina the blood flow decreased to 50% of control on both sides.

5 The administration of TRH in animals pretreated with L-NAME caused a significant increase in blood pressure and cerebral blood flow.

6 In L-NAME-treated animals the CBF was not affected when the mean arterial blood pressure was increased by ligation of the abdominal aorta.

7 The CAVOD increased from 56.0 ± 5.2 to $73.6 \pm 3.5\%$, 20 min after the administration of L-NAME. In animals given 1 mg kg^{-1} TRH after L-NAME the CAVOD decreased to $54.6 \pm 4.6\%$, 5 min after the injection of TRH.

8 The results of the present study indicate that endogenous NO is involved in the regulation of regional blood flow and blood pressure in the anaesthetized rabbit. The reduction in cerebral blood flow that was caused by L-NAME was not due to a reduction in cerebral metabolism. An interaction between the NO synthesis/release/effect and the sympathetic nervous system was found in the uvea. There was no evidence for a major involvement of NO in the cardiovascular responses to TRH and autoregulation of cerebral blood flow was not abolished by L-NAME.

Keywords: Blood flow; blood pressure; brain; eye; cardiovascular control; cerebral autoregulation; N^G-nitro-L-arginine methyl ester (L-NAME); nitric oxide; thyrotropin releasing hormone (TRH); rabbit

Introduction

The neuropeptide thyrotropin-releasing hormone (TRH) and its receptors have been found in various sites in the brain, spinal cord and in some peripheral organs (Hökfelt *et al.*, 1975; Leppäluoto *et al.*, 1978). TRH has been found to influence several important physiological functions including those of the autonomic nervous system (Feuerstein *et al.*, 1983; Koskinen, 1986a; Seligsohn & Koskinen, 1991). Complex effects on the cardiovascular system have been demonstrated (Feuerstein *et al.*, 1983; Sirén *et al.*, 1988). In the rabbit, TRH has been found to elicit cerebral vasodilatation by activation of an intrinsic cerebral vasodilating neural pathway (Koskinen, 1986a) which involves a yohimbine-sensitive mechanism (Seligsohn & Koskinen, 1991). In some peripheral organs TRH causes vasoconstriction and in others vasodilatation (Koskinen & Bill, 1984; Hugoson-Seligsohn & Koskinen, 1989; Seligsohn, 1992a). Recently, evidence has been found for the involvement of endogenous TRH in cardiovascular regulation during bleeding (Okuda *et al.*, 1988) and in the regulation of the blood pressure in the spontaneously hypertensive rat (Nurminen, 1992).

Various effects of TRH on several neurotransmitter systems have been proposed and its effects on neuronal activity have been described (Yarbrough, 1979; Horita *et al.*, 1986). The mechanisms by which TRH produces cerebral vasodilatation are not fully understood. The potent vasodilator substance, nitric oxide (NO), which is synthesized from L-arginine by vascular endothelial cells (Palmer *et al.*, 1988), has been found to influence cerebral blood flow in rats (Tanaka *et al.*, 1991) and induce vasodilatation of bovine cerebral arteries *in vitro* (González & Estrada, 1991). The administration of stable arginine analogues such as N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methyl ester (L-NAME) has been shown to cause a dose-dependent inhibition of the formation of NO from L-arginine (Rees *et al.*, 1990).

The aim of the present study was to investigate the involvement of endogenous NO in the cardiovascular system of the anaesthetized rabbit by using a stable L-arginine analogue, N^G-nitro-L-arginine methyl ester (L-NAME), which inhibits NO synthesis (Rees *et al.*, 1990), as well as ascertain whether the cardiovascular effects of TRH are modified by pretreatment with L-NAME. It was also of interest to investigate whether the cerebral and ocular vascular effects of the drugs were influenced by cervical sympathotomy.

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The results were reported in part at the Scandinavian Physiological Society Meeting, Copenhagen, Denmark (Seligsohn, 1992b).

Methods

New Zealand white rabbits of either sex, weighing between 1.9–4.2 kg, were used. The general procedure has previously been described (Seligsohn, 1992a). In brief, the animal was anaesthetized with urethane (7 ml mg^{-1}, i.v., of a 25% solution), tracheotomized and artificially ventilated. Both femoral arteries were cannulated and used for measurements of mean arterial blood pressure (MAP), heart rate (HR) and blood sampling. One vein was cannulated and used for drug delivery. The left ventricle was retrogradely cannulated for injections of 15 μm labelled spheres (NEN Chemicals, Boston, Mass., U.S.A.) and the tracer microspheres method was used for determining regional blood flows. Blood flow was calculated according to the free flow method (Alm & Bill, 1972).

Arterial blood gases (P_{O_2}, P_{CO_2}) and pH were determined regularly with an ABL 300 acid-base analyser (Radiometer, Copenhagen, Denmark). Any deviations in the acid-base balance were corrected by administering sodium bicarbonate and/or changing the ventilation rate. Heparin (Kabi Vitrum, Stockholm, Sweden) (500 iu kg^{-1}) was given intravenously (i.v.) to prevent clotting. Tubocurarine (Tubocuran, Nordisk Droge, Copenhagen, Denmark) (0.5–1 mg kg^{-1}) was administered i.v. to induce skeletal muscle relaxation. Body temperature was recorded by a rectal thermistor and maintained at about 38°C with a heating pad.

Unilateral sectioning of the cervical sympathetic chain was performed about 1 cm below the upper cervical ganglion. Blood flow data for the sectioned side were compared with data for the intact side. The following experiments were performed (Figure 1).

(I) In the first series of experiments ($n = 6$) a control blood flow measurement preceded the i.v. administration of 300 mg kg^{-1} of L-NAME, which was followed 12 min later by the second microsphere injection. The third microsphere injection was performed 20 min after the administration of L-NAME. In this study a high dose of L-NAME (300 mg kg^{-1}) was used to ensure a total inhibition of the NO synthesis and maximal effect on regional blood flow. This high dose of L-NAME was chosen after preliminary experiments where the administration of 30 mg kg^{-1} of L-NAME elicited an increase in MAP which was sustained for at least 20 min. However, in these experiments the decrease in CBF_{tot} measured after the administration of L-NAME was maintained only for a relatively short period of time (<20 min). It has recently been shown that in the rabbit, administration of L-NAME caused a dose-dependent reduction of microvascular diameters in the dose-interval 1–100 mg kg^{-1} while a maximal increase in MAP was already achieved at a dose of 30 mg kg^{-1} (Persson *et al.*, 1991).

(II) In the second series of experiments ($n = 5$) the first two blood flow determinations were made in the same manner as in the first series. Then TRH was given, in a dosage of 1 mg kg^{-1}, i.v., 15 min after the administration of L-NAME. A third blood flow measurement was performed 5 min after the TRH injection (20 min after L-NAME).

(III) In order to investigate if the cerebral autoregulatory response was affected by treatment with L-NAME, a third series of experiments was performed. In these experiments ($n = 5$) the procedure differed in some respects from that described above. Blood pressure recording was performed through an ear artery and a brachial artery was cannulated in order to collect blood samples. A brachial vein was cannulated for drug injections. The abdomen was opened and a loose ligature was placed around the abdominal part of the aorta. A blood flow measurement, which was regarded as the control, was performed 12 min after the injection of L-NAME. Then blood pressure was elevated by ligation of the aorta and blood flow was measured 4–5 min later. In all the experiments the animals were killed by intracardial administration of saturated KCl after the last microsphere injection. Organs and tissue samples were autopsied and placed in preweighed plastic tubes. The radioactivity of the samples was analysed by gammaspectrometry. Total cerebral blood flow (CBF_{tot}) was calculated to include all parts of the brain, except medulla oblongata and cerebellum. Cardiac output (CO) was calculated as $CO = Q_r \times CPM_i \times CPM_r^{-1}$, where Q_r = reference blood flow, CPM_i = injected radioactivity and CPM_r = reference radioactivity. CO was further indexed by unit of weight (CO_W) (g min^{-1} kg^{-1}). Peripheral vascular

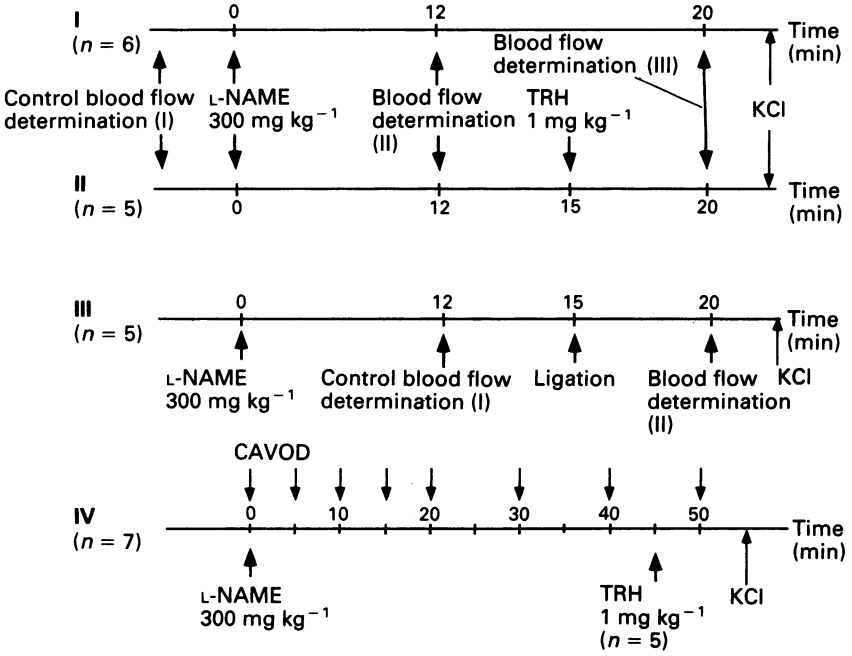


Figure 1 Experimental protocols in experiments with blood flow determination (I-III) and in experiments with measurements of the cerebral arteriovenous oxygen saturation difference (CAVOD) (IV).

resistance (VR) was calculated as $VR = MAP \times Q_t^{-1}$ where MAP is given in mmHg and Q_t = tissue blood flow in g min^{-1} 100 g^{-1} tissue and values expressed as % of control value.

(IV) In the fourth series of experiments ($n = 7$) the arteriovenous O_2 saturation difference in the brain (CAVOD) was measured in order to investigate whether the vasoconstriction observed after the L-NAME injection was due to a decrease in the O_2 demand of the brain. The animal was placed in a stereotaxic instrument (David Kopf Instrument, Tujunga, CA, U.S.A.). A hole was drilled in the midline of the skull between the coronal and lamboid suture. Indomethacin (Sigma Chemical Company, St. Louis, MO, U.S.A.) was administered i.v. in a dosage of 20 mg kg^{-1} in order to inhibit the cyclo-oxygenase activity and thus reduce the adhesion of platelets to the vessel wall. The superior sagittal sinus was cannulated under visual control with a thin polyethylene catheter (pp50). The tip of the cannula was inserted into the vein in the downstream direction. The arterial and venous O_2 saturations were determined with an ABL 300 acid-base analyser (Radiometer, Copenhagen, Denmark) in both a control situation and at different times after the administration of 300 mg kg^{-1} L-NAME (Figure 1). In 5 animals, 1 mg kg^{-1} TRH was given i.v. at the end of the experiment and the CAVOD was measured 5 min after the administration of the peptide.

The following drugs were used: TRH, lot no. 125F-59201 (Sigma Chemical Company, St. Louis, MO, U.S.A.) and N^G-nitro-L-arginine methyl ester (L-NAME) lot no. 3640692 (Sigma Chemical Company, St. Louis, MO, U.S.A.). Drugs were freshly dissolved in saline.

A statistical evaluation of the results was made with the two-tailed Student's t test for paired or unpaired observations or with an analysis of variance (ANOVA) with repeated measurements. Fischer's PLSD test was used as a post ANOVA test for comparison within treatments. The level of significance was set at $P < 0.05$ and was adjusted with Bonferroni's correction. The results are expressed as means ± s.e.

Results

There was no difference in CBF between the side with an intact sympathetic supply and the sympathectomized side (in both the control situation and after drug administration); therefore only the values for the side with an intact sympathetic supply are presented.

I. Effects of N^G-nitro-L-arginine methyl ester (L-NAME)

There were no major changes in arterial blood gases after the administration of L-NAME. The injection of L-NAME caused an increase in MAP from 84.0 ± 4.5 to 95.3 ± 7.5 mmHg (11.2 ± 0.6 to 12.7 ± 1.0 kPa) ($P < 0.05$) ($n = 11$). There was also a decrease in HR from 289 ± 7 to 277 ± 8 beats min^{-1} ($P < 0.05$) and in CO_W from 230 ± 22 to 122 ± 11 g min^{-1} kg^{-1} ($P < 0.001$). The administration of L-NAME caused an increase in total VR to 216 ± 12% of the control ($P < 0.001$). In several peripheral organs such as heart, gastric mucosa, duodenum, triceps muscle, kidney and adrenal gland, L-NAME caused an increase in VR ranging between 80–200% (data not shown). In the spleen a marked increase in VR of 627 ± 179% ($P < 0.01$) was noted after the administration of L-NAME. In all organs investigated the increases in VR were sustained 20 min after the L-NAME injection.

In the uvea, administration of L-NAME caused a reduction in blood flow. Basal values in the choroid were 1101 ± 257 mg min^{-1} on the intact side and 1256 ± 332 mg min^{-1} on the sympathectomized side. Corresponding values in the iris were 39 ± 10 mg min^{-1} and 69 ± 20 mg min^{-1} and in the ciliary body 99 ± 23 mg min^{-1} and 97 ± 19 mg min^{-1}, respectively. There were no significant differences in control situation between the intact and the sympathectomized side. The reduction in blood flow was greater on the side with an intact sympathetic supply and this difference in blood flow reduction was statistically significant, measured 12 min after the L-NAME injection, in both the choroid ($P < 0.01$) and the iris ($P < 0.05$). In the retina the blood flow decreased from 18 ± 2 to 8 ± 1 mg min^{-1} on the intact side and from 23 ± 3 to 10 ± 1 on the sympathectomized side (Figure 2).

The administration of L-NAME caused a decrease in blood flow ranging between 10–30% (Figure 3) in several cerebral regions investigated. Total cerebral blood flow (CBF_{tot}) decreased from 65 ± 4 to 48 ± 3 g min^{-1} 100 g^{-1} ($P < 0.05$). This decrease in CBF was maintained 20 min after the administration of L-NAME (Figure 3). No significant effects were found in gray matter, the hippocampal region and the caudate nucleus.

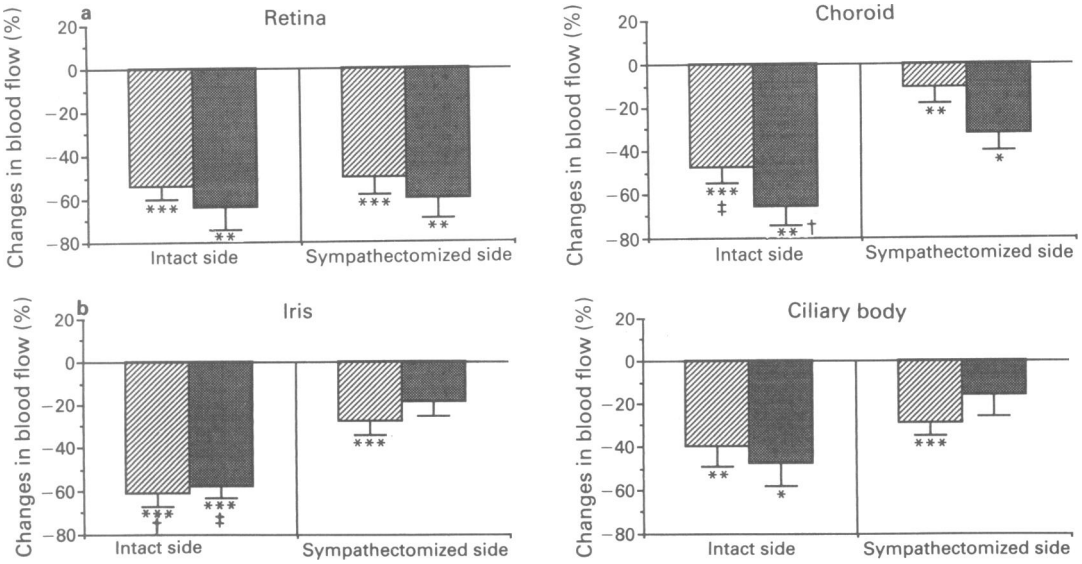


Figure 2 Ocular blood flow at 12 min (hatched columns, $n = 11$) and 20 min (stippled columns, $n = 6$) after the administration of 300 mg kg^{-1} N^G-nitro-L-arginine methyl ester on the intact side and on the sympathectomized side; (a) retina and choroid; (b) iris and ciliary body. Values are given as % of controls. $*P < 0.05$; $**P < 0.01$, $***P < 0.001$ as compared to controls. $\dagger P < 0.05$; $\ddagger P < 0.,01$ as compared with sympathectomized side (Student's t test, paired data).

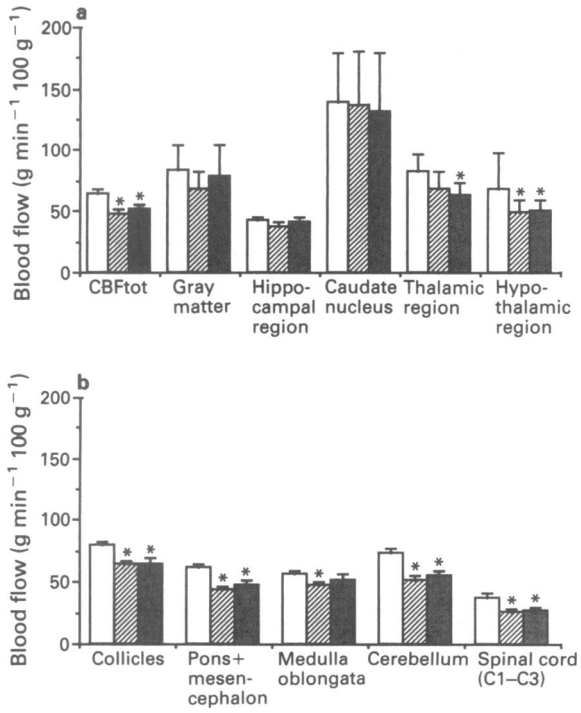


Figure 3 Total (CBF_{tot}) and regional cerebral blood flow ($n = 6$) in control situation (open columns), 12 min (hatched columns) and 20 min (stippled columns) after the administration of 300 mg kg^{-1} N^G-nitro-L-arginine methyl ester. Values are given in g min^{-1} 100 g^{-1} tissue. *$P<0.05$, as compared to controls (ANOVA).

II Effects of TRH in L-NAME pretreated animals

In this series of experiments the cardiovascular parameters measured 12 min after the administration of L-NAME are referred to as controls. The arterial blood gases and pH were not affected during the experiments. After the administration of TRH there was an increase in MAP from 87.8 ± 5.3 to 123.8 ± 11.3 mmHg ($P<0.05$). HR and CO_W were not significantly affected by TRH.

The administration of TRH caused an increase in cerebral blood flow in several regions investigated (Table 1). Blood flow in the hippocampal region increased to 208 ± 28% of the control and in the caudate nucleus the corresponding value was 209 ± 17%. CBF_{tot} increased to 189 ± 20% of the control ($P<0.05$) (Figure 4).

Table 1 Regional cerebral blood flows in animals pretreated with 300 mg kg^{-1} N^G-nitro-L-arginine methyl ester (L-NAME) before and after the administration of 1 mg kg^{-1} of thyrotropin-releasing hormone (TRH) ($n = 5$)

	12 min post-L-NAME	*L-NAME + TRH*
Gray matter	41 ± 7	90 ± 12**
Hippocampal region	38 ± 2	79 ± 12*
Caudate nucleus	74 ± 9	152 ± 19**
Thalamic region	47 ± 6	108 ± 14**
Hypothalamic region	48 ± 4	91 ± 11**
Collicles	60 ± 7	75 ± 5*
Pons + mesencephalon	43 ± 2	65 ± 3***
Medulla oblongata	44 ± 2	61 ± 5**
Cerebellum	54 ± 4	64 ± 7
Spinal cord (C1-C3)	23 ± 2	28 ± 2

Values are given in g min^{-1} 100 g^{-1} tissue.
*$P<0.05$; **$P<0.01$; ***$P<0.001$ as compared with blood flow determination after L-NAME pretreatment (Student's t test, paired data).

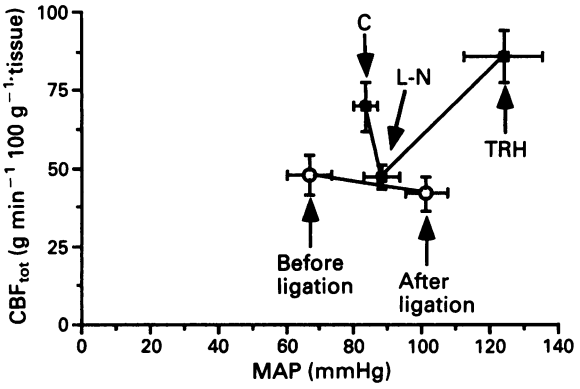


Figure 4 Total (CBF_{tot}) cerebral blood flow and mean arterial blood pressure (MAP) (mmHg) in (■) control situation (C), after the administration of 300 mg kg^{-1} N^G-nitro-L-arginine methyl ester (L-NAME, L-N) and after the administration of 1 mg kg^{-1} thyrotropin-releasing hormone (TRH) ($n = 6$), and (○) before and after aortic ligation ($n = 5$) in animals pretreated with 300 mg kg^{-1} L-NAME.

III Effects of increased mean arterial blood pressure in L-NAME pretreated animals

In these experiments the blood flow after the administration of L-NAME is referred to as the control. Control values for blood gases and arterial pH were in the same range as in the earlier experiments. After the ligation of the abdominal aorta there was a decrease in PCO_2 in arterial blood from 4.56 ± 0.21 to 3.72 ± 0.27 kPa ($P<0.01$). The blood pressure, measured through an ear artery, was increased from 66.8 ± 6.8 to 101.3 ± 6.0 mmHg ($P<0.01$) by ligation of the abdominal part of the aorta. In these experiments only cerebral tissues were analysed. Total (CBF_{tot}) (Figure 4) and regional cerebral blood flows were not affected in these experiments.

IV Cerebral arteriovenous oxygen saturation difference (CAVOD)

There were no significant changes in arterial pH or PCO_2 during the experiments. The CAVOD in the brain increased

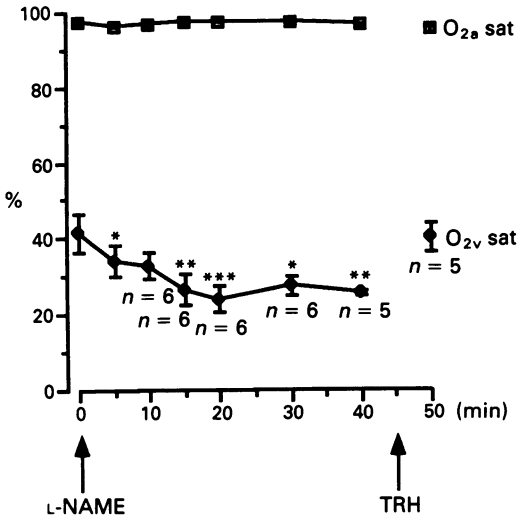


Figure 5 Arterial (O_{2a}) and venous (O_{2v}) oxygen saturation in the brain in control situation and after the administration of 300 mg kg^{-1} N^G-nitro-L-arginine methyl ester (L-NAME) ($n = 7$) and 5 min after the administration of 1 mg kg^{-1} thyrotropin-releasing hormone (TRH) ($n = 5$). *$P<0.05$; **$P<0.01$; ***$P<0.001$ (Student's t test).

from 56.0 ± 5.2% to 62.3 ± 4.0% ($P<0.05$) 5 min after the injection of L-NAME and then gradually increased to 73.6 ± 3.5 20 min after the L-NAME delivery (Figure 5). Measured 40 min after the administration of L-NAME, the CAVOD was 71.0 ± 0.7%. The injection made in 5 experiments of 1 mg kg^{-1} of TRH caused a decrease in the CAVOD from 71.3 ± 0.6 to 54.6 ± 4.6% ($P<0.05$).

Discussion

Effects of L-NAME and TRH

In the present study, the inhibition of the NO synthesis by administration of L-NAME caused a reduction in blood flow in many tissues including the eye as well as several cerebral regions. This indicates an involvement of endogenous NO in the regulation of regional blood flow in rabbits under urethane anaesthesia. There was also an increase in MAP after the administration of L-NAME, a decrease in CO and a concurrent decrease in heart rate.

Several mechanisms may be involved in the vascular effects of L-NAME. NO is released under basal physiological conditions and in response to receptor stimulation (Moncada *et al.*, 1991) and the L-arginine:NO pathway is among the mechanisms whereby cells regulate their own function or communicate with others (Moncada *et al.*, 1991). The importance of this L-arginine:NO pathway in the nervous system is not yet fully clarified. However, NO synthase is distributed in the CNS in various cell types, including brain neurones, retina and vascular endothelial cells (Bredt *et al.*, 1990) and it seems likely that this pathway is associated with other mediators in the central nervous system. In the cardiovascular system, basal release of NO seems to play an important role and acts as a general adaptive mechanism whereby the vascular endothelium responds to changes in its environment and regulates blood flow and blood pressure through an action on the vascular smooth muscle.

Lacolley *et al.* (1991) recently showed that the pressor response induced by L-arginine analogues was diminished after ganglionic blockade. This indicates an interaction between the sympathetic nervous system and the system using NO as a mediator. Interestingly, in some tissues in our study the effect on the sympathetically intact side was greater than on the sympathetically deprived side. Taken together, these results suggest that NO modulates vascular resistance both by a peripheral action and by an effect on the central regulation of sympathetic tone, which is in agreement with recent results by Sakuma *et al.* (1992). The reduction in blood flow in the brainstem caused by L-NAME is one factor that may activate the sympathetic nervous system.

TRH has an influence on the autonomic nervous system and interacts with both its sympathetic and parasympathetic parts (McCann *et al.*, 1989; Koskinen & Bill, 1984; Seligsohn, 1992a). There is evidence for the involvement of endogenous TRH in the regulation of blood pressure in spontaneously hypertensive rats (Nurminen, 1992). In the rabbit, TRH elicits both vasoconstriction in the gastric mucosa, pancreas and spleen and vasodilatation in the brain (Koskinen, 1986b; Seligsohn, 1992a). The vasoconstriction in the rabbit seems mainly to be due to an α_1-adrenoceptor mechanism (Seligsohn & Koskinen, 1991). However, the mediator of the vasodilatation is unknown.

Effects of L-NAME and TRH in the eye

In the eye the administration of L-NAME caused a reduction in blood flow of about 50–60%. In the rabbit the blood flow through the uvea is not autoregulated and is affected by changes in blood pressure as well as in intraocular pressure (Bill, 1974). Sympathetic stimulation reduces blood flow through all parts of the rabbit uvea, although retinal blood flow is unaffected (Bill, 1984). The results of the present study show that the reduction in blood flow in the uvea was more prominent on the side with an intact sympathetic supply. This could indicate an activation of sympathetic vasoconstrictor fibres as discussed above or could be due to an enhanced vasoconstrictor effect of noradrenaline. Another possibility is that there is a facilitation of the release of noradrenaline due to removal of NO-induced prejunctional inhibition (Lacolley *et al.*, 1991). Interestingly, in the retina, where there are no sympathetic nerves, there was no difference in response to L-NAME between the intact side and the sympathetically deprived side. The importance of NO formation in the ophthalmic artery has recently been shown in *in vitro* experiments (Yao *et al.*, 1991; Haefliger *et al.*, 1992). Our results support the concept that the ophthalmic circulation is in a state of vasodilatation as a result of NO production (Haefliger *et al.*, 1992; Granstam *et al.*, 1993). In this study TRH did not have any major effects on the ocular blood flow, which is in agreement with previously reported results (Seligsohn & Koskinen, 1991).

Effects of L-NAME and TRH in the brain

There is evidence for the involvement of NO or a NO-like substance in the transmission of information from vasodilator nerves to cerebral artery smooth muscle (Toda & Okamura, 1991). In addition, inhibition of NO synthesis with L-NMMA has been shown to cause a constriction of bovine cerebral arteries *in vitro* (Gonzáles & Estrada, 1991). *In vivo*, it was recently shown by Ko'zniewska *et al.* (1992) that inhibition of endogenous NO caused a decrease in cerebral blood flow in the rat. These results also suggest that there might be an involvement of NO in the cerebral autoregulatory response. However, no evidence was found in the experiments reported here for a major involvement of NO in the autoregulation of CBF; the results indicate rather that autoregulation is established at a lower level of CBF after L-NAME. This is in agreement with recent observations in the rat, made by Wang *et al.* (1992).

It seems clear that several peptide mechanisms are involved in the regulation of cerebral and spinal blood flow (Uddman & Edvinsson, 1989). TRH has been shown to cause a cerebral vasodilatation in various species (Koskinen, 1989; Hugoson-Seligsohn & Koskinen, 1989; Seligsohn, 1992c). The cerebrovasodilator effect of the peptide does not seem to be due to an increase in cerebral metabolism (Koskinen & Bill, 1984; Koskinen & Sperber, 1986; Hugoson-Seligsohn & Koskinen, 1989). TRH seems to elicit its effects by activation of an intracerebral vasodilator pathway (Koskinen, 1986a). The cerebrovasodilator effect of TRH was abolished by yohimbine pretreatment (Seligsohn & Koskinen, 1991), indicating that α_2-adrenoceptors are involved. Anatomical details of the pathway are unknown. However, it has recently been shown that stimulation of a medullary region elicits cerebral vasodilatation and that this effect was abolished in animals pretreated with the α_2-receptor antagonist, yohimbine (Saeki *et al.*, 1989). This pathway might be identical to that activated by TRH. The demonstration of NO synthase in the endothelial layers of various cerebral vessels and in nerve fibres surrounding the adventitia of brain vessels suggests that regulation of cerebral circulation might involve release of NO. However, the results presented here suggest that the vasodilator mechanisms involving TRH do not operate via release of NO.

After administration of TRH there was an increase in MAP in the animals pretreated with L-NAME. This made it difficult to interpret the increase in CBF observed. It might be due to increased MAP if autoregulation was affected by L-NAME or it might be a 'normal' response to TRH. The experiments with ligation of the aorta were performed to elucidate this problem and demonstrated intact autoregulation.

The arteriovenous difference in oxygen saturation (CAVOD) increased after L-NAME administration. This increase was

about 25%, which is similar to the decrease in CBF found at the same time. This result indicates that the vasoconstriction could not be explained, at least entirely, by a decrease in oxygen demand in the brain due to changes in cerebral metabolism. The administration of TRH in L-NAME-pretreated animals caused a marked increase in cerebral blood flow in the same range as has previously been reported and the arteriovenous difference in oxygen saturation decreased towards the control level (Koskinen & Bill, 1984; Seligson, 1992a). The increase in CBF is not likely to be due to the rise in MAP since, as discussed above, L-NAME seems to leave autoregulatory mechanisms intact.

Interestingly, TRH seemed to have a pronounced cerebrovasodilator effect in regions where the arginine:NO pathway appeared to play a relatively smaller role.

In conclusion, the results of the present study indicate that endogenous nitric oxide is involved in the regulation of regional blood flow and blood pressure in the rabbit. There seems to be an interaction between the NO-mediated mechanism and the sympathetic nervous system, at least in the control of uveal blood flow. No evidence was found for any major involvement of endogenous nitric oxide in the cardiovascular response to TRH. However, TRH restored CBF and CAVOD to normal levels in animals given L-NAME.

We wish to thank Ms Kristina Andersson for excellent technical assistance. This study was supported by Grant No. B90-14x-00147 from the Swedish Medical Research Council and Grant No. 5 RO1 Ey 00475 from the National Eye Institute, U.S. Public Health Service.

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(Received September 4, 1992
Revised March 3, 1993
Accepted April 6, 1993)

Br. J. Pharmacol. (1993), **109**, 1226–1231 © Macmillan Press Ltd, 1993

Inhibition of the ATP-sensitive potassium channel by class I antiarrhythmic agent, cibenzoline, in rat pancreatic β-cells

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1 Cibenzoline, a class I antiarrhythmic agent, was investigated for its effect on the ATP-sensitive K^+ channel of pancreatic β-cells by the patch clamp technique.

2 In perforated patch clamp experiments, cibenzoline depolarized the membrane of single β-cells and thereafter, caused firing of action potentials in the presence of 2.8 mM glucose.

3 Cibenzoline inhibited the activity of the ATP-sensitive K^+ channel in cell-attached recordings in the presence of 2.8 mM glucose and evoked repetitive fluctuations of the baseline current, apparently reflecting the action potentials of the β-cell.

4 In whole-cell clamp experiments, time-independent outward current was induced by depleting cytoplasmic ATP with 0.1 mM ATP and 0.1 mM ADP in the solution contained in the pipette. The outward current was inhibited by cibenzoline in a dose-dependent manner in the concentration range of 1 μM to 100 μM and half maximum inhibition occurred at 1.5 μM.

5 Cibenzoline blocked substantially the ATP-sensitive K^+ channel current when applied at the inner side of the membrane in isolated inside-out membrane patches.

6 It is concluded that cibenzoline blocks the ATP-sensitive K^+ channel of pancreatic β-cells and, thereby, stimulates insulin secretion at sub-stimulatory levels of glucose.

Keywords: Cibenzoline; ATP-sensitive K^+ channel; pancreatic β-cells; insulin secretion

Introduction

Stimulation of insulin secretion from pancreatic β-cells is evoked physiologically by an elevation of the plasma glucose level, via metabolic production of ATP which then inhibits the ATP-sensitive K^+ channel (K_{ATP} channel) (Cook & Hales, 1984; Ashcroft *et al.*, 1984). It has been established that the activity of the K_{ATP} channel is a major determinant of the resting potential of β-cells. Thereby, inhibition of the channel results in membrane depolarization followed by an increase in the cytosolic Ca^{2+} concentration through Ca^{2+} entry via voltage-dependent Ca^{2+} channels (Yada *et al.*, 1992) and eventual exocytosis of insulin (for reviews see Henquin & Meissner, 1984; Ashcroft & Rorsman, 1989).

A newly-synthesized class I antiarrhythmic agent, cibenzoline, is known to have an extracardiac side-effect of hypoglycaemia (Hilleman *et al.*, 1987; Gachot *et al.*, 1988; Jeandel *et al.*, 1988; Lefort *et al.*, 1988; Houdent *et al.*, 1991). Recently Bertrand *et al.* (1992) reported that cibenzoline directly stimulates pancreatic β-cells and potentiates insulin secretion. It has been reported that other antiarrhythmic agents, such as quinidine or disopyramide, also may induce hypoglycaemia (Panisko & Keystone, 1990; Kim & Benowitz, 1990). However, the mechanism by which these drugs produce hypoglycaemia, remains to be elucidated. Sulphonylureas, which are used to increase the ability of the pancreatic β-cell to release insulin in response to glucose stimulation, are known to inhibit specifically the K_{ATP} channel (Sturgess *et al.*, 1985; Trube *et al.*, 1986). It has also been reported that the K_{ATP} channel is blocked by quinidine in cardiac myocytes (Undrovinas *et al.*, 1990) and by sparteine, an antiarrhythmic agent with oxytocic action, in the insulin-secreting β-cell line, HIT-T15 (Ashcroft *et al.*, 1991). Similarly, it is likely that the K_{ATP} channel in pancreatic β-cells may be involved in antiarrhythmic agent-induced hypoglycaemia. In the present study, we have investigated the effect of cibenzoline on the activity of the K_{ATP} channel in rat pancreatic β-cells.

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Methods

Islets of Langerhans were isolated from Wistar rats weighing 200–300 g by collagenase digestion (Type 4, Funakoshi Co. Ltd., Japan) as described elsewhere (Sutton *et al.*, 1986). The animal was anaesthetized by an intraperitoneal injection of pentobarbitone (80 mg kg^{-1}). The abdomen was opened and collagenase (3 mg ml^{-1}) dissolved in 5 ml of 5 mM Ca^{2+}-containing Krebs-Ringer bicarbonate buffer (KRB) solution was injected into the common bile duct at the distal end after ligation of the duct proximal to the pancreas. Then, the pancreas was dissected out and incubated for 17 min at 37°C. The islets of Langerhans were examined under the biological microscope and were dispersed mechanically into single cells in Ca^{2+}-free KRB solution. The isolated islet cells were plated on coverslips (18 × 24 mm, 0.12 mm in thickness) in 35 mm tissue culture dishes and cultured for 1–4 days in Eagle's MEM medium containing 5.6 mM glucose supplemented with 10% foetal bovine serum, 100 μg ml^{-1} streptomycin and 100 u ml^{-1} penicillin at 37°C in an atmosphere of 95% air plus 5% CO_2. The coverslip was cut into small pieces, placed in the recording chamber (0.3 ml) and superfused with the KRB solution containing 2.8 mM glucose at least 20 min before either whole-cell voltage clamp, perforated current clamp or cell-attached patch recordings, or superfused with the standard internal solution in the inside-out membrane patch experiments.

Standard whole-cell or patch-clamp techniques (Hamill *et al.*, 1981) were used to record the K_{ATP} channel currents. Patch pipettes were pulled from hard glass tubing (Narishige Scientific Inst. Lab., Tokyo, Japan), coated with silicon resin to reduce their electrical capacitance and fire-polished just before use. The pipettes had resistances between 5 and 10 MΩ when filled with the solution (140 mM KCl) in either the cell-attached patch or inside-out patch configuration and resistances less than 3 MΩ with the internal solution in the perforated or ordinary whole-cell clamp configuration, respectively. The current was recorded by an amplifier (List, EPC7) and stored on a PCM digital data recorder (TEAC, RD-1117Z). Replayed data were then low-pass filtered

(24 dB/octave. NF, E-3201A) at a cut-off frequency indicated in the figure legends and digitized by a 12-bit AD converter to be analysed with a computer (Hewlett Packard, HP-9816).

The perforated whole-cell current clamp mode was used to record the membrane potential by using 0.01% nystatin dissolved in 0.2% dimethylsulphoxide in the pipette (Horn & Marty, 1988).

In whole-cell clamp experiments, the pipette solution contained 0.1 mM ATP and 0.1 mM ADP in order to evoke the ATP-sensitive K^+ channel current (Trube *et al.*, 1986). After formation of the whole-cell clamp configuration, the resting membrane conductance progressively increased due to the reduction of cytoplasmic ATP concentration by dialysis between the cell interior and the pipette solution and reached a steady state level within 10 min. To construct the dose-response relations between cibenzoline and the K_{ATP} channel current, the membrane potential was voltage-clamped to a holding potential of − 70 mV and depolarized by 10 mV with 100 ms duration every 5 s throughout the experiment. The current through the K_{ATP} channels was determined by its time- and voltage-independent kinetics and sensitivity to the sulphonylurea, 0.1 mM tolbutamide. We then tested effects of cibenzoline on this tolbutamide-sensitive current fraction. The current amplitude during exposure to cibenzoline was normalized with respect to that of the tolbutamide-sensitive current fraction, and plotted as a function of cibenzoline concentration. Relative current was fitted to the Hill equation to obtain the half maximal inhibition (IC_{50}) of the K_{ATP} channel activity by a least squares method:

$$\text{relative current} = 1/\{1 + ([L]/K_{1/2})^n\}$$

where L is a ligand concentration (cibenzoline). $K_{1/2}$ is the half-maximal concentration of cibenzoline on the channel activity and n is the Hill coefficient.

To calculate the mean K_{ATP} current we constructed an amplitude histogram with a current level of 0.04 pA/bin on the abscissae during superfusion with each test solution for at least 2 min. The mean current was obtained by integrating open channel distributions on the histogram and by averaging over time. The mean amplitude of the single channel event was determined as the difference between the current level of closed channel distribution and that of the first-step channel events.

Solutions

The composition of the pipette solution used in both cell-attached and inside-out membrane patch experiments was (in mM): KCl 140, $CaCl_2$ 2 and HEPES 5 (pH 7.2 adjusted with KOH). The KRB solution contained (in mM): NaCl 129, KCl 4.7, $CaCl_2$ 2.0, $MgCl_2$ 1.2, KH_2PO_4 1.2 and $NaHCO_3$ 5.0; the pH was ajusted to 7.4 with 10 mM HEPES-NaOH. Glucose was added to the KRB solution as required. The pipette solution used in perforated whole-cell clamp contained (in mM): K_2SO_4 40, KCl 50, HEPES 10, $MgCl_2$ 2, EGTA 0.5 (pH 7.2 adjusted with KOH). The standard internal solution for whole-cell clamp experiments contained (in mM): KCl 50, K_2SO_4 35, $MgCl_2$ 2.0, EGTA 11, $CaCl_2$ 1, HEPES 11 (pH 7.2 adjusted with KOH; final K^+ concentration 140 mM). The internal solution used for the inside-out patch experiments contained (in mM): KCl 113, $MgCl_2$ 2.0, EGTA 11, $CaCl_2$ 1.0, and HEPES 11 (pH 7.2 adjusted with KOH). Adenosine-5′-triphosphate (ATP-2Na) and adenosine-5′-diphosphate (ADP-K) were purchased from Boehringer and added to intracellular solutions as required. Cibenzoline, a gift from Fujisawa Pharmaceutical Co., Ltd. (Tokyo, Japan), was dissolved in distilled water as a 10 mM stock solution and diluted to the required concentration in the bathing solution before use. All the experiments were performed at room temperature (22–25°C).

Results

The effect of cibenzoline on the membrane potential of a single pancreatic β-cell superfused with the KRB solution containing 2.8 mM glucose, recorded by the perforated whole-cell current clamp technique is shown in Figure 1. Initially in formation of the whole-cell current clamp, the membrane potential was − 70 mV (control) at 2.8 mM glucose. The membrane potential was stable as long as the 2.8 mM glucose superfusion continued and this gradually depolarized approximately 2 min after the start of exposure to 100 μM cibenzoline, and eventually evoked action potentials. The membrane potential was then partially hyperpolarized after washout of the agent with the KRB solution containing 2.8 mM glucose but depolarization was sustained for further 30 min without returning to the control level. We observed cibenzoline-induced depolarization and poor reversibility on washout in all of 16 cells at concentrations exceeding 30 μM.

We then performed cell-attached experiments to record the K_{ATP} channel current and explored the effect of cibenzoline on channel activity (Figure 2). In the presence of 2.8 mM glucose, we observed openings of a K^+ channel which had a unit amplitude of 4.3 pA (Figure 2, upper trace). The single channel conductance was estimated to be 61 pS, assuming the resting membrane potential to be − 70 mV and intracellular potassium concentration to be 140 mM.

The channel openings were characterized by rapid open-close transitions separated with long closed intervals. These characteristics agreed well with those of the ATP-sensitive K^+ channel previously described in pancreatic β-cells (Cook & Hales, 1984; Ashcroft *et al.*, 1984; Rorsman & Trube, 1985).

The K_{ATP} channel was substantially inhibited by 100 μM cibenzoline within 3 min of exposure of the cell to this agent, following which biphasic deflections of the base-line current were observed (Figure 2, middle trace). It has been suggested that this phenomenon is caused by the action potential of the β-cell (Ashcroft *et al.*, 1984; Rorsman & Trube, 1985). Thus

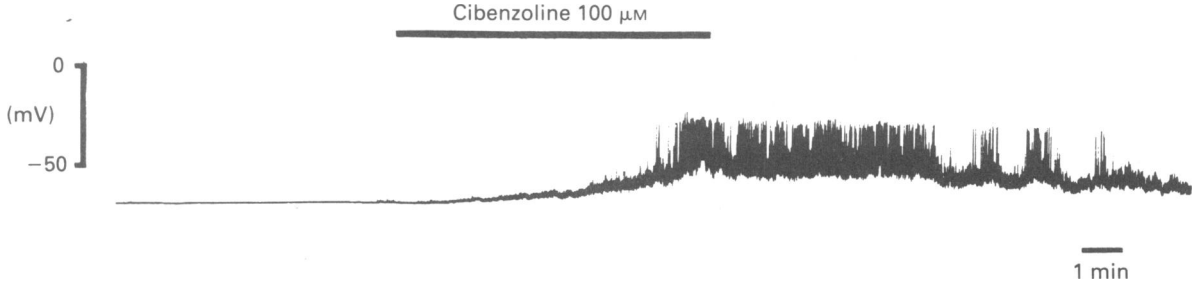


Figure 1 The effect of 100 μM cibenzoline on the membrane potential of a single pancreatic β-cell. The cell was superfused with the KRB solution containing the agent during the period indicated by the bar above the trace. The perforated current clamp technique was used. The glucose concentration in the KRB solution was maintained at 2.8 mM throughout this experiment.

