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Possible action of cyclopiazonic acid on myocardial sarcoplasmic reticulum: inotropic effects on neonatal and adult rat heart

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Cyclopiazonic acid (CPA), a mycotoxin from *Aspergillus* and *Penicillium*, has been described as a highly selective inhibitor of Ca^{2+}-ATPase in the sarcoplasmic reticulum (SR) in skeletal and smooth muscles but no reports at present deal with the effect of CPA in cardiac muscle. In the present study, we examined the inotropic effect of CPA on adult and neonatal rat myocardia, the contractions of which are known to be highly dependent on Ca^{2+}-release from the sarcoplasmic reticulum and trans-sarcolemmal Ca^{2+}-influx, respectively. CPA (30 μM) produced a negative inotropic effect in adult preparations, accompanied by marked prolongation of the contraction duration. In contrast, CPA had minimum effects on neonatal myocardium. Thus we have demonstrated that CPA exerts negative inotropic effects on adult myocardium probably through inhibition of SR function.

Keywords: Cyclopiazonic acid; myocardium; inotropism; rat neonate

Introduction Cyclopiazonic acid (CPA) is a mycotoxin from *Aspergillus* and *Penicillium*, the effects of which have been examined in skeletal and smooth muscles. CPA is considered to be a specific inhibitor of Ca^{2+}-ATPase in the sarcoplasmic reticulum (SR) based on experiments with SR vesicles (Goeger *et al.*, 1988), intact smooth muscle (Bourreau *et al.*, 1991) and skinned muscle fibres from skeletal (Kurebayashi & Ogawa, 1991) and smooth (Ueyama *et al.*, 1992) muscles. Although a specific inhibitor of SR Ca^{2+}-ATPase would be a useful pharmacological tool to study the mechanism of kinetics of SR Ca^{2+}-uptake and storage not only in skeletal and smooth muscles but also in cardiac muscles, no reports at present deal with the effect of CPA in cardiac muscle.

The contraction of mammalian myocardium is known to be dependent on both trans-sarcolemmal Ca^{2+}-influx and intracellular Ca^{2+}-release from the SR. The relative importance of the two calcium sources varies among species and the developmental stage of the animal (Fabiato & Fabiato, 1978). Physiological and pharmacological studies indicate that Ca^{2+}-release from the SR plays an especially important role in the contraction of the adult rat myocardium, while the role of SR is much smaller in the neonate. Thus, the neonatal and adult rat myocardium provides a useful model to study the role of SR in excitation-contraction coupling and the mechanism of drug action (Tanaka & Shigenobu, 1989; Agata *et al.*, 1992).

Therefore, in the present study, we applied CPA to myocardial preparations from neonatal and adult rats, which differ greatly in their dependence on SR function (Tanaka & Shigenobu, 1989), to examine (1) whether CPA has negative inotropic effects on cardiac muscle, and (2) whether a highly developed SR is essential for its action.

Methods Right ventricular free wall strips and right ventricular papillary muscles were rapidly isolated from 1-day old neonatal (male and female) and adult (male) (8 week old, 250–300 g) Wistar strain rats, respectively. Preparations were placed horizontally in a 20 ml organ bath containing physiological salt solution of the following composition (mM concentration): NaCl 135, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 15 and glucose 5.5 (pH 7.4). The nutrient solution was aerated with 95% O_2:5% CO_2 and maintained at 36.5°C. Preparations were driven by a pair of platinum plate electrodes (field stimulation) with rectangular current pulses (1 Hz, 5 ms. 1.2 × threshold voltage) generated from an electronic stimulator (Dia Medical System, DPS-165B). The resting tension applied to each preparation was adjusted so that the muscle was stretched to the peak of its length-tension curve. Developed tension was recorded isometrically with a force-displacement transducer (Nihon Kohden TB-611T) connected to a minipolygraph (Nihon Kohden RM-6100). CPA was added after preparations were allowed to equilibrate for more than 40 min, after which the developed tension of the preparations was well maintained. The contractile force after CPA addition was expressed as a percentage of the contractile force in the absence of drugs. Statistical significance between neonatal and adult preparations were determined with Student's *t* test and a *P* value less than 0.05 was considered significant.

Results CPA 30 μM produced a decrease in contractile force in the adult (Figure 1). The contractile force reached its minimum at about 60 min after CPA application, which was 19.8 ± 4.1% of the control value (Figure 2). Prolongation of

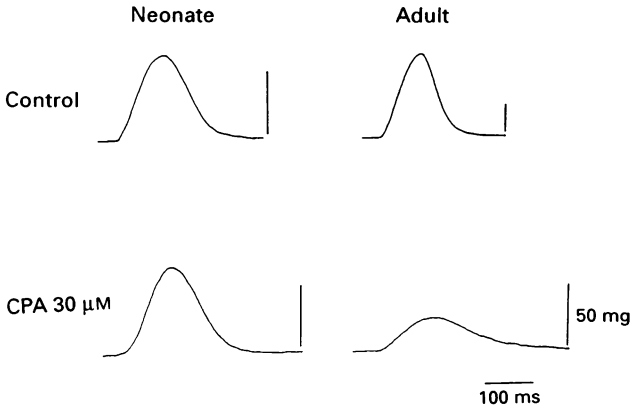


Figure 1 Effects of cyclopiazonic acid (CPA) on the contraction of neonatal and adult cardiac muscles. Typical traces in the presence and absence of 30 μM CPA. Vertical bars indicate 50 mg of contractile force, and horizontal bars indicate 100 ms.

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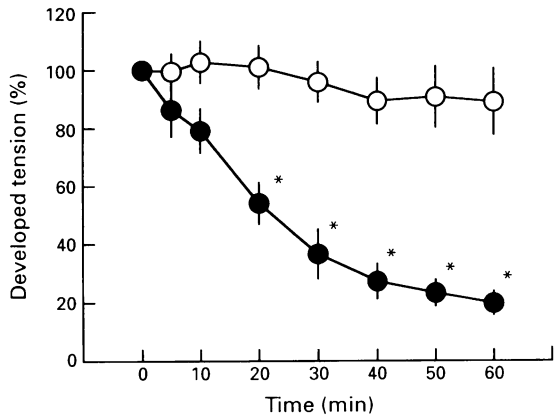


Figure 2 Time course of the inotropic responses to 30 μM CPA in the neonate (○) and adult (●). Mean ± s.e. (vertical lines) of 5 preparations for each group. Ordinate scale: contractile force expressed as a percentage of the value before the addition of CPA. Abscissa scale: time after addition of CPA in min. $*P<0.05$ vs neonate.

the relaxation phase of contraction was also observed. The time required for 90% relaxation (RT_{90}) was 74.0 ± 6.2 ms under control conditions and was 276.0 ± 29.3 ms at 60 min after the addition of CPA. In contrast, in neonatal preparations, CPA affected neither the size nor the time course of contraction (Figures 1 and 2). The contractile force at 60 min after the addition of CPA was 89.2 ± 11.4% of control values. RT_{90} was 95.6 ± 5.8 ms under control condition and was 128.2 ± 5.0 ms at 60 min after the addition of CPA.

Discussion When compared with other species, the myocardium of the adult rat is resistant to the slow current antagonist D600 (Willerson *et al.*, 1978), while ryanodine and caffeine markedly decrease the contractile force and increase the time required for relaxation (Sutko & Willerson, 1980; Vornanen, 1985), suggesting that Ca^{2+}-release from the SR plays an especially important role in the contraction and relaxation of the adult rat myocardium. In contrast, the neonatal rat myocardium is more sensitive to nicardipine and resistant to ryanodine and caffeine than the adult, suggesting a much smaller role for SR in neonatal myocardium (Vornanen, 1985; Tanaka & Shigenobu, 1989). Thus, the neonatal and adult rat myocardium provide a pair of preparations from the same species, the contractions of which differ greatly in their dependence on SR function and, therefore, serve as a useful model to study the role of SR in excitation-contraction coupling and the mechanism of drug action (Tanaka & Shigenobu, 1989; Agata *et al.*, 1992).

In the adult rat preparations, CPA reduced the contractile force and prolonged the relaxation phase. These results are similar to the effect of ryanodine (Tanaka & Shigenobu, 1989) and caffeine (Vornanen, 1985) on the same preparation, suggesting that CPA reduces the amount of Ca^{2+} available for contraction and increases the time required for Ca^{2+}-removal through the inhibition of SR function. That the effects of CPA were minimal in neonatal preparations also suggests its action on the SR. In skinned smooth muscles, CPA was demonstrated to inhibit ATP-dependent Ca^{2+}-uptake into SR and subsequently inhibit Ca^{2+}-release (Ueyama *et al.*, 1992). Whether CPA acts through the same mechanism in cardiac muscle awaits further investigation. Although the effects of CPA on neonatal and adult myocardium are similar to that of caffeine, which inhibits Ca^{2+}-uptake into SR, ryanodine, which interferes with the Ca^{2+}-release from SR, also produced similar effects. Our tentative conclusion is that the effects of CPA are due to inhibition of Ca^{2+}-release and Ca^{2+}-uptake into the SR. In smooth muscle, CPA was demonstrated not to affect extracellular Ca^{2+}-influx or the Ca^{2+}-sensitivity of contractile proteins (Ueyama *et al.*, 1992). Although there is no direct evidence at present, CPA is also unlikely to affect these general mechanisms of contraction in cardiac muscle because it had minimal effects in neonatal myocardium.

In conclusion, we have demonstrated that CPA exerts a negative inotropic effect on adult rat myocardium probably through inhibition of SR function. CPA appears to be a useful pharmacological tool for the study of the role of SR in excitation-contraction coupling not only in skeletal and smooth muscles but also in cardiac muscle.

References

AGATA, N., TANAKA, H. & SHIGENOBU, K. (1992). Effect of Mn^{2+} on neonatal and adult rat heart: initial depression and late augmentation of contractile force. *Eur. J. Pharmacol.*, (in press).

BOURREAU, J.P., ABELA, A.P., KWAN, C.Y. & DANIEL, E.E. (1991). Acetylcholine Ca^{2+} stores refilling directly involves a dihydropyridine-sensitive channel in dog tracheas. *Am. J. Physiol.*, **261**, C497–C505.

FABIATO, A. & FABIATO, F. (1978). Calcium-induced release of calcium from the sarcoplasmic reticulum of skinned cells from adult human, dog, cat, rat and frog hearts and from fetal and newborn rat ventricles. *Ann. N.Y. Acad. Sci.*, **307**, 491–522.

GOEGER, D.E., RIELEY, R.T., DORNER, J.W. & COLE, R.J. (1988). Cyclopiazonic acid inhibition of the Ca^{2+}-transport ATPase in rat skeletal muscle sarcoplasmic reticulum vesicles. *Biochem. Pharmacol.*, **37**, 978–981.

KUREBAYASHI, N. & OGAWA, Y. (1991). Discrimination of Ca^{2+}-ATPase activity of the sarcoplasmic reticulum from acto-myosin-type ATPase activity of myofibrils in skinned mammalian skeletal muscle fibers: distinct effects of cyclopiazonic acid on the two ATPase activities. *J. Muscle Res. Cell Motil.*, **12**, 355–365.

SUTKO, J.L. & WILLERSON, J.T. (1980). Ryanodine alteration of the contractile state of rat ventricular myocardium. Comparison with dog, cat, and rabbit ventricular tissues. *Circ. Res.*, **46**, 332–343.

TANAKA, H. & SHIGENOBU, K. (1989). Effect of ryanodine on neonatal and adult rat heart: developmental increase in sarcoplasmic reticulum function. *J. Mol. Cell. Cardiol.*, **21**, 1305–1313.

UEYAMA, Y., IMAIZUMI, Y. & WATANABE, M. (1992). Effects of cyclopiazonic acid, a novel Ca^{2+}-ATPase inhibitor, on contractile responses in skinned ileal smooth muscle. *Br. J. Pharmacol.*, **106**, 208–214.

VORNANEN, M. (1985). Effect of strontium on the contractile properties of postnatally developing rat heart ventricles. *Can. J. Physiol. Pharmacol.*, **63**, 9–17.

WILLERSON, J.T., WHEELAN, S., ADCOCK, R.C., TEMPLETON, G.H. & WILDENTHAL, K. (1978). Species differences in responses to hyperosmolarity and D600 in cat and rat heart. *Am. J. Physiol.*, **235**, H276–280.

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SPECIAL REPORT

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Anti-inflammatory actions of an N-terminal peptide from human lipocortin 1

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An acetylated polypeptide corresponding to residues 2–26 of human lipocortin 1 was synthesized and the anti-inflammatory activity assessed in three models of acute inflammation in rat and mouse. In the carrageenin rat paw oedema test, the peptide produced a maximal inhibition of approximately 41% at the 3 h time point with a 10 μg dose. When rat paw oedema was induced by the injection of venom phospholipase A_2, the peptide produced a significant inhibition (31%) at the top dose of 20 μg per paw. In the mouse air-pouch model, systemic treatment with the peptide produced a dramatic reduction in cytokine-induced leukocyte migration with an ID_{50} of approximately 40 μg per mouse.

The N-terminal peptide 2–26 shares the actions of lipocortin 1 in these acute models of inflammation.

Keywords: Lipocortin 1/annexin 1; acute inflammation; N-terminus; anti-inflammatory actions

Introduction Human recombinant lipocortin 1 suppresses the release of proinflammatory eicosanoids *in vitro* (Cirino & Flower, 1987; Cirino *et al.*, 1987) and exhibits strong anti-inflammatory actions in rat (Cirino *et al.*, 1989) and mouse (Perretti & Flower, 1993). An intact C-terminus is apparently not necessary for biological activity since the 1–188 recombinant fragment suppresses cytokine induced fever (Carey *et al.*, 1990) and ischaemic brain damage in the rat (Relton *et al.*, 1991). Conversely, removal of the 3kDa N-terminal fragment from lipocortin 1 by proteolytic plasmin cleavage inactivated the molecule (Cirino, Browning & Flower unpublished results).

To elucidate further the role of the N-terminus in lipocortin 1 action we have synthesized a peptide representing the entire N-terminus (residues 2–26). To retard proteolytic degradation, this peptide was synthesized with an acetyl blocked N-terminus.

Methods *Carrageenin and phospholipase A_2 paw oedema* Oedema was induced in male wistar rats weighing 130–150 g (Nossan, Italy) by a single subplantar injection of either 100 μl of a 1% (w/v) solution of carregeenin or 5 μg per paw of phospholipase A_2 from *Naja mocambique mocambique* into the right hind paw (Cirino *et al.*, 1989). The peptide was dissolved in 100 μl sterile saline while the control received saline only (100 μl). Paw volume was measured with a water plethysmometer (Basile, Italy) immediately before and after the injection and at appropriate intervals thereafter.

Interleukin 1β induced neutrophil accumulation in mouse air pouch Male Swiss albino mice (22–25 g; Tuck U.K.) were injected with 2.5 ml of air subcutaneously on day 0 and day 3. On day six, animals received either PBS or the peptide (10–400 μg per mouse) in different doses prepared in 200 μl of PBS by intravenous injection, 20–30 min before interleukin-1β (20 ng) injection into the air-pouch. Pouches were washed with 2 ml of PBS containing heparin and counted after staining in Turk's solution (1/10 dilution). The data are expressed as a percentage of the control migration observed in the PBS-treated groups (Perretti & Flower, 1993).

Materials The peptide 2–26 (N-acetyl-AMVSEFLKQAWFIENEEQEVVQTVK) was obtained from three different sources Bachem (U.S.A.), Multiple Peptide Systems (U.S.A.) and Biogen (U.S.A.). The latter was a generous gift of Dr J.L. Browning. The purity was always greater than 95%. Phospholipase A_2 from *Naja mocambique mocambique* (EC 3.1.1.4.), carrageenin and other routine buffers and salts were purchased from the Sigma Chemical Company. Interleukin-1β was a generous gift from Dr L. Parente (I.R.I.S., Siena, Italy).

Results Figure 1a shows that the peptide produced a dose-related inhibition of the carrageenin paw oedema at 3 h. Indeed 5 μg and 10 μg per paw produced 20% and 41% inhibition respectively. However 20 μg did not produce any further inhibitory activity (data not shown). At doses of 5 μg and 10 μg per paw the peptide was without effect on the oedema produced by phospholipase A_2 (data not shown). However subplantar injection of 20 μg of the peptide produced a weak (31%) but significant inhibition of the inflammation (Figure 1b).

Figure 2 shows that the peptide produced a dose-related reduction of polymorphonuclear leukocyte (PMN) migration into the pouch with an IC_{50} of approximately 40 μg per mouse.

No overt toxicity of the peptide was observed at the doses used in this experiment in either the rat or mouse.

Discussion We have tested a peptide corresponding to residues 2–26 of lipocortin 1 in three models of inflammation which are known to respond to the inhibitory action of the full length protein. In the carrageenin paw oedema test and phospholipase A_2-induced oedema, human recombinant lipocortin 1 has an ID_{50} of 10–20 μg respectively (Cirino *et al.*, 1989). Thus on a molar basis the peptide is at least one order of magnitude less effective than the native species. In the mouse neutrophil migration test, lipocortin 1 itself has an ID_{50} of approximately 4 μg per mouse when given intravenously (Perretti & Flower, 1993) and the peptide is approximately two orders of magnitude less potent on a molar basis than the parent molecule.

It is interesting to note that the N-terminal peptide shares some of the actions of lipocortin 1 in these models of

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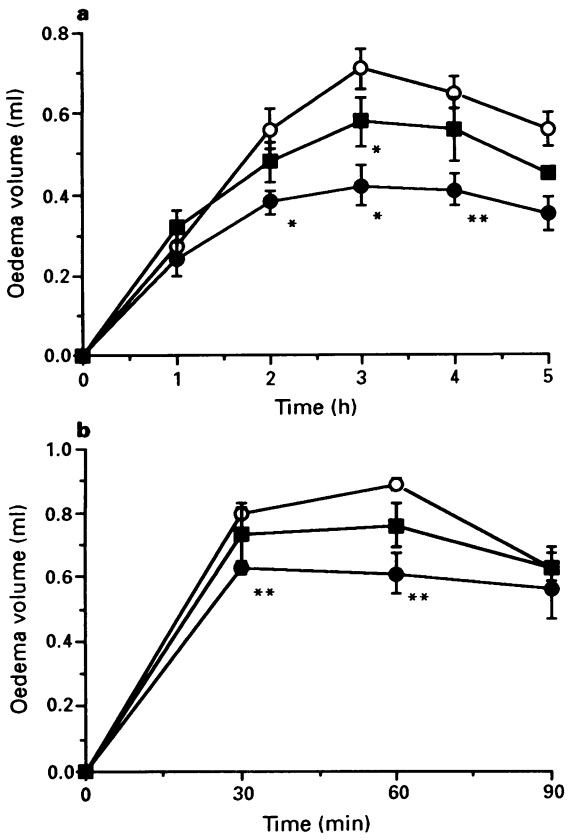


Figure 1 (a) The inhibitory effect of N-acetyl 2–26 lipocortin 1 on the oedema (ml) produced in rat paw by subplantar injection of carrageenin: (○) 0.1 ml 0.1% carrageenin; (■) carrageenin + 5 μg N-acetyl 2–26; (●) carrageenin + 10 μg N-acetyl 2–26. Each point is a mean value ± s.e.mean (vertical bars); $n = 15$ rats per group. (b) The inhibitory effect of N-acetyl 2–26 lipocortin 1 on the oedema (ml) produced in rat paw by subplantar injection of phospholipase A_2: (○) 5 μg phospholipase A_2 only; (■) phospholipase A_2 + 10 μg N-acetyl 2–26; (●) phospholipase A_2 + 20 μg N-acetyl 2–26. Each point is the mean ± s.e.mean.
Significance (Student's t test) is indicated by $*P<0.05$; $**P<0.01$.

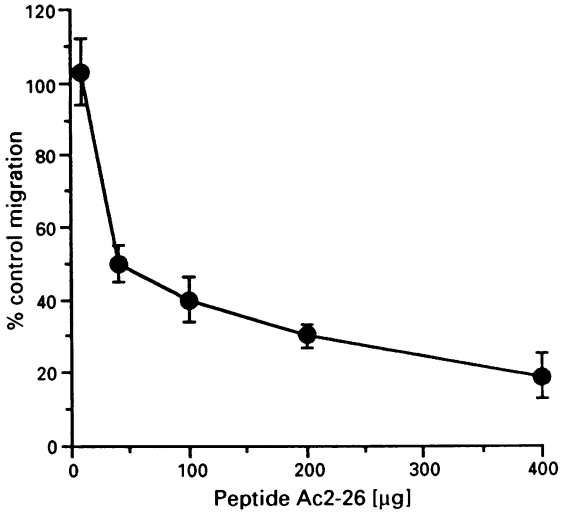


Figure 2 The inhibitory effect of N-acetyl 2–26 lipocortin 1 on neutrophil migration into the mouse air pouch elicited by interleukin 1. The control migration ($9.63 \pm 0.84 \times 10^6$ per mouse; $n = 15$) was measured in mice treated with intravenous saline only and the effect of the peptide tested at different concentrations by i.v. injection. All points are significantly different from control with a minimum significance of $P<0.05$.

inflammation. That it does so is consistent with our previous unpublished observations that N-terminal clipped molecule is without biological activity and with work demonstrating that lipocortin 1-188 seems fully effective in several models (Carey *et al.*, 1990; Relton *et al.*, 1991). All these data now point to the N-terminus as a functionally important domain and suggest that the remaining core sequence, which is able to bind calcium and phospholipid, is somehow important for the attachment of the molecule to its target cell.

Phosphorylation of the N-terminus at Tyr-21 leads to a change in the physico-chemical properties of the protein with enhanced proteolysis and biological inactivation (Chuah & Pallen, 1989). Interestingly, several peptides with a homologous structure to the lipocortin 1 N-terminus, such as those of middle T-antigen and synthetic peptides from pp60, have previously been shown to alter neutrophil chemotactic properties and to posses lipocortin-like effects on phospholipase A_2 (Notsu *et al.*, 1985).

The most likely explanation for the activity of the peptide is that it interacts with the cell surface lipocortin 1 binding protein (Goulding *et al.*, 1990) thus bringing about a biological effect similar to the native protein. However, the peptide could also be interfering with the phosphorylation or turnover of naturally occurring lipocortin 1 thus prolonging and producing a *de facto* anti-inflammatory effect.

Further studies will examine this possibility as well as testing other peptides from different regions of the N-terminus to define more closely the active site.

References

CAREY, F., FORDER, R., EDGE, M.D., GREENE, A.R., HORAN, M.A., STRIJBOS, P.J.L.M. & ROTHWELL, N.J. (1990). Lipocortin 1 fragment modifies pyrogenic actions of cytokines in rats. *Am. J. Physiol.*, **259**, R266–R269.

CHUAH, S.Y. & PALLEN, C.J. (1989). Calcium dependent and phosphorylation-stimulated proteolysis of lipocortin 1 by an endogenous A431 cell membrane protease. *J. Biol. Chem.*, **264**, 21160–21166.

CIRINO, G., FLOWER, R.J., BROWNING, J.L., SINCLAIR, L.K. & PEPINSKY, R.B. (1987). Recombinant human lipocortin 1 inhibits thromboxane release from guinea-pig isolated perfused lung. *Nature*, **328**, 270–272.

CIRINO, G. & FLOWER, R.J. (1987). Human recombinant lipocortin 1 inhibits prostacyclin production by human umbilical artery *in vitro*. *Prostaglandins*, **34**, 59–62.

CIRINO, G., FLOWER, R.J., BROWNING, J.L., SINCLAIR, L.K. & PEPINSKY, R.B. (1989). Human recombinant lipocortin 1 has acute local anti-inflammatory properties in the rat paw oedema test. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 3428–3432.

GOULDING, N.J., LUYING, P. & GUYRE, P.M. (1990). Characteristics of lipocortin 1 binding to the surface of human peripheral blood leukocytes. *Biochem. Soc. Trans.*, **18**, 1237–1238.

NOTSU, Y., NAMIUCHI, S., HATTORI, T., MATSUDA, K. & HIRATA, F. (1985). Inhibition of phospholipase by met-leu-phe-ile-leu-ile-lys-arg-ser-arg-his-phe, C-terminus of middle-sized tumour antigen. *Arch. Biochem. Biophys.*, **236**, 195–204.

PERRETTI, M. & FLOWER, R.J. (1993). Modulation of Il-1-induced neutrophil migration by dexamethasone and lipocortin 1. *J. Immunol.*, (in press).

RELTON, J.K., STRIJBOS, P.J.L.M., O'SHAUGHNESSY, C.T., CAREY, F., FORDER, R.A., TILDERS, F.J.H. & ROTHWELL, N.J. (1991). Lipocortin 1 is an endogenous inhibitor of ischemic damage in rat brain. *J. Exp. Med.*, **174**, 305–310.

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SPECIAL REPORT

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Active secretion of the fluoroquinolone ciprofloxacin by human intestinal epithelial Caco-2 cell layers

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The bidirectional transepithelial fluxes of ciprofloxacin, an antibacterial fluoroquinolone, across the human intestinal epithelial Caco-2 cell-line show marked asymmetry. Basal-to-apical flux of ciprofloxacin (10 μM) exceeds apical-to-basal flux indicating net secretion. Net ciprofloxacin secretion is abolished by azide/2-deoxy-D-glucose treatment, displays saturation kinetics (K_m = 0.89 ± 0.23 mM, V_{max} 44.3 ± 4.9 nmol cm^{-2}.h) and competition by other fluoroquinolones. A specific, active secretion in Caco-2 epithelia may explain the transintestinal elimination of ciprofloxacin observed in pharmacokinetic studies in man.

Keywords: Caco-2 cells; ciprofloxacin; epithelia; fluoroquinolone; intestinal secretion

Introduction In human volunteers, 18% of an intravenous dose of ciprofloxacin, an antibacterial fluoroquinolone, is eliminated by a non-renal process across the intestine (Sorgel *et al.*, 1989; 1991). In renal failure patients, transintestinal ciprofloxacin elimination was increased to approximately 40% of an intravenous dose (Rohwedder *et al.*, 1990). Since biliary elimination of the drug is small and only a minor component of ciprofloxacin in faeces is metabolites (Sorgel *et al.*, 1989; Rohwedder *et al.*, 1990) it is apparent that an unidentified intestinal secretory mechanism for this drug must exist.

The Caco-2 cell-line is being used increasingly as an *in vitro* model for absorption studies (Hidalgo *et al.*, 1989). This human colonic cell-line expresses a number of brush-border membrane hydrolases and membrane transport systems consistent with a predominantly small intestinal phenotype (Inui *et al.*, 1992). Here we report that ciprofloxacin undergoes substantial active secretion in Caco-2 epithelial layers. The relationship between ciprofloxacin secretion and other identified transport systems in Caco-2 cells is discussed.

Methods *Cell culture* Caco-2 cells (85–100 serial passages) were obtained from Dr I. Hassan (Ciba-Geigy Pharmaceuticals, Horsham, Sussex) and grown in Dulbecco's modified Eagle's medium (DMEM) containing D-glucose (4.5 g l^{-1}) supplemented with foetal calf serum (10%), glutamine (2 mM), non-essential amino acids (1%) and gentamycin (50 μg ml^{-1}). Epithelial layers were obtained by high-density seeding (3 × 10^6 cells) onto permeable matrices (Anocells, Nunc 25 mm culture inserts) and used in experiments after 11 days.

Transepithelial [^{14}C]-ciprofloxacin fluxes: Cell monolayers were washed in Krebs buffer (composition all mmol l^{-1}) NaCl 137, KCl 5.4, $CaCl_2$ 2.8, $MgSO_4$ 1.2, NaH_2PO_4 0.3, KH_2PO_4 0.3, Tris base 14, HCl 12, glucose 5 (pH 7.4 at 37°C), placed in fresh 6-well plates containing 2 ml of Krebs (basal solution). Krebs solution (2 ml) was added to the apical side and [^{14}C]-ciprofloxacin (10 μM at 61.24 μCi mg^{-1}, or with increasing concentrations of ciprofloxacin) was present in either apical or basal solution. Unlabelled ciprofloxacin was included in the contralateral solution. Bidirectional fluxes (apical-to-basal, J_{a-b}; basal-to-apical J_{b-a}) of ciprofloxacin were measured at 1 h time intervals for up to 3 h (200 μl samples) with at least three replicates for each condition. Cell-associated radioactivity was determined following extensive washing of culture inserts (4 changes of 1 l buffer at 4°C) and removal of filters to counting vials. As an indicator of monolayer integrity, [^{3}H]-mannitol fluxes were determined simultaneously with [^{14}C]-ciprofloxacin. ^{14}C- and ^{3}H-activities were determined by scintillation counting, appropriate corrections being made for quench and cross-channel spill-over.

Materials [^{14}C]-ciprofloxacin (61.24 μCi mg^{-1}) and unlabelled ciprofloxacin were generous gifts of Bayer (Wuppertal, Germany). [^{3}H]-mannitol (30 Ci mmol^{-1}) was from Amersham International plc (Little Chalfont, Amersham, Bucks). Scintillation cocktail 'Optiphase safe' was from Pharmacia LKB Biotechnology Ltd (Milton Keynes, Bucks). All tissue culture media and reagents (Gibco BRL), tissue culture plastics and tissue culture inserts (Nunc) were supplied by Life Technologies Ltd (Paisley, Scotland). All other reagents/chemicals were obtained from Sigma Chemical Co. (Poole, Dorset) or BDH Chemicals Ltd (Poole, Dorset).

Statistics Flux rates (pmol cm^{-2}.h) were obtained by linear regression of time courses (each hour over 1–3 h) ± s.e., where d.f. denotes the degree of freedom (3–6 monolayers per condition). Net ciprofloxacin flux, $J_{net} = J_{b-a} - J_{a-b}$.

Results The bidirectional transepithelial fluxes for ciprofloxacin (10 μM) were linear up to 3 h. Basal-to-apical flux (J_{a-b}, 451 ± 52.5 pmol cm^{-2}.h, d.f. = 22) was greater than apical-to-basal flux (J_{a-b}, 126 ± 10 pmol cm^{-2}.h, d.f. = 22) indicating a substantial net secretion of ciprofloxacin from basal to apical surfaces (J_{net}, 325 pmol cm^{-2}.h). Simultaneous measurements of bidirectional mannitol fluxes showed no marked asymmetry (J_{a-b} and J_{b-a} were 52.3 and 47.5 fmol cm^{-2}.h, respectively). In order to test the dependence of ciprofloxacin fluxes upon cellular ATP, fluxes were determined in the presence of 15 mM Na azide and 50 mM 2-deoxy-D-glucose in both apical and basal solutions. The substantial net flux observed in control epithelial layers (325 pmol cm^{-2}.h) was virtually eliminated (to 27 pmol cm^{-2}.h) both by a reduction in J_{b-a} from 434 ± 55 (d.f. = 10) to 233 ± 28 (d.f. = 10) pmol cm^{-2}.h and an increment in J_{a-b} from 104 ± 11 (d.f. = 10) to 214 ± 40 (d.f. = 10) pmol cm^{-2}.h. The increment in J_{a-b} is likely to result from an increased paracellular flux as mannitol flux is increased concurrently (from control J_{a-b} of 36.3 ± 11.6 to 85.3 ± 12.6 fmol cm^{-2}.h in azide/2-deoxy-D-glucose).

Figure 1 shows the concentration-dependence of the bidirectional ciprofloxacin fluxes. Whereas J_{a-b} is a simple linear function of ciprofloxacin concentration, J_{a-b} is non-linear. J_{net}

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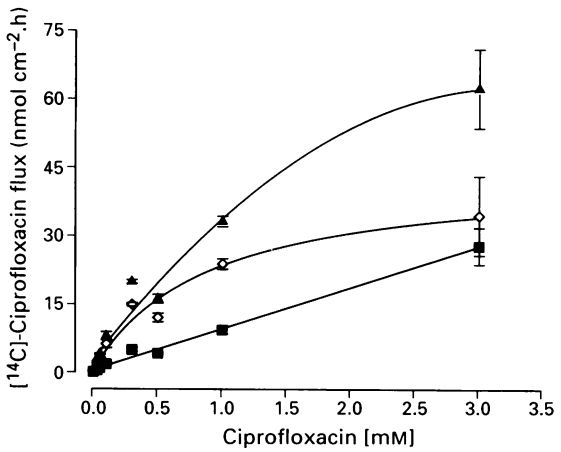


Figure 1 Concentration-dependence of [^{14}C]-ciprofloxacin fluxes; (■) J_{a-b} (apical-to-basal flux), (▲) J_{b-a} (basal-to-apical flux), (◇) J_{net} (net flux = $J_{b-a} - J_{a-b}$). Solid line for J_{a-b} is the linear regression line for the data $y = 9.1x + 0.44$, $r = 0.99$, d.f. = 22, $P < 0.001$. Solid line for J_{net} was calculated by non-linear regression assuming Michaelis-Menten kinetic fit for the data; $K_m = 0.89 \pm 0.23$ nM, $V_{max} = 44.3 \pm 4.9$ nmol cm^{-2}.h.

demonstrates saturation kinetics with half-maximal saturation being observed at 0.89 ± 0.23 mM (d.f. = 22) and a maximal secretory capacity of 44.3 ± 4.9 nmol cm^{-2}.h. Solubility of ciprofloxacin limited the use of higher concentrations.

Competitive inhibition of 10 μM [^{14}C]-ciprofloxacin net flux (J_{net}) was observed by other unlabelled fluoroquinolones added to the same solution as the tracer [^{14}C]-ciprofloxacin: J_{net} calculated at 3 h (n values = paired cell layers), control (10 μM ciprofloxacin) 255 ± 34 pmol cm^{-2}.h ($n = 10$); with 1 mM flumequine 175 ± 10 pmol cm^{-2}.h (32% inhibition; $n = 6$); with 1 mM enoxacin 162 ± 15 pmol cm^{-2}.h (36% inhibition; $n = 6$); with 1 mM norfloxacin 227 ± 2 pmol cm^{-2}.h (11% inhibition; $n = 5$). Verapamil (100 μM) reduced [^{14}C]-ciprofloxacin net secretion by 38% compared to control, from 152 ± 10 to 94 ± 11 pmol cm^{-2}.h ($n = 3$). Indomethacin (100 μM) was without effect on [^{14}C]-ciprofloxacin transport; net secretory flux 378 ± 12 and 392 ± 32 pmol cm^{-2}.h ($n = 3$) with and without indomethacin, respectively.

Discussion The present data show that Caco-2 cells possess a specific mechanism for the transepithelial secretion of ciprofloxacin from basolateral to apical bathing media. Active transport is indicated since this net ciprofloxacin secretion is generated in the absence of an external driving force and is abolished by azide/2-deoxy-D-glucose implying a dependence upon cellular metabolism. Ciprofloxacin secretion exhibits saturation kinetics and is subject to competitive inhibition by compounds of a similar structure. That ciprofloxacin secretion is observed in human intestinal cells in culture suggests that this system may be useful in elucidating the exact nature of transintestinal elimination of this class of antibacterials.

Transintestinal elimination as a route for xenobiotic clearance has been noted previously for a diverse range of substances including cardiac glycosides, quinine and erythromycin (Israili & Dayton, 1984). Recently it has been suggested that functional expression of P-glycoprotein within the epithelial cells of the intestinal tract may mediate the elimination (from plasma to the intestinal lumen) of numerous drugs (e.g., vinblastine) shown to be substrates for this ATP-dependent pump (Hunter *et al.*, 1991a). Transepithelial vinblastine secretion in a variety of cell lines is virtually abolished by verapamil at 100 μM, with an IC_{50} of around 2–20 μM (Hunter *et al.*, 1991a,b). The incomplete inhibition of ciprofloxacin secretion by 100 μM verapamil may suggest the lack of involvement of P-glycoprotein. In addition recent work has demonstrated that the anionic fluorochrome BCECF may also be the subject of ATP-dependent export across the apical (luminal border) of Caco-2 cells mediated by an indomethacin-sensitive mechanism distinct from P-glycoprotein (Collington *et al.*, 1992). The absence of indomethacin inhibition suggests that the transporters involved in ciprofloxacin secretion are distinct from those mediating BCECF export.