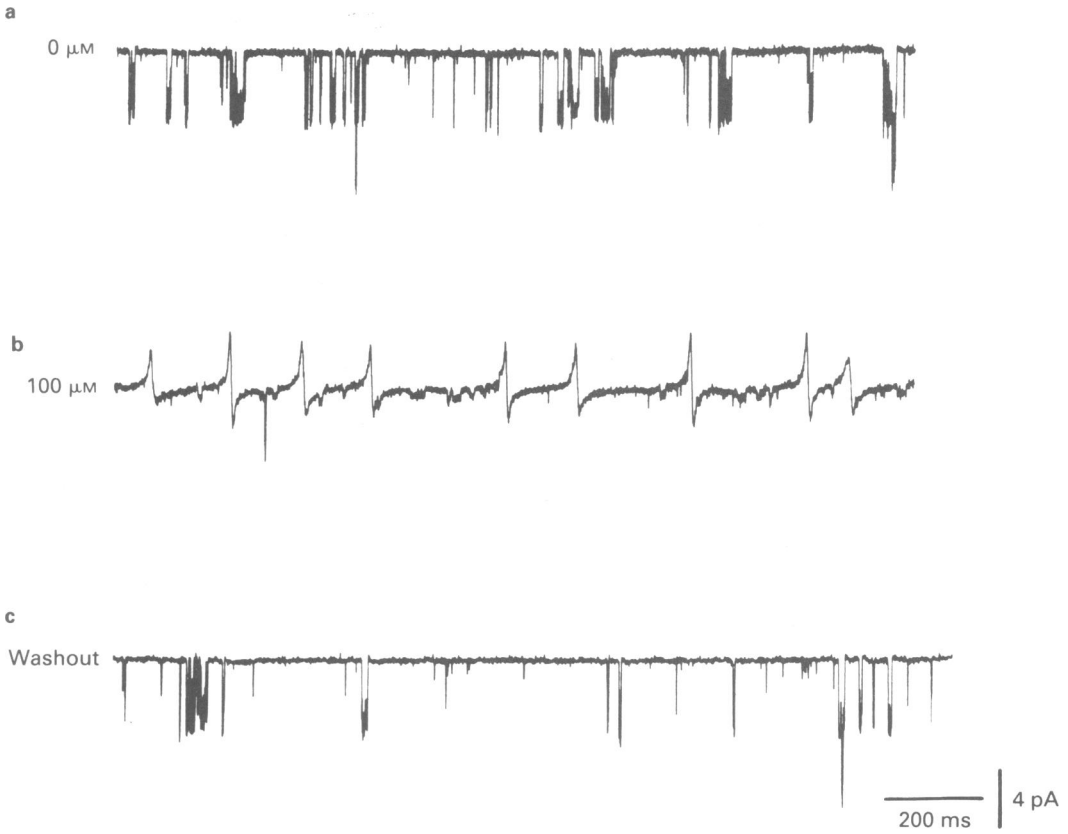


Figure 2 Effects on single K_{ATP} channel currents of 100 μM cibenzoline in the cell-attached membrane patch. The cell was superfused with 2.8 mM glucose containing KRB solution with 5 mM K^+ and the pipette solution contained 140 mM K^+. Since the pipette potential was 0 mV, the K_{ATP} channel current is driven by the resting membrane potential (− 70 mV), resulting in inward current as downward deflections of channel openings (a). Cibenzoline was contained in the bathing solution outside the patch pipette and as a consequence of the K_{ATP} channel inhibition, biphasic deflections of the baseline current level due to the action potentials of β-cells were seen (b). The lower trace (c) shows the K_{ATP} channel current recorded 15 min after washout. The current was low-pass filtered at 800 Hz and plotted on the chart recorder.

we conclude that cibenzoline induces action currents by depolarizing the membrane following inhibition of the K_{ATP} channel. During this action current, openings of a channel with a small amplitude were observed. We did not determine if this channel activity represents openings of the K_{ATP} channel with a decreased driving force due to membrane depolarization or is that of voltage-dependent potassium channels, as reported by Rorsman & Trube (1986). Mean patch current of the K_{ATP} channel was 0.12 pA in control and partially recovered to 0.06 pA after the inhibition by washing out cibenzoline in the cell-attached membrane patch (Figure 2, lower trace). We observed the reversible inhibition of channel activity in 4 out of 7 membrane patches. In other patches the channel activity did not reverse from the inhibition by cibenzoline in the concentration-range between 10 and 100 μM on washout.

The effect of cibenzoline on the membrane current was examined in the whole-cell clamp experiment. After disrupting the patch membrane at the pipette tip, a progressive increase in the time-independent outward current in response to 10 mV depolarization from the holding potential of − 70 mV was observed and the current level reached a steady state in 10 min (control in Figure 3a). This time-independent whole-cell current seemed to reflect activation of the K_{ATP} channel because of depletion of cytosolic ATP with the pipette solution containing less ATP (Trube *et al.*, 1986). This is supported by the fact that subsequent application of 0.1 mM tolbutamide reversibly decreased the current. We used a pipette solution with both 0.1 mM ATP and 0.1 mM ADP because the sensitivity of the K_{ATP} channel to ATP is decreased in the presence of ADP (Kakei *et al.*, 1986). Since 0.1 mM tolbutamide inhibits the K_{ATP} channel by approximately 90% of control (Trube *et al.*, 1986), the difference in current before and during the exposure to tolbutamide should be due to the current through the K_{ATP} channels.

In Figure 3a, the membrane current recorded at indicated voltage before, during and after an application of 100 μM tolbutamide (upper trace) and 30 μM cibenzoline (lower trace) are shown. Application of 100 μM tolbutamide reversibly depressed the membrane current as illustrated in the upper left panel in Figure 3a. Subsequent exposure of 30 μM cibenzoline inhibited the K_{ATP} channel current at both − 60 mV and + 30 mV. Cibenzoline inhibited the initial current level at the beginning of the depolarized pulse. The time-dependent outward current remained during the superfusion of cibenzoline (Figure 3a, right panel). In the present study, we did not examine the effects of cibenzoline on this time-dependent outward current.

Since the K_{ATP} channel current was poorly dependent on the membrane potential (Figure 3a, control), current-voltage relationships between initial current and each voltage applied were plotted in Figure 3b. The membrane conductance measured at − 70 mV was 2.7 nS at 100 μM tolbutamide and 1.7 nS at 30 μM cibenzoline in contrast to 28 nS in control. Both current-voltage relations obtained in the presence of tolbutamide or cibenzoline were superimposable at all the potentials tested and they showed linear relationship against the membrane potential. Thus we suggest that the channel block by cibenzoline or tolbutamide is not dependent on membrane voltage. In the whole-cell clamp experiments, the inhibition of the K_{ATP} channel current by cibenzoline was not reversible ($n = 12$) when tested at concentrations greater than 1 μM.

In Figure 4, the relative whole-cell current decreased by

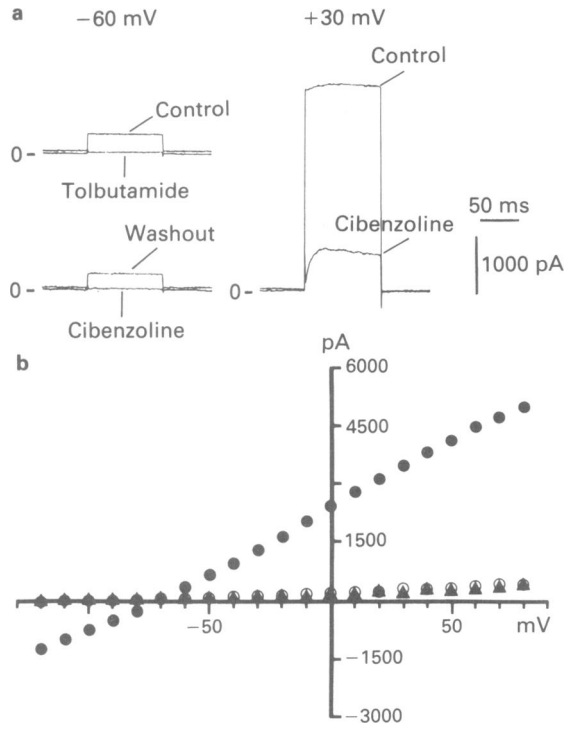


Figure 3 Effect of 30 μM cibenzoline on the membrane current and current-voltage relationship in whole-cell clamp experiments. The membrane was voltage-clamped at the holding potential of − 70 mV and depolarized to various potentials with a pulse duration of 100 ms every 5 s. The pipette solution contained 0.1 mM ATP and 0.1 mM ADP. (a) The membrane currents produced by a depolarized pulse to − 60 mV (left panel) and + 30 mV (right panel) before (Control), during (Tolbutamide) and after (Washout) exposure to 100 μM tolbutamide are illustrated. Cibenzoline 30 μM was then superfused (Cibenzoline). (b) Current-voltage relationships between initial current and membrane potential in the absence (control; ●) and in the presence of either 100 μM tolbutamide (○) or 30 μM cibenzoline (▲) are plotted.

cibenzoline is plotted as a function of its concentration between 1 and 100 μM. The mean membrane capacitance of single β-cells was 6.7 ± 0.6 pF ($n = 9$) and the input conductance, when measured from the current amplitude produced by the 10 mV depolarization pulses in the presence of 0.1 mM ATP and 0.1 mM ADP, was 2400 ± 430 pS/pF ($n = 9$). Input membrane conductance was measured after reaching a steady state level during the superfusion of various concentrations of cibenzoline (usually 1, 3, 10, 30 and 100 μM as a sequence) and the normalized conductance was plotted in Figure 4.

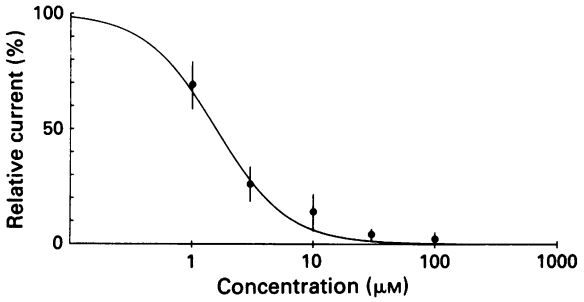


Figure 4 Dose-response relations of cibenzoline on the whole-cell K_{ATP} channel current produced by a reduction of cytosolic ATP concentration. The whole-cell membrane current was monitored during application of 10 mV depolarized pulses with 100 ms duration from the holding potential of − 70 mV every 5 s. The pipette solution containing 0.1 mM ATP and 0.1 mM ADP was used to evoke the K_{ATP} channel current. The membrane conductance between − 70 mV and − 60 mV was measured in control and test solutions containing various concentrations of cibenzoline. The conductance measured during exposure of cibenzoline was normalized with respect to the fraction of tolbutamide-sensitive conductance determined by difference between the conductance in control and that on superfusing 100 μM tolbutamide at the beginning of experiments. The data at required concentration of cibenzoline were collected from the separate membrane patches ($n = 12$) and then plotted as a function of the concentration of cibenzoline. Vertical bars represent the mean ± s.e.mean. The curve was drawn according to the Hill equation with IC_{50} of 1.5 μM and the Hill coefficient of 1.5.

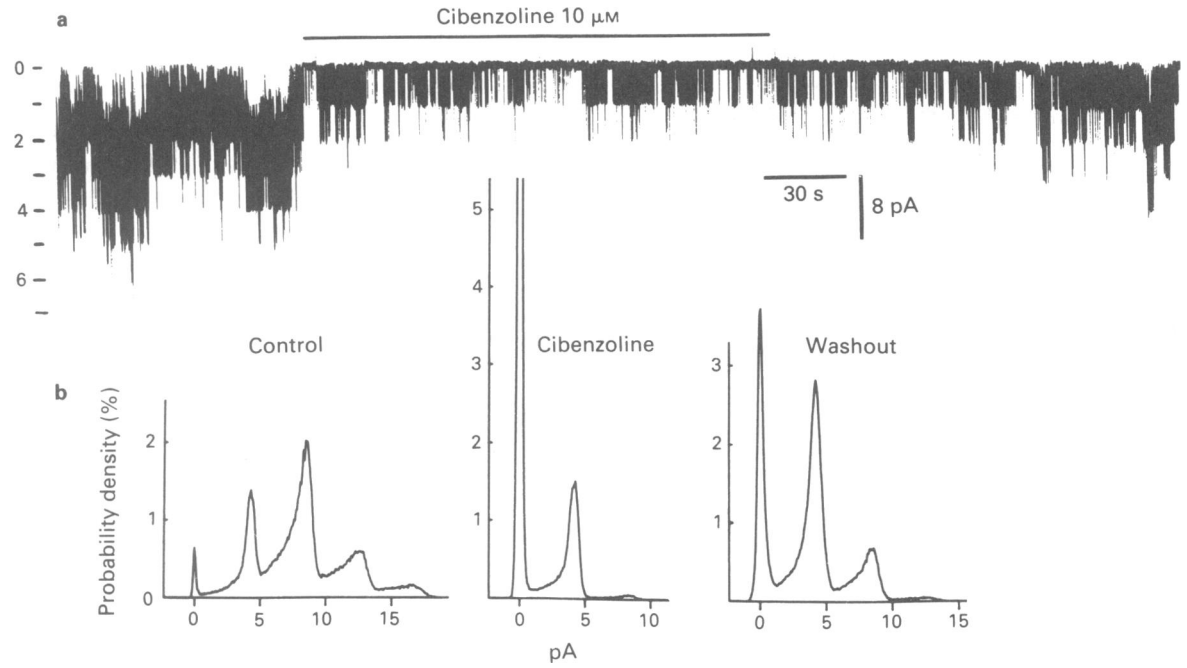


Figure 5 The effect of cibenzoline on the K_{ATP} channel in the inside-out patch. The K^+ concentration of both internal and pipette solutions was 140 mM and the patch membrane potential was held at − 60 mV. The bathing solution contained 0.3 mM ATP and 0.1 mM ADP. (a) Single K_{ATP} channel current was recorded in an inside-out membrane patch in the presence and absence of 10 μM cibenzoline. The number beside the current trace indicates the open channel level. (b) The amplitude histograms corresponding to that in the control (left), during cibenzoline exposure (middle) and after washout of the agent (right). The ordinate scale expresses the percentage of total sampled points and the abscissa scale the bin size in units of 0.04 pA. The data were low-pass filtered at 1 kHz and sampled at 5 kHz.

Cibenzoline inhibited the K_{ATP} channel in a dose-dependent manner. The curve was drawn by fitting the data to the equation (see Methods) with an IC_{50} of 1.5 μM and a Hill coefficient of 1.5 by using a least squares method.

Cibenzoline was also an effective inhibitor of K_{ATP} channel activity when exposed to the channels via the inside-out membrane configuration. In Figure 5a, the chart recording of the opening of K_{ATP} channels is illustrated, where at least 7 channels were present in this membrane patch and 10 μM cibenzoline inhibited these channels. The inhibitory effect of cibenzoline was partially reversible as described later at a concentration of 10 μM but irreversible at 100 μM ($n = 5$) for at least 30 min superfusion with washout solution. Amplitude histograms constructed by continuous sampling for more than 30 s before, during and after applying 10 μM cibenzoline are shown (Figure 5b). The single channel amplitude was unaffected and was 4.3 pA in the presence and absence of cibenzoline. The conductance of the single K_{ATP} channel was also unchanged by cibenzoline (not shown). The mean patch current was 8.1 pA, 1.0 pA and 3.6 pA before, during and after exposure of cibenzoline, respectively. Consequently the open probability of the K_{ATP} channel was 0.27 in control, assuming the total number of the channels to be 7. Cibenzoline decreased the mean patch current to 12% of control and this was in good agreement with that found in the whole-cell clamp experiment with respect to the channel inhibition by the agent (Figure 4). It can be suggested that cibenzoline decreased the probability of the channel being open or that this agent decreased the number of the channels in the membrane patch. In the presence of cibenzoline the number of openings of overlapped K_{ATP} channels was 2 (the middle histogram in Figure 5b). When we attempted to fit the probability that a given number of channels were open by using binominal analysis, we found that channel inhibition by cibenzoline could be due to both a reduction of the number of channels and open probability or due to an equal decrease in open probability without a change in the number of channels.

Discussion

In the present paper we have demonstrated that the ATP-sensitive K^+ channels in pancreatic β-cells are inhibited by the antiarrhythmic agent, cibenzoline. It is likely that such inhibition of the K_{ATP} channel results in depolarization of the β-cell membrane and evokes an increase in the cytosolic Ca^{2+} concentration followed by stimulation of insulin secretion even at subthreshold glucose levels. We suggest that this effect is, in part, a cause of hypoglycaemia with hyperinsulinemia induced by this agent (Houdent *et al.*, 1991; Bertrand *et al.*, 1992). Bertrand *et al.* (1992) demonstrated that cibenzoline directly stimulates insulin secretion from an isolated pancreas. They showed that cibenzoline at 2 μM did not increase insulin release when the glucose concentration was 4.2 mM but 6 μM cibenzoline did. Since the concentrations they used were located on the steepest part of the dose-inhibition curve of the K_{ATP} channel for cibenzoline (Figure 4), these concentrations seem to be critical for the stimulation of insulin secretion.

Since cibenzoline is used clinically as an antiarrhythmic drug, it certainly affects ionic channels other than the K_{ATP} channel. In cardiac myocytes, the voltage-dependent Ca^{2+} channel has been reported to be inhibited by cibenzoline (Holck & Osterrieder, 1986; Matsuoka *et al.*, 1991). The 50% inhibition for the peak Ca^{2+} currents was 14 μM (Holck & Osterrieder, 1986) or 30 μM (Matsuoka *et al.*, 1991). These values are greater than that found (1.5 μM) in the present study.

Cibenzoline blocked the K_{ATP} channel from both sides of the membrane and can reach its site of action in the channel even when applied via the bathing solution in the cell-attached mode without direct exposure to the patch membrane. These observations suggest that cibenzoline can diffuse within the lipid membrane. These actions of the agent resemble those of tolbutamide which inhibits the K_{ATP} channel from both the outside and inside of the β-cell membrane (Trube *et al.*, 1986). We observed poor reversibility of the channel inhibition by cibenzoline over 1 μM in whole-cell voltage clamp experiments. Bertrand *et al.* (1992) demonstrated a sustained increase in insulin release by 6 μM cibenzoline during washout of the agent. We speculate that cibenzoline may be retained in the lipid membrane and may not diffuse out of the membrane. The ability of cibenzoline to block the channel was also similar to that of tolbutamide in that 50% inhibition occurred at 6–10 μM (Trube *et al.*, 1986).

It has been reported that cibenzoline-induced hypoglycaemia is associated with hyperinsulinemia (Lefort *et al.*, 1988; Houdent *et al.*, 1991). In such cases, cibenzoline may potentiate insulin secretion in response to the plasma glucose level and induce inappropriate secretion of insulin even at non-stimulatory concentrations of plasma glucose. In clinical use, it is known that the plasma cibenzoline concentration in normal man can reach a maximum of approximately 1.0 μM (Massarella *et al.*, 1986). This level may be exceeded when renal function is reduced as the kidney is the major organ by which this agent is excreted (Aronoff *et al.*, 1991). However, we do not exclude the possibility that the extra-islet actions of cibenzoline, such as enhanced glucose utilization, may cause cibenzoline-induced hypoglycaemia with normal insulin levels as has been reported elsewhere (Hilleman *et al.*, 1987; Gachot *et al.*, 1988; Jeandel *et al.*, 1988; Houdent *et al.*, 1991).

This work was supporterd by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan and Japan Diabetic Foundation.

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(Received December 8, 1992
Revised March 16, 1993
Accepted April 7, 1993)

Br. J. Pharmacol. (1993), **109**, 1232–1238 © Macmillan Press Ltd, 1993

Induction by endogenous noradrenaline of an α_1-adrenoceptor-mediated positive inotropic effect in rabbit papillary muscles

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1 The possible involvement of α_1-adrenoceptors in the inotropic and electrophysiological responses to endogenous noradrenaline released by tyramine was examined in rabbit papillary muscles.

2 A concentration-dependent positive inotropic effect was produced by tyramine. This effect of tyramine was not observed in muscles from rabbits pretreated with reserpine.

3 The positive inotropic effect of tyramine was greatly inhibited by propranolol, but not altered by prazosin. However, when β-adrenoceptors were blocked by pretreatment with propranolol, tyramine still produced a positive inotropic effect, an effect which was antagonized by prazosin.

4 Tyramine caused a decrease in action potential duration (APD) and an increase in action potential amplitude in a concentration-dependent manner. Isoprenaline also produced the same electrophysiological effects. These electrophysiological effects of both agents were inhibited by propranolol.

5 When β-adrenoceptors were blocked by propranolol, the observed prazosin-sensitive positive inotropic effect of tyramine was not accompanied by any change in APD. In contrast, APD was markedly prolonged by α_1-adrenoceptor stimulation with phenylephrine in the presence of propranolol, in association with the positive inotropic effect.

6 It is concluded that in rabbit papillary muscles, endogenous noradrenaline causes a positive inotropic effect predominantly mediated by β-adrenoceptors, but can still evoke a positive inotropic effect through α_1-adrenoceptors when β-adrenoceptor stimulation is eliminated. This suggests that the α_1-adrenoceptor-mediated positive intropic mechanism(s) may be masked by simultaneous activation of β-adrenoceptors. In addition, this study indicates that APD prolongation is not involved in the α_1-adrenoceptor-mediated inotropic responses to endogenous noradrenaline.

Keywords: Tyramine; endogenous noradrenaline; α_1-adrenoceptors; β-adrenoceptors; positive inotropic effect; action potential

Introduction

It is generally accepted that stimulation of myocardial α_1-adrenoceptors results in a positive inotropic effect in various mammalian species (for reviews, see Scholz, 1980; Schümann, 1980; Benfey, 1982). The question has remained unsettled as to whether myocardial α_1-adrenoceptors have a functional or physiological role in the regulation of myocardial contractility. Earlier studies have usually failed to demonstrate that the positive inotropic response to noradrenaline is antagonized by the α-adrenoceptor antagonists such as phentolamine in rabbit papillary muscles (Schümann *et al.*, 1974; Endoh *et al.*, 1976), in human atria (Schümann *et al.*, 1978; Wagner *et al.*, 1980) and in human papillary muscles (Jakob *et al.*, 1988). These authors have concluded that noradrenaline, in contrast to adrenaline and phenylephrine, does not evoke a significant α_1-adrenoceptor-mediated inotropic response. However, Osnes and his collaborators (Aass *et al.*, 1983; Skomedal *et al.*, 1988) have found that when β-adrenoceptors are extensively blocked, noradrenaline is capable of evoking a positive inotropic effect through α_1-adrenoceptors in rat and rabbit papillary muscles. Thus, demonstration of the α_1-adrenoceptor-mediated positive inotropic response to noradrenaline appears to require extensive β-adrenoceptor blockade. It was therefore assumed that the expression of the inotropic response to α_1-adrenoceptor stimulation might be restricted by simultaneous stimulation of β-adrenoceptors when both adrenoceptors are activated (Skomedal *et al.*, 1988; 1990).

The positive inotropic effect of α_1-adrenoceptor stimulation is associated with sarcolemmal Ca^{2+} influx and a resultant elevation of peak Ca^{2+} transients (Endoh & Blinks, 1988). Recently, stimulation of myocardial α_1-adrenoceptors has been shown to cause an inhibition of the transient outward current (Apkon & Nerbonne, 1988; Fedida *et al.*, 1989; Tohse *et al.*, 1990). Inhibition of this current could result in prolongation of APD, leading to an elevation of $[Ca^{2+}]_i$ by increased Ca^{2+} influx during depolarization. Whereas the mechanism of the β-adrenoceptor-mediated positive inotropic effect is related to an increase in Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Tsien, 1977; Kameyama *et al.*, 1985) and to an increase in the accumulation rate of Ca^{2+} into the sarcoplasmic reticulum (Tada & Katz, 1982), it is known that activation of myocardial β-adrenoceptors causes a marked decrease in APD (Kass & Wiegers, 1982; Nakaya *et al.*, 1990) possibly due to activation of the delayed rectifier potassium current and/or the chloride current (Walsh & Kass, 1988; Harvey & Hume, 1989). Thus, it is possible that this opposite action on APD to α_1-adrenoceptor stimulation might mask the positive inotropic effect mediated by α_1-adrenoceptors during simultaneous stimulation of α_1- and β-adrenoceptors by noradrenaline.

The present study was undertaken to determine whether endogenous noradrenaline can reveal a positive inotropic effect through α_1-adrenoceptor stimulation in rabbit papillary muscles. For this purpose, we used tyramine which releases endogenous noradrenaline by stoichiometric displacement after being taken up into nerve endings (Trendelenburg, 1972). The results indicate that endogenous noradrenaline released by tyramine produced a positive inotropic response mediated by α_1-adrenoceptors, only when the effect was unopposed by β-adrenoceptor stimulation, as seen with exogenous noradrenaline (Aass *et al.*, 1983; Skomedal *et al.*, 1988). Electrophysiological experiments were further carried out to address the possibility that the opposite effect of β-adrenoceptor stimulation on APD might contribute to the restricted expression of the α_1-adrenoceptor-mediated ino-

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tropic response during simultaneous adrenoceptor activation by endogenous noradrenaline. For comparison, we also characterized the inotropic and electrophysiological effects of phenylephrine (in the presence of propranolol) and isoprenaline as representative exogenous stimulation of α_1- and β-adrenoceptors, respectively.

Methods

Measurement of inotropic responses

New Zealand White rabbits of either sex weighing 2–3 kg were killed by a blow on the head. The hearts were quickly removed and transferred to a dissection bath filled with oxygenated warm Krebs-Henseleit solution. The composition of the solution was (mM): NaCl 119, $CaCl_2$ 2.5, KCl 4.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.9 and glucose 10.0. The right ventricular papillary muscles were carefully dissected from the heart. The muscle was mounted under 0.5 g of resting tension in a water-jacketed organ bath containing 10 ml of Krebs-Henseleit solution. The solution in the bath was bubbled with 95% O_2 and 5% CO_2 and its temperature was maintained at 30 ± 1°C. The muscle was stimulated by rectangular pulses of 5 ms duration and 1.5 times the threshold voltage of 0.5 Hz frequency. The pulses were delivered by a pair of spiral platinum electrodes connected to an electronic stimulator (Sanei-Sokki, 3F46, Tokyo, Japan) through an isolation unit (Sanei-Sokki, 5361). Isometric tension developed in the preparation was measured with a force transducer (Nihon Kohden, TB-612T, Tokyo, Japan) and recorded on a pen recorder (Nihon Kohden, RJG-3026) through a preamplifier (Nihon Kohden, RP-5). The preparations were allowed to equilibrate for at least 60 min before the experiments were begun.

The concentration-response curves for the positive inotropic effects of isoprenaline, phenylephrine and tyramine were determined in a cumulative manner by increasing the concentration of the tested agonists in steps of 0.5 log units (van Rossum, 1963). When propranolol and prazosin were used, they were added to the bath 60 min before the administration of the tested agents.

In some experiments, the papillary muscles were obtained from the rabbits pretreated with reserpine. The animals received reserpine, 1 mg kg^{-1} i.p., for 3 days and were used on the 4th day. Reserpine was dissolved in distilled water containing 10% ascorbic acid.

Measurement of electrophysiological responses

Rabbit right ventricular papillary muscles were prepared as described above. The muscle was transferred to a tissue bath of 5 ml in volume and superfused at a rate of 10 ml min^{-1} with Krebs-Henseleit solution gassed with 95% O_2 and 5% CO_2. The temperature of perfusate was kept constant at 30 ± 1°C.

One end of the muscle was hooked to the lever arm of a force transducer (Nihon Kohden, TB-651T) mounted on a micromanipulator and the other was pinned to the bottom of the tissue chamber. Resting tension of the preparation was increased to 200 mg gradually. The muscle was stimulated by rectangular pulses of 0.5 Hz frequency, 1 ms duration and 1.5 times threshold voltage, delivered through platinum field electrodes connected to an electronic stimualtor (Nihon Kohden, SEN3101) and an isolation unit (Nihon Kohden, 22101J). Transmembrane potentials were recorded using glass microelectrodes filled with 3 M KCl (tip resistance, 10–30 MΩ). The microelectrode was coupled via an Ag/AgCl junction to a high impedance capacitance neutralizing amplifier (Nihon Kohden, MEZ8301). An agar bridge containing 3 M KCl was used as a reference electrode. An electronic differentiator whose output was linear from 50–1000 V s^{-1} was used for measuring the maximum rate of rise ($\dot{V}_{max}$) of action potentials. These amplified signals were displayed on an oscilloscope (Nihon Kohden, VC-10), photographed on 35 mm film and recorded on a chart recorder (Watanabe Sokki, WR3101, Tokyo, Japan). Further details of the experimental procedure have been described elsewhere (Hattori *et al.*, 1990).

The preparations were allowed to equilibrate for at least 90 min before impalement by the microelectrode. We included in the study only the experiments in which a stable impalement was maintained throughout the protocol.

Drugs

The following compounds were used: (−)-isoprenaline HCl, (−)-phenylephrine HCl and (±)-propranolol HCl (Sigma, St. Louis, MO, U.S.A.); tyramine HCl (Wako, Osaka, Japan); prazosin HCl (Taito-Pfizer, Tokyo, Japan); and reserpine (Aldrich, Milwaukee, WI, U.S.A.). All drugs except reserpine (see above) were dissolved in distilled water and further dilutions were made with Krebs-Henseleit solution. Ascorbic acid (0.1 mM) was added to the solution of isoprenaline to retard its auto-oxidation.

Statistics

All values are presented as means ± s.e.mean. Analysis by Student's *t* test was performed for paired and unpaired observations. When appropriate, statistically significant differences were assessed by analysis of variance with repeated measurements. A *P* value less than 0.05 was considered an indication of statistical significance.

Results

Inotropic and electrophysiological effects of isoprenaline and phenylephrine

Figure 1 shows concentration-response curves for the positive inotropic effects of isoprenaline and phenylephrine in rabbit right ventricular papillary muscles electrically driven at 0.5 Hz. The inotropic response to phenylephrine was evaluated in the presence of 1 μM propranolol to eliminate its weak β-adrenoceptor agonistic action. Propranolol (1 μM)

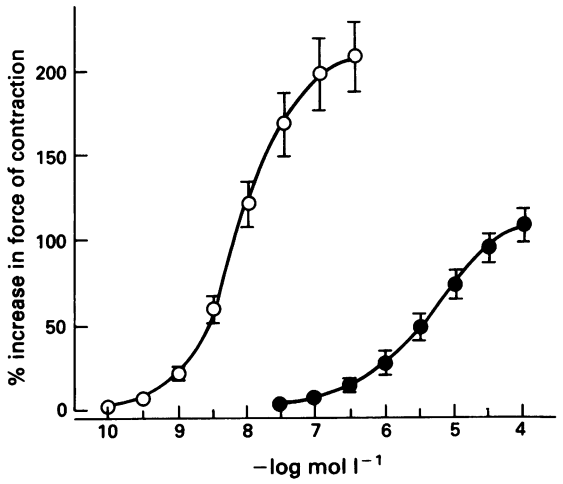


Figure 1 Concentration-response curves for the positive inotropic effects of isoprenaline (○) and phenylephrine (●). Propranolol at 1 μM was added 60 min before the addition of phenylephrine. Points (mean ± s.e.mean, $n = 9–10$) represent the net increases in force of contraction expressed as a percentage of basal values recorded before the addition of the agonists. The force of contraction was measured when it reached the maximum in response to the respective concentration of the agonists. Basal force of contraction was 441 ± 53 mg ($n = 19$).

itself did not affect force of contraction. The positive inotropic effect produced by phenylephrine after β-adrenoceptor blockade with 1 μM propranolol was exclusively mediated by α_1-adrenoceptors, since its concentration-response curve was displaced rightward by prazosin in an apparently competitive manner (data not shown). The maximum increases in force of contraction induced by β-adrenoceptor stimulation (isoprenaline) and by α_1-adrenoceptor stimulation (phenylephrine) were 211 ± 21% ($n = 9$) and 109 ± 10% ($n = 10$), respectively. Thus, the maximum positive inotropic effect of α_1-adrenoceptor stimulation was about 52% of that of β-adrenoceptor stimulation.

Representative results showing the effects of isoprenaline and phenylephrine on action potential configuration and force of contraction are depicted in Figure 2. The positive inotropic effect of 1 nM isoprenaline was accompanied with a decrease in APD. On the other hand, APD was prolonged in association with the positive inotropic effect of 1 μM phenylephrine in the presence of 1 μM propranolol. At a concentration of 1 μM, propranolol had no significant effect on action potential parameters.

As shown in Figure 3, isoprenaline caused concentration-dependent decreases in APD_{20}, APD_{50} as well as APD_{90}. In addition, isoprenaline produced an increase in action potential amplitude (APA). At a concentration of 1 nM isoprenaline significantly increased APA from 113.7 ± 1.6 to 116.4 ± 1.5 mV ($n = 7$, $P < 0.01$). These electrophysiological effects of isoprenaline were blocked by 1 μM propranolol (data not shown). Neither resting membrane potential nor $\dot{V}_{max}$ was altered by isoprenaline at concentrations up to 10 nM.

In contrast to isoprenaline, phenylephrine in the presence of propranolol increased APD_{20}, APD_{50} and APD_{90} in a concentration-dependent manner (Figure 3). It also caused a significant increase in APA: APA was increased from 112.4 ± 1.8 to 114.0 ± 1.8 mV ($n = 5$, $P < 0.05$) by 5 μM phenylephrine. The increasing effects of phenylephrine on APDs and APA were antagonized by 1 μM prazosin (data not shown). Resting membrane potential and $\dot{V}_{max}$ remained unchanged with phenylephrine.

Inotropic and electrophysiological effects of tyramine

Tyramine produced an increase in force of contraction in a concentration-dependent manner (Figure 4). The maximum increase by tyramine was about 37% of that by isoprenaline. The positive inotropic response to tyramine was abolished in papillary muscles obtained from the reserpine-treated rabbits: tyramine (100 μM) did not induce significant change in force of contraction in reserpinized muscles (1.4 ± 2.0% of basal force of contraction, $n = 5$). This finding indicates that the inotropic response to tyramine is attributable to endogenous noradrenaline released from reserpine-sensitive pools. The concentration-response curve for the positive inotropic effect of tyramine was not affected by pretreatment with 1 μM prazosin (Figure 4). On the other hand, pretreatment with 1 μM propranolol markedly inhibited the positive inotropic

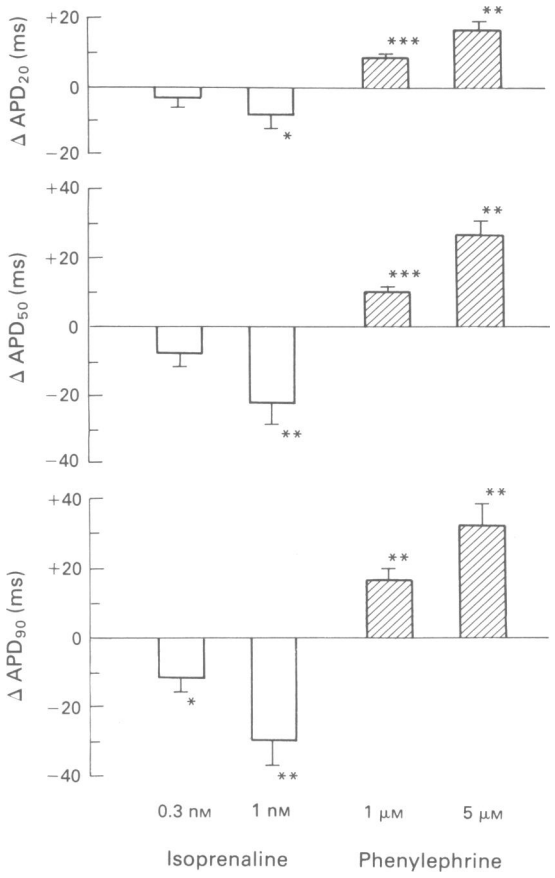


Figure 3 Concentration-dependent effects of isoprenaline and phenylephrine of APD_{20}, APD_{50} and APD_{90}. Phenylephrine was added in the presence of 1 μM propranolol. The APD changes induced by the agonists were measured 10 min after the addition. Columns represent means ± s.e.mean of 5–7 experiments. Basal APD_{20}, APD_{50} and APD_{90} were 98 ± 6, 159 ± 8 and 208 ± 9 ms ($n = 13$), respectively. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs the corresponding values before the addition of the agonists.

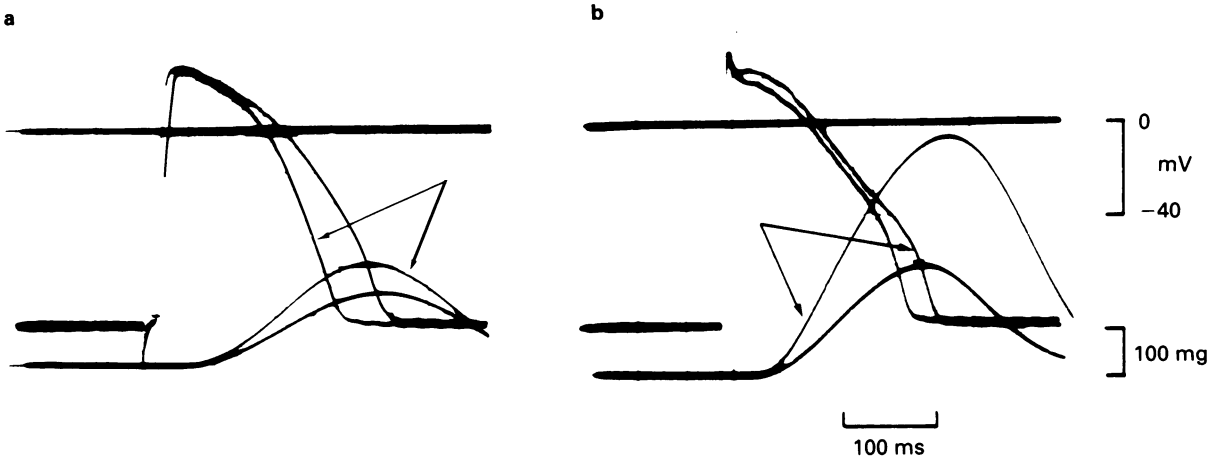


Figure 2 Typical effects of 1 nM isoprenaline (a) and 1 μM phenylephrine (b) on action potential and force of contraction. The effect of phenylephrine was measured in the presence of 1 μM propranolol. Superimposed records before and 10 min after the addition of isoprenaline (a) and phenylephrine (b) are shown. Arrows indicate traces after application of the agonists.

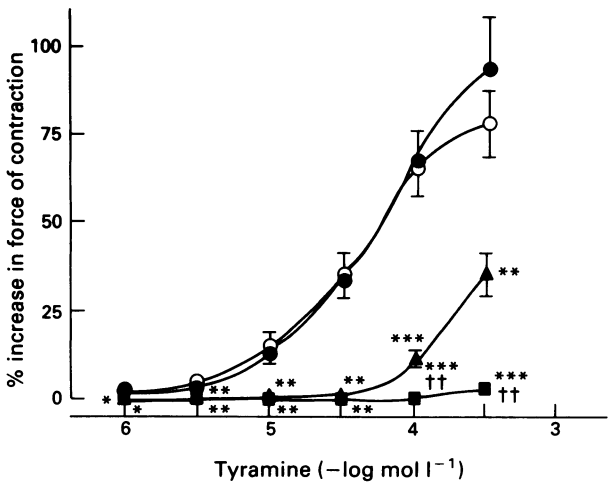


Figure 4 Concentration-response curves for the positive inotropic effect of tyramine in the absence (○) and presence of 1 μM prazosin (●), 1 μM propranolol (▲) and 1 μM prazosin and 1 μM propranolol (■). Points (means ± s.e.mean, $n = 6-7$) represent the net increase in force of contraction expressed as a percentage of basal values recorded before the addition of tyramine. The force of contraction was measured when it reached the maximum in response to the respective concentration of tyramine. Basal force of contraction was 730 ± 83 mg ($n = 26$). $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ vs the corresponding values with tyramine alone. ††$P<0.01$ vs the values obtained in the presence of propranolol.

response to tyramine (Figure 4). Thus, the increase in force of contraction in response to endogenous noradrenaline released by tyramine appears to be mediated predominantly by β-adrenoceptors. However, tyramine at higher concentrations (⩾ 100 μM) produced a substantial increase in force of contraction even in the presence of propranolol. Further addition of prazosin to propranolol completely eliminated the positive inotropic effect of tyramine at concentrations of 100 and 300 μM (Figure 4).

The concentration-dependent effects of tyramine on action potential parameters are summarized in Figure 5. Tyramine produced concentration-dependent decreases in APD_{20}, APD_{50} and APD_{90}. APA was increased by tyramine in a concentration-dependent fashion. These electrophysiological effects of tyramine were similar to those of isoprenaline. Resting membrane potential was significantly decreased by tyramine at a high concentration (100 μM). This appears to be due to excess stimulation of β-adrenoceptors, because we have previously demonstrated that isoprenaline at higher concentrations (⩾ 30 nM) produces a membrane depolarization in guinea-pig papillary muscles (Nakaya *et al.*, 1990). Tyramine did not produce a significant change in $\dot{V}_{max}$.

As expected, the tyramine-induced decreases in APDs were significantly inhibited by pretreatment with 1 μM propranolol (Figures 6 and 7). In the presence of propranolol, tyramine still produced a positive inotropic effect, an affect which was completely abolished by further pretreatment with prazosin (Figure 6). However, any increase in APDs was not observed with tyramine in the presence of propranolol (Figure 7).

Discussion

From our study the results indicate that the positive inotropic effect of endogenous noradrenaline released by tyramine in rabbit papillary muscles is predominantly mediated by β-adrenoceptors. Support for this is provided by the findings that the concentration-response curve for the positive inotropic effect of tyramine was displaced downward and to the right by propranolol but remained unchanged with prazosin. However, when β-adrenoceptors were adequately blocked by propranolol, tyramine produced a prazosin-sensitive positive inotropic effect. In recent work, we have demonstrated that 5-hydroxytryptamine is capable not only of inducing a release of noradrenaline from nerve endings but also of blocking β-adrenoceptors in rabbit papillary muscles, resulting in an appearance of the α_1-adrenoceptor-mediated positive inotropic effect in response to released noradrenaline (Hattori *et al.*, 1992). Thus, endogenous noradrenaline

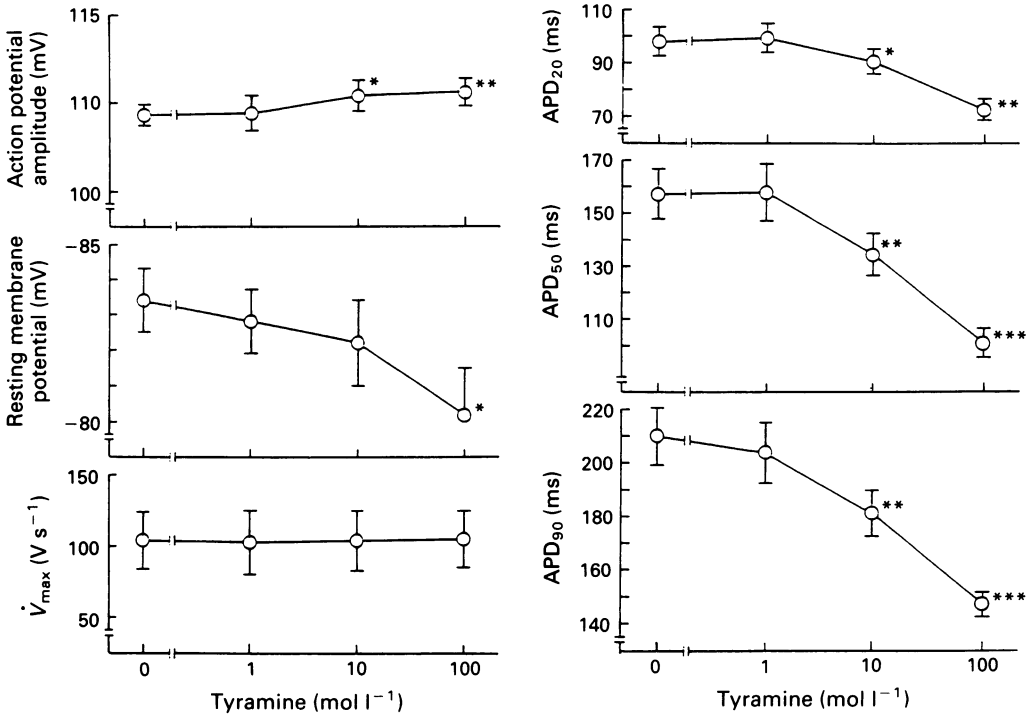


Figure 5 Concentration-response curves for the effects of tyramine on action potential parameters. Tyramine was added to the organ bath in a cumulative way. The effects of tyramine were measured 10 min after its addition. Points are means ± s.e.mean of 5 experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs the corresponding control values.

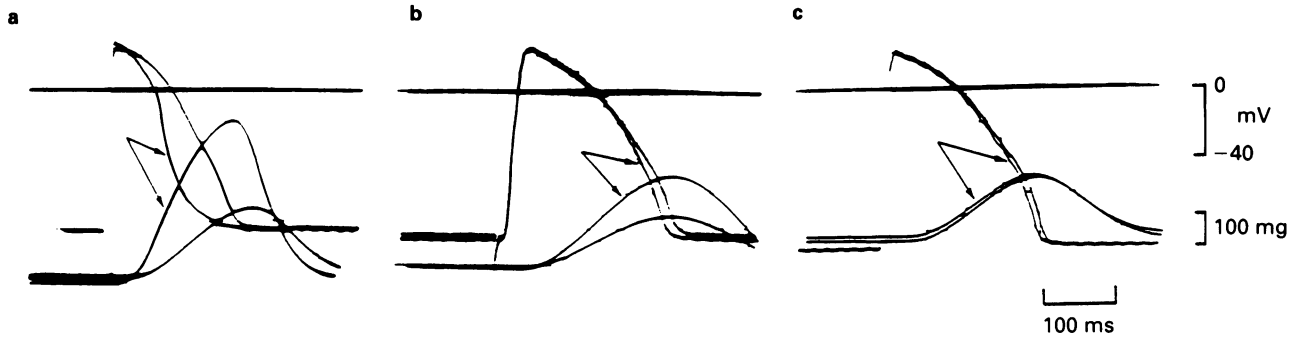


Figure 6 Typical effects of tyramine on action potential and force of contraction in the absence (a) and presence of 1 μM propranolol (b) and 1 μM propranolol plus 1 μM prazosin (c). Propranolol and prazosin were added to the organ bath 30 min before application of tyramine. Superimposed records before and 10 min after the addition of 100 μM tyramine are shown. Arrows indicate traces after application of tyramine.

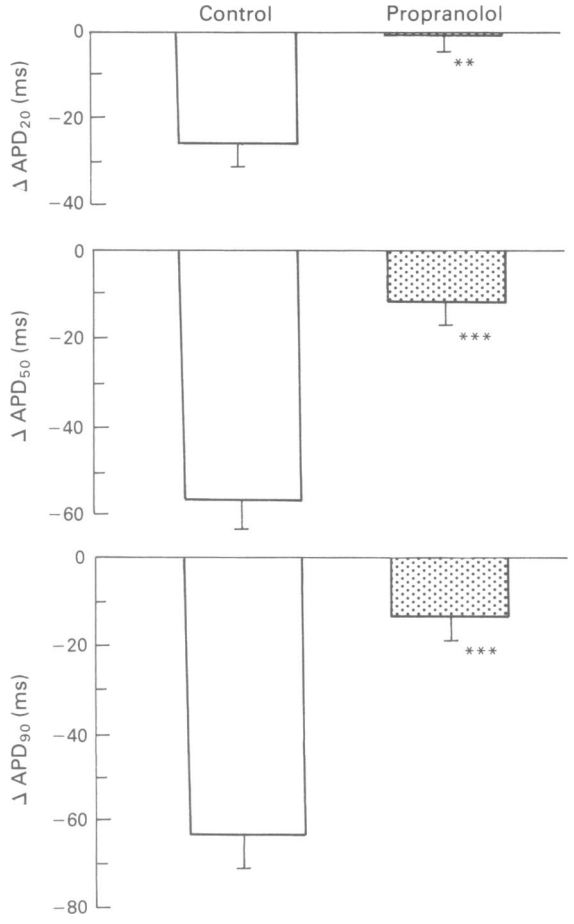


Figure 7 Influence of propranolol on the effect of tyramine on APD_{20} APD_{50} and APD_{90}. The APD changes induced by 100 μM tyramine were measured 10 min after its administration. Propranolol at 1 μM was added to the organ bath 30 min before tyramine. Columns represent means ± s.e.mean of 5–7 experiments. Basal APD_{20}, APD_{50} and APD_{90} were 97 ± 5, 156 ± 6 and 205 ± 6 ms ($n = 12$), respectively. $^{**}P < 0.01$; $^{***}P < 0.001$ vs the corresponding values with tyramine alone.

appears to cause an increase in force of contraction through activation of α_1-adrenoceptors only when β-adrenoceptor stimulation is eliminated. Our results obtained with tyramine and 5-hydroxytryptamine are consistent with and extend earlier findings that exogenous noradrenaline can exhibit an α_1-adrenoceptor-mediated positive inotropic effect after extensive blockade of β-adrenoceptors (Aass *et al.*, 1983; Skomedal *et al.*, 1988). The potencies of noradrenaline are reported to be equivalent in inhibiting the α-adrenoceptor ligand [^{3}H]-dihydroergocriptine binding and the β-adrenoceptor ligand (−)-[^{3}H]-alprenolol binding to cardiac membranes (Alexander *et al.*, 1975; Williams & Lefkowitz, 1978). Furthermore, the number of α_1-adrenoceptors is quite similar to that of β-adrenoceptors in rabbit heart (Mukherjee *et al.*, 1983). Therefore, we assume that, when β-adrenoceptors are activated concomitantly with α_1-adrenoceptors, the mechanism(s) underlying the positive inotropic response to α_1-adrenoceptors may be counteracted by simultaneous stimulation of β-adrenoceptors.

Tyramine caused a concentration-dependent decrease in APD. The same effect could be obtained with isoprenaline and could be suppressed with propranolol. Thus, the APD shortening in response to endogenous noradrenaline released by tyramine is likely to be due to mediation through β-adrenoceptors. In contrast to β-adrenoceptor stimulation, activation of α_1-adrenoceptors is known to prolong APD in various cardiac tissues (Pappano, 1971; Giotti *et al.*, 1973; Miura & Inui, 1984; Dukes & Vaughan-Williams, 1984; Tohse *et al.*, 1987). We also found that α_1-adrenoceptor stimulation with phenylephrine (in the presence of propranolol) produced significant APD prolongation in rabbit papillary muscles. The opposing effects of α_1- and β-adrenoceptor stimulation on APD appear to involve different ionic mechanisms. Activation of the delayed rectifier potassium current and/or the chloride current contributes to the β-adrenoceptor-mediated APD shortening (Walsh & Kass, 1988; Harvey & Hume, 1989). Suppression of the transient outward current is related to the α_1-adrenoceptor-mediated APD prolongation (Apkon & Nerbonne, 1988; Fedida *et al.*, 1989; Tohse *et al.*, 1990). However, when β-adrenoceptors are activated concomitantly with α_1-adrenoceptors, the β-adrenoceptor-mediated APD shortening might apparently disturb the α_1-adrenoceptor-mediated APD prolongation, and the balance between these opposing actions could ultimately determine APD. Since prolongation of APD would lead to an elevation of $[Ca^{2+}]_i$ by increasing Ca^{2+} influx during the plateau phase, it was suggested that the α_1-adrenoceptor-mediated APD prolongation may be important and responsible for an increase in force of contraction (Miura & Inui, 1984; Dukes & Vaughan-Williams, 1984). If the APD prolongation is primarily involved in the positive inotropic response mediated by α_1-adrenoceptors, the APD shortening elicited by β-adrenoceptor stimulation might mask full expression of the α_1-adrenoceptor-mediated positive inotropic response during simultaneous activation of both adrenoceptors.

After β-adrenoceptor blockade with propranolol, endogenous noradrenaline released by tyramine evoked a prazosin-sensitive positive inotropic effect, i.e. an α_1-adrenoceptor-mediated one. However, the α_1-adrenoceptor-mediated positive inotropic effect was not accompanied by APD prolongation. Even in the presence of propranolol, tyramine still exhibited a small decrease in APD (see Figures 6 and 7). It is possible that

a substance other than noradrenaline might be released from cardiac nerve endings in response to tyramine and could be involved in the propranolol-resistant APD shortening. Nevertheless, it should be emphasized that tyramine can evoke a positive inotropic effect through α_1-adrenoceptors without producing APD prolongation. Alternatively, the α_1-adrenoceptor-mediated positive inotropic effect induced by endogenous noradrenaline seems to be independent of changes in APD. Furthermore, these results exclude the possibility that the β-adrenoceptor-mediated APD shortening might be involved in the restricted expression of the α_1-adrenoceptor-mediated positive inotropic response when both adrenoceptors are simultaneously activated.

It has been postulated that the mechanisms underlying the positive inotropic effect of α_1-adrenoceptor stimulation in rabbit papillary muscles are related to at least two Ca^{2+}-mediated processes: an increase in the intracellular Ca^{2+} transient and an increase in Ca^{2+} sensitivity of contractile proteins (Endoh & Blinks, 1988). The amplitude of the intracellular Ca^{2+} transient could be increased by the increased Ca^{2+} influx as a result of APD prolongation when α_1-adrenoceptors are stimulated with phenylephrine. However, the increase in the amplitude of the Ca^{2+} transient produced by α_1-adrenoceptor stimulation is much smaller than that by increased $[Ca^{2+}]_o$, when compared at the same level of inotropic responses (Endoh & Blinks, 1988). In this context, it seems very likely that the increase in Ca^{2+} sensitivity of contractile proteins may largely account for the α_1-adrenoceptor-mediated positive inotropic response to endogenous noradrenaline, since we found that endogenous noradrenaline evoked this inotropic response with little influence on APD. Resent study on skinned rat ventricular myocytes has also demonstrated that α_1-adrenoceptor stimulation leads to an increase in responsiveness of the myofilaments to Ca^{2+} (Puceat *et al.*, 1990). On the other hand, stimulation of β-adrenoceptors results in a decrease in the Ca^{2+} sensitivity of contractile proteins (Endoh & Blinks, 1988; Puceat *et al.*, 1990). It therefore is likely that the decrease in myofibrillar Ca^{2+} sensitivity caused by β-adrenoceptor simulation could mask the α_1-adrenoceptor-mediated positive inotropic response. This may explain the reason why β-adrenoceptors must be extensively blocked in order to reveal the α_1-adrenoceptor-mediated inotropic response.

Recently, Guse *et al.* (1991) have reported that stimulation of β-adrenoceptors attenuates the α_1-adrenoceptor-mediated formation of inositol phosphates in cultured rat ventricular myocytes. If there is a close association of phosphoinositide hydrolysis and its resultant products with the α_1-adrenoceptor-mediated positive inotropic effect, the inhibitory effect of β-adrenoceptor stimulation on the α_1-adrenoceptor-mediated phosphoinositide response might be responsible for the restricted positive inotropic response to α_1-adrenoceptor stimulation. It has been suggested that the positive inotropic effect of α_1-adrenoceptor stimulation may be mediated by inositol 1,4,5-trisphosphate through mobilization of intracellular Ca^{2+} and/or by 1,2-diacylglycerol through activation of protein kinase C (Otani *et al.*, 1988). However, the role of inositol 1,4,5-trisphosphate as an intracellular Ca^{2+} mobilizagent in cardiac muscles is still unclear (Movsesian *et al.*, 1985; Nosek *et al.*, 1986; Ehrlich & Watras, 1988). Also, direct activation of protein kinase C with tumour-promoting phorbol esters rather causes a negative inotropic action (Yuan *et al.*, 1987; Kushida *et al.*, 1988). Finally, the relation between the acceleration of phosphoinositide hydrolysis and the positive inotropic effect in response to α_1-adrenoceptor stimulation is still the subject of controversy (Endou *et al.*, 1991; Endoh *et al.*, 1991).

In conclusion, endogenous noradrenaline elicits an α_1-adrenoceptor-mediated positive inotropic effect in rabbit papillary muscles that is unmasked by extensive blockade of β-adrenoceptors. The α_1-adrenoceptor-mediated positive inotropic mechanism(s) may be masked when β-adrenoceptors are simultaneously activated. The present data also suggest that the APD prolongation which is usually induced by α_1-adrenoceptor agonists such as phenylephrine does not appear to be a primary mechanism of the α_1-adrenoceptor-mediate positive inotropic effect in rabbit papillary muscles.

The authors thank Mr Y. Shirakura for his excellent technical assistance and Miss R. Yamazaki for her secretarial assistance. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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(Received December 22, 1992
Revised March 30, 1993
Accepted April 7, 1993)

Br. J. Pharmacol. (1993), **109**, 1239–1246 © Macmillan Press Ltd, 1993

Differential inhibition of a transient K^+ current by chlorpromazine and 4-aminopyridine in neurones of the rat dorsal root ganglia

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1 K^+ currents were recorded from neurones of the newborn rat cultured dorsal root ganglia, by a whole cell variation of the patch-clamp technique.

2 Chlorpromazine (CPZ), a neuroleptic, reversibly reduced the amplitude of the transient K^+ current (referred to as 'I_T' hereafter) with a dissociation constant (K_d) of 4.5 μM. The inhibition of the delayed rectifier K^+ current (I_{DR}) was much less potent (K_d, 120 μM). CPZ (100 μM) had no effect on the inward rectifier K^+ current.

3 The blocking action of CPZ on I_T was about seven times more potent than that of 4-aminopyridine (4-AP) which had a K_d of 31 μM. The inhibition of I_T followed one-to-one binding stoichiometry with both drugs.

4 The decay time course of I_T was not affected by CPZ, whereas 4-AP markedly accelerated the decay phase of I_T.

5 The steady-state inactivation curve of I_T was shifted in the negative direction (about 5 mV) by CPZ, whereas the curve was shifted in the positive direction (about 13 mV) by 4-AP.

6 The recovery from inactivation as measured by a conventional double pulse protocol was described by two exponential components in the control solution. CPZ markedly reduced the first component and slowed down the recovery from inactivation. In contrast, in the presence of 4-AP, the peak amplitude of I_T was rather increased by a preceding I_T possibly through voltage-dependent unbinding of 4-AP molecules.

7 These results indicate that CPZ has a preferential blocking action on I_T and the mechanism underlying this block is markedly different from the mechanism underlying the blocking action of 4-AP.

Keywords: Chlorpromazine; psychotropic drugs; neuroleptic; potassium channel; dorsal root ganglion; transient potassium current; aminopyridine; channel block; cultured neurone

Introduction

The neuroleptic drugs are chemically diverse compounds which are widely prescribed for the treatment of psychiatric disorders such as schizophrenia. Their antischizophrenic actions are thought to involve blockade of dopamine receptors in the brain (Seeman, 1981). In addition, it is well known that neuroleptics have quinidine-like direct membrane effects in the heart which may be related to some of their cardiovascular side actions (Risch *et al.*, 1981). A potent blocking action of chlorpromazine (CPZ), a phenothiazine neuroleptic, on cardiac Na^+ (Ogata & Narahashi, 1989; Ogata *et al.*, 1989), neuronal Na^+ (Ogata & Tatebayashi, 1989; Ogata *et al.*, 1990) and neuronal Ca^{2+} channels (Ogata & Narahashi, 1990) may explain the quinidine-like direct membrane effects of the neuroleptic drugs.

With the exception of a few drugs such as tetrodotoxin (TTX) for Na^+ channels, many of the blockers of ion channels are not absolutely selective in their blocking action. A number of papers reported a direct action of CPZ on Ca^{2+}-activated K^+ channels (e.g., Dinan *et al.*, 1987; McCann & Welsh, 1987). However, a direct action of CPZ on other types of K^+ channels remains to be demonstrated. An activation of voltage-gated K^+ channels plays a pivotal role as the 'neuronal brake' in regulation of cellular excitability, since K^+ currents counteract inward currents through Na^+ or Ca^{2+} channels. Voltage-gated K^+ channels form the largest and most diversified class of ion channels. Therefore, it seems of particular interest to examine responses to CPZ of different types of K^+ channels, since each channel type has different roles in cellular functioning (Adams & Nonner, 1990). We, therefore, investigated the action of CPZ on different types of K^+ currents in cultured neurones from rat dorsal root ganglia (DRG).

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Methods

Culture procedures

Dissociated cultures of rat DRG neurones were prepared as described by Tatebayashi & Ogata (1992). Rats (1 to 3 days old) were killed by decapitation under ether anaesthesia. DRG were dissected out and incubated at 36°C for 30–40 min in Ca^{2+}- and Mg^{2+}-free saline, containing 0.25% trypsin (Type XI, Sigma). The ganglia were then mechanically dissociated with a fire-polished Pasteur pipette. The cells were plated on glass cover-slips coated with poly-L-lysine (Sigma) and maintained in a humidified incubator containing 5% CO_2 in air at 35°C in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (GIBCO), penicillin (40 iu ml^{-1}), and streptomycin (40 ng ml^{-1}). After 1–2 days in culture, cytosine β-(+)-arabinofuranoside (Sigma) was added to cultures to suppress the growth of non-neuronal cells. Subsequent medium changes were done at 3–4 day intervals. Cells between 2–10 days in culture were used.

Electrical recording

The methods for electrical recording used were similar to those previously described (Ogata *et al.*, 1990). Membrane currents were recorded with the whole-cell patch-clamp tech-

nique (Hamill *et al.*, 1981). The d.c. resistance of suction electrodes was 1.5–3 MΩ. The pipette solution contained (in mM): K-glutamate 130, Mg-ATP 3, $MgCl_2$ 2.5, glucose 5, HEPES 5 and EGTA 5. The pH of the pipette solution was adjusted to 7.0 with KOH. The external solution contained (in mM): tetramethylammonium-Cl 120, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5 and glucose 25. The pH of the external solution was adjusted to 7.4 with NaOH. TTX at a concentration of 0.2 μM was added to block inward TTX-sensitive Na^+ current. Cells having TTX-insensitive Na^+ current (Ogata & Tatebayashi, 1992) were not used for experiments. Since the external solution was Na^+-free, a possible involvement of Na^+-dependent K^+ current (Coraboeuf & Carmeliet, 1982; Escande *et al.*, 1987; Bader *et al.*, 1990) was eliminated. Ca^{2+} currents were eliminated by 50 μM Cd^{2+} which completely blocked Ca^{2+} currents in rat DRG (Tatebayashi & Ogata, 1992). Thus, Ca^{2+}-dependent components of K^+ currents could be eliminated. Membrane currents passing through the pipette were recorded by a current-to-voltage converter designed by M. Yoshii (Ogata *et al.*, 1990). Compensation for the series resistance was performed by adding a part of the output voltage of the current recording to the command pulse.

All the experiments were performed with an on-line system which has been developed by M. Yoshii and N. Ogata, using a personal computer (PC-286V, EPSON, Tokyo, Japan). Capacitative and leakage currents were subtracted digitally by the P-P/4 procedure (Ogata *et al.*, 1990). Exponential fits were determined by computer using a non-linear sum of the least squares fitting routine. The liquid junction potential between internal and external solutions was about 11 mV. The data shown here were compensated for this effect by adjusting the zero-current potential to the liquid junction potential. Only cells showing an adequate voltage and space clamp (Ogata *et al.*, 1989) were used. All experiments were done at room temperature (21–23°C). Results are expressed as mean ± s.e.mean, and n represents the number of cells.

Drugs

Drugs were applied through a rapid microsuperfusion system (Ogata & Tatebayashi, 1991). This system uses a fine polyethylene needle (20 μm tip internal diameter) placed near (about 50 μm) the cell. Electromagnetic valves were used to apply test solutions. This microsuperfusion system enabled us a rapid (latency, less than 0.5 s) and localized application of

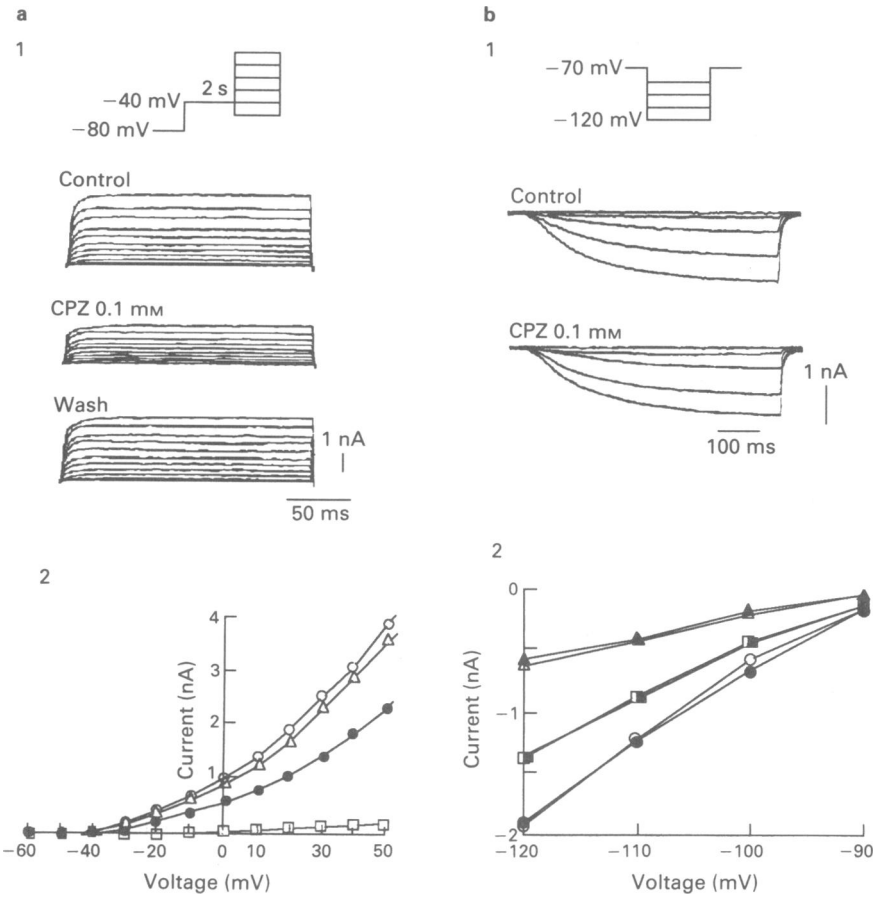


Figure 1 Effects of chlorpromazine (CPZ) on the delayed rectifier K^+ current, I_{DR}, (a) and on the inward (anomalous) rectifier K^+ current, I_{IR}, (b). Insets in a_1 and b_1 illustrate pulse protocols for selective activation of I_{DR} and I_{IR}, respectively. (a_1) From a holding potential (V_h) of − 80 mV, a prepulse to − 40 mV was given for 2 s to inactivate I_T, and immediately following the prepulse, a test pulse to potential levels positive to − 40 mV was given. Only a period of the test pulse is illustrated. (b_1) From V_h of − 70 mV, test pulses to potential levels negative to − 90 mV was given. To further eliminate a possible contamination by transient outward K^+ currents, 4-aminopyridine (4-AP) in a concentration of 1 mM was added to the medium in recordings of I_{DR}. I_{IR} in b_1 was recorded in 50 mM K^+ (by replacing NaCl with equimolar amounts of KCl). a_2 and b_2 plot current-voltage curves for I_{DR} and I_{IR}, respectively. In a_2, amplitudes of I_{IR} in the control solution (○), in the presence of 0.1 mM CPZ (●) or 25 mM TEA (□) and after washing (△) were plotted against test pulse potential. TEA was applied by replacing TMA-Cl with equimolar amounts of TEA-Cl. The current-voltage curves for I_{IR} were obtained from the same neurone in different external K^+ concentrations. (△, ▲), (□, ■) and (○, ●) were recorded in 5 mM, 25 mM and 50 mM K^+, respectively; open symbols, in the control solution; filled symbols, in the presence of 0.1 mM CPZ.

test solutions and their rapid washout. Solutions containing chlorpromazine-HCl (CPZ, Sigma) 4-aminopyridine (4-AP, Sigma) or α-dendrotoxin (α-DTX, Alomone Labs, Jerusalem, Israel) were prepared immediately before the experiment. The pH of the solution containing 4-AP was re-adjusted to 7.4 before application.

Results

K^+ currents in DRG neurones

In DRG, we identified four types of voltage-gated K^+ currents, namely, the delayed rectifier (I_{DR}), inward (anomalous) rectifier (I_{IR}), transient K^+ (refered to as 'I_T' hereafter), and Ca^{2+}-activated K^+ currents ($I_{K(Ca)}$). $I_{K(Ca)}$ was not studied, because direct interaction between $I_{K(Ca)}$ and CPZ could only be investigated with single channel recordings from excised patches of the membrane, and such an investigation has already demonstrated direct interaction between $I_{K(Ca)}$ and CPZ (McCann & Welsh, 1987).

The three types of K^+ currents were easily differentiated on the basis of their pharmacological and kinetic properties (see below). I_{DR} and I_{IR} were observed in most of the DRG cells examined. However, I_T was observed preferentially in a certain group of cells having relatively larger diameters, i.e. I_T was found in 68 cells out of 80 cells having diameters larger than 40 μm, whereas majority of the cells having smaller diameters (less than 20 μm) were devoid of any transient type of K^+ currents. Among the 40 smaller diameter cells examined, I_T was found in only 9 cells. Thus, we used cells having diameters larger than 40 μm.

Effects of CPZ on I_{DR} or I_{IR}

Figure 1 shows the effect of CPZ on I_{DR} or I_{IR}. CPZ at a concentration of 0.1 mM reversibly reduced the amplitude of I_{DR} to about 50% of the control (Figure $1a_{(1)}$). A large portion of I_{DR} was blocked by 25 mM TEA (Figure $1a_{(2)}$). The time course of I_{DR} and its voltage-dependence of activation as evaluated with current-voltage curves in Figure $1a_{(2)}$ were not affected by CPZ. Figure 1b shows the lack of effect of CPZ on I_{IR}. From a holding potential (V_h) of − 70 mV, test pulses to potential levels negative to − 90 mV induced a time-dependent inward current ($b_{(1)}$). This current, I_{IR}, was readily blocked by 1 mM Cs^+ (not illustrated), and its amplitude (consequently, conductance) was augmented by an increase in external concentration of K^+ ($b_{(2)}$). These features of I_{IR} were identical to those of the typical inward (anomalous) rectifier current in marine egg cells (Hagiwara & Yoshii, 1979). I_{IR} was not affected by a high concentration (0.1 mM) of CPZ.

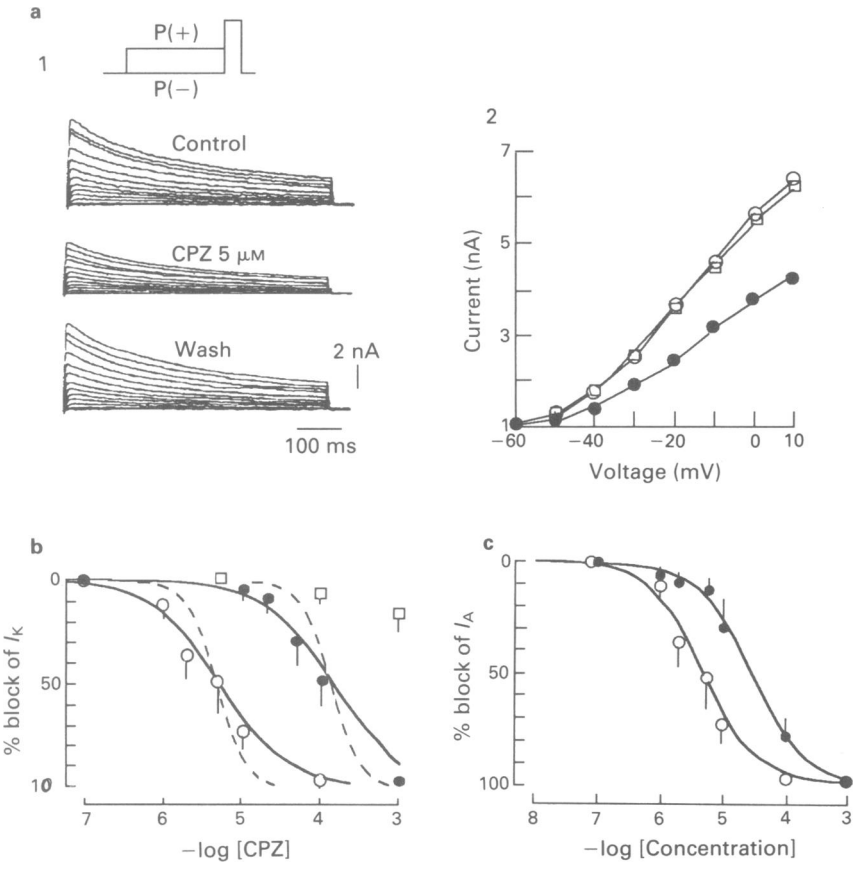


Figure 2 Effects of chlorpromazine (CPZ) on the slowly inactivating K^+ current, I_T. Inset in a_1 illustrates pulse protocol. K^+ currents were evoked by command pulses applied directly from V_h of − 80 mV ($P_{(-)}$) or applied subsequent to a prepulse to − 40 mV ($P_{(+)}$). I_T was obtained by subtracting the current produced by $P_{(+)}$ from the current produced by $P_{(-)}$. (a_2) Current-voltage curves. Amplitudes of I_T in the control solution (○), in the presence of CPZ (●) and after washing (□) were plotted against test pulse potential. (b) Concentration-response curves for CPZ block of different types of K^+ currents. Each point represents the mean ± s.e.mean from 5 experiments. Curves were drawn according to the following equation:

$$I_K = 1/\{1 + ([CPZ]/K_d)^n\}$$

where K_d is the dissociation constant of the binding reaction (4.5 μM for I_T and 120 μM for I_{DR}), [CPZ] is the concentration of CPZ, and n is 1 (solid line) or 2 (broken line). (○) I_T; (●) I_{DR}; (□) I_{IR}. (c) Concentration-response curves for block of I_T by CPZ (○) and 4-aminopyridine (4-AP) (●). Lines were drawn according to the above equation (4.5 μM for CPZ and 31 μM for 4-AP).

Characterization of I_T

I_T (Figure 2a$_{(1)}$) could be evoked in the absence of I_{DR}. A holding potential of -40 mV inactivates I_T (see Figure 4) and appreciable amount of I_{DR} was not activated at this potential (see Figure 1). A hyperpolarizing prepulse to -120 mV from V_h of -40 mV removed the inactivation of I_T, and a step back to V_h evokes I_T in isolation. A conventional double pulse protocol showed that a hyperpolarizing prepulse longer than 2 s was required to obtain the maximal amplitude of I_T (data not illustrated). It was also possible to isolate I_T using subraction between currents obtained with or without depolarizing prepulse to -40 mV. To exclude further a possible contamination by I_{DR}, recordings of I_T were carried out in the presence of 5 mM TEA which had no detectable effect on I_T.

I_T activated rapidly at potentials positive to -60 mV but shows only slow (inactivation time constant, τ_{intact}, an order of seconds, see Figure 3) and incomplete inactivation, I_T was inhibited by low concentrations of 4-AP (2–100 μM, see Figure 2c) and α-DTX (44 ± 8% inhibition by 10 nM, $n = 5$). This current was noticeably different from the 'classical' transient K^+ current (collectively called 'I_A' or 'A-current') which rapidly inactivates ($\tau_{inact.}$ less than 100 ms) and is generally insensitive to 4-AP at concentrations below 100 μM (Rogawski, 1985). A similar slowly inactivating K^+ current has been reported in a subpopulation (A-cell) of rat nodose ganglion (visceral afferent) neurones (Stansfeld *et al.*, 1986). This nodose K^+ current was also inhibited by low concentrations of 4-AP (1–30 μM) and DTX (3–10 nM). Thus, I_T recorded in DRG may be analogous to the slowly inactivating K^+ current recorded in A-cells of nodose ganglia. However, there are differences in channel kinetics (e.g., an activation threshold of the nodose slowly inactivating K^+ current was about -70 mV) which may be due to differences in cell type (dorsal root vs nodose), preparation (cultured cell vs freshly-dissociated cell), or recording conditions (patch-clamp vs microelectrode voltage-clamp).

Effects of CPZ on I_T

As shown in Figure 2a$_{(1)}$, CPZ at a concentration of 5 μM reduced the amplitude of I_T to 60% of the control. The effect was completely reversible. The time course of the current was not affected by CPZ. Figure 2a$_{(2)}$ shows the current-voltage curves for I_T obtained in the presence or absence of CPZ. The curve was proportionally reduced by CPZ at each potential level. The effects of CPZ on three types of K^+ currents are summarized in Figure 2b. The concentration required for 50% block (dissociation constant, K_d) was 120 μM for I_{DR} and 4.5 μM for I_T, respectively. Figure 2c shows the concentration-response curves for the block of I_T by CPZ and 4-AP. K_d for 4-AP was 31 μM. In each case, the concentration-response curves were fitted by a 1:1 stoichiometry.

Effects of CPZ and 4-AP on the decay time-course of I_T

It is well-established that 4-AP has a preferential blocking action on the transient K^+ currents (Gustafsson *et al.*, 1982; Thompson, 1982), and thus this agent has been utilized as a pharmacological tool in studies of K^+ channels. Therefore, we compared the action of CPZ on I_T with the action of 4-AP. Figure 3a illustrates changes in current envelope during applications of CPZ and 4-AP. The currents in the presence of drugs (filled arrows) were normalized to the currents measured before application of drugs (open arrows). The current in the presence of CPZ completely overlapped with the control current, whereas the current in the presence of 4-AP had a faster decay phase as compared with the control current. The decay phase of I_T in the control solution was usually described by three exponential components (b). The three time constants of 70, 405 and 2067 ms in the control solution were reduced by 4-AP to 53, 240 and

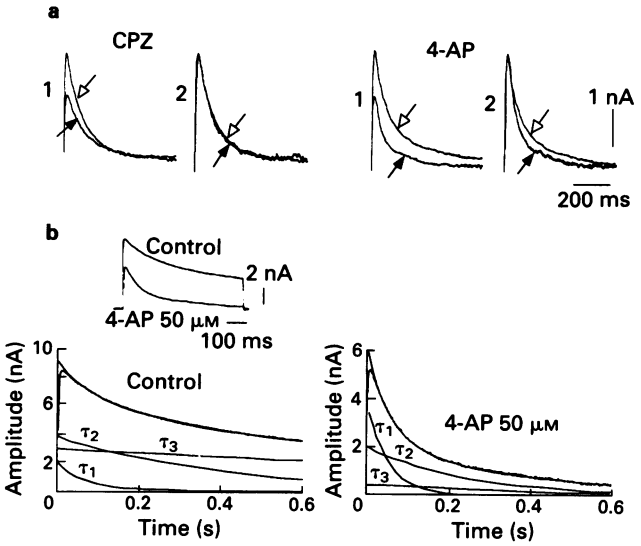


Figure 3 Effects of chlorpromazine (CPZ) and 4-aminopyridine (4-AP) on the decay phase of I_T. (a) I_T was recorded before and during applications of drugs. The currents in the presence of CPZ or 4-AP (filled arrows in traces labelled 1) were scaled to the currents measured before application of drugs (open arrows in traces labelled '1') and shown superimposed (traces labelled '2'). (b) The decay phase of I_T in the control solution or in the presence of 4-AP was fitted by exponential functions. τ_1, τ_2 and τ_3 in Control, 70, 405 and 2067 ms; τ_1, τ_2 and τ_3 in 4-AP 50 μM, 53, 240 and 450 ms. For further explanation, see text.

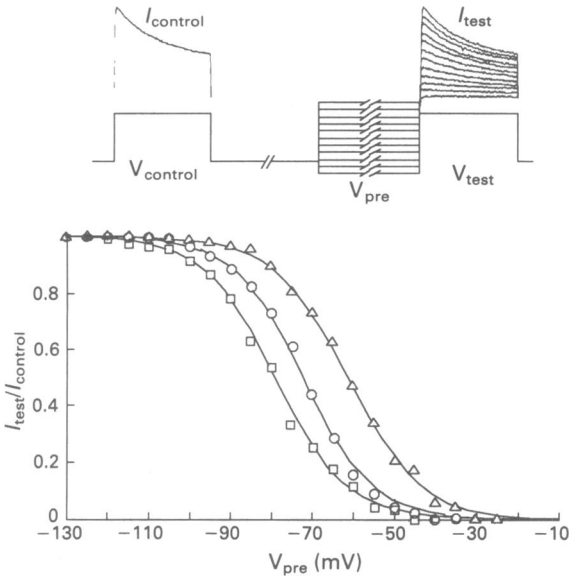


Figure 4 Steady-state inactivation curves for I_T measured in the control solution (○) and in the presence of 5 μM chlorpromazine (□) or 50 μM 4-aminopyridine (△). Inset illustrates pulse protocol. Two identical step depolarizations to -40 mV for 0.6 s were applied 20 s before ($V_{control}$) and immediately subsequent (V_{test}) to the prepulse (V_{pre}) from V_h of -120 mV. The potential level of V_{pre} was changed from -130 mV to -25 mV in 5 mV steps. The peak amplitude of I_{test} was normalized to that of $I_{control}$ and plotted as a function of V_{pre}. The smooth curves were drawn according to the equation:

$$I_{test}/I_{control} = 1/(1 + \exp[(V_{pre} - V_i)/\kappa])$$

where V_i is the prepulse potential when I_T is one-half maximal, and κ is the slope factor.

450 ms, respectively. Such an acceleration of the decay phase by 4-AP was observed in all of the 10 cells examined, and the slower phase of the decay was more sensitive to 4-AP.

Effects of CPZ and 4-AP on the steady-state inactivation of I_T

Figure 4 shows the steady-state inactivation curves for I_T measured in the control solution and in the presence of CPZ or 4-AP. The steady-state inactivation curve in the control solution (circles) had $V_{\frac{1}{2}}$ of -74 mV. CPZ and 4-AP had opposite effects on the steady-state inactivation characteristics. The steady-state inactivation curve was shifted 5 mV (5.5 ± 1.2 mV, $n = 4$) in the negative direction by CPZ (squares), whereas it was shifted by 13 mV (13.1 ± 2.1 mV, $n = 4$) in the positive direction by 4-AP (triangles). The slope factor was not affected by either drug. These observations strongly suggest different modulatory actions of these drugs on the inactivation process of I_T.

Effects of CPZ and 4-AP on the recovery process of I_T

I_T fully recovered from inactivation after a recovery period (ΔT) of 6400 ms in the control solution (Figure 5a, open circles). When the medium contained CPZ, the recovery process was considerably delayed at relatively shorter ΔT (squares). However, an overall period required for recovery of I_T was similar in either the control solution or in the presence of CPZ. The recovery process of I_T was fitted by exponential functions (Figure 5b). In the control solution, the recovery proceeded as the sum of two exponentials having time constants of 260 and 1400 ms. In the presence of CPZ, a third exponential function with a time constant of 800 ms was required to give a best fit in addition to the two time constants used for the control time course. Essentially similar results were obtained in two additional experiments.

In contrast to CPZ, 4-AP increased the peak amplitude of I_T evoked subsequently to the prepulse (Figure 5a, triangles). Such a paradoxical increase of I_T by 4-AP reached a maximum at ΔT of 160 ms and declined very slowly with a time constant of 7.2 ± 1.7 s ($n = 5$). The increase was no longer observed at ΔT of 20 s. These observations were reproducible in all of the 4 cells examined. These results indicate that in the presence of 4-AP, use-dependent unblocking of I_T is induced by a preceding activation of I_T.

Effects of CPZ and 4-AP on the frequency-dependent modulation of I_T

A striking difference between CPZ and 4-AP was noted in their actions on frequency modulation of I_T. Figure 6a shows typical examples of current traces recorded during ten repet-

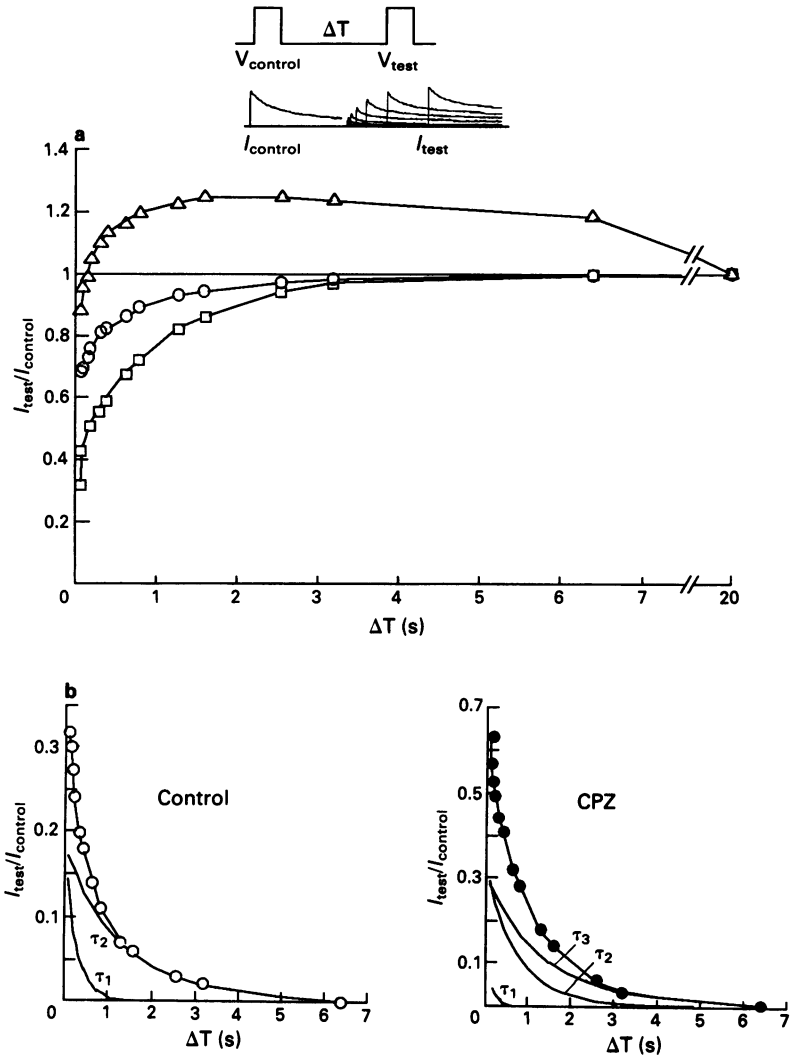


Figure 5 Effects of chlorpromazine (CPZ) and 4-aminopyridine (4-AP) on the recovery process of I_T from inactivation. (a) Two identical test pulses ($V_{control}$ and V_{test}) to -30 mV for 0.5 s were given with varying inter-pulse interval (ΔT) from V_h of -90 mV. The amplitude of I_{test} normalized to that of $I_{control}$ were plotted against ΔT in the control solution (○) and in the presence of 5 μM CPZ (□) or 50 μM 4-AP (△). (b) The value, $1\text{-}I_{test}/I_{control}$, was plotted against ΔT. The time course of $(1\text{-}I_{test}/I_{control})$ was fitted by exponentials. τ_1 and τ_2 in Control, 260 and 1400 ms; τ_1, τ_2 and τ_3 in CPZ, 260, 800 and 1400 ms. For further explanation, see text.

itive test pulses applied with inter-pulse interval (ΔT) of 100 ms, in the control solution and in the presence of CPZ or 4-AP. The peak current amplitude for each pulse in the train was normalized to that of the first pulse (I_T) and plotted against the pulse number (Figure 6b).

In the control solution, the peak amplitude of I_T was progressively decreased during ten repetitive test pulses. The decrease was smaller when ΔT became more prolonged from 80 ms to 3200 ms, and with ΔT of 6400 ms, there was no longer any detectable decrease in I_T amplitude throughout the ten test pulses. When the same pulse protocol was examined in the presence of CPZ, I_T was successively attenuated as in the case of the control solution. The degree of the attenuation of I_T induced by test pulses having comparable ΔT was always greater when compared with the decrease in the control solution.

In the presence of 4-AP, I_T was rather increased in most cases. The maximal increase was obtained following the third pulse. It should be noted that, in the presence of 4-AP, the I_T evoked subsequently to a 5 s pause after the ten repetitive pulses (I_{11} in Figure 6a) was much larger than I_T evoked during ten repetitive pulses. Thus, it is suggested that, during repetitive pulses in the presence of 4-AP, there was an interplay between the inactivation process resultant from the preceding activation and the increased channel availability due to unbinding of 4-AP molecules from the channel. At the time of I_{11} activation, the inactivation process could be recovered while the increased channel availability is still present (see Figure 5a). In contrast to the case of 4-AP, the peak amplitude of I_{11} in the control solution or in the presence of CPZ was identical to that of I_1.

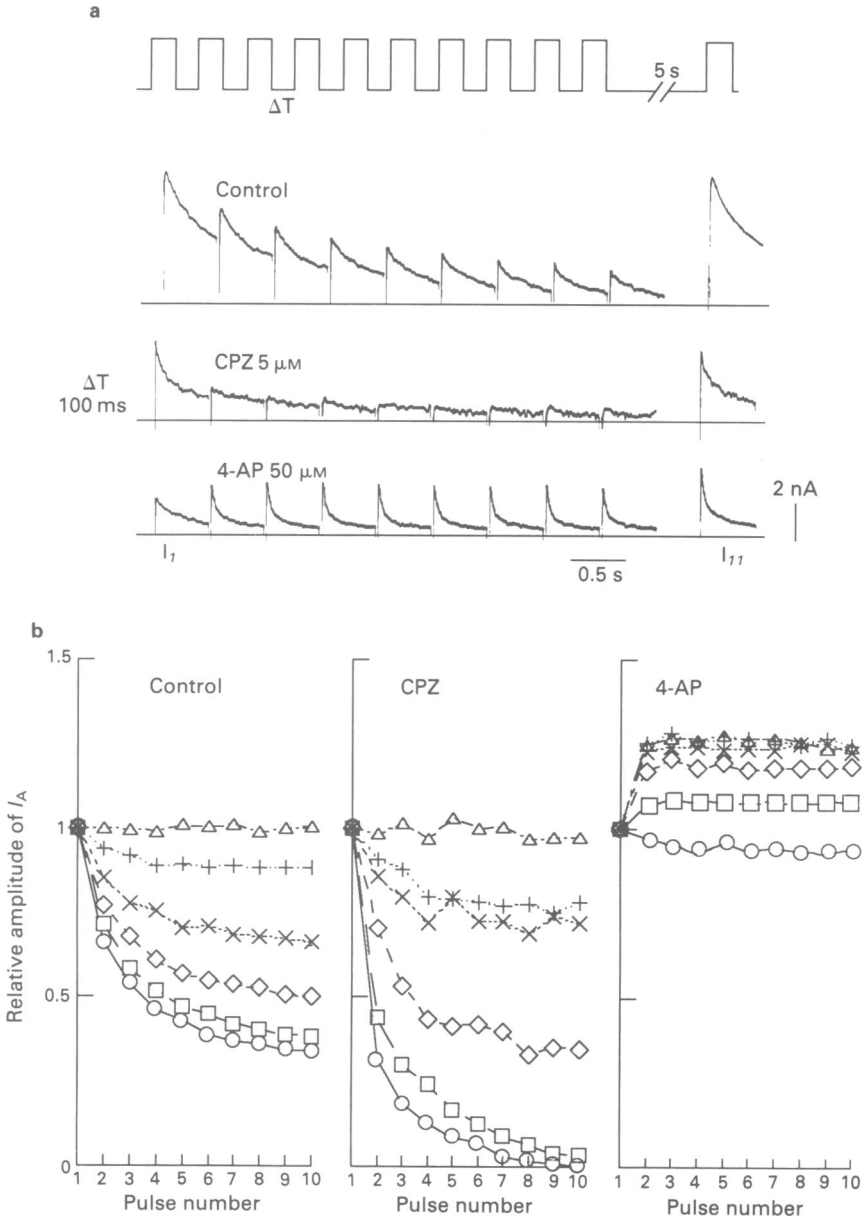


Figure 6 Effects of chlorpromazine (CPZ) and 4-aminopyridine (4-AP) on the frequency modulation of I_T. (a) Inset illustrates pulse protocol. From V_h of − 90 mV, ten consecutive test pulses to − 30 mV for 0.5 s were applied with varying inter-pulse interval (ΔT), and an additional test pulse (I_{11}) was given after 5 s pause at V_h. Typical examples of current traces in the control solution (upper) and in the presence of CPZ (middle) or 4-AP (lower) evoked with ΔT of 100 ms are illustrated. (b) The peak current amplitude for each pulse in the train was normalized to that of the first pulse (I_1) and plotted against the pulse number. Left, in the control solution: middle, in the presence of 5 μM CPZ: right, in the presence of 50 μM 4-AP: (○) 80 ms; (□) 160 ms; (◇) 640 ms; (×) 1600 ms; (+) 3200 ms and (△) 6400 ms.

Discussion

The present results demonstrate that CPZ blocks I_T (K_d, 4.5 μM) in preference to two other voltage-gated K^+ currents, I_{DR} and I_{IR}. Although I_{DR} was weakly sensitive (K_d, 120 μM) to CPZ, I_{IR} was practically insensitive to CPZ. It has been shown that $I_{K(Ca)}$ is depressed by 1–2 μM CPZ in rat hippocampal neurones (Dinan *et al.*, 1987) and in dog airway smooth muscle (McCann & Welsh, 1987). Thus, it appears that CPZ has a preferential depressant action on particular types of voltage-gated K^+ channels.

It is difficult to equate concentrations of CPZ in the experimental perfusion system with those in the serum or cerebrospinal fluid of humans, due to various factors that influence the tissue concentrations of CPZ such as the prolonged trapping of CPZ in a closed enterohepatic circuit or strong binding of CPZ to plasma or tissue proteins (Dingell *et al.*, 1961; Baldessarini, 1990). Nevertheless, concentrations used in this study appear to be well within the range of concentrations attained in schizophrenic patients treated with this drug (9–60 μM, Hugang & Ruskin, 1964; 3 μM, Surawicz & Lasseter, 1970).

Neuroleptics also inhibit calmodulin; however, an involvement of calmodulin inhibition appears to be unlikely, because N- (6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7), a calmodulin antagonist (Hidaka *et al.*, 1981), did not influence I_T. In addition, the blocking action of CPZ was observed when we used fluoride as an intracellular impermeant anion which disrupts cellular metabolism by chelating Ca^{2+} ions (data not shown). A similar conclusion has been drawn in CPZ-induced blocks of neuronal Na^+ (Ogata *et al.*, 1990) and Ca^{2+} (Ogata & Narahashi, 1990) channels or of $I_{K(Ca)}$ in airway smooth muscle (McCann & Welsh, 1987).

It is well known that neuroleptic drugs lower the seizure threshold and induce discharge patterns in the EEG that are associated with epileptic seizure disorders, and therefore, neuroleptic agents should be used with extreme caution in epileptic patients and in patients undergoing withdrawal from central depressants such as alcohol or barbiturates (Baldessarini, 1990). There has been no rational explanation for this side effect of the neuroleptic drugs.