Ciprofloxacin secretion is thus mediated by an active transport system. Whether this represents a novel active secretion system of an as yet unidentified physiological substrate remains to be elucidated.

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References

COLLINGTON, G.K., HUNTER, J., ALLEN, C.N., SIMMONS, N.L. & HIRST, B.H. (1992). Polarised efflux of BCECF from cultured epithelial cell monolayers. *Biochem. Pharmacol.*, **44**, 417–424.

HIDALGO, I.J., RAUB, T.J. & BORCHARDT, R.T. (1989). Characterisation of the human colon carcinoma cell-line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology*, **96**, 734–749.

HUNTER, J., HIRST, B.H. & SIMMONS, N.L. (1991a). Epithelial secretion of vinblastine by human intestinal adenocarcinoma cell (HCT-8 and T84) layers expressing P-glycoprotein. *Br. J. Cancer*, **64**, 437–444.

HUNTER, J., HIRST, B.H. & SIMMONS, N.L. (1991b). Transepithelial vinblastine secretion mediated by P-glycoprotein is inhibited by forskolin derivatives. *Biochem. Biophys. Res. Commun.*, **181**, 671–676.

INUI, K.I., YAMAMOTO, M. & SAITO, H. (1992). Transepithelial transport of oral cephalosporins by monolayers of intestinal cell-line Caco-2: specific transport systems in the apical and basolateral membranes. *J. Pharmacol. Exp. Ther.*, **261**, 195–201.

ISRAILI, Z.H. & DAYTON, P.G. (1984). Enhancement of xenobiotic elimination: role of intestinal excretion. *Drug Metab. Rev.*, **15**, 1123–1159.

ROHWEDDER, R., BERGAN, T., THORSTEINSSON, S.B. & SCHOLL, H. (1990). Transintestinal elimination of ciprofloxacin. *Chemotherapy*, **36**, 77–84.

SORGEL, F., NABER, K.G., JAEHDE, U., REITER, A., SEELMAN, R. & SIGL, G. (1989). Gastrointestinal secretion of ciprofloxacin. *Am. J. Med.*, **87**, 63–65S.

SORGEL, F., NABER, K.G., MAHR, G. & MUTH, P. (1991). Comparative pharmacokinetics of ciprofloxacin and temafloxacin in humans: a review. *Am. J. Med.*, **91**, 51–68S.

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Inhibition of [^{3}H]-(+)-MK 801 binding to rat brain sections by CPP and 7-chlorokynurenic acid: an autoradiographic analysis

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1 The regional binding of [^{3}H]-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo(*a*,*d*)cyclohepten-5,10-imine maleate ([^{3}H]-(+)-MK 801) to sections of rat brain was measured by an *in vitro* quantitative autoradiographic technique. A heterogeneous distribution of binding sites was observed.

2 High values of binding were detected in the hippocampal formation and cerebral cortex, while very low binding was found in cerebellum. [^{3}H]-(+)-MK 801 binding was not detectable in white matter tracts or in the brain stem.

3 [^{3}H]-(+)-MK 801 binding was inhibited by increasing concentrations of both 7-chlorokynurenate (1–1000 μM) and ((+)-2-carboxypiperazine-4-yl)propyl-1-phosphonic acid (CPP) (0.1–100 μM). High concentrations of both drugs were able to inhibit completely specific [^{3}H]-(+)-MK 801 binding.

4 IC_{50} values calculated for both 7-chlorokynurenate and CPP-induced [^{3}H]-(+)-MK 801 binding inhibition were similar in all brain regions analyzed.

5 The inhibitory action of 7-chlorokynurenate and that of CPP on [^{3}H]-(+)-MK 801 binding were reversed by addition of glycine and glutamate respectively.

6 It is concluded that activation of glycine and N-methyl-D-aspartate (NMDA) receptors is obligatory for the binding of [^{3}H]-(+)-MK 801 to occur in all of the brain regions examined in the present study. Furthermore, on the basis of the similar regional sensitivities of [^{3}H]-(+)-MK 801 binding to the inhibitory action of 7-chlorokynurenate and CPP, a single pharmacological classification of the NMDA receptor complex in brain is suggested. The cerebellum was not included in the study due to the very low level of [^{3}H]-(+)-MK 801 binding detected under the experimental conditions used.

Keywords: Excitatory amino acids; NMDA receptors; receptor autoradiography; [^{3}H]-(+)-MK 801; 7-chlorokynurenate; CPP

Introduction

Current evidence suggests that excitatory amino acid (EAA) receptors are the main neurotransmitter receptors mediating synaptic excitation in the mammalian central nervous system (CNS) (Watkins & Evans, 1981; McLennan, 1983; Monaghan *et al.*, 1989). The physiological role of EAA transmission is wide, ranging from processing of sensory information, to learning, memory and cognitive functions (Flood *et al.*, 1990). It has been suggested that alterations in EAA transmission are critical factors in the development of neurological disorders such as Alzheimer's disease and Huntington's chorea (Olney, 1990). Furthermore EAA(s) seem to play a pivotal role in the development of epileptic seizures (Meldrum *et al.*, 1983) and in eliciting and sustaining neurodegeneration in hypoglycaemia (Simon *et al.*, 1986), ischaemia and stroke (Rothman & Olney, 1987).

The presence of EAA receptor subtypes has become evident with the development of selective agonists and antagonists. So far five pharmacologically different EAA receptors have been defined: the NMDA, kainate, AMPA, L-AP4 and Metabotropic subtypes (Monaghan *et al.*, 1989). Of these the NMDA receptor is the most well characterized and appears to be involved in certain types of memory (Harris *et al.*, 1984) and in neurodegenerative processes in pathological conditions (Simon *et al.*, 1984). This EAA receptor is associated with a cation-selective ion channel that gates K^+, Na^+ and Ca^{2+} ions and it is blocked in a voltage-sensitive manner by Mg^{2+}.

The NMDA receptor is a complex organization of different interrelated functional domains. In addition to the L-glutamate recognition site, a strychnine-insensitive glycine recognition site is present, where glycine acts as a positive allosteric modulator or as a co-agonist (Kleckner & Dingledine, 1988), as it enhances the effect of glutamate in electrophysiological assays (Johnson & Ascher, 1987) and in [^{3}H]-(+)-5-methyl-10, 11-dihydro-5*H*-dibenzo(*a*,*d*) cyclohepten-5, 10-imine maleate ([^{3}H]-(+)-MK 801) binding experiments (Reynolds *et al.*, 1987). Non-competitive antagonists of the NMDA receptor, such as phencyclidine (PCP), MK 801 and phencyclidine-like compounds, bind to a recognition site presumed to be located within the ion channel of the receptor complex (Loo *et al.*, 1986). Since the binding of ligands selective for the PCP recognition site is proportional to the degree of channel activation, binding can therefore be regarded as an indirect measure of the NMDA receptor complex function (Huettner & Bean, 1988; Javitt & Zukin, 1989). The ability of the competitive NMDA antagonists, such as ((+)-2-carboxypiperazine-4-yl)propyl-1-phosphonic acid (CPP) and D-amino-5-phosphonopentanoate (D-AP5), and strychnine-insensitive glycine receptor antagonists, such as 7-chlorokynurenate (7-Cl-kyn), to inhibit [^{3}H]-(+)-MK 801 binding in rat brain membrane preparations has provided further evidence to support this notion (Sircar *et al.*, 1989; Hood *et al.*, 1990).

Autoradiographic studies on rat and gerbil brain have demonstrated similar distributions of MK 801, NMDA-sensitive L-glutamate and strychnine-insensitive glycine recognition sites (Gundlach *et al.*, 1986; Bowery *et al.*, 1988).

In the present study we have determined the pattern of inhibition of [^{3}H]-(+)-MK 801 binding to rat brain slices, induced by the selective NMDA receptor competitive antagonist, CPP and by the selective strychnine-insensitive glycine receptor antagonist 7-chlorokynurenic acid. Autoradiography coupled with computer assisted image analysis techniques were used to determine the regional distribution and density

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of radioligand binding. A preliminary report of part of the study has been presented to the British Pharmacological Society (Tacconi *et al.*, 1991).

Methods

Receptor autoradiography

Male Sprague-Dawley rats (Bantin & Kingman U.K.) weighing 200–250 g were decapitated and the brains quickly removed and immediately frozen in isopentane pre-cooled in liquid nitrogen. Frozen brains were stored at − 80°C for up to 2 weeks or cryostat sections (12 μm thickness) were cut the same day at − 20°C and thaw-mounted on glass microscope slides before storing at − 20°C for up to 3 weeks. On the day of the experiment, slide-mounted sections were dried at room temperature and pre-incubated for 60 min in Tris-HCl (50 mM, pH = 7.40). Sections were then dried at room temperature, and 100 μl of binding assay solution was pipetted as a droplet onto the brain sections. The assay solution comprised 40 nM [^{3}H]-(+)-MK 801 (53.1 Ci mmol^{-1}) in Tris-HCl (50 mM, pH = 7.40), containing different concentrations of unlabelled compounds. Sections were incubated for 20 min at room temperature, washed twice for 30 s in Tris-HCl and rinsed quickly in twice distilled water to remove the excess of buffer salts before drying. Adjacent sections, accounting for non-specific binding (or 'background'), were incubated in the same medium containing 100 μM unlabelled (+)-MK 801. Dried sections were apposed to tritium-sensitive film (Hyperfilm-[^{3}H], Amersham) and exposed in X-ray cassettes for 9–20 days depending on the amount of total binding present on the sections as determined by liquid scintillation spectrometry of entire sections. High-level methacrylate strip ^{3}H standards (RPA 506 or RPA 507, Amersham) were exposed to the same films. After exposure, films were developed in Kodak D-19 for 1.5 min at room temperature, fixed in Kodak Unifix for 5 min and then washed in tap water. Autoradiograms were analyzed with a computer-assisted video image based instrument (Quantimet-970, Cambridge Instruments). Regional film densities were converted to the corresponding ligand concentration by reference to the tritium standards on the same film. IC_{50} values for the inhibition of [^{3}H]-(+)-MK 801 binding by test compounds were calculated by means of non-linear fitting procedures ('ALLFIT') (DeLean *et al.*, 1978).

Materials

[^{3}H]-(+)-MK 801 maleate salt, (53.1 Ci mmol^{-1}, TRQ 5489) was custom labelled by Amersham U.K.; (+)-MK 801 maleate salt was obtained from RBI U.S.A.; CPP and 7-chlorokynurenic acid were obtained from Tocris-Neuramin U.K. All other salts and reagents used were of analytical grade.

Results

Distribution of [^{3}H]-(+)-MK 801 binding sites

Autoradiography revealed a heterogeneous distribution of [^{3}H]-(+)-MK 801 binding throughout the brain (Table 1, Figure 1). Areas with the highest binding included subregions of the hippocampus (CA1 radiatum) and the frontal cortex. Intermediate levels were observed in the caudate putamen and thalamic nuclei. Because binding in cerebellum was very low, even in the presence of glutamate and glycine (data not shown), it was not possible to determine regional differences within this structure. Specific binding in the brain stem as well as in white matter tracts was not quantifiable.

Table 1 Distribution of [^{3}H]-(+)-MK 801 binding sites in rat brain

*Area	*Specific [^{3}H]-(+)-MK 801 binding* (fmol mg^{-1} brain tissue)	*s.e.mean*
CA1 oriens	591.3	35.0
CA1 pyram.	453.5	31.7
CA1 radiatum	651.0	35.1
CA3 oriens	402.5	37.6
CA3 pyram.	251.9	23.3
CA3 radiatum	498.0	34.4
Mol	590.6	36.0
GRDG	245.1	14.2
Hif	256.3	17.3
S	291.9	19.7
LD	331.1	24.8
DLG	302.5	18.4
VPM	314.7	27.1
VPL	294.4	24.7
CPu	347.4	18.9
Fr Cx ext. l.	526.6	21.9
Fr Cx med. l.	426.4	22.8
Fr Cx int. l.	302.6	20.2
Oc Cx ext. l.	465.0	26.7
Oc Cx med. l.	421.0	20.5
Oc Cx int. l.	359.2	10.5
cc	ND	ND
Cerebellum	76.2	18.2
Brain stem	ND	ND

Values represent the mean of 5 to 6 animals ($n = 5–6$) (6 determinations per section, 6 sections per animal).
*Abbreviations used are from Paxinos & Watson (1982).
ND = not detectable.

Inhibition of [^{3}H]-(+)-MK 801 binding by CPP and 7-chlorokynurenic acid

CPP (0.1–100 μM) and 7-chlorokynurenic acid (1.0–1000 μM) inhibited [^{3}H]-(+)-MK 801 in all brain regions examined (Table 2). There were no significant differences in the IC_{50} values of each compound obtained in the different regions. No correlation between the IC_{50} values obtained by CPP inhibition and the IC_{50} values obtained by 7-chlorokynurenic acid inhibition was apparent ($r = 0.32$; $P > 0.05$). Both CPP and 7-chlorokynurenic acid, when tested at high concentrations (100 and 1000 μM respectively), were able to inhibit completely specific binding of [^{3}H]-(+)-MK 801 (data not shown). Representative displacement curves for three brain regions are shown in Figures 2 and 3.

The inhibition obtained in the presence of 10 μM CPP or 100 μM 7-chlorokynurenic acid was reversed by 100 μM glutamate and 100 μM glycine, respectively (Table 3 and 4, Figure 4).

Discussion

[^{3}H]-(+)-MK 801 binding to rat brain membrane preparations has been shown to be a reliable index of the activity of the NMDA receptor complex (Huettner & Bean, 1988). This model can thus be useful for assessing the specificity and potency of drugs interacting on the NMDA receptor com-

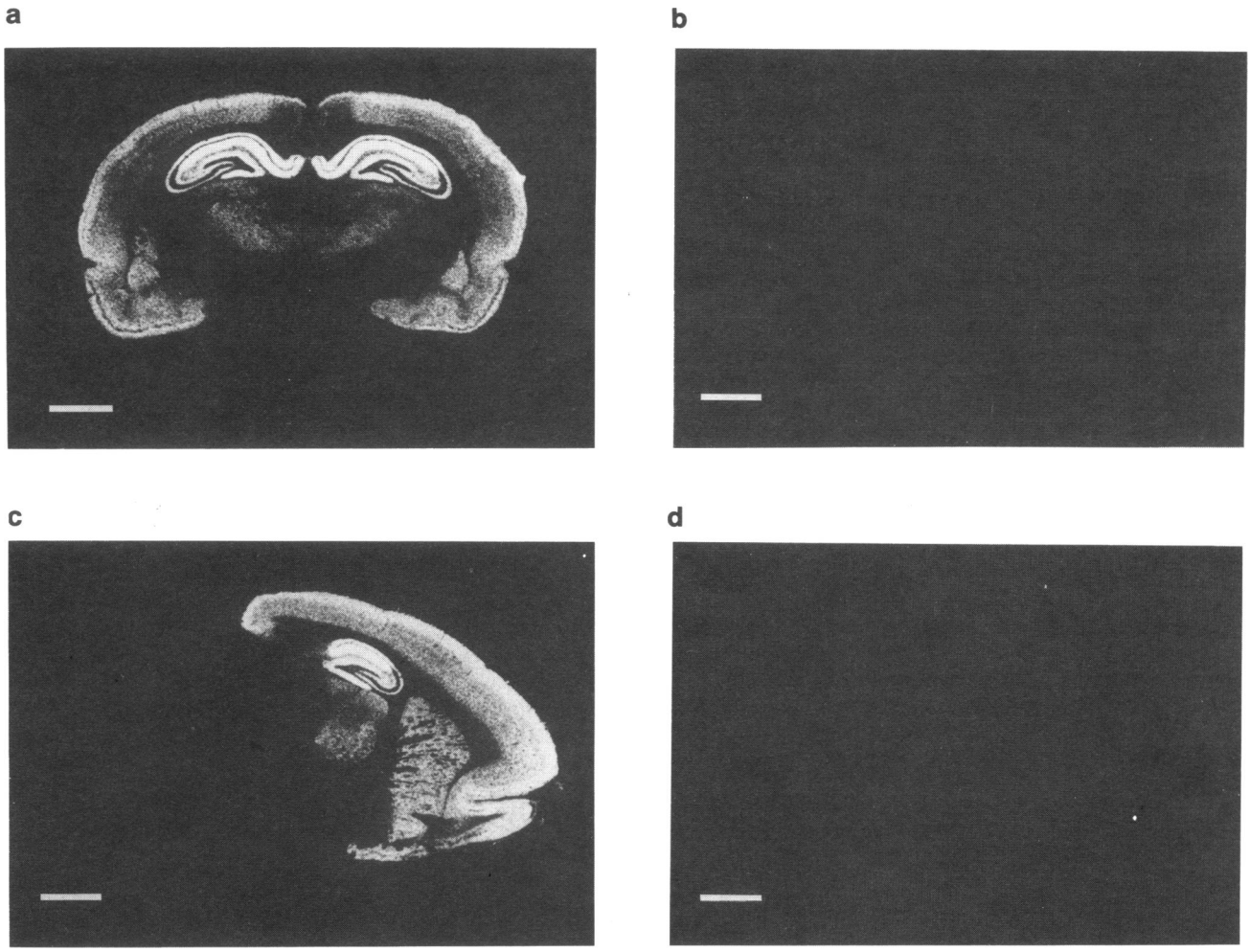


Figure 1 Distribution of [^{3}H]-(+)-MK 801 binding in rat brain sections. Coronal sections (corresponding approximately to Table 32 of Paxinos & Watson, 1982): (a) Total binding; (b) non specific binding in the presence of 100 μM (+)-MK 801. Parasagittal sections (corresponding approximately to Table 82 of Paxinos & Watson, 1982): (c) Total binding; (d) non specific binding in the presence of 100 μM (+)-MK 801. Calibration bar corresponds to 2.0 mm.

plex, either at the glutamate recognition site or at allosteric modulatory sites such as the glycine site or polyamine site. The regional distribution of [^{3}H]-(+)-MK 801 binding sites in our study confirms data previously reported using the same ligand (Bowery *et al.*, 1988) or [^{3}H]-*N*-(1-[2-thienyl] cyclohexyl)3,4-piperidine (TCP), a PCP-like drug that binds to the same recognition site as MK 801 and dissociative anaesthetics (e.g. ketamine) (Gundlach *et al.*, 1986; Hosford *et al.*, 1990). Confirming the results of these studies, we found that the areas of rat brain displaying the highest levels of binding were CA1 radiatum, the frontal cortex and the caudate putamen, areas in which NMDA sensitive [^{3}H]-glutamate binding is also high (Bowery, 1987). Binding in cerebellum was low. Although recent evidence has suggested a different type of NMDA receptor to be present in synaptic membranes isolated from rat cerebellum (Ebert *et al.*, 1991), the experimental procedure used in the present autoradiography study was not optimal for investigating binding in this area.

Binding of [^{3}H]-(+)-MK 801 to the brain regions studied was not enhanced by exogenously added glycine (10 μM), glutamate (10 μM), or the simultaneous administration of both amino acids (data not shown). This led us to believe that the endogenous concentrations of both amino acids are saturating in the present experimental conditions, despite the 60 min pre-incubation procedure.

In the present study the inhibitory actions of 7-chlorokynurenate and CPP on [^{3}H]-(+)-MK 801 binding have been studied by means of receptor autoradiography. These two compounds were chosen because of their respective potencies and selectivity toward the glycine and glutamate sites of the NMDA receptor complex: 7-chlorokynurenate selectively antagonizes [^{3}H]-glycine binding to strychnine-insensitive receptors (Kemp *et al.*, 1988), while CPP is a selective antagonist at the NMDA recognition site (Harris *et al.*, 1986). 7-Chlorokynurenate was able to inhibit [^{3}H]-(+)-MK 801 binding in a dose-dependent manner in all brain regions examined. That the inhibitory effect can be reversed by co-incubation with 100 μM glycine is further evidence for a direct interaction at the glycine recognition site (Sircar *et al.*, 1989) rather than a non-specific interference at the [^{3}H]-(+)-MK 801 binding site. CPP also inhibited in a concentration-dependent manner the binding of [^{3}H]-(+)-MK 801. The reversal by glutamate is consistent with an action at the NMDA recognition site. CPP inhibition of [^{3}H]-(+)-MK 801 binding to rat brain sections also confirms the previous finding that another NMDA antagonist, D-AP5 inhibits the basal binding of [^{3}H]-TCP (a PCP recognition site radioligand) in rat brain membrane preparations (Hosford *et al.*, 1990). In the present study, CPP was used in preference to D-AP5 as the glutamate site antagonist since the latter has been shown to have direct effects at the glycine recognition site (Danysz *et al.*,

Table 2 Regional inhibition of [^{3}H]-(+)-MK 801 binding by 7-chlorokynurenate (7-Cl-Kyn) and CPP

*Area	[a]CPP IC_{50} μM ± s.e.mean		[a]7-Cl-Kyn IC_{50} μM ± s.e.mean	
CA1 oriens	28.12	7.53	32.34	8.54
CA1 pyram.	20.27	5.73	23.61	8.14
CA1 radiatum	28.79	7.55	40.97	8.84
CA3 oriens	27.31	13.5	21.50	8.87
CA3 pyram.	13.49	1.89	16.63	8.07
CA3 radiatum	17.00	1.42	29.83	11.6
Mol	22.15	3.36	43.40	16.6
GRDG	12.70	1.88	27.03	9.58
Hif	15.73	2.18	30.03	11.4
S	17.72	8.03	15.17	6.57
LD	13.04	4.21	34.63	13.7
DLG	15.69	2.45	34.57	11.7
VPM	13.98	3.97	38.47	11.0
VPL	20.47	6.48	41.83	18.6
CPu	34.68	7.30	25.17	9.10
Fr Cx ext. 1.	21.08	3.43	21.47	7.00
Fr Cx med. 1.	12.70	0.45	24.27	6.72
Fr Cx int. 1.	15.62	2.87	18.40	6.15
Oc Cx ext. 1.	18.77	1.18	25.57	9.01
Oc Cx med. 1.	12.80	2.06	18.13	7.84
Oc Cx int. 1.	19.46	1.14	15.80	5.44
cc	ND		ND	
Cerebellum	ND		ND	
Brain stem	ND		ND	

Values represent the mean of 3 animals ($n = 3$) (6 determinations per section, 6 sections per animal).
*Abbreviations used are from Paxinos & Watson (1982).
ND = not detectable.
[a]Multiple t test revealed no significant differences between areas.

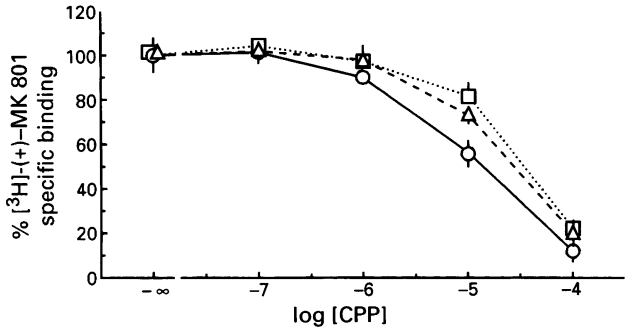


Figure 2 Inhibition of [^{3}H]-(+)-MK 801 specific binding to rat brain areas by ((+)-2-carboxypiperazine-4-yl) propyl-1-phosphonic acid (CPP): (○) frontal cortex medium layer; (□) caudate putamen; (△) CA1 radiatum layer. Bars represent s.e.mean ($n = 3$).

1989).

Analysis of regional IC_{50} values for 7-chlorokynurenate and CPP indicate that there are no significant differences in inhibitory potency between brain regions. In a recent autoradiography study, Monaghan (1991) has reported a significantly greater enhancement of [^{3}H]-(+)-MK 801 binding in the medial striatum and dorsal lateral septum compared to the internal and external layers of the cerebral cortex, while no significant differences were apparent in other brain regions examined. Our study did not include an examination of the septum and only the lateral regions of the caudate putamen which were revealed in sagital sections could be measured. Thus, we cannot exclude a differential effect of glutamate and glycine antagonists in these two brain regions.

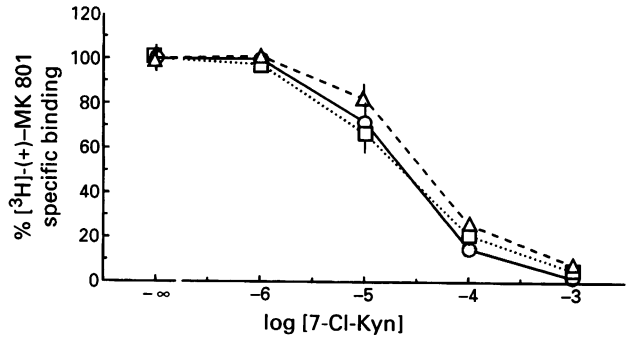


Figure 3 Inhibition of [^{3}H]-(+)-MK 801 specific binding to rat brain areas by 7-chlorokynurenate: (○) frontal cortex medium layer; (□) caudate putamen; (△) CA1 radiatum layer. Bars represent s.e.mean ($n = 3$).

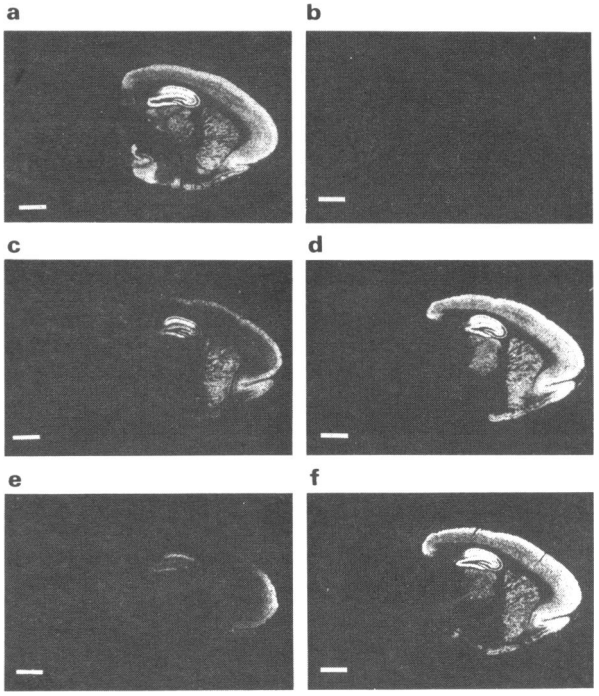


Figure 4 Reversal of [^{3}H]-(+)-MK 801 binding inhibition: (a) Total binding; (b) non specific binding; (c) + 10 μM ((+)-2-carboxypiperazine-4-yl) propyl-1-phosphonic acid (CPP); (d) + 10 μM CPP + 100 μM glutamate; (e) + 100 μM 7-chlorokynurenate (7-Cl-Kyn); (f) + 100 μM 7-Cl-Kyn + 100 μM glycine. Calibration bar corresponds to 2.0 mm.

On the basis of the present results, it is concluded that glycine as well as glutamate are obligatory requirements for the NMDA receptor to be activated, as reported in different experimental models (e.g. Kleckner & Dingledine, 1988). This would appear to be the case for all brain regions examined in the present study. In addition the similar sensitivities to the inhibitory action of 7-chlorokynurenate and CPP *in vitro* suggests that there are no regional differences between NMDA receptors, on the basis of their regulation by glycine and glutamate.

Table 3 CPP-induced inhibition of regional [^{3}H]-(+)-MK 801 binding and reversal by glutamate

*Area	*Total binding* (% ± s.e.mean)	+ *CPP* (10 μM) (% ± s.e.mean)	+ *CPP* (10 μM) + *glutamate* (100 μM) (% ± s.e.mean)
CA1 oriens	100 1.1	70.1* 1.5	92.5 6.6
CA1 pyram.	100 7.2	64.3** 5.9	89.9 7.7
CA1 radiatum	100 1.2	72.4** 3.3	95.0 1.6
CA3 oriens	100 1.9	66.3** 7.9	89.9 6.1
CA3 pyram.	100 3.1	60.9** 6.4	86.6 7.3
CA3 radiatum	100 5.8	64.5** 2.2	91.4 4.5
Mol	100 6.2	64.9** 3.8	96.2 2.2
GRDG	100 1.0	59.8** 4.7	107 8.4
Hif	100 3.9	59.1** 6.1	99.7 2.0
S	100 8.1	48.5** 2.5	85.7 3.7
LD	100 3.3	54.3** 8.1	90.0 3.8
DLG	100 0.8	59.9** 1.8	94.0 0.4
VPM	100 2.3	56.3** 5.2	85.6 3.4
VPL	100 3.6	70.2* 11.3	81.1 4.8
CPu	100 8.0	75.8* 1.5	87.0 4.8
Fr Cx ext. l.	100 1.4	68.2** 2.9	98.2 3.4
Fr Cx med. l.	100 8.4	56.7** 1.3	97.5 2.7
Fr Cx int. l.	100 2.2	64.2** 6.4	88.8 2.6
Oc Cx ext. l.	100 1.4	64.9** 1.5	92.1 2.1
Oc Cx med. l.	100 2.4	56.9** 3.5	92.0 0.3
Oc Cx int. l.	100 2.3	50.4** 2.4	89.3 7.3
cc	ND		
Cerebellum	ND		
Brain stem	ND		

Values represent the mean of 3 animals ($n = 3$) (6 determinations per section, 6 sections per animal).
*Abbreviations used are from Paxinos & Watson (1982).
ND = not detectable.
*$P < 0.05$; **$P < 0.01$; Student's t test.

Table 4 7-Chlorokynurenate (7-Cl-Kyn)-induced inhibition of regional [^{3}H]-(+)-MK 801 binding and reversal by glycine

*Area	*Total binding* (% ± s.e.mean)	+ *7-Cl-Kyn* (100 μM) (% ± s.e.mean)	+ *7-Cl-Kyn* (100 μM) + *glycine* (100 μM) (% ± s.e.mean)
CA1 oriens	100 8.8	22.4** 3.2	99.6 3.8
CA1 pyram.	100 2.2	17.2** 3.8	114.5 6.3
CA1 radiatum	100 2.8	26.4** 2.2	96.9 7.2
CA3 oriens	100 5.5	15.6** 3.8	112.1 4.9
CA3 pyram.	100 10.7	8.2** 5.5	107.5 12.9
CA3 radiatum	100 5.4	19.6** 3.6	110.5 5.5
Mol	100 2.8	21.0** 5.5	109.8 3.6
GRDG	100 4.8	19.4** 5.5	116.9 12.5
Hif	100 0.03	16.9** 6.1	94.3 2.8
S	100 4.0	12.2** 5.6	110.2 8.0
LD	100 1.8	21.9** 5.0	100.9 1.7
DLG	100 6.9	22.2** 5.7	116.3 3.7
VPM	100 4.3	27.6** 3.6	100.6 4.5
VPL	100 11.9	28.3** 5.8	113.6 3.7
CPu	100 6.4	20.9** 4.2	109.5 1.0
Fr Cx ext. l.	100 2.1	15.9** 2.0	102.5 1.5
Fr Cx med. l.	100 2.8	14.9** 2.9	107.7 2.8
Fr Cx int. l.	100 3.1	14.5** 2.0	106.7 4.7
Oc Cx ext. l.	100 3.9	20.7** 4.3	111.2 1.1
Oc Cx med. l.	100 4.9	14.7** 2.1	110.9 3.9
Oc Cx int. l.	100 3.3	12.2** 1.6	111.2 5.0
cc	ND		
Cerebellum	ND		
Brain stem	ND		

Values represent the mean of 3 animals ($n = 3$) (6 determinations per section, 6 sections per animal).
*Abbreviations used are from Paxinos & Watson (1982).
ND = not detectable.
*$P < 0.05$; **$P < 0.01$; Student's t test.

References

BOWERY, N.G. (1987). Glycine binding sites and NMDA receptors in brain. *Nature*, **326**, 338.

BOWERY, N.G., WONG, E.H.F. & HUDSON, A.L. (1988). Quantitative autoradiography of [^{3}H]-+-MK 801 binding sites in mammalian brain. *Br. J. Pharmacol*,. **93**, 944–954.

DANYSZ, W., FADDA, E., WROBLEWSKI, J.T. & COSTA, E. (1989). Kynurenate and 2-amino-5-phosphonovalerate interact with multiple binding sites of the N-methyl-D-aspartate-sensitive glutamate receptor domain. *Neurosci. Lett.*, **96**, 340–344.

DELEAN, A., MUNSON, P.J. & RODBARD, D. (1978). Simultaneous analysis of families of sigmoidal dose-response curves. *Am. J. Physiol.*, **235**, E97–E102.

EBERT, B., WONG, E.H.F. & KROGSGAARD-LARSEN, P. (1991). Identification of a novel NMDA receptor in rat cerebellum. *Eur. J. Pharmacol.-Mol. Pharmacol. Sect.*, **208**, 49–52.

FLOOD, J.F., BAKER, M.L. & DAVIS, J.L. (1990). Modulation of memory processing by glutamic acid receptor agonist and antagonists. *Brain Res.*, **521**, 197–202.

GUNDLACH, A.L., LARGENT, B.L. & SNYDER, S.H. (1986). Phencyclidine (PCP) receptors: autoradiographic localization in brain with the selective ligand [^{3}H]TCP. *Brain Res.*, **386**, 266–279.

HARRIS, E.W., GANONG, A.H. & COTMAN, C.W. (1984). Long term potentiation in the hippocampus involves activation of N-methyl-D-aspartate receptors. *Brain Res.*, **323**, 132–137.

HARRIS, E.W., GANONG, A.H., MONAGHAN, D.T., WATKINS, J.C. & COTMAN, C.W. (1986). Action of 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP): a new and highly potent antagonist of N-methyl-D-aspartate receptors in the hippocampus. *Brain. Res.*, **382**, 174.

HOOD, W.F., COMPTON, R.P. & MONAHAN, J.B. (1990). N-methyl-D-aspartate recognition site ligands modulate activity at the coupled glycine recognition site. *J. Neurochem*,. **54**, 1040–1046.

HOSFORD, D.A., BONHAUS, D.W. & MCNAMARA, J.O. (1990). A radiohistochemical measure of [^{3}H]TCP binding to the activated NMDA-receptor-gated ion channel in rat brain. *Brain Res.*, **516**, 192–200.

HUETTNER, J.E. & BEAN, B.P. (1988). Block of N-methyl-D-aspartate-activated current by the anticonvulsant MK 801: selective binding to open channels. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 1303–1311.

JAVITT, D.C. & ZUKIN, S.R. (1989). Interaction of [^{3}H]-MK 801 with multiple sites of NMDA receptor complex of rat brain. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 740–744.

JOHNSON, J.W. & ASCHER, P. (1987). Glycine potentiates the NMDA response in cultured mouse brain neurones. *Nature*, **325**, 529–531.

KEMP, J.A., FOSTER, A.C., LEESON, P.D., PRIESTLEY, T., TRIDGETT, R., IVERSEN, L.L. & WOODRUFF, G.N. (1988). 7-Chloro-kynurenic acid is a selective antagonist at the glycine modulatory site of the N-methyl-D-aspartate receptor complex. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 6547–6550.

KLECKNER, N.W. & DINGLEDINE, R. (1988). Requirement for glycine in activation of NMDA receptors expressed in *Xenopus* oocytes. *Science*, **241**, 835–837.

LOO, P., BRAUNWALDER, A., LEHMAN, J. & WILLIAMS, M. (1986). Radioligand binding to central phencyclidine recognition site is dependent on excitatory aminoacid receptor agonist. *Eur. J. Pharmacol.*, **123**, 467–468.

MCLENNAN, H. (1981). Receptors for the excitatory aminoacids in the mammalian central nervous system. *Prog. Neurobiol.*, **20**, 251–271.

MELDRUM, B.S., CROUCHER, M.J., BORDMAN, G. & COLLINS, J.F. (1983). Antiepileptic action of excitatory aminoacid antagonists in the photosensitive baboon *Papio papio*. *Neurosci. Lett.*, **39**, 101–104.

MONAGHAN, D.T. (1991). Differential stimulation of [^{3}H]-MK 801 binding to subpopulations of NMDA receptors. *Neurosci. Lett.*, **122**, 21–24.

MONAGHAN, D.T., BRIDGES, R.J. & COTMAN, C.W. (1989). The excitatory amino acid receptors: their classes, pharmacology and distinct properties in the function of the central nervous system. *Annu. Rev. Pharmacol. Toxicol.*, **29**, 365–402.