$I_{K(Ca)}$ is considered to underlie the long-lasting after-hyperpolarization (AHP) observed in several types of neurones which acts as a powerful neuronal brake (Alger & Nicoll, 1980). I_T which is activated in the subthreshold range of cellular excitation is thought to reduce the excitatory effect of depolarizing membrane currents in a time-dependent manner. In addition, a recent investigation suggested that an inactivating type of K^+ current may also be involved in the generation of the AHP (Ruppersberg *et al.*, 1991). Thus, CPZ may affect cellular excitability through inhibition of $I_{K(Ca)}$ and I_T.

I_T had properties similar to those of the slowly inactivating K^+ current found in nodose A-cells (Stansfeld *et al.*, 1986), e.g., these two currents are highly sensitive to 4-AP and DTX whereas they are relatively insensitive to TEA. A comparable K^+ current has been shown also in rat hippocampal neurones (I_D, Storm, 1988). The slowly inactivating K^+ currents in nodose and hippocampal neurones have been shown to play an important role in spike adaptation, such that inhibition of these currents induces striking repetitive firing (Standfeld *et al.*, 1986; Storm, 1988). In addition, it has been shown that 4-AP induces a similar repetitive firing in rat dorsal root (but not ventral root) fibres (Baker *et al.*, 1985). Thus, there is a possibility that the slowly inactivating K^+ current is a major target underlying the proconvulsant side effect of CPZ.

The blocking action of CPZ on I_T was strikingly different from that of 4-AP which blocks inactivating K^+ currents in a variety of tissues (Gustafsson *et al.*, 1982; Thompson, 1982; Stansfeld *et al.*, 1986; Storm, 1988). The action of 4-AP on I_T was characterized by a prominent acceleration of the decay phase of I_T (see Figure 3), whereas such a kinetic effect was not observed in the case of CPZ. Two possible explanations might be considered for the acceleration of the I_T decay. One possibility is that 4-AP binds more strongly to the open (activated) channel state than to the closed state ('open-channel block'). Indeed, the open-channel block of the inactivating K^+ current by 4-AP has been reported in molluscan neurones (Thompson, 1982) or in neuroblastoma x glioma hybrid cells (Robbins & Sim, 1990). The other possibility is that 4-AP preferentially blocks the slower component of I_T. I_T may be composed of multiple components, since the decay phase of I_T was fitted best, by three exponential components (Figure 3b). Two types of transient K^+ currents have been reported in cardiac muscle cells (Coraboeuf & Carmeliet, 1982; Escande *et al.*, 1987). These possibilities could be distinguished by use of single channel recordings.

An additional striking difference between actions of CPZ and 4-AP was the contrasting effects of these drugs on the steady-state inactivation characteristics (Figure 4). The positive shift of the steady-state inactivation curve by 4-AP suggests a possible voltage-dependent unblocking action of this drug as has been suggested for the A-currents in molluscan neurones (Thompson, 1982) and in dog ventricular muscle (Simurda *et al.*, 1989). This notion is further strengthened by the use-dependent facilitation of I_T in the presence of 4-AP (Figures 5 and 6).

In contrast to 4-AP, CPZ shifted the steady-state inactivation curve in the negative direction, i.e., the block was increased by membrane depolarization (Figure 4). This effect of CPZ was observed also in blocks of Na^+ or Ca^{2+} channels and attributed to the greater affinity of CPZ for the inactivated state of channels than for the resting channels (Ogata *et al.*, 1990) consistent with the 'modulated receptor model' (Hille, 1977; Hondeghem & Katzung, 1977). The slowed recovery time course (Figure 5) and the use-dependent depression of I_T (Figure 6) in the presence of CPZ may be due to slow repriming of CPZ-bound channels from inactivation.

In conclusion, CPZ causes preferential block of I_T in neurones of the rat dorsal root ganglia. Although its channel selectivity was low as compared with the channel selectivity of 4-AP, the concentration of CPZ required to produce a comparable inhibition of I_T was much lower than the concentration of 4-AP. Since CPZ also depresses the neuronal Na^+ (Ogata *et al.*, 1990) and Ca^{2+} (Ogata & Narashi, 1990) channels, its overall effect on the neuronal activity may be diverse depending on the types of neurone. These direct membrane effects of CPZ appear to be related to some aspects of clinical efficacy of this drug.

We thank Professor Hirosi Kuriyama for pertinent support and advice. This study was supported by the Japanese Ministry of Education (Scientific Research 63570096).

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(Received February 16, 1993
Revised April 6, 1993
Accepted April 8, 1993)

Br. J. Pharmacol. (1993), **109**, 1247–1253 © Macmillan Press Ltd, 1993

Effects of heparin on the vasodilator action of protamine in the rabbit mesenteric artery

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1 The effects of protamine on the rabbit isolated small mesenteric artery were investigated both in the presence and in the absence of heparin, by the isometric tension-recording method.

2 The dissociation constant for the binding of heparin to protamine has never been previously reported, so in order to minimize the effects of protamine, known to have a vasodilator action, and to examine only the effects of a heparin-protamine complex, the experiments with heparin were performed in the presence of high concentrations of heparin (21–700 u ml^{-1}), concentrations at which heparin itself does not affect the vascular tone.

3 Protamine (15–500 $\mu g\ ml^{-1}$), in the absence of heparin, was found to inhibit ($P<0.05$) noradrenaline (1 μM)-induced contractions both in endothelium-intact and in endothelium-denuded tissues.

4 Such vasodilator action of protamine in either endothelium-intact or -denuded tissues continued, even in the presence of excess heparin at a heparin/protamine (H/P) ratio of 1.4 u μg^{-1}, but was almost completely blocked in the presence of a much greater excess of heparin (H/P ratio $\geq$ 4.7 u μg^{-1}): heparin was present both before and during the application of protamine.

5 The vasodilator action of protamine in the absence of heparin was prolonged both in the endothelium-intact and -denuded tissues after protamine had been washed out from the bath with Krebs solution. Although this washing out with a Krebs solution containing excess heparin (4.7 u ml^{-1}) readily reversed such prolonged vasodilator action of protamine both in the endothelium-denuded strips and in the endothelium-intact strips which had been pretreated with inhibitors of the endothelium-derived relaxing factor (EDRF) pathway, it did not affect the prolonged vasodilator action of protamine in the endothelium-intact strips which received no pharmacological intervention.

6 These results suggest that: (1) only protamine, not a heparin-protamine complex, exerts vasodilator action *in vitro*; (2) the vasodilator action of protamine presumably has an EDRF-mediated component; and (3) protamine probably exerts its direct vasodilator action without entering the smooth muscle cell.

Keywords: Blood; anticoagulants; heparin; protamine; hypotension; arginine-rich basic polypeptide; smooth muscle; endothelium-derived relaxing factor

Introduction

Protamine has been widely used for the neutralization of heparin-induced anticoagulation in the clinical practice of cardiovascular surgery and radiology. Various adverse haemodynamic responses have been reported during the neutralization of heparin anticoagulation with protamine, including systemic hypotension, pulmonary hypertension, and bradycardia (Horrow, 1985; Hobbhahn *et al.*, 1988; Fisher *et al.*, 1989; Morel *et al.*, 1990). However, the precise mechanisms of these adverse haemodynamic effects have not yet been fully elucidated (Horrow, 1985).

Previous *in vitro* studies have demonstrated that free protamine, which has not formed a complex with heparin, also exists side by side with a heparin-protamine complex in plasma where the heparin-induced anticoagulation has been adequately neutralized with protamine, as judged from the clotting time, implying that both a heparin-protamine complex and free protamine exist together in the blood during the adequate *in vivo* neutralization of heparin-induced anticoagulation with protamine (Kitani *et al.*, 1980; Shanberge *et al.*, 1987). If this is so, when the adverse haemodynamic responses which occur during the neutralization of heparin-induced anticoagulation with protamine are discussed, the effects of a heparin-protamine complex and of free protamine on the cardiovascular system should both be considered.

It has recently been reported that protamine, in the absence of heparin, exerts a vasodilator action on isolated arteries (Rapoport *et al.*, 1989; Akata *et al.*, 1991). However, little information is available on the effects of a heparin-protamine complex on vascular smooth muscle. In order to address this issue, we investigated how heparin affects the vasodilator effects of protamine in small mesenteric arteries, which are known to play an important role in the regulation of systemic blood pressure (Guyton, 1986; Stephenson, 1989).

Methods

Tissue preparation

After receiving institutional approval for this animal study, male albino rabbits (2.0–2.5 kg) were given sodium pentobarbitone (40 mg kg^{-1}, i.v.) and were then exsanguinated. The mesentery in the jejunal region was immediately placed in a dissecting chamber filled with a preoxygenated Krebs solution, and the third-order branches of the mesenteric artery (0.2–0.3 mm in diameter) were rapidly excised. Under a binocular microscope, the fat and adhering connective tissue were carefully removed, and thin circular strips (rabbit; 0.4–0.7 mm long, 0.15–0.2 mm wide) were prepared from the distal portion of the third-order branches for tension-recording. In some experiments, the endothelium was also removed [E(-) strips] as previously reported (Akata *et al.*, 1991), and its removal was verified by the disappearance of the acetylcholine (ACh)-induced, endothelium-dependent relaxant response.

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Recording of mechanical activity

Mechanical responses were measured by attaching the strips to a strain gauge (UL-2 type, Shinko Co., Tokyo, Japan) in a chamber of 0.9 ml capacity. The strips were stretched to about 1.1 times their original resting length in order to maintain resting tension (1 mg): under these conditions, the contractile response to high K^+ was maximal (optimal point). The solution was changed by rapid perfusion from one end and simultaneous aspiration with a water pump from the other end (Akata *et al.*, 1991; Itoh *et al.*, 1987). Since our previous study on the rabbit mesenteric arteries suggested that there is no significant difference regarding the vasodilator action of protamine observed between 32°C and 37°C, all the experiments in this study were performed at 32°C in order to prevent any early deterioration of the strips (Akata *et al.*, 1991).

Solutions

The ionic concentration of the Krebs soltuions was as follows (mM): NaCl 137.4, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.6, $NaHCO_3$ 15.5, KH_2PO_4 1.2, glucose 11.4. The high K^+ solution was prepared by substituting NaCl with equimolar KCl. The solution was bubbled with a 95% O_2/5% CO_2 mixture (pH 7.3–7.4).

Drugs

Noradrenaline HCl, methaemoglobin (Bovine), methylene blue, N^G-nitro L-arginine, and tetrodotoxin (TTX) were obtained from the Sigma Chemical Co., St. Louis, Mo, U.S.A. The other chemicals used were guanethidine HCl (Tokyo Kasei Kogyo, Tokyo, Japan), ethyleneglycol-*bis*-(β-amino-ethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), protamine sulphate and heparin sodium (Nakalai Tesque, Kyoto, Japan).

Protamine was prepared in normal saline solution just before use. The concentration of protamine was expressed as $\mu g\ ml^{-1}$, because its molecular weight (mol. wt.) varied from 2 to 12 kDa (mean mol. wt.: approximately 4.5 kDa).

Heparin (unfractionated) was prepared in distilled water just before use, and its concentration was expressed as $u\ ml^{-1}$.

Oxyhaemoglobin was prepared by reducing commercial bovine Hb containing 75% methaemoglobin as described by Martin *et al.* (1985).

Experimental design

Test stimulants

The effects of protamine on noradrenaline (NA; 1 μM, submaximal)- or high K^+ (40 mM, submaximal)-induced contractions were studied either in the presence or absence of heparin: each test stimulant (NA, high K^+) was applied to the vascular strips either with or without endothelium, for a period of 2 min, every 8 min, with protamine (15–500 $\mu g\ ml^{-1}$) being applied cumulatively after the amplitude of each contraction became constant, either in the presence or absence of heparin: in this artery, constant responses to the NA or high K^+ of the vascular strips could be obtained for at least 140 min. Since both NA- and high K^+-induced contractions consist of two components, an initial phasic and a subsequent tonic component as previously observed (Akata *et al.*, 1991; Itoh *et al.*, 1987), the effects of protamine on both components were assessed. Moreover, since the sympathetic vasoconstrictor system is well known for playing a significant role in maintaining the vasomotor tone (Guyton, 1986), NA, the substance secreted at the endings of the sympathetic vasoconstrictor nerves, was chosen as the test stimulant for most of the experiments. In the remainder of the experiments, however, high K^+ was chosen as the test stimulant in order to examine the effects of protamine on the voltage-dependent Ca^{2+} influxes: in order to suppress sympathetic nerve activity, the experiments with high K^+ were performed in the presence of 0.1 μM tetrodotoxin and 3 μM guanethidine. The absolute tension levels of the NA- or high K^+-induced contractions are summarized in Table 1.

Heparin/protamine ratio

The dissociation constant for the binding of heparin to protamine has never been previously reported. Furthermore, the difficulty in actually determining the constant was described recently by Jones *et al.* (1986). Thus, it is impossible to know how much free protamine, free heparin or heparin-protamine complex exist in a Krebs solution, in which heparin and protamine are mixed at a clinically relevant ratio (e.g. 0.1 $u\ \mu g^{-1}$). It is thus quite difficult to interpret the effects observed in the *in vitro* experiments with a clinical heparin/protamine (H/P) ratio: under such experimental conditions, it would surely be difficult to distinguish the effects of a heparin-protamine complex from the effects of free protamine or free heparin, because both free protamine and free heparin are known to affect smooth muscle tone (Kobayashi *et al.*, 1988; Rapoport *et al.*, 1989; Akata *et al.*, 1991). Accordingly, it may well be unreasonable to choose a clinically relevant ratio for the mixture of heparin and protamine in order to simulate the *in vivo* situation in our experimental setting. Consequently, in order to minimize the effect of free protamine and to examine only the effect of a heparin-protamine complex, the present experiments with heparin were performed in the presence of the highest possible concentrations of heparin, concentrations at which heparin itself did not have any vasodilator action in this artery.

Since the preliminary experiments showed that concentrations of heparin greater than 700 $u\ ml^{-1}$ (> 700 $u\ ml^{-1}$) exert vasodilator action in this artery, the concentrations of heparin used in this experiment were less than 700 $u\ ml^{-1}$ (≤ 700 $u\ ml^{-1}$), levels at which heparin itself does not exert any vasodilator action: heparin (21–700 $u\ ml^{-1}$) did not significantly inhibit the NA (1 μM)-induced contractions either in E(+) or E(−) tissues (not shown).

Vasodilator action of protamine in the absence of heparin

The effects of protamine (15–500 $\mu g\ ml^{-1}$) on the NA (1 μM)-induced contractions evoked in the Krebs solution

Table 1 The absolute tension levels (mg) of both phasic and tonic components of the noradrenaline (NA, 1 μM)- or high potassium (40 mM)-induced contractions either in the endothelium-intact (E(+))- or -denuded (E(−))-strips of rabbit mesenteric artery

	NA (1 μM)		*K* (40 mM)	
	Phasic	*Tonic*	*Phasic*	*Tonic*
E(+)	55.5 (27.6) [30]	102.0 (56.7) [30]	–	–
E(−)	54.0 (28.7) [8]	81.5 (28.7) [8]	78.7 (25.1) [8]	82.3 (25.1) [8]

Values are mean with s.d. in parentheses [*n*] = number of strips.

both in the E(+) and E(−) tissues were first studied in the absence of heparin. Since even low concentrations of protamine exerted their submaximal or maximal vasodilator effects after incubation for 45–55 min as previously shown (Akata *et al.*, 1991) and preliminarily observed in this study, the maximal application time for each concentration of protamine was 45 min.

Effects of heparin on the vasodilator action of protamine

The effects of excess heparin (H/P ratio = 1.4–14 u μg^{-1}) on the vasodilator action of protamine were then studied: heparin was present both before and during the application of protamine, and protamine was applied to the vascular tissues as a Krebs solution in which protamine and excess heparin were premixed. The maximal application time for each concentration of protamine in this study was also 45 min, for the reasons described above.

Ability of heparin to reverse the vasodilator action of protamine

Since the vasodilator action of protamine both in the E(−) and E(+) strips was found to be prolonged after the washing out of protamine from the bath, as has been previously demonstrated (Akata *et al.*, 1991), the effects of heparin on the prolonged vasodilator action of protamine (150 $\mu g\ ml^{-1}$) under the following various conditions were examined: (1) the inhibition of the NA-induced contractions in the E(+) strips; (2) the inhibition of the NA-induced contractions in the presence of N^G-nitro L-arginine (L-NOARG, 100 μM, Bult *et al.*, 1990), methylene blue (MB, 10 μM, Martin *et al.*, 1985) and oxyhaemoglobin (HbO_2, 10 μM, Martin *et al.*, 1985) in the E(+) strips; and (3) the inhibition of the NA- and high K^+-induced contractions in the E(−) strips. The preliminary experiments showed that it usually took 25 min for 150 $\mu g\ ml^{-1}$ of protamine to exert its submaximal or maximal vasodilator action. Therefore, in this study, protamine was washed out from the experimental bath with a Krebs solution, either with or without heparin, after the application of protamine to vascular tissue for 28 min. The inhibitors of the EDRF pathway (HbO_2, MB, L-NOARG) were applied to the strips for 20 min both before and during the experiments.

Calculations and statistical analysis

Protamine-induced inhibitions of the NA- or high K^+-induced contractions were assessed at the point where the inhibitions reached a submaximum or maximum, and are expressed as a percentage (%) of change from the amplitudes of each contraction before the application of protamine. The concentrations of protamine or heparin to produce a half-maximal response (IC_{50} values for protamine or heparin) were calculated by the four-parameter logistic equation described by DeLean *et al.* (1978). These IC_{50} values are expressed as mean ± s.e.mean, while all other results are expressed as mean ± s.d. The statistical assessment of the data was made by one- or two-factor analysis of variance with repeated measures followed by Scheffé's F test (for multiple comparison) or by a two-tailed, unpaired Student's t test (for comparison between two treatments) (Wampold *et al.*, 1990). The level of $P<0.05$ was considered significant.

Results

Pretreatmental effects of heparin on the vasodilator action of protamine

Protamine (15–500 $\mu g\ ml^{-1}$), in the absence of heparin, inhibited both phasic and tonic components of the NA (1 μM)-induced contractions both in the E(+) and E(−) strips. Its inhibition developed gradually, particularly at the lower concentrations ($\leq$ 50 $\mu g\ ml^{-1}$) (Figures 1 and 2). The presence of excess heparin at an H/P ratio of 1.4 u μg^{-1} was found to slightly, although significantly, inhibit the protamine-induced inhibition of the NA-induced tonic contraction evoked in the E(+) strips, but it did not significantly affect such inhibition of the NA-induced phasic contraction in the E(+) strips, or such inhibition of the NA-induced phasic and tonic contractions in the E(−) strips (Figure 2: the data on phasic contractions are not shown): the IC_{50} values for the protamine inhibition of the NA-induced phasic and tonic contractions both in the absence and in the presence of heparin (H/P ratio = 1.4 u μg^{-1}) are shown in Table 2. On the other hand, the protamine inhibition of the NA-induced contractions was almost completely blocked by the presence of a much greater excess of heparin at a higher H/P ratio ($\geq$ 4.7 u μg^{-1}): the effects of higher concentrations (H/P ratio = 4.7 or 14 u μg^{-1}) of heparin on the 150 or 500 $\mu g\ ml^{-1}$ protamine-induced inhibitions could not be studied because of the inhibitory effects of heparin itself which are described above (Figure 2: the data on the phasic component are not shown). The IC_{50} values for the inhibitory effects of heparin on the protamine (50 $\mu g\ ml^{-1}$) inhibition of the NA-induced phasic and tonic contractions in the E(+) strips were 100.8 ± 0.02 and 68.8 ± 8.82 u ml^{-1}, respectively, while the IC_{50} values for the inhibitory effects of heparin on the protamine (50 $\mu g\ ml^{-1}$) inhibition of the NA-induced phasic and tonic contractions in the E(−) strips were 107.4 ± 7.9 and 47.5 ± 5.0 u ml^{-1}, respectively.

Effects of heparin on the prolonged vasodilator action of protamine

The following inhibitory actions of protamine (150 $\mu g\ ml^{-1}$) on the NA- or high K^+-induced contractions were prolonged after the washing out of protamine from the bath with a Krebs solution which did not contain any heparin (Figures 3–5): (1) inhibition of the NA (1 μM)-induced contractions in the E(+) strips (Figure 3); (2) inhibition of the NA (1 μM)-induced contractions in the E(+) strips pretreated with the various inhibitors of the EDRF pathway (HbO_2 + MB + L-NOARG) (Figure 4); (3) inhibition of the NA (1 μM)-induced contraction in the E(−) strips (Figure 5a); and (4) inhibition of the high K^+ (40 mM)-induced contractions in the E(−) strips (Figure 5b).

On the other hand, the following inhibitory actions of

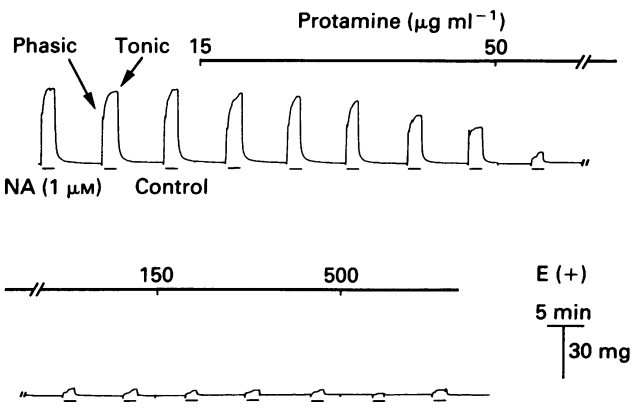


Figure 1 An example of the inhibitory effects of protamine (15–500 $\mu g\ ml^{-1}$) on both the phasic and tonic components of the noradrenaline (NA, 1 μM)-induced contractions in the endothelium-intact strips. E(+): endothelium-intact.

protamine were readily reversed after the washing out of protamine from the bath with a Krebs solution containing excess heparin (700 u ml^{-1}, H/P ratio = 4.7 u μg^{-1}): (1) inhibition of the NA (1 μM)-induced contractions in the E(+) strips pretreated with the EDRF pathway inhibitors (Figure 4); and (2) inhibitions of both the NA (1 μM)- and high K^+ (40 mM)-induced contractions in the E(−) strips (Figure 5). However, the inhibitory action of protamine on the NA (1 μM)-induced contractions in the E(+) strips which did not receive any pharmacological intervention remained prolonged, even after the washing out of protamine from the bath with a Krebs solution containing excess heparin (700 u ml^{-1}): as regards the time course for the protamine-induced inhibition of the NA-induced contractions in the E(+) strips which did not receive any pharmacological intervention, there was no significant difference ($P > 0.05$) between those experiments which used heparin, and those which did not (Figure 3).

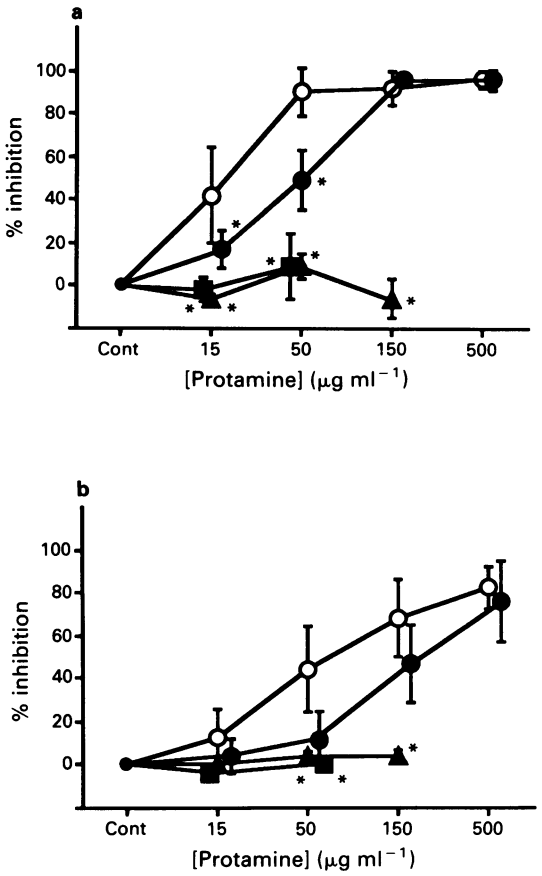


Figure 2 Dose-dependent effects of protamine on the noradrenaline (NA, 1 μM)-induced tonic contractions both in the presence of heparin (closed symbols) and in its absence (○: control) in either endothelium-intact (a) or -denuded (b) strips. Heparin (heparin/protamine ratio; 1.4–14 u μg^{-1}, the maximal concentration; 700 u ml^{-1}) was present both before and during the application of protamine. The effects of protamine are shown in the presence of heparin at an H/P ratio of 1.4 (●); 4.7 (▲) and 14 u μg^{-1} (■). All values are the means ± s.d., $n = 3$–12. *$P < 0.05$ compared to the control value at each concentration.

Table 2 IC_{50} values for the inhibitory effects of protamine on the noradrenaline-induced phasic and tonic contractions both in the absence (control) and in the presence of heparin (H/P ratio = 1.4 u μg^{-1}) either in the endothelium-intact or -denuded strips of rabbit mesenteric artery

		Treatment	*IC_{50}*[a]
Endothelium-intact	Phasic	Control	18.1 ± 0.1
		Heparin	32.2 ± 3.9
	Tonic	Control	16.2 ± 0.5
		Heparin	43.3 ± 5.5*
Endothelium-denuded	Phasic	Control	35.8 ± 3.5
		Heparin	36.7 ± 5.1
	Tonic	Control	41.8 ± 1.8
		Heparin	111.7 ± 10.0

[a]IC_{50} values for protamine (dose of protamine eliciting 50% of its maximum inhibitory effects) were calculated by the four-parameter logistic equation as described in Methods. Values represent mean ± s.e.mean.
*Significantly different from controls, $P < 0.05$ as determined by two-factor (treatment, concentration) analysis of variance.

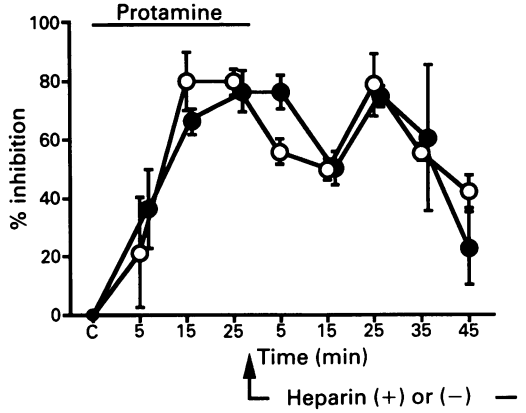


Figure 3 The effects of heparin (700 u ml^{-1}) on the prolonged inhibitory actions of protamine (150 μg ml^{-1}) on the noradrenaline (NA, 1 μM)-induced tonic contractions in the endothelium-intact strips. (○) Show the data obtained in the experiments without heparin (control), whilst (●) show the data obtained in the experiments with heparin, where protamine was washed out with a Krebs solution containing heparin. Identical results were obtained in the experiments on the effects of heparin on the protamine inhibition of the NA-induced phasic contractions (not shown). All values are means ± s.d., $n = 3$.

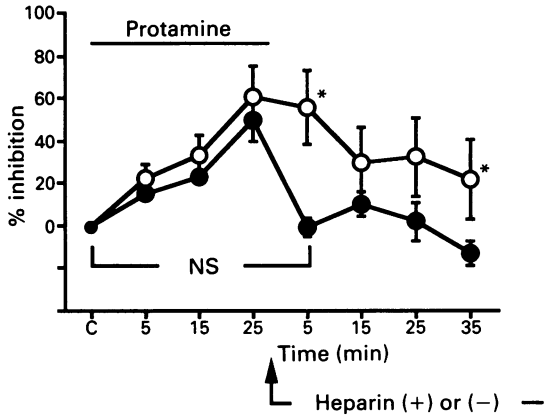


Figure 4 The effects of heparin (700 u ml^{-1}) on the prolonged inhibitory actions of protamine (150 μg ml^{-1}) on the noradrenaline (NA, 1 μM)-induced tonic contractions in the unrubbed strips pretreated with the EDRF pathway inhibitors (L-NOARG + MB + HbO_2). (○) Show the data obtained in the experiments without heparin (control), whilst (●) show the data obtained in the experiments with heparin, where protamine was washed out with a Krebs solution containing heparin. Identical results were obtained in the experiments on the effects of heparin on the protamine inhibition of the NA-induced phasic contractions (not shown). All values are means ± s.d., $n = 3$. *$P < 0.05$ compared to the value obtained in the experiments with heparin at each time. NS, not significantly different from the control value within each group.

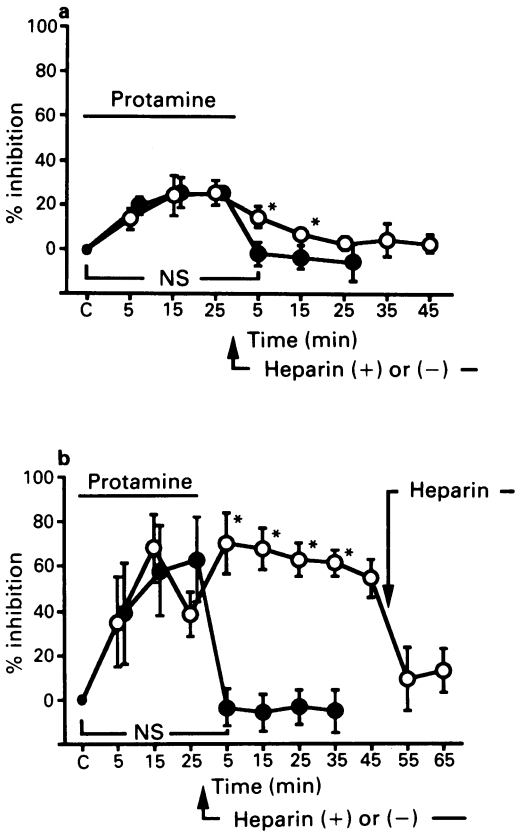


Figure 5 The effects of heparin (700 u ml^{-1}) on the prolonged inhibitory actions of protamine (150 $\mu g\ ml^{-1}$) on the noradrenaline (NA, 1 μM)-induced contractions (a) or on the high K^+-induced contractions (b) in the endothelium-denuded strips. (○) Show the data obtained in the experiments without heparin (control), whilst (●) show the data obtained in the experiments with heparin, where protamine was washed out with a Krebs solution containing heparin. Identical results were obtained in the experiments on the effects of heparin on the protamine inhibition of the NA-induced phasic contractions (not shown). All values are means ± s.d., $n = 3-7$. *$P < 0.05$ compared to the value obtained in the experiments with heparin at each time. NS, not significantly different from the control value within each group.

Discussion

In the present study, protamine (15–500 $\mu g\ ml^{-1}$), in the absence of heparin, inhibited NA-induced contractions both in the E(+) and E(−) strips. This vasodilator action of protamine was less affected by the presence of excess heparin at an H/P ratio of 1.4 u μg^{-1} but was almost completely blocked by the presence of a much greater excess of heparin at an H/P ratio of more than 4.7 u μg^{-1} (4.7 and 14 u μg^{-1}). Since the dissociation constant for the binding of heparin to protamine in a Krebs solution has never been previously reported, it is impossible for us to know how much free protamine (that which has not formed a complex with heparin) exists in a Krebs solution in which protamine and heparin were premixed at an H/P ratio of 1.4, 4.7, or 14 u μg^{-1}. However, we would like to speculate that the slightly attenuated but persisting vasodilator action of protamine, even in the presence of excess heparin with an H/P ratio of 1.4 u μg^{-1}, was probably due to the existence of free protamine at concentrations sufficient for it to exert vasodilator action, and that the disappearance of the vasodilator action of protamine in the presence of a much greater excess of heparin with an H/P ratio of more than 4.7 u μg^{-1} ($\geq$ 4.7 u μg^{-1}) was probably due to a significant decrease in the concentrations of free protamine in the Krebs solution. The above observations lead us to conclude that only protamine itself, rather than a heparin-protamine complex, produces vasodilator action in the splanchnic small arteries.

Although it was recently proposed that the vasodilator action of protamine may have an EDRF-mediated component besides the endothelium-independent component in this artery, this was not fully convincing because of the subtleties of the methodology employed (Akata *et al.*, 1991). However, our present findings may support the above proposal: heparin readily reversed the vasodilator action of protamine both in the E(−) strips and the E(+) strips which had been pretreated with various inhibitors of the EDRF pathway, while such reversing effects of heparin on the vasodilator action of protamine were not observed in the E(+) strips which had received any pharmacological intervention. Such differences in the effects of heparin on the prolonged vasodilator action of protamine between the E(+) strips which had not received any pharmacological intervention and the E(−) strips probably suggest the existence of an endothelium-dependent component in the vasodilator action of protamine. Furthermore, the differences in the effects of heparin on the prolonged vasodilator action of protamine between the E(+) strips which did not receive any pharmacological intervention and the E(+) strips pretreated with the inhibitors of the EDRF pathway, and the similarities in these effects between the E(−) strips and the E(+) strips pretreated with the inhibitors of the EDRF pathway suggest that the endothelium-dependent component in the vasodilator action of protamine is mediated through EDRF, which has recently been identified as nitric oxide or its related compounds (Furchgott & Zawadzki, 1980; Palmer *et al.*, 1988; Myers *et al.*, 1990).

It has been demonstrated that endothelial denudation or the application of inhibitors of the EDRF pathway enhanced the contractile responses in various isolated arteries, including rabbit mesenteric arteries (Nishiye *et al.*, 1989; Ignarro *et al.*, 1989; Akata *et al.*, 1991; Nakashima *et al.*, 1992). However, in the present study, the endothelium was removed mechanically before mounting the sample in the bath, and so it is impossible to know how the endothelial denudation affected the contractile responses to NA. Nevertheless, considering the previous observations cited above, it is quite conceivable that the contractile responses to NA could have been enhanced by this prior endothelial denudation. If so, the vasodilator actions of protamine in the E(−) strips may possibly be underestimated because of the enhanced contractile responses to NA, when the vasodilator action is estimated according to the percentage decrement from the control response. Because of this, we are reluctant to compare the present data on the vasodilator action of protamine between the E(+) and E(−) strips in order to discuss its endothelium-dependence. Furthermore, we consider it unreasonable to compare the data between the E(+) and E(−) strips from the viewpoint of the absolute tension level in our experimental setting, because technically it is extremely difficult for us to obtain constant samples consisting of the same number of vascular smooth muscle cells with the same contractile ability due to the extraordinarily small size of our vascular samples.

Although it has been previously reported that protamine enters the cell (De Robertis & De Robertis, 1980), the present findings on the reversing effects of heparin on the vasodilator action of protamine may suggest that protamine exerts its direct vasodilator action without entering the smooth muscle cell: the vasodilator action of protamine in the E(−) strips and in the E(+) strips pretreated with the EDRF pathway inhibitors was prolonged after the washing out of protamine with a Krebs solution which did not contain any heparin, while such prolonged action was readily reversed by the washing out of protamine with a Krebs solution containing heparin. It is generally accepted that heparin cannot easily enter the cell because of its poor lipid-solubility and large molecular weight (Stoelting, 1987). The present results actually support this idea: although 'cytosolic'

heparin has been reported to inhibit the IP_3-induced Ca^{2+} release from the intracellular Ca^{2+} store (Kobayashi *et al.*, 1988; 1989; Tones *et al.*, 1989), even an extraordinarily high concentration (700 u ml^{-1}) of heparin did not inhibit the NA-induced 'phasic' contraction, which has been proposed as being dependent on IP_3-induced Ca^{2+}-release in this artery (Hashimoto *et al.*, 1986; Itoh *et al.*, 1987). Thus, if protamine exerts its direct vasodilator action after entering the smooth muscle cell, it would surely be impossible for heparin, which cannot enter the cell, to gain access to intracellular protamine and to reverse its vasodilator action readily and completely. Therefore, the ability of heparin to reverse the vasodilator action of protamine in the E(−) strips or in the E(+) strips pretreated with the inhibitors of the EDRF pathway suggests that protamine exerts its direct vasodilator actions without entering the smooth muscle cell. Accordingly, protamine presumably exerts its 'direct' vasodilator action by acting on the cell membrane rather than by directly acting on the intracellular contractile protein apparatus.

It has been proposed (Uruno *et al.*, 1985; Akata *et al.*, 1991) that the direct vasodilator action of protamine may be attributable to its positive charge: protamine may inhibit the Ca^{2+} influxes by replacing the superficially bound Ca^{2+}, known to be essential for Ca^{2+} influx (Ishiyama *et al.*, 1975; Loutzenheiser *et al.*, 1983; Karaki & Weiss, 1984), at the 'anionic' superficial Ca^{2+}-binding sites, and protamine may inhibit the IP_3-induced Ca^{2+} release from the intracellular store by binding with 'anionic' phosphatidyl-inositol 4,5-bisphosphate (PIP_2) and by preventing PIP_2 breakdown. Our present observation in the E(−) strips may support the above previous proposals, i.e. heparin probably prevented and also reversed the vasodilator action of protamine in the E(−) strips by neutralizing the positive charge of protamine with its anionic charge as a result of the formation of ionic complexes. On the other hand, the inability of heparin to reverse the vasodilator action of protamine in the E(+) strips may imply that protamine exerts its endothelium-dependent vasodilator action by binding strongly to some anionic sites of the endothelial cell membrane and/or by entering the endothelial cell: the affinity of protamine for endothelium might be much stronger than its affinity for heparin. Further investigations are needed to clarify the mechanisms behind the vasodilator action of protamine in the endothelium-intact strips.

Since it is quite conceivable from previous reports (Kitani *et al.*, 1980; Shanberge *et al.*, 1987) that free protamine (that which has not formed a complex with heparin) can also exist in the blood side by side with a heparin-protamine complex even during the adequate neutralization of heparin-induced anticoagulation with protamine, it might be possible that the vasodilator action of protamine observed in the present study may play some role in its hypotensive effects: however, the amount of free protamine that actually exists in the blood during neutralization has never been previously reported. From the viewpoint of the present data, we would like to recommend that in order to minimize the vasodilator action of free protamine, in other words, in order to prevent any unnecessary increase in the blood concentration of free protamine, the absolute minimum possible amount of protamine should be administered slowly for the neutralization of heparin-induced anticoagulation, because only protamine, not a heparin-protamine complex, has vasodilator action, and the vasodilator action of protamine in the presence of intact endothelium cannot be readily reversed by heparin. Furthermore, it may well be advisable to recommend an evaluation of heparin-induced anticoagulation (measurement of activated clotting time, etc.) before the administration of an additional amount of protamine when systemic hypotension occurs during the neutralization of heparin anticoagulation, because under such conditions, there is the possibility that the heparin anticoagulation has already been neutralized by protamine and that the plasma concentration of the protamine which has not formed a complex with heparin, has increased to a level where protamine exerts its vasodilator action. Finally, our present data strongly suggest the importance of the determination of the amount of protamine required for the precise neutralization of heparin-induced anticoagulation.

To conclude, we would like to point out that the disappearance of the vasodilator action of protamine after a pretreatment application of excess heparin suggests that only protamine, not a heparin-protamine complex, exerts vasodilator action in the rabbit isolated mesenteric arteries. In addition, the differences in the ability of heparin to reverse the vasodilator action of protamine between the E(+) strips and the E(−) strips or the E(+)-strips pretreated with the inhibitors of the EDRF pathway, and the similarities in the ability of heparin to reverse the vasodilator action of protamine between the E(−) strips and the E(+) strips pretreated with the EDRF inhibitors, suggest that the *in vitro* vasodilator action of protamine probably has an EDRF-mediated component besides the endothelium-independent component. Moreover, the ability of heparin to reverse readily the vasodilator action of protamine in the E(−) strips may suggest that protamine probably exerts its direct vasodilator action without entering the vascular smooth muscle cell.

The authors are grateful to Miss K. Miller (Royal English Language Centre, Fukuoka, Japan) for correcting the English used in this manuscript. This work was supported by a Grant-in-Aid (C) (No. 02670687) from the Ministry of Education, Science and Cultures, Japan.

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(Received November 19, 1992
Revised April 5, 1993
Accepted April 8, 1993)

Br. J. Pharmacol. (1993), **109**, 1254–1262 © Macmillan Press Ltd, 1993

Vascular mode of action of kinin B_1 receptors and development of a cellular model for the investigation of these receptors

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1 Kinins exert a contractile effect on rabbit aortic rings via the stimulation of B_1 receptors. Des-Arg^9-bradykinin (BK) is more potent than BK on this receptor type. The mode of action of des-Arg^9-BK on rabbit aortic tissue has been studied by both the aortic ring contractility assay and a cellular model using cultured aortic smooth muscle cells (SMCs).

2 The des-Arg^9-BK-induced contractions in rabbit aortic rings were unaffected by pretreatments with nifedipine, indomethacin, REV-5901 (a 5-lipoxygenase blocker) and LY-83583 (a guanylyl cyclase inhibitor); however, the protein kinase inhibitors H-7 and H-9 significantly reduced the maximal effect of des-Arg^9-BK.

3 The contractile responses to des-Arg^9-BK in calcium-free Krebs solution were slightly but not significantly attenuated in amplitude, as compared to paired control tissues bathed in Krebs solution, and sustained plateaus of contraction were observed in the absence of Ca^{2+}. However, Ca^{2+} replenishment further increased the kinin-induced contraction measured in Ca^{2+}-free bathing fluid.

4 Despite the lack of evidence of a mediating role for prostaglandin in the mechanical response to des-Arg^9-BK, the kinin stimulated the release of prostacyclin from rabbit aorta rings measured as immunoreactive 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$).

5 Smooth muscle cells (SMCs) derived from the rabbit aorta exhibit functional responses to des-Arg^9-BK in acute release of 6-keto-$PGF_{1\alpha}$ and of inositol phosphate turnover which were inhibited by pretreatment with the B_1 receptor antagonist, Lys[Leu^8]des-Arg^9-BK, but not by the B_2 receptor antagonist, Hoe-140. Preincubation of the cells with interleukin-1 (IL-1) 20 h before stimulation with the kinin had no effect on basal inositol phosphate turnover, but potentiated the acute effect of des-Arg^9-BK.

6 These results suggest that second mesengers derived from the action of phospholipase C are produced by SMCs when B_1 receptors are activated in rabbit aortic tissue. Intracellular calcium stores are primarily mobilized by des-Arg^9-BK, although receptor-controlled calcium influx has not been ruled out, and may contribute to initiate the contractile responses. The maintenance of the contractile state involves protein kinase C activity and is consistent with a current model of SMC function. The cell model retains some of the cardinal properties of B_1 receptor-mediated vascular responses: endothelium-independent PGI_2 release and up-regulation by the cytokine IL-1. PGI_2 is not involved in the mechanical response, possible because the rabbit aorta is refractory to this prostaglandin.

Keywords: des-Arg^9-bradykinin; B_1 receptors for kinins; rabbit aortic preparation; signal transduction

Introduction

While the classification of receptor for bradykinin (BK) still awaits the molecular cloning of all the relevant receptor molecules, considerable pharmacological evidence supports the broad classification into the B_1 and B_2 types (Regoli & Barabé, 1980; Farmer & Burch, 1992). The B_1 receptor is stimulated selectively by kinin fragments of BK and Lys-BK without the C-terminal arginine residue such as des-Arg^9-BK and Lys-des-Arg^9-BK. The development of competitive, potent and selective antagonists (prototype [Leu^8]des-Arg^9-BK) has consolidated the pharmacological identity of the receptors of the B_1 type (Regoli *et al.*, 1977). Agonists and antagonists for B_1 receptors are conveniently assayed on the rabbit aortic preparation, which exhibits a prostaglandin-independent contractile response to kinins solely via this receptor type (Regoli *et al.*, 1977). The B_2 receptor type is optimally stimulated by the native kinins, BK or Lys-BK, and is antagonized by numerous sequence-related synthetic peptides, of which Hoe-140 is an excellent example (Hock *et al.*, 1991). Hoe-140 is a very high affinity antagonist that has no affinity for the B_1 receptors, and hence, does not inhibit kinin-induced contractions in the rabbit isolated aorta (Hock *et al.*, 1991). Conversely Hoe-140 is probably effective on all preparations containing the B_2 receptors, providing some uniformity to this pharmacological entity. The two kinin receptors cloned to-date from rat and human tissues belong to the B_2 class; they are highly homologous and the human type can be functionally inhibited by Hoe-140, but not by [Leu^8]des-Arg^9-BK (McEachern *et al.*, 1991; Hess *et al.*, 1992).

The B_1 receptors for kinins are usually not sufficiently dominant to account for the cardiovascular effects of kinins, such as hypotension, vasodilatation and increased vascular permeability. However, careful analyis has revealed that they account for a significant part of the effect of kinins in normal dogs (Toda *et al.*, 1987; Müller-Schweinitzer, 1988; Rhaleb *et al.*, 1989; Lorti *et al.*, 1992), and that they co-exist with B_2 receptors following an apparent up-regulation process in the cardiovascular system of rabbits or pigs injected with bacterial material or experiencing experimental arthritis (Regoli *et al.*, 1981; Drapeau *et al.*, 1991; Siebeck *et al.*, 1989; Farmer *et al.*, 1991). In chronic models of inflammation in the rat, kinin-mediated hyperalgia is reportedly largely dependent on B_1 receptors (Perkins *et al.*, 1992). We have shown that the contractile response to B_1 receptor agonists is acquired *in vitro* in the prototype preparation for B_1 receptor definition, the rabbit isolated aorta; this process is dependent on protein synthesis and can be further amplified by the

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cytokine interleukin-1 (IL-1) (Bouthillier *et al.*, 1987; deBlois *et al.*, 1988; 1991). In other preparations, such as the rabbit mesenteric and dog renal arteries, the dominant relaxation response to the stimulation of B_1 receptors is a prostaglandin-dependent and endothelium-independent process (Churchill & Ward, 1986; deBlois & Marceau, 1987; Rhaleb *et al.*, 1989). A significant portion of the hypotensive effect of B_1 agonists in lipopolysaccharide-treated rabbits is also accounted for by prostaglandin release (Drapeau *et al.*, 1991).

Data on the cardiovascular effects dependent on the B_1 receptors are lacking because no systematic studies on the signal transduction pathways or on receptor binding assay have been reported to date. The direct contractile effect of kinins on the rabbit isolated aorta is unaffected by indomethacin, endothelium removal and tyrosine kinase inhibitors (deBlois *et al.*, 1992), but is partially inhibited by the removal of extracellular calcium, or by treatments with $NiCl_2$ (used as a Ca^{2+} channel inhibitor), or by protein kinase C inhibitors (Calixto & Medeiros, 1992). In the porcine isolated coronary artery, the contractile effect of des-Arg^9-BK did not occur simultaneously with a significant change in membrane potential (Beny *et al.*, 1987). These observations are compatible with the activation of the phospholipase C signalling pathway and also, possibly, with the activation of receptor-controlled calcium channels following B_1 receptor stimulation. Direct evidence of phospholipase C activation by B_1 receptor stimulation was obtained in cultured rat mesangial cells, one of the numerous cultured cell types that express this pharmacological entity (Issandou & Darbon, 1991). Agonists that activate membrane associated tyrosine kinase activities, such as insulin and epidermal growth factor, have been reported to act synergistically with B_1 receptor agonists in the rat mesengial cells and in rabbit aortic strips, respectively (Issandou & Darbon, 1991; deBlois *et al.*, 1992).