OLNEY, J.W. (1990). Excitotoxic aminoacids and neuropsychiatric disorders. *Annu. Rev. Pharmacol. Toxicol.*, **30**, 47–71.

PAXINOS, G. & WATSON, C. (1982). *The Rat Brain in Stereotaxic Coordinates*. Sydney: Academic Press.

REYNOLDS, I.J., MURPHY, S.N. & MILLER, R.J. (1987). [^{3}H]-labelled MK 801 binding to the excitatory aminoacid receptor complex from rat brain is enhanced by glycine. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 7744–7748.

ROTHMAN, S.M. & OLNEY, J.W. (1987). Excitotoxicity and the NMDA receptor. *Trend Neurosci.*, **10**, 299–302.

SIMON, R.P., SCHMIDLEY, J.W., MELDRUM, B.S., SWAN, J.H. & CHAPMAN, A.G. (1986). Excitotoxic mechanisms in hypoglycaemic hippocampal injury. *Neuropath. Appl. Neurobiol.*, **12**, 567–576.

SIMON, R.P., SWAN, J.H., GRIFFITHS, T. & MELDRUM, B.S. (1984). Blockade of N-methyl-D-aspartate receptors may protect against ischemic damage in the brain. *Science*, **226**, 850–852.

SIRCAR, R., FRUSCIANTE, M.J., JAVITT, D.C. & ZUKIN, S.R. (1989). Glycine reverses 7-Cl-kynurenic acid-induced inhibition of [^{3}H]-MK 801 binding. *Brain Res.*, **504**, 325–327.

TACCONI, S., RATTI, E., MARIEN, M.R., GAVIRAGHI, G. & BOWERY, N.G. (1991). Regional inhibition of [^{3}H]-(+)MK 801 binding in rat brain by CPP and 7-Cl-kynurenate: a receptor autoradiography study. *Br. J. Pharmacol.*, **102**, 62P.

WATKINS, J.C. & EVANS, R.H. (1981). Excitatory amino acid transmitters. *Annu. Rev. Pharmacol. Toxicol.*, **21**, 165–204.

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5-HT loss in rat brain following 3, 4-methylenedioxymethamphetamine (MDMA), *p*-chloroamphetamine and fenfluramine administration and effects of chlormethiazole and dizocilpine

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1 The present study has investigated whether the neurotoxic effects of the relatively selective 5-hydroxytryptamine (5-HT) neurotoxins, 3,4-methylenedioxymethamphetamine (MDMA or 'Ecstasy'), *p*-chloroamphetamine (PCA) and fenfluramine on hippocampal and cortical 5-HT terminals in rat brain could be prevented by administration of either chlormethiazole or dizocilpine.

2 Administration of MDMA (20 mg kg^{-1}, i.p.) resulted in an approximate 30% loss of cortical and hippocampal 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) content 4 days later. Injection of chlormethiazole (50 mg kg^{-1}) 5 min before and 55 min after the MDMA provided complete protection in both regions, while dizocilpine (1 mg kg^{-1}, i.p.) protected only the hippocampus.

3 Administration of a single dose of chlormethiazole (100 mg kg^{-1}) 20 min after the MDMA also provided complete protection to the hippocampus but not the cortex. This regime also attenuated the sustained hyperthermia (approx + 2.5°C) induced by the MDMA injection.

4 Injection of PCA (5 mg kg^{-1}, i.p.) resulted in a 70% loss of 5-HT and 5-HIAA content in hippocampus and cortex 4 days later. Injection of chlormethiazole (100 mg kg^{-1}, i.p.) or dizocilpine (1 mg kg^{-1}, i.p.) 5 min before and 55 min after the PCA failed to protect against the neurotoxicity, nor was protection afforded by chlormethiazole when a lower dose of PCA (2.5 mg kg^{-1}, i.p.) was given which produced only a 30% loss of 5-HT content. Chlormethiazole did prevent the hyperthermia induced by PCA (5 mg kg^{-1}), while the lower dose of PCA (2.5 mg kg^{-1}) did not produce a change in body temperature.

5 Neither chlormethiazole nor dizocilpine prevented the neurotoxic loss of hippocampal or cortical 5-HT neurones measured 4 days following administration of fenfluramine (25 mg kg^{-1}, i.p.).

6 In general, chlormethiazole and dizocilpine were effective antagonists of the 5-HT-mediated behaviours of head weaving and forepaw treading which appeared following injection of all three neurotoxins.

7 Both chlormethiazole and dizocilpine have previously been shown to prevent the neurotoxic effects of a high dose of methamphetamine on cerebral 5-HT and dopamine pathways. These drugs also prevent MDMA-induced neurotoxicity of 5-HT pathways, but not that induced by injection of PCA or fenfluramine. This suggests that the mechanisms of neurotoxic damage to 5-HT pathways produced by substituted amphetamines cannot be identical. The monoamine loss does not appear to result from the hyperthermia produced by the neurotoxic compounds.

Keywords: 5-Hydroxytryptamine; *p*-chloroamphetamine; fenfluramine; 3,4-methylenedioxymethamphetamine; neurotoxicity; hyperthermia; Ecstasy; neurodegeneration; chlormethiazole; dizocilpine

Introduction

Administration of several high doses of methamphetamine induces neurotoxicity and a subsequent marked decrease in dopamine (Koda & Gibb, 1973) and 5-hydroxytryptamine (5-HT) (Hotchkiss & Gibb, 1980) content in several regions of rodent brain. Evidence for the neuronal damage following methamphetamine has been obtained by use of biochemical (e.g. Stone *et al.*, 1986; 1988) and morphological (e.g. Jonsson & Nzanze, 1982; Ricuarto *et al.*, 1982) techniques and has been reviewed recently elsewhere (Gibb *et al.*, 1990).

Other substituted amphetamines are also neurotoxic when given either as a high single dose or several lower doses administered over a relatively short period of time. Some of these compounds are relatively selective neurotoxins of 5-HT pathways, particularly *p*-chloroamphetamine (Sanders-Bush *et al.*, 1972; Fuller *et al.*, 1975), fenfluramine (Harvey *et al.*, 1975; Neckers *et al.*, 1976) and 3, 4-methylenedioxymethamphetamine (Schmidt, 1987), the last named drug being of particular current interest because, under the name of 'Ecstasy' it has become a commonly used drug of abuse (for review see McKenna & Peroutka, 1990).

The loss in 5-HT content following all three of the more 5-HT selective neurotoxins mentioned above follows a two phase pattern, an initial rapid release (1–2 h following injection) followed by recovery by 24 h and then a long term decrease which is marked by 3–4 days and then lasts for several months. These changes have been reported for 3, 4-methylenedioxymethamphetamine (MDMA; Schmidt, 1987), *p*-chloroamphetamine (PCA; Sanders-Bush *et al.*, 1972; Fuller *et al.*, 1975; Neckers *et al.*, 1976) and fenfluramine (Harvey *et al.*, 1975; Neckers *et al.*, 1976). The second phase is marked by loss of monoamine content, and loss of tryptophan hydroxylase activity. Recent immunocytochemical and visualization studies have reported that neurodegenerative changes are seen in the second phase and include increased 5-HT axon calibre, huge swollen varicosities, frag-

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mentation and dilated proximal axon stumps with forebrain 5-HT axon terminals being lost (see review of Molliver et al., 1990). All indications are therefore that all three drugs, like methamphetamine (Ricuarto et al., 1982), produce neurodegeneration and that the biochemical markers reflect the neurodegenerative changes.

Recently Sonsalla and her colleagues provided evidence that excitatory amino acids were involved in methamphetamine-induced neurotoxicity since they found that dizocilpine (Sonsalla et al., 1989) and other competitive and non-competitive N-methyl-D-aspartate (NMDA) antagonists (Sonsalla et al., 1991) prevented methamphetamine-induced neurotoxicity of striatal dopamine terminals. Johnson et al. (1989) extended this work and showed that dizocilpine (or MK 801) also prevented the toxic effect of methamphetamine on striatal 5-HT content. Green et al. (1992) recently confirmed the protective effect of dizocilpine against methamphetamine-induced neurotoxicity of striatal dopamine and also showed that this drug protected against the neurotoxic loss of 5-HT pathways in the cortex and hippocampus. In addition, it was also shown that chlormethiazole was a very effective drug in protecting against methamphetamine-induced neurodegeneration of striatal dopamine and hippocampal and cortical 5-HT pathways (Green et al., 1992).

This work has now been extended to examine whether dizocilpine and chlormethiazole will protect against the loss of 5-HT content in the cortex and hippocampus produced by administration of PCA, MDMA and fenfluramine.

In general a dose of chlormethiazole of 50 mg kg^{-1}, i.p., was given since this afforded complete protection against methamphetamine-induced neurotoxicity (Green et al., 1992). However, it should be noted that this dose is near the ED_{50} of the drug to protect against ischaemia-induced neurodegeneration in the gerbil (Cross et al., 1991) and as an anticonvulsant drug (Green & Murray, 1989). The dizocilpine dose of 1 mg kg^{-1}, i.p., was again chosen because it was an effective dose against methamphetamine-induced toxicity (Sonsalla et al., 1989; Green et al., 1992) and is neuroprotective in the gerbil model of ischaemic neurodegeneration (Gill et al., 1987; Cross et al., 1991).

A preliminary account of some of the work has been given to the British Pharmacological Society (Colado et al., 1992).

Methods

Animals and drug administration

Adult male Lister Hooded rats (Harlan Olac, Bicester) weighing 200–300 g were used. They were housed in groups of 5 in conditions of constant temperature (21°C) and a 12 h light/dark cycle (lights on 07 h 00 min) and given free access to food and water.

All drugs were given i.p. and because various administration protocols were used, these are detailed in the results section. The rats were always killed 4 days following the drug administration for measurement of brain monoamine concentrations.

Measurement of monoamines

Rats were killed by cervical dislocation and decapitation, the brains removed and cortex and hippocampus dissected out.

Tissue was homogenized in perchloric acid (0.2 M), containing sodium metabisulphite (0.1%), cysteine (0.1%) and ethylenediamine tetraacetic acid (EDTA, 0.01%). Homogenates were centrifuged at 13,000 r.p.m. for 10 min in an Eppendorf bench centrifuge. A 50 µl sample of the supernatant was injected into the high performance liquid chromatography (h.p.l.c.) system.

The chromatographic system comprised as ACS isocratic pump and Rheodyne 7125 injector with 50 µl loop. Residual micro-particulate material was removed by use of guard column (20 × 4.6 mm, ODS, 5 µm). The analytical column was a reversed phase 150 × 4.6 mm, ODS1, 5 µm (Spherisorb) and detection was by a Coulochem electrochemical cell. The mobile phase (flow rate 1 ml min^{-1}) comprised sodium-1-octane sulphonic acid (0.75 mM), EDTA (0.1 mM), methanol (15%) and KH_2PO_4 (0.1 M), which had been adjusted to pH 3.0 with phosphoric acid, filtered and degassed. Standards contained known concentrations of 5-HT and 5-HIAA in the perchloric acid mixture.

Temperature measurement

Temperature was measured by use of a rectal probe inserted 2.5 cm and a thermocouple with digital readout.

Behavioural assessment

The two major components of the 5-HT behavioural syndrome, headweaving and forepaw treading, were scored as described by Deakin & Green (1978) on a severity score of 0–3 (0-absent, 1-just present, 2-definite, 3-severe) every 10 min for 80 min. The total scores of each animals have been reported. The same scoring system was used for evaluating the backward walking behaviour seen after fenfluramine. The observer was, where possible, blind to the treatment condition although in practice this was difficult given the sedative nature of chlormethiazole.

Drugs

Drugs were obtained from the following (sources in brackets): *p*-chloroamphetamine, fenfluramine and 3,4-methylenedioxymethamphetamine (all from Sigma Chemical Co., Poole): dizocilpine (Semat Technical (UK) Ltd, St. Albans), di-chlormethiazole ethane disulphonate (Astra Arcus, Södertälje, Sweden). Drugs were dissolved in saline (0.9% w/v NaCl), and all doses refer to the concentration of the base. All other chemicals were obtained from Sigma Chemical Co. or E. Merck (Dagenham).

Statistics

The effet of the neurotoxin on indole concentration has always been compared with the saline (control) treated group using an unpaired Student's *t* test. The effect of drugs on the neurotoxicity has been likewise compared with the drug alone group. Behavioural data have been evaluated by the Mann Whitney rank order 'U' test.

Results

Effect of chlormethiazole and dizocilpine on the loss of 5-HT and 5-HIAA in cortex and hippocampus caused by MDMA administration

Rats were injected with either saline, chlormethiazole (50 mg kg^{-1}) or dizocilpine (1 mg kg^{-1}). Five minutes later all groups were injected with MDMA (20 mg kg^{-1}). After a further 55 min, the groups were given a further injection of saline, chlormethiazole (50 mg kg^{-1}) or dizocilpine (1 mg kg^{-1}).

Administration of MDMA resulted in the appearance of a behavioural syndrome which included forepaw treading and headweaving. Pretreatment with chlormethiazole decreased the median 5-HT-mediated behavioural score, albeit not significantly (Table 1) also producing reduction in the locomotor activity for around 40 min after administration, while dizocilpine abolished both the behavioural changes and locomotion (Table 1). MDMA administration produced around a 30% decrease in hippocampal and cortical 5-HT and 5-HIAA (Table 2). Chlormethiazole when given pre- and post-MDMA injection afforded complete protection against the

Table 1 The effect of administration of 3,4-methylenedioxymethamphetamine (MDMA), *p*-chloroamphetamine (PCA) or fenfluramine on the 5-hydroxytryptamine (5-HT)-mediated behaviour and the effect on the behaviour of chlormethiazole or dizocilpine

			Median behavioural score		
Neurotoxin	*Behaviour*	Injected:	*Saline*	*Chlormethiazole*	*Dizocilpine*
MDMA	5-HT syndrome		15[−7, +3]	9[−5, +2]	0*
PCA	5-HT syndrome		25[−1, +4]	0*	3[−2, +3]*
Fenfluramine	5-HT syndrome		5[−2, +2]	0*	2[−2, +1]†
	Backward walking		13.5[−2.5, +3.5]	0*	0*

The behavioural score (see methods) of rats following administration of MDMA (20 mg kg^{-1}), PCA (5 mg kg^{-1}) of fenfluramine (25 mg kg^{-1}) are shown as a total in the 70 min following the injection of the neurotoxin as a median ± interquartile range ($n = 10$). Also shown is the score of animals given either dizocilpine (1 mg kg^{-1}) 5 min before and 55 min after the neurotoxin or chlormethiazole on the same time schedule at a dose of either 100 mg kg^{-1} (in the PCA experiment) or 50 mg kg^{-1} in the fenfluramine and MDMA experiments.
Different from saline injected groups: *$P < 0.002$; †$P < 0.05$ (Mann Whitney U test).

Table 2 The effect of chlormethiazole and dizocilpine on the neurotoxic action of 3, 4-methylenedioxymethamphetamine (MDMA) on cortical and hippocampal 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) concentrations

	Hippocampus		*Cortex*	
Injected	*5-HT*	*5-HIAA*	*5-HT*	*5-HIAA*
Saline	323 ± 9 (8)	325 ± 7 (8)	314 ± 14 (8)	218 ± 17 (8)
Chlormethiazole	369 ± 18 (6)	327 ± 16 (6)	314 ± 21 (6)	212 ± 11 (6)
Dizocilpine	371 ± 6 (6)	327 ± 13 (6)	323 ± 13 (6)	178 ± 5 (6)
MDMA	234 ± 14 (16)*	249 ± 13 (16)*	227 ± 16 (16)*	150 ± 7 (16)*
MDMA + chlormethiazole	325 ± 13 (6)	288 ± 16 (6)	294 ± 13 (5)	182 ± 10 (6)
MDMA + dizocilpine	325 ± 17 (4)	328 ± 12 (4)	260 ± 7 (4)‡	178 ± 4 (4)‡

The concentration of 5-HT and 5-HIAA in the hippocampus and cortex of rats 4 days following injection of MDMA (20 mg kg^{-1}) is shown in ng g^{-1} tissue (n), together with the effect of these parameters of injection of chlormethiazole (50 mg kg^{-1}) or dizocilpine (1 mg kg^{-1}) given 5 min before and 55 min after the MDMA.
*Significantly different from saline-injected control rats, $P < 0.01$. MDMA + chlormethiazole and MDMA + dizocilpine groups are not different from the appropriate control group in hippocampus. ‡Not different from MDMA alone group.

neurotoxic effect of MDMA in both brain regions (Table 2) while dizocilpine only protected the hippocampus.

In the next experiment we examined whether a single dose of chlormethiazole, given shortly after the MDMA would also be protective.

Rats were injected with MDMA (20 mg kg^{-1}) and 20 min later injected with a single dose of chlormethiazole (100 mg kg^{-1}). Injection of chlormethiazole at this time inhibited the 5-HT-mediated behaviour and locomotion but this was not quantified. Both groups were killed and regional brain indole concentrations examined 4 days later. Chlormethiazole was again protective in the hippocampus as indicated by the lack of 5-HT and 5-HIAA loss (Figure 1). The protection of the cortex was less clear. While the 5-HIAA concentration was not significantly different from control values in the MDMA/chlormethiazole treated rats, the 5-HT content was lower than saline-injected animals (Figure 1).

Effect of chlormethiazole and dizocilpine on the neurotoxic effects of PCA and fenfluramine

In the study on the neurotoxic effect of PCA, rats were injected with saline, chlormethiazole (100 mg kg^{-1}) or dizocilpine (1 mg kg^{-1}) followed 5 min later by PCA (5 mg kg^{-1}). After a further 55 min the animals were injected with another dose of the saline, chlormethiazole (100 mg kg^{-1}) or dizocilpine (1 mg kg^{-1}). In the case of fenfluramine, the protocol was essentially as above with the dose of fenfluramine being 25 mg kg^{-1}. However while the dizocilpine dose remained at 1 mg kg^{-1} the dose of chlormethiazole given was 50 mg kg^{-1} as the high dose of chlormethiazole was not well-tolerated in animals also given fenfluramine.

Administration of PCA produced clear and marked head weaving and forepaw treading which were abolished by chlormethiazole or markedly reduced by dizocilpine (Table 1). The behavioural changes seen following fenfluramine were rather different. There was a weak expression of head weav-

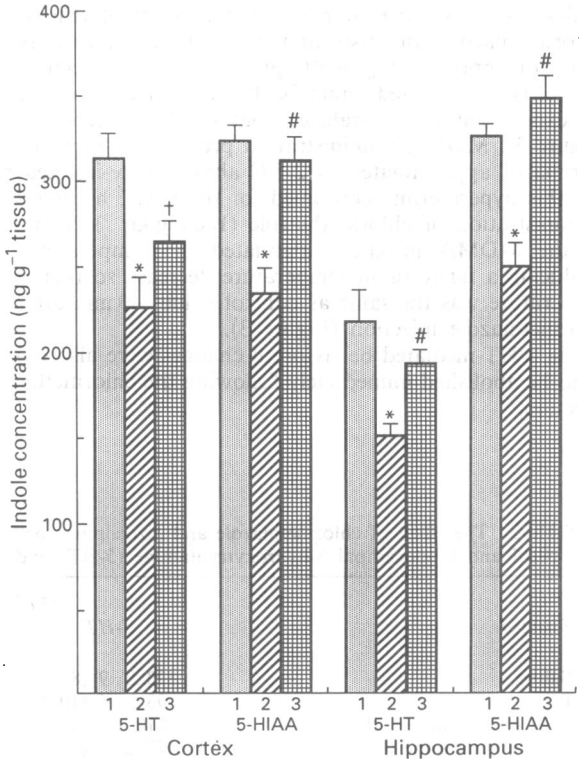


Figure 1 The effect of a single dose of chlormethiazole (100 mg kg^{-1}) given 20 min after 3, 4-methylenedioxymethamphetamine (MDMA, 20 mg kg^{-1}) on the 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) content of the rat hippocampus and cortex 4 days later: (1) Saline; (2) MDMA; (3) MDMA plus chlormethiazole. *Significantly different from saline injected control group, $P < 0.01$ or better; †significantly different from MDMA-treated group $P < 0.01$ or better; #not different from saline-treated group.

ing and forepaw treading (Table 1) which was reduced with dizocilpine and abolished by chlormethiazole but the predominant behaviour was backward-walking which was abolished by both drugs (Table 1).

Neither chlormethiazole nor dizocilpine protected against the neurotoxic consequences of fenfluramine or PCA in either brain region, as judged by the loss of 5-HT or 5-HIAA content (Table 3).

The decrease in 5-HT content produced by both fenfluramine and PCA was considerably greater than that seen after MDMA administration (Tables 2 and 3). In order therefore to confirm that the lack of protection was not merely because chlormethiazole and dizocilpine were ineffective against drugs producing a substantial monoamine loss, we next lowered the dose of PCA to achieve 5-HT losses similar to those seen after MDMA and mimic the MDMA study as closely as possible. Rats were injected with chlormethiazole (50 mg kg^{-1}) followed 5 min later by PCA (2.5 mg kg^{-1}) with a further dose of chlormethiazole (50 mg kg^{-1}) after a further 55 min. There was no indication for chlormethiazole producing any neuroprotection in this study (Figure 2).

The effect of chlormethiazole on the hyperthermia produced by MDMA administration

MDMA administration to rats results in marked hyperthermia (Nash *et al.*, 1988). We therefore examined whether this effect was altered by chlormethiazole injection. Rats were injected with saline or MDMA (20 mg kg^{-1}). Twenty minutes later both groups were injected with either saline or chlormethiazole (100 mg kg^{-1}), the protocol was thus exactly as described in the second neuroprotection experiment (results of which are shown in Figure 1).

The rectal temperature of the saline injected group declined modestly through the course of the experiment (Figure 3). Chlormethiazole administration produced a temperature decrease of approximately 0.5°C greater than that seen in the control (saline-injected) animals. The further decrease during the experiment thus paralleled that of the control animals (Figure 3). MDMA administration produced a rapid hyperthermia of approximately + 2.5°C above saline-injected rats and this hyperthermia continued for the next 3 h (Figure 3). Administration of chlormethiazole (100 mg kg^{-1}) 20 min after the MDMA injection attenuated the temperature rise producing a fairly rapid temperature decrease so that rectal temperature was the same as control values 40 min after the chlormethiazole injection (Figure 3).

The 5-HT-mediated behavioural changes were also observed to be abolished immediately following the chlormethiazole injection.

Effect of chlormethiazole on the hyperthermia produced by p-chloroamphetamine administration

Groups of rats were injected with either saline or chlormethiazole (50 mg kg^{-1}). Both groups were injected 5 min later with PCA (5 mg kg^{-1}). After a further 55 min the animals were injected with, saline or chlormethiazole (50 mg

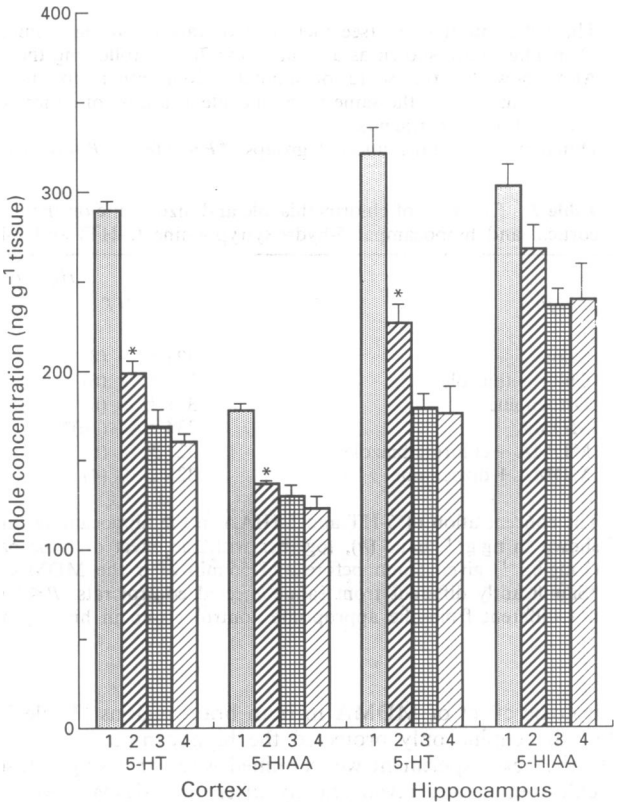


Figure 2 The effect of *p*-chloroamphetamine (PCA, 2.5 mg kg^{-1}) on the 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) content of hippocampus and cortex 4 days later, together with the lack of effect of chlormethiazole (50 mg kg^{-1}) or dizocilpine (1 mg kg^{-1}) given 5 min before and 55 min after the PCA injection: (1) Saline; (2) PCA; (3) PCA plus chlormethiazole; (4) PCA plus dizocilpine. *Significantly different from saline injected control group, $P<0.001$. The 5-HT and 5-HIAA concentrations in the chlormethiazole and dizocilpine-treated rats were not in general significantly different from the PCA alone groups, although hippocampal 5-HT values were lower than PCA alone group ($P<0.005$).

Table 3 The effect of chlormethiazole and dizocilpine on the neurotoxic effect of *p*-chloroamphetamine (PCA) and fenfluramine on cortical and hippocampal 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) concentrations

	Hippocampus		*Cortex*	
Injected	*5-HT*	*5-HIAA*	*5-HT*	*5-HIAA*
Saline	323 ± 9 (8)	325 ± 7 (8)	314 ± 14 (8)	218 ± 17 (8)
PCA	93 ± 5 (10)*	122 ± 6 (10)*	72 ± 3 (10)*	67 ± 5 (10)*
PCA + chlormethiazole	81 ± 1 (4)†	102 ± 12 (4)†	77 ± 3 (4)†	68 ± 8 (4)†
PCA + dizocilpine	77 ± 11 (3)†	138 ± 18 (3)†	76 ± 7 (3)†	82 ± 11 (3)†
Fenfluramine	97 ± 5 (9)*	138 ± 9 (10)*	76 ± 4 (10)*	79 ± 5 (10)*
Fenfluramine + chlormethiazole	82 ± 5 (6)†	92 ± 5 (6)†	59 ± 4 (6)†	52 ± 3 (6)†
Fenfluramine + dizocilpine	108 ± 13 (3)†	155 ± 23 (4)†	110 ± 20 (4)‡	84 ± 9 (4)†

The concentration of 5-HT and 5-HIAA in the hippocampus and cortex of rats 4 days following injection of either PCA (5 mg kg^{-1}) or fenfluramine (25 mg kg^{-1}) is shown in ng g^{-1} tissue (*n*), together with the effect on these parameters of injection of chlormethiazole (100 mg kg^{-1} in the PCA experiments; 50 mg kg^{-1} in the fenfluramine experiment) or dizocilpine (1 mg kg^{-1}) given 5 min before and 55 min after the neurotoxin.

*Significantly different from saline-injected control rats, $P<0.001$. †Not significantly different from neurotoxin alone treated group. ‡Different from neurotoxin alone group, $P<0.05$ or better.

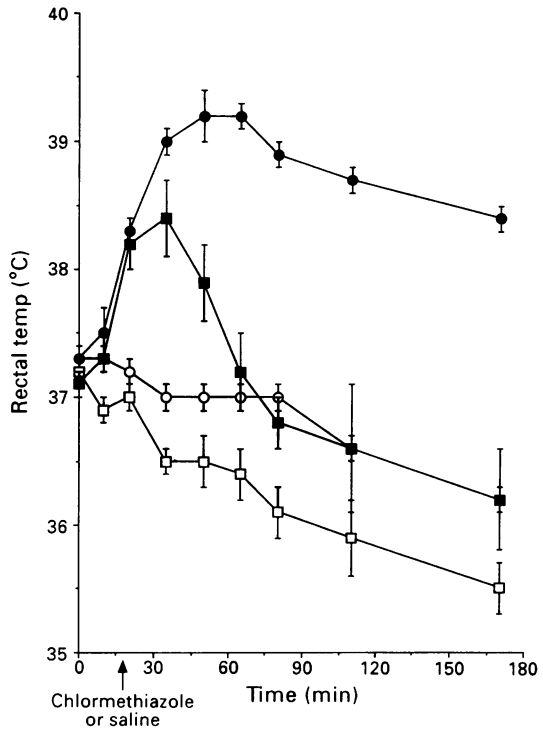


Figure 3 Rectal temperature in rats ($n = 5$) injected with saline (○); 3,4-methylenedioxymethamphetamine (MDMA), 20 mg kg^{-1} (●); chlormethiazole (CMZ), 100 mg kg^{-1} (□); MDMA followed 20 min later by CMZ (■). Temperatures shown immediately before the MDMA or saline injection with the time of the CMZ injection marked by the arrow. Results shown as mean ± s.e.mean (vertical bars).

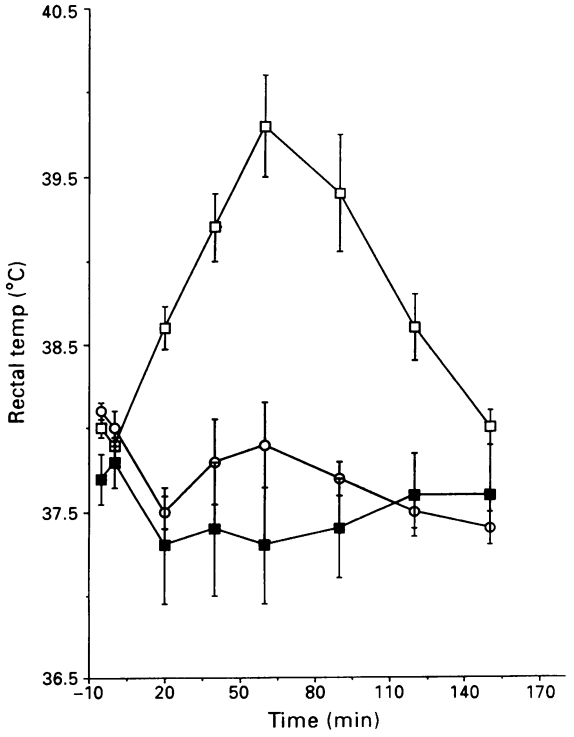


Figure 4 Rectal temperature in rats ($n = 5$) injected with *p*-chloramphetamine (PCA), 5 mg kg^{-1} (□); PCA 2.5 mg kg^{-1} (○) or PCA, 5 mg kg^{-1} with chlormethiazole 50 mg kg^{-1} being given 5 min before and 55 min after the PCA injection (■). Results shown as mean ± s.e.mean (vertical bars).

kg^{-1}) respectively. The dose protocol was exactly as in the neuroprotective study shown in Table 2. Administration of PCA (5 mg kg^{-1}) resulted in an increase of rectal temperature of around + 2.5°C, peaking 60 min after administration (Figure 4). Chlormethiazole administration abolished this hyperthermic response (Figure 4). The lower dose of PCA (2.5 mg kg^{-1}) which also produced monoamine loss (Figure 2) did not have a significant effect on rectal temperature (Figure 4).

Discussion

The current study demonstrates that chlormethiazole or dizocilpine when given immediately before and shortly after the MDMA injection both protect against the delayed neurotoxic loss of hippocampal 5-HT and 5-HIAA. Chlormethiazole, but not dizocilpine, also protected the cortex. Additionally it was demonstrated that a single dose of chlormethiazole, given shortly after the MDMA, provided total protection to the hippocampus and some protection to the cortex (Figure 1). These results are therefore somewhat analogous to our earlier observation that both chlormethiazole and dizocilpine protect both cortical and hippocampal 5-HT neurones from neurotoxic damage due to methamphetamine administration (Green *et al.*, 1992).

In contrast, neither chlormethiazole nor dizocilpine were able to prevent the neurotoxic damage to the cortex or hippocampus induced by injection of the neurotoxins PLA and fenfluramine. However, as the loss of 5-HT induced by PCA and fenfluramine was considerably greater (around 75%) than that seen following MDMA (around 30%) it seemed possible that chlormethiazole or dizocipline were unable to protect the brain from this considerable insult, but might protect if the 5-HT depletion were less, although this seemed unlikely given that both compounds were active against methamphetamine-induced toxicity when amine losses of around 75% were also observed (Green *et al.*, 1992). Since we found that chlormethiazole was also ineffective in protecting against the effects of a smaller dose of PCA which lowered cerebral 5-HT content by only 30% (and thus to the same degree as MDMA) our results suggest strongly that chlormethiazole is ineffective against PCA and fenfluramine-induced neurotoxicity.

It has recently been suggested that the mechanism of the initial 5-HT loss induced by injection of methamphetamine, MDMA, PCA and fenfluramine is the same (Berger *et al.*, 1992). Our data nevertheless indicate that the neurotoxic effects of these drugs are unlikely to be via a common mechanism given the protection from the neurotoxic effects of methamphetamine and MDMA by chlormethiazole and dizocilipine, but failure of these drugs to protect against the consequences of administration of PCA and fenfluramine. However, it is difficult to state this unequivocally given the current lack of information on other drugs that do, or do not, provide protection and the conflicting information in the literature concerning studies that have been performed.

While there is general agreement that dizocilpine protects against methamphetamine-induced loss of striatal dopamine and 5-HT in several brain receptors (Sonsalla *et al.*, 1989; 1991; Gibb *et al.*, 1990; Green *et al.*, 1992), there are conflicting data regarding the effect of N-methyl-D-aspartate (NMDA) antagonists on MDMA neurotoxicity. Johnson *et al.* (1989) have reported that dizocilpine does not protect against MDMA-induced toxicity of 5-HT, while Finnegan *et al.* (1990) have shown that these effects of MDMA are prevented by dextromethorphan. Our data are clearly consistent with those of Finnegan *et al.* (1990) since we found dizocilpine to be an effective protective agent against MDMA toxicity. However this same group has also reported that dextromethorphan (which also blocks neuronal cell damage induced by NMDA in cell cultures) prevented PCA-induced neurotoxicity *in vivo* in hippocampus and cortex (Finnegan *et*

al., 1991) while we found no neuroprotective effect of dizocilpine. Studies do not appear to have been made on the effect of dizocilpine on fenfluramine-induced neurotoxicity, although there is at least some evidence that long term effects of this compound on the 5-hydroxytryptaminergic system may differ from MDMA, PCA and methamphetamine (Schmidt et al., 1991).

The present experiments appear to rule out any direct relationship between the neurotoxic effects of the substituted amphetamines studied and their acute effects on either body temperature or behaviour. In the previous study (Green et al., 1992) a relationship between methamphetamine-induced hyperthermia and neurotoxicity appeared to be unlikely as the hyperthermia was antagonized by both chlormethiazole and diazepam, while only the former drug prevented neurotoxicity. The current study reinforces the view that body temperature and neurotoxicity are unrelated. MDMA (20 mg kg^{-1}) did produce a sustained hyperthermia of about 2.5°C above normal and this effect was antagonized by chlormethiazole given at a dose and administrative regime that was protective (Figures 1 and 3). However PCA (5 mg kg^{-1}) produced a similar degree of hyperthermia which chlormethiazole prevented, while not altering the neurotoxicity (Table 3 and Figure 4). Additionally the lower dose of PCA of 2.5 mg kg^{-1} had no effect on rectal temperature (Figure 4) but was neurotoxic (Figure 2).

Administration of PCA resulted in the appearance of the 5-HT-induced behavioural syndrome (for review of the syndrome, see Green & Heal, 1985), confirming several earlier reports (e.g. Green & Kelly, 1976; Curzon et al., 1979). These behaviours were antagonized by both chlormethiazole and dizocilpine, but the PCA-induced monamine loss still occurred. MDMA-treated animals also weakly displayed the 5-HT-mediated behavioural syndrome together with marked hyperactivity. Fenfluramine induced a rather different behavioural syndrome with a pronounced backward walking component, confirming the report of Curzon et al. (1979). These behaviours were again prevented by dizocilpine and chlormethiazole while the neurotoxicity still occurred. It was, however, difficult to assess accurately the behavioural changes since chlormethiazole was immediately sedative and dizocilpine itself induced many abnormal behaviours (see Tricklebank et al., 1989).