The present study has been undertaken to characterize further the mode of action of B_1 receptor agonists in the rabbit aortic preparation. Ultimately, we hope to develop a cellular model that would retain the essential characteristics of the vascular preparations to study the cardiovascular actions of B_1 receptor-mediated effects of kinins. Such a model would allow the analysis of possible signalling pathways (calcium, eicosanoids and phospholipase C) and of receptor population regulation subsequent to immunological stimulation.

Methods

Organ bath pharmacology of B_1 receptors

The thoracic aorta was isolated from New Zealand White rabbits of either sex (1.5–2 kg). The vessels were cut into rings (2–3 mm width), suspended between a metal hook and a thread loop under a basal tension of 2 g in 5 ml organ chambers containing oxygenated (95% O_2:5% CO_2) and warmed (37°C) Krebs solution (Marceau *et al.*, 1991). Their responses to agents were recorded isometrically as described previously (Bouthillier *et al.*, 1987).

In order to analyze the mechanism of the contractile effect of the selective B_1 receptor agonist des-Arg^9-BK, a nearly maximal concentration (1.7 μM) of this agent was applied to tissues after 3.5 to 4.5 h of equilibration. This time was kept constant in treated tissues and their paired controls because the maximal effect of this kinin changes considerably as a function of the incubation time. The mechanism for the increase of tissue response to B_1 receptor agonists has been documented (Bouthillier *et al.*, 1987; deBlois *et al.*, 1988; 1989; 1991). Inhibitory drugs were applied 30 min before the kinin and then continuously in treated groups; paired control tissues were used to evaluate the effect of the drug vehicle, if any. The same tissues were also challenged at later times with other submaximal contractile stimuli for comparison purpose: KCl (30 mM) and phorbol myristate acetate (PMA; 1.1 μM). The inhibitory drugs under study were nifedipine, an L-type Ca^{2+} channel blocker (Murad, 1990), the protein kinase inhibitors H-7 and H-9 (Hidata *et al.*, 1984), indomethacin, the 5-lipoxygenase blocker (REV-5901 (McMillan & Walker, 1992) and the guanylyl cyclase inhibitory LY-83583 (Mulsch *et al.*, 1989). The concentrations of the drugs were chosen on the basis of their reported activity in various *in vitro* systems and, when possible, relevant controls were obtained in rabbit aortic tissue. In some experiments involving H-7, full cumulative concentration-effect curves were established for KCl and des-Arg^9-BK to document further the effect of the kinase inhibitor on a wide range of contraction amplitudes for both agents. Another protocol consisted of replacing the Krebs solution by a calcium-free formulation of Krebs solution, 5–10 min before each contractile stimulation; this was done to evaluate the contribution of extracellular calcium to the effect of each type of stimulation. Between tests, the tissue preparation was maintained in regular Krebs solution with calcium to avoid depletion of the intracellular pools.

In some experiments, the possible stimulation of eicosanoid release by des-Arg^9-BK was tested by measuring the concentration of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), the major cyclo-oxygenase product in this tissue (Förstermann *et al.*, 1984), in the bathing fluid. Some tissues were exposed to indomethacin (2.8 μM) 30 min before the effect of des-Arg^9-BK was examined. The aortic rings were washed and three aliquots of the bathing fluid were successively taken at 10 min intervals. Immediately after the second sampling, des-Arg^9-BK (1.7 μM) was applied to each tissue. 6-Keto-$PGF_{1\alpha}$ release results are expressed as the mass secreted per aortic ring during two consecutive 10-min periods, firstly a basal secretion period followed by a treatment period. These values were calculated by subtracting the concentration at the beginning of a period from the concentration at the end of that period. The prostaglandin concentration was determined by a specific radioimmunoassay (Lebel & Grose, 1986).

Cell culture

Several distinct lines of rabbit smooth muscle cells were established from rabbit aortae aseptically removed from freshly killed animals. The tissues (approximate weight 200 mg) were incubated at 37°C for 1.5 h in medium 199 (Gibco) supplemented with 10% foetal bovine serum (FBS; Gibco), antibiotics (streptomycin, 50 μg ml^{-1}, and penicillin, 50 u ml^{-1}; Gibco), bacterial collagenase (type IA, 3 mg ml^{-1}) and elastase (type III, 1 mg ml^{-1}); all enzymes were obtained from Sigma. Dispersed cells and partly digested tissue fragments were centrifuged and resuspended in medium 199 supplemented with 10% FBS and antibiotics. This suspension was seeded directly in 25 cm^2 flasks coated with 0.2% gelatin. Cells were passaged on gelatin-coated surfaces at confluency with a brief trypsin (0.05%)-EDTA (0.53 mM) treatment. The cells were studied at passages 1 to 7 only; their identity as smooth muscle cells was checked by indirect immunofluorescence for α-actin, a smooth muscle marker (monoclonal antibody 1A4 from Sigma) with a Bio-Rad MRC-600 scanning confocal microscope.

Experiments were conducted with the same type of cells to evaluate the turnover of phosphoinositides and the production of prostaglandin in response to B_1 receptor stimulation.

Inositol phosphate production

Labelling and stimulation of rabbit aorta smooth muscle cells were carried out according to Balla *et al.* (1988) with some modifications. Briefly, cells were seeded at a density of 2.5×10^5 in wells of 2 cm^2 in the culture medium described above and cultured for 24 h. The cells were then washed three times with serum-free medium. The incorporation medium (500 μl) consisted of Medium 199 containing 20 μCi ml^{-1} of *myo*-[^{3}H]-inositol (Amersham, specific activity 82 Ci

mmol^{-1}) and IL-1β (5 ng ml^{-1}), when indicated, was added to the wells for a labelling period of 16–20 h. After incubation at 37°C in a humidified atmosphere containing 5% CO_2, each well was washed twice with Earle's balanced salt solution (EBSS). The cells were further incubated for 20 min in EBSS with 10 mM LiCl and with a kinin antagonist, when indicated. The cells were then challenged with des-Arg^9-BK (1.7 μM) or its vehicle for exactly 5 min. Stimulations were terminated by the addition of perchloric acid (final concentration 5%).

Inositol phosphates were extraced and separated as previously described (Guillemette *et al.*, 1989). At the end of the experiment, the cells were kept on ice for 30 min and then scraped from the wells and transferred into tubes. The wells were rinsed with additional perchloric acid (5% w/v) and the resulting media was centrifuged at 10,000 *g* for 5 min. Perchloric acid was extracted from the supernatant with a 1:1 mixture of Freon and tri-n-octylamine (Downes *et al.*, 1986; reagents from Sigma). After neutralization, the samples were applied to anion-exchange resin (Dowex AG 1-X8 formate, 200–400 mesh, Bio-Rad No. 140–1450) columns (1 ml bed volume) for the separation of the inositol phosphates according to Berridge *et al.* (1983). The inositol phosphates were sequentially eluted with 3 × 5 ml of distilled water (free inositol), 3 × 5 ml of 0.2 M ammonium formate/0.1 M formic acid (inositol monophosphates, IP_1), 3 × 5 ml of 0.5 M ammonium formate/0.1 M formic acid (inositol biphosphates, IP_2), 3 × 5 ml of 0.8 M ammonium formate/0.1 M formic acid (inositol trisphosphates, IP_3) and 3 × 5 ml of 1.0 M ammonium formate/0.1 M formic acid (inositol tetrakisphosphates, IP_4). The fractions were collected, mixed with Ecolite + scintillation fluid (ICN Biomedicals, Irvine CA, U.S.A.) and counted for radioactivity in a liquid scintillation counter (Beckman LS 8000).

Smooth muscle cells were used to evaluate the kinin-induced release of 6-keto-$PGF_{1\alpha}$. Cells were seeded in gelatin-coated 12-well plates (Linbro, Rockville MD, U.S.A.; well diameter 24 mm) at a density of 3 × 10^4 per well and cultured for 24 h in the complete medium described above. The culture medium was then removed, the wells were washed once and replaced with 1 ml of equilibrated (37°C, 5% CO_2) EBSS containing the test substances (a kinin agonist, antagonist or both together). After a 20 min incubation period, the supernatants were collected for measurement of 6-keto-$PGF_{1\alpha}$ as described above.

Drugs

The following drugs were purchased from Sigma (St-Louis MO, U.S.A.): nifedipine, H-7 (1-(5-isoquinolinesulphonyl)-2-methylpiperazine), H-9 (N-(2-aminoethyl)-5-isoquinolinesulphonamide), indomethacin and PMA (phorbol 12-myristate 13-acetate). REV-5901 (α-pentyl-3-(2-quinolinylmethoxy)-benzene methanol, *para* isomer) was purchased from Biomol (Plymouth Meeting PA, U.S.A.) and LY-83583 (6-anilino-5,8-quinolinedione), from Calbiochem (La Jolla CA, U.S.A.). Hoe-140 (D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK; icatiban) was a gift from Dr K. Wirth, (Hoechst AG, Frankfurt, Germany). Des-Arg^9-BK was purchased from Bachem (Torrance CA, U.S.A.) and Lys[Leu^8]des-Arg^9-BK was synthesized in our laboratories (Drapeau & Regoli, 1988). Human recombinant interleukin-1β was a gift of Dr D.E. Tracey (Upjohn Co., Kalamazoo MI, U.S.A.).

Results

Contractility assays

Comparative experiments were performed with des-Arg^9-BK, a very selective B_1 receptor agonist, and with other contractile agents in rabbit aortic rings (Table 1). A nearly maximal concentration (ED_{95}) of the kinin was chosen for these experiments, whereas the effect of KCl (30 mM) and of PMA (1.1 μM) was submaximal in each case. Two or three contractile responses were recorded at the same time for control and treated rings after at least 3.5 h of equilibration; this pairing is necessary because the effect of des-Arg^9-BK varies as a function of the time of *in vitro* incubation and because there is a large inter-animal variation for these responses (Bouthillier *et al.*, 1987). Inhibitory drugs were applied 30 min before recording contractile responses to the kinin.

The voltage-operated calcium channel blocker, nifedipine, had no significant effect on the contractile response to des-Arg^9-BK. In the same tissues, it inhibited very extensively the contractile effect of the depolarizing agent KCl, providing an internal control of the effectiveness of the drug at the concentration tested. Protein kinase inhibitors were tested in particular to evaluate the possible role of protein kinase C following the stimulation of B_1 receptors. Both protein kinase blockers H-7 (10 μM) and H-9 (30 μM) partially inhibited the

Table 1 Pharmacological analysis of the mode of action of the B_1 receptor agonist des-Arg^9-BK in the rabbit aortic preparation

		Contractile responses (g)		
Treatment	n	*des-Arg^9-BK* (1.7 μM)	*KCl* (30 mM)	*PMA* (1.1 μM)
Nifedipine 100 nM	6	0.84 ± 0.09	0.16 ± 0.02***[a]	NT[b]
Vehicle (ethanol)	6	1.03 ± 0.08	2.71 ± 0.26	NT
H-7 10 μM	8	1.0 ± 0.3*	3.3 ± 0.3	0.14 ± 0.01***
Vehicle (saline)	8	1.7 ± 0.3	3.6 ± 0.1	0.27 ± 0.02
H-9 30 μM	6	0.57 ± 0.15**	2.65 ± 0.34	0.09 ± 0.01***
Vehicle (HCl)	6	1.34 ± 0.17	4.10 ± 0.67	0.30 ± 0.03
Indomethacin 2.8 μM	14	0.70 ± 0.12	2.87 ± 0.35	NT
Vehicle (Na_2CO_3)	14	0.89 ± 0.19	2.78 ± 0.33	NT
REV-5901 500 nM	6	1.19 ± 0.29	3.78 ± 0.49	NT
Vehicle (ethanol)	6	0.98 ± 0.09	3.24 ± 0.47	NT
LY-83583 10 μM	5	0.74 ± 0.22	3.03 ± 0.49	NT
Vehicle (DMSO)	5	0.79 ± 0.02	3.13 ± 0.09	NT
Calcium-free Krebs[c]	10	1.39 ± 0.48	0.24 ± 0.04***	NT
Control	10	1.63 ± 0.30	4.62 ± 0.30	NT

[a]Values in treated groups were compared to controls from the same animals by Student's *t* test for paired samples: *$P<0.05$; **$P<0.01$; ***$P<0.001$.

[b]NT, not tested.

[c]Tissues were exposed to calcium-free Krebs solution only 10 min before each recording and were then placed in normal Krebs for recovery.

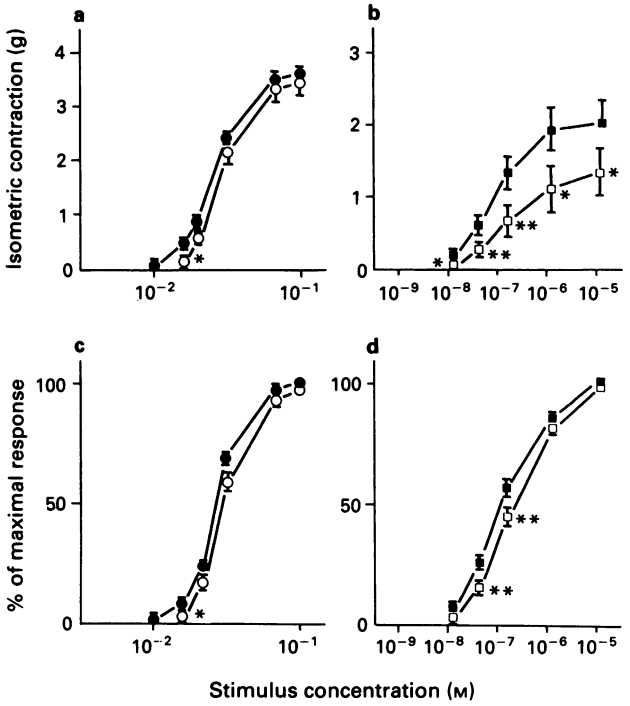


Figure 1 Effect of H-7 (10 μM) on the cumulative concentration-effect curves for KCl (a, c) and des-Arg^9-BK (b, d) in rabbit aortic rings. In (a) and (b) results are expressed in g of developed contraction; the same data are plotted as a percentage of the maximal response in (c) and (d). Abcissa scale: – log of molar concentration. Values are the means ± s.e.mean of 6 determinations for KCl, 8 for des-Arg^9-BK. Open symbols: values recorded in the presence of H-7; closed symbols, controls. Student's *t* test for paired samples was used to assess the effect of H-7: $*P<0.05$; $**P<0.01$.

effect of des-Arg^9-BK and of PMA, but not that of KCl. These results may indicate a role for one of the susceptible protein kinases in the contractile response to des-Arg^9-BK. The selectivity of the effect of the drug H-7 (10 μM) was further analyzed by constructing full cumulative concentration-effect curves for des-Arg^9-BK and KCl in its presence (Figure 1). The contractile effect of all concentration levels of the kinin were significantly depressed by H-7 (Figure 1b), whereas only one of the smaller concentration of KCl was affected by the drug (Figure 1a). The EC_{50} for KCl was not significantly affected by H-7 ($2.7 \pm 0.1 \times 10^{-2}$ M in the presence of H-7; control value $2.5 \pm 0.05 \times 10^{-2}$ M). H-7 shifted the curve slightly to the right for the kinin (EC_{50} of $1.9 \pm 0.3 \times 10^{-7}$ M in the presence of H-7; control value of $1.1 \pm 0.2 \times 10^{-7}$ M; $P<0.01$ by Student's *t* test for paired samples).

As reported previously (Regoli *et al.*, 1977; deBlois *et al.*, 1992), indomethacin did not inhibit the effect of des-Arg^9-BK in the rabbit aortic preparation; the drug was also not effective on the KCl-induced contractions (Table 1). The 5-lipoxygenase blocker, REV-5901, failed to influence the effects of the contractile agents, des-Arg^9-BK or KCl. The inhibitor of the soluble form of guanylyl cyclase, LY-83583, also failed to inhibit the contractile effect of any of the two stimuli at a concentration that reportedly inhibits guanosine 3′:5′-cyclic monophosphate (cyclic GMP) accumulation in the acetylcholine-stimulated rabbit aorta (Diamond, 1987).

The major eicosanoid produced by the isolated aorta, either in the basal or stimulated conditions is PGI_2 (Förstermann *et al.*, 1984). We have measured the concentration of immunoreactive 6-keto-$PGF_{1\alpha}$, the PGI_2 breakdown product, in the bathing fluid of rabbit aortic rings challenged with des-Arg^9-BK (Table 2). The paired tissues exposed to indomethacin are the same preparations for which the mechanical responses are reported in Table 1. Des-Arg^9-BK treatment increased sharply the release of the eicosanoid during the experimental 10 min period, as compared to a similar basal release period. However, tissues treated with indomethacin released less 6-keto-$PGF_{1\alpha}$ in the basal period and did not respond to the kinin stimulation. This suggests that eicosanoid release occurs in the rabbit isolated aorta in response to B_1 receptor stimulation, but does not affect the mechanical response to the same stimulus.

A group of experiments was designed to evaluate the role of extracellular calcium in the B_1 receptor-mediated contractile events. Tissues incubated in Krebs solution were rinsed extensively with calcium-free Krebs 10 min before their response to des-Arg^9-BK or to control stimulus was recorded (Table 1, statistical analysis). After the maximal effect was recorded, the tissues were washed and further incubated in regular Krebs solution with calcium. This procedure was developed to avoid long-term leakage of intracellular calcium

Table 2 Release of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) from rabbit aortic rings during two consecutive 10 min basal and des-Arg^9-BK-stimulated (1.7 μM) periods

		6-Keto-$PGF_{1\alpha}$ (pg released/10 min)	
Pretreatment	n	*Basal period*	*des-Arg^9-BK*
None (control)	14	39 ± 12	211 ± 68*[a]
Indomethacin 2.8 μM	14	16 ± 4	8 ± 2

[a]Values obtained in the treated periods were compared to those measured in the control periods by Student's *t* test for paired samples: $*P<0.05$. The mechanical responses of these tissues to des-Arg^9-BK are documented in Table 1.

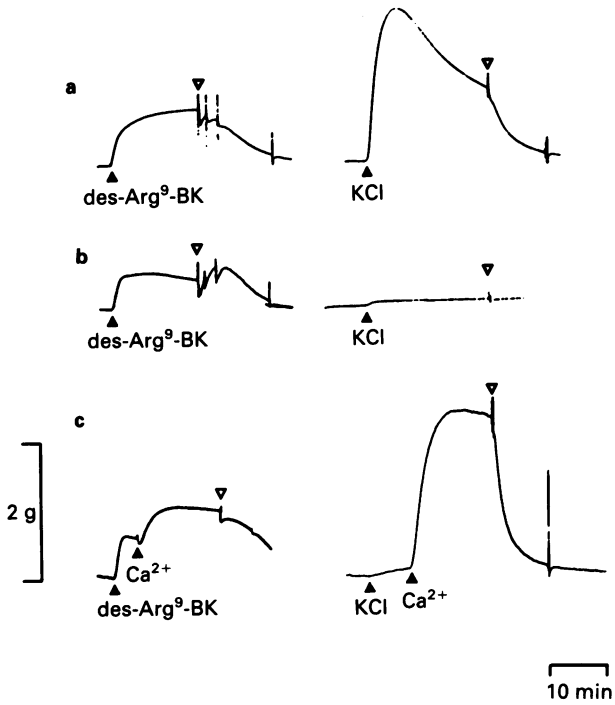


Figure 2 Representative tracings of the contraction profiles recorded from different aortic rings from a single rabbit. Tissues were successively stimulated with des-Arg^9-BK (1.7 μM) and KCl (30 nM). (a) Control tracing, in regular Krebs solution; (b) effect of calcium removal 5 min before pharmacological stimulation; (c) effect of calcium concentration restoration (2.5 mM) on contraction developed in calcium-free Krebs. Closed symbols refer to stimulus application and open symbols, to the first of a series of tissue washing with normal Krebs solution. Abscissa scale: time; ordinate scale: isometric contraction, g.

stores and is validated by the extensive inhibition of KCl-induced contraction, a stimulus which is essentially dependent on extracellular calcium (Cauvin *et al.*, 1983). Under these conditions, there was some inhibition of the maximal effect of des-Arg^9-BK, although it was not statistically significant in this series of experiments. This protocol is illustrated by representative tracings (Figure 2). It can be seen that tissues contracted with the kinin in the absence of extracellular calcium maintain a good contraction plateau over a 10 min period (Figure 2b). In tissues contracted with des-Arg^9-BK in the absence of calcium, replenishment of calcium when the effect had developed further increased the contractile response, whereas the KCl-induced contraction was initiated only when Ca^{2+} concentration was restored (Figure 2c). The same experiment conducted in tissues pretreated with nifedipine produced identical results for des-Arg^9-BK: there was an additional tension that developed following the introduction of Ca^{2+} (tracings not shown).

Cellular model of the vascular effect of des-Arg^9-BK

Cells derived from the digestion of rabbit aortic tissue without endothelium exhibited a narrow, pointed morphology; when the cultures approached confluence, parallel bundles of cells were observed under phase contrast microscopy (Figure 3a). Most of the cells stained positive for the smooth muscle marker α-actin as cytosolic fibrillar patterns, (Figure 3b,c), but the intensity of the staining was variable from one cell to another.

The cultured smooth muscle cells derived from the rabbit aorta retained the capacity to respond to kinins by increased release of 6-keto-$PGF_{1\alpha}$ (Figure 4, Table 3). The selective B_1 agonist des-Arg^9-BK elicited a concentration-dependent stimulant effect, with statistical significance at and above 10 nM (Figure 4). BK was not statistically active in this respect up to 10 μM. The kinin antagonists Hoe-140 and Lys[Leu^8]des-Arg^9-BK had no direct effect on basal 6-keto-$PGF_{1\alpha}$ release at 1.7 μM (Table 3). Only the latter antagonist, selective for the B_1 receptors, reduced the influence of des-Arg^9-BK when co-administered.

Evidence was also obtained for des-Arg^9-BK-induced IP turnover in this type of cell (Table 4). In experiment 1, des-Arg^9-BK (1.7 μM) acutely induced the accumulation of inositol phosphates in cells maintained in both the regular medium, which significantly increased the concentration of IP_2 and IP_4, and in the presence of IL-1β, which significantly increased levels of IP_1, IP_2 and IP_3. In the absence of the kinin, IL-1β alone had no effect, but there appears to be an increased incorporation efficiency of *myo*-inositol into the cells. However, IL-1β pretreatment potentiated significantly the des-Arg^9-BK effect on the cellular IP_3 concentration. It was previously shown that the cytokine increased the maximal contractile effect of des-Arg^9-BK considerably in fresh aortic tissue over several hours (deBlois *et al.*, 1988; 1991).

All the cells were pretreated with IL-1β in experiment 2 to maximize the effect of the kinin. As shown in Table 4, the kinin antagonist Lys[Leu^8]des-Arg^9-BK (1.7 μM) failed to increase IP metabolism significantly in cultured SMCs, but it inhibited the stimulatory effect of des-Arg^9-BK on IP_1 and IP_3 concentrations, suggesting that the influence of this kinin on phospholipase C activity is related to B_1 receptor-mediated events. The B_2 antagonist, Hoe-140, did not inhibit des-Arg^9-BK-induced alterations in IP concentrations (Student's *t* test); at 1.7 μM, the antagonist peptide may have stimulated IP production directly and thus potentiated the kinin-induced IP_2 production, but the changes were not statistically significant.

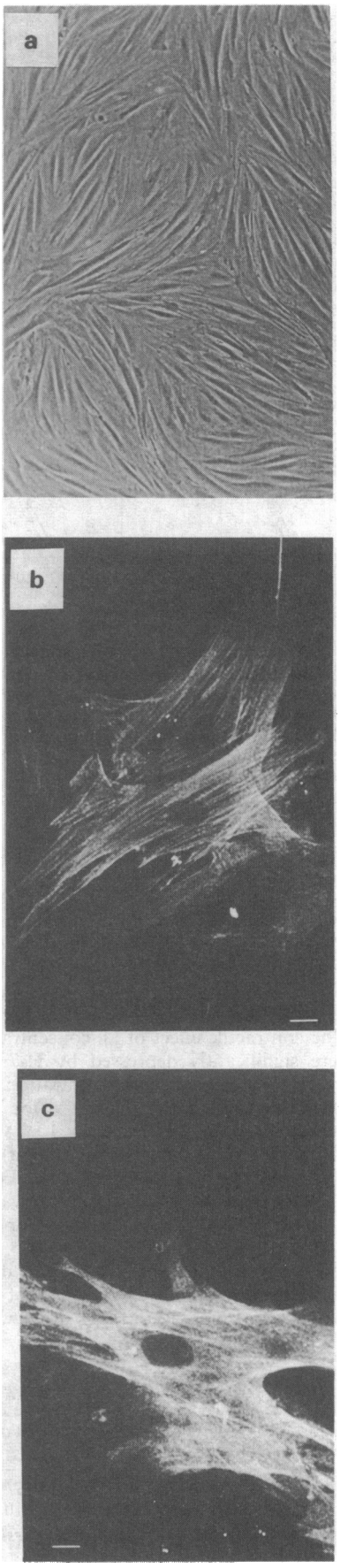


Figure 3 Cultured smooth muscle cells from rabbit aortic tissue. (a) Phase contrast appearance passage 1 (× 100); (b and c) indirect immunofluorescence for the smooth muscle specific α-actin (passages 1 and 4, respectively). Horizontal bar: 10 μm scale.

Discussion

Complementary approaches were used to analyze the mode of action of des-Arg^9-BK employing the rabbit aortic prep-

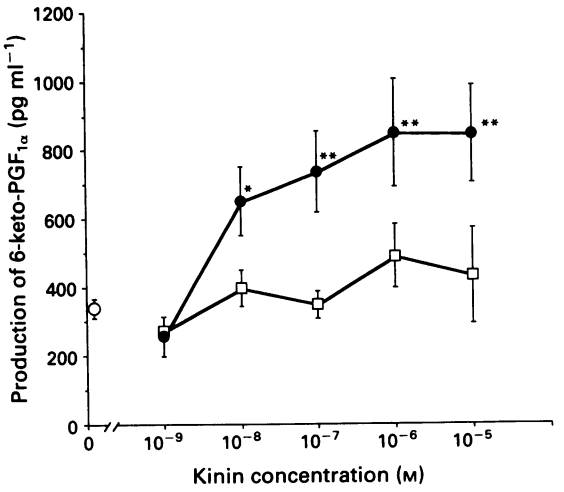


Figure 4 Concentration-effect relationship for des-Arg^9-BK- (●) and bradykinin (BK)-induced (□) production of 6-keto-$PGF_{1\alpha}$ from cultured smooth muscle cells derived from rabbit aortae. Values are the means ± s.e.mean of 15 values composed of data from 6 cell lines (passages 3 to 7). The basal release value (○) is the mean ± s.e.mean of 36 values from the same cell lines. One-way analysis of variance indicated that the groups were not homogeneous ($P<0.01$). Dunnett's test was then applied to compare the effect of each treatment with the controls (Tallarida & Murray, 1987): $*P<0.05$; $**P<0.01$.

aration, the prototype bioassay for this receptor type (Regoli & Barabé, 1980). Using contractility as the end point, it was shown that rabbit aortic rings are less dependent on extracellular calcium when stimulated with des-Arg^9-BK than with KCl. A relatively sustained contraction was observed with the kinin, even in the absence of extracellular calcium in the bathing fluid. Another group of investigators has suggested that the BK-induced contraction, also mediated by B_1 receptors, is highly dependent on extracellular calcium; this conclusion is based on experiments conducted in a Ca^{2+}-free physiological medium and on the modest inhibitory effect of $NiCl_2$ (Calixto & Medeiros, 1992). In that report, however, there was a 30 min pre-incubation period in a Ca^{2+}-free buffer before pharmacological stimulation in the form of a cumulative concentration scale was carried out. Our discrepant findings may be due to the selection of a single peptide concentration and of a shorter preincubation period of only 5–10 min; this approach may prevent the time-dependent 'leakage' of intracellular calcium pools, and at the same time was sufficiently effective to inhibit 95% of the contractile effect of KCl, which is a depolarizing stimulus dependent on extracellular Ca^{2+} (Cauvin *et al.*, 1983). Restoration of the Ca^{2+} concentration in a tissue precontracted with des-Arg^9-BK in Ca^{2+}-free Krebs solution further increased contraction; this experiment suggests that during the des-Arg^9-BK-induced stimulations influx of Ca^{2+} occurs. However, this component which was not inhibited by nifedipine, is relatively minor. It may be due to receptor-controlled Ca^{2+} channels not sensitive to dihydropyridines. Nifedipine had no effect on kinin-induced contractions, but it abolished the KCl-induced contraction, which is consistent with the inhibitory effect of this drug on the voltage-operated channels (Murad, 1990). The lack of effect of des-Arg^9-BK on membrane potential in porcine coronary arteries has been reported previously (Beny *et al.*, 1987) and is consistent with the lack of effect of nifedipine noted in the present study, and of nicardipine in a recent publication (Calixto & Medeiros, 1992).

Contractility data obtained with aortic rings also suggest the involvement of protein kinase(s) in des-Arg^9-BK-induced contraction. Protein kinase C is susceptible to inhibition by both H-7 and H-9 (Hidata *et al.*, 1984) and, accordingly, both compounds suppressed effectively the small contractile effect due to the protein kinase C activator, PMA. Also, the two inhibitors partially but significantly reduced the contractile effect of des-Arg^9-BK, suggesting that the signal transduction of B_1 receptors may be physiologically regulated by diacylglycerol derived from the phospholipase C pathway. H-7 exhibited some selectivity in its action as it inhibited only minimally equicontractile concentrations of KCl (Figure 1). Des-Arg^9-BK is not likely to increase the intracellular Ca^{2+} concentration in a sustained manner, especially in the absence of extracellular Ca^{2+}; however, the observed contraction plateaus are very stable (Figure 2). According to a concept of smooth muscle contraction (Rasmussen *et al.*, 1987), relative to the mechanical response, the rise in intracellular calcium concentration and the resulting activation of the myosin light chain kinase are only transient in SMCs, but these components are necessary to initiate a strong contractile response. Protein kinase C activation is involved in the

Table 3 Pharmacological characterization of the kinin-induced release of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) from cultured smooth muscle cells from the rabbit aorta

Agonist	*Antagonist*	*6-keto-$PGF_{1\alpha}$ release* (pg/well)	*Dunnett's test*[a]
None	None	421 ± 27	—
	Lys[Leu^8]des-Arg^9-BK 1.7 μM	472 ± 52	NS
	Hoe-140 1.7 μM	456 ± 66	NS
des-Arg^9-BK 1.7 μM	None	948 ± 85	<0.01
	Lys[Leu^8]des-Arg^9-BK 1.7 μM	567 ± 67*	NS
	Hoe-140 1.7 μM	878 ± 78	<0.01
BK 1.7 μM	None	542 ± 59	NS
	Lys[Leu^8]des-Arg^9-BK 1.7 μM	451 ± 39	NS
	Hoe-140 1.7 μM	629 ± 105	NS

[a]Values are the means ± s.e.mean of 9 values composed of triplicate determinations from 3 cell lines (passages 4 to 6), except for the control group (first line), which composed of 26 values from the same cell lines. One-way analysis of variance was performed ($P<0.01$). Dunnett's test was then applied to compare the effect of each treatment with the baseline prostaglandin release (first line) (Tallarida & Murray, 1987). The effect of each antagonist was compared to the corresponding controls exposed to the same agonist by Student's *t* test ($*P<0.01$).

Table 4 Inositol phosphates measurements in rabbit aortic smooth muscle cells 5 min after exposure to des-Arg^9-BK (1.7 μM), to a kinin antagonist (1.7 μM of either one), or to the combination of des-Arg^9-BK and an antagonist[a]

Pretreatments[b]	*Stimulus*	*Inositol phosphates* (c.p.m. per well)[c] IP_1	IP_2	IP_3	IP_4	*Cell-associated myo-inositol*
Experiment 1						
None	Control	2295 ± 459	300 ± 58	188 ± 22	148 ± 9	126869 ± 7021
None	des-Arg^9-BK	3631 ± 673	785 ± 199*[d]	270 ± 40	179 ± 11*	115241 ± 13124
IL-1β (− 20 h)	Control	1619 ± 356	328 ± 35	155 ± 10	150 ± 14	187081 ± 30785
IL-1β (− 20 h)	des-Arg^9-BK	5686 ± 882***	1073 ± 182**	518 ± 82***†	231 ± 43	198577 ± 29783†
Experiment 2						
IL-1β (− 20 h)	Control	1250 ± 173	145 ± 31	88 ± 15	61 ± 11	270932 ± 23808
IL-1β (− 20 h)	des-Arg^9-BK	2567 ± 437*	298 ± 48	166 ± 29*	84 ± 11	291997 ± 61488
IL-1β (− 20 h)	Lys-[Leu^8]-des-Arg^9-BK	1264 ± 244	203 ± 31	102 ± 10	69 ± 11	284019 ± 44703
IL-1β (− 20 h)	Hoe-140	1656 ± 318	212 ± 31	118 ± 11	76 ± 9	243001 ± 26440
IL-1β (− 20 h)	des-Arg^9-BK + Lys-[Leu^8]-des-Arg^9-BK	1115 ± 112	174 ± 50	93 ± 16	64 ± 9	267460 ± 23933
IL-1β (− 20 h)	des-Arg^9-BK + Hoe-140	2354 ± 460	481 ± 80**	175 ± 31*	89 ± 10	304648 ± 64560
Analysis of variance[e]		$P<0.01$	$P<0.01$	$P<0.01$	NS	NS

[a]Smooth muscle cells from different cell lines were used at passage 2, 3 and 4 for experiment 1 (values are the means ± s.e.mean of 9 values composed of triplicate determinations from 3 cell lines) and 4 or 5 for experiment 2 (values are the means ± s.e.mean of 12 values composed of triplicate determinations from 4 cell lines).
[b]Human recombinant IL-1β (5 ng ml^{-1}) was present during the 20 h incubation period with *myo*-[^{3}H] inositol.
[c]In experiment 1, one of the variables (IL-1 presence) influenced significantly *myo*-inositol incorporation; therefore results are expressed directly as c.p.m. per well. In experiment 2, IL-1 was uniformly applied and the *myo*-inositol incorporation was homogeneous in all groups (ANOVA). Therefore the results have been normalized as c.p.m. of IP per 100,000 c.p.m. of the remaining cell-associated *myo*-inositol in each cell well.
[d]For experiment 1, the effect of des-Arg^9-BK was compared with controls by Student's *t* test: *$P<0.05$; **$P<0.01$; ***$P<0.001$. The effect of IL-1 pretreatment on experimental values was also assessed by comparing IL-1-treated groups with the corresponding group (with or without kinin challenge) by Student's *t* test: †$P<0.05$.
[e]For experiment 2, one-way analysis of variance was performed on columns of data (NS, not significant). In three columns the test indicated that groups were significantly different from each other. Dunnett's test was then applied to compare the effect of each treatment with the control cells pretreated with IL-1β only (first line) (Tallarida & Murray, 1987): *$P<0.05$; **$P<0.01$.

maintenance of the contractile state, but if it is not preceded by a Ca^{2+} concentration increase, as with PMA, it leads only to a very small and slow developing contraction (Rasmussen *et al.*, 1987).

The use of protein kinase C inhibitors has some limitations, which include lack of selectivity and they may also effect several distinct protein kinases. For example, the affinity of H-9 for rabbit protein kinase C is supported by the use of this drug in affinity chromatography to isolate this kinase from rabbit tissues (Inagaki *et al.*, 1985). However, H-9 and H-7 also inhibit cyclic nucleotide-dependent protein kinases (Hidata *et al.*, 1984). The lack of effect of the guanylyl-cyclase inhibitor LY-83583, as well as the known relaxant effect of both cyclic AMP and cyclic GMP in vascular smooth muscle suggest that the B_1 receptors are not linked directly to cyclic nucleotide metabolism in the rabbit aortic tissue. Several tyrosine kinase inhibitors had no effect on des-Arg^9-BK-induced contraction in the aortic preparation, but they do inhibit the potentiation of the kinin effect by epidermal growth factor (deBlois *et al.*, 1992). Indomethacin at low concentrations reportedly inhibits a cyclic AMP-dependent protein kinase, in addition to suppressing the synthesis of prostanoids (Kantor & Hampton, 1978). This drug and the 5-lipoxygenase blocker, REV-5901, an inhibitor of leukotriene synthesis (McMillan & Walker, 1992), had no effect on des-Arg^9-BK-induced contractions in rabbit aortic rings. Therefore, the results tend to eliminate eicosanoids, notably the contractile compounds (thromboxane A_2, PGH_2, sulphidopeptide leukotrienes) from consideration as secondary mediators of kinin action.

Two types of mechanical responses have been reported following B_1 kinin receptor stimulation in rabbit isolated blood vessels: a sustained contraction, as in the aorta (Regoli *et al.*, 1977; present results) and the mesenteric vein (Regoli *et al.*, 1978), and a relaxation, sometimes preceded by a brief contraction, in the mesenteric and coeliac arteries (Churchill & Ward, 1986; Ritter *et al.*, 1989). The relaxations are not prevented by removing the endothelium in the latter tissues (deBlois & Marceau, 1987; Ritter *et al.*, 1989), but the relaxations can be reversed to a B_1-receptor-mediated contraction in the presence of indomethacin. These observations suggest that the contractile events produced by B_1 receptor stimulation are masked by prostaglandin-induced relaxations in the mesenteric and coeliac arteries. The contractile effect of des-Arg^9-BK in the rabbit aorta and mesenteric vein is resistant to many pharmacological antagonists (Regoli *et al.*, 1977; 1978) and is apparently not dependent on secondary released mediators. We provide evidence here for the existence of increased PGI_2 formation in the aortic preparation stimulated with des-Arg^9-BK (Table 2). However, the inhibition of this release by indomethacin did not modify the mechanical response of the treated tissues (Table 1). It is possible that the increased PGI_2 production is a uniform response of large rabbit vessels to B_1 receptor stimulation, but that the local sensitivity to prostanoids varies from one preparation to another. Indeed, the rabbit aorta does not exhibit a relaxant response to PGI_2 or PGE_2, the major eicosanoids produced by rabbit isolated vessels under basal or stimulated conditions (Förstermann *et al.*, 1984). On the contrary, the mesenteric and coeliac arteries are relaxed by low concentrations of these prostaglandins (Simmet & Hertting, 1980; Förstermann *et al.*, 1984; Demirel & Türker, 1991), and their dominant mechanical relaxation response to des-Arg^9-BK is probably dependent on prostaglandin receptor stimulation.

SMCs derived from aortic tissue without endothelium cultured over weeks retained the distinctive capacity to respond to kinins via B_1 receptors. An acute release of PGI_2 was observed in response to des-Arg^9-BK, and the use of antag-

onists confirmed the receptor identity (Figure 4, Table 3). The phosphoinositide response to des-Arg^9-BK was also attributed to B_1 receptors, according to the effect of antagonists selective for the B_1 and B_2 receptor types. The increased IP concentration after 5 min exposure to des-Arg^9-BK, evidence of the activation of phospholipase C, was relatively weak under basal conditions, but was potentiated in cells pretreated with IL-1β. This cytokine pretreatment has not been tested for PGI_2 release, because IL-1 is known to be a powerful prostaglandin release stimulus in rabbit vascular tissue (Marceau *et al.*, 1991). The activation of phospholipase C following the activation of B_1 receptors is consistent for the participation of diacylglycerol-regulated protein kinase C in the contractile effect of des-Arg^9-BK that was proposed in the paragraphs above, in reference to the effect of protein kinase inhibitors. Significant increases in IP_3 concentrations were also recorded 5 min after stimulation with des-Arg^9-BK in IL-1 pretreated cells (Table 4). The IP_3 (inositol 1,4,5-triphosphate)-mediated release of intracellular calcium stores in the cells could also be a distinctive feature of B_1 receptor pharmacology in blood vessels, and is also consistent with the involvement of intracellular calcium inferred from the contractility studies. Thus, at least two second messengers derived from phosphatidylinositol lipids, IP_3 and diacylglycerol, may be important in the B_1 receptor signal transduction process.

Hoe-140, at the concentration of 1.7 μM, occasionally stimulated IP turnover in cultured SMCs, either alone or as a potentiating agent for des-Arg^9-BK (Table 4). However, these effects did not reach statistical significance, and the phenomenon may be related to a non-specific effect at this relatively high drug concentration. The B_2 receptor antagonist, Hoe-140, consistently failed to inhibit the effects of kinins in SMCs. The pharmacological analyses based on the order of potency of agonists (des-Arg^9-BK > BK) and on the effect of selective antagonists indicate that bradykinin B_1 receptors mediate the biochemical responses to kinins in this cell type.

The tissue source of cultured arterial SMCs apparently determines the type of kinin receptors that are expressed. In the present study, only B_1 receptors were detected in cells derived from the rabbit aorta, but SMCs derived from the rat mesenteric artery expressed predominantly B_2 receptors (Dixon *et al.*, 1990). However, functional responses to the B_1 receptor agonist, des-Arg^9-BK, were present at 10^{-7}–10^{-6} M, with an intensity of about 20% of the maximal effect of BK in rat SMCs (Dixon *et al.*, 1990), which is suggestive of B_1 receptor co-expression. Thus, cultured SMCs derived from the rabbit aorta provide a relevant model pertinent to the investigation of the vascular effects of B_1 receptor stimulation, the release of vasorelaxant prostaglandin and direct vascular contraction. Unlike the freshly isolated rabbit aorta, but similar to aortic tissues incubated for several hours, the cultured cells retain a certain level of responsiveness to des-Arg^9-BK after weeks of culture. The potentiation of des-Arg^9-BK-induced phospholipase C activation by IL-1β provides preliminary evidence for an immunological regulation of B_1 receptor function, as observed in the cardiovascular system of the rabbit (Bouthillier *et al.*, 1987; deBlois *et al.*, 1988; 1991). The cellular model will be very useful for analysing the effect of cytokines and growth factors on the receptor population itself, and in identifying whether these factors regulate the kinin response through modifications of receptor density and/or affinity, or at the post-receptor level.

Note added in proof

While this manuscript was being processed, a report (Tropea, Gummelt, Herzig & Fredrik Leeb-Lundberg, 1993) appeared showing that des-Arg^9-BK promotes the formation of inositol phosphate and release of arachidonic acid in cultured rabbit superior mesenteric artery smooth muscle cells.

Supported by the Medical Research Council of Canada (grant MA-9358) and the Fonds de la recherche en Santé du Québec (FRSQ). G.D. and F.M. are Scholars of the FRSQ. We thank Ms Johanne Bouthillier and Mr Claude Villeneuve for technical assistance, Mr Claude Chamberland for assistance with fluorescence microscopy and Dr Gaétan Guillemette for advice on the technical aspects of cellular phosphoinositide measurements.

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(Received January, 28, 1993
Revised April 7, 1993
Accepted April 8, 1993)

Br. J. Pharmacol. (1993), **109**, 1263–1267 © Macmillan Press Ltd, 1993

Protection by nicorandil against the dysfunction of the central vagal baroreflex system following transient global cerebral ischaemia in dogs

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1 A possible cerebroprotective effect of nicorandil was investigated in a canine model of 5 min global cerebral ischaemia, and compared with protective effects of nitroglycerin and nicardipine.

2 Cerebral ischaemia was produced by occlusion of the left subclavian and the brachiocephalic arteries with preceding ligation of the intercostal arteries. The decrease in baroreflex sensitivity (BRS), measured by phenylephrine-induced reflex bradycardia, was used to assess the cerebroprotective effect.

3 Nicorandil (10 or 30 $\mu g\,kg^{-1}\,min^{-1}$, i.v.), nitroglycerin (3 $\mu g\,kg^{-1}\,min^{-1}$, i.v.) or nicardipine (0.3 $\mu g\,kg^{-1}\,min^{-1}$, i.v.) were infused for 60 min just before ischaemia. Nitroglycerin and nicardipine decreased mean arterial blood pressure to an extent similar to that induced by the lower dose of nicorandil. Blood flow in the dorsal medulla oblongata was increased by nicorandil and nicardipine, but not by nitroglycerin.

4 Nicorandil at both doses and nitroglycerin prevented the post-ischaemic decrease in BRS. In these cases, bilateral vagotomy during the reperfusion period decreased BRS, indicating that the vagal component of BRS was protected from ischaemia. On the other hand, nicardipine failed to exert a cerebroprotective effect.

5 The present study suggests that nicorandil may possess a direct cerebroprotective effect and that its property as a nitrate might, at least in part, be important for the observed cerebral protection.

Keywords: Cerebral ischaemia; baroreceptor reflex; nicorandil; nitroglycerin; nicardipine

Introduction

Nicorandil, N-(2-hydroxyethyl) nicotinamide nitrate (ester), is an antianginal vasodilator with a dual mechanism of action as a nitrate and a K^+ channel opener (Taira, 1989). Thus, nicorandil has been shown to increase guanosine 3':5'-cyclic monophosphate (cyclic GMP) in blood vessels (Holtzmann, 1983; Kukovetz *et al.*, 1991) and to increase K^+ conductance in vascular smooth muscle (Furukawa *et al.*, 1981) and myocardium (Nakayama *et al.*, 1991). Additionally, nicorandil possesses a direct cardioprotective effect that may be independent of its haemodynamic effects and attributable to the opening of myocardial K^+ channels (Ohta *et al.*, 1991; Auchampach *et al.*, 1992), inhibition of oxygen free radical production by neutrophils and scavenging of hydroxyl radicals (Pieper & Gross, 1992), and/or reduction in free fatty acid uptake during ischaemia (Pieper & Gross, 1987). However, such a cytoprotective effect of nicorandil has yet to be established in organs other than the heart.

In this context, the present study was conducted to investigate a possible cerebroprotective effect of nicorandil in a canine model of transient global cerebral ischaemia in which we have already confirmed the cerebroprotective effects of some drugs including flunarizine (Kurihara *et al.*, 1989b; 1990b), ifenprodil (Kurihara *et al.*, 1990b), beraprost (Kurihara *et al.*, 1990a) and halothane (Kurihara *et al.*, 1992). The results were compared with those obtained with nitroglycerin and nicardipine.

Methods

Preparation of animals

Mongrel dogs of either sex weighing 6 to 16 kg were anaesthetized with sodium pentobarbitone (32 mg kg^{-1}, i.v. followed by an infusion of 3.2 mg $kg^{-1}\,h^{-1}$, i.v.). Under artificial ventilation with room air (a tidal volume of 20 ml kg^{-1} at a rate of 20 breaths min^{-1}) animals were immobilized with suxamethonium chloride (2 mg kg^{-1}, i.v. followed by an infusion of 1 mg $kg^{-1}\,h^{-1}$, i.v.). Appropriate volumes of O_2 and CO_2 were provided to maintain arterial P_{O_2} and P_{CO_2} at about 100 mmHg and 35 mmHg, respectively. Rectal temperature was maintained at about 38°C. Arterial blood pressure was measured from the left femoral artery by means of a pressure transducer (Nihon Kohden, TP-200T, Japan) and heart rate was measured by a heart rate counter (Nihon Kohden, AT-600G) triggered by lead II ECG.