Recent work of Molliver et al. (1990) has demonstrated that the monoamines loss following PCA, MDMA and fenfluramine is associated with neurodegenerative changes. The preserved monoamine concentrations which occur in MDMA-treated rats given chlormethiazole and dizocilpine may therefore reflect a neuroprotective action of these compounds. While this cannot be stated with authority until morphological studies have been performed, it would not be surprising in view of the fact that both chlormethiazole and dizocilpine are effective neuroprotective agents in the gerbil model of global forebrain ischaemia (Gill et al., 1987; 1988; Cross et al., 1991).

Chlormethiazole and dizocilpine share a similar profile in protecting against neurotoxic damage induced by either methamphetamine or MDMA but not that produced by PCA or fenfluramine administration. It would seem likely therefore that the neurotoxicity induced by both MDMA and methamphetamine involves excitotoxic damage since dizocilpine is a non-competitive NMDA antagonist (Wong et al., 1986; Kemp et al., 1987). While there is no evidence for chlormethiazole acting at any site on the NMDA receptor complex (Cross et al., 1992), it is nevertheless an effective antagonist of NMDA-induced convulsions (Cross et al., 1992) so can clearly antagonize NMDA function in some way, possibly through potentiating γ-aminobutyric acid (GABA) function (Harrison & Simmonds, 1983; Cross et al., 1989; Green & Murray, 1989). One is also left with the conclusion that the neurotoxic damage produced to 5-HT pathways by PCA and fenfluramine is unlikely to involve NMDA receptor-induced changes.

There is reasonable evidence to support the proposition that dopamine release in the striatum occurs after administration of the neurotoxic compounds under study, and that prevention of this change can prevent the degeneration of 5-HT pathways not only in the striatum but also elsewhere in the brain (Schmidt et al., 1985; 1991; Schmidt & Kehne, 1990; Johnson et al., 1991). Nevertheless the current data indicate that this cascade of events cannot be identical in the case of PCA and fenfluramine, compared with MDMA and methamphetamine and also possibly amphetamine, given the recent report (Hemrick-Luecke et al., 1992) that dizocilpine prevents the neurotoxic effect of this compound on dopamine neurones.

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References

BERGER, U.V., GU, X.F. & AZMITIA, E.C. (1992). The substituted amphetamines 3, 4-methylenedioxymethamphetamine, methamphetamine, p-chloroamphetamine and fenfluramine induce 5-hydroxy-tryptamine release via a common mechanism blocked by fluoxetine and cocaine. *Eur. J. Pharmacol.*, **215**, 153–160.

COLADO, M.I., MURRAY, T.K. & GREEN, A.R. (1992). Prevention by chlormethiazole of some of the neurotoxic consequences of MDMA administration to rats. *Br. J. Pharmacol.*, **107**, 72P.

CROSS, A.J., JONES, J.A., BALDWIN, H.A. & GREEN, A.R. (1991). Neuroprotective action of chlormethiazole following transient forebrain ischaemia in the gerbil. *Br. J. Pharmacol.*, **104**, 406–411.

CROSS, A.J., MISRA, A., SNAPE, M.F., MURRAY, T.K. & GREEN, A.R. (1992). Chlormethiazole inhibits the effects of N-methyl-DL-aspartate *in vivo* but does not inhibit NMDA receptor binding. *Br. J. Pharmacol.*, **105**, 20P.

CROSS, A.J., STIRLING, J.M., ROBINSON, T.N., BOWEN, D.M., FRANCIS, P.T. & GREEN, A.R. (1989). The modulation by chlormethiazole of the $GABA_A$ receptor complex in rat brain. *Br. J. Pharmacol.*, **98**, 284–290.

CURZON, G., FERNANDO, J.C.R. & LEES, A.J. (1979). Backward walking and circling: behavioural responses induced by drug treatments which cause simultaneous release of catecholamines and 5-hydroxytryptamine. *Br. J. Pharmacol.*, **66**, 573–579.

DEAKIN, J.F.W. & GREEN, A.R. (1978). The effects of putative 5-hydroxytryptamine antagonists on the behaviour produced by administration of tranylcypromine and L-tryptophan or L-dopa to rats. *Br. J. Pharmacol.*, **64**, 201–209.

FINNEGAN, K.T., KERR, J.T. & LANGSTON, J.W. (1991). Dextromethorphan protects against the neurotoxic effects of p-chloroamphetamine in rats. *Brain Res.*, **558**, 109–111.

FINNEGAN, K.T., SKRATT, J.J., IRWIN, I. & LANGSTON, J.W. (1990). The N-methyl-D-aspartate (NMDA) receptor antagonist, dextrorphan, prevents the neurotoxic effects of MDMA in rats. *Neurosci. Lett.*, **105**, 300–706.

FULLER, R.W., PERRY, K.W. & MOLLOY, B.B. (1975). Reversible and irreversible phase of serotonin depletion by 4-chloroamphetamine. *Eur. J. Pharmacol.*, **33**, 119–124.

GIBB, J.W., JOHNSON, M., STONE, D. & HANSON, G.R. (1990). MDMA: historical perspectives. *Ann. N.Y. Acad. Sci.*, **600**, 601–611.

GILL, R., FOSTER, A.C. & WOODRUFF, G.N. (1987). Systemic administration of MK-801 protects against ischaemia-induced hippocampal neurodegeneration in the gerbil. *J. Neurosci.*, **7**, 3343–3349.

GILL, R., FOSTER, A.C. & WOODRUFF, G.N. (1988). MK-801 is neuroprotective in gerbils when administered during the post-ischaemic period. *Neuroscience*, **24**, 847–855.

GREEN, A.R. & HEAL, D.J. (1985). The effect of drugs on serotonin-mediated behavioural models. In *Neuropharmacology of Serotonin* ed. Green, A.R. pp. 326–365. Oxford: Oxford University Press.

GREEN, A.R. & KELLY, P.H. (1976). Evidence concerning the involvement of 5-hydroxytryptamine in the locomotor activity produced by amphetamine or tranylcypromine plus L-DOPA. *Br. J. Pharmacol.*, **57**, 141–147.

GREEN, A.R. & MURRAY, T.K. (1989). A simple intravenous infusion method in rodents for determining the potency of anticonvulsants acting through GABAergic mechanisms. *J. Pharm. Pharmacol.*, **41**, 879–880.

GREEN, A.R., DE SOUZA, R.J., WILLIAMS, J.L., MURRAY, T.K. & CROSS, A.J. (1992). The neurotoxic effects of methamphetamine on 5-hydroxytryptamine and dopamine in brain: evidence for the protective effect of chlormethiazole. *Neuropharmacology*, **31**, 315–321.

HARRISON, N.L. & SIMMONDS, M.A. (1983). Two distinct interactions of barbiturates and chlormethiazole with the GABA receptor complex in rat cuneate nucleus *in vitro*. *Br. J. Pharmacol.*, **80**, 387–394.

HARVEY, J.A. & MCMASTER, S.E. (1975). Fenfluramine: evidence for a neurotoxic action in midbrain and a long term depletion of serotonin. *Pharmacol. Commun.*, **1**, 217–228.

HEMRICK-LUECKE, S.K., HENDERSON, M.G. & FULLER, R.W. (1992). MK 801 antagonism of the prolonged depletion of striatal dopamine by amphetamine in iprindole-treated rats. *Life Sci.*, **50**, PL31–PL33.

HOTCHKISS, A. & GIBB, J.W. (1980). Long term effects of multiple doses of methamphetamine on tryptophan hydroxylase and tyrosine hydroxylase in rat brain. *J. Pharmacol. Exp. Ther.*, **214**, 257–262.

JOHNSON, M.P., HUANG, X. & NICHOLS, D.E. (1991). Serotonin neurotoxicity in rats after combined treatment with a dopaminergic agent followed by a non-neurotoxic 3, 4-methylenedioxymethamphetamine (MDMA) analogue. *Pharmacol. Biochem. Behav.*, **40**, 915–922.

JOHNSON, M., HANSON, G.R. & GIBB, J.W. (1989). Effect of MK 801 on the decrease in tryptophan hydroxylase induced by methamphetamine and its methylene dixoy analog. *Eur. J. Pharmacol.*, **165**, 315–318.

JONSSON, G. & NZANZE., E. (1982). Selective (+)-amphetamine neurotoxicity on striatal dopamine nerve terminals in the mouse. *Br. J. Pharmacol.*, **77**, 335–345.

KEMP, J.A., FOSTER, A.C. & WONG, E.H.F. (1987). Non-competitive antagonists of excitatory amino acid antagonists. *Trends Pharmacol. Sci.*, **10**, 294–298.

KODA, L.Y. & GIBB, J.W. (1973). Adrenal and striatal tyrosine hydroxylase activity after methamphetamine. *J. Pharmacol. Exp. Ther.*, **185**, 42–48.

MCKENNA, D.J. & PEROUTKA, S.J. (1990). Neurochemistry and neurotoxicity of 3, 4-methylenedioxymethamphetamine (MDMA, 'Ecstasy'). *J. Neurochem.*, **54**, 14–22.

MOLLIVER, M.E., BERGER, U.V., MAMOUNAS, L.A., MOLLIVER, D.L., O'HEARN, E. & WILSON, M.A. (1990). Neurotoxicity of MDMA and related compounds: anatomic studies. *Ann. N.Y. Acad. Sci.*, **600**, 640–661.

NASH, J.F., MELTZER, H.Y. & GUDELSKY, G.A. (1988). Elevation of serum prolactin and corticosterone concentrations in the rat after administration of 3, 4-methylenedioxymethamphetamine. *J. Pharmacol. Exp. Ther.*, **245**, 873–879.

NECKERS, N.M., BERTILSSON, L. & COSTA, E. (1976). The action of fenfluramine and p-chloroamphetamine on serotonergic mechanisms: a comparable study in rat brain nuclei. *Neurochem. Res.*, **1**, 29–35.

RICUARTO, G.A., GUILLERY, R.W., SEIDEN, L.S., SCHASTER, C.R. & MOORE, R.Y. (1982). Dopamine nerve terminal degeneration produced by high dose of methylamphetamine. *Brain Res,*. **235**, 93–103.

SANDERS-BUSH, E., BUSHING, J.A. & SULSER, F. (1972). Long term effects of p-chloroamphetamine on tryptophan hydroxylase activity and on the levels of 5-hydroxytryptamine and 5-hydroxyindole acetic acid in brain. *Eur. J. Pharmacol.*, **20**, 385–388.

SCHMIDT, C.J. (1987). Neurotoxicity of the psychedelic amphetamine, methylenedioxy methamphetamine. *J. Pharmacol. Exp. Ther.*, **240**, 1–7.

SCHMIDT, C.J., BLACK, C.K. & TAYLOR, V.L. (1991). L-Dopa potentiation of the serotonergic deficits due to a single administration of 3, 4-methylenedioxyamphetamine, p-chloroamphetamine or methamphetamine to rats. *Eur. J. Pharmacol.*, **203**, 41–49.

SCHMIDT, C.J. & KEHNE, J.H. (1990). Neurotoxicity of MDMA: neurochemical effects. *Ann. N.Y. Acad. Sci.*, **600**, 665–681.

SCHMIDT, C.J., RITTER, J.K., SONSALLA, P.K., HANSON, G.R. & GIBB, J.W. (1985). Role of dopamine in the neurotoxic effects of methamphetamine. *J. Pharmacol. Exp. Ther.*, **233**, 539–544.

SONSALLA, P.K., NICKLAS, W.J. & HEIKKILA, R.E. (1989). Role for excitatory amino acids in methamphetamine-induced nigrostriatal dopaminergic toxicity. *Science, Wash.*, **243**, 398–400.

SONSALLA, P.K., RIORDAN, D.E. & HEIKKILA, R.E. (1991). Competitive and non-competitive antagonists at N-methyl-D-aspartate receptors protect against methamphetamine-induced dopaminergic damage in mice. *J. Pharmacol. Exp. Ther.*, **256**, 506–512.

STONE, D.M., JOHNSON, M., HANSON, G.R. & GIBB, J.W. (1988). Role of endogenous dopamine in the central serotonergic deficits induced by 3, 4-methylenedioxymethamphetamine. *J. Pharmacol. Exp. Ther.*, **247**, 79–87.

STONE, D.M., STAHIL, D.C., HANSON, G.R. & GIBB, J.W. (1986). The effects of 3, 4-methylenedioxymethamphetamine (MDMA) and 3, 4-methylenedioxyamphetamine (MDA) on monoaminergic systems in the rat brain. *Eur. J. Pharmacol,*. **128**, 41–48.

TRICKLEBANK, M.D., SINGH, L., OLES, R.J., PRESTON, L. & IVERSEN, S.D. (1989). The behavioural effects of MK 801; a comparison with antagonists acting non-competively at the NMDA receptor. *Eur. J. Pharmacol,*. **167**, 127–135.

WONG, E.H.F., KEMP, J.A., PRIESTLY, T., KNIGHT, A.R., WOODRUFF, G.N. & IVERSEN, L.L. (1986). The anticonvulsant MK 801 is a potent N-methyl-D-aspartate antagonist. *Proc. Natl. Acad. Sci. USA*, **83**, 7104–7108.

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Striatal dopamine release *in vivo* following neurotoxic doses of methamphetamine and effect of the neuroprotective drugs, chlormethiazole and dizocilpine

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1 Administration to rats of methamphetamine (15 mg kg^{-1}, i.p.) every 2 h to a total of 4 doses resulted in a neurotoxic loss of striatal dopamine of 36% and of 5-hydroxytryptamine (5-HT) in the cortex (43%) and hippocampus (47%) 3 days later.

2 Administration of chlormethiazole (50 mg kg^{-1}, i.p.) 15 min before each dose of methamphetamine provided complete protection against the neurotoxic loss of monoamines while administration of dizocilpine (1 mg kg^{-1}, i.p.) using the same dose schedule provided substantial protection.

3 Measurement of dopamine release in the striatum by *in vivo* microdialysis revealed that methamphetamine produced an approximate 7000% increase in dopamine release after the first injection. The enhanced release response was somewhat diminished after the third injection but still around 4000% above baseline. Dizocilpine (1 mg kg^{-1}, i.p.) did not alter this response but chlormethiazole (50 mg kg^{-1}, i.p.) attenuated the methamphetamine-induced release by approximately 40%.

4 Dizocilpine pretreatment did not influence the decrease in the dialysate concentration of the dopamine metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) produced by administration of methamphetamine while chlormethiazole pretreatment decreased the dialysate concentration of these metabolites still further.

5 The concentration of dopamine in the dialysate during basal conditions increased modestly during the course of the experiment. This increase did not occur in chlormethiazole-treated rats. HVA concentrations were unaltered by chlormethiazole administration.

6 Chlormethiazole (100–1000 μM) did not alter methamphetamine (100 μM) or K^+ (35 mM)-evoked release of endogenous dopamine from striatal prisms *in vitro*.

7 Several NMDA antagonists prevent methamphetamine-induced neurotoxicity; however chlormethiazole is not an NMDA antagonist. Inhibition of striatal dopamine function prevents methamphetamine-induced toxicity of both dopamine and 5-HT pathways. Therefore the attenuation of the enhanced dopamine release which occurs in animals given chlormethiazole may be associated with the protective action of this drug against methamphetamine-induced neurotoxicity.

Keywords: Dopamine release *in vivo*; chlormethiazole; dizocilpine; methamphetamine; neurotoxicity; 5-hydroxytryptamine; dopamine; *in vivo* microdialysis; striatum

Introduction

It has been known for some time that large doses of methamphetamine are neurotoxic and result in a long term loss of dopamine content in the striatum (Koda & Gibb, 1973). Subsequent research established that 5-hydroxytryptamine (5-HT) is also lost, not only in the striatum (Hotchkiss & Gibb, 1980), but also in cortex and hippocampus (see Gibb *et al.*, 1990). Immunocytochemical and visualisation studies have indicated that the loss of biochemical markers such as enzyme activity and monoamine content reflect neurodegenerative changes that have occurred (Ricuarto *et al.*, 1982; Molliver *et al.*, 1990).

Recently Sonsalla *et al.* (1989) demonstrated that the non-competitive N-methyl-D-aspartate antagonist, dizocilpine, protected dopaminergic neurones in the striatum from methamphetamine-induced neurotoxicity and this work was recently extended to show that this protective property was shared by other competitive and non-competitive NMDA antagonists (Sonsalla *et al.*, 1991). Dizocilpine has also been found to prevent the toxic effects of methamphetamine on striatal 5-HT systems (Johnson *et al.*, 1989).

Green *et al.* (1992) confirmed the protective effect of dizocilpine against methamphetamine-induced loss of striatal dopamine and extended the work of Johnson *et al.* (1989) to demonstrate that dizocilpine would also protect against 5-HT loss in the hippocampus and cortex. However this study also demonstrated that chlormethiazole was a very effect protective agent against methamphetamine-induced toxicity to both dopamine in the striatum and 5-HT in cortex and hippocampus (Green *et al.*, 1992).

While there is now considerable evidence to show that chlormethiazole potentiates the action of γ-aminobutyric acid (GABA) by interacting with the $GABA_A$ receptor complex (Harrison & Simmonds, 1983; Ogren, 1986; Cross *et al.*, 1989; Moody & Skolnick, 1989; Vincens *et al.*, 1989) there is no evidence that the drug interacts directly with the NMDA receptor complex (Cross *et al.*, 1992b). The protective action of chlormethiazole could not therefore be ascribed to a direct action involving decreased NMDA-receptor-mediated events.

It is well established that inhibiting dopamine function in the striatum either with dopamine antagonists (Buening & Gibb, 1974) or dopamine synthesis inhibitors (Gibb & Kogan, 1979) prevents methamphetamine-induced neurotoxicity of striatal dopamine and 5-HT (Schmidt *et al.*, 1985). Therefore, using a methamphetamine dose paradigm which we show produces neurotoxicity, we have examined whether protective doses of chlormethiazole and dizocilpine alter

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dopamine release by using *in vivo* microdialysis in the striatum. This has not been previously examined in the case of chlormethiazole, while such data as exist with dizocilpine have been conflicting, with Weihmuller *et al.* (1991; 1992) reporting that the drug attenuated dopamine release and Kashihara *et al.* (1991) finding no alteration of release. In addition we have examined the concentration of the major dopamine metabolites in the dialysate and the effect of chlormethiazole on methamphetamine- and potassium-evoked release of dopamine *in vitro* using striatal slices.

The doses of protective drugs given in the current study were 50 mg kg^{-1} i.p. for chlormethiazole and 1 mg kg^{-1} i.p. for dizocilpine. These doses have previously been shown to be effective against neurotoxic damage induced by methamphetamine in this laboratory (Green *et al.*, 1992). They are also effective doses in protecting against neurotoxicity produced by injection of 3,4-methylenedioxymethamphetamine (MDMA or 'Ecstasy') (Colado *et al.*, 1993) and ischaemia-induced neurodegeneration (Gill *et al.*, 1987; Cross *et al.*, 1991).

A preliminary account of some of this work has been reported to the British Pharmacological Society (Baldwin *et al.*, 1992).

Methods

Animals and drug treatment

Adult male Lister Hooded rats (Harlan Olac, Bicester) weighing 250–350 g were used. They were housed in groups of 5 in conditions of constant temperature (21°C) and controlled lighting (light period: 07 h 00 min–19 h 00 min) and given free access to food and water.

Neurotoxicity was induced by the injection of methamphetamine (15 mg kg^{-1}, i.p.) at 2 h intervals to a total of four injections. In the studies on the effects of neuroprotective drugs, chlormethiazole (50 mg kg^{-1}, i.p.) or dizocilpine (1 mg kg^{-1}, i.p.) were given 15 min before each dose of methamphetamine, control rats being given saline (NaCl 0.9% w/v) in place of the neuroprotective drug.

Measurement of monoamines and metabolites

Three days after methamphetamine administration, rats were killed by cervical dislocation, brains removed and cortex, hippocampus and striatum dissected out. Subsequent measurement of the monoamines (dopamine and 5-hydroxytryptamine) and their metabolites: 5-hydroxyindoleacetic acid (5-HIAA), dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) was by high performance liquid chromatography (h.p.l.c.) with electrochemical detection as described previously (Green *et al.*, 1992).

Implantation of dialysis probes

Rats were implanted with microdialysis probes (3.5 mm × 200 μm; Hospal) in the striatum as described by Baldwin *et al.* (1991). On the following day probes were perfused with artificial cerebrospinal fluid (aCSF) at 1 μl min^{-1} and samples were collected from the freely moving animals at 20 min intervals (Baldwin *et al.*, 1991). The 'dead space' volume of the system was approximately 10 μl. This means that there was an approximate 10 min delay between any response and its detection. Samples were stored at −70°C until analysis for dopamine, DOPAC and HVA by h.p.l.c. with electrochemical detection (Green *et al.*, 1992).

In the study on basal concentrations of dopamine and its metabolites, dopamine concentrations were some 200 times lower than DOPAC. There was therefore marked interference with the dopamine peak unless the DOPAC was eliminated with the solvent front. It was felt that dopamine was the important measure in the study and DOPAC could not therefore be determined.

Measurement of dopamine release from tissue slices

The effect of chlormethiazole on endogenous dopamine release from rat brain tissue prisms was performed by a modification of the method of Auerbach & Lipton (1985) as described in detail by Robinson *et al.* (1989). Briefly, tissue prisms (300 × 300 μm, 45°C) were prepared by use of a McIlwain tissue chopper. Slices were suspended in Krebs-Ringer bicarbonate buffer containing nomifensine (10 μM) and pargyline (10 μM). Tissue suspension was distributed between plastic test tubes with nylon mesh bases. These baskets were incubated in vials containing buffer (basal release) followed by vials containing KCl (35 mM) for K^+-evoked release or methamphetamine (100 μM). Supernatants from the incubation were analysed for dopamine content. In the current study basal release (absence of K^+ or methamphetamine) was measured during a 2 min rather than 5 min period as in the study by Robinson *et al.* (1989).

Drugs

Drugs were obtained from the following (sources in parentheses): (+)-methamphetamine (Sigma Chemical Co, Poole), dichlormethiazole ethanedisulphonate (Astra Arcus, Södertälje, Sweden), dizocilpine hydrogen maleate (Semat Technical (UK) Ltd, St Albans). All other chemicals were obtained from Sigma Chemical Co. or Merck Ltd, Dagenham.

Drugs were dissolved in saline (0.9% w/v NaCl) and all doses refer to the concentration of the base.

Statistics

In vivo dialysis data were analysed by Analysis of Variance (ANOVA). The effects of methamphetamine on dialysate concentrations of dopamine, DOPAC and HVA were determined by separate one-way ANOVAs with 'time' as the repeated measure. The effects of chlormethiazole and dizocilpine on the basal and methamphetamine-induced concentrations of dopamine, DOPAC and HVA were determined by separate two-way ANOVAs with 'drug' as the between groups factor and 'time' as the repeated measure. Comparison of cerebral concentrations of monoamines and their metabolites following drug treatments was by Student's *t* test (unpaired).

Results

Effect of repeated doses of methamphetamine on monoamine content of striatum, hippocampus and cortex and effect of chlormethiazole or dizocilpine pretreatment

Rats were injected with methamphetamine (15 mg kg^{-1}, i.p.) every 2 h to a total of four doses. Three days later the striatal dopamine content was decreased by 36% and the 5-HT content decreased in both hippocampus and cortex by 47% and 43% respectively (Table 1). The concentration of the major 5-HT metabolite, 5-HIAA, was similarly decreased in both regions (Table 1).

Administration of chlormethiazole (50 mg kg^{-1}, i.p.) or dizocilpine (1 mg kg^{-1}, i.p.) every 2 h to a total of four doses did not alter either striatal dopamine content or the indole content in hippocampus or cortex 3 days later except for a modest rise in hippocampal 5-HT following dizocilpine (Table 1). However, administration of chlormethiazole (50 mg kg^{-1}) 15 min before each dose of methamphetamine totally prevented the catecholamine and indoleamine loss (Table 1). Dizocilpine (1 mg kg^{-1}) given 15 min before each dose of methamphetamine, was a reasonably effective protec-

Table 1 The effect of methamphetamine (15 mg kg^{-1}) given 4 times at 2 hourly intervals on brain monoamine concentrations 3 days later and the effects of chlormethiazole (50 mg kg^{-1}) or dizocilpine (1 mg kg^{-1}) given 15 min before each methamphetamine dose

Injected	*Striatum* dopamine	*Hippocampus* 5-HT	*Hippocampus* 5-HIAA	*Cortex* 5-HT	*Cortex* 5-HIAA
Saline	5776 ± 289 (8)	294 ± 12 (8)	355 ± 15 (8)	261 ± 12 (8)	181 ± 8 (8)
Chlormethiazole	6300 ± 463 (8)	321 ± 22 (7)	349 ± 13 (8)	276 ± 13 (8)	186 ± 7 (8)
Dizocilpine	6751 ± 2161 (8)	379 ± 13 (8)‡	335 ± 7 (8)	283 ± 9 (8)	178 ± 4 (8)
Methamphetamine	3721 ± 317 (4)†	155 ± 16 (4)†	201 ± 17 (4)†	150 ± 9 (4)†	104 ± 6 (4)†
Methamphetamine + chlormethiazole	5983 ± 346 (7)	312 ± 20 (7)	354 ± 21 (7)	243 ± 13 (7)	184 ± 12 (7)
Methamphetamine + dizocilpine	6518 ± 231 (6)	277 ± 21 (7)*ø	252 ± 14 (7)*	208 ± 15 (6)*ø	134 ± 8 (7)*ø

Results shown as mean ± s.e.mean (*n*) ng g^{-1} tissue (wet weight).
Different from saline injected; †$P < 0.001$; ‡$P < 0.01$. Different from dizocilpine injected; *$P < 0.001$. Different from methamphetamine injected; ø$P < 0.03$ or better.

tive agent but, unlike chlormethiazole did not always produce monoamine concentrations in the brain of methamphetamine-treated rats that were not different from control values (Table 1).

Effect of chlormethiazole and dizocilpine on methamphetamine-induced release of dopamine in the striatum

Three baseline samples of dialysate were collected from all rats. One group was then injected with saline followed 15 min later by methamphetamine (15 mg kg^{-1}). This procedure was repeated two further times at 2 h intervals. Further groups were injected with chlormethiazole (50 mg kg^{-1}) or dizocilpine (1 mg kg^{-1}) 15 min before each dose of methamphetamine and the procedure repeated twice more at 2 h intervals. Sample collections were made over an 8 h period.

The first doses of methamphetamine produced a massive release of dopamine of somewhat greater than 7000% above baseline (Figure 1). At the end of 2 h the dialysate dopamine was still more than 4000% above baseline. The second methamphetamine injection produced another peak which was similar to the first. The third injection however produced a somewhat smaller peak, albeit still around 4500% above basal (Figure 1).

Pretreatment with dizocilpine before each dose of methamphetamine did not influence the pattern of dopamine release (Figure 1). Chlormethiazole pretreatment did not alter the first peak concentration but did produce a more rapid return towards baseline and a lower dopamine dialysate concentration than the methamphetamine alone group (Figure 1). The second peak was attenuated and the third methamphetamine-induced peak nearly abolished (Figure 1). Statistical analysis indicated that chlormethiazole had markedly inhibited the dopamine release ($P < 0.0001$, see legend to Figure 1). Whilst not quantified, observation of the behaviour of the animals during the dialysis experiment was undertaken. Injection of methamphetamine led to prolonged and severe locomotor activity which was diminished by the time the next dose was given. Both chlormethiazole and dizocilpine abolished the behavioural excitation, the animals being apparently sedated most of the time. Some behavioural excitation was beginning to appear in the 20 min before the next injection of chlormethiazole or dizocilpine.

Effect of methamphetamine on dialysate HVA and DOPAC concentration and the effect of chlormethiazole and dizocilpine

The first methamphetamine injection resulted in a marked decrease in dialysate DOPAC concentration (Figure 2a) and a modest decrease in HVA concentration (Figure 2b). The diminished concentration of these metabolites continued throughout the collection period with the subsequent methamphetamine injections not inducing any further major effects (Figure 2). Pretreatment with dizocilpine before each methamphetamine injection did not influence the HVA or DOPAC concentration at all (Figure 2). Chlormethiazole pretreatment however produced a further decrease in the dialysate concentration of both HVA and DOPAC compared with methamphetamine (Figure 2).

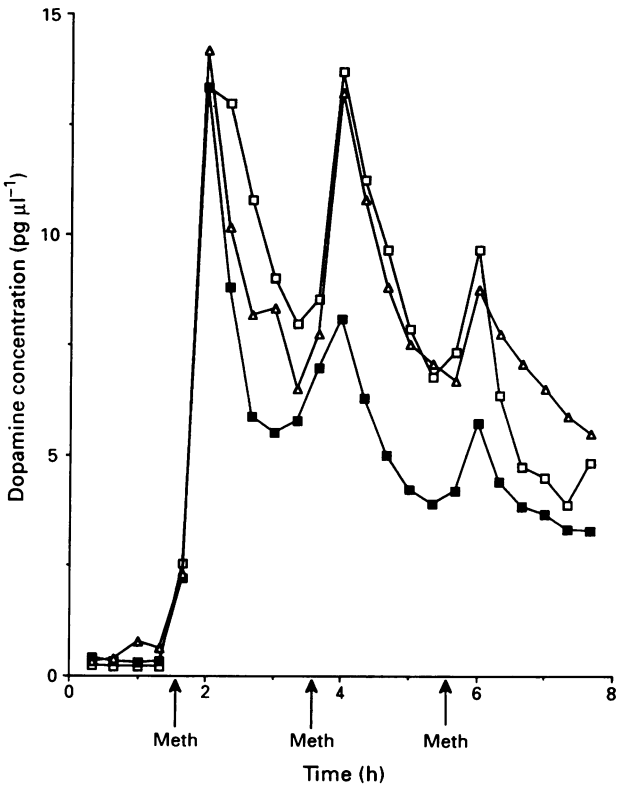


Figure 1 Effect of chlormethiazole (CMZ; 50 mg kg^{-1}, i.p.) and dizocilpine (Diz; 1 mg kg^{-1}, i.p.) given 15 min before each dose of methamphetamine (Meth; 15 mg kg^{-1}, i.p.) on dopamine release in the striatum: (□) Meth; (■) Meth/CMZ; (△) Meth/Diz. Values are mean dopamine concentrations in the dialysate (pg µl^{-1}). There was a highly significant effect of Meth on dopamine release (one way ANOVA F (18, 36) = 24.2, $P < 0.0001$). A separate 2-way ANOVA on the dopamine concentrations following the first methamphetamine injection only showed that there was no effect of CMZ on this peak. However, ANOVA on the remaining data showed that CMZ significantly prevented the dopamine release following the 2nd and 3rd Meth injections (2 way ANOVA 'time' × 'CMZ' interaction F (9, 54) = 2.8, $P < 0.01$). Dizocilpine had no effect on dialysate dopamine concentrations. Mean ± s.e.mean basal concentrations were: Meth 0.22 ± 0.05 (n = 4); Meth/CMZ 0.36 ± 0.05 (n = 5); Meth/Diz 0.35 ± 0.09 (n = 5).

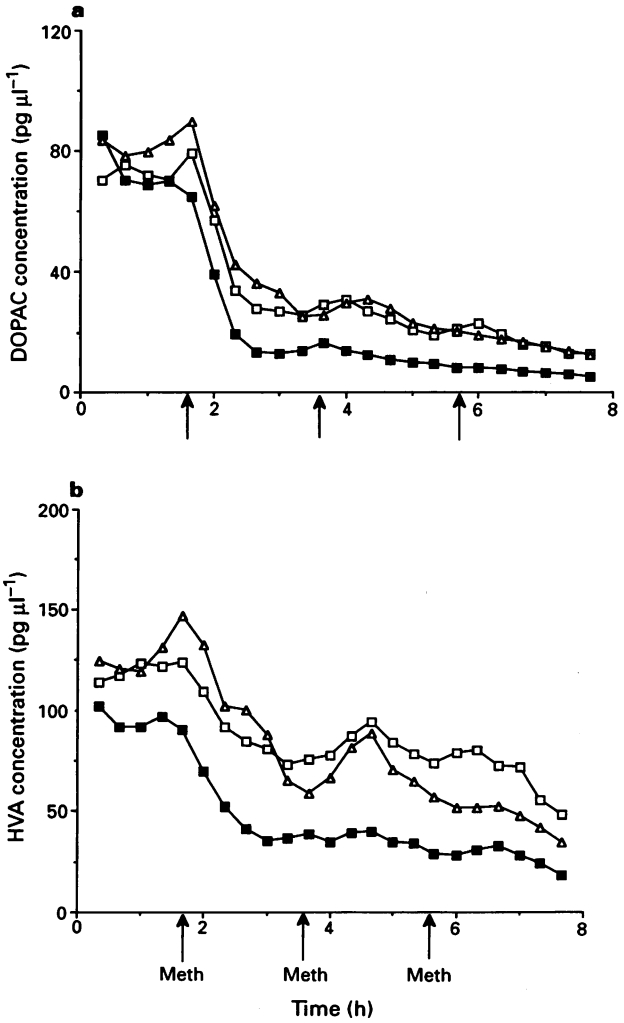


Figure 2 (a) Effect of chlormethiazole (CMZ; 50 mg kg^{-1}, i.p.) and dizocilpine (Diz; 1 mg kg^{-1}, i.p.) on dihydroxyphenylacetic acid (DOPAC) concentrations in the striatum following methamphetamine (Meth; 15 mg kg^{-1}, i.p.): (□) Meth; (■) Meth/CMZ; (△) Meth/Diz. Values are mean DOPAC concentrations in the dialysate (pg µl^{-1}). There was a significant reduction in DOPAC concentrations following methamphetamine administration (one-way ANOVA F (19, 59) = 58.6, $P < 0.0001$). Chlormethiazole further reduced DOPAC concentrations (two-way ANOVA 'CMZ' F (1, 6) = 13.3, $P < 0.05$) whilst dizocilpine had no effect on DOPAC levels in the dialysate. Mean ± s.e.mean basal concentrations were: Meth 72.45 ± 2.22 (n = 4); Meth/CMZ 69.45 ± 3.76 (n = 5); Meth/Diz 80.56 ± 5.82 (n = 5). (b) Effect of chlormethiazole (CMZ; 50 mg kg^{-1}, i.p.) and dizocilpine (Diz; 1 mg kg^{-1}, i.p.) on homovanillic acid (HVA) concentrations in the striatum following methamphetamine (Meth; 15 mg kg^{-1}, i.p.): (□) Meth; (■) Meth/CMZ; (△) Meth/Diz. Values are mean HVA concentrations in the dialysate (pg µl^{-1}). There was a significant reduction in HVA concentrations following methamphetamine administration (one-way ANOVA F (19, 38) = 2.0, $P < 0.05$). Chlormethiazole further reduced HVA concentrations (two-way ANOVA 'CMZ' F (1, 6) = 11.3, $P < 0.05$) whilst dizocilpine had no effect on HVA levels in the dialysate. Mean ± s.e.mean basal concentrations were: Meth 118.18 ± 7.90 (n = 4); Meth/CMZ 91.01 ± 12.08 (n = 5); Meth/Diz 115.92 ± 21.38 (n = 5).

Effect of chlormethiazole and dizocilpine on basal concentrations of dopamine and HVA in the dialysate

Rats were injected with saline in place of the methamphetamine but injected with either chlormethiazole (50 mg kg^{-1}), dizocilpine (1 mg kg^{-1}) or saline as before. Saline-injected animals showed a small rise in basal dopamine over time which was prevented by chlormethiazole (Figure 3a) but not dizocilpine (data not shown). There was no significant effect of chlormethiazole (Figure 3b) or dizocilpine (data not shown) injections on basal HVA concentrations. DOPAC could not be measured in this part of the study for reasons detailed in the Methods.

Effect of chlormethiazole on methamphetamine- or K^+-evoked release of endogenous dopamine from brain tissue prisms

The release of endogenous dopamine from tissue prisms prepared from the striatum induced by the addition of either K^+ (35 mM) or methamphetamine (100 µM) to the medium was unaffected by the presence of 100 µM or 1000 µM chlormethiazole (Table 2).