Thoracotomy was performed at the fifth intercostal space and approximately 14 intercostal arteries descending along the thoracic aorta were permanently ligated. Pneumatic vascular occluders were placed around the left subclavian artery and the brachiocephalic artery as close as possible to the aortic arch.

The animals were finally placed in a prone position and the head was fixed in a stereotaxic frame. The head was tilted down at an angle of about 30 degrees to help in the manipulation of the brainstem. The foramen magnum was enlarged by removal of the caudal edge of the occipital bone. A plate type thermocouple electrode was placed on the surface of the right fasciculus cuneatus in the dorsal medulla oblongata proximal to the obex, and the regional blood flow was continuously measured by a tissue flow monitor (Unique Medical, UMW-101, Japan). The cortical EEG was recorded by means of stainless steel electrodes screwed bilaterally into the parietal skull, and continuously analyzed by a frequency analyzer (Nihon Kohden, OEE-7102) to confirm the steady level of anaesthesia throughout the experiment.

Measurement of baroreflex sensitivity (BRS)

Five or more bolus injections of (−)-phenylephrine hydrochloride were performed for each BRS measurement. The

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doses which increase mean arterial blood pressure by the range of about 5 to 25 mmHg were used; the dose-range was usually 0.3 to 10 $\mu g\,kg^{-1}$, i.v. The pressor response to phenylephrine was accompanied by a transient reflex bradycardia. Thus, the increase in pulse interval (ms) was correlated with the increase in mean arterial blood pressure (mmHg) by the method of least squares. The linearity of the regression line was always statistically significant, judging from the correlation coefficient. Then, the slope of the regression line (ms $mmHg^{-1}$) was used as a measure of BRS, because ischaemia did not displace the regression line, but altered the slope (Kurihara *et al.*, 1989a).

According to a previous study (Kurihara *et al.*, 1990b), an ischaemic insult of 5 min duration produces a selective dysfunction of the vagal component of BRS. Thus, the influence of bilateral section of the cervical vagosympathetic trunk (vagotomy) on BRS was investigated during the reperfusion period, following 5 min ischaemia, in order to evaluate the extent of the residual function of the vagal component of BRS. The practical timing of BRS measurement was as follows: BRS was measured before ischaemia and during the reperfusion period of 60 to 100 min. Then, the vagotomy was performed just after the second BRS measurement, and the third BRS measurement was started about 15 min later.

Production of cerebral ischaemia

Transient global cerebral ischaemia was produced by inflating the pneumatic occluders for 5 min. Since the severity of ischaemia in the dorsal medulla oblongata has been shown to be a good determinant of the post-ischaemic dysfunction of the vagal baroreflex (Kurihara *et al.*, 1990b), it was assessed by calculating the mean residual blood flow during ischaemia as described previously (Kurihara *et al.*, 1989c; 1990b).

Drugs

Nicorandil was supplied by Chugai Pharmaceutical Co., Ltd., Japan and dissolved in saline (1 or 3 $mg\,ml^{-1}$) just before use. Nitroglycerin solution (Nippon Kayaku, Japan) was diluted with saline to 0.3 $mg\,ml^{-1}$. Nicardipine hydrochloride (Sigma Chemical Company, U.S.A.) was dissolved in distilled water and diluted with saline to 30 $\mu g\,ml^{-1}$. The solutions of these three drugs or saline were infused into the right saphenous vein of 6 animals in each group with a syringe infusion pump (Harvard Apparatus, 2400–003, U.S.A.) at a rate of 10 $\mu l\,kg^{-1}\,min^{-1}$ for 60 min just prior to ischaemic insult. (−)-Phenylephrine hydrochloride (Sigma Chemical Company, U.S.A.) and suxamethonium chloride (Tokyo Kasei, Japan) were dissolved in saline and administered into the cephalic vein.

Statistics

Results are expressed as means ± s.e.mean. Values in different groups were analysed by the one-way analysis of variance followed by Bonferroni's multiple-comparison test. Differences between the values before and after ischaemia were analyzed by Dunnett's t test. Differences giving $P<0.05$ were regarded as statistically significant.

Results

Effects on blood pressure and heart rate

As shown in Table 1, all drugs tested decreased arterial blood pressure and the hypotensive effect of nicorandil was dose-dependent. The magnitude of the decrease in systolic blood pressure induced by nicorandil and nitroglycerin was greater than the decrease in diastolic blood pressure. Although the hypotensive effect of nitroglycerin developed more rapidly than that of nicorandil and reached a steady state within 10 min of infusion, the magnitude of the decrease in blood pressure at the end of infusion was similar to that produced by the lower dose of nicorandil. On the other hand, nicardipine preferentially decreased diastolic blood pressure. At the end of the infusion, the magnitude of the decrease in diastolic blood pressure produced by nicardipine was similar to that caused by the higher dose of nicorandil, while the decrease in systolic blood pressure was similar to that produced by the lower dose of nicorandil.

Heart rate was increased by the drugs, and there was no statistically significant difference in the maximum increase in heart rate. In all groups, tachycardia tended to recover during the infusion period despite the consistent decrease in blood pressure. In particular, in 4 animals which received the higher dose of nicorandil, tachycardia reversed to bradycardia by the end of infusion.

Effects on cerebral circulation

Regional arterial resistance (rAR) was calculated by dividing mean arterial blood pressure by regional cerebral blood flow in the dorsal medulla oblongata (rCBF). As shown in Table 2, nicorandil decreased rAR in a dose-dependent manner and increased rCBF at the higher dose. Nitroglycerin failed to produce a significant change in rAR and decreased rCBF. Nicardipine decreased rAR and increased rCBF to an extent similar to that induced by the higher dose of nicorandil.

Cerebroprotective effects

Changes in BRS following ischaemia and vagotomy were as shown in Figure 1. There was no statistically significant

Table 1 Effects of nicorandil, nitroglycerin and nicardipine on arterial blood pressure and heart rate

	Blood pressure (mmHg)								
	Systolic		*Mean*		*Diastolic*		*Heart rate* (beats min^{-1})		
Drugs	*Before*	*Change*[a]	*Before*	*Change*[a]	*Before*	*Change*[a]	*Before*	*Change*[a]	*(Max)*
Saline									
10 $\mu l\,kg^{-1}\,min^{-1}$	154 ± 8	− 4 ± 3	130 ± 6	− 3 ± 2	113 ± 5	− 1 ± 2	160 ± 7	1 ± 3	
Nicorandil									
10 $\mu g\,kg^{-1}\,min^{-1}$	167 ± 8	− 24 ± 5	139 ± 7	− 17 ± 3	124 ± 7	− 12 ± 1	158 ± 8	9 ± 2	(11 ± 2)
30 $\mu g\,kg^{-1}\,min^{-1}$	163 ± 6	− 56 ± 7**††	134 ± 6	− 45 ± 6**††	118 ± 6	− 38 ± 5**††	161 ± 9	− 2 ± 5	(10 ± 2)
Nitroglycerin									
3 $\mu g\,kg^{-1}\,min^{-1}$	162 ± 9	− 35 ± 6**	134 ± 6	− 23 ± 5*‡	117 ± 5	− 14 ± 5‡‡	150 ± 8	9 ± 4	(16 ± 3)
Nicardipine									
0.3 $\mu g\,kg^{-1}\,min^{-1}$	162 ± 8	− 19 ± 6‡‡	136 ± 7	− 27 ± 4**	123 ± 6	− 32 ± 5**†	157 ± 9	16 ± 5‡	(19 ± 4)

Results from 6 animals in each group are indicated as means ± s.e.mean.
[a]Change at the end of infusion period. Numbers in parentheses indicate the maximum increase in heart rate during infusion.
*$P<0.05$; **$P<0.01$; significantly different from saline.
†$P<0.05$; ††$P<0.01$; significantly different from the lower dose of nicorandil.
‡$P<0.05$; ‡‡$P<0.01$; significantly different from the higher dose of nicorandil.

Table 2 Effects of nicorandil, nitroglycerin and nicardipine on regional cerebral blood flow (rCBF) and regional arterial resistance (rAR) in the dorsal medulla oblongata

	Change in rCBF (%)	*Change in rAR (%)*
Saline		
10 μg kg^{-1} min^{-1}	0.1 ± 3.4	− 2.3 ± 3.2
Nicorandil		
10 μg kg^{-1} min^{-1}	0.2 ± 1.8	− 12.5 ± 2.3**
30 μg kg^{-1} min^{-1}	25.1 ± 7.8*	− 45.2 ± 4.7**
Nitroglycerin		
3 μg kg^{-1} min^{-1}	− 10.0 ± 3.3*	− 7.2 ± 4.3
Nicardipine		
0.3 μg kg^{-1} min^{-1}	17.9 ± 5.2*	− 30.6 ± 2.2**

Changes at the end of infusion are indicated as means ± s.e.mean of results from 6 animals in each group. *$P<0.05$; **$P<0.01$; statistically significant changes.

difference in pre-ischaemic BRS values and mean residual blood flow during ischaemia between the five groups. In animals which received saline or nicardipine prior to ischaemia, BRS during the reperfusion period was about 50% of the value before ischaemia. Subsequent vagotomy failed to affect BRS in these animals, indicating that the vagal component of BRS had been functionally abolished and that nicardipine failed to exert a cerebroprotective effect in the present experimental model. On the other hand, there was no significant decrease in BRS following ischaemia in animals which received nicorandil or nitroglycerin. Vagotomy decreased BRS in these animals, indicating that nicorandil and nitroglycerin protected the vagal component of BRS from ischaemia.

Figure 2 shows the effects of drugs on the correlation between the severity of ischaemia and the extent of the

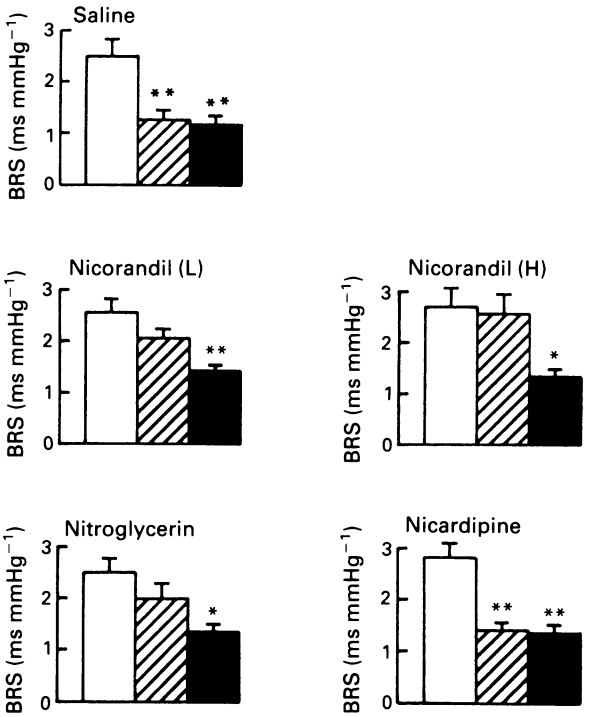


Figure 1 Effects of pretreatment with drugs on changes in baroreflex sensitivity (BRS) following 5 min global cerebral ischaemia. Open, hatched and closed columns indicate the BRS values ± s.e.mean before ischaemia, during reperfusion (before vagotomy) and after vagotomy, respectively ($n = 6$ for each group). The doses of the drugs are as indicated in Table 1. Nicorandil (L); the lower dose, Nicorandil (H); the higher dose. *$P<0.05$; **$P<0.01$; significantly different from the value before ischaemia.

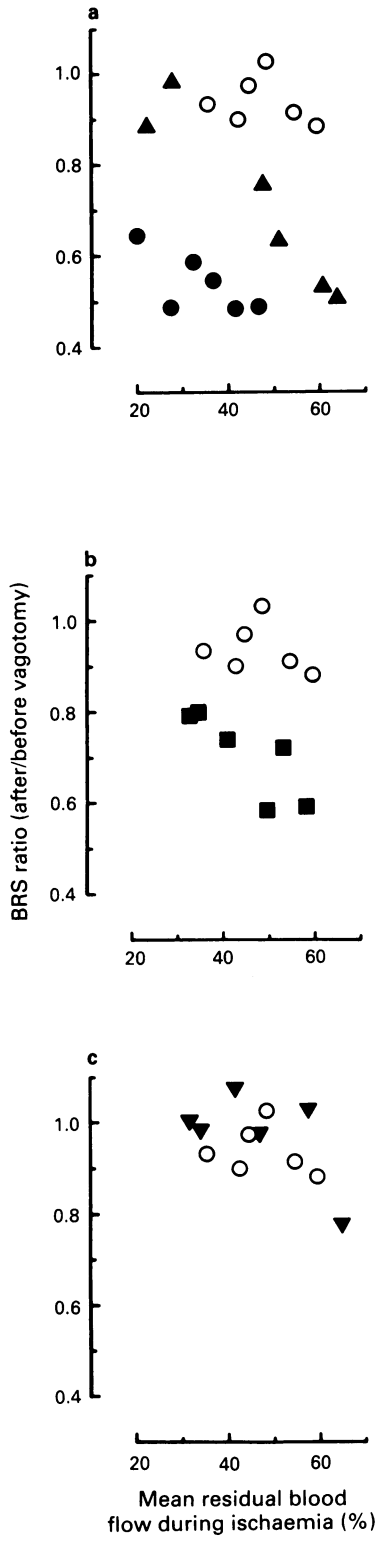


Figure 2 Correlation between the severity of ischaemia and the extent of post-ischaemic dysfunction of the vagal baroreflex. The data from animals treated with the lower (▲ in a) and higher (● in a) dose of nicorandil, nitroglycerin (■ in b) and nicardipine (▼ in c) are compared with those from saline-treated animals (○ in a, b and c). Abscissa scale; severity of ischaemia is indicated as the mean residual blood flow in the dorsal medulla oblongata during ischaemia. Thus, the severity of ischaemia increases as the value decreases. Ordinate scale: the extent of the influence of vagotomy is indicated as the ratio of the baroreflex sensitivity after vagotomy to that before vagotomy. Thus, the value 1 indicates that the vagal component of baroreflex is virtually absent. Additionally, the BRS ratio in sham-operated animals, which suffered no ischaemic insult, has been shown to be about 0.5 on average (see Kurihara *et al.*, 1990b).

post-ischaemic dysfunction of the vagal baroreflex. The data from saline-treated animals were consistent with a previous result that an ischaemic insult which reduced the mean residual blood flow by more than about 50% abolished the vagal component of baroreflex (Kurihara *et al.*, 1990b). The cerebroprotective effect of nitroglycerin was similar to that of the equihypotensive dose of nicorandil ($10\,\mu g\,kg^{-1}\,min^{-1}$, i.v.). In both cases, the extent of cerebral protection was dependent on the severity of ischaemia; the drugs were less effective against the severe ischaemia. The effect of nicorandil was dose-dependent. The higher dose of nicorandil was considered to be able to protect the vagal component of the baroreflex completely from the reduction in mean residual blood flow to about 30%, as the BRS ratio in sham-operated animals, which suffered no ischaemic insult, has been shown to be about 0.5 (Kurihara *et al.*, 1990b). In contrast, the data from nicardipine-treated animals shows that nicardipine failed to provide cerebral protection even against a mild ischaemia. Additionally, it was confirmed in preliminary experiments with sham-operated animals that nicorandil ($30\,\mu g\,kg^{-1}\,min^{-1}$, i.v.), nitroglycerin ($3\,\mu g\,kg^{-1}\,min^{-1}$, i.v.) and nicardipine ($0.3\,\mu g\,kg^{-1}\,min^{-1}$, i.v.) failed to affect the BRS ratio during the period from 60 min after the cessation of the 60 min infusion (data not shown).

Discussion

In the present study, nicorandil prevented the post-ischaemic dysfunction of the central vagal baroreflex system at doses similar to those shown to protect the heart from ischaemia/reperfusion injuries in dogs (Lamping *et al.*, 1984; Shimshak *et al.*, 1986; Gross *et al.*, 1987; Grover *et al.*, 1990; Auchampach *et al.*, 1992). The protective effect of nicorandil was dose-dependent and was dependent on the severity of ischaemia. In addition, the present study demonstrates that nicorandil produces cerebral vasodilatation and increases blood flow in the dorsal medulla oblongata. The latter results are consistent with those obtained by Nakagawa *et al.* (1979), who observed that nicorandil increased cerebral venous outflow in dogs. Nicardipine increased cerebral blood flow to an extent similar to that observed with the higher dose of nicorandil, while it failed to prevent the post-ischaemic cerebral dysfunction. On the other hand, nitroglycerin exerted a cerebroprotective effect similar to that of the lower dose of nicorandil, producing a small decrease in cerebral blood flow. Therefore, the observed cerebral protection by nicorandil and nitroglycerin may be independent of their effects on cerebral circulation, but rather ascribed to a direct protective effect.

Neichi *et al.* (1980) demonstrated that nicorandil increased prostacyclin (PGI_2) formation in the coupled system of platelets and aortic microsomes. PGI_2 has been shown to be cerebroprotective (Pluta, 1985; Masuda *et al.*, 1986) and our previous study demonstrated that beraprost, a chemically stable PGI_2 analogue, possesses a protective effect in the same experimental model as used in the present study (Kurihara *et al.*, 1990a). These results may indicate a contribution of PGI_2 to the cerebroprotective effect of nicorandil in the present study. However, at least in a canine model of myocardial ischaemia, it has been shown that nicorandil failed to increase PGI_2 release into coronary venous blood and that indomethacin failed to affect the cardioprotective effect of nicorandil (Gross *et al.*, 1989). Furthermore, the protective effect of beraprost in our previous study (Kurihara *et al.*, 1990a) was observed only at a dose higher than that required to inhibit platelet aggregation. Therefore, it seems to be unlikely that the cerebroprotective effect of nicorandil is due to an increase in PGI_2 formation.

The mechanism of the cytoprotective effect of nicorandil, which is independent of its haemodynamic effect, has been investigated in several models of myocardial ischaemia/reperfusion injuries (Gross *et al.*, 1989; Ohta *et al.*, 1991; Auchampach *et al.*, 1992). The recent study by Auchampach *et al.* (1992) clearly demonstrated that glibenclamide blocked the protective effect of nicorandil against myocardial stunning induced by ischaemia/reperfusion in dogs, suggesting that activation of the ATP-sensitive K^+ channel (K^+_{ATP} channel) may be the dominant mechanism of the cardioprotective effect of nicorandil. Since a similar K^+_{ATP} channel seems to be present in the brain (Ashford *et al.*, 1988; Mourre *et al.*, 1989; Krnjević, 1990), it is possible that nicorandil might protect the brain through activation of K^+_{ATP} channels with a resultant inhibition of excessive excitation of neurones during ischaemia, providing nicorandil readily penetrates the blood-brain barrier. According to unpublished data of the Chugai Pharmaceutical Co., Ltd., the ratio of the concentration of nicorandil in cerebrospinal fluid to that in plasma reaches about 1 after a 1 h infusion of $30\,\mu g\,kg^{-1}\,min^{-1}$ i.v., to anaesthetized dogs. Thus, it can be expected that at least the higher dose of nicorandil used in the present study might be enough to activate K^+_{ATP} channels in the brain, although further study is necessary to confirm this.

On the other hand, the present study also suggests that the nitrate properties of nicorandil may contribute to cerebral protection, as the cerebroprotective effect of nicorandil was mimicked by nitroglycerin at an equihypotensive dose. This result is in contrast with that from the canine model of myocardial ischaemia (Gross *et al.*, 1989), in which nitroglycerin exerted only a minimal protective effect.

It has been shown that the pharmacological effects of nitroglycerin are mediated by nitric oxide (NO), which is released from its $-ONO_2$ moiety and subsequently increases cytosolic cyclic GMP through activation of soluble guanylate cyclase (Ahlner *et al.*, 1991). Nicorandil also possesses the $-ONO_2$ moiety and its vasodilator effect may be, at least in part, mediated through cyclic GMP (Holtzmann, 1983; Kukovetz *et al.*, 1991). Concerning the relationship between cyclic GMP and neuronal damage, Garthwaite & Garthwaite (1988) suggested that guanlyate cyclase/cyclic GMP may be part of a protective mechanism against oxygen free radicals in rat cerebellar slices. Since oxygen free radicals may play an important role in the development of neuronal damage by cerebral ischaemia/reperfusion (Siesjö *et al.*, 1989), one can speculate that the increase in cyclic GMP by nicorandil and nitroglycerin might be favourable for cerebral protection in the present study. In this context, Pieper & Gross (1992) recently suggested that the nitrate action could, at least in part, explain the inhibition by nicorandil of free radical production in neutrophils.

In conclusion, the present study suggests that nicorandil may exert a direct cerebroprotective effect at doses which effectively protect the heart from ischaemia/reperfusion injuries. Although the precise mechanism of cerebral protection by nicorandil is still obscure, its property as a nitrate might, at least in part, be important in addition to activation of K^+ channels.

The authors express their thanks to Chugai Pharmaceutical Co., Ltd. for providing an opportunity to perform the present study, and to Mr Daisuke Aoki and Miss Miki Ogouchi for their technical assistance.

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(Received January 4, 1993
Revised April 1, 1993
Accepted April 13, 1993)

Br. J. Pharmacol. (1993), **109**, 1268–1271 © Macmillan Press Ltd, 1993

Blockade of adenosine receptors unmasks a stimulatory effect of ATP on cardiac contractility

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1 The effects of ATP, α,β-methylene ATP and β,γ-methylene ATP on the contractile tension of guinea-pig isolated left atria were evaluated.

2 ATP (1– 100 μM) produced a concentration-dependent negative inotropic effect; this response was converted to a positive inotropic effect in the presence of the antagonist of adenosine A_1 receptors, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; 0.1 μM), and in the presence of 8-phenyltheophylline (10 μM), an antagonist of A_1 and A_2 receptors.

3 The positive inotropic effect of ATP was antagonized by the P_2 receptor antagonist, suramin (500 μM). Reactive blue 2 (30– 500 μM), a putative P_{2y} receptor antagonist, concentration-dependently reduced and finally abolished the effect of ATP.

4 In the presence of 8-phenyltheophylline, the stable analogues of ATP, α,β-methylene ATP and β,γ-methylene ATP (1– 30 μM), produced a concentration-dependent increase in atrial contractility of a lesser degree than that induced by ATP.

5 The results suggest that when inhibitory adenosine receptors are blocked, ATP produces a positive inotropic effect, probably mediated by P_{2y} receptor stimulation.

Keywords: ATP; heart; positive inotropism; P_2-purinoceptors

Introduction

Adenine nucleotides exert well-known depressant effects in the mammalian heart, consisting of a negative chronotropic and inotropic response, an anti-adrenoceptor effect and an inhibition of adrenergic neurotransmission (for two reviews see Burnstock, 1980; Pelleg *et al.*, 1990). These effects are mediated by stimulation of adenosine receptors, since they are antagonized by the non-selective adenosine receptor antagonist, 8-phenyltheophylline. According to some reports, adenosine and ATP, at high concentrations, induce a positive inotropic effect in ventricular preparations (Legssyer *et al.*, 1988; Dorigo *et al.*, 1988; Scamps *et al.*, 1990; Szentmiklosi *et al.*, 1990). In chick ventricular myocytes pretreated with either 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), a selective A_1 receptor antagonist, or the G_i ribosylating agent, pertussis toxin, adenosine induces a stimulation of adenylate cyclase activity and an increase in contractile amplitude (Xu *et al.*, 1992). On the other hand, ATP produces a small positive inotropic effect in rat papillary muscle, but a negative inotropic effect in rat atria (Scamps *et al.*, 1990). The inhibitory response observed in rat atria is converted to a positive effect in preparations obtained from pertussis toxin-pretreated animals (Scamps *et al.*, 1990). A biphasic, negative and positive effect for ATP in spontaneously beating guinea-pig atria has also been reported by Dorigo *et al.* (1988); the negative phase is abolished by 8-phenyltheophylline, thus suggesting that adenosine receptors are not involved in this positive inotropic effect. In the present study we have evaluated the influence of ATP on cardiac contractility in guinea-pig left atria, and have attempted to characterize the receptors involved in the observed effects.

Methods

Male guinea-pigs (250–400 g) were killed by a blow on the head. The hearts were rapidly removed and left atria were isolated and vertically mounted in an organ bath containing Tyrode solution of the following composition (mM): NaCl 115, KCl 4.7, $CaCl_2$ 1.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 10. The solution was oxygenated with 95% O_2 and 5% CO_2, kept at 30°C and had a pH of 7.4. The preparations were electrically driven at 1 Hz by point stimulation with square wave pulses of 0.5 ms duration and supra-threshold voltage (5 V). Resting tension was adjusted at 0.5–0.7 g and developed tension was recorded by an isometric transducer on a pen recorder (Battaglia Rangoni KV 135). Atria were allowed to equilibrate for 1 h before drug administration. ATP and analogues were added to the bath solution in a cumulative way at intervals of 3–4 min. Antagonists were present in Tyrode solution for the duration of the experiment, or added to the bath at least 15 min before ATP administration. Since ATP caused desensitization, only one concentration-response curve for ATP was determined in a single preparation. Therefore concentration-response curves in the absence and in the presence of antagonists were carried out in different preparations. The effect induced by ATP and analogues was evaluated as the difference between the basal contractile tension and the tension after drug administration (net increase in mg).

Drugs

Adenosine 5'-triphosphate (ATP), lithium salt of α,β-methylene ATP, sodium salt of β,γ-methylene ATP, reactive blue 2 and prazosin hydrochloride were all obtained from Sigma Chemical Co., St. Louis, U.S.A.; suramin (Bayer, Milan, Italy), 8-phenyltheophylline (Calbiochem, San Diego, U.S.A.), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; Research Biochemical Inc., Natick, MA, U.S.A.), cimetidine hydrochloride (Smith, Kline Beecham, Welwyn Garden City, U.K.); 2-hydroxy-5-{2-[hydroxy-3-(4-[(1-methyl-4-trifluoromethyl) 1H-imidazol-2-yl-]-phenoxy)-propyl]aminoethoxyl}-benzamide (CGP 20712A) was kindly supplied by Ciba Geigy, Basel, Switzerland.

Drugs were prepared fresh daily in distilled water. A stock solution of 8-phenyltheophylline was made up in 80% ethanol containing 0.2 mM NaOH; further dilutions were made in distilled water.

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Statistics

The values reported are means ± s.e.mean. Statistical significance was calculated with Student's *t* test for unpaired data. A P value <0.05 was considered significant.

Results

Guinea-pig left atria, electrically stimulated at 1 Hz, developed a basal contractile tension of 530.3 ± 36.3 mg ($n = 30$). Concentrations of ATP ranging from 1 to 100 μM were tested. The nucleotide produced a fast developing negative inotropic effect at concentrations higher than 3 μM (Figure 1). The maximum negative inotropic effect, obtained with 100 μM ATP, consisted of a reduction of cardiac contractility of 44.6 ± 1.4% ($n = 4$; Table 1). The selective A_1 receptor antagonist, DPCPX (0.1 μM), did not affect the atrial contractile tension for a period of 40 min (Figure 1). However, in the presence of the antagonist, the negative inotropic effect of ATP was converted to a concentration-dependent positive effect. Records obtained from a typical experiment showing that the A_1 antagonist induced a reversal of the ATP effect are shown in Figure 1. The maximum positive inotropic effect induced by 30 μM ATP in the presence of DPCPX, consisted of an increase in contractile tension of 32.0 ± 7% ($n = 6$). The positive inotropic response to ATP was not modified by pretreatment of the preparations with 1 μM CGP 20712A, 1 μM prazosin, or 1 μM cimetidine, selective antagonists of β_1-adrenoceptors, α_1-adrenoceptors and H_2-receptors, respectively (data not shown in figure). In order to test whether the positive inotropic effect detected in the presence of the A_1 receptor antagonist was mediated by stimulation of A_2 adenosine receptors, further experiments were performed in the presence of 10 μM 8-phenyltheophylline, a non-selective adenosine receptor antagonist. A concentration-dependent positive inotropic response to ATP was also detected in the presence of the antagonist; the maximum effect was observed with a concentration of 30 μM ATP, and consisted of a net increase in contractile tension of about 120 mg ($n = 24$; Figure 2). A further increase in the ATP concentration to 100 μM did not induce another increase in contractile tension; moreover, in some experiments, the increase in ATP concentration pro-

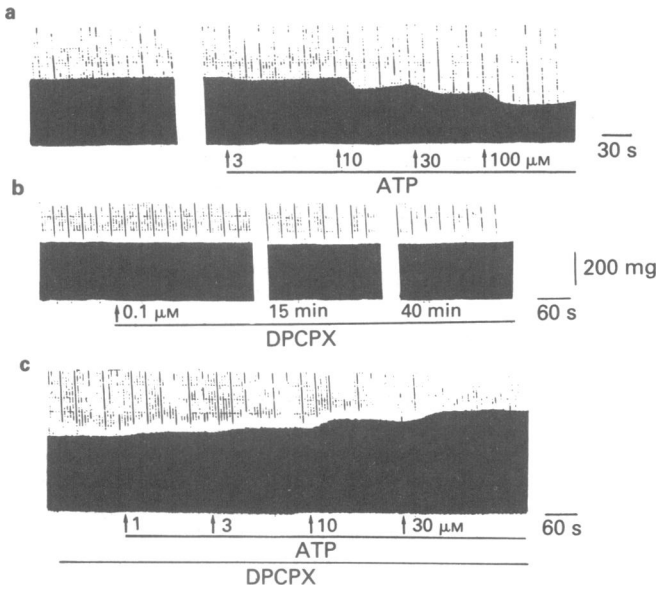


Figure 1 (a) Negative inotropic effect of ATP in guinea-pig left atria, electrically stimulated at 1 Hz; (b) lack of effect of 0.1 μM 1, 3-dipropyl-8-cyclopentylxanthine (DPCPX) on the contractile tension during an observation period of 40 min; (c) positive inotropic effect of ATP in the presence of 0.1 μM DPCPX.

Table 1 Negative inotropic effect induced by ATP in guinea-pig left atria electrically stimulated at 1 Hz

ATP concentration (μM)	*% reduction of contractility*
1	0 ± 0
3	4.2 ± 1.4
10	15.2 ± 2.3
30	27.7 ± 1.7
100	44.6 ± 1.4

Values are mean ± s.e.means of 4 experiments.

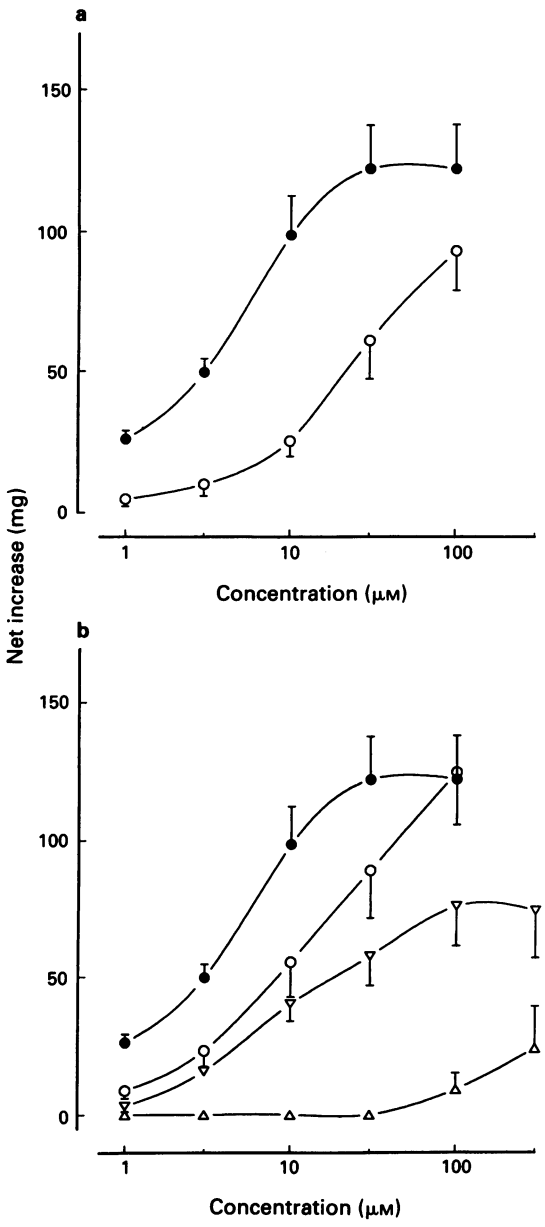


Figure 2 Concentration-response curves for the effect of ATP in the absence and in the presence of antagonists on force of contraction of guinea-pig isolated left atria preincubated with 10 μM 8-phenyltheophylline and electrically stimulated at 1 Hz. (a) (●) ATP alone ($n = 24$); (○) ATP in the presence of 500 μM suramin ($n = 10$). (b) (●) ATP alone ($n = 24$); (○) ATP in the presence of 30 μM reactive blue 2 ($n = 10$); (▽) ATP in the presence of 100 μM reactive blue 2 ($n = 6$); (△) ATP in the presence of 500 μM reactive blue 2 ($n = 6$). The effects are expressed as net increase in mg over the baseline value. Symbols indicate means ± s.e.mean.

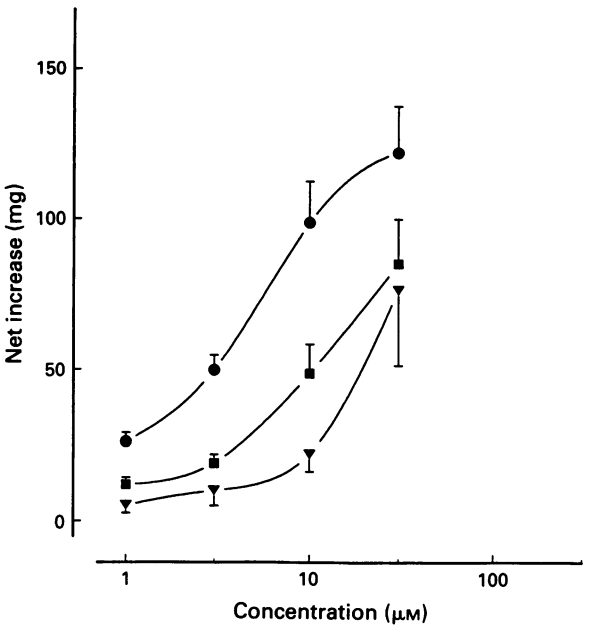


Figure 3 Concentration-response curves for the effect of ATP (●, $n = 24$), α,β-methylene ATP (■, $n = 8$) and β,γ-methylene ATP (▼, $n = 6$) on force of contraction of guinea-pig isolated left atria preincubated with 10 μM 8-phenyltheophylline and electrically stimulated at 1 Hz. The effects are expressed as net increase in mg over the baseline value. Symbols indicate means ± s.e.mean.

duced a slight reduction of tension, probably because of the displacement of the competitive antagonist 8-phenyltheophylline from A_1 receptors. The concentration-response curve for ATP was shifted to the right in a statistically significant manner by 500 μM suramin, an antagonist of P_2 purinoceptors ($n = 10$; Figure 2a). Moreover, reactive blue 2 (30–500 μM), a dye which has been reported active as a P_{2y} antagonist, significantly reduced the effect of ATP in a concentration-dependent manner. The shift of the curve observed in the presence of reactive blue 2 suggested a non-competitive type of antagonism since, in the presence of the dye, 100 μM ATP was no longer able to produce its maximum positive inotropic effect, and the ATP response was completely abolished by 500 μM reactive blue (Figure 2b). The effect of reactive blue 2 was not due to a nonspecific effect on muscular cells since the highest concentration of the antagonist (500 μM) did not affect the ability of the preparations to develop an inotropic effect in response to increasing concentrations of either calcium or noradrenaline (not shown in figure).

In addition, the stable analogues of ATP, α,β-methylene ATP and β,γ-methylene ATP, produced a positive inotropic effect in the concentration range 1–30 μM, in the presence of 8-phenyltheophylline. The curves showing the effects of the analogues, in comparison with that of ATP, are shown in Figure 3. The analogues were less potent than ATP, α,β-methylene ATP being more active than β,γ-methylene ATP.

Discussion

The results of the present study demonstrate that the usual negative inotropic effect of ATP can be converted to a positive inotropic response by blockade of adenosine receptors. The receptors involved in the cardiodepressant effects of adenine nucleotides have been identified as adenosine receptors of the A_1 subtype, which are linked to different intracellular pathways. The negative inotropic and chronotropic effects, which are predominant in atrial tissue, are due to an increase in K^+ current (Belardinelli & Isemberg, 1983; Bruckner *et al.*, 1985); the antiadrenoceptor effect, which is better observed in ventricular preparations, is due to an inhibition of β-receptor stimulation-induced cyclic AMP accumulation (Dobson, 1983). Both these kinds of A_1 receptor-mediated effects are linked to pertussis toxin-sensitive G proteins (Endoh *et al.*, 1983). After inactivation of G proteins by pertussis toxin, or blockade of A_1 receptors, both adenosine (Bohm *et al.*, 1988; Xu *et al.*, 1992) and ATP (Scamps *et al.*, 1990) can induce a positive inotropic effect on cardiac preparations. The cardiac stimulant response to adenosine and ATP had been tentatively attributed to different mechanisms: a stimulation of cyclic AMP by adenosine in the presence of DPCPX has been reported in chick ventricular myocytes (Xu *et al.*, 1992). It has also been demonstrated that ATP is able to increase calcium current in rat ventricular preparations (Bjornsson *et al.*, 1989; De Young & Scarpa, 1989; Scamps *et al.*, 1990) and to produce inositoltrisphosphate formation in the rat ventricle (Legssyer *et al.*, 1988). The above mentioned effects are not antagonized by adenosine receptor antagonists, isobutylmethylxanthine (IBMX) or 8-phenyltheophylline, thus suggesting that specific ATP receptors, (P_2 receptors) are involved in the excitatory response of ATP.

In agreement with the previously reported observations, a positive inotropic response to ATP was observed in the present study, after blockade of inhibitory adenosine receptors. The increase in cardiac tension produced by ATP was not modified by pretreatment with CGP 20712A, prazosin or cimetidine, selective antagonists of β_1-adrenoceptors, α_2-adrenoceptors and H_2-receptors, respectively. These findings clearly indicate that the cardiac stimulant response to ATP was not attributable to noradrenaline or histamine release. These results also demonstrate that adenosine A_2 receptors, which have been reported able to stimulate cyclic AMP production in several preparations (Londos *et al.*, 1980; Paton, 1984), were not involved in the positive inotropic response to ATP. Furthermore, the increase in cardiac contractility produced by ATP was antagonized by drugs reported as antagonists of P_2 receptors, such as suramin and reactive blue 2 (Kennedy, 1990), indicating that the effect of the nucleotide was mediated by stimulation of specific ATP receptors. A positive inotropic effect, even if to a lesser degree than ATP, was also produced by the two more stable analogues, α,β-methylene ATP and β,γ-methylene ATP. ATP receptors have been recently classified into P_{2x} and P_{2y} subtypes, on the basis of agonist potency order and of the ability to produce different functional responses in vascular preparations, i.e. a vasoconstrictor and a vasodilator effect, respectively (Burnstock & Kennedy, 1985; Kennedy, 1990). The order of potency of the agonists tested in the present study, (ATP > α,β-methylene ATP > β,γ-methylene ATP), is indicative of a P_{2y} receptor subtype. The concentration-positive inotropic response curve for ATP was shifted to the right by suramin, an antagonist of ATP receptors which is not able to discriminate between P_{2x} and P_{2y} receptors (Kennedy, 1990; Blakeley *et al.*, 1991). Moreover, the concentration-response curve to the agonist was also shifted in a concentration-dependent fashion by reactive blue 2, an anthraquinone-sulphonic acid derivative which has been reported as able to antagonize selectively the effects mediated by P_{2y} receptor stimulation (Burnstock & Warland, 1987; Taylor *et al.*, 1989). According to previous reports, the stimulation of P_{2y} receptors by ATP induces a rapid and transient accumulation of inositol 1,4,5-trisphosphate in a variety of cultured cells, such as endothelial cells, hepatocytes, leukocytes and erythrocytes. This effect is associated with a rapid increase in cytoplasmic calcium concentration (Boeynaems & Pearson, 1990). An increase in calcium concentration by ATP has also been reported in ventricular rat myocytes (De Young & Scarpa, 1989), associated with enhanced contractility (Danziger *et al.*, 1988). Moreover it has been demonstrated that ATP and other analogues increase the intracellular calcium concentration in rat myocytes with an order of potency of the agonists indicative of the P_{2y} receptor subtype (Bjornsson *et al.*, 1989).

The concentration-range of ATP able to increase the intracellular calcium level in this last study is comparable to the concentrations found able to increase the contractility in atrial preparations. Thus, the hypothesis can be advanced that the ATP-induced positive inotropic effect observed in the present study was mediated by stimulation of specific P_2 receptors, presumably belonging to the P_{2y} subtype, and that, according to previous observations, this effect was mediated by an increase in intracellular calcium.

This work was supported by a grant from the Italian Ministry of Scientific and Technological Research. We are grateful to Mary Forrest for revising the English.

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(Received February 3, 1993
Revised April 8, 1993
Accepted April 15, 1993)

Br. J. Pharmacol. (1993), **109**, 1272–1275 © Macmillan Press Ltd, 1993

The effects of α-adrenoceptor agonists on intracellular Ca^{2+} levels in freshly dispersed single smooth muscle cells from rat tail artery

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1 The presence of functional α-adrenoceptors in freshly dispersed single smooth muscle cells from rat tail arteries was investigated by use of selective α-adrenoceptor agonists and antagonists.

2 Cirazoline, a selective α_1-adrenoceptor agonist, caused a prazosin-sensitive, rapid but transient increase in intracellular Ca^{2+}, which was partially inhibited by the voltage-dependent Ca^{2+} channel blocker, nifedipine.

3 TL99, an α_2-adrenoceptor agonist, in the presence of prazosin, initiated a slow and sustained increase in intracellular Ca^{2+} which was partially inhibited by yohimbine and almost completely blocked by nifedipine.

4 In rat tail artery, the effects (dose-response and time-response curves) of cirazoline and TL99 on intracellular Ca^{2+} levels in freshly dispersed single smooth muscle cells were comparable with those obtained with organ bath studies of ring preparations of artery.

5 In freshly dispersed single smooth muscle cells, the time-course response curves induced by the selective α_1-adrenoceptor agonist, phenylephrine and the selective α_2-adrenoceptor agonist, UK14304, were similar to those observed with cirazoline and TL99, respectively.

6 These results indicate that: (a) functional α_1- and α_2-adrenoceptors are present in freshly dispersed single smooth muscle cells from rat tail artery and (b) α_1- and α_2-adrenoceptors are coupled to different cellular processes that lead to an increase in intracellular Ca^{2+}.

Keywords: Intracellular Ca^{2+}; single smooth muscle cells; α-adrenoceptors; rat tail artery

Introduction

An extensive literature from *in vivo* studies supports a role for both postjunctional α_1- and α_2-adrenoceptors in mediating pressor responses to α-adrenoceptor agonists. In contrast, with *in vitro* techniques, only a few studies have demonstrated α_2-mediated contractile responses in arterial smooth muscle (see Timmermans & van Zwieten, 1982). Furthermore, although there are very few reports of α-mediated responses in freshly dispersed vascular smooth muscle cells or cell cultures, responses to peptides seem to be well preserved (Bukoski, 1990; Papageorgiou & Morgan, 1991). In the rat tail artery, *in vitro* studies and radioligand binding assays indicate the presence of both postjunctional α_1- and α_2-adrenoceptors (Abe *et al.*, 1987; Cheung & Triggle, 1988). The present study describes the effects of α-adrenoceptor agonists on intracellular Ca^{2+} levels in freshly dispersed single smooth muscle cells from rat tail artery. The major objectives of the present study were to determine (a): whether α_1- and α_2-adrenoceptors could be demonstrated in freshly dispersed vascular smooth muscle cells and (b): by use of fura-2 Ca^{2+}-measurement techniques, the cellular pathways for Ca^{2+} entry/mobilization utilized by α_1- and α_2-adrenoceptors.

Methods

Smooth muscle cell dispersion

Male Sprague Dawley rats (300–400 g) were stunned by a blow to the head and exsanguinated. The ventral tail artery was excised, cleaned and cut into segments of approximately 3–4 cm length. The technique for cell dispersion was essentially that described by Bolzon & Cheung (1989). Each segment was tied at both ends onto polythylene tubing (PE 50) and perfused with a physiological salt solution I (PSS I) (composition in mM: NaCl 137, KCl 5.4, KH_2PO_4 0.4, $NaHCO_3$ 4.2, NaH_2PO_4 0.4, glucose 5.6, HEPES 10.0 and pH was adjusted to 7.4 with 1 M NaOH) at a rate of 0.05 ml min^{-1} at 37°C. After a 90 min equilibration period, the perfusate was changed to one containing 0.13% papain (BDH INC., Canada) and 0.04% collagenase (Yakult Corp., Japan) and 4 mM dithiothreitol (Sigma, U.S.A.). After 45 min of enzyme perfusion, the segment was cut free and transferred to enzyme-free solution, a longitudinal incision was made, and the tissue was gently pipetted to release the smooth muscle cells. The smooth muscle cells were characteristically oblong in shape, and fura-2 signals were collected from only those cells that responded with a contractile response to the agonists (as described below).

Intracellular Ca^{2+} measurement

Intracellular Ca^{2+} levels were assessed with the fluorescent calcium indicator fura-2. The smooth muscle cells were incubated with 8 μM fura-2-acetoxy methylester at room temperature for 30 min and then transferred to a perfusion chamber, the bottom of which consisted of a glass coverslip. After the cells settled in the chamber, they were perfused with physiological salt solution II (PSS II) (+/− antagonists or blocker) for 5–10 min and then perfused with agonist-containing-PSS II. PSS II consisted of (mM); NaCl 120, KCl 5.0, NaH_2PO_4 1.0, $NaHCO_3$ 25.0, $CaCl_2$ 2.5, $MgSO_4$ 1.0, glucose 11.0, and HEPES 5.0. Changes in fluorescence intensity of fura-2 at excitation wavelengths of 340 and 380 nm

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were monitored in single cells at room temperature, with an SLM-Aminco 1000 fluorometer (SLM Instruments Inc., U.S.A.) interfaced with a Nikon Diaphot inverted microscope. After subtracting the background intensity at 340 and 380 nm, $[Ca^{2+}]_i$ was determined as described by Grynkiewicz *et al.* (1985) from the equation:

$$[Ca^{2+}]_i = K_d\{(R-R_{min})/(R_{max}-R)\}(Fo/Fs)$$

where R is the ratio of emission (510 nm) intensity at 340 and 380 nm excitation after background correction, K_d is the dissociation constant for fura-2 and Ca^{2+} (assumed to be 200 nM, Williams *et al.*, 1987). Fo and Fs are the fluorescence intensity at 380 nm excitation for free and calcium-saturated dye, respectively. Rmin and Fo were determined by exposure of the cells to Br-A-23187 (17 μM) in Ca^{2+}-free PSS II containing 4 mM EGTA, and R_{max} and Fs by exposure of the cells to Br-A-23187 (17 μM) in 10 mM Ca^{2+}-PSS II, respectively.

Organ bath studies

Ventral tail arteries were cut into rings of approximately 5–6 mm in length. Each ring was suspended between platinum hooks and mounted under 1.5 g of passive tension in 10 ml organ baths containing Krebs solution, maintained at room temperature and bubbled with 5% CO_2 in O_2 (pH 7.4). Tissues were allowed to equilibrate in this medium for 90 min, during which the bathing Krebs solution was routinely changed every 20 min. Fifteen minutes before preparation of dose-response or time-response curves, 10^{-6} M cocaine and 10^{-6} M propranolol were added to all tissue baths. Isometric tension was recorded with a force displacement transducer coupled to a Grass polygraph model 7D. The Krebs solution had the following composition (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 12.5 and glucose 11.1.

Cirazoline was a generous gift from Synthelabo, France; UK14304 (5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline), a gift from Pfizer, U.K.; prazosin hydrochloride from Pfizer,

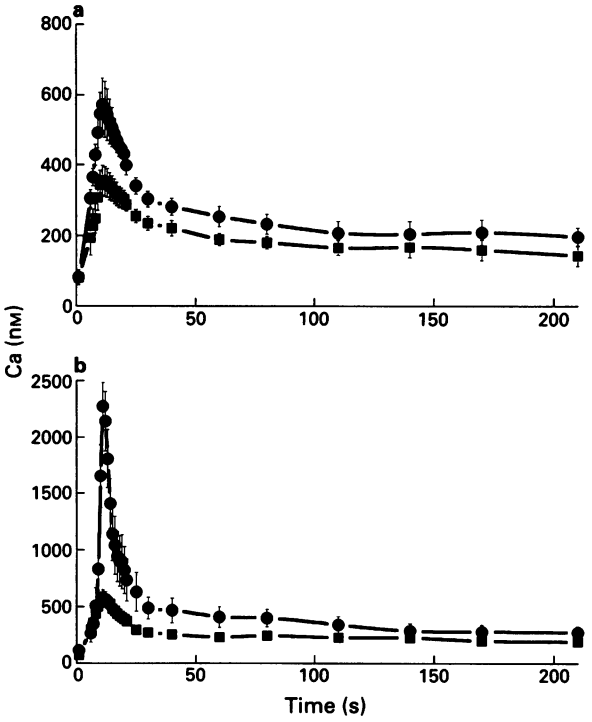


Figure 2 The effects of cirazoline on intracellular Ca^{2+} concentration in fura-2 loaded freshly dispersed single smooth muscle cells from rat tail artery. (a) Time course for cirazoline (0.1 μM) in the absence (●, $n = 6$) or presence of 0.1 μM prazosin (■, $n = 6$). (b) Time course for cirazoline (1 μM) in the absence (●, $n = 6$), or presence of 0.01 μM prazosin (■, $n = 6$). Each data point is plotted as mean ± s.e.mean.

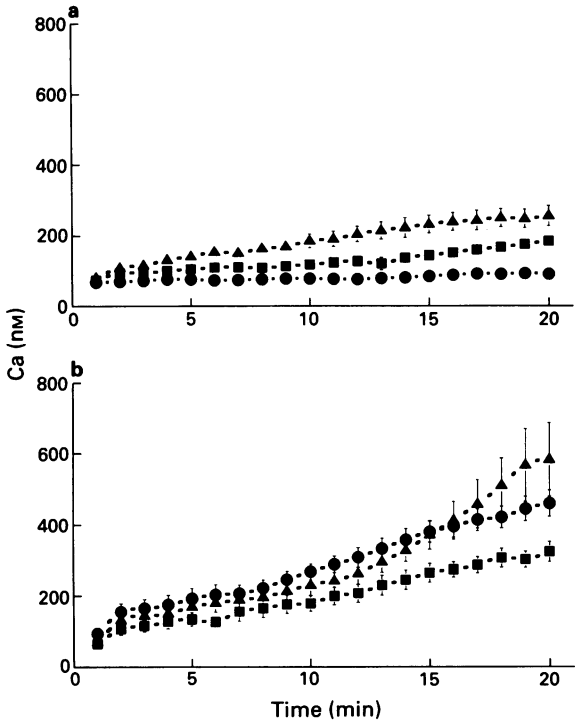


Figure 1 The effects of TL99 on intracellular Ca^{2+} concentrations in fura-2 loaded freshly dispersed single smooth muscle cells from rat tail artery. (a) Time course for 5 μM TL99 (+ 0.05 μM prazosin) in the absence (▲, $n = 6$) or presence of 1 μM yohimbine (■, $n = 8$) and control (perfusing the cells with PSS II) (●, $n = 4$). (b) Time course for 50 μM TL99 in the absence (▲, $n = 6$), and presence of 0.05 μM prazosin (●, $n = 6$), or 0.05 μM prazosin plus 1 μM yohimbine (■, $n = 6$). Every 60th data point is plotted as mean ± s.e.mean.

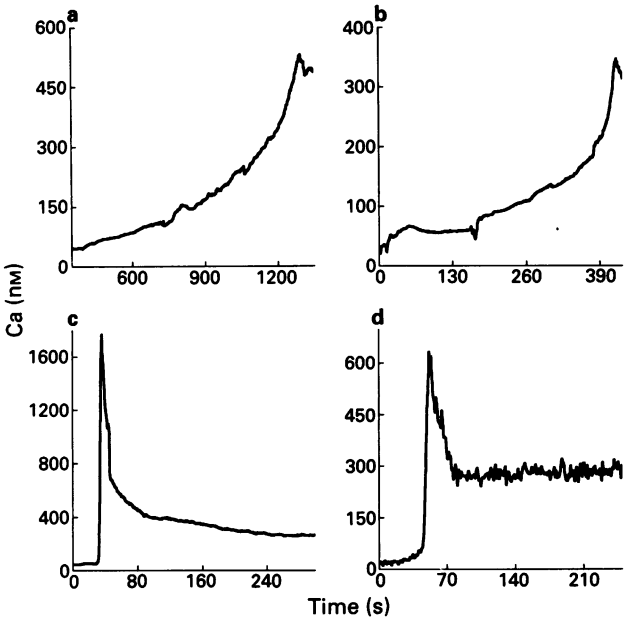


Figure 3 Original traces of intracellular Ca^{2+} content from fura-2-loaded freshly dispersed single smooth muscle cells from rat tail artery stimulated with (a) TL99 (50 μM), (b) UK14304 (10 μM), (c) cirazoline (1 μM) and (d) phenylephrine (1 μM).

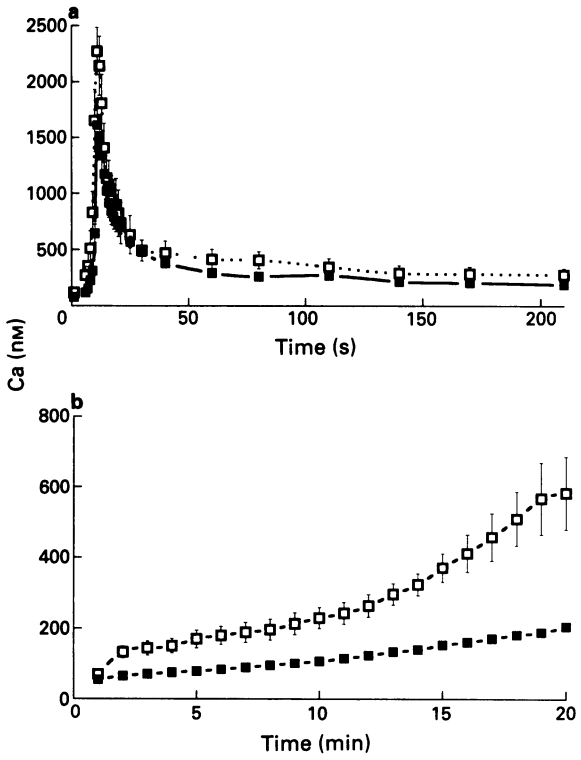


Figure 4 The effects of nifedipine on cirazoline and TL99-induced changes in intracellular Ca^{2+} levels in fura-2-loaded freshly dispersed single smooth muscle cells from rat tail artery. (a) Time course for cirazoline (1 μM) in the absence (□, $n = 6$), or presence of 0.1 μM nifedipine, (■, $n = 6$). (b) Time course for TL99 (50 μM) in the absence, (□, $n = 6$) or presence of 0.1 μM nifedipine (■, $n = 6$). Each data point is plotted as mean ± s.e.mean.

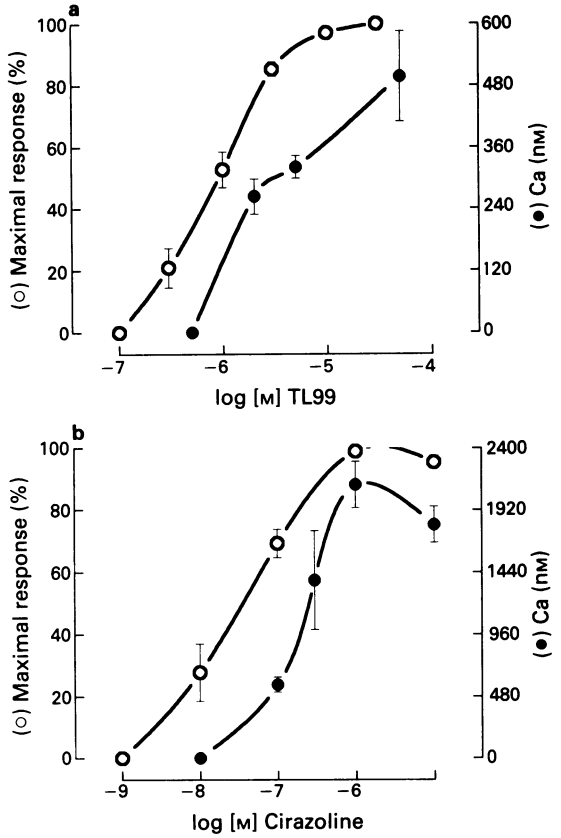


Figure 5 The concentration-dependent changes in intracellular Ca^{2+} stimulated by TL99 and cirazoline in fura-2-loaded freshly dispersed single smooth muscle cells compared with contractile responses to TL99 and cirazoline in artery rings. (a) Concentration-response curves for TL99 in artery ring preparations (○, $n = 4$), and single cells (●, $n = 5-6$). (b) Concentration-response curves for cirazoline in artery ring preparations (○, $n = 4$), and single cells (●, $n = 5-6$). Each data point is plotted as mean ± s.e.mean.

Canada; TL99 [2-(N,N-dimethyl)amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene] from RBI, U.S.A.; yohimbine hydrochloride and phenylephrine hydrochloride from Sigma, U.S.A.; Br-A-23187 (4-bromo A-23187) and fura-2/AM (fura-2-acetoxy methylester) from Molecular Probes Inc., U.S.A. All values are expressed as means ± s.e.mean. Differences between the mean values were determined by Student's t test for unpaired observations, and were regarded as significant when $P < 0.05$.

Results

Studies with single smooth muscle cells

Exposures to cirazoline, phenylephrine and TL99, UK14304 under the conditions described below resulted in >40–50% decreases in length and sometimes blebbing. The average baseline $[Ca^{2+}]_i$ for the smooth muscle cells studied here was 84.5 ± 4.2 nM ($n = 108$). As shown in Figure 1, TL99 (5 μM and 50 μM), in the presence of prazosin (0.05 μM), initiated a slow, dose-dependent increase in $[Ca^{2+}]_i$ and yohimbine (1 μM) inhibited these responses. Perfusion with PSS II alone did not contract the cells and $[Ca^{2+}]_i$ levels remained stable. Figure 2 summarizes the results with cirazoline (0.1 μM and 1 μM), indicating that this selective α_1-adrenoceptor agonist induced a rapid, transient and prazosin-sensitive (0.1 μM) $[Ca^{2+}]_i$ signal.

The time-courses of the response curves for cirazoline and TL99 were different, and the response to TL99, compared with cirazoline, developed more slowly (it took about 20 min to reach maximal response), and resulted in a smaller increase in maximal $[Ca^{2+}]_i$. We also tried different agonists, and found that the time-course response curves induced by phenylephrine (1 μM) and UK14304 (10 μM) were similar to those induced by cirazoline (1 μM) and TL99 (50 μM), respectively (Figure 3). Thus α_1-adrenoceptor agonists produce a fast, comparatively large and transient response, whereas α_2-adrenoceptor agonists induce a slow, sustained and small response. In order to address whether different cellular pathways for Ca^{2+} mobilization were utilized by α_1- and α_2-adrenoceptors, we studied the effects of nifedipine on cirazoline and TL99-induced responses. Figure 4 shows that 0.1 μM nifedipine, almost abolished the increase in intracellular Ca^{2+} induced by 50 μM TL99. In contrast, the same concentration of nifedipine had minimal effects on responses initiated by 1 μM cirazoline.

Studies comparing single cells with ring preparations of rat tail artery

Concentration-response relationships were established for TL99 and cirazoline, and compared to those obtained for contraction of artery rings (organ bath). As shown in Figure 5, TL99 and cirazoline resulted in intracellular Ca^{2+} increases in a concentration-dependent manner. The shapes of concentration-response curves and time-course response curves (data not shown) obtained from freshly dispersed single cells correlated well with those obtained from artery rings.

In single smooth muscle cells, EC_{50} values for cirazoline and TL99 were 1.5×10^{-7} M ($n = 5-6$), and 4.0×10^{-6} M ($n = 5-6$), respectively; in ring preparations, they were $4.3 \pm 1.2 \times 10^{-8}$ M ($n = 4$), and $9.2 \pm 0.9 \times 10^{-7}$ M ($n = 4$), respectively.

Discussion

In the present study, the presence of postjunctional α-adrenoceptors in freshly dispersed single smooth muscle cells from rat rail artery was determined by use of selective α-adrenoceptor agonists and antagonists. Cirazoline, a selective α_1-adrenoceptor agonist (Ruffolo & Waddel, 1982), initiated a $[Ca^{2+}]_i$ signal which was more rapid and transient than that observed for α_2-adrenoceptor agonists. Treatment of the cells with the α_1-adrenoceptor antagonist, prazosin, greatly depressed responses to cirazoline. It can thus be concluded that functional α_1-adrenoceptors are present in freshly dispersed single smooth muscle cells from rat tail artery. Exposure to TL99, an α_2-adrenoceptor agonist (Hicks & Cannon, 1980), resulted in a slowly developing Ca^{2+} signal, that was not affected by the α_1-adrenoceptor antagonist, prazosin (0.05 μM). Treatment of the cells with the α_2-adrenoceptor antagonist, yohimbine greatly inhibited the responses to TL99. The reported K_d and K_i values for prazosin and yohimbine, as determined from radioligand binding studies with crude plasma membrane preparation from the rat tail artery, are 0.17 nM and 1.64 nM, respectively (Cheung & Triggle, 1988), and thus our use of 0.05–0.1 μM prazosin and 1 μM yohimbine should result, respectively, in the inhibition of responses at α_1- and α_2-adrenoceptors.

It is also noted that time-response curves for the fura-2 Ca^{2+} signals following α_1- and α_2-adrenoceptor stimulation were different and that the α_1-mediated Ca^{2+} signal is comparatively fast. Treatment with nifedipine, almost abolished the TL99-induced increase in intracellular Ca^{2+}, but only partially inhibited the cirazoline-induced response. It is clear that activation of α_2-adrenoceptors induces a slow, sustained intracellular Ca^{2+} increase subsequent to the opening of L-type voltage-dependent Ca^{2+} channels, whereas, α_1-adrenoceptors mediate a fast and transient increase in intracellular Ca^{2+}, which is likely to result from the mobilization of intracellular Ca^{2+} and Ca^{2+} influx through receptor-operated channels.

In order to evaluate whether the enzymatic dispersal of the cells results in functional impairment, we compared the concentration and time-course response curves for cirazoline and TL99 obtained from freshly dispersed single cells to those from artery rings. Both the time-course and the concentration-response curves from isolated tissues and single cells were similar. These data suggest that our cell dispersal technique does not result in functional impairment.

These results demonstrate that freshly dispersed vascular smooth muscle cells can be used for the study of signal-transduction pathways coupled to α-adrenoceptor activation. Furthermore, preliminary studies with this technique indicate that, although the activation of either α_1- or α_2-adrenoceptors leads to an increase in $[Ca^{2+}]_i$, the translocation/release processes involved are different.

The support of the Alberta Heart & Stroke Foundation is gratefully acknowledged.

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(Received February 2, 1993
Revised March 29, 1993
Accepted April 9, 1993)

Br. J. Pharmacol. (1993), **109**, 1276–1281 © Macmillan Press Ltd, 1993

Altered cardiac adrenergic neurotransmission in streptozotocin-induced diabetic rats

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1 Functional alterations of the sympathetic neuroeffector junction of the left atria were studied in rats with streptozotocin-induced diabetes.

2 Eight to 12 weeks of diabetes resulted in a marked decrease in the positive inotropic response of left atria to electrical field stimulation (EFS).

3 The overflow of [^{3}H]-noradrenaline from diabetic left atria caused by EFS was much less than that from control preparations.

4 The concentration-response curves showed no change in sensitivities of the left atria to exogenous noradrenaline and tyramine in diabetic rats. The maximum positive inotropic response to these agents were similar in diabetic and control animals.

5 The left atrial content of noradrenaline was not significantly changed in diabetic rats. The cocaine-sensitive uptake of [^{3}H]-noradrenaline was also unaltered.

6 Atropine enhanced the positive inotropic response and [^{3}H]-noradrenaline overflow induced by EFS in control left atria. Similarly, yohimbine caused an enhancement of EFS-evoked inotropic response in control atria. However, these effects of the antagonists were not observed in diabetic left atria.

7 It is concluded that the decrease in the positive inotropic response of the left atria to EFS in diabetic rats is caused by an impairment of noradrenaline release from the sympathetic nerve terminals through a calcium-dependent exocytotic mechanism. The present results also indicate that presynaptic α_2-adrenoceptors and muscarinic receptors that are linked to inhibition of the noradrenaline release during nerve stimulation may be functionally impaired in diabetic animals.

Keywords: Diabetes; heart; electrical field stimulation; positive inotropic effect; noradrenaline release; presynaptic inhibitory receptors

Introduction

Autonomic neuropathy is a significant complication of diabetes and is considered to be responsible for orthostatic hypotension, skin ulceration, arterial calcification and abnormal temperature regulation (Sharpey-Schaffer & Taylor, 1960; Moorhouse *et al.*, 1966; Goebel & Fuessl, 1983; Edomonds *et al.*, 1986). Clinical studies have revealed that there is less orthostatic increment in noradrenaline release in diabetic patients (Cryer & Weiss, 1980; Hilsted, 1982).

Ultrastructural study has revealed many abnormal, degenerated adrenergic nerve profiles in diabetic right atria (Tomlinson & Yusof, 1983). Furthermore, a decreased noradrenaline content in the heart of diabetic patients (Neubauer & Christensen, 1976) and reduced noradrenaline turnover in diabetic rat hearts (Yoshida *et al.*, 1985) have been reported. Functional studies have shown that the positive chronotropic and inotropic responses to sympathetic transmural nerve stimulation were markedly attenuated in diabetic rat atria (Sato *et al.*, 1989; Hashimoto *et al.*, 1990).

On the other hand, cardiac noradrenaline levels have been shown to increase in diabetes (Paulson & Light, 1981; Fushimi *et al.*, 1984). Ganguly *et al.* (1986) have demonstrated that cardiac noradrenaline in the diabetic rat heart is maintained at a higher level by an increased synthesis and uptake of noradrenaline in the adrenergic nerve terminals and have indicated higher sympathetic activity in the diabetic myocardium. It is possible that elevated noradrenaline levels may result in decreased density of β-adrenoceptors and defective β-adrenoceptor-adenylate cyclase coupling (Savarese & Beikowitz, 1979; Heyliger *et al.*, 1982; Smith *et al.*, 1984; Atkins *et al.*, 1985). Therefore, it remains uncertain whether the decreased response of diabetic rat atria to transmural nerve stimulation results from a decreased noradrenaline release from the sympathetic nerve terminals or from a decreased postsynaptic response to noradrenaline.

The purpose of the present study was to examine the function of the sympathetic neuroeffector mechanism in the left atria from rats with streptozotocin-induced diabetes. The aim was to determine how diabetes affects the responsiveness of cardiac muscle to endogenously released noradrenaline and to determine how diabetes affects the content, release and uptake of noradrenaline in cardiac sympathetic nerves.

Methods

Induction of diabetes

Male Wistar rats, 16 weeks old, weighing 450–520 g, were anaesthetized with diethyl ether and received a single intravenous injection of streptozotocin, 45 mg kg^{-1}, via the tail vein. Streptozotocin was dissolved in a citrate solution (0.1 M citric acid and 0.2 M sodium phosphate, pH 4.5). Control rats received the citrate buffer solution alone. All rats were fed on normal rat chow and water *ad libitum* until they were used. On the day of the experiment, a blood sample was collected and serum glucose level was determined by Rapid Blood Analyzer Super using Uni-Kit (Chugai, Tokyo, Japan). All animals injected with streptozotocin were severely hyperglycaemic (range 473–865 mg dl^{-1}). The general features of diabetic rats and age-matched control animals were summarized in Table 1.

Organ bath experiments

Eight to 12 weeks after treatment, rats were anaesthetized with diethyl ether. The hearts were rapidly excised and transferred to dissection baths filled with oxygenated Krebs-

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Table 1 General characteristics of control and streptozotocin-induced diabetic rats

	Control	*Diabetic*
Body weight (g)	690 ± 14	381 ± 5***
Plasma glucose (mg dl^{-1})	183 ± 4	617 ± 16***
Heart weight (mg)	940 ± 86	660 ± 56***

All values are means ± s.e.mean ($n = 45$ for each group). The values in diabetic animals are compared with their corresponding controls and the level of significant difference is indicated by ***$P < 0.001$.

Henseleit solution at room temperature (composition mM): NaCl 143.9, KCl 4.8, $CaCl_2$ 1.3, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.9 and glucose 10.0, pH 7.4. The left atrium was carefully dissected and mounted vertically in a 50 ml water-jacketed bath containing Krebs-Henseleit solution bubbled with 95% O_2 and 5% CO_2 at 33°C. The lower end of the left atrium was pinned on a pair of hook electrodes for stimulation and the other end was connected by a silk thread to a force transducer (Nihon Kohden, TB-612T). Isometric tension was recorded with a force-transducer and displayed on a pen recorder (Nihon Kohden, RTG-3026) through a preamplifier (Nihon Kohden, RP-5). The resting tension was adjusted to 2 g. The atria were electrically paced by rectangular pulses of 5 ms duration and an intensity of 1.5 times threshold current at 1 Hz frequency. The pulses were delivered from an electronic stimulator (Sanei-Sokki, 3F46) through an isolation unit (Sanei-Sokki, 5361). Each preparation was equilibrated for 60 min before the start of the experiments. Details of the experimental procedure have been published elsewhere (Hattori & Kanno, 1985; Hattori *et al.*, 1987).

Electrical field stimulation methods during the myocardial refractory period (which were described by Blinks, 1966), were used to stimulate sympathetic nerves. The stimuli for electrical field stimulation (EFS) were delivered by a second electrical stimulator (50 mA, 5 ms duration) through platinum electrodes placed on either side of the atrium. A graded stimulus-inotropic response curve was obtained by a stepwise increase in the number of pulses.

The concentration-response curves for the positive inotropic effects of noradrenaline and tyramine were determined in a cumulative manner by increasing the concentrations in steps of 0.5 log units.

[^{3}H]-noradrenaline efflux measurements

The left atria were incubated at 2 ml oxygenated Krebs-Henseleit solution containing 0.75 mg ml^{-1} ascorbic acid and 1.5×10^{-7} M (−)-[^{3}H]-noradrenaline (New England Nuclear, Boston, U.S.A., specific activity 41 Ci mmol^{-1}) for 15 min at 33°C and then transferred to a superfusion chamber. The atrium was pinned on the bottom of the chamber and superfused at a rate of 2.0 ml min^{-1} with Krebs-Henseleit solution gassed with 95% O_2 and 5% CO_2. The temperature of the perfusate was maintained at 33°C. The left atrium was electrically stimulated by rectangular pulses of 1 Hz frequency, 5 ms duration and 1.5 times threshold current, delivered by a pair of platinum wire electrodes connected to the electronic stimulator through the isolator. After a washout period of 90 min to establish a stable basal efflux of radioactivity, superfusate samples were collected at a 1 min interval. EFS was applied for 15 min through platinum field electrodes on either side of the atrium. At the end of the experiment the preparations were frozen with liquid nitrogen and weighed. Evaluation of EFS-evoked tritium overflow was made by calculating the difference between the overflow for 5 min before EFS and the overflow for 5 min after the start of EFS.

[^{3}H]-noradrenaline uptake measurements

[^{3}H]-noradrenaline uptake was determined according to the methods described by Williams (1991). Briefly, the left atria were preincubated in Krebs-Henseleit solution containing 0.75 mg ml^{-1} ascorbic acid, 3×10^{-5} M hydrocortisone and 10^{-5} M pargyline in the presence or absence of 3×10^{-6} M cocaine for 30 min at 33°C. Then (−)-[^{3}H]-noradrenaline (final concentration 1.5×10^{-7} M) was added to the incubation medium. After incubation for 60 min, the tissue was removed, immediately blotted with Whatman No. 5 filter paper, rinsed by placing the tissue consecutively into four beakers containing Krebs-Henseleit solution without radioligand, blotted again, and weighed. Subsequently, the tissue was treated with 1.0 ml tissue solubilizer (Solvable, New England Nuclear) for 3 to 6 h at 50°C. Finally, 0.1 ml 30% H_2O_2 was added and radioactivity in the solubilized tissue was determined. The difference in the [^{3}H]-noradrenaline accumulation in the absence and presence of cocaine was defined as cocaine-sensitive neuronal uptake.

Catecholamine assays

Each of the left atria obtained from control and diabetic rat hearts were frozen with clamps which had been cooled with liquid nitrogen. The frozen muscle samples were weighed and then homogenized in 2.5 ml of 0.4 N $HClO_4$ containing 2 mM EDTA by means of a micro homogenizer (Nition, NS 310E) for 30 s. The homogenate was centrifuged at 10,000 r.p.m. for 10 min at 4°C. The supernatant was collected and subjected to the determination of noradrenaline by a high performance liquid chromatographic procedure (Yui *et al.*, 1980).

Drugs

The drugs used were as follows: atropine sulphate, (−)-noradrenaline bitartrate and physostigmine sulphate (Wako, Osaka, Japan), cocaine hydrochloride (Takeda, Osaka, Japan), prazosin hydrochloride (Taito-Pfizer, Tokyo, Japan), (±)-propranolol hydrochloride, yohimbine hydrochloride, hydrocortisone, pargyline, and streptozotocin (Sigma Chemical, St. Louis, MO, U.S.A.). All compounds were dissolved in Krebs-Henseleit solution or distilled water with the exception of streptozotocin (see above).

Statistics

All data were expressed in terms of the mean ± s.e.mean. For statistical evaluation, Student's paired and unpaired t tests were used and a significant difference was assumed to exist if the P value was less than 0.05.

Results

The basal force of contraction of left atria isolated from diabetic rats (446 ± 29 mg; $n = 41$) was not significantly different from that of left atria from age-matched control animals (469 ± 40 mg; $n = 41$). Trains of 1 to 64 field pulses applied during the refractory period of the atrial muscle evoked an increase in force of contraction in both control and diabetic atria (Figure 1). Diabetic atria developed a lower force of contraction in response to EFS than control atria and the difference was statistically significant at every level of stimulation (Figure 1). In the presence of 3×10^{-7} M atropine or 10^{-6} M yohimbine, the positive inotropic response to EFS was significantly enhanced in control atria (Figure 2). On the other hand, none of the antagonists augmented the inotropic response to EFS in diabetic atria (Figure 2). In both control and diabetic left atria, pretreatment with 3×10^{-6} M propranolol completely abolished the positive inotropic response caused by EFS. After blocking α_1- and β-adre-

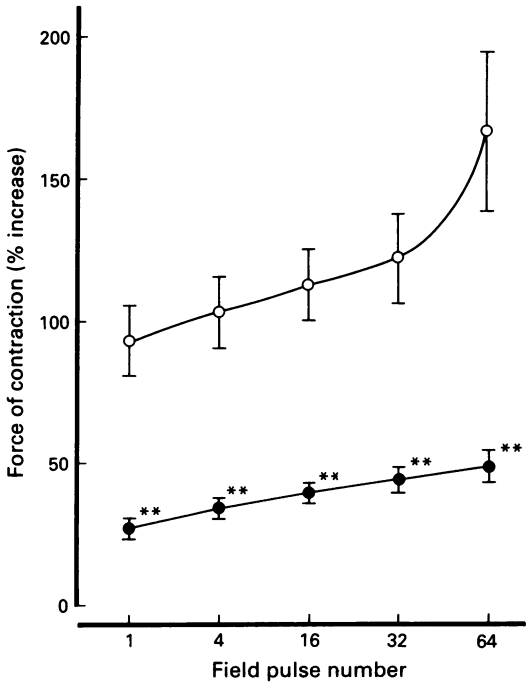


Figure 1 Positive inotropic responses to electrical field stimulation of left atria isolated from control (○, $n = 5$) and diabetic (●, $n = 5$) rats. All values are means ± s.e.mean and are expressed as % increase in basal force of contraction prior to application of field stimulation. Abscissa scale: number of pulses. $^{**}P < 0.01$ indicates a significant difference from the corresponding control values.

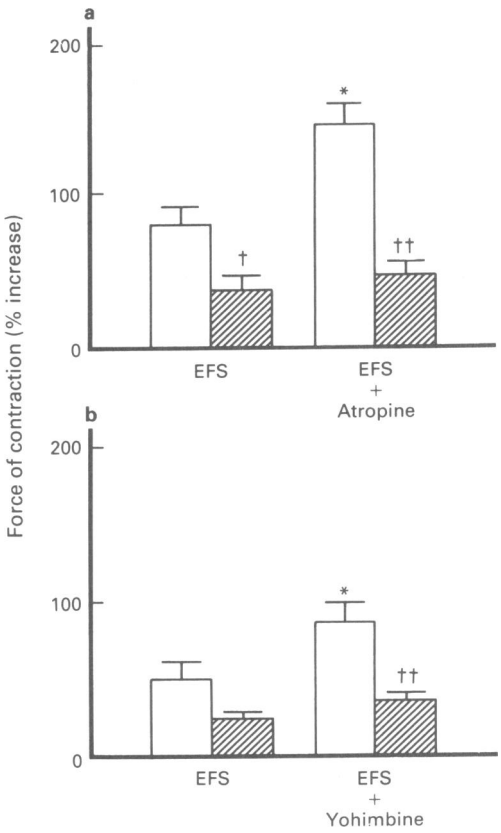


Figure 2 Influences of 3×10^{-7} M atropine (a) and 10^{-6} M yohimbine (b) on the positive inotropic response to electrical field stimulation (EFS) of left atria isolated from control (open columns) and diabetic rats (hatched columns). EFS was by application of a current of 50 mA and 5 ms duration. Values are means ± s.e.mean of five experiments and expressed as % increase in basal force of contraction prior to application of EFS. $^{*}P < 0.05$ indicates a significant difference from EFS in the absence of the antagonists: $\dagger P < 0.05$; $\dagger\dagger P < 0.01$ indicate significant differences from the corresponding control values.

noceptors with 10^{-6} M prazosin and 3×10^{-6} M propranolol, EFS did not produce a decrease in force of contraction even in the presence of 10^{-4} M physostigmine in control and diabetic atria.

Noradrenaline and tyramine produced a positive inotropic effect in a concentration-dependent manner in both control and diabetic atria (Figure 3). The maximum responses to noradrenaline and tyramine were not significantly different between control and diabetic atria. The pD_2 values for

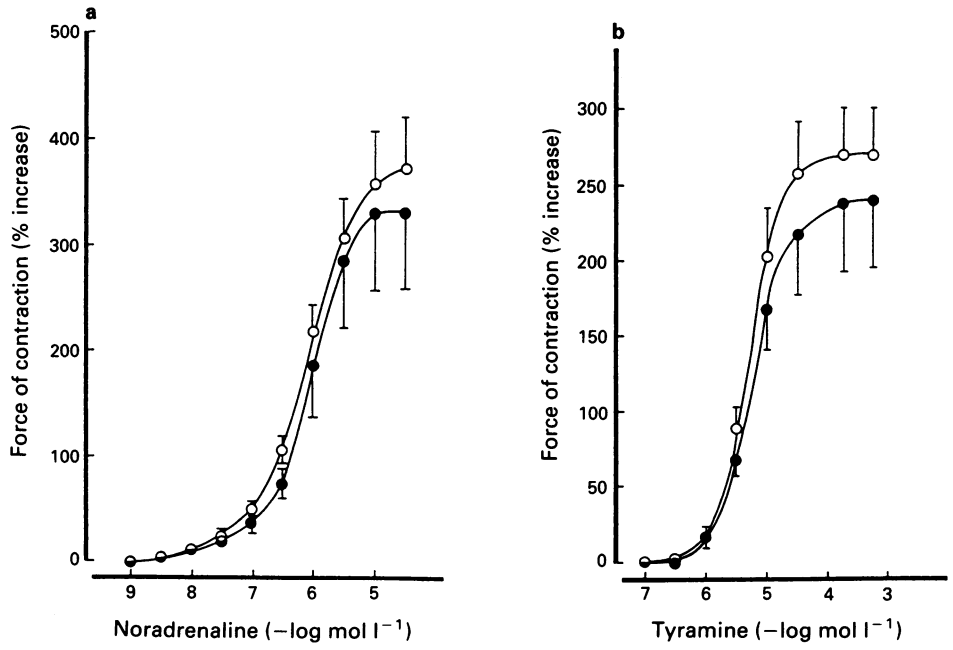


Figure 3 Concentration-response curves for the positive inotropic effects of noradrenaline (a) and tyramine (b) in left atria isolated from control (○) and diabetic (●) rats. All values are means ± s.e.mean of six to seven experiments.

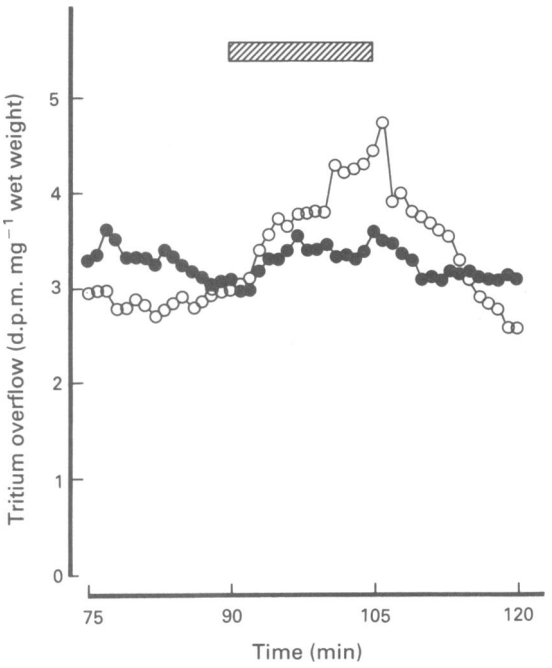


Figure 4 Representative examples of the effect of electrical field stimulation on [^{3}H]-noradrenaline overflow from left atria isolated from control (○) and diabetic (●) rats. Electrical field stimulation was by application of a current of 50 mA and 5 ms duration and is indicated by hatched bar.

noradrenaline (6.16 ± 0.07) and tyramine (5.97 ± 0.16) in diabetic atria were almost the same as the control values for noradrenaline (6.13 ± 0.07) and tyramine (5.81 ± 0.11).

The amounts of basal efflux of [^{3}H]-noradrenaline from control and diabetic atria (~75 min after superfusion) were 0.497 ± 0.06 and 0.768 ± 0.14 d.p.m. mg^{-1} wet wt. min^{-1}, respectively ($n = 5$ for each group); the difference between these values was not statistically significant. As shown in Figure 4, EFS increased the overflow of [^{3}H]-noradrenaline from control atria. However, EFS failed to increase [^{3}H]-noradrenaline efflux in diabetic atria. The efflux of [^{3}H]-noradrenaline in response to EFS was significantly greater in control atria than in diabetic atria (141.9 ± 9.4 *vs* 103.8 ± 11.8% of the basal tritium overflow, $n = 5$ for each group, $P < 0.01$). In the presence of 3×10^{-7} M atropine, the increase in [^{3}H]-noradrenaline efflux induced by EFS was markedly enhanced in control atria but not in diabetic atria (data not shown).

In the presence of hydrocortisone and pargyline, the uptake of [^{3}H]-noradrenaline was not significantly different between control and diabetic atria (2627 ± 241 *vs* 2656 ± 238 d.p.m.; mg^{-1} wet wt., $n = 5$ for each group). Cocaine inhibited the majority of the uptake of [^{3}H]-noradrenaline in both groups (1303 ± 166 *vs* 1759 ± 264 d.p.m. mg^{-1} wet wt., $n = 5$ for each group). There was also no statistically significant difference between these values. The difference in neuronal uptake of [^{3}H]-noradrenaline (defined as the difference in uptake of [^{3}H]-noradrenaline in the absence and presence of cocaine) was also insignificant between both groups.

Noradrenaline contents of the left atria were not significantly different between control and diabetic animals (633 ± 31 *vs* 632 ± 56 ng ml^{-1} wet wt., $n = 6$ for each group).

Discussion

In the present study we showed that the positive inotropic response to EFS was markedly attenuated in the left atria obtained from diabetic rat. The findings that the positive inotropic response to exogenous noradrenaline and tyramine were unaffected in the left atria from diabetic rats indicate that the attenuation of the positive inotropic response to EFS may be caused by presynaptic derangement of neurochemical transmission. The release of noradrenaline by tyramine is reported to be independent of extracellular calcium (Bönisch & Trendelenburg, 1988), whereas EFS releases noradrenaline from the stores in sympathetic nerve endings by a calcium-dependent exocytotoxic mechanism (Rubin, 1970; Brasch, 1989). The most probable explanation for the observation that the atrial response to EFS, but not tyramine, was depressed in diabetic rats is that the release of noradrenaline by calcium-sensitive exocytosis may be specifically impaired in left atria from diabetic rats.

In contrast to our findings that diabetic left atria responded normally to exogenous noradrenaline, a reduction in the inotropic response to β-adrenoceptor stimulation has been reported in ventricular myocytes (Horackova & Murphy, 1988), in spontaneously beating atria (Foy & Lucas, 1978) and in left atria (Sato *et al.*, 1989; Hashimoto *et al.*, 1990) from diabetic rats, and a decrease in the number of the β-adrenoceptors has been demonstrated (Savarese & Berkowitz, 1979; Atkins *et al.*, 1985). It has been suggested that the decline in β-adrenoceptor density may be responsible for the decreased inotropic response to β-adrenoceptor stimulation in diabetic hearts (Heyliger *et al.*, 1982). However, Durante *et al.* (1989) have found no difference in the positive inotropic response of left atria to isoprenaline between control and diabetic rats, despite the fact that β-adrenoceptor density is reduced by 64% in left atria from diabetic rats, indicating that the decrease in cardiac β-adrenoceptor density in diabetes does not necessarily result in an altered β-adrenoceptor-mediated inotropic response. Our findings are in agreement with other studies showing no change in the inotropic effect of isoprenaline in left atria and papillary muscle (McCullough & McNeill, 1983) or isolated hearts (Ingebretsen *et al.*, 1980; Vadlamudi & McNeil, 1984) from diabetic rats. Moreover, Austin & Chess-Willims (1991) have observed an enhanced sensitivity to isoprenaline in left atria and papillary muscles from diabetic rats. Thus, it seems very unlikely that changes in the number of β-adrenoceptors and β-adrenoceptor-adenylate cyclase coupling are involved in the diminished positive inotropic response to EFS in diabetic left atria.

In diabetes, the noradrenaline concentration in the myocardium has been found to increase (Paulson & Light, 1981; Fushimi *et al.*, 1984; Ganguly *et al.*, 1986), decrease (Neubauer & Christensen, 1976) or to be unchanged (Kaul & Grewal, 1980; Akiyama *et al.*, 1989). Noradrenaline turnover has been reported to be depressed in diabetic rats hearts, indicating a reduction in sympathetic nerve activity (Yoshida *et al.*, 1985; 1987). In contrast, Ganguly *et al.* (1986) have shown an increased turnover, uptake and synthesis of noradrenaline in diabetic hearts, implying an increased sympathetic activity. The apparent discrepancy in these results may be related to difference in experimental models, i.e., strain and age of animals, duration and severity of diabetes. In this study we found that the content of noradrenaline in the left atrium was not significantly changed in diabetic rats. In addition, in the presence of hydrocortisone and pargyline, which inhibit extraneuronal uptake and monoamine oxidase, respectively, the cocaine-sensitive accumulation of [^{3}H]-noradrenaline in the left atrium was not significantly different between diabetic and control atria. However the overflow of [^{3}H]-noradrenaline produced by EFS was significantly less in diabetic left atria than controls. Since the diminished overflow of [^{3}H]-noradrenaline reflects a decreased noradrenaline release rather than enhancement of its uptake or retention with the tissue, an impairment of the presynaptic functional process for releasing noradrenaline from the nerve endings could explain the decreased inotropic response to EFS in diabetic left atria.

In the left atria from control rats yohimbine significantly

enhanced the positive inotropic effect induced by EFS, which is considered to be due to blockade of presynaptic α_2-adrenoceptors which are activated by released noradrenaline (Langer, 1981; Starke, 1987). Similarly, atropine caused significant potentiation of the positive inotropic response to EFS in control left atria. It seems unlikely that this potentiating effect of atropine resulted from inhibition of a negative inotropic response to acetylcholine released from cholinergic nerve stimulation, as we did not detect a negative inotropic effect elicited by EFS even in the presence of propranolol, prazosin and physostigmine, but rather through inhibition of presynaptic inhibitory muscarinic receptors (Starke, 1977; Langer, 1981). It appears that atropine is blocking presynaptic inhibitory muscarinic receptors resulting in potentiation of the positive inotropic response to EFS, as atropine facilitated the overflow of [^{3}H]-noradrenaline induced by EFS in control left atria.

In contrast to their effects in nondiabetic atrial preparations, neither yohimbine nor atropine potentiated the positive inotropic response to EFS in diabetic left atria. It is suggested that activation of presynaptic inhibitory receptors reduces transmitter release by decreasing the availability of calcium for stimulus-secretion coupling (Starke, 1977; Langer, 1981). Therefore, if the availability of calcium necessary for stimulus-secretion coupling in the sympathetic nerve endings is primarily impaired in diabetes, actual effects of the experimental procedures of reducing calcium availability such as stimulation of presynpatic inhibitory receptors would be minimized. Alternatively, the amounts of noradrenaline and acetylcholine released from diabetic nerve endings by EFS may be too small to activate efficiently presynaptic inhibitory receptors. Moreover, we cannot exclude the possibility that the densities of presynaptic inhibitory receptors might be depressed in diabetic left atria. Whatever mechanisms are involved in the lack of effect of yohimbine and atropine on the positive inotropic response to EFS in diabetic left atria, our results indicate that regulation of noradrenaline release through presynaptic inhibitory receptors may be altered in diabetic animals.

In summary, the present study shows that the positive inotropic response to EFS is specifically depressed in left atria from diabetic rats. This appears to be due to a decrease in noradrenaline release from the sympathetic nerve terminals through calcium-dependent exocytosis. In addition, it is suggested that presynaptic inhibitory receptors on diabetic sympathetic endings may lose a functional role in modulating adrenergic neurotransmission.

The authors thank Miss R. Yamazaki for her secretarial assistance. This work was supported in part by a Grant-in-Aid for Science Research from the Ministry of Education, Science and Culture of Japan and by the Akiyama Foundation.

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(Received January 25, 1993
Revised March 25, 1993
Accepted April 14, 1993)

Br. J. Pharmacol. (1993), **109**, 1282–1289 © Macmillan Press Ltd, 1993

Alfuzosin, a selective α_1-adrenoceptor antagonist in the lower urinary tract

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1 Phenylephrine-induced contractions of rabbit isolated trigone and urethra were antagonized in a competitive manner by alfuzosin (pA_2 7.44 and 7.30, respectively) and prazosin.

2 The characteristics of [^{3}H]-prazosin binding to human prostatic adenoma tissue were evaluated. [^{3}H]-prazosin was potently displaced by α_1-adrenoceptor specific agents including alfuzosin, its (+)- and (−)-enantiomers and prazosin (IC_{50} 0.035, 0.023, 0.019 and 0.004 μM, respectively), but only weakly by α_2-adrenoceptor selective agents, for example, yohimbine (IC_{50} = 6.0 μM).

3 In the pithed rat, alfuzosin (0.03–0.3 mg kg^{-1}, i.v.) markedly inhibited pressor responses produced by the α_1-selective agonist, cirazoline but inhibited only slightly responses to the α_2-selective agonist, UK 14,304. Alfuzosin (1 mg kg^{-1}, i.v.) had minimal effects against responses mediated by stimulation of prejunctional α_2-receptors (UK 14,304-induced inhibition of sympathetic tachycardia).

4 In the anaesthetized cat, alfuzosin (0.001–1 mg kg^{-1}, i.v.) and prazosin (0.001–0.3 mg kg^{-1}, i.v.) produced dose-related inhibition of the increases in urethral pressure caused by stimulation of sympathetic hypogastric nerves. Prazosin was approximately 5 fold more potent than alfuzosin. When phenylephrine was employed to induce urethral and vascular α_1-mediated tone simultaneously, prazosin inhibited both stimuli with similar potency whereas alfusozin was 3–5 fold more potent against elevated urethral pressure. This functional uroselectivity of alfuzosin was more evident by the intraduodenal route, since doses of 0.03 and 0.1 mg kg^{-1} alfuzosin inhibited urethral pressure with minimal effects on arterial blood pressure.

5 Alfuzosin is a potent selective α_1-adrenoceptor antagonist in tissues of the lower urinary tract including the human prostate. This provides a pharmacological basis for its use in the treatment of benign prostatic hypertrophy.