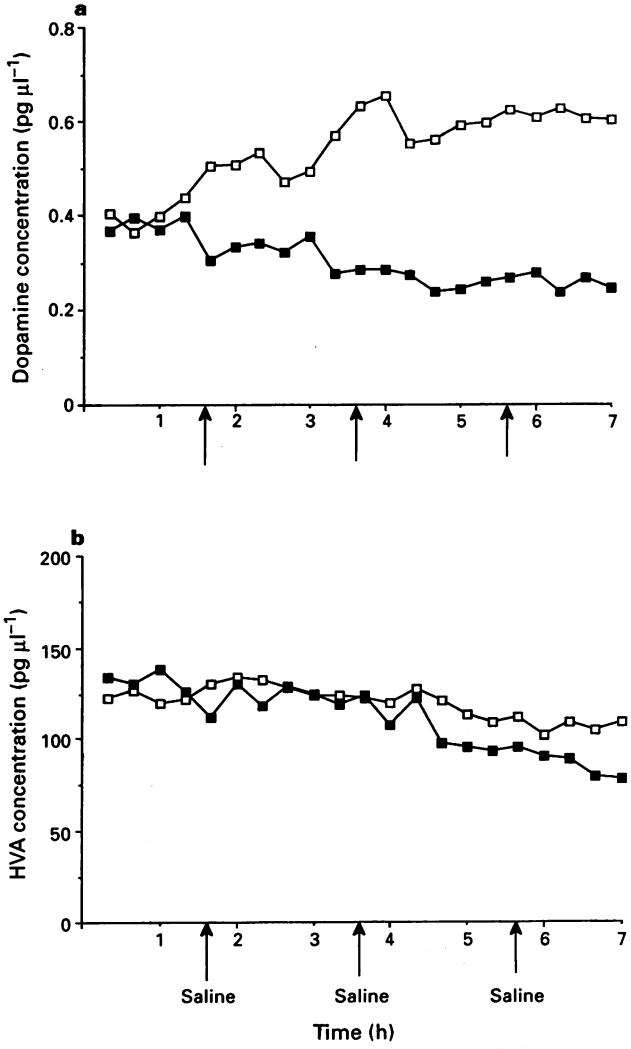


Figure 3 (a) Effect of chlormethiazole (CMZ; 50 mg kg^{-1}, i.p.) given 15 min before each saline injection on basal dopamine release in the striatum: (□) saline; (■) CMZ. Values are mean dopamine concentrations in the dialysate (pg µl^{-1}). Chlormethiazole significantly reduced dialysate dopamine concentrations compared with saline injections alone (2 way ANOVA 'time' × 'CMZ' interaction F (19, 133) = 3.2, $P < 0.0001$). Mean ± s.e.mean basal concentrations were: saline 0.39 ± 0.05 (n = 5); CMZ 0.38 ± 0.07 (n = 4). (b) Effect of chlormethiazole (CMZ; 50 mg kg^{-1}, i.p.) given 15 min before each saline injection on basal homovanillic acid (HVA) levels in the striatum: (□) saline; (■) CMZ. Values are mean HVA concentrations in the dialysate (pg µl^{-1}). There was no significant effect of chlormethiazole on HVA concentrations compared with saline injections alone. Mean ± s.e.mean basal concentrations were: saline 119.96 ± 13.50 (n = 5); CMZ 138.68 ± 4.52 (n = 4).

Table 2 Endogenous dopamine release from striatal slices. Effect of chlormethiazole (CMZ) on methamphetamine (100 μM) and potassium (35 mM) stimulated release

Condition	CMZ concentration (μM)	Dopamine concentration (ng mg^{-1} protein)
Basal	–	3.0 ± 0.6
Methamphetamine	–	16.2 ± 0.9
Methamphetamine	100	14.9 ± 2.8
Methamphetamine	1000	14.1 ± 3.9
K^+	–	18.5 ± 0.6
K^+	100	15.2 ± 3.3
K^+	1000	16.2 ± 0.6
Basal	1000	3.2; 3.1

Results shown as mean ± s.e.mean of 3 observations except basal in presence of chlormethiazole which shows the two observation values. The presence of chlormethiazole did not alter either methamphetamine- or potassium-evoked release.

Discussion

There is substantial evidence to support the contention that the loss of monoamines which occurs in several brain regions following high dose methamphetamine is a reflection of neurodegeneration. This has been demonstrated using biochemical (Stone *et al.*, 1986; 1988) and importantly morphological (Jonsson & Nzanze, 1982; Ricuarto *et al.*, 1982; Molliver *et al.*, 1990) techniques.

In our previous study (Green *et al.*, 1992) four doses of methamphetamine (15 mg kg^{-1}) were given at 3 h intervals. However, the logistics of this protocol when applied to collection of microdialysis samples was difficult and it was therefore decided to administer the methamphetamine at 2 h intervals in the current investigation. The neurotoxic effects of substituted amphetamines has been shown to be dependent on both the dose and treatment regime (see McKenna & Peroutka, 1990) and so the first part of the study examined the severity of monoamine loss following the protocol to be used in the rest of the investigation.

Three days after the repeated methamphetamine injection there was around a 45% loss of striatal dopamine and cortical and hippocampal 5-HT (Table 1). This decrease was somewhat less than seen when the methamphetamine was given at 3 h intervals (65–75%; Green 1992) but was nevertheless a substantial neurotoxic effect. Consistent with the earlier study was the fact that the loss of both 5-HT and dopamine was similar. This part of the study confirmed both the protective effect of dizocilpine against methamphetamine-induced neurotoxicity as previously reported (Sonsalla *et al.*, 1989; Johnson *et al.*, 1989; Green *et al.*, 1992) and also that, at the doses used, chlormethiazole was a more effective protective agent than dizocilpine (Green *et al.*, 1992).

Administration of methamphetamine produced a massive release of dopamine in the striatum. This response was attenuated somewhat by the third injection as one might expect if neuronal stores were being depleted. Dizocilpine pretreatment failed to have any effect on this release. Weihmuller *et al.* (1991, 1992) in contrast, have reported that dizocilpine administration inhibited dopamine release after every injection of methamphetamine. Two points should perhaps be made here. The methamphetamine dose in their investigation was lower (4 mg kg^{-1}) than the current study and produced a rise in dialysate dopamine concentrations around 10 times less than our study. This might explain why the final injection in their investigation was able to produce the largest rise (Weihmuller *et al.*, 1992). In contrast, in our study, methamphetamine produced a much smaller increase by the third injection, presumably as stores had become depleted. This dosing difference makes direct comparison difficult. However, the second point is that the Weilmuller *et al.* (1991, 1992) studies do not agree with Kashihara *et al.* (1991) who used a similar dose of methamphetamine (6.25 mg kg^{-1}) and saw no evidence for an inhibition of dopamine release after a single injection of methamphetamine, consistent with our failure to detect any inhibition at all. The fact remains that in our investigation dizocilpine was an effective protective agent against the consequences of high dose methamphetamine and no inhibition of dopamine release was seen at any time.

Chlormethiazole, in contrast, produced a substantial decrease in dopamine release throughout the whole experiment (around 35%) and, with the exception of the first peak around a 40% decrease in the subsequent peaks (above basal).

The effect of chlormethiazole, but not dizocilpine, on dopamine release was further illustrated by the measures of dopamine metabolites in the dialysate. The concentrations of HVA and DOPAC were unaffected by dizocilpine while they declined further after chlormethiazole. These data confirm the observation of Kashihara *et al.* (1991) that the DOPAC concentration in the dialysate after dizocilpine plus methamphetamine is the same as that following methamphetamine alone.

The question arose as to what effect chlormethiazole would have on extracellular striatal dopamine concentrations in untreated rats. Basically it appeared to induce a small inhibition of the modest rise that occurred during the study, consistent with the drug being a GABA-mimetic agent since other GABA-mimetic agents are known to inhibit dopamine release (Fuxe *et al.*, 1979). However this effect on release cannot explain the inhibition of methamphetamine-induced dopamine release since the inhibition of release by GABA-mimetic drugs is presumably due to a presynaptic effect involving nerve-impulse flow. In contrast, amphetamine-evoked release involves uptake of the amphetamine, probable reversal of the uptake pump and release of newly synthesized transmitter (see McMillen, 1983). This is a Ca^{2+}-independent mechanism not requiring nerve impulse flow (see McMillen, 1983; Robinson *et al.*, 1989). Dizocilpine was not observed to alter basal dopamine release consistent with the report of Weihmuller *et al.* (1992).

Interestingly, we were unable to show any effect of chlormethiazole on either K^+- or methamphetamine-evoked release of endogenous dopamine from tissue prisms *in vitro*. These *in vitro* data therefore indicate that chlormethiazole does not have a direct effect on dopamine release at the nerve ending and is acting through neuronal loops requiring intact anatomical architecture. Dizocilpine has previously been shown not to alter methamphetamine-induced release of dopamine from brain slices (Bowyer *et al.*, 1991). These studies further indicate that chlormethiazole has no direct effect on uptake of methamphetamine into nerve terminals *in vitro*. It is reasonable therefore to conclude that the inhibition of dopamine release after methamphetamine and chlormethiazole *in vivo* cannot be because the drug is interfering with the uptake of methamphetamine into the nerve ending.

Both chlormethiazole and dizocilpine inhibited the methamphetamine-induced behavioural changes and dizocilpine at least is not doing so by directly decreasing dopamine release. It therefore seems likely that dizocilpine and possibly also chlormethiazole (given that there is still considerable

dopamine release) are acting 'downstream' to attenuate the behavioural responses. These data again conflict with Weihmuller *et al.* (1991) who reported that dizocilpine did not inhibit the methamphetamine-induced behaviour but not the report of Farfel *et al.* (1991) who noted an attenuation by dizocilpine of methamphetamine-induced stereotyped behaviour. We, have also recently observed that dizocilpine and chlormethiazole inhibit the behavioural abnormalities induced by administration of three other neurotoxic substituted amphetamines namely, *p*-chloramphetamine, fenfluramine and 3,4-methylenedioxy-methamphetamine (MDMA or Ecstasy) albeit only the MDMA neurotoxicity of 5-HT pathways was prevented by chlormethiazole and dizocilpine (Colado *et al.*, 1993).

Whilst the concentration of chlormethiazole used in the medium might appear high, the IC_{50} value for inhibition of [^{3}H]-TPBS binding to the chloride channel is over 10^{-4} M (Cross *et al.*, 1989) and peak plasma and brain concentrations are also greater than 10^{-4} M following an injection of chlormethiazole of 50–100 mg kg^{-1} (Kalant & Khanna, 1986; Cross *et al.*, unpublished observations).

What the current experiments do provide is partial explanation for why chlormethiazole prevents methamphetamine-induced neurotoxicity. There is substantial evidence that inhibiting striatal dopamine function by use of either dopamine synthesis inhibitors or receptor antagonists prevents not only the subsequent damage to striatal dopamine (Buening & Gibb, 1974; Gibb & Kogan, 1979; Gibb *et al.*, 1990) but also brain 5-hydroxytryptaminergic systems (Schmidt *et al.*, 1985; Gibb *et al.*, 1990). The ability of chlormethiazole to attenuate the dopamine release caused by methamphetamine may well therefore be involved in the protective effect. Dizocilpine and other NMDA antagonists, in contrast, presumably act by preventing NMDA receptor 'overactivity' downstream to the dopamine release. That having been said, the degree of inhibition of dopamine release by chlormethiazole was only partial. There is now increasing evidence from both experiments on N-methyl-DL-aspartate-induced seizures (Cross *et al.*, 1992b) and harmaline-induced guanosine 3':5'-cyclic monophosphate (cyclic GMP) formation in the cerebellum (Cross *et al.*, 1992a) that chlormethiazole will inhibit NMDA-mediated functional responses even though it does not interact with the NMDA receptor directly (Cross *et al.*, 1992b). It is possible therefore that the protective effect of chlormethiazole involves *both* the inhibition of dopamine release and antagonism of NMDA receptor-mediated function. Consistent with this proposal is the fact that chlormethiazole is also an effective protective agent against MDMA-induced neurotoxicity of 5-HT terminals in the cortex and hippocampus (Colado *et al.*, 1993). MDMA is a weak releaser of striatal dopamine (see Schmidt & Kehne, 1990) so inhibition of dopamine release is probably an unsatisfactory proposal in order to explain entirely the neuroprotection by chlormethiazole in this model.

While these data allow one to propose that chlormethiazole is protective in methamphetamine-induced neurotoxicity because of it inhibiting dopamine release, in addition to its reported effect of inhibiting at least some NMDA-mediated effects, no explanation can be given at present as to the mechanism by which it inhibits methamphetamine-induced dopamine release other than to say that this does not appear to be due to a direct effect at dopamine nerve terminals.

Both chlormethiazole (Cross *et al.*, 1991) and dizocilpine (Gill *et al.*, 1987; 1988; Cross *et al.*, 1991) are protective agents in the gerbil model of global ischaemia and both are protective against methamphetamine- (Green *et al.*, 1992; this paper) and MDMA-induced (Colado *et al.*, 1993) neurotoxicity. The involvement of dopamine as well as glutamate in ischaemia-induced neurodegeneration has been proposed (Globus *et al.*, 1988) so the current investigation may assist in the search for the mechanisms by which chlormethiazole is neuroprotective against ischaemia-induced damage.

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References

AUERBACH, S. & LIPTON, P. (1985). Regulation of serotonin release from the *in vitro* rat hippocampus: effects of alterations in the levels of depolarisation and in rates of serotonin metabolism. *J. Neurochem.*, **44**, 1116–1130.

BALDWIN, H.A., COLADO, M.I., MURRAY, T.K. & GREEN, A.R. (1992). Chlormethiazole, but not dizocilpine, inhibits dopamine release in rat striatum induced by high dose methamphetamine. *Br. J. Pharmacol.*, (Proc Suppl; in press).

BALDWIN, H.A., DE SOUZA, R.J., SARNA, G.S., MURRAY, T.K., GREEN, A.R. & CROSS, A.J. (1991). Measurements of tacrine and monoamines in brain by *in vivo* microdialysis argue against release of monoamines by tacrine at therapeutic doses. *Br. J. Pharmacol.*, **103**, 1946–1950.

BOWYER, J.F., SCALLET, A.C., HOLSON, R.R., KIPE, G.W., SLIKKER, W. & ALI, S.F. (1991). Interactions of MK-801 with glutamate-, glutamine- and methamphetamine-evoked release of [^{3}H]-dopamine from striatal slices. *J. Pharmacol. Exp. Ther.*, **257**, 262–270.

BUENING, M.K. & GIBB, J.W. (1974). Influence of methamphetamine and neuroleptic drugs on tyrosine hydroxylase activity. *Eur. J. Pharmacol.*, **26**, 30–34.

COLADO, M.I., MURRAY, T.K. & GREEN, A.R. (1993). 5-HT loss in rat brain following 3,4-methylenedioxy-methamphetamine (MDMA), *p*-chloramphetamine and fenfluramine administration and effects of chlormethiazole and dizocilpine. *Br. J. Pharmacol.*, **108**, 583–589.

CROSS, A.J., JONES, J.A., BALDWIN, H.A. & GREEN, A.R. (1991). Neuroprotective action of chlormethiazole following transient forebrain ischaemia in the gerbil. *Br. J. Pharmacol.*, **104**, 406–411.

CROSS, A.J., MISRA, A., SANDILANDS A. & GREEN, A.R. (1992a). Inhibition by chlormethiazole of the harmaline-induced increase in cyclic GMP content in rat cerebellum. *Br. J. Pharmacol.*, (Proc Suppl; in press).

CROSS, A.J., MISRA, A., SNAPE, M.F., MURRAY, T.K. & GREEN, A.R. (1992b). Chlormethiazole inhibits the effects of N-methyl-DL-aspartate in vivo but does not inhibit NMDA receptor binding. *Br. J. Pharmacol.*, **105**, 20P.

CROSS, A.J., STIRLING, J.M., ROBINSON, T.N., BOWEN, D.M., FRANCIS, P.T. & GREEN, A.R. (1989). The modulation by chlormethiazole of the $GABA_A$ receptor complex in rat brain. *Br. J. Pharmacol.*, **98**, 284–290.

FARFEL, G.M., FERGUSON, N.E. & SEIDEN, L.A. (1991). MK801 partially antagonises the locomotor and stereotypic effects of methamphetamine. *Soc. Neurosci.* Abstract. 567.15.

FUXE, K., ANDERSSON, K., OGREN, S.O., PEREZ DE LA MORA, M., SCHWARCZ, R., HÖKFELT, T., ENEROTH, P., GUSTAFSSON, J.-Å. & SKETT, P. (1979). GABA neurones and their interaction with monamine neurones. An anatomical, pharmacological and functional analysis. In *GABA neurotransmitters – Pharmacochemical, Biochemical and Pharmacological Aspects* ed. Krogsgaard-Larsen, P., Scheel-Kruger, J.E. & Kofod, K. pp. 74–94. Copenhagen: Munksgaard.

GIBB, J.W., JOHNSON, M., STONE, D. & HANSON, G.R. (1990). MDMA: historical perspectives. *Ann. N.Y. Acad. Sci.*, **600**, 601–611.

GIBB, J.W. & KOGAN, F.J. (1979). Influence of dopamine synthesis on methamphetamine-induced changes in striatal and adrenal tyrosine hydroxylase. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **310**, 185–187.

GILL, R., FOSTER, A.C. & WOODRUFF, G.N. (1987). Systemic administration of MK-801 protects against ischaemia-induced hippocampal neurodegeneration in the gerbil. *J. Neurosci.*, **7**, 3343–3349.

GILL, R., FOSTER, A.C. & WOODRUFF, G.N. (1988). MK-801 is neuroprotective in gerbils when administered during the post-ischaemic period. *Neuroscience*, **24**, 847–855.

GLOBUS, M.Y.-T., BUSTO, R., DIETRICH, W.D., MARTINEZ, E., VALDES, I. & GINSBERG, M.D. (1988). Effect of ischaemia on the *in vivo* release of striatal dopamine, glutamate and γ-aminobutyric acid studied by intracerebral microdialysis. *J. Neurochem*, **51**, 1455–1464.

GREEN, A.R., DE SOUZA, R.J., WILLIAMS, J.L., MURRAY, T.K. & CROSS, A.J. (1992). The neurotoxic effects of methamphetamine on 5-hydroxytryptamine and dopamine in brain: evidence for the protective effect of chlormethiazole. *Neuropharmacology*, **31**, 315–321.

HARRISON, N.L. & SIMMONDS, M.A. (1983). Two distinct interactions of barbiturates and chlormethiazole with the GABA receptor complex in rat cuneate nucleus *in vitro*. *Br. J. Pharmacol.*, **80**, 387–394.

HOTCHKISS, A. & GIBB, J.W. (1980). Long term effects of multiple doses of methamphetamine on tryptophan hydroxylase and tyrosine hydroxylase in rat brain. *J. Pharmacol. Exp. Ther.*, **214**, 257–262.

JOHNSON, M., HANSON, G.R. & GIBB, J.W. (1989). Effect of MK 801 on the decrease in tryptophan hydroxylase induced by methamphetamine and its methylene dioxy analog. *Eur. J. Pharmacol.*, **165**, 315–318.

JONSSON, G. & NZANZE, E. (1982). Selective (+)-amphetamine neurotoxicity on striatal dopamine nerve terminals in the mouse. *Br. J. Pharmacol.*, **77**, 335–345.

KASHIHARA, K., OKUMURA, K., ONISHI, M. & OTSUKI, S. (1991). MK-801 fails to modify the effect of methamphetamine on dopamine release in the rat striatum. *NeuroReport*, **2**, 236–238.

KALANT, H. & KHANNA, J.M. (1986). Metabolic and functional aspects of tolerance to chlormethiazole and cross-tolerance to ethanol in the rat. *Acta. Psychiat. Scand.*, **73**, (Suppl 329) 45–53.

KODA, L.Y. & GIBB, J.W. (1973). Adrenal and striatal tyrosine hydroxylase activity after methamphetamine. *J. Pharmacol. Exp. Ther.*, **185**, 42–48.

MCKENNA, D.J. & PEROUTKA, S.J. (1990). Neurochemistry and neurotoxicity of 3,4-methylenedioxymethamphetamine (MDMA, 'Ecstasy'). *J. Neurochem.*, **54**, 14–22.

MCMILLEN, B.A. (1983). CNS stimulants: two distinct mechanisms of action for amphetamine-like drugs. *Trends Pharmacol Sci.*, **4**, 429–432.

MOLLIVER, M.E., BERGER, U.V., MAMOUNAS, L.A., MOLLIVER, D.L., O'HEARN, E. & WILSON, M.A. (1990). Neurotoxicity of MDMA and related compounds: anatomic studies. *Ann. N.Y. Acad. Sci.*, **600**, 640–661.

MOODY, E.J. & SKOLNICK, P. (1989). Chlormethiazole: neurochemical actions at the gamma-aminobutyric acid receptor complex. *Eur. J. Pharmacol.*, **164**, 153–158.

OGREN, S.-O. (1986). Chlormethiazole-mode of action. *Acta Psychiat. Scand.*, **73**, Suppl. 329, 13–27.

RICUARTO, G.A., GUILLERY, R.W., SEIDEN, L.S., SCHASTER, C.R. & MOORE, R.Y. (1982). Dopamine nerve terminal degeneration produced by high dose methylamphetamine. *Brain Res.*, **235**, 93–103.

ROBINSON, T.N., DE SOUZA, R.J., CROSS, A.J. & GREEN, A.R. (1989). The mechanism of tetrahydroaminoacridine-evoked release of endogenous 5-hydroxytryptamine and dopamine from rat brain tissue prisms. *Br. J. Pharmacol.*, **98**, 1127–1136.

SCHMIDT, C.J. & KEHNE, J.H. (1990). Neurotoxicity of MDMA: neurochemical effects. *Ann. N.Y. Acad Sci.*, **600**, 665–680.

SCHMIDT, C.J., RITTER, J.K., SONSALLA, P.K., HANSON, G.R. & GIBB, J.W. (1985). Role of dopamine in the neurotoxic effects of methamphetamine. *J. Pharmacol. Exp. Ther.*, **233**, 539–544.

SONSALLA, P.K., NICKLAS, W.J. & HEIKKILA, R.E. (1989). Role for excitatory amino acids in methamphetamine-induced nigrostriatal dopaminergic toxicity. *Science, Wash.*, **243**, 398–400.

SONSALLA, P.K., RIORDAN, D.E. & HEIKKILA, R.E. (1991). Competitive and non-competitive antagonists at N-methyl-D-aspartate receptors protect against methamphetamine-induced dopaminergic damage in mice. *J. Pharmacol. Exp. Ther.*, **256**, 506–512.

STONE, D.M., JOHNSON, M., HANSON, G.R. & GIBB, J.W. (1988). Role of endogenous dopamine in the central serotonergic deficits induced by 3,4-methylene-dioxymethamphetamine. *J. Pharmacol. Exp. Ther.*, **247**, 79–87.

STONE, D.M., STAHIL, D.C., HANSON, G.R. & GIBB, J.W. (1986). The effects of 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyamphetamine (MDA) on monoaminergic systems in the rat brain. *Eur. J. Pharmacol.*, **128**, 41–48.

VINCENS, M., ENJALABERT, A., LLOYD, K.G., PAILLARD, J.J., THIRET, F., KORDON, C. & LECHAT, P. (1989). Evidence that chlormethiazole interacts with the macromolecular $GABA_A$-receptor complex in the central nervous system and in the anterior pituitary gland. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **339**, 397–402.

WEIHMULLER, F.B., O'DELL, S.J., COLE, B.N. & MARSHALL, J.F. (1991). MK-801 attenuates the dopamine-releasing but not the behavioural effects of methamphetamine: an in vivo microdialysis study. *Brain Res.*, **549**, 230–235.

WEIHMULLER, F.B., O'DELL, S.J. & MARSHALL, J.F. (1992). MK801 protection against methamphetamine-induced striatal dopamine terminal injury is associated with attenuated dopamine overflow. *Synapse*, **11**, 155–163.

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Chronic treatment with the monoamine oxidase inhibitors clorgyline and pargyline down-regulates non-adrenoceptor [^{3}H]-idazoxan binding sites in the rat brain

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1 The binding of [^{3}H]-idazoxan in the presence of 10^{-6} M (−)-adrenaline was used to quantitate non-adrenoceptor idazoxan binding sites (NAIBS) in the rat brain after treatment with various psychotropic drugs.

2 Chronic treatment (14 days) with the monoamine oxidase (MAO) inhibitors clorgyline (0.3–10 mg kg^{-1}, i.p.) and pargyline (10 mg kg^{-1}, i.p.), but not with Ro 41-1049 (1 mg kg^{-1}, i.p.), markedly decreased (30–50%) the density of NAIBS in the cerebral cortex without any apparent change in the affinity of the radioligand.

3 Acute (1 day) and/or chronic treatments (14 days) with other psychotropic drugs such as desipramine (3 mg kg^{-1}, i.p.), cocaine (10 mg kg^{-1}, i.p.), reserpine (0.12 mg kg^{-1}, s.c.), haloperidol (1 mg kg^{-1}, i.p.) and diazepam (10 mg kg^{-1}, i.p.) did not alter the density of NAIBS in the cerebral cortex.

4 *In vitro*, the propargylamines clorgyline, pargyline and deprenyl displaced the binding of [^{3}H]-idazoxan to NAIBS from two distinct sites, but only clorgyline displayed an apparent very high affinity for a relevant population of NAIBS (K_{iH} = 40 pM; K_{iL} = 10.6 μM). The structurally diverse MAO inhibitors Ro 16-6491 (selective for MAO-B) and Ro 41-1049 (selective for MAO-A), as well as the other psychotropic drugs (desipramine, cocaine, reserpine and haloperidol) displaced the binding of [^{3}H]-idazoxan to NAIBS monophasically and with very low potencies. As expected, the MAO inhibitors clorgyline and Ro 41-1049 displaced the binding of [^{3}H]-Ro 41-1049 to MAO-A monophasically and with high potencies (K_i values: 0.18 nM and 22 nM, respectively). In contrast, idazoxan displayed very low affinity (K_i = 40 μM) against the binding of [^{3}H]-Ro 41-1049 to MAO-A. These results disprove a direct interaction between [^{3}H]-idazoxan and the enzyme MAO.

5 Preincubation of cortical membranes with clorgyline (10^{-9} M or 10^{-6} M for 30 min) or pargyline (10^{-6} M or 10^{-5} M for 30 min), reduced by 30–50% and by 17–30%, respectively, the total density of NAIBS without any apparent change in the affinity of the radioligand. Preincubation with 10^{-6} M clorgyline did not alter the affinity of cirazoline for the two populations of NAIBS, but reduced by 60% the binding of [^{3}H]-idazoxan to the high affinity site without affecting the binding of the radioligand to the low affinity site. These results indicate that the two MAO inhibitors irreversibly block the binding of [^{3}H]-idazoxan to NAIBS.

6 *In vivo*, however, various acute treatments with clorgyline (1–20 mg kg^{-1}, i.p.) for different time intervals (6–48 h) did not alter the density of NAIBS. *In vivo*, only very high doses of clorgyline (40 and 80 mg kg^{-1}, i.p.) induced modest decreases (21–28%) in the density of NAIBS in the cerebral cortex.

7 Together the results indicate that the irreversible binding of clorgyline and pargyline to NAIBS found *in vitro* does not fully explain the marked decreases in the density of NAIBS found *in vivo* after the chronic treatments. It is suggested that the down-regulation of NAIBS induced *in vivo* by clorgyline and pargyline, through a direct or indirect mechanism, may have functional implications.

Keywords: Non-adrenoceptor idazoxan binding sites; NAIBS; [^{3}H]-idazoxan; rat brain; MAO inhibitors; psychotropic drugs

Introduction

Evidence has accumulated that imidazoli(di)ne compounds such as idazoxan and *p*-aminoclonidine bind with high affinity not only to α_2-adrenoceptors, but also to non-adrenoceptor sites in a variety of species and tissues, including the brain (for a review see Michel & Insel, 1989; Atlas, 1991; Kilpatrick *et al.*, 1992). Non-adrenoceptor [^{3}H]-idazoxan binding sites (NAIBS) appear to be pharmacologically different from those labelled by [^{3}H]-*p*-aminoclonidine (Michel & Insel, 1989; Hieble & Ruffolo, 1992; Ernsberger, 1992) and also differ from α_2-adrenoceptors in in their biochemical properties and anatomical distribution (Boyajian *et al.*, 1987; Mallard *et al.*, 1992). Additional distinction between NAIBS and α_2-adrenoceptors refers to the subcellular localization of NAIBS on the mitochondrial outer membrane (Tesson & Parini, 1991; Tesson *et al.*, 1991) as well as on a different position of the cell surface in some tissues (Diamant *et al.*, 1992).

Endogenous depression appears to be associated with increased brain α_2-adrenoceptors ([^{3}H]-clonidine and [^{3}H]-UK 14304 binding) and down-regulation of these inhibitory receptors has been involved in the mechanism of action of various antidepressant drugs (Meana *et al.*, 1992 and other references therein). Recently, NAIBS as well as non-adrenoceptor binding sites labelled by [^{3}H]-*p*-aminoclonidine have also been found increased in postmortem brains of depressed suicide victims (Barturen *et al.*, 1992 and unpub-

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lished results) and in platelet membranes from depressed patients (Piletz et al., 1990; 1991). These imidazoline-preferring receptors also appear to mediate a presynaptic inhibition on the release of noradrenaline (NA) in adrenergic nerve terminals (Göthert & Molderings, 1991; Molderings et al., 1991). Therefore, enhanced imidazoline-preferring receptor (and α_2-adrenoceptor) activity and with it decreased NA release could be involved in the pathogenesis of endogenous depression.

In this context, the present study was designed to assess the effects of chronic antidepressant drugs (clorgyline and other monoamine oxidase (MAO) inhibitors, and desipramine) and other psychotropic drug treatments on NAIBS and also to investigate *in vitro* the pharmacological properties of the interaction of these drugs with the imidazoline binding sites.

Methods

Animals and treatments

Male Sprague-Dawley rats (250–300 g) were used. The animals received a standard diet with water freely available and were housed at 20 ± 2°C with a 12 h light/dark cycle. In the chronic (14 days) treatments the animals received i.p. every 12 h either 0.9% saline vehicle, the MAO-A inhibitors clorgyline (0.3–10 mg kg^{-1}) or Ro 41-1049 (1 mg kg^{-1}), the MAO-B inhibitor pargyline (10 mg kg^{-1}), and the NA re-uptake blockers desipramine (3 mg kg^{-1}) or cocaine (10 mg kg^{-1}). Rats were also treated with reserpine (0.12 mg kg^{-1} s.c., every 48 h for 14 days) to assess the effect of depletion of NA on NAIBS. In another series of experiments, rats were treated i.p. every 12 h for 14 days with haloperidol (1 mg kg^{-1}) or diazepam (10 mg kg^{-1}) for comparison. In the acute (1 day) treatments, the rats received two doses (12 h apart) of clorgyline (1 mg kg^{-1}) or desipramine (3 mg kg^{-1}). Both in the acute and chronic treatments the rats were killed 48 h after the last injection. In some experiments, the acute effects of various doses of clorgyline (10–80 mg kg^{-1}) on NAIBS were assessed at different time intervals (6, 12, 24 and 48 h).

Preparation of membranes

Neural membranes (P_2 fractions) were prepared by established methods (Giralt & García-Sevilla, 1989) from the parietooccipital cortex (frozen). Briefly, the tissue samples were homogenized in 5 ml of ice-cold Tris-sucrose buffer (5 mM Tris-HCl; 250 mM sucrose; 1 mM $MgCl_2$; pH 7.4). The homogenates were centrifuged at 1,100 *g* for 10 min, and the supernatants were then recentrifuged at 40,000 *g* for 10 min. The resulting pellet was washed twice with 2 ml of fresh incubation buffer (50 mM Tris-HCl, 0.1% ascorbic acid, pH 7.5) for [^{3}H]-idazoxan binding assays and 50 mM Tris-HCl, 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM EGTA, pH 7.4 for [^{3}H]-Ro 41-1049 binding assays (Cesura *et al.*, 1990a). The final pellet was resuspended in an appropriate volume of this buffer to a final protein content of 800–1000 μg ml^{-1}. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

[^{3}H]-idazoxan binding assay

Total [^{3}H]-idazoxan binding was measured in 1.1 ml-aliquots (50 mM Tris-HCl, 0.1% ascorbic acid, pH 7.5) of the neural membranes which were incubated with shaking for 30 min at 25°C. Binding of [^{3}H]-idazoxan to NAIBS was always done in the presence of 10^{-6} M (−)-adrenaline to prevent the binding of the radioligand to α_2-adrenoceptors. Nonspecific binding was determined in the presence of 10^{-4} M naphazoline, as previously described (Olmos *et al.*, 1992). In the saturation studies, cortical membranes were incubated with eight concentrations of [^{3}H]-idazoxan (6×10^{-10} M to 5×10^{-8} M) as above. Specific binding (97% to 30%) was defined as the difference between total non-adrenoceptor binding (in the presence of 10^{-6} M adrenaline) and nonspecific binding (determined with 10^{-4} M naphazoline) and was plotted as a function of increasing concentrations of the radioligand. In the drug competition studies, cortical membranes were incubated as above with [^{3}H]-idazoxan (4×10^{-9} M, in the presence of 10^{-6} M adrenaline) and in the absence or presence of various concentrations of the competing drugs (10^{-14} M to 10^{-3} M; 15–24 concentrations). Total binding was determined as above and plotted as a function of the drug concentration.

In some experiments, the effects of *in vitro* preincubation with clorgyline or pargyline on [^{3}H]-idazoxan binding to NAIBS were tested. The pellet resulting from the last centrifugation (see preparation of membranes) was resuspended in 10 ml of fresh incubation buffer and incubated for 30 min at 25°C in the presence of two concentrations of clorgyline (10^{-9} M and 10^{-6} M) or pargyline (10^{-6} M and 10^{-5} M). Then neural membranes were washed twice with 10 ml of fresh incubation buffer and the final pellet was resuspended as above and used in the saturation and competition binding experiments.

Incubations were terminated by diluting the samples with 5 ml of ice-cold Tris incubation buffer (4°C). Membrane-bound [^{3}H]-idazoxan was measured by vacuum filtration, using a Brandel 48R cell harvester (Biomedical Research & Development Laboratories, U.S.A.), through Whatman GF/C glass fibre filters, which had been presoaked with 0.5% polyethylenimine (Bruns *et al.*, 1983). Then the filters were rinsed twice with 5 ml of incubation buffer, air-dried, transferred to minivials containing 5 ml of OptiPhase 'HiSafe' II cocktail (LKB, England) and counted for radioactivity by liquid scintillation spectrometry at 50% efficiency (Packard model 300 C).

[^{3}H]-Ro 41-1049 binding assay

Binding of [^{3}H]-Ro 41-1049 was performed as previously described (Cesura *et al.*, 1990a) with modifications (e.g. bound/free radioligand was separated by filtration instead of centrifugation). These assays were similar to those described above for [^{3}H]-idazoxan except in the buffer composition (50 mM Tris-HCl, 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM EGTA, pH 7.4) and in the incubation conditions (60 min at 37°C). Drug competition studies were performed as above with [^{3}H]-Ro 41-1049 (4.5×10^{-9} M) in the absence or presence of various concentrations of the competing drugs (10^{-14} M to 10^{-2} M; 21 concentrations). The specific binding of [^{3}H]-Ro 41-1049, defined with 10^{-6} M clorgyline, represented about 98% of total binding.

Analyses of binding data and statistics

Analyses of saturation isotherms (K_d, dissociation constant; B_{max}, maximum density of binding sites) and competition experiments (K_i, inhibition constant) as well as the fitting of data to the appropriate binding models were performed by computer-assisted nonlinear regression using the EBDA-LIGAND (Munson & Rodbard, 1980; McPherson, 1985) or GraFit (Leatherbarrow, 1990) programmes. All experiments were initially analysed assuming a one-site model of radioligand binding and then assuming a two-site binding model. The selection between the different binding models was made statistically by the extra sum of squares principle (F test) as outlined by Munson & Rodbard (1980). The more complex model was accepted if the P value resulting from the F test was less than 0.05. For other details of the computer analyses see Olmos *et al.* (1992).

Results are expressed as mean ± s.e.mean. One-way analysis of variance (ANOVA), followed by Scheffé's test, was

used for the statistical evaluations. The level of significance was $P = 0.05$.

Drugs

[^{3}H]-idazoxan (specific activity, 40–48 Ci mmol^{-1}) was purchased from Amersham International plc (U.K.). [^{3}H]-Ro 41-1049 (specific activity, 30.8 Ci mmol^{-1}), was a generous gift from Dr J.G. Richards and Dr J. Saura (F. Hoffmann-La Roche Ltd., Switzerland). Both radioligands were stored at − 30°C. For the binding assays, appropriate amounts of the stock solutions were diluted with distilled and purified water (Milli-Q) containing 2.5 mM HCl and 6% ethanol. Other drugs (and their sources) included: cirazoline HCl (Synthélabo Recherche, France); clorgyline HCl (Sigma Chemical Co., U.S.A.); cocaine HCl (Alcaloides Abelló S.A., Spain); deprenyl (RBI, U.S.A.); desipramine HCl (Rorer Central Research, U.S.A.); diazepam (S.A. Lasa Laboratories, Spain); haloperidol HCl (Sigma); idazoxan HCl (synthesized by Dr F. Geijo at S.A. Lasa Laboratories); pargyline HCl (Sigma); reserpine (Serpasil) (Ciba-Geigy, Spain); Ro 41-1049 [N-(2-aminoethyl)-5-(3-fluorophenyl)-4-thiazole carboxamide] HCl, Ro 16-6491 [N-(2-aminoethyl-*p*-chlorobenzamide] HCl (F. Hoffmann-La Roche Ltd.). Other reagents were obtained from Sigma Chemical Co. (U.S.A.).

Results

In vivo *effects of MAO inhibitors and other psychotropic drugs on NAIBS*

Chronic treatment (14 days) with the irreversible MAO-A inhibitor clorgyline (0.3–10 mg kg^{-1}) markedly decreased the density of NAIBS in the rat cerebral cortex (Table 1 and Figure 1a). This effect appeared to be dose-dependent, at least in the range of 1, 3 and 10 mg kg^{-1}, and the total density of NAIBS decreased by 28%, 40% and 51% ($P<0.01$), respectively. In contrast, acute (1 day) treatment with clorgyline (1 mg kg^{-1}) did not alter the density of NAIBS in the brain (Table 1). Chronic treatment (14 days) with pargyline (10 mg kg^{-1}), an irreversible non-selective MAO inhibitor *in vivo* particularly after repeated treatment (see Fowler *et al.*, 1981), also markedly decreased (46%; $P<0.01$) the density of NAIBS in the cerebral cortex (Table 1 and Figure 1b). In contrast, chronic treatment (14 days) with Ro 41-1049 (1 mg kg^{-1}), a selective but reversible MAO-A inhibitor, did not decrease the density of NAIBS in the cerebral cortex (Table 1).

Acute (1 day) and/or chronic (14 days) treatments with the NA reuptake blockers desipramine (3 mg kg^{-1}) or cocaine (10 mg kg^{-1}) did not modify significantly the binding parameters of [^{3}H]-idazoxan to NAIBS in the cerebral cortex (Table 2). Moreover, chronic depletion of brain NA with reserpine (0.12 mg kg^{-1} for 14 days) resulted in similar negative results (Table 2). Other chronic treatments (14 days) with haloperidol (1 mg kg^{-1}) or diazepam (10 mg kg^{-1}) did not alter the density of NAIBS in the rat brain (Table 2).

In vitro *effects of MAO inhibitors and other psychotropic drugs on [^{3}H]-idazoxan and [^{3}H]-Ro 41-1049 binding*

Since clorgyline and pargyline irreversibly bind to the enzyme MAO, the possibility that the chronic *in vivo* effects of both drugs on NAIBS could be related, at least in part, to an interaction of [^{3}H]-idazoxan with the MAO was investigated. In the rat cerebral cortex, *in vitro* competition curves for clorgyline against [^{3}H]-idazoxan binding (in the presence of 10^{-6} M adrenaline) were clearly biphasic and nonlinear analyses revealed the existence of two distinct binding sites, one of them with very high affinity (40 picomolar) for clorgyline (Table 3 and Figure 2a). Interestingly, the MAO inhibitors pargyline and deprenyl, which like clorgyline possess a propargylamine structure (Youdim *et al.*, 1988), also displaced the binding of [^{3}H]-idazoxan from two distinct sites but with much lower potency (Table 3). In contrast, the structurally diverse compounds Ro 16-6491 (selective MAO-B inhibitor) and Ro 16-6491 (selective MAO-B inhibitor) and Ro 41-1049 (selective MAO-A inhibitor) displaced the binding of [^{3}H]-idazoxan to NAIBS monophasically and with very low potencies (Table 3 and Figure 2a).

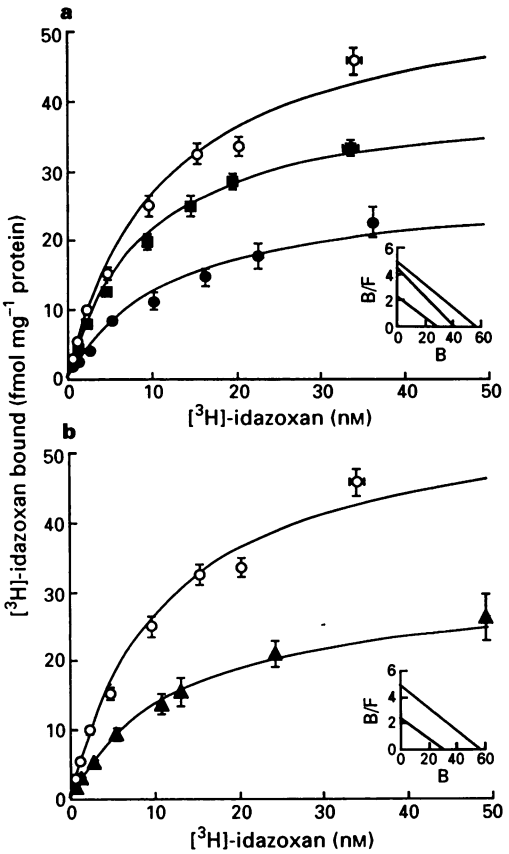


Figure 1 Specific binding of [^{3}H]-idazoxan (in the presence of 10^{-6} M adrenaline) to NAIBS in the rat cerebral cortex after saline (○) or chronic treatment with (a) clorgyline 1 mg kg^{-1} (■) or 10 mg kg^{-1} (●) and (b) pargyline 10 mg kg^{-1} (▲) i.p., every 12 h for 14 days. Each point of the saturation curve is the mean ± s.e.mean (vertical and horizontal bars) of 5 to 20 experiments. Inset: Scatchard plots (same data) showing K_d and B_{max} values similar to those obtained by nonlinear analysis. See Table 1 for changes in binding parameters and other details.

As expected, competition curves for idazoxan against [^{3}H]-idazoxan binding were monophasic and the best fit was to a single population of high affinity binding sites (Table 3 and Figure 2a). The various psychotropic drugs used in the chronic treatments (desipramine, reserpine, haloperidol and cocaine) also competed but with very low affinities against [^{3}H]-idazoxan binding to NAIBS (K_i values in the high micromolar range) (Table 3).

Competition curves for clorgyline and Ro 41-1049 against [^{3}H]-Ro 41-1049 binding to MAO-A were clearly monophasic and the best fit was to a single population of high affinity binding sites (K_i values: 0.18 nM and 22 nM, respectively) (Figure 2b). In contrast, idazoxan displayed very low affinity (K_i: 40 μM) against the binding of [^{3}H]-Ro 41-1049 to MAO-A (Figure 2b).

With these experiments a direct interaction of [^{3}H]-idazoxan with the active centre of MAO-A and B isoenzymes was discounted.

In vitro *preincubation with clorgyline and pargyline on NAIBS*

To further assess the mechanism of action involved in the *in vivo* down-regulation of NAIBS induced by clorgyline and pargyline, incubation experiments were performed to determine if, consistent with their actions on MAO, these compounds could also irreversibly bind to NAIBS. Thus, clorgyline and less potently pargyline, irreversibly blocked the binding of [^{3}H]-idazoxan to NAIBS. Preincubation of cortical membranes with 10^{-9} M or 10^{-6} M clorgyline reduced by 30% and 49% ($P<0.01$), respectively, the total density of NAIBS (Figure 3a). Similarly, preincubation with 10^{-6} M or 10^{-5} M pargyline also reduced by 17% and 32% ($P<0.01$), respectively, the density of NAIBS (data not shown). These preincubation procedures did not affect the affinity (K_d) of [^{3}H]-idazoxan binding to NAIBS (Figure 3a).

Next, the question was addressed whether in cortical membranes preincubated with 10^{-6} M clorgyline, the affinity of a potent and selective drug for NAIBS, cirazoline, as well as the proportion of high- and low-affinity sites of NAIBS were altered. Both in membranes preincubated in buffer only (control) and in those preincubated with 10^{-6} M clorgyline, competition curves for cirazoline against [^{3}H]-idazoxan binding (in the presence of 10^{-6} M adrenaline) were biphasic and, as expected (Olmos *et al.*, 1992), curve fitting with the LIGAND programme revealed that a two-site fit was significantly better than a one-site binding model (Figure 3b). Preincubation with 10^{-6} M clorgyline did not alter the affinity of cirazoline for the two populations of NAIBS, but significantly reduced (60%; $P<0.01$) the binding of [^{3}H]-idazoxan to the high affinity population (B_{maxH}) of NAIBS, without affecting the binding of the radioligand to the low affinity population (B_{maxL}) (Figure 3b).

In vivo *acute effects of clorgyline on NAIBS*

Chronic but not acute treatment with clorgyline (both with a 48 h period of drug washout) was found to induce down-regulation of NAIBS. However, since clorgyline and pargyline appeared to bind irreversibly *in vitro* to NAIBS, the effects of various acute treatments with high doses of clorgyline (10–80 mg kg^{-1}) were studied at shorter time intervals (6–48 h) to assess further whether this drug can induce a rapid and irreversible blockade of [^{3}H]-idazoxan binding to NAIBS *in vivo*. Clorgyline at doses of 10 mg kg^{-1} (the

Table 1 Effects of acute and/or chronic treatments with monoamine oxidase (MAO) inhibitors on NAIBS in the rat cerebral cortex

		[^{3}H]-idazoxan		
Treatment	Dose (mg kg^{-1})	K_d (nM)	B_{max} (fmol mg^{-1} protein)	n
Saline	–	11.3 ± 0.6	57 ± 2	20
Clorgyline				
Acute	1	15.8 ± 1.3	57 ± 2	4
Chronic	0.3	10.1 ± 1.2	34 ± 2*	6
	1	9.2 ± 1.0	41 ± 2*	8
	3	13.3 ± 1.9	34 ± 3*	6
	10	12.5 ± 1.8	28 ± 3*	6
Pargyline				
Chronic	10	12.9 ± 1.9	31 ± 5*	5
Ro 41-1049				
Chronic	1	11.6 ± 0.7	61 ± 4	5

Each drug was administered i.p., every 12 h for 14 days, except for the acute clorgyline treatment which was for only one day. The rats were killed 48 h after the last injection. Neural membranes were incubated at 25°C for 30 min with eight concentrations of [^{3}H]-idazoxan (6×10^{-10} M to 5×10^{-8} M). The specific binding of [^{3}H]-idazoxan to NAIBS was defined as the difference between binding in the presence of 10^{-6} M adrenaline (total nonadrenoceptor binding) and 10^{-4} M naphazoline (nonspecific binding). Binding parameters (K_d, B_{max}) were determined directly by computer-assisted nonlinear analysis from untransformed data using the EBDA-LIGAND programmes. Each value represents the mean ± s.e.mean of n experiments per group with an animal per experiment. One-way ANOVA followed by a multiple comparison test detected a significant decrease in B_{max} after chronic treatment with clorgyline (all doses) and pargyline, but not after acute clorgyline or chronic Ro 41-1049 treatments ($F[7,52] = 20.03$, $P = 0.0001$). *$P<0.01$ as compared with saline-treated group (ANOVA followed by Scheffés test).

Table 2 Effects of acute and/or chronic treatments with psychotrophic drugs on NAIBS in the rat cerebral cortex

		[^{3}H]-idazoxan		
Treatment	Dose (mg kg^{-1})	K_d (nM)	B_{max} (fmol mg^{-1} protein)	n
Saline	–	11.3 ± 0.6	57 ± 2	20
Desipramine				
Acute	3	13.4 ± 1.1	67 ± 5	4
Chronic	3	8.0 ± 0.3	61 ± 3	6
Cocaine				
Chronic	10	9.1 ± 0.3	54 ± 1	5
Reserpine				
Chronic	0.12	9.3 ± 0.7	57 ± 2	5
Haloperidol				
Chronic	1	11.5 ± 2.0	57 ± 5	3
Diazepam				
Chronic	10	9.8 ± 0.8	57 ± 2	5

Each drug, except reserpine, was administered i.p., every 12 h for 14 days or for only one day in the acute desipramine treatment. Reserpine was administered s.c., every 48 h for 14 days. The rats were killed 48 h after the last injection. Other details as for Table 1. Each value represents the mean ± s.e.mean of n experiments per group with an animal per experiment. One-way ANOVA did not detect any significant change in the binding parameters of [^{3}H]-idazoxan to NAIBS after the various treatments.

Table 3 Binding parameters of various drugs to NAIBS in the rat cerebral cortex

	[^{3}H]-idazoxan			
Drug	K_{iH} (nM)	K_{iL} (nM)	$\%R_H$	n
MAO inhibitors				
Clorgyline	0.04	10,600	38	10
Pargyline	150	35,000	35	3
Deprenyl	1,400	55,000	76	3
Ro 16-6491	1,900	–	100	2
Ro 41-1049	79,000	–	100	2
Other drugs				
Idazoxan	14	–	100	3
Desipramine	6,400	–	100	2
Reserpine	12,100	–	100	2
Haloperidol	13,400	–	100	3
Cocaine	338,000	–	100	2

Cortical membranes were incubated at 25°C for 30 min with [^{3}H]-idazoxan (4×10^{-9} M in the presence of 10^{-6} M adrenaline) and in the absence or the presence of the competing drugs (10^{-14} M or 10^{-10} M to 10^{-3} M). Binding parameters (K_{iH}, K_{iL} and $\%R_H$, defined as the percent of high affinity sites for a given drug) were determined directly by simultaneous analysis of n independent experiments for each drug using the EBDA-LIGAND or the GraFit programmes. For the propargylamines (clorgyline, pargyline and deprenyl), computer-assisted curve fitting demonstrated that a two-site fit was significantly better than a one-site binding model ($P<0.001$, F test).

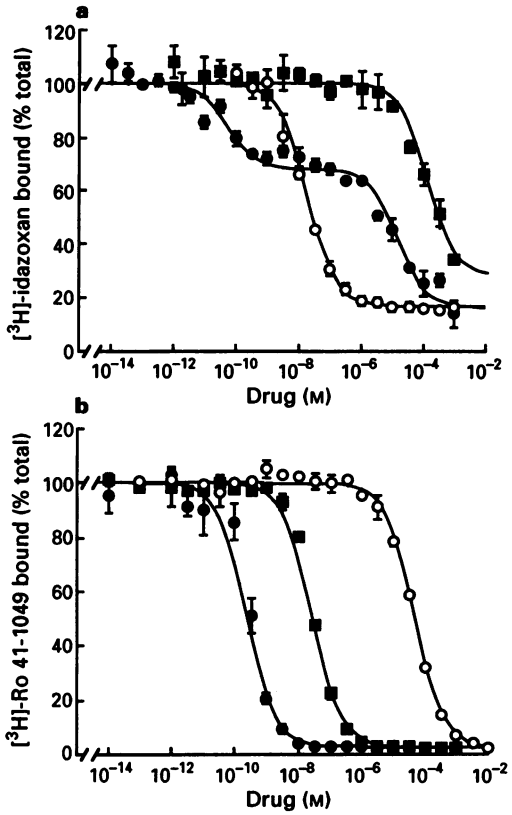


Figure 2 (a) Inhibition of binding of [^{3}H]-idazoxan (in the presence of 10^{-6} M adrenaline) to NAIBS in the rat cerebral cortex by clorgyline (●), idazoxan (○) and Ro 41-1049 (■). Cortical membranes were incubated at 25°C for 30 min with [^{3}H]-idazoxan (4×10^{-9} M) in the absence or presence of various concentrations of the competing drugs. Total control binding was 1,900 d.p.m. Data shown are mean ± s.e.mean (vertical bars) of 2 to 10 experiments per drug. See Table 3 for K_i values and other details. (b) Inhibition of binding of [^{3}H]-Ro 41-1049 to MAO-A in the rat cerebral cortex by clorgyline (●), Ro 41-1049 (■) and idazoxan (○). Cortical membranes were incubated at 37°C for 60 min with [^{3}H]-Ro 41-1049 (4.5×10^{-9} M) in the absence or presence of various concentrations of the competing drugs. Total control binding was 27,000 d.p.m. Data shown are mean ± s.d. of 2 experiments per drug. The K_i values were calculated as above by simultaneous analysis with the EBDA-LIGAND programmes: clorgyline, $K_i = 0.18$ nM; Ro 41-1049, $K_i = 22.4$ nM; and idazoxan, $K_i = 40{,}400$ nM.

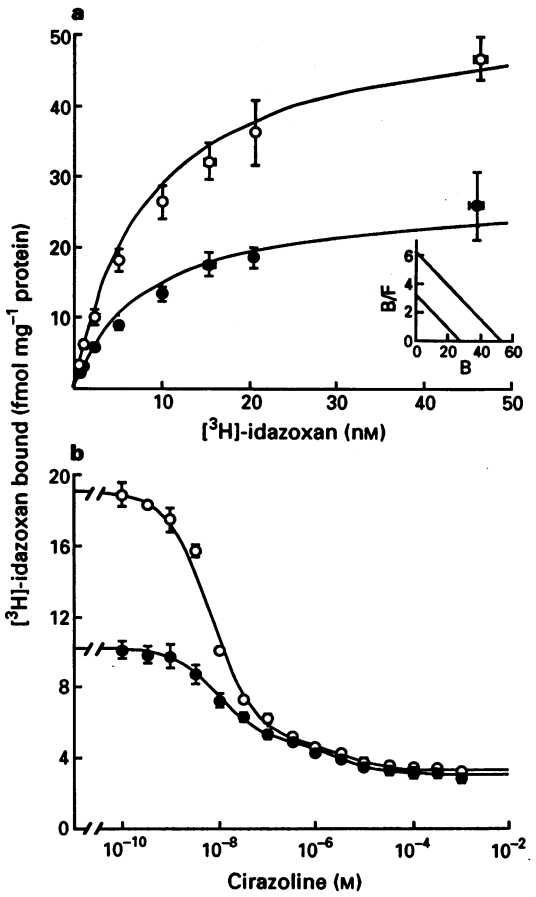


Figure 3 Specific binding of [^{3}H]-idazoxan (in the presence of 10^{-6} M adrenaline) to NAIBS in the rat cerebral cortex after preincubation (30 min at 25°C) of neural membranes in buffer only (○) or buffer containing 10^{-6} M clorgyline (●). (a) Saturation curves of [^{3}H]-idazoxan binding to NAIBS. Nonlinear analysis (EBDA-LIGAND programmes) of untransformed data yielded $K_d = 8.6 \pm 0.5$ nM; $B_{max} = 53 \pm 5$ fmol mg^{-1} of protein for control membranes ($n = 6$) and $K_d = 8.1 \pm 0.9$ nM; $B_{max} = 27 \pm 4$ fmol mg^{-1} of protein for membranes preincubated with clorgyline ($n = 6$) ($P < 0.01$ for B_{max} values). Each point is the mean ± s.e.mean (vertical bars) of n experiments per group. (b) Competition curves for cirazoline against [^{3}H]-idazoxan (4×10^{-9} M) binding to NAIBS. Computer-assisted curve fitting (EBDA-LIGAND programmes) demonstrated that in both sets of membranes a two-site fit was significantly better than a one-site binding model ($P < 0.0001$; F test). The binding parameters obtained were $K_{iH} = 5.2$ nM; $K_{iL} = 2{,}900$ nM; $B_{maxH} = 51$ fmol mg^{-1} of protein; $B_{maxL} = 6$ fmol mg^{-1} of protein for control membranes ($n = 3$) and $K_{iH} = 6.6$ nM; $K_{iL} = 1{,}300$ nM; $B_{maxH} = 20$ fmol mg^{-1} of protein; $B_{maxL} = 7$ fmol mg^{-1} of protein for clorgyline-treated membranes ($n = 6$). Simultaneous analysis of both sets of data with the GraFit programme yielded significant differences between the two groups only with respect to the B_{maxH} ($P < 0.01$; F test). Each point is the mean ± s.e.mean (vertical bars) of n experiments per group.

highest dose used in the chronic studies) and 20 mg kg^{-1} for 6, 12, 24 and 48 h did not induce any reduction of NAIBS in the cerebral cortex (Figure 4). In fact, only with very high doses of clorgyline (40 and 80 mg kg^{-1} for 6 and 48 h) was a modest decrease (21–28%) in the density of NAIBS in the cerebral cortex found (Figure 4).

Discussion

The binding of [^{3}H]-idazoxan to NAIBS has been shown to be markedly heterogeneous (Olmos *et al.*, 1992) and the existence of two affinity states for these sites has been proposed (Wikberg *et al.*, 1992). In this context, the results of the competition experiments for clorgyline (as well as for pargyline and deprenyl) against [^{3}H]-idazoxan binding to NAIBS indicate a biphasic interaction between these propargylamines and these sites, with an apparent very high affinity (40 picomolar) displayed by clorgyline for a relevant population of NAIBS in the brain. These results could be explained, as the incubation experiments with these MAO-inhibitors suggested, by an irreversible interaction of clorgyline and pargyline with only one population of NAIBS, and then by a competitive low affinity binding to the remaining population of non-adrenoceptor sites. Thus, clorgyline has been shown to bind irreversibly to a high affinity population of NAIBS (B_{maxH}) that appears to represent a 90% of total non-adrenoceptor [^{3}H]-idazoxan binding (see Figure 3b). In saturation experiments the reduction of [^{3}H]-idazoxan binding after preincubation with 10^{-6} M clorgyline (total density of NAIBS reduced by about 40%, see Figure 3a) was similar to the decrease (53%) of [^{3}H]-idazoxan binding to only the high affinity population of NAIBS found in competition experiments with cirazoline (Figure 3b), which in addition was also similar to the 40% inhibition induced by clorgyline (10^{-6} M) in competition experiments (Figure 2a). Therefore, clorgyline *in vitro* appears to interact irreversibly with the high affinity population of NAIBS in the brain.

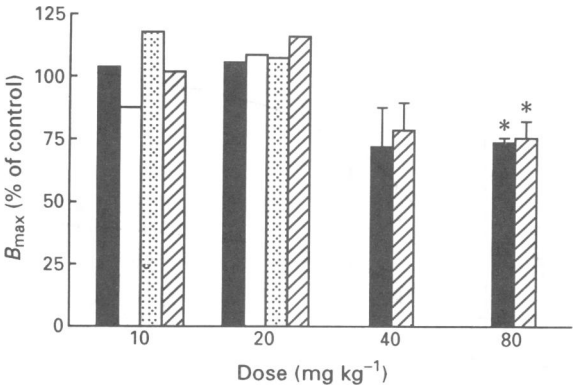


Figure 4 Effect of acute clorgyline administration on the density of NAIBS in the rat cerebral cortex expressed as a percentage of that in saline-treated rats. Total density of NAIBS (B_{max}) was determined through the specific binding of [^{3}H]-idazoxan (in the presence of 10^{-6} M adrenaline) at 6 h (solid column), 12 h (open column), 24 h (stippled column) and 48 h (hatched column) after a single administration (i.p.) of clorgyline at the doses indicated. Values for the doses of 10 and 20 mg kg^{-1} are from one single experiment and for the doses of 40 and 80 mg kg^{-1} represent the mean ± s.d. of two experiments with an animal per experiment. One-way ANOVA followed by a multiple comparison test detected a significant decrease in B_{max} at 6 and 48 h after the administration of 80 mg kg^{-1} of clorgyline ($F[2,6] = 9.19$; $P = 0.015$). *$P < 0.05$ as compared to saline-treated group (ANOVA followed by Scheffé's test).

Since the molecular structure of clorgyline does not resemble that of imidazoli(di)nes, it raises the question as to whether the high affinity found for this MAO-inhibitor on NAIBS is the result of further interactions of [^{3}H]-idazoxan with other protein/binding sites. In this respect, NAIBS appear to be associated with mitochondrial outer membranes and the density of these sites correlates well with MAO activity (Tesson *et al.*, 1991). However, the low affinity on NAIBS displayed by the selective (not propargylamines) MAO-A and MAO-B inhibitors, Ro 41-1049 (Cesura *et al.*, 1990a; Da Prada *et al.*, 1990) and Ro 16-6491 (Cesura *et al.*, 1988; Cesura *et al.*, 1990b), respectively; the lack of down-regulation of NAIBS induced by chronic treatment with Ro 41-1049; and the much higher densities (40 to 75 times) of the two MAO isoenzymes (Saura *et al.*, 1992) with respect to that of NAIBS in the rat cerebral cortex, makes it unlikely that the portion of binding of [^{3}H]-idazoxan blocked with very high affinity by clorgyline could be to enzyme MAO.

Recently, it has been reported that clorgyline also displays high affinity for the σ (sigma) binding sites in the mouse brain (Itzkak & Kassim, 1990). However, the possibility of a direct link/interaction between [^{3}H]-idazoxan and the σ site was also discarded. Firstly, the subcellular localization of the σ binding sites is different from that of NAIBS (Samovilova & Vinogradov, 1992). Secondly, haloperidol, which is a non selective, high affinity σ_1 and σ_2 ligand (Ferris *et al.*, 1991; Quirion *et al.*, 1992; Barnes *et al.*, 1992) displayed very low affinity for NAIBS (K_i = 13,400 nM); and thirdly, chronic treatment with haloperidol decreases the density of σ binding sites in the mouse and rat brains (Itzhak & Alerhand, 1989; Jansen *et al.*, 1992) but it does not affect the density of NAIBS in the rat brain (see Tables 2 and 3).

In addition, the autoradiographic distribution of NAIBS in the rat brain was recently found to be similar to that of peripheral type benzodiazepine sites (Mallard *et al.*, 1992). This site is also a mitochondrial protein which can be labelled by [^{3}H]-diazepam and other selective ligands (for a review see Gavish *et al.*, 1992). Also in this case a hypothetical link between NAIBS and this site was discarded. Thus, the compound PK 11195, a highly selective drug for the peripheral type benzodiazepine sites, displayed very low affinity (K_i > 10,000 nM) competing with the binding of [^{3}H]-idazoxan in human and rabbit liver mitochondria (Tesson *et al.*, 1991). Moreover, chronic treatment with diazepam did not alter the density of NAIBS in the rat brain (see Table 2).

Together these results suggest that, *in vitro*, clorgyline and to a lesser extent pargyline, directly bind to a high affinity population of NAIBS or alternatively that the binding of these MAO-inhibitors to a different protein (MAO, σ site or others) provokes an interaction of this protein with NAIBS that blocks the binding of [^{3}H]-idazoxan to these non-adrenoceptor sites.

The present study also demonstrates that chronic treatment (14 days) with clorgyline (0.3–10 mg kg^{-1}) and pargyline (10 mg kg^{-1}) markedly down-regulate the density of NAIBS in the rat brain. In contrast, various acute treatments with clorgyline (1–20 mg kg^{-1}) for different time intervals (6–48 h) did not alter the density of NAIBS. In the acute treatments only very high doses of clorgyline (40 and 80 mg kg^{-1}) produced significant but modest decreases in the density of NAIBS. These results suggest that the irreversible binding of clorgyline and pargyline to NAIBS found *in vitro* is not entirely responsible for the decrease found *in vivo* after chronic treatment with low/moderate doses of both drugs, and it raises the question as to whether these drugs can act as 'direct agents' upon these non-adrenoceptor sites and thus are able to decrease their density after sustained activation, or if the observed down-regulation of NAIBS is the result of an indirect mechanism. Although chronic treatment with the MAO inhibitor clorgyline has been shown to down-regulate brain α_2-adrenoceptors probably by increasing synaptic NA availability (Giralt & García-Sevilla, 1989 and other references therein), the integrity and/or parallel modulation of the α_2-adrenoceptor is not necessary for the regulation of NAIBS (Olmos *et al.*, 1992). Moreover, the effect of clorgyline on the density of NAIBS in the brain does not appear to be dependent on NA availability since chronic treatment with other drugs (Ro 41-1049, desipramine, cocaine and reserpine) which also potently modify the intraneuronal and/or synaptic NA pools did not induce any significant effect on NAIBS. Another possibility is that some non identified metabolite(s) of the propargylamines clorgyline and pargyline could be active on NAIBS, but very little is known about the metabolism of these compounds (Fowler *et al.*, 1981).

Together the results are compatible with the view that clorgyline and pargyline, through an as yet unknown *in vivo* mechanism, can modulate the density of NAIBS in the brain. Although the native ligand and functions of NAIBS are also unknown, the observed down-regulation of NAIBS induced specially by clorgyline could be related to the antidepressant effects of this drug. In this context, the findings of increased NAIBS in the brain of depressed suicide victims (Barturen *et al.*, 1992) as well as elevated non-adrenoceptor binding sites labelled by [^{3}H]-*p*-aminoclonidine in platelet from depressed patients that can be down-regulated by desipramine (Piletz *et al.*, 1991), provide further evidence that these putative imidazoline receptors may be involved in the pathophysiology of certain forms of depression. In contrast, NAIBS do not appear to be involved with the chronic effects of other psychotropic drugs such as haloperidol, diazepam and cocaine.

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References

ATLAS, D. (1991). Clonidine-displacing substance (CDS) and its putative imidazoline receptor. New leads for further divergence of α_2-adrenergic receptor activity. *Biochem. Pharmacol.*, **41**, 1541–1549.

BARNES, J.M., BARNES, N.M., BARTER, P.C., CHAMPANEIRA, S., COSTALL, B., HORUSBY, C.D., IRONSIDE, J.W. & NAYLOR, R.J. (1992). Pharmacological comparison of the sigma recognition site labelled by [^{3}H]haloperidol in human and rat cerebellum. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **345**, 197–202.

BARTUREN, F., MEANA, J.J., MARTIN, I. & GARCIA-SEVILLA, J.A. (1992). Increased non-adrenoceptor [^{3}H] idazoxan binding sites (NAIBS) in postmortem brains of depressed suicide victims. *Fundam. Clin. Pharmacol.*, **6** (Suppl 1), 57s, P27.

BOYAJIAN, C.L., LOUGHLIN, S.E. & LESLIE, F.M. (1987). Anatomical evidence for alpha-2 adrenoceptor heterogeneity: differential autoradiographic distributions of [^{3}H]rauwolscine and [^{3}H]-idazoxan in rat brain. *J. Pharmacol. Exp. Ther.*, **241**, 1079–1091.

BRUNS, R.F., LAWSON-WENDLING, K. & PUGSLEY, T.A. (1983). A rapid filtration assay for soluble receptors using polyethylenimine-treated filtres. *Anal. Biochem.*, **132**, 74–81.

CESURA, A.M., IMHOF, R., TAKACS, B., GALVA, M.D., PICOTTI, G.B. & DA PRADA, M. (1988). [^{3}H]Ro 16-6491, a selective probe for affinity labelling of monoamine oxidase type B in human brain and platelet membranes. *J. Neurochem.*, **50**, 1037–1043.

CESURA, A.M., BÖS, M., GALVA, M.D., IMHOF, R., DA PRADA, M. (1990a). Characterization of the binding of [^{3}H]Ro 41-1049 to the active site of human monoamine oxidase-A. *Mol. Pharmacol.*, **37**, 358–366.

CESURA, A.M., IMHOF, R., MUGGLI-MANIGLIO, D., PICOTTI, G.B. & DA PRADA, M. (1990b). Characterization of [^{3}H]Ro 16-6491 binding to digitonin solubilized monoamine oxidase-B and purification of the enzyme from human platelets by affinity chromatography. *Biochem. Pharmacol.*, **39**, 216–230.

DA PRADA, M., KETTLER, R., KELLER, H.H., CESURA, A.M., RICHARDS, J.G., SAURA MARTI, J., MUGGLI-MANIGLIO, D., WYSS, P.-C., KYBURZ, E. & IMHOF, R. (1990). From moclobemide to Ro 19-6327 and Ro 41-1049: the development of a new class of reversible, selective MAO-A and MAO-B inhibitors. *J. Neural. Transm.*, **29**, 279–292.

DIAMANT, S., ELDAR-GEVA, T. & ATLAS, D. (1992). Imidazoline binding sites in human placenta: evidence for heterogeneity and a search for physiological function. *Br. J. Pharmacol.*, **106**, 101–108.

ERNSBERGER, P. (1992). Heterogeneity of imidazoline binding sites: proposed I_1 and I_2 subtypes. *Fundam. Clin. Pharmacol.*, **6** (Suppl 1), 55s, P18.

FERRIS, C.D., HIRSCH, D.J., BROOK, B.P. & SNYDER, S.H. (1991). σ receptors: from molecule to man. *J. Neurochem.*, **57**, 729–737.

FOWLER, C.J., ORELAND, L. & CALLINGHAM, B.A. (1981). The acetylenic monoamine oxidase inhibitors clorgyline, deprenyl, pargyline and J-508: their properties and applications. *J. Pharm. Pharmacol.*, **33**, 341–347.

GAVISH, M., KATZ, Y., BAR-AMI, S. & WEIZMAN, R. (1992). Biochemical, physiological, and pathological aspects of the peripheral benzodiazepine receptor. *J. Neurochem.*, **58**, 1589–1601.

GIRALT, M.T. & GARCIA-SEVILLA, J.A. (1989). Acute and long-term regulation of brain α_2-adrenoceptors after manipulation of noradrenergic transmission in the rat. *Eur. J. Pharmacol.*, **164**, 455–466.

GÖTHERT, M. & MOLDERINGS, G.J. (1991). Involvement of presynaptic imidazoline receptors in the α_2-adrenoceptor-independent inhibition of noradrenaline release by imidazoline derivatives. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **343**, 271–282.

HIEBLE, J.P. & RUFFOLO, R.R. Jr. (1992). Imidazoline receptors: historical perspective. *Fundam. Clin. Pharmacol.*, **6** (Suppl 1), 7s–13s.

ITZHAK, Y. & ALERHAND, S. (1989). Differential regulation of σ and PCP receptors after chronic administration of haloperidol and phencyclidine in mice. *FASEB J.*, **3**, 1868–1972.

ITZHAK, Y. & KASSIM, C. (1990). Clorgyline displays high affinity for σ binding sites in C57BL/6 mouse brain. *Eur. J. Pharmacol.*, **176**, 107–108.

JANSEN, K.L.R., ELLIOT, M. & LESLIE, R.A. (1992). σ Receptors in rat brain and testes show similar reductions in response to chronic haloperidol. *Eur. J. Pharmacol.*, **214**, 281–283.

KILPATRICK, A.T., BROWN, C.C. & MACKINNON, A.C. (1992). Non-α_2-adrenoceptor idazoxan binding sites: a new target for drug development. *Biochem. Soc. Trans.*, **20**, 113–118.

LEATHERBARROW, R.J. (1990). *GraFit* Version 2.0., Erithacus Software Ltd., Staines, U.K.

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

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

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

MEANA, J.J., BARTUREN, F. & GARCIA-SEVILLA, J.A. (1992). α_2-Adrenoceptors in the brain of suicide victims: increased receptor density associated with major depression. *Biol. Psychiatry*, **31**, 471–490.

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

MOLDERINGS, G.J., HENTRICH, F. & GÖTHERT, M. (1991). Pharmacological characterization of the imidazoline receptor which mediates inhibition of noradrenaline release in the rabbit pulmonary artery. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **344**, 630–638.

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

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

PILETZ, J.E., HALARIS, A., SARAN, A. & MARLER, M.R. (1990). Elevated ^{3}H-para-aminoclonidine binding to platelet purified plasma membranes from depressed patients. *Neuropsychopharmacology*, **3**, 201–210.