Keywords: Alfuzosin; α_1-adrenoceptor antagonist; lower urinary tract; benign prostatic hypertrophy

Introduction

Benign prostatic hypertrophy (BPH), a condition which causes disturbance of micturition in a relatively high proportion of elderly men, has conventionally been treated by surgical resection of the enlarged prostate gland. However, more recently, alternative pharmacological treatment options have been developed, prominent amongst which is the use of selective α_1-adrenoceptor antagonists e.g. alfuzosin.

The outflow obstruction which accompanies abnormal prostate growth and which is reponsible for the observed urinary symptoms (difficulty in urination, incontinence, etc.) is considered to have two components, a static one related to physical compression of the urethra caused by prostatic hypertrophy and a dynamic component which derives from changes in smooth muscle tone within the prostate, bladder neck and urethra and is determined by the level of activation of the sympathetic nervous system (Caine, 1986). This dynamic and potentially reversible neurogenic factor contributes significantly to the total observed urethral obstruction (47%, Furuya *et al.*, 1982) and is probably responsible for the characteristic fluctuations in symptoms of voiding dysfunction experienced by BPH patients. A therapeutic strategy based on antagonism of the increased sympathetic drive to the lower urinary tract has added significantly to the recent management of BPH (Hieble & Caine, 1986).

It is known that three key tissues concerned in micturition in the context of BPH, i.e. the bladder base (trigone), the urethra and the prostate itself, contain smooth muscle with sympathetic innervation through the hypogastric nerve and contract in response to sympathetic activation. The receptor type primarily involved, and thus responsible for sympathetically-mediated bladder outflow obstruction in man, is the α_1-subtype. Although both α_1- and α_2-receptors have been identified within the human prostate, the latter appear to predominate within vascular and glandular tissue rather than in the smooth muscle-containing element, the stroma (James *et al.*, 1989). Hence, the selective α_1-agonist, phenylephrine, contracts human isolated prostatic strips (Kitada & Kumazawa, 1987) and responses to the sympathetic neurotransmitter noradrenaline are antagonized potently by selective α_1-antagonists but only weakly by α_2-antagonists (Hedlund *et al.*, 1985; Hieble *et al.*, 1985) demonstrating the functional importance of the α_1-subtype. Similarly, in human urinary bladder base and urethral preparations noradrenaline-induced contractions are α_1-mediated (Kunisawa *et al.*, 1985).

As a final test of this hypothesis, a number of clinical trials have demonstrated that α_1-antagonists, prazosin, alfuzosin and terazosin (Hedlund *et al.*, 1983; Lepor *et al.*, 1990; Jardin *et al.*, 1991) are effective at improving the symptoms of BPH. In a large placebo-controlled trial, alfuzosin, the subject of this paper, significantly reduced the incidence of obstructive and irritative symptoms and increased urinary flow rates (Jardin *et al.*, 1991). Although agents of this type are also capable of causing vascular effects (potentially undesirable in the context of BPH), alfuzosin was well-tolerated and its efficacy was maintained over a 6-month period. In this paper we describe the animal pharmacology of alfuzosin (N-[3-[(4-amino-6, 7-dimethoxyquinazolin-2-yl) methylamino] propyl] tetrahydro-2-furancarboxamide), a quinazoline derivative which differs structurally from prazosin in the absence of a piperidine moiety, with particular emphasis on its α_1-antagonist properties in the lower urinary tract *in vitro* and *in vivo*. Some of these data have already been presented in abstract form (Cavero *et al.*, 1984b; 1985; Pimoule *et al.*, 1989).

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Methods

Phenylephrine-contracted rabbit isolated trigone and urethra

Male rabbits (3–4 kg) were killed by cervical dislocation and strips (5 × 2 mm) of trigone muscle or urethral rings (5 mm), prepared as described by Ueda *et al.* (1984), were set up under 1 g tension in Krebs buffer containing (mM): NaCl 114, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.7 and ascorbic acid 1.1. Propranolol (1 μM) was routinely included to eliminate possible effects mediated by stimulation of β-receptors. After a 2 h stabilization period, cumulative concentration-response curves to phenylephrine were determined before and after a 30 min incubation with alfuzosin or one of its optical isomers (0.03–3 μM) or prazosin (0.03–3 μM). Each preparation received only a single antagonist concentration or vehicle. Concentration-response curves to phenylephrine in the presence of antagonist or vehicle were expressed as a percentage of the maximal response of the control curve. The antagonist potency was evaluated by Schild analysis. For alfuzosin, (+)-alfuzosin and prazosin, pA_2 values and slopes were calculated from regression analysis of all available data points (log[DR − 1]). In the case of (−)-alfuzosin, because of limited data, a pA_2 estimate was calculated directly from the Schild equation.

Displacement of [^{3}H]-prazosin binding in human prostatic adenomyofibroma membrane preparations

Human prostatic adenomas were obtained during surgery for BPH. After dissection, the cranial and periurethral regions were stored at − 80°C until use. The tissue was homogenized in 30 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.5) with the use of a Polytron homogenizer. The homogenate was washed by two successive centrifugations for 10 min at 38,000 *g*, with intermittent resuspension of the resulting pellet in an identical volume of fresh ice-cold Tris-HCl buffer. The final pellet was resuspended to 18 ml of Tris-HCl buffer g^{-1} original wet tissue weight and filtered over 2 × 4 layers of cheesecloth. A 900 μl aliquot of this membrane suspension, the equivalent of 50 mg original wet tissue weight, was incubated with [^{3}H]-prazosin (specific activity 30 Ci $mmol^{-1}$, DuPont de Nemours/NEN Products, Boston, MA, U.S.A.) in a final volume of 1 ml Tris-HCl buffer (pH 7.5) in the presence or absence of competing drugs. Following a 30 min incubation at 25°C, membranes were recovered from the homogenate by vacuum filtration over Whatman GF/B filters and washed with three 5 ml aliquots of ice-cold 50 mM Tris-HCl buffer. Filter-retained radioactivity was quantified by liquid scintillation spectrometry at an efficiency of 40–45%. Specific [^{3}H]-prazosin binding was defined as the decrease in the amount of filter-retained radioactivity after incubation in the presence of 100 μM phentolamine. The abilities of alfuzosin, prazosin and a variety of other reference compounds to displace [^{3}H]-prazosin binding were studied at a radioligand concentration of 0.5 nM and expressed as an IC_{50} value (concentration providing 50% inhibition of specific binding).

Functional responses to α_1- and α_2-adrenoceptor stimulation in pithed rats

Effects on postjunctional α_1- and α_2-adrenoceptor mediated vasoconstriction Male rats (250 g) were anaesthetized with sodium pentobarbitone (55 mg kg^{-1}, i.p.), pithed and placed under artifical respiration. Blood pressure and heart rate were measured from the carotid artery and recorded on a polygraph. The compounds were administered via a femoral vein. Pressor responses to cumulative doses of cirazoline (α_1-adrenoceptor agonist: 0.125 to 128 μg kg^{-1}, i.v.) were studied 5 min after a 5 min i.v. infusion of alfuzosin (0.03, 0.1 and 0.3 mg kg^{-1}), prazosin (0.01, 0.03 and 0.1 mg kg^{-1}) or solvent (isotonic NaCl solution: 0.4 ml kg^{-1}). In animals of a second group receiving alfuzosin (0.3 and 1.0 mg kg^{-1}), prazosin (0.1 and 0.3 mg kg^{-1}) or solvent intravenously during a 5 min period, the pressor effects of UK-14,304 (α_2-adrenoceptor agonist: 0.31 to 1280 μg kg^{-1}, i.v. dosed cumulatively) were studied 5 min after each treatment. Each agonist dose was injected when the maximal pressor effect of the preceding dose was reached. Dose-response curves were constructed and the doses of agonist producing a 50 mmHg increase in mean blood pressure (ED_{50} values) were determined in the presence of antagonist or solvent.

Effects on prejunctional α_2-adrenoceptor stimulation Animals were prepared as described above. The thoracic (C_6–T_5) spinal cord was continuously stimulated at low-frequency (0.1–0.2 Hz, 60 V, 0.5 ms) in order to induce a sustained increase in heart rate of about 100 beats min^{-1}. The metal pithing rod was varnished except for 1 cm approximately 6 cm from the caudal extremity. Cumulative doses of the prejunctional α_2-adrenoceptor agonist UK-14,304 (0.125 to 256 μg kg^{-1}, i.v.) were administered; each dose was injected when the maximal effect of the previous one had been attained. The inhibitory dose-response curve to UK-14,304 on experimental sympathetic tachycardia was determined for animals pretreated with alfuzosin (1.0 mg kg^{-1}), prazosin (0.3 mg kg^{-1}), idazoxan (0.3 mg kg^{-1}) or solvent (isotonic NaCl); these drugs were administered by a 5 min intravenous infusion. Results are expressed as mean ± s.e.mean and significance was determined by Student's *t* test for unpaired series.

Measurement of urethral pressure elevated by sympathetic nerve stimulation or phenylephrine infusion in anaesthetized cats

Sympathetic nerve stimulation Adult cats of either sex weighing 2.5–3.5 kg were anaesthetized with sodium pentobarbitone (42 mg kg^{-1}, i.p. + 6 mg h^{-1}, i.v. throughout the duration of the experiment) and artificially ventilated with room air. A catheter was introduced into the brachial artery for measurement of arterial pressure and heart rate using a cardiotachometer triggered by the aortic pulse wave. A catheter was introduced into the bladder initially at the level of the trigone and subsequently advanced into the first half of the urethra for measurement of intraurethral pressure after ligature of the bladder neck. Catheters were placed in the cephalic and femoral veins for drug administration. Cardiovascular and urological parameters were recorded with a polygraph. The right hypogastric nerve was isolated and prepared for electrical stimulation (5 V; 20 Hz; 2 ms; 15 s). In order to eliminate autonomic neural activity other than that evoked through α-adrenoceptors, all animals were treated with chlorisondamine (0.5 mg kg^{-1}, i.v.), atropine (0.5 mg kg^{-1}, i.v.) and propranolol (0.75 mg kg^{-1}, i.v.). Alfuzosin was administered in cumulative doses (1–1000 μg kg^{-1}, i.v., using dose steps of 1, 3, 10, 20, 55, 100, 300, 500 and 1000 μg kg^{-1} each dose being given over 5 min), and its effects upon smooth muscle contraction triggered by electrical stimulation of the hypogastric nerve were studied 5 min after each dose. Comparisons were made with prazosin (cumulative doses: 1–300 μg kg^{-1}, i.v. using the same dose steps for alfuzosin) or solvent (5% glucose). Previous studies had shown that the activity of alfuzosin or prazosin was maximal 5 min after treatment. The doses of α-antagonist necessary to cause 50% inhibition of urethral hypertonia were determined (ED_{50}).

In a separate series of experiments alfuzosin (0.1–3 mg kg^{-1}) was administered via the intraduodenal route and its effects on elevated intraurethral pressure monitored during the 3 h period which followed drug administration.

Phenylephrine infusion Anaesthetized male cats (3–4.5 kg) were prepared for recording blood pressure and urethral pressure as described above. Urethral pressure and arterial

blood pressure were elevated in a sustained fashion by continuous i.v. infusion of phenylephrine (9–12 $\mu g\ kg^{-1}\ min^{-1}$). Once the preparation was stable, alfuzosin (1–1000 $\mu g\ kg^{-1}$, i.v.), prazosin (1–1000 $\mu g\ kg^{-1}$, i.v.) or vehicle (5% glucose) was administered cumulatively, doses being added once the effects of the previous dose had reached maximum. The ED_{50} and ED_{80} values (doses of antagonist necessary to produce 50% or 80% inhibition of urethral pressure and blood pressure) were calculated by linear regression analysis. This procedure involved fitting a straight line to data points in the linear region of each dose-response curve, checking for linearity, deriving the appropriate regression equation and using this equation to calculate the doses which corresponded to each of the set response levels (50% or 80% inhibition). In a separate series of experiments using the same phenylephrine infusion protocol, alfuzosin was administered as a single intraduodenal dose (0.03 or 0.1 $mg\ kg^{-1}$) and its effects monitored for 60 min following administration.

Drugs used

Alfuzosin hydrochloride, (+)-alfuzosin hydrochloride, (−)-alfuzosin hydrochloride, prazosin hydrochloride, cirazoline, idazoxan hydrochloride and UK-14,304 (5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline) were synthesized in the Department of Chemistry, Synthélabo Recherche. Sources of other drugs used were as follows: phenylephrine hydrochloride, noradrenaline bitartrate, yohimbine hydrochloride, papaverine hydrochloride (Sigma); chlorisondamine chloride, phentolamine hydrochloride (Ciba-Geigy); atropine sulphate (Prolabo); propranolol (ICI); phenoxybenzamine hydrochloride (SK&F); pentobarbitone sodium (Sanofi).

Results

Rabbit isolated trigone and urethra

The selective α_1-adrenoceptor agonist, phenylephrine, caused concentration-dependent contractions of the isolated trigone and urethra and was virtually equipotent in both preparations. The pD_2 (− log molar EC_{50}) for phenylephrine was 4.9 ± 0.07 in the urethra ($n = 26$) and 5.2 ± 0.07 in the trigone ($n = 26$).

Alfuzosin (0.1–3 μM) or prazosin (0.03–3 μM) caused significant parallel rightward shifts of the phenylephrine concentration-response curve in both trigone and urethra. The maximum contractile response to phenylephrine was unaffected by the presence of alfuzosin or prazosin, except at the highest concentration of each where a reduction of maximum of approximately 20%–30% was observed.

The α_1-adrenoceptor antagonist potencies of alfuzosin and prazosin were evaluated by Schild analysis and the pA_2 values are shown in Table 1. The slopes of the Schild plots were not significantly different from unity, indicating that both drugs were apparently competitive antagonists against phenylephrine-induced responses in these preparations.

Table 2 Pharmacological profile of [^{3}H]-prazosin binding to cranial human prostate adenoma

Drug	IC_{50} (μM)
Prazosin	0.004 ± 0.001
Phentolamine	0.018 ± 0.001
Alfuzosin	0.035 ± 0.008
(+)-Alfuzosin	0.023 ± 0.002
(−)-Alfuzosin	0.019 ± 0.006
Phenoxybenzamine	0.038 ± 0.016
Idazoxan	3.5 ± 1.0
Yohimbine	6.0 ± 2.0
Phenylephrine	7.0 ± 1.5
UK 14,304	36 ± 14
Propranolol	37 ± 9
Papaverine	60 ± 15
Noradrenaline	1.1 ± 0.46

Drug concentrations producing a 50% inhibition of specific [^{3}H]-prazosin binding (IC_{50}) are shown as the mean ± s.e. of at least three experiments.

Both enantiomers of alfuzosin also inhibited responses to phenylephrine in trigone and urethra with a potency similar to that of racemic alfuzosin (Table 1).

[^{3}H]-prazosin binding in human prostatic adenomyofibroma tissue

Using a radioligand concentration-range of 0.01–10 nM non-specific [^{3}H]-prazosin binding, determined in the presence of 100 μM phentolamine, increased linearly as a function of the radioligand concentration. Specific [^{3}H]-prazosin binding, in contrast, increased non-linearly with the radioligand concentration, and at 0.8 nM [^{3}H]-prazosin specific binding represented approximately 55% of the total amount of [^{3}H]-prazosin retained by the filter. Non-linear regression analysis indicated that the data are adequately described by a model of [^{3}H]-prazosin binding to a single homogeneous class of binding sites ($K_D = 0.17 \pm 0.02$ nM) with a maximal binding capacity (B_{max}) of 41 ± 3 $fmol\ mg^{-1}$ protein ($n = 18$).

As shown in Table 2, [^{3}H]-prazosin binding was inhibited with high affinity by the specific α_1-adrenoceptor antagonists, prazosin, alfuzosin and both enantiomers of alfuzosin but with only low affinity by idazoxan, a selective α_2-antagonist. The relative potencies of various other commonly used adrenoceptor ligands are shown in Table 2 and confirm that [^{3}H]-prazosin labels the α_1-adrenoceptor in this model.

α_1 and α_2-adrenoceptor mediated responses in pithed rats

Effects on postjunctional α_1- and α_2-adrenoceptor mediated vasoconstriction Cirazoline caused dose-related increases in mean blood pressure with an ED_{50} value of 0.9 ± 0.05 $\mu g\ kg^{-1}$, i.v., in the presence of solvent. Figure 1 shows that both alfuzosin and prazosin produced marked parallel right-

Table 1 Antagonist potency of alfuzosin, its enantiomers and prazosin against phenylephrine-induced contractions of the rabbit trigone and urethra preparations

	Trigone			*Urethra*		
Antagonist	pA_2	*Slope*	n	pA_2	*Slope*	n
Alfuzosin	7.44 (7.25–7.69)	0.87	24	7.30 (7.04–7.75)	0.78	17
(+)-Alfuzosin	7.74 (7.44–8.55)	0.76	7	7.77 (7.53–8.24)	0.74	8
(−)-Alfuzosin	7.55 (7.47–7.63)	–	5	7.69 (7.52–7.86)	–	5
Prazosin	7.88 (7.33–8.43)	1.13	9	7.96 (7.11–8.81)	1.17	7

pA_2 values are shown with 95% confidence limits. None of the slopes differed significantly from 1. n = number of dose-ratios.

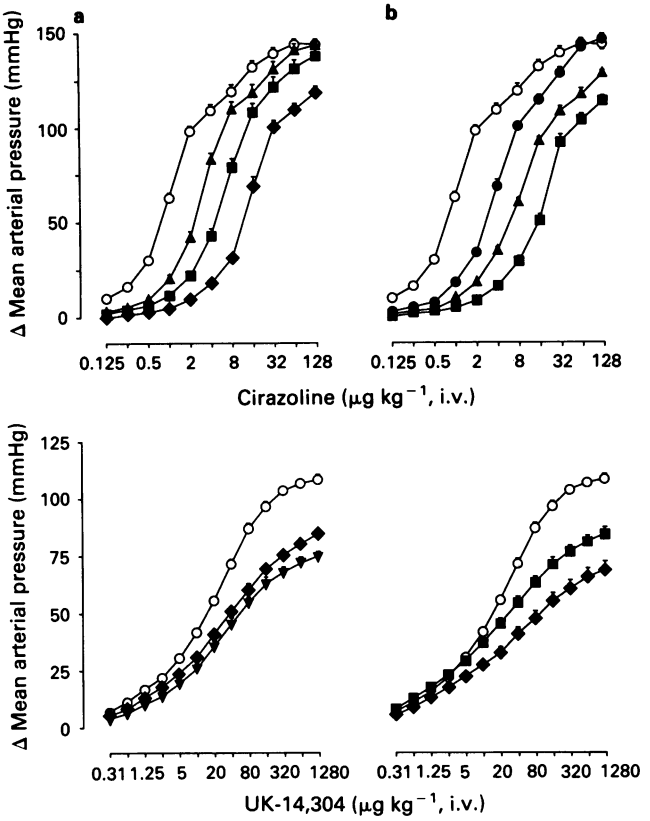


Figure 1 Effects of (a) alfuzosin and (b) prazosin on pressor responses induced by the selective α_1-agonist cirazoline (upper panel) and the selective α_2-agonist UK 14,304 (lower panel) in pithed rats. Responses were obtained under control conditions (○) and following antagonist doses of 0.01 (●), 0.03 (▲), 0.1 (■), 0.3 (◆) or 1 mg kg^{-1}, i.v. (▼). Values shown are means and s.e.mean (n = 5–7).

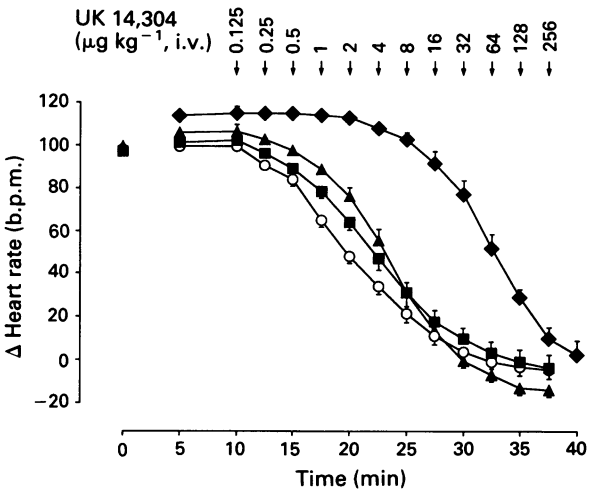


Figure 2 Effects of alfuzosin 1.0 mg kg^{-1}, i.v. (▲), prazosin 0.3 mg kg^{-1}, i.v. (■), idazoxan 0.3 mg kg^{-1}, i.v. (◆) and vehicle (○) on the inhibitory dose-response curve to the selective α_2-agonist, UK 14,304, against tachycardia produced by sustained stimulation of sympathetic nerves in the pithed rat. Electrical stimulation of thoracic spinal cord was performed continuously between − 10 and + 40 min. UK 14,304 was administered as cumulative i.v. bolus doses (0.125–256 μg kg^{-1}) at 2.5 min intervals commencing at + 10 min. Antagonists or vehicle were administered between 0 and + 5 min. Values shown are means ± s.e.mean (n = 4–5).

ward displacements of the cirazoline curve in a dose-related fashion at all antagonist doses tested. The maximum response to cirazoline appeared unchanged except possibly at the highest dose of each antagonist. The minimum effective antagonist doses were $\leqslant$ 0.03 mg kg^{-1}, i.v. for alfuzosin and $\leqslant$ 0.01 mg kg^{-1}, i.v. for prazosin. To quantitate antagonist potency we have calculated in each case the mean antagonist dose necessary to produce a 10 fold increase in the ED_{50} value of cirazoline (DR_{10} value). The DR_{10} values for alfuzosin and prazosin were 0.22 mg kg^{-1} and 0.043 mg kg^{-1}, respectively indicating that alfuzosin was approximately 5 times less potent than prazosin.

To establish the selectivity of alfuzosin and prazosin for postjunctional α_1-receptors, high concentrations of each antagonist were tested against pressor responses produced by the α_2-selective agonist, UK-14,304 (Figure 1). At antagonist doses which exceeded the DR_{10} doses versus cirazoline, alfuzosin (0.3–1 mg kg^{-1}, i.v.) and prazosin (0.1–0.3 mg kg^{-1}, i.v.) produced only slight antagonism of the UK-14,304 response curve with some depression of maximum. Dose ratios (DR) calculated against UK-14,304 using the ED_{50} values were 2.6 ± 0.3 and 3.6 ± 0.4 for alfuzosin at 0.3 and 1.0 mg kg^{-1} and 2.2 ± 0.3 and 6.7 ± 1.0 following prazosin 0.1 and 0.3 mg kg^{-1}, respectively.

Effects on cardiac prejunctional α_2-adrenoceptor stimulation Low frequency continuous sub-maximal stimulation of sympathetic nerves innervating the cardiac region caused a sustained increase in heart rate (101 ± 1 beats min^{-1}, n = 19) which was stable for the duration of the experimental procedure.

Cumulative administration of UK-14,304 produced a dose-related inhibition of sympathetically-induced tachycardia which was complete at a dose between 32–64 μg kg^{-1}, i.v. Figure 2 shows that the selective α_2-antagonist idazoxan (0.3 mg kg^{-1}, i.v.) elevated heart rate by approximately 15 beats min^{-1} and markedly antagonized the anti-accelerator responses to UK-14,304, the agonist dose-response curve being displaced to the right approximately 20 fold. In contrast, neither prazosin (0.3 mg kg^{-1}, i.v.) nor alfuzosin (1 mg kg^{-1}, i.v.) significantly altered responsiveness to UK-14,304. ED_{50} values for UK-14,304 were 1.27 ± 0.23 μg kg^{-1} (vehicle), 2.15 ± 0.4 μg kg^{-1} after prazosin, 2.25 ± 0.39 μg kg^{-1} after alfuzosin and 27.11 ± 4.3 μg kg^{-1} after idazoxan. Only the idazoxan value was significantly different from vehicle ($P < 0.05$).

Elevated urethral pressure in the anaesthetized cat

Sympathetic nerve stimulation Stimulation of the hypogastric sympathetic nerves caused reproducible increases in urethral pressure within the range of 30–45 cmH_2O which did not differ significantly between groups of animals. None of the treatments significantly changed basal blood pressure during the course of the experiments. Pre- and post-treatment values in mmHg in the three groups were, vehicle 74 ± 4, 72 ± 2, alfuzosin 76 ± 6, 70 ± 5, prazosin 68 ± 2, 64 ± 3.

Intravenous administration of both alfuzosin and prazosin (Figure 3) produced dose-related inhibition of sympathetically mediated increases in urethral pressure, whereas the solvent was without significant effect. ED_{50} values for alfuzosin and prazosin were 0.077 ± 0.003 mg kg^{-1} and 0.016 ± 0.001 mg kg^{-1}, respectively, showing that alfuzosin was approximately 5 times less potent than prazosin. The α_2-antagonist, yohimbine (0.3 mg kg^{-1}, i.v.) did not modify the urethral response to sympathetic nerve stimulation (data not shown).

When given by the intraduodenal route alfuzosin (0.1–3 mg kg^{-1}) produced sustained dose-related inhibition of the increases in urethral pressure (Figure 3) with an ED_{50} of 0.36 ± 0.06 mg kg^{-1}. The inhibitory effect was rapid in onset, reached maximum within 45 min–1 h after dosing and was still evident at 3 h.

Phenylephrine infusion The infusion of phenylephrine produced sustained increases in arterial blood pressure and

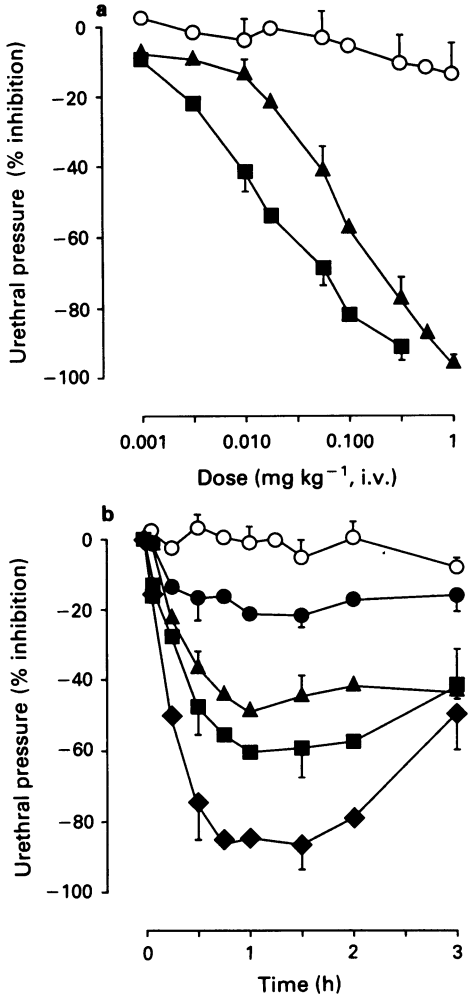


Figure 3 (a) Effects of cumulative i.v. bolus doses of alfuzosin (▲), prazosin (■) or vehicle (○) on the increase in urethral pressure induced by electrical stimulation of sympathetic hypogastric nerves in the anaesthetised cat. Values shown are means ± s.e.mean (n = 6–13). (b) In the same model, time course of inhibition of urethral pressure produced by single intraduodenal administrations of alfuzosin 0.1 (●), 0.3 (▲), 1 (■) or 3 mg kg^{-1} (◆), or vehicle (○). Values shown are means ± s.e.mean (n = 4–5).

urethral pressure. The respective increases observed for each parameter were 127.3 ± 4.4 mmHg and 28.1 ± 2.7 cmH_2O (n = 15, i.v. series) and 126.5 ± 2.9 mmHg and 30.4 ± 1.8 cmH_2O (n = 16, intraduodenal series).

In the i.v. antagonist studies the solvent did not lead to any significant changes in urethral or blood pressures (Figure 4). However, alfuzosin and prazosin caused dose-related decreases in both parameters. Whereas prazosin was approximately equipotent against urethral and vascular stimuli, alfuzosin demonstrated approximately 3–5 fold higher potency against elevated urethral pressure (ED_{50} and ED_{80} response levels) when compared with the arterial pressure response. In addition, alfuzosin but not prazosin inhibited urethral pressure by >100% i.e. to a greater extent than the original stimulation evoked by phenylephrine. The ED_{50} values (with 95% confidence limits) for urethral pressure were 0.031 (0.028–0.033) mg kg^{-1} and 0.014 (0.011–0.016) mg kg^{-1}, and for arterial pressure, 0.079 (0.068–0.090) mg kg^{-1} and 0.018 (0.016–0.020) mg kg^{-1}, for alfuzosin and prazosin respectively. The corresponding ED_{80} values for urethral pressure were 0.106 (0.101–0.110) mg kg^{-1} and 0.053 (0.041–0.081) mg kg^{-1}, and for arterial pressure, 0.465 (0.362–0.660) mg kg^{-1} and 0.091 (0.071–0.128) mg kg^{-1}, for alfuzosin and prazosin, respectively. Thus alfuzosin was only two times less potent than prazosin against urethral hypertonia but was five times weaker than prazosin for effects on arterial pressure. Statistical analysis indicated that the preferential effect of alfuzosin on urethral pressure, relative to arterial pressure, was significant (P<0.01, three way analysis of variance).

For intraduodenal studies with alfuzosin (Figure 5) the duration of the experiments was limited to the 60 min period following dosing due to a tendency for urethral and arterial pressures to decline in the control group. Alfuzosin (0.03 and 0.1 mg kg^{-1} intraduodenally) caused a rapid and marked decrease in elevated urethral pressure reaching a plateau 30–40 min after dosing. In contrast, the changes in arterial pressure following 0.03 mg kg^{-1} alfuzosin were similar to those observed in the control group and, at the higher dose (0.1 mg kg^{-1}), alfuzosin produced only slight inhibition. Hence, given by the intraduodenal route alfuzosin demonstrated a potent and relatively selective inhibition of phenylephrine-induced elevation of urethral pressure.

Discussion

The data presented show conclusively that alfuzosin is a potent competitive antagonist of α_1-adrenoceptors in the

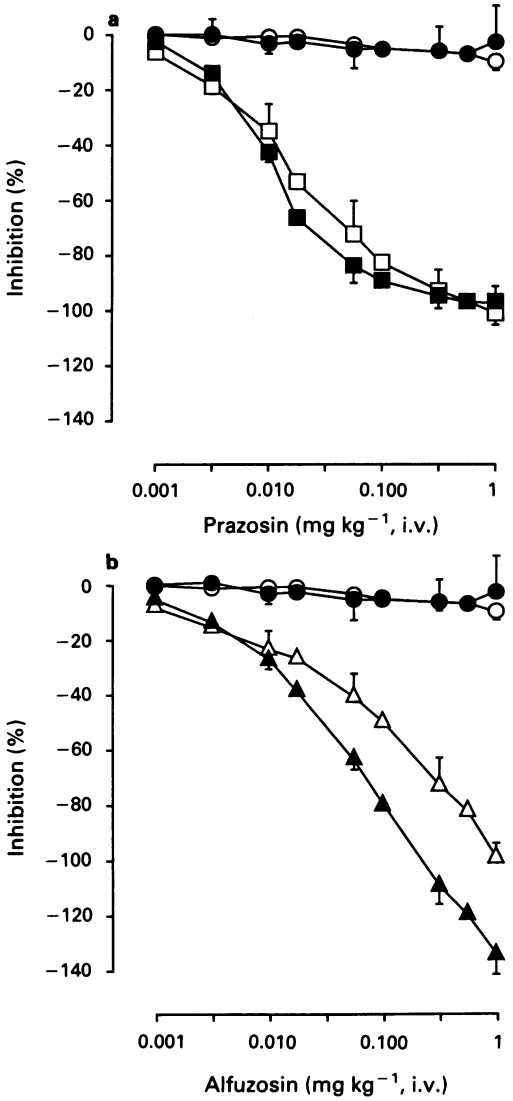


Figure 4 Anaesthetized cat preparations having arterial blood pressure (open symbols) and urethral pressure (filled symbols) elevated by continuous i.v. infusion of the α_1-agonist, phenylephrine. Percentage inhibition of these parameters by cumulative i.v. bolus doses of prazosin (■, □, a), alfuzosin (▲, △, b) or vehicle (●, ○). Values shown are means ± s.e.mean (n = 4–6).

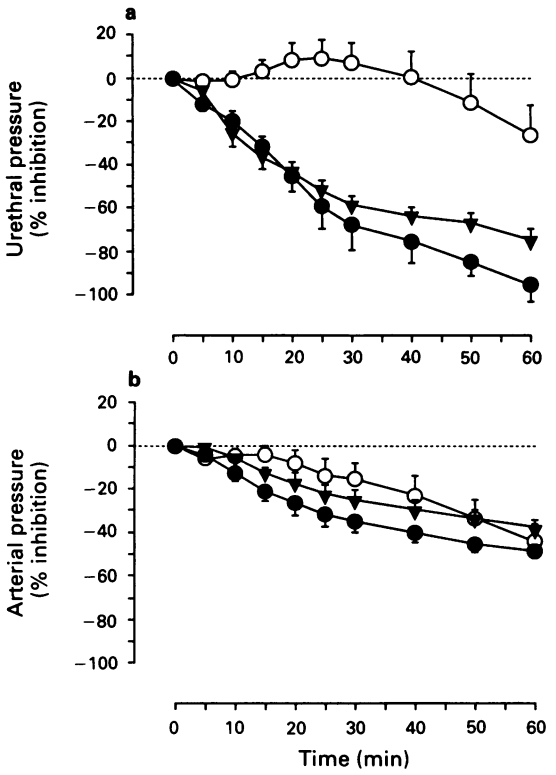


Figure 5 Anaesthetized cat preparations having urethral pressure (a) and arterial blood pressure (b) elevated by continuous i.v. infusion of the α_1-agonist, phenylephrine. Time course of the inhibitory effects of these two parameters of intraduodenal administration of alfuzosin 0.03 mg kg^{-1} (▼), 0.1 mg kg^{-1} (●) or its vehicle (○). Values shown are means ± s.e.mean ($n = 5-6$).

lower urinary tract. In this respect, its profile was similar to that of prazosin although alfuzosin was in general slightly less potent. The affinity of alfuzosin for α_1-receptors in the lower urinary tract is of the same order as that reported for α_1-receptors in other isolated tissues, e.g. rabbit pulmonary artery: pA_2 7.47 versus phenylephrine, displacement of [^{3}H]-prazosin binding in rat cerebral cortex: IC_{50} 15 nM (Cavero *et al.*, 1984a). Ligand binding and functional studies demonstrate that the α_1-antagonist properties of alfuzosin reside equally in its two enantiomers. This absence of stereoselectivity distinguishes alfuzosin from certain other α_1-antagonists, for example IP66 (Manzini *et al.*, 1991) and YM-12617 (Yamada *et al.*, 1987).

The separation of α-adrenoceptors into α_1- and α_2-subtypes (Langer, 1974) and the subsequent extension of this classification to allow for the presence of α_2-receptors at both postjunctional and prejunctional sites (Langer *et al.*, 1980) is well recognised. Taken in the context of the treatment of BPH the α_1/α_2 selectivity ratio may be a significant factor in determining clinical utility amongst α-antagonists. Hence, since functional sympathetic tone to the smooth muscle of the bladder trigone, urethra and prostatic stroma in man is largely or exclusively mediated via α_1-receptors (Kunisawa *et al.*, 1985; Hieble *et al.*, 1985) there would be little additional advantage to be gained by simultaneously blocking postjunctional α_2-receptors. Furthermore, antagonism of prejunctional α_2-receptors on sympathetic nerve terminals would inhibit negative feedback by neurotransmitter noradrenaline thus augmenting neurotransmitter release. This would tend to counteract the beneficial effects of postjunctional α_1-blockade and exacerbate sympathetically mediated bladder outflow obstruction. Although these theoretical arguments favour the use of an α_1-selective antagonist in BPH, it has to be recognised that the non-selective α-blockers, phentolamine and phenoxybenzamine, have been reported to show clinical efficacy in this condition although neither is a drug of choice (Christmas & Kirby, 1991). The present results demonstrate that, in *in vivo* functional studies, alfuzosin shows a high selectivity for α_1- versus α_2-receptors. In a direct comparison of its ability to block postjunctional α_1- and α_2-mediated responses in the pithed rat, alfuzosin was approximately 30 fold more potent as an antagonist of cirazoline-induced pressor responses by comparison with those produced by the α_2-selective agonist UK-14,304. In addition, since the α_2-selectivity of UK-14,304 is not absolute (Beckeringh *et al.*, 1984) it is conceivable that the weak inhibitory effects of alfuzosin, which were most evident at high doses of UK-14,304, actually reflect an α_1-agonist component in the vasopressor response. Prazosin, considered to have a large selectivity margin between α_1- and α_2-receptors, showed a similar profile in this model. Prejunctional α_2-antagonist properties were evaluated by testing alfuzosin and prazosin against UK-14,304-induced antiaccelerator effects in the presence of a tachycardiac response to cardiac sympathetic nerve stimulation. Although this response was highly sensitive to the α_2-antagonist idazoxan, alfuzosin was without significant inhibitory effect at 1 mg kg^{-1}, i.v., a dose 300 times greater than its threshold α_1-antagonist dose based on inhibition of urethral hypertonia in anaesthetized cats using phenylephrine as agonist.

Using [^{3}H]-prazosin as a selective radioligand for the α_1-adrenoceptor, we have identified high affinity [^{3}H]-prazosin recognition sites in the cranial region of the human prostatic adenoma that display the characteristics expected of the α_1-adrenoceptor. Thus, [^{3}H]-prazosin binding was potently inhibited by the α_1-adrenoceptor antagonists, alfuzosin, prazosin and phentolamine, with IC_{50} values in the low nanomolar range. In contrast, the α_2-adrenoceptor antagonists, idazoxan and yohimbine, or the β-adrenoceptor antagonist, propranolol, only affect [^{3}H]-prazosin binding with IC_{50} values in the micromolar range. Our data thus confirm other reports (Lepor & Shapiro, 1984; Yamada *et al.*, 1987) demonstrating specific α_1-adrenoceptor binding sites in the human prostate, the 'target tissue' in BPH, and demonstrates that alfuzosin shows high affinity for such sites. There is also evidence that the number of α_1-receptors in the abnormal prostate (BPH) is higher than that found in normal prostatic tissue (Yamada *et al.*, 1987) possibly as a direct result of hypertrophy of the muscular stroma, although other studies do not support this (Gup *et al.*, 1990). Studies in isolated prostate smooth muscle preparations demonstrate that the maximum contractile response to the α_1-agonist, phenylephrine, is doubled in tissues from BPH patients (Kitada & Kumazawa, 1987). A recent report shows that alfuzosin-displaceable [^{125}I]-HEAT binding sites (α_1-receptor) are exclusively associated with the muscular stroma of the prostate and are globally elevated in sections from prostatic adenomas as compared with normal prostate tissue (Benavides *et al.*, 1991).

In vivo experiments in the anaesthetized cat confirm the α_1-antagonist properties of alfuzosin. The compound produced dose-related and complete inhibition of the rise in urethral pressure resulting from postganglionic stimulation of hypogastric sympathetic nerves. This can be considered to represent an animal model of the dynamic sympathetic constriction of urethral smooth muscle which is regarded as a contributory factor in the micturition disorders which characterize BPH. Furthermore, as in man, the urethral hypertonic response in the cat is mediated exclusively by stimulation of α_1-receptors since it was completely inhibited by prazosin but unaffected by the α_2-antagonist yohimbine. Alfuzosin was also effective by the intraduodenal route producing dose-related and prolonged inhibition of urethral responses.

We were interested to establish the relationship between the ability of alfuzosin to inhibit sympathetically-mediated increases in urethral tone and its effect on sympathetically-mediated vasoconstriction *in vivo* since both responses involve activation of postjunctional α_1-receptors. Such comparsions may be relevant to the therapeutic margin of α_1-antagonists

in BPH with respect to unwanted vascular effects (e.g. orthostatic hypotension). In view of the technical difficulties involved in stimulating both vascular and urethral sympathetic nerves simultaneously in the same animal in an equal fashion, we designed, as an alternative, experiments using i.v. infusion of the α_1-agonist phenylephrine. Phenylephrine infusion caused sustained elevations of urethral pressure and arterial blood pressure. The dose of phenylephrine was selected with a view to producing responses of the two parameters in question which were as large as possible although still submaximal and within the linear region of the dose-response curve. Arterial pressure was slightly more sensitive to phenylephrine than urethral pressure ($\simeq$80% and $\simeq$65% of their respective maxima). These α_1-agonist urethral and vascular stimuli were equally sensitive to prazosin which produced dose-related and complete inhibition of both parameters with similar potency. In addition, the inhibitory potency of prazosin against phenylephrine induced increases in urethral pressure was virtually identical to its potency in inhibiting urethral hypertonic response to sympathetic nerve stimulation (ED_{50} values 0.014 mg kg^{-1}, i.v. and 0.016 mg kg^{-1}, i.v., respectively).

Alfuzosin also caused dose-related inhibition of phenylephrine-induced responses consistent with its α_1-antagonist properties; however, in contrast to prazosin, certain observations did not fit the expected pattern. In the first place, alfuzosin was moderately but significantly more potent in inhibiting the urethral hypertonic response compared with the pressor response. This separation between urethral and vascular indices in the presence of phenylephrine appeared at low doses of alfuzosin $\simeq$20 μg kg^{-1} and increased progressively with dose to give a uroselectivity ratio of $\simeq$5 at the 80% response level. Interestingly, alfuzosin but not prazosin decreased urethral pressure by >100%, i.e. by an amount greater than the total tone induced with phenylephrine. Alfuzosin was approximately three times more potent against urethral responses to phenylephrine than against those to sympathetic nerve stimulation. The ability of alfuzosin to inhibit selectively the urethral response to phenylephrine was even more evident when the compound was given by the intraduodenal route. Although the stability of this preparation did not allow the full time course of each response to be monitored, at maximum effect (achieved within 30–40 min of dosing) alfuzosin caused a marked inhibition of elevated urethral pressure but had little effect on the phenylephrine pressor response. It is noteworthy that these effects were observed at relatively low doses of alfuzosin (30 and 100 μg kg^{-1} intraduodenally) and that alfuzosin was approximately 10 times more potent in this series of experiments than when tested against nerve stimulation-induced increases in urethral pressure using the same route of administration.

A number of possible explanations for this preferential effect of alfuzosin on urethral responses to phenylephrine can be considered. It is now recognised that multiple subtypes of the α_1-receptor exist (α_{1A}, α_{1B}, α_{1C}, α_{1D} – Lomasney *et al.*, 1991; Faure-Halley *et al.*, 1992) although the functional relevance of these subtypes in terms of contraction of urethral and vascular smooth muscle is far from being fully understood. However, since alfuzosin, like prazosin, does not show marked selectivity for any particular α_1-subtype (D. Graham, C. Pimoule and H. Schoemaker, unpublished observations) this is not likely to be a significant factor. Although the presence of an additional relaxant mechanism in alfuzosin cannot be entirely excluded, testing to date suggests that the compound is a selective α_1-antagonist. In unpublished studies, alfuzosin shows no affinity for histamine, muscarinic, β-adrenoceptor, dopamine, 5-HT_1, 5-HT_2 or benzodiazepine receptors *in vitro* at concentrations up to 10^{-5} M and does not inhibit vasopressor responses to angiotensin, vasopressin or 5-HT in the pithed rat after the high dose of 3 mg kg^{-1}, i.v. Weak affinity for calcium channels (IC_{50} 4.5×10^{-5} M in [^{3}H]-diltiazem binding) and even weaker spasmolytic activity (IC_{50} 9×10^{-4} M in depolarized rabbit aorta) have been identified. However, since these effects occur at concentrations of alfuzosin at least 1000 times greater than those necessary to block α_1-receptors, it is difficult to believe that they could contribute over the dose-range used in the studies performed in the anaesthetized cat. *In vitro*, the α_1-antagonist potency of alfuzosin against phenylephrine is similar in vascular and urinogenital smooth muscle preparations. Thus, the uroselectivity shown by alfuzosin in the cat may therefore be a reflection of an *in vivo* phenomenon. In this context, it is interesting to note that pharmacokinetic studies in the rat have demonstrated that alfuzosin is preferentially distributed in the prostate gland after systemic administration giving a prostate: plasma concentration ratio of $\simeq$3 fold. Therefore, tissue distribution could influence the *in vivo* pharmacological profile of alfuzosin. Clearly, additional studies will be necessary to develop a fuller understanding of these observations including the difference noted between alfuzosin and prazosin. Other relevant examples of *in vivo* differences between alfuzosin and prazosin are reports that alfuzosin causes a lesser effect on the orthostatic reflex in conscious dogs (Cavero *et al.*, 1984b) and is much less potent as an acute antihypertensive agent in spontaneously hypertensive rats (Lefèvre-Borg *et al.*, 1992).

In summary, the data presented demonstrate that alfuzosin is a potent and selective α_1-adrenoceptor antagonist in the lower urinary tract *in vitro* and *in vivo* and has a high affinity for the α_1-receptor of the human prostate. Since these represent the key target tissues in BPH, there is a good pharmacological rationale for expecting alfuzosin to alleviate the dynamic component of urinary obstruction attributable to sympathetic tone in this condition. Clinical studies confirm the efficacy of alfuzosin in BPH and suggest that it is well tolerated with respect to vascular side effects.

We are grateful to Drs A. Jardin and H. Bensadoun (Hôpital Pitié Salpétrière, Paris) and Dr M.O. Bitker (Hôpital du Kremlin-Bicêtre, Paris) for their assistance in obtaining the human prostate tissue samples.

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(Received January 25, 1993
Revised April 15, 1993
Accepted April 19, 1993)

British Journal of Pharmacology

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The *British Journal of Pharmacology* is published monthly by the Scientific & Medical Division, Macmillan Press Ltd. The journal is covered by *Current Contents*, *Excerpta Medica* and *Index Medicus*.

All business correspondence and reprint requests should be addressed to the Scientific & Medical Division, Macmillan Press Ltd., Houndmills, Basingstoke, Hampshire RG21 2XS, UK. Telephone: (0256) 29242; Fax: (0256) 810526.

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Overseas subscribers may make payments into UK Post Office Giro Account No. 5192455. Full details must accompany the payment.

British Journal of Pharmacology (ISSN 0007–1188) is published monthly by Macmillan Publishers Ltd, c/o Mercury Airfreight International Ltd, 2323 Randolph Avenue, Avenel, NJ 07001, USA. Subscription price is $923.00 per annum. 2nd class postage is paid at Rahway NJ. *Postmaster*: send address corrections to Macmillan Publishers, c/o Mercury Airfreight International Ltd, 2323 Randolph Avenue, Avenel NJ 07001.

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Typeset by Elite Typesetting Techniques, Southampton, Hampshire
and printed in Great Britain by Unwin Brothers Ltd., The Gresham Press, Old Woking, Surrey

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