PILETZ, J.E., HALARIS, A., SARAN, A. & MARLER, M.R. (1991). Desipramine lowers tritiated para-aminoclonidine binding in platelets of depressed patients. *Arch. Gen. Psychiatry*, **48**, 813–820.

QUIRION, R., BOWEN, W.D., ITZHAK, Y., JUNIEN, J.L., MUSACCHIO, J.M., ROTHMAN, R.B., SU, T.-P., TAM, W. & TAYLOR, D.P. (1992). A proposal for the classification of sigma binding sites. *Trends Pharmacol. Sci.*, **13**, 85–86.

SAMOVILOVA, N.N. & VINOGRADOV, V.A. (1992). Subcellular distribution of (+)-[^{3}H]SKF 10,047 binding sites in rat liver. *Eur. J. Pharmacol.-Mol. Pharmacol. Sect.*, **225**, 69–74.

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

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

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

WIKBERG, J.E.S., UHLÉN, S. & CHHAJLANI, V. (1992). Evidence that drug binding to non-adrenergic [^{3}H]-idazoxan binding sites (I-receptors) occurs to interacting or interconvertible affinity forms of the receptor. *Pharmacol. Toxicol.*, **70**, 208–219.

YOUDIM, M.B.H., FINBERG, J.P.M. & TIPTON, K.F. (1988). Monoamine Oxidase. In *Catecholamines I.* ed. Trendelenburg, U. & Weiner, N. pp. 119–191. Berlin: Springer-Verlag.

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Immediate inhibitory effect of methylprednisolone suleptanate (U-67590A) on antigen-induced cutaneous and airway anaphylactic responses in guinea-pigs

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1 Inhibitory effects of water-soluble glucocorticoids administered intravenously were examined on skin and airway reactions caused by antigen challenge or chemical mediators in guinea-pigs.

2 Methylprednisolone suleptanate (U-67590A) which is an analogue of methylprednisolone, produced immediate inhibition of 3-h and 7-day homologous passive cutaneous anaphylaxis (PCA) reactions, but not of histamine- or bradykinin-induced cutaneous vascular permeability, when administered 10 min before antigen challenge. In contrast, methylprednisolone succinate (MP) or dexamethasone (DXM) administered 10 min before antigen challenge failed to show an immediate inhibitory effect on the PCA or mediator-induced reactions. When administered 1 to 5 h before antigen challenge, all the steroids used in this study reduced both PCA and mediator-induced reactions.

3 Pretreatment with cycloheximide almost completely abolished the late inhibition of 3-h PCA and histamine reactions produced by U-67590A or MP, but it did not affect the immediate inhibition of 3-h PCA produced by U-67590A.

4 U-67590A also demonstrated immediate inhibitory effects on antigen-induced bronchoconstriction in guinea-pigs actively sensitized with ovalbumin even when administered 10 min before antigen challenge, whereas MP and DXM failed to show the immediate inhibitory effect. When administered 3 h before antigen challenge, all the steroids used in this study reduced the response to antigen.

5 The late inhibitory effect of U-67590A administered 3 h before antigen challenge was almost completely abolished by treatment with cycloheximide or 17α-methyltestosterone, whereas the immediate inhibition produced by U-67590A administered 10 min before challenge was not affected by this treatment.

6 U-67590A administered 10 min or 3 h before challenge did not affect the bronchoconstriction induced by histamine or leukotriene D_4.

7 Release of histamine from lung fragments of sensitized guinea-pigs *in vitro* was inhibited by U-67590A.

8 The present experiments indicate that U-67590A demonstrated dual, immediate and late, inhibitory effects. The former are independent of protein synthesis and may be associated with non-genomic direct action on the mediator-releasing process without affecting mediator-induced reactions. The latter share common inhibitory actions with other glucocorticoids which are dependent on protein synthesis through gene expression.

Keywords: Methylprednisolone; dexamethasone; glucocorticoids; passive cutaneous anaphylaxis; vascular permeability; airway resistance; protein synthesis

Introduction

Glucocorticoids are an important type of therapeutic agent for the treatment of such allergic diseases as bronchial asthma and allergic dermatitis. It is well-established that a latent period is required for their anti-inflammatory action which takes place due to binding to their intracellular receptors and the induction of synthesis of functional proteins (Flower & Blackwell, 1979; Tsurufuji *et al.*, 1979; 1980; Hirata *et al.*, 1980). Lipocortin (Di Rosa *et al.*, 1984) is one such protein that inhibits phospholipase A_2 activity, resulting in the decreased formation of lipid mediators, including leukotrienes, thromboxane A_2, prostaglandins, and platelet activating factor (PAF) (Flower, 1988). The actual mechanism involved in the anti-allergic action of glucocorticoids, however, is still not clear.

The protective effects of glucocorticoids on antigen-induced airway responses in guinea-pigs (Hicks, 1970; Anderson & Brattsand, 1982; Dahlén *et al.*, 1985; Vargas *et al.*, 1990), in rats (Church *et al.*, 1972; Ufkes & Ottenhof, 1984) and in allergic sheep (Delehunt *et al.*, 1984) have been studied. A latent period (usually 2.5–24 h) or repeated daily administration has also been reported to be necessary for glucocorticoids to inhibit anaphylactic bronchoconstriction. As prompt intravenous treatment with methylprednisolone (Littenberg & Gluck, 1981) or hydrocortisone (Fanta *et al.*, 1983) is needed to terminate an asthmatic attack and to alleviate symptoms during the emergency care of acute asthma, it is worth investigating whether derivatives of glucocorticoids act immediately on antigen-induced allergic reactions.

U-67590A, methylprednisolone 21-suleptanate, was found as a 21-ester substitute in a physical-organic approach to the design of solution-stable, water-soluble analogues of the corticosteroid methylprednisolone (Anderson *et al.*, 1985a,b). In the present study, we examined the inhibitory effects of U-67590A on skin and airway responses caused by antigen and chemical mediators, as compared to other water-soluble

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glucocorticoids, methylprednisolone 21-succinate and dexamethasone 21-phosphate. Furthermore, we investigated whether the observed effects were dependent on protein synthesis through binding with the glucocorticoid receptor.

Methods

Animals

Male Hartley guinea-pigs weighing 250–300 g at the time of sensitization, purchased from Japan SLC Inc. (Hamamatsu, Japan), were used throughout these experiments.

Preparation of guinea-pig IgGγ1- or IgE-like serum

Guinea-pig IgGγ1-like serum was prepared by subcutaneous immunization with 1 ml of 1 mg ovalbumin ml^{-1} in saline, emulsified 1:1 in Freund's complete adjuvant. Four weeks after immunization, blood samples were collected from the abdominal vein under light ether anaesthesia and serum was separated. The titer of pooled antiserum was 1:1024 as determined by a 3-h passive cutaneous anaphylaxis (3-h PCA) assay.

Guinea-pig IgE-like serum was prepared by the method of Levine *et al.* (1971). Guinea-pigs were immunized by intraperitoneal injection of a mixture of ovalbumin (2 μg) and aluminum hydroxide gel (alum: about 7.5 mg). Booster injections of the same amount of the ovalbumin-alum mixture were given at 3, 6 and 9 weeks after the primary immunization. One week after the last booster injection, blood samples were collected from the abdominal vein under light ether anaesthesia and serum was separated. The antibody titer of the pooled serum was 1:256 as determined by the 7-day PCA assay. The anti-serum contained IgE-like antibody because heating at 56°C for 2 h almost completely destroyed the 7-day PCA activity. Both anti-sera were stored at −20°C before use.

Measurement of vascular permeability caused by PCA reactions, histamine or bradykinin in guinea-pig dorsal skin

The PCA reaction was elicited in the shaved dorsal skin of the guinea-pig. One hundred microlitres of diluted IgE (1:32) anti-ovalbumin serum in saline was injected intradermally 7 days before the antigen challenge (7-day PCA). IgG (1:256) was injected 3 h before the antigen challenge (3-h PCA). A solution of 3 mg kg^{-1} of the ovalbumin solution and 2 ml kg^{-1} of 1% Evans blue was injected intravenously. Thirty minutes later, the animals were killed by exsanguination and a blueing site on the dorsal skin was punched out. The skin was dissolved with 2 ml of 2 N KOH. The extravasated dye was extracted with 6 ml of acetone after the sample was neutralized with 2 ml of 1.24 N phosphoric acid. The amount of dye was measured spectrophotometrically (620 nm) and was expressed as μg per site.

The increase in vascular permeability caused by intradermal injection of 0.1 ml histamine (100 μg ml^{-1}) or bradykinin (100 μg ml^{-1}) was also evaluated by determination of Evans blue dye extravasated into the dorsal skin of the same animals. Anti-inflammatory steroids were administered intravenously at a specified time before the injection of antigen and mediators.

Measurement of airway resistance

Animals were sensitized by subcutaneous injection of a 1 ml solution containing 1 mg ovalbumin ml^{-1} in saline and emulsified 1:1 in Freund's complete adjuvant. Four to 6 weeks after sensitization, they were anaesthetized with Nembutal-Na (50 mg kg^{-1}, i.p.) and their tracheae cannulated. The jugular vein was cannulated for intravenous drug administration and antigen challenge. Airway resistance was measured by the overflow technique according to the method of Konzzet & Rössler (1940). Animals were ventilated mechanically with a rodent respirator (Model SN-480-7, Shinano, Tokyo, Japan) at 40 strokes min^{-1} at a load pressure of 10 cmH_2O. Ventilation overflow through a side arm of the tracheal cannula was monitored with a Bronchospasm Transducer 7020 (Ugo Basil, Comerio-Varese, Italy) and amplified with a Bioelectric Amplifier AB-621G (Nihonkoden, Tokyo, Japan). The increase in ventilation overflow was taken as an index of increased airway resistance and was expressed as a percentage of the maximum overflow obtained by clamping off the trachea at the end of the experiment. When antigen over the dose-range of 0.01 to 1 mg kg^{-1} was injected intravenously into sensitized guinea-pigs, a dose-related increase in ventilation overflow was observed that reached a maximum at 1 mg kg^{-1}. Antigen challenge with 1 mg kg^{-1} ovalbumin in 0.9% saline was, therefore, carried out by injection into the right jugular vein, unless otherwise stated. In another experiment, antigen aerosol, prepared by ultrasonic nebulization (Model NE-U11B, Omron, Tokyo, Japan) of 10 ml of ovalbumin solution (10 mg ml^{-1}), was inhaled through the air inlet channel of the respirator for 1 min. Intravenous injection of leukotriene D_4 (LTD_4) or histamine into non-sensitized guinea-pigs induced bronchoconstriction and the potencies were measured by the procedure described above. The glucocorticoids were given intravenously through the jugular vein or the dorsal vein of the penis at a specified time before the antigen challenge.

Release of histamine from lung fragments of sensitized guinea-pigs

Guinea-pigs were sensitized passively with an intravenous injection of anti-ovalbumin guinea-pig IgE serum (1 ml). Seven days later, the animals were exsanguinated and their lungs were perfused with 40 ml of calcium-free Hank's balanced salt solution (HBSS) via the right ventricle of the heart into the pulmonary artery to remove blood. Lung tissue was dissected from the trachea and major bronchioles and was cut into cubes of about 0.5 mm^3 by a McIlwain mechanical tissue chopper (Mickle Laboratory Engineering, Nr. Guildford, Surrey). After being washed with calcium-free HBSS on gauze, 100 mg aliquots of the lung fragments were suspended in 0.8 ml of normal HBSS per tube. After incubation with U-67590A at 37°C for 10 or 60 min, the lung fragments were challenged with 0.1 ml of ovalbumin solution (3 mg ml^{-1}). Thirty minutes later, the reaction was terminated by cooling on ice and the supernatant assayed for histamine after filtration through gauze. The histamine released was extracted and the amount was determined by a fluorometric assay according to the method of Shore (1971). The fluorospectrometer used was a Titertek Fluoroskan II (excitation 355 nm, emission 460 nm, Flow Laboratories Japan, Tokyo, Japan). Histamine release was expressed as a percentage of the total histamine content in the tissue determined for the supernatant after boiling 100 mg of lung tissue for 10 min.

Drugs and chemicals

U-67590A (methylprednisolone, 21-[8-[methyl(2-sulphoethyl) amino]-8-oxooctanoate], sodium salt; The Upjohn Company, Kalamazoo, MI, U.S.A.) was dissolved in 0.34% NaCl at a concentration of 180 mg ml^{-1} (equivalent to 100 mg ml^{-1} methylprednisolone), then diluted with 0.9% saline. Methylprednisolone 21-succinate sodium salt (Solu-Medrol, The Upjohn Company) was prepared in 0.9% saline. U-67590A and methylprednisolone sodium 21-succinate (methylprednisolone) are expressed as methylprednisolone-equivalent doses (mg kg^{-1}). The other compounds used were FPL-55712 (sodium 7-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylate), ovalbumin, dexamethasone 21-phosphate disodium salt

(dexamethasone), mepyramine maleate salt, cycloheximide, 17α-methyltestosterone and bradykinin acetate salt (Sigma, St. Louis, MO, U.S.A.), Evans blue, histamine dihydrochloride, LTD_4 (Wako Pure Chemicals, Osaka, Japan), Freund's complete adjuvant (Difco, Detroit, MI, U.S.A.) and Nembutal-Na (Abbot Lab., North Chicago, IL, U.S.A).

Statistics

Dunnett's multiple comparison test was used after ANOVA. Results are expressed as means ± s.e.mean, *n* representing the number of animals used.

Results

Time course of the inhibitory effects of U-67590A and methylprednisolone on the vascular permeability caused by histamine and the 3-h PCA reaction

Figure 1 shows the effects of U-67590A and methylprednisolone, administered 10, 30, 60, 120 or 300 min before histamine or antigen challenge, on the vascular permeability caused by histamine and the 3-h PCA reaction.

U-67590A, administered 10 min before the antigen challenge, clearly inhibited the vascular permeability caused by the 3-h PCA reaction (Figure 1a). At 30 to 60 min after administration, the degree of inhibition had decreased but was still significant. A second peak of inhibitory activity was observed at 180 min. U-67590A also inhibited histamine-induced vascular permeability, but this inhibition was seen only after a latent period of at least 60 min.

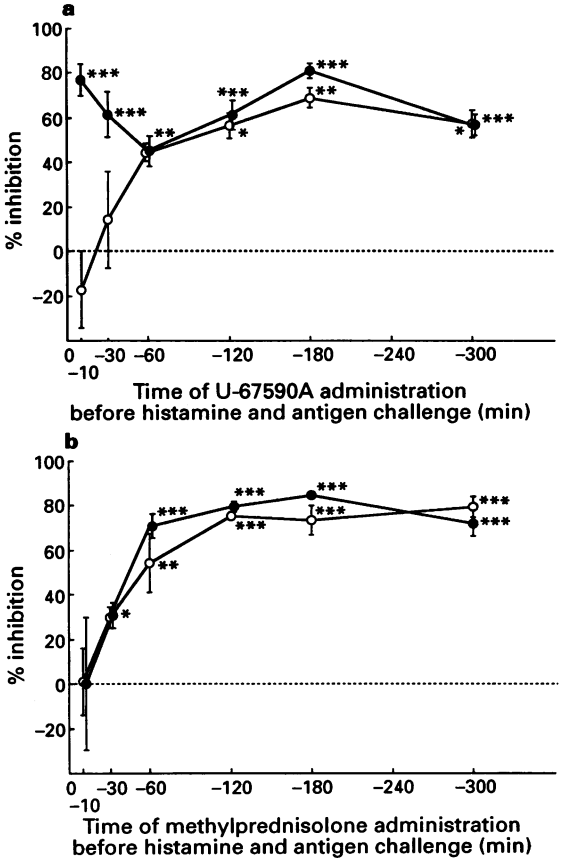


Figure 1 Time course of the inhibitory effects of U-67590A (a) and methylprednisolone (b) on the vascular permeability caused by histamine (○) and 3-h PCA (●) reactions in guinea-pig dorsal skin. The abscissa scale indicates the time of intravenous administration of U-67590A (30 mg kg^{-1}) and methylprednisolone (100 mg kg^{-1}) before the antigen and histamine challenges. Values are expressed as the percentage inhibition of the amount of dye extravasated in the treated groups when compared to the amount in vehicle control. Each point represents the mean ± s.e.mean (vertical bars) ($n = 4-5$). The amounts of dye leakage (μg per site) for the control animals were 3-h PCA: 91.5 ± 13.0 (control for U-67590A), 108.0 ± 15.3 (control for methylprednisolone) and histamine: 95.7 ± 17.6 (control for U-67590A), 109.3 ± 14.3 (control for methylprednisolone). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$: when compared with vehicle control.

In contrast to U-67590A, methylprednisolone administered 10 min before the challenge did not inhibit the vascular permeability induced by either the 3-h PCA or histamine (Figure 1b). Thirty minutes after administration, inhibition of both reactions by methylprednisolone was apparent and significant inhibition was seen for the 3-h PCA. Inhibition of both responses reached a plateau at 120 min after methylprednisolone administration.

Thus, U-67590A, but not methylprednisolone, demonstrated an early onset of inhibition of the 3-h PCA reaction, whereas a latent period was necessary for these glucocorticoids to inhibit histamine-induced vascular permeability.

Effect of cycloheximide pretreatment on the inhibitory effects of U-67590A and methylprednisolone on the vascular permeability caused by histamine and the 3-h PCA reaction

To determine whether the inhibitory effects of U-67590A or methylprednisolone on the cutaneous reactions were mediated by functional protein synthesis, cycloheximide (5 mg kg^{-1}), a protein synthesis inhibitor, was given subcutaneously 1 h before steroid treatment (Figure 2).

Pretreatment with cycloheximide completely abolished the late inhibitory effect of U-67590A, when administered 60 to 180 min before challenge, for both the 3-h PCA reaction and histamine-induced vascular permeability (Figure 2a). In contrast, the immediate inhibitory effect of U-67590A, administered less than 30 min before the challenge, was not affected by cycloheximide pretreatment. The inhibitory effects of methylprednisolone on both the 3-h PCA and the histamine reactions were almost completely abolished by cycloheximide pretreatment (Figure 2b). Cycloheximide alone did not affect either the 3-h PCA reaction or histamine-induced vascular permeability (data not shown).

Effects of glucocorticoids and mepyramine on the vascular permeability caused by histamine, bradykinin, 3-h PCA and 7-day PCA reactions

The immediate effects of U-67590A, methylprednisolone, dexamethasone and mepyramine, administered 10 min before the challenge, on the increase in vascular permeability caused by chemical mediators and PCA reactions are summarized in Table 1. None of the glucocorticoids used inhibited the histamine- or bradykinin-induced vascular permeabiltiy. In contrast, U67590A inhibited both the 3-h and 7-day PCA reactions, whereas neither methylprednisolone nor dexamethasone inhibited these reactions. Although mepyramine almost completely inhibited histamine-induced vascular permeability, the inhibition of the 3-h and 7-day PCA reactions by mepyramine, which was observed in the same dose-range as for histamine-induced reactions, was not complete.

As shown in Table 2, the glucocorticoids, administered 3 h before challenge, suppressed significantly the cutaneous responses provoked by the 3-h and 7-day PCA reactions as well as by histamine or bradykinin at the highest dose used, and in some instances, at lower doses. The inhibition by U-67590A of the 7-day PCA reaction occurred at lower doses than those required to inhibit the histamine or bradykinin reactions, whereas methylprednisolone and dexamethasone inhibited the 3-h and 7-day PCA, histamine and bradykinin reactions at similar doses.

Thus, the immediate and late effects of U-67590A on PCA reactions were observed in similar dose-ranges. The doses of methylprednisolone and dexamethasone which caused a late

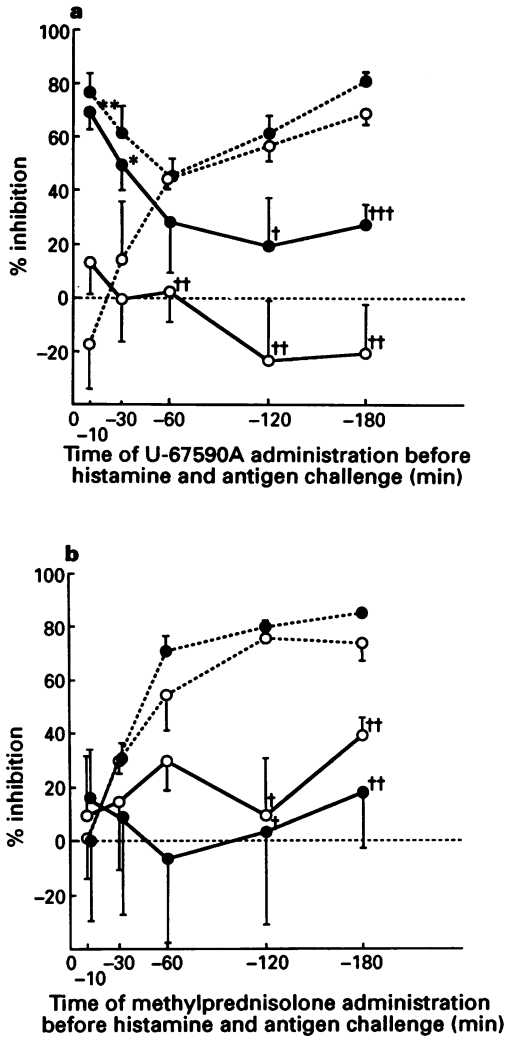


Figure 2 Effect of cycloheximide (CHI) pretreatment on the inhibitory effects of U-67590A (a) and methylprednisolone (b) on the vascular permeability caused by histamine (○) and 3-h PCA (●) reactions in guinea-pig dorsal skin (solid lines). The abscissa scale indicates the times of intravenous administration of U-67590A (30 mg kg^{-1}) and methylprednisolone (100 mg kg^{-1}) before the antigen and histamine challenges. CHI (5 mg kg^{-1}) was given subcutaneously 1 h before steroid treatment. Values are expressed as the percentage inhibition of the amount of dye extravasated in the treated groups when compared to the amount in vehicle control. Each point represents the mean ± s.e.mean (vertical bars) ($n = 4-5$). The amounts of dye leakage (μg per site) for the control animals were 3-h PCA: 85.0 ± 10.1 (control for U-67590A), 39.1 ± 14.6 (control for methylprednisolone) and histamine: 51.6 ± 7.9 (control for U-67590A) 56.5 ± 18.3 (control for methylprednisolone). The results from Figure 1 (broken lines) have been superimposed, to indicate effects without cycloheximide treatment. $^{*}P < 0.05$; $^{**}P < 0.01$: when compared with vehicle control. †$P < 0.05$; ††$P < 0.01$; †††$P < 0.001$: when compared with the group without cycloheximide treatment (broken lines).

inhibition of histamine and PCA reactions did not, however, have the immediate effects on the PCA reactions.

As the doses of U-67590A and methylprednisolone were 10 fold higher than those of dexamethasone, the immediate effects of high doses of dexamethasone were also investigated to determine whether the immediate inhibitory effect of U-67590A may be induced by a non-specific action at high doses. Dexamethasone did not affect the 3-h PCA or histamine reactions at doses up to 30 mg kg^{-1} and slightly (but not significantly) inhibited both responses to almost the same extent at 100 mg kg^{-1}, which was about 30 fold higher than the dose for the late inhibitory effect (Table 3). Moreover, the degree of inhibition was considerably smaller (14–18% *vs* 69–79%) and was not affected by pretreatment with cycloheximide.

Intravenous injection of the ester side chain of U-67590A, suleptanate (8-oxo-8-[(2-sulphoethyl) methylamino] octanoic acid), which is the only difference between methylprednisolone and U-67590A, did not affect the 3-h PCA reaction and histamine-induced vascular permeability even at 100 mg kg^{-1} (3-h PCA, vehicle: 39.1 ± 14.6, $n = 5$, *vs* suleptanate: 42.3 ± 10.4, $n = 4$; histamine, vehicle: 51.6 ± 20.1, $n = 5$ *vs* suleptanate: 63.0 ± 11.6, $n = 4$).

Inhibitory effects of glucocorticoids on the antigen-induced bronchial response

The onset of the inhibitory effect of U-67590A, administered 10 min to 24 h before antigen challenge, was investigated for the bronchial response (Figure 3). A significant reduction in airway resistance was found for U-67590A even when administered 10 min before antigen challenge. Inhibition was greatest 3 h after treatment, had decreased slightly at 5 h and was no longer apparent 24 h later.

As shown in Figure 4, methylprednisolone (100 mg kg^{-1}, i.v.) and dexamethasone (10 mg kg^{-1}, i.v.) significantly inhibited the bronchial response to antigen challenge when administered 3 h before antigen challenge. When administered 10 min before antigen challenge, however, these two glucocorticoids did not suppress the response.

Suleptanate, the ester side chain of U-67590A, had no effect on airway resistance increased by the antigen challenge even at the high dose of 300 mg kg^{-1}, i.v. (vehicle: 97.6 ± 2.4, $n = 5$ *vs* suleptanate: 93.7 ± 4.2, $n = 4$).

Effects of cycloheximide and 17α-methyltestosterone on the inhibition of antigen-induced bronchial response caused by U-67590A

Cycloheximide or 17α-methyltestosterone (a glucocorticoid antagonist) was given subcutaneously 1 h before treatment with U-67590A to test for functional protein synthesis and for receptor-mediated events. As shown in Figure 5a, pretreatment with cycloheximide substantially reduced the late inhibitory effect produced by U-67590A administered 3 h before the antigen challenge (solid columns), but failed to abolish the immediate inhibition caused by U-67590A administered 10 min before the challenge (hatched columns). Similar results were obtained for pretreatment with 17α-methyltestosterone (Figure 5b). Cycloheximide alone at 5 mg kg^{-1} had a slight inhibitory effect (15.5%) on the antigen-induced bronchoconstriction and 17α-methyltestosterone alone, even at a high dose (200 mg kg^{-1}), had no effect (data not shown).

Effect of U-67590A on histamine- and leukotriene D_4-induced bronchoconstriction

Combined treatment of guinea-pigs with the H_1 histamine antagonist mepyramine (1 mg kg^{-1}, i.v.) and the sulphidopeptide leukotriene antagonist FPL-55712 (10 mg kg^{-1}, i.v.) 10 min before antigen challenge, almost completely abolished the bronchial response in this model (data not shown). The direct inhibitory action of U-67590A on histamine and LTD_4 was, therefore, examined in non-sensitized guinea-pigs. As shown in Figure 6, histamine at 3–100 μg kg^{-1} and LTD_4 at 0.04–0.2 μg kg^{-1} induced dose-dependent bronchoconstriction. Administration of U-67590A 10 min or 3 h before the challenge neither shifted the dose-response curves of these bronchoconstrictors nor reduced the degree of bronchoconstriction.

Effect of U-67590A on the bronchoconstriction induced by antigen inhalation

U-67590A clearly inhibited the bronchoconstriction induced by antigen inhalation when administered 10 min (immediate

Table 1 Immediate effect of glucocorticoids and mepyramine administered 10 min prior to mediators and antigen challenge, on the vascular permeability caused by histamine, bradykinin, 3-h PCA and 7-day PCA reactions in guinea-pig dorsal skin

Steroids	Dose (mg kg^{-1} i.v.)	% inhibition Histamine	Bradykinin	3-h PCA	7-day PCA
U-67590A	3	− 2.3 ± 10.0	− 7.0 ± 23.1	26.8 ± 14.4	79.6 ± 10.5***
	10	− 37.8 ± 17.1	− 40.0 ± 30.0	55.2 ± 21.6	66.5 ± 13.9***
	30	− 17.2 ± 17.2	4.4 ± 10.8	76.8 ± 6.9**	69.3 ± 7.5***
Methylprednisolone	10	30.9 ± 10.3	− 17.3 ± 34.8	26.1 ± 13.6	23.7 ± 36.9
	30	16.6 ± 26.8	20.4 ± 27.4	− 14.8 ± 31.7	− 16.2 ± 51.8
	100	1.0 ± 15.0	13.9 ± 26.6	0.2 ± 29.7	− 34.1 ± 25.3
Dexamethasone	0.3	− 9.3 ± 45.7	24.4 ± 9.9	22.6 ± 14.4	− 10.4 ± 27.7
	1	− 5.4 ± 30.0	36.3 ± 9.6	31.8 ± 13.1	0.4 ± 27.7
	3	− 34.8 ± 18.3	23.2 ± 3.4	− 25.3 ± 44.0	− 2.3 ± 21.0
Mepyramine	0.03	13.1 ± 16.0	− 12.4 ± 25.1	− 21.1 ± 41.0	− 5.9 ± 25.6
	0.3	83.1 ± 5.1**	54.4 ± 11.7	63.6 ± 9.9*	51.5 ± 2.8*
	3	98.5 ± 0.4**	53.3 ± 8.7	59.5 ± 8.5*	57.3 ± 9.5*

Values are means ± s.e.mean ($n = 4-5$) percentage inhibition of the amount of dye extravasated for the treated groups as compared to the amounts for the respective vehicle controls. The amounts of dye leakage for the control animals were histamine: 39.4 ± 4.5 to 81.2 ± 14.1; bradykinin: 15.1 ± 2.9 to 33.8 ± 2.1; 3-h PCA: 16.2 ± 2.4 to 60.4 ± 13.3; 7-day PCA: 13.1 ± 3.4 to 40.6 ± 5.8 (μg per site). *$P<0.05$; **$P<0.01$; ***$P<0.001$; when compared with vehicle control.

Table 2 Late effect of glucocorticoids administered 3 h prior to mediators and antigen challenge, on the vascular permeability caused by histamine, bradykinin, 3-h PCA and 7-day PCA reactions in guinea-pig dorsal skin

Steroids	Dose (mg kg^{-1} i.v.)	% inhibition Histamine	Bradykinin	3-h PCA	7-day PCA
U-67590A	3	4.4 ± 20.4	− 4.6 ± 30.4	53.2 ± 13.9	63.1 ± 9.1**
	10	42.1 ± 11.3	53.0 ± 9.7*	54.3 ± 14.3	76.8 ± 6.8***
	30	83.5 ± 4.3**	80.3 ± 4.9**	77.9 ± 5.7**	91.0 ± 5.9***
Methylprednisolone	10	20.1 ± 11.7	13.3 ± 9.3	3.5 ± 9.8	15.4 ± 37.5
	30	59.8 ± 11.7**	40.5 ± 16.9	48.2 ± 7.1	21.6 ± 9.3
	100	64.5 ± 10.0**	76.4 ± 7.9**	54.8 ± 19.0*	82.5 ± 3.6*
Dexamethasone	0.3	− 18.5 ± 26.1	28.4 ± 8.4	59.1 ± 8.5	− 40.5 ± 57.1
	1	57.2 ± 7.7	68.6 ± 8.4***	56.5 ± 13.0	33.3 ± 12.2
	3	69.1 ± 11.4*	71.9 ± 10.1***	78.7 ± 3.9*	85.4 ± 4.4*

Values are means ± s.e.mean ($n = 4-5$) percentage inhibition of the amount of dye extravasated for the treated groups as compared to the amounts for the respective vehicle control. The amounts of dye leakage for the control animals were histamine: 39.4 ± 4.5 to 81.2 ± 14.1; bradykinin: 15.1 ± 2.9 to 33.8 ± 2.1; 3-h PCA: 16.2 ± 2.4 to 60.4 ± 13.3; 7-day PCA: 13.1 ± 3.4 to 40.6 ± 5.8 (μg per site). *$P<0.05$; **$P<0.01$; ***$P<0.001$; when compared with vehicle control.

Table 3 Immediate effect of high doses of dexamethasone, administered 10 min prior to histamine and antigen challenge, on the vascular permeability caused by histamine and 3-h PCA reactions in guinea-pig dorsal skin

Steroids	Dose (mg kg^{-1})	n	Amoung of dye leakage (μg per site) Histamine	3-h PCA
Vehicle		6	52.9 ± 8.0 (0)	47.5 ± 3.8 (0)
Dexamethasone (i.v.)	10	5	58.3 ± 4.9 (− 10.2)	53.3 ± 7.2 (− 12.3)
	30	6	62.8 ± 8.4 (− 18.7)	50.7 ± 3.9 (− 6.8)
	100	5	45.3 ± 7.0 (14.3)	39.0 ± 3.5 (17.8)
Dexamethasone (i.v.) + Cycloheximide (s.c.)	100 + 5	6	45.2 ± 5.3 (14.6)	34.0 ± 3.5 (28.3)

Cycloheximide (5 mg kg^{-1}) was injected subcutaneously 1 h before dexamethasone treatment. Values represent means ± s.e.mean ($n = 5-6$). Figures in parentheses indicate the percentage inhibition compared with vehicle control values.

inhibition) or 3 h (late inhibition) prior to the challenge (Figure 7). This result was similar to that obtained by intravenous challenge with antigen.

Effect of U-67590A on histamine release from lung fragments of sensitized guinea-pigs in vitro

As shown in Table 4, U-67590A (0.01–1 mM) significantly inhibited antigen-induced histamine release from lung fragments of sensitized guinea-pigs in a concentration-dependent manner. The same inhibitory effect was obtained after incubation of lung fragments with U-67590A for 10 or 60 min.

Histamine release was not significantly inhibited by 0.1 mM suleptanate but was inhibited by 49.6% at 1 mM. However, this was 10 times the concentration of U-67590A that produced almost complete inhibition.

Discussion

It was established in the 1960s that steroids exert their actions through intracellular steroid hormone receptors and

regulate gene expression. It was also reported that glucocorticoids produce not only lipocortin as an inhibitory protein of phospholipase A_2 (Di Rosa *et al.*, 1984), but vasoregulin

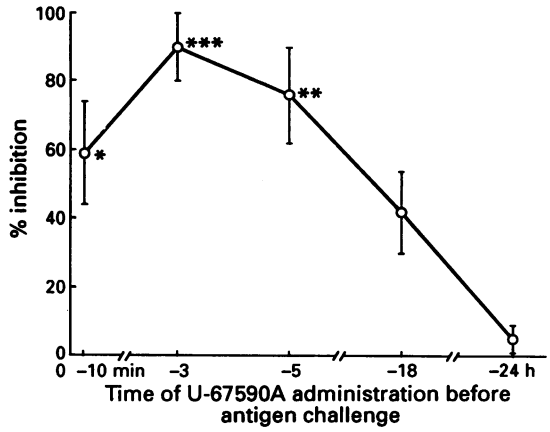


Figure 3 Time course of the inhibitory effect of U-67590A on antigen-induced bronchoconstriction in guinea-pigs sensitized with ovalbumin. The abscissa scale indicates the times of intravenous administration of U-67590A (30 mg kg^{-1}) before the antigen challenge. Each point indicates the percentage inhibition of airway resistance and represents the mean ± s.e.mean (vertical bars) ($n = 7-8$). $*P<0.05$; $**P<0.01$; $***P<0.005$: when compared with vehicle control.

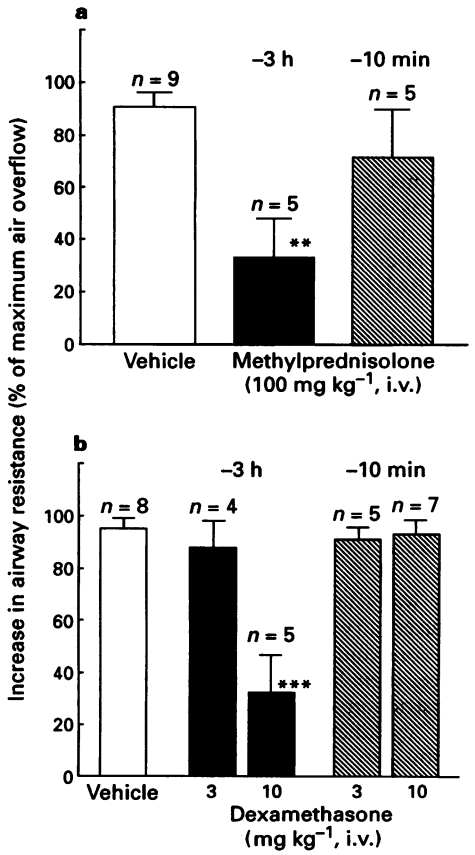


Figure 4 Effects of methylprednisolone (a) and dexamethasone (b) on antigen-induced bronchoconstriction in sensitized guinea-pigs. Each steroid was administered intravenously 3 h (solid columns) or 10 min (hatched columns) before the antigen challenge. Open columns indicate the responses of vehicle controls. Airway resistance is expressed as a percentage of maximum overflow. Vertical bars represent the s.e.mean. $**P<0.01$; $***P<0.005$: when compared with vehicle control.

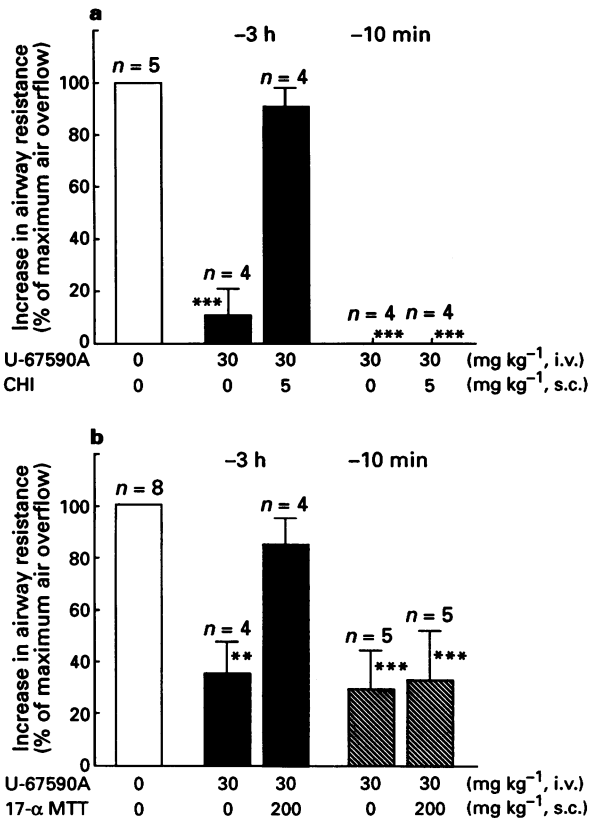


Figure 5 Effects of cycloheximide (CHI; a) and 17α-methyltestosterone (17α-MTT; b) on the inhibitory effect of U-67590A on the antigen-induced bronchoconstriction in sensitized guinea-pigs. CHI or 17α-MTT was given 1 h before U-67590A treatment and the animals were challenged with antigen 3 h (solid columns) or 10 min (hatched columns) later. Open columns indicate the responses of the vehicle controls, which were 100% of the maximum airflow in all the animals. Airway resistance is expressed as a percentage of maximum overflow. Vertical bars represent the s.e.mean. $**P<0.01$; $***P<0.005$: when compared with vehicle control.

(Oyanagui & Suzuki, 1985) and vasocortin (Di Rosa *et al.*, 1985) as well, although the latter two proteins do not inhibit phospholipase A_2. These proteins are responsible for some of the anti-inflammatory actions of glucocorticoids through gene expression. The late inhibitory action of glucocorticoids, therefore, is considered to be exerted through gene expression, because protein synthesis takes longer than 1 h (Blackwell *et al.*, 1982).

The present study, however, demonstrated that intravenous injection of U-67590A, a novel methylprednisolone analogue, caused immediate inhibition of antigen-induced reactions in guinea-pig skin and airways. This effect is not considered to be mediated by gene expression.

The present experiments showed that the inhibitory effects of dexamethasone, methylprednisolone and U-67590A on the vascular permeability induced by 3-h and 7-day PCA reactions or by histamine and bradykinin were mediated by the synthesis of anti-inflammatory proteins since cycloheximide reduced these effects, when the steroids were administered more than 60 min before the challenge. In addition, U-67590A showed a specific characteristic, in that it was effective in reducing the antigen-induced vascular permeability, even when administered as little as 10 min prior to the challenge. This immediate inhibition is in marked contrast to the actions of other anti-inflammatory steroids and it was not inhibited by cycloheximide. Thus, U-67590A has dual inhibitory actions on inflammatory responses (Figure 1). The doses of glucocorticoids used in our experiments were relatively high because of the low sensitivity

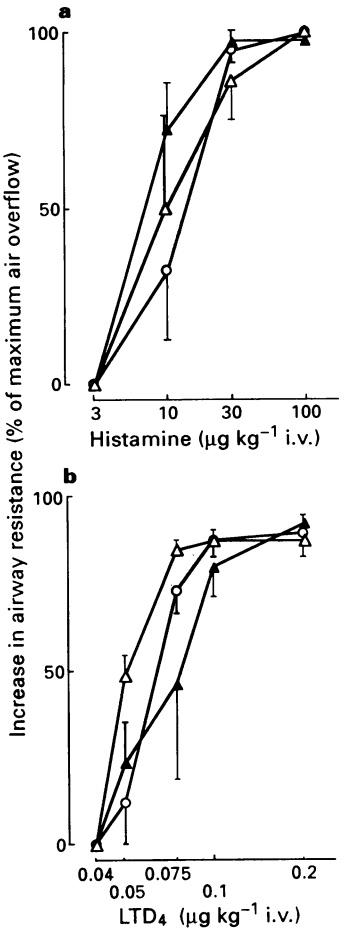


Figure 6 Effect of U-67590A on histamine- (a) and leukotriene D_4 (LTD_4) (b)-induced bronchoconstriction in non-sensitized guinea-pigs. U-67590A (30 mg kg^{-1}) was administered intravenously 10 min (△) or 3 h (▲) before the intravenous injection of the bronchoconstrictors; (○) responses of vehicle control. Airway resistance is expressed as a percentage of maximum overflow. Each point represents the mean ± s.e.mean (vertical bars) ($n = 3-4$).

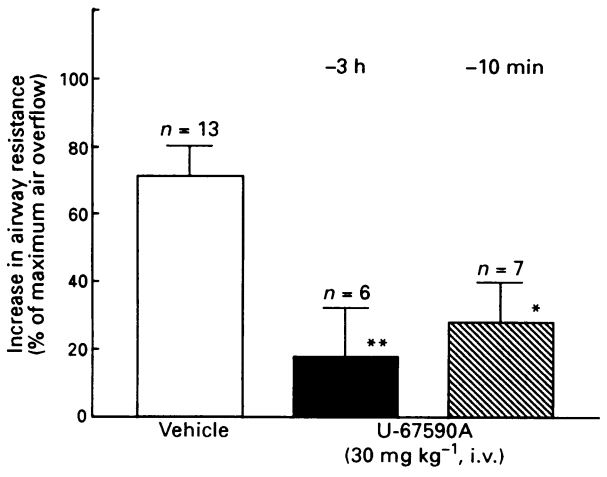


Figure 7 Effect of U-67590A on bronchoconstriction induced by antigen inhalation in sensitized guinea-pigs. U-67590A was administered intravenously 3 h (solid columns) or 10 min (hatched columns) before ovalbumin inhalation. The open column indicates the response of vehicle control. Airway resistance is expressed as a percentage of maximum overflow. Vertical bars represent the s.e.mean. $*P<0.05$; $**P<0.01$: when compared with vehicle control.

Table 4 Effect of U-67590A on histamine release from lung fragments of sensitized guinea-pigs *in vitro*

U-67590A (mM)	*Antigen*	*% histamine release* 10 min[a]	60 min[a]
0	–	4.9 ± 0.9***	6.2 ± 0.8***
0	+	34.0 ± 4.0	32.9 ± 4.3
0.01	+	27.1 ± 3.3 (23.7)	28.0 ± 2.8 (18.4)
0.1	+	11.9 ± 1.0***(75.9)	9.3 ± 1.8***(88.4)
1	+	5.0 ± 1.1***(99.7)	6.6 ± 2.0***(98.5)

Histamine release is expressed as the mean ± s.e.mean percentage of the total histamine in the lung 30 min after antigen stimulation (ovalbumin 0.3 mg ml^{-1}). $n = 4-5$. [a]Incubation period with U-67590A. Figures in parentheses indicate the percentage inhibition of histamine release corrected for spontaneous release with no antigen stimulation. $***P<0.001$ when compared with antigen (+) vehicle control.

of guinea-pigs to glucocorticoids in general (Claman, 1975). The immediate inhibition by U-67590A of the PCA reaction was not, however, considered to be cytotoxic, because (1) U-67590A did not inhibit the vascular permeability caused by histamine or bradykinin in the same dose ranges as for PCA; (2) the dose ranges of U-67590A used did not differ for the immediate and late inhibitions; (3) dexamethasone did not show immediate inhibition of the vascular permeability produced by 3-h PCA reactions and histamine at doses 30 times those causing late inhibition.

PCA is an *in vivo* skin reaction commonly used to evaluate the immediate hypersensitivity caused by homocytotropic antibody (Ovary, 1958). In guinea-pigs, both $IgG\gamma_1$ and IgE are homocytotropic (Bloch & Ohman, 1971; Perini & Mota, 1972) and the respective activities of $IgG\gamma_1$ and IgE could be assessed by the 3-h and 7-day PCA (Parish, 1970; Andersson, 1981). Mepyramine inhibited both the 3 h and 7-day PCA reactions, indicating that histamine may play an important role in plasma leakage in cutaneous anaphylaxis. As its inhibition was not marked, however, other compounds are likely to be involved in the guinea-pig PCA reaction. Recently, Weg *et al.* (1991) reported that not only histamine but LTD_4 and PAF may be involved in the guinea-pig PCA reaction.

The dye leakage in the skin induced by histamine and bradykinin was strongly inhibited by U-67590A, methylprednisolone or dexamethasone given 3 h before challenge with the mediators. None of the glucocorticoids used was effective, however, when administered 10 min prior to these challenges, and only mepyramine at this time inhibited the dye leakage produced by histamine. This indicates that the glucocorticoid applied a short time before the challenge did not inhibit the vascular leakage induced by the mediators which have been generated.

The same was true for the effect of glucocorticoids on the antigen-induced airway response. When dexamethasone, methylprednisolone and U-67590A were administered 3 h before the challenge, their late inhibitory effects on antigen-induced bronchoconstriction were mediated by protein synthesis and reduced by cycloheximide and by 17α-methyltestosterone which is known to compete with the steroid in binding with its receptor in hepatoma cells (Samuels & Tompkins, 1970). Moreover, the immediate inhibition by U-67590A was also observed in the antigen-induced bronchial response as well as in the PCA reaction and this inhibition was not affected by cycloheximide or 17α-methyltestosterone. In guinea-pigs, histamine and sulphidopeptide leukotrienes are thought to be important mediators in antigen-induced bronchoconstriction (Forsberg & Sorenby, 1981; Ritchie *et al.*, 1981; Anderson *et al.*, 1983; Hedqvist *et al.*, 1985). U-67590A did not, however, affect the bronchoconstriction induced by histamine or LTD_4 *in vivo*. Therefore, the immediate inhibition of antigen-

induced bronchonstriction by U-67590A was not associated with a decrease in the responsiveness of the airway smooth muscle to contractile agonists. It was probably due to a reduction in the release of mediators.

The mechanism of immediate inhibition by U-67590A is not clear, but we could show that U-67590A inhibited *in vitro* histamine release from lung fragments of sensitized guinea-pigs. The concentrations used in this experiment were somewhat high, but as a plasma concentration of U-67590A of 108 μM has been measured in dogs after i.v. administration of 5 mg kg^{-1} U-67590A (unpublished data), the concentrations used in this study may be sufficient to inhibit histamine release *in vivo*. This would explain the quantitative inhibition of the 3-h and 7-day PCA reactions and the lack of effect on vascular permeability caused by histamine and bradykinin. The difference in the action of U-67590A and methylprednisolone has been attributed to the ester side chain of U-67590A, suleptanate. It may cause early transport of this steroid to the cell membrane although suleptanate itself did not possess any anti-inflammatory effects. This may be related to the accumulating evidence, as described by McEwen (1991), that steroids also affect the cell surface and alter ion permeability, as well as the release of neurohormones and neurotransmitters and they affect neuronal activity through non-genomic and genomic effects.

In conclusion, U-67590A, a novel water-soluble analogue of methylprednisolone, showed dual inhibitory effects, causing both immediate and late inhibition of the antigen-induced skin and airway reactions. Although the late inhibitory action is common to other steroids, immediate inhibition is probably not mediated by gene expression via cytoplasmic glucocorticoid receptors and may be partly explained by the direct inhibition of mediator release. Thus, this novel glucocorticoid may be added as a useful drug for therapeutic strategies against allergic diseases, including asthmatic attacks, in which an immediate inhibitory effect is called for.

References

ANDERSON, B.D., CONRADI, R.A. & KNUTH, K.E. (1985a). Strategies in the design of solution-stable, water-soluble prodrugs I: a physical-organic approach to pro-moiety selection for 21-esters of corticosteroids. *J. Pharm. Sci.*, **74**, 365–374.

ANDERSON, B.D., CONRADI, R.A., KNUTH, K.E. & NAIL, S.L. (1985b). Strategies in the design of solution-stable, water-soluble prodrugs II: properties of micellar prodrugs of methylprednisolone. *J. Pharm. Sci.*, **74**, 375–381.

ANDERSON, W.H., O'DONNELL, M., SIMKO, B.A. & WELTON, A.F. (1983). An *in vivo* model measuring antigen-induced SRS-A-mediated bronchoconstriction and plasma SRS-A level in the guinea-pig. *Br. J. Pharmacol.*, **78**, 67–74.

ANDERSSON, P. (1981). Antigen-induced bronchial anaphylaxis in actively sensitized guinea pigs. The effect of booster injection and cyclophosphamide treatment. *Int. Archs. Allergy Appl. Immunol.*, **64**, 249–258.

ANDERSSON, P. & BRATTSAND, R. (1982). Protective effect of the glucocorticoid, budesonide, on lung anaphylaxis in actively sensitized guinea-pigs: inhibition of IgE- but not IgG-mediated anaphylaxis. *Br. J. Pharmacol.*, **76**, 139–147.

BLACKWELL, G.J., CARNUCCIO, R., DI ROSA, M., FLOWER, R.J., LANGHAM, C.S.J., PARENTE, L., PERSICO, P., RUSSELL-SMITH, N.C. & STONE, D. (1982). Glucocorticoids induce the formation and release of anti-inflammatory and anti-phospholipase proteins into the peritoneal cavity of the rat. *Br. J. Pharmacol.*, **76**, 185–194.

BLOCH, K.J. & OHMAN, J.L. (1971). The stable homocytotropic antibodies of guinea pig, mouse and rat; and some evidence for the *in vivo* interaction of homocytotropic antibodies of two different rat immunoglobulin classes at a common receptor on target cells. In *Biochemistry of Acute Allergic Reactions, Second International Symposium.* ed. Austen, K.F. & Becker, E.L. pp. 45–60. Oxford: Blackwell Scientific Publications.

CHURCH, M.K., COLLIER, H.O.J. & JAMES, W.L. (1972). The inhibition by dexamethasone and disodium cromoglycate of anaphylactic bronchoconstriction in the rat. *Br. J. Pharmacol.*, **46**, 56–65.

CLAMAN, H.N. (1975). How corticosteroids work. *J. Allergy Clin. Immunol.*, **55**, 145–151.

DAHLÉN, S.-E., PALMERTZ, U. & HEDQVIST, P. (1985). Methylprednisolone inhibition of leukotriene-dependent airway anaphylaxis in the guinea-pig *in vivo*. *Agents Actions*, **16**, 310–311.

DELEHUNT, J.C., YENGER, B.S., AHMED, T. & ABRAHAM, W.M. (1984). Inhibition of antigen-induced bronchoconstriction by methylprednisolone succinate. *J. Allergy Clin. Immunol.*, **73**, 479–483.

DI ROSA, M., FLOWER, R.J., PARENTE, L. & RUSSO-MARRIE, F. (1984). Anti-phospholipase proteins: nomenclature announcement. *Prostaglandins*, **28**, 441–442.

DI ROSA, M., CALIGNANO, A., CARNUCCIO, R., IALENTI, A. & SAUTEBIN, L. (1985). Multiple control of inflammation by glucocorticoids. *Agents Actions*, **17**, 284–289.

FANTA, C.H., ROSSING, T.H. & MCFADDEN, E.R.Jr. (1983). Glucocorticoids in acute asthma: a critical controlled trial. *Am. J. Med.*, **74**, 845–851.

FLOWER, R.J. (1988). Lipocortin and the mechanism of action of the glucocorticoids. *Br. J. Pharmacol.*, **94**, 987–1015.

FLOWER, R.J. & BLACKWELL, G.J. (1979). Anti-inflammatory steroids induce biosynthesis of a phospholipase A_2 inhibitor which prevents prostaglandin generation. *Nature*, **278**, 456–459.

FORSBERG, K. & SÖRENBY, L. (1981). The influence of a new corticosteroid, budesonide, on anaphylactic bronchoconstriction and SRS-A release in the guinea-pig. *Agents Actions*, **11**, 391–395.

HEDQVIST, P., DAHLÉN, S.-E. & PALMERTZ, U. (1985). Leukotriene-dependent airway anaphylaxis in guinea-pigs. *Prostaglandins*, **28**, 605–608.

HICKS, R. (1970). The influence of corticosteroid pretreatment on anaphylactic bronchoconstriction in the guinea-pig. *J. Pharm. Pharmacol.*, **22**, 170–179.

HIRATA, F., SCHIFFMAN, E., VENKATASUBRAMANIAN, K., SALOMON, D. & AXELROD, J. (1980). A phospholipase A_2 inhibitory protein in rabbit neutrophils induced by glucocorticoids. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 2533–2536.

KONZETT, H. & RÖSSLER, R. (1940). Versuchsanordnung zu untersuchungen an der bronchialmuskulatur. *Naunyn-Schmiederbergs Arch. Exp. Path. Pharmakol.*, **195**, 71–74.

LEVINE, B.B., CHANG, H.Jr. & VAZ, N.M. (1971). The production of hapten specific reaginic antibodies in the guinea-pig. *J. Immunol.*, **106**, 29–33.

LITTENBERG, B. & GLUCK, E.H. (1981). A controlled trial of methylprednisolone in the emergency treatment of acute asthma. *N. Engl. J. Med.*, **305**, 783–789.

MCEWEN, B.S. (1991). Non-genomic and genomic effects of steroids on neural activity. *Trends Pharmacol. Sci.*, **12**, 141–147.

OVARY, Z. (1958). Passive cutaneous anaphylaxis in the mouse. *J. Immunol.*, **81**, 355–357.

OYANAGUI, Y. & SUZUKI, S. (1986). Vasoregulin, a glucocorticoid-inducible vascular permeability inhibitory protein. *Agents Actions*, **17**, 270–277.

PARISH, W.E. (1970). Homologous serum passive cutaneous anaphylaxis in guinea pigs mediated by two γ_1 or γ_1-type heat-stable globulins and a non-γ_1 heat-labile reagin. *J. Immunol.*, **105**, 1296–1298.

PERINI, A. & MOTA, I. (1972). Heterogeneity of guinea-pig homocytotropic antibodies. *Immunology*, **22**, 915–923.

RITCHIE, D.M., SIERCHIO, J.N., CAPETOLA, R.J. & ROSENTHALE, M.E. (1981). SRS-A-mediated bronchospasm by pharmacologic modification of lung anaphylaxis *in vivo*. *Agents Actions*, **11**, 396–401.

SAMUELS, H.H. & TOMPKINS, G.M. (1970). Relation of steroid structure to enzyme induction in hepatoma tissue culture cells. *J. Mol. Biol.*, **52**, 57–74.

SHORE, P.A. (1971). Fluorometric assay histamine. In *Methods in Enzymology*, vol. 17/b. ed. Tabor, H. & Tabor, C.W. pp. 842–845. New York: Academic Press.

TSURUFUJI, S., SUGIO, K. & TAKEMASA, F. (1979). The role of glucocorticoid receptor and gene expression in the anti-inflammatory action of dexamethasone. *Nature*, **280**, 408–410.

TSURUFUJI, S., SUGIO, K., TAKEMASA, F. & YOSHIZAWA, S. (1980). Blockade by antiglucocorticoids, actinomycin D and cycloheximide of anti-inflammatory action of dexamethasone against bradykinin. *J. Pharmacol. Exp. Ther.*, **212**, 225–231.

UFKES, J.G.R. & OTTENHOF, M. (1984). Characterization of various anti-allergic agents using a new method for inducing systemic anaphylaxis in the rat. *J. Pharmacol. Meth.*, **11**, 219–226.

VARGAS, M.H., MONTAÑO, L.M., VANDA, B., SEGURA, P. & SELMAN, M. (1990). Pharmacological characterization of mediators and vagal influence in the acute allergic bronchoconstriction in guinea pigs. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **342**, 278–283.

WEG, V.B., WATSON, M.L., CORDEIRO, R.S.B. & WILLIAMS, T.J. (1991). Histamine, leukotriene D_4 and platelet-activating factor in guinea pig passive cutaneous anaphylaxis. *Eur. J. Pharmacol.*, **204**, 157–163.

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Protein kinase regulation of muscarinic receptor signalling in colonic smooth muscle

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1 We have previously demonstrated that M_2 and M_3 muscarinic receptors coexist in the circular smooth muscle of canine promixal colon. Activation of receptors of the M_2 subtype leads to inhibition of adenylyl cyclase activity through the GTP-binding protein, G_i, while M_3 receptors are coupled to a pertussis toxin-insensitive GTP-binding protein and mediate phosphoinositide hydrolysis.

2 In the present study, the interactions between these second messenger systems were examined. Activation of either protein kinase C or adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase attenuated carbachol-stimulated phosphoinositide hydrolysis without affecting basal activity. Activation of both protein kinases produced greater attenuation of inositol 1,4,5-trisphosphate formation than activation of either kinase alone.

3 In contrast to its inhibitory effect on phosphoinositide hydrolysis, activation of protein kinase C had no effect on adenylyl cyclase activity.

4 Activation of protein kinase C by phorbol ester treatment resulted in the sequestration of M_3 muscarinic receptors from the cell surface without effecting the M_2 muscarinic receptor population. Sequestered M_3 muscarinic receptors were not rapidly degraded.

5 In contrast, elevation of cellular cyclic AMP decreased the affinity of cell surface muscarinic receptors for an antagonist radioligland without affecting their density.

6 Muscarinic agonist binding was not affected by either activation of protein kinase C or elevation of cellular cyclic AMP.

7 These data support the notion of negative feedback by protein kinase C and cyclic AMP-dependent protein kinase on phosphoinositide hydrolysis. In canine colonic circular smooth muscle this negative feedback regulation of inositol phosphate generation by muscarinic receptor stimulation does not appear to involve the guanine nucleotide binding protein:receptor interaction.

Keywords: Muscarinic receptors; protein kinase C, cyclic AMP-dependent protein kinase; phosphoinositide hydrolysis; smooth muscle, colon

Introduction

Parasympathetic innervation of the colon resulting in the action of acetylcholine at postsynaptic muscarinic receptors on the smooth muscle provides the principal stimulus for normal colonic motility. We have demonstrated the coexistence of muscarinic receptors of the M_2 and M_3 subtype in canine colonic circular smooth muscle (Zhang *et al.*, 1991). Activation of M_2 receptors leads to inhibition of adenylyl cyclase activity through a pertussis toxin-sensitive guanine nucleotide binding protein (G-protein), G_i, while M_3 receptors are coupled to a pertussis toxin-insensitive G-protein and mediate phosphatidylinositol (PI) hydrolysis (Zhang & Buxton, 1991). While a precise role for the M_2 receptor in the contraction of gastrointenstinal smooth muscle has not been conclusively established (McDaniel *et al.*, 1991), removing the relaxation produced by adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent phosphorylations is likely to be necessary, if not sufficient, for contraction (Candell *et al.*, 1990; Fernandes *et al.*, 1992). On the other hand, hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate inositol 1,4,5-trisphosphate (Ins-1,4,5-P_3) and diacyglycerol (DAG) following activation of the M_3 receptor is likely to play a direct role in initiation of contraction of the muscle (Candell *et al.*, 1990). Ins-1,4,5-P_3 is known to release Ca^{2+} from sarcoplasmic stores in smooth muscle (Somlyo & Himpens, 1989), while DAG together with Ca^{2+}, would be expected to activate protein kinase C (PKC) (Nishizuka, 1986).

It is generally agreed that protein phosphorylations mediated by specific cellular kinases, such as PKC and cyclic AMP-dependent protein kinase (cyclic AMP-PK), play a critical role in cellular physiology, including regulation of receptor responsiveness. Activation of PKC, an expected consequence of PI hydrolysis, blocks muscarinic agonist-induced stimulation of phospholipase C (PLC) in the brain as well as other cell types (Orellana *et al.*, 1985; Blackmore & Exton, 1986; Lai & El-Fakahany, 1988); while the action of cyclic AMP-PK leads to inhibition of inositol phosphate formation mediated by α_1-adrenoceptors in rat aorta (Neylon & Summers, 1988; Manolopoulos *et al.*, 1991). Such protein kinase regulation of PI turnover could occur at any of a number of possible steps. The most probable of these are: (i) phosphorylation of receptors leading to their sequestrian (Scherer & Nathanson, 1990; Abdallah *et al.*, 1990; Hu *et al.*, 1991); (ii) phosphorylation of the G-protein coupling receptors to PLC activation (Brostom *et al.*, 1989; Scherer & Nathanson, 1990; Imaizumi *et al.*, 1991); or (iii) phosphorlylation of the PLC enzyme itself (Kim *et al.*, 1989; Rhee *et al.*, 1989; Ryu *et al.*, 1990). The functions of many members of the G-protein-linked receptor family, including muscarinic receptors, are regulated by receptor phosphorylation. It has been shown that purified M_1 muscarinic receptors can be phosphorylated *in vitro* by both PKC and cyclic AMP-PK and that purified M_2 receptors can be phosphorylated by cyclic AMP-PK (Rosenbaum *et al.*, 1987; Haga *et al.*, 1988). In this regard, it is intriguing that phosphorylation of muscarinic receptors shows subtype specificity with M_1 and M_3 receptor

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subtypes being more sensitive to the effects of PKC (Scherer & Nathanson, 1990; Hu *et al.*, 1991).

Since M_2 and M_3 muscarinic receptors coexist on the same smooth muscle cell in colonic circular muscle and mediate their effects through distinct signal transduction pathways, it is of interest to determine the potential interactions between these second messenger systems. In the present study, we employ the phorbol ester PKC agonist, phorbol 12,13-dibutyrate (PDBu) and the adenylyl cyclase agonist, forskolin (FSK), to examine the effects of activation of cyclic AMP-PK and PKC on PI hydrolysis stimulated by occupation of muscarinic receptors in canine colonic circular smooth muscle. In addition, we extend our observations of muscarinic receptor heterogeneity on individual smooth muscle cells to include the fraction of receptors present on the cell surface.

Methods

Tissue and cell preparation

Mongrel dogs of either sex were anaesthetized with pentobarbitone sodium (30 mg kg^{-1}). A segment of proximal colon (6–14 cm from the ileocecal spincter) was removed and cut into 2 × 3 cm pieces in a dissecting dish bathed with Krebs-Ringer bicarbonate solution of the following composition (in mM): Na^+ 142.3, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 125.6, HCO_3^- 23.8, $H_2PO_4^-$ 1.2, dextrose 11.0. This solution had a pH of 7.4 at 37°C when bubbled to equilibrium with 95% O_2/5% CO_2. The longitudinal smooth muscle was carefully removed and the circular smooth muscle separated from the submucosa by the method of Smith *et al.* (1987). Isolated circular smooth muscle cells were prepared as described previously (Zhang *et al.*, 1991). Briefly, isolated circular muscle strips were minced and digested in calcium-free Hank's solution containing (mg ml^{-1}): collagenase type II (1.3); bovine serum albumin (2.0); trypsin inhibitor (2.0); and adenosine triphosphate (0.56). Digestion was carried out with gentle agitation at 37°C for 30 min. The supernatant was collected and replaced alternatively with calcium-free Hank's solution and the buffer containing enzyme, every 20 min until the tissue was totally digested. Intact cells were found in the supernatant to which a final concentration of 10% foetal calf serum was added. Final purification of cells with an anisodiametric appearance (>90%) excluding trypan blue (0.3%) was achieved by unit gravity sedimentation. Cells not excluding dye were stubby or rounded and represented dead or dying cells. The final preparation was devoid of small debris present in the original digestion. The use of unit gravity sedimentation in the preparation of cells is believed to purify in favour of the relatively more dense smooth muscle cells over other cells types present in the original digestion.

Radioligand binding in membrane homogenates

Tissues prepared as described above were washed three times with ice-cold hypotonic buffer A (50 mM Tris base, 10 mM $MgCl_2$, 1 mM EGTA, pH 7.4) and homogenized in 10 vol. of the same buffer. The homogenate was centrifuged as 2,500 × *g* for 20 min, the supernatant collected and the pellet again homogenized and centrifuged under the same conditions. This second pellet, containing nuclei and mitochondria, was discarded and the supernatants combined and centrifuged at 50,000 *g* for 60 min at 4°C. The resulting supernatant was discarded and the pellet quickly frozen in liquid nitrogen and stored as a frozen powder at −80°C for 1–2 weeks until employed in radioligand binding studies. Competition between the non selective muscarinic receptor antagonist radioligand [^{3}H]-QNB and the muscarinic agonist, carbachol (CCh) was measured as previously described (Zhang *et al.*, 1991). Protein was determined by the method of Bradford (1976).

Radioligand binding to intact cells

Binding of ligands to freshly dispersed intact colonic circular smooth muscle cells was measured by a rapid filtration method similar to that described previously (Zhang *et al.*, 1991). Cells were washed three times with oxygenated Krebs solution (composition mM: NaCl 118, KCl 4.7, KH_2PO_4 0.6, Na_2HPO_4 0.6, $MgCl_2$ 1.2, destrose 20, $CaCl_2$ 1, HEPES 25, pH 7.4). Following incubation with the permeant phorbol ester, PDBu, FSK or diluent at 32°C for the time period indicated, equilibrium binding of [^{3}H]-NMS and [^{3}H]-QNB to intact cells was carried out in Krebs solution at 32°C for 60 min. For saturation binding experiments, cells were incubated with [^{3}H]-NMS (25 to 3200 pM) in the presence and absence of 10 μM atropine to define nonspecific binding. Specific binding of [^{3}H]-NMS averaged approximately 90% of total binding at its K_D. Bound and free radioligand were separated by rapid filtration over Whatman GF/C filters washed with two 5 ml aliquots of ice-cold 0.9% NaCl and counted at 45% efficiency in a Beckman LS 6000IC liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA, U.S.A.). All determinations were performed in triplicate, and specific binding of [^{3}H]-NMS and [^{3}H]-QNB at 0.2 nM was examined in competition studies employing increasing concentrations of unlabelled competitor.

Measurement of protein kinase activity

Circular muscles were homogenized in ice-cold 20 mM Tris/HCl, pH 7.4, containing 2 mM EDTA, 0.5 mM EGTA, and 2 mM phenyl methyl-sulphonylfluoride and centrifuged at 100,000 *g* for 30 min at 4°C. Assay of the supernatant was defined as cytoplasmic PKC activity. Membrane-bound PKC activity was extracted from the pellet by resuspension in 20 mM Tris/HCl, pH 7.4, containing 1% Triton X-100 (4°C for 60 min) and assayed in the supernatant following centrifugation at 100,000 *g* for 30 min. Both cytosolic and membrane-bound fractions were purified over DEAE cellulose columns washed with 5 ml of 20 mM Tris/HCl, pH 7.4, and eluted with 3 ml of the same buffer containing 120 mM NaCl. Fractions were assayed for PKC activity by the method of Secrest *et al.* (1991) using phosphorylation of histone (Type VII-S). DEAE fractions (35 μl) were incubated at 30°C for 10 min in 20 mM Tris HCl buffer (pH 7.4) containing: 750 μg ml^{-1} histone, 5 mM magnesium acetate, 20 mg ml^{-1} phosphatidylserine, 30 mM $CaCl_2$ and 20 mM ATP (~4–6 × 10^6 d.p.m. [^{32}P]-ATP) in a total volume of 150 μl. The incubation was stopped on ice for 3 min, spotted onto 2.1 cm circles of Whatman P-81 phosphocellulose paper and immediately placed into hot (60°C) 10% trichloroacetic acid (TCA)/2% sodium phosphate for 5 min. Filters were removed and washed in two changes of fresh TCA/sodium phosphate solution at 21°C. Incorporation of [^{32}P]-ATP into histone was determined by counting radioactivity remaining on the filter. Protein kinase activity other than PKC activity was determined in the absence of phosphatidylserine and $CaCl_2$ and the presence of 1 mM EGTA. Specific PKC activity was calculated by sub-tracting activity in the absence of lipid and Ca^{2+}. Data are expressed as pmol-PO_4 incorporated min^{-1} mg^{-1} protein.

Phosphoinositide hydrolysis

Circular smooth muscle was dissected into 50 mg (wet wt.) pieces and placed into oxygenated labeling buffer of the following composition (mM): NaCl 118.0, KCl 4.7, KH_2PO_4 0.6, Na_2HPO_4 0.6, $MgCl_2$ 1.2, dextrose 5.0, $CaCl_2$ 0.5, HEPES 10.0, $NaHCO_3$ 5.0 (pH 7.4). Tissues were incubated with [^{3}H]-*myo*-inositol (200 μCi ml^{-1}) for 3 h at 35°C and washed with six changes of fresh buffer over 30 min to remove unincorporated radioactivity. At this concentration of the radiolabel, incorporation of [^{3}H]-*myo*-inositol into the inositol containing phospholipids, phosphatidylinositol (PI),

phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) reached apparent steady state by 3 h (Zhang & Buxton, 1991). Following various treatments determined by experimental design, tissues were challenged with the muscarinic agonist carbachol (CCh) in the presence of Li^+ (10 mM) and the reactions terminated by quick freezing in liquid N_2. Tissues were then homogenized in 2 ml ice-cold $CHCl_3$:CH_3OH:HCl (66:33:1), centrifuged at 400 *g* for 10 min and the aqueous phase collected and lyophilized to dryness. [^{3}H]-inositol phosphates (InsPs) were separated by high performance liquid chromatography (h.p.l.c.) over Whatman Partisil 10 SAX eluted with an ammonium phosphate gradient as described previously (Zhang & Buxton, 1991). The identities of the [^{3}H]-InsPs were verified by comparison with authentic standards. Quantification was achieved by determining peak area as recorded in counts per min by the radioactive flow scintillation counter (^{3}H Eff. = 26%).

Cyclic AMP assay

Cyclic AMP content of circular smooth muscle was determined by the method of Gilman (1970). Freshly dissected tissues (30 mg wet wt.) were incubated with the cyclic AMP-phosphodiesterase inhibitor, isobutylmethyl xanthine (1 mM) for 30 min prior to treatment with agonists for the determination of cyclic AMP. Control experiments (data not shown) revealed that without the presence of this submaximal block of cyclic AMP metabolism, changes in cyclic AMP content following either β-receptor or FSK stimulated adenylyl cyclase activity could not be reproducibly measured. After treatment, tissues were homogenized in 5% TCA and the deproteinized supernatant purified over Dowex AG 50W-X4 (200–400 mesh). The recovery of cyclic AMP after purification was determined by the addition of 0.25 pmol [^{3}H]-cyclic AMP to the sample before column purification. Values are expressed as pmol cyclic AMP mg^{-1} protein.

Materials

Drugs and chemicals were purchased from the following sources: carbachol, pirenzepine, atropine, isoprenaline, PDBu, FSK, ATP, cyclic AMP, GTP, creatine phosphate, creatine phosphokinase, cyclic AMP-PK, phosphatidylserine, HEPES, EGTA, EDTA, histone VII-S, bovine serum albumin, soybean trypsin inhibitor, and isobutylmethyl xanthine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); 4α-phorbol-12,13-dideconoate was from Calbiochem (San Diego, CA, U.S.A.); collagenase type II was from Worthington (Freehold, NJ, U.S.A.); anion exchange resin from BioRad Laboratories (Richmond, CA, U.S.A.); DEAE-Sephacel was obtained from Pharmacia LKB (Piscataway, NJ, U.S.A.); P-81 phosphocellulose paper from Whatman (Hillsboro, OR, U.S.A.); [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB; 45.7 Ci $mmol^{-1}$), [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS, 70 Ci $mmol^{-1}$), [^{3}H]-cyclic AMP (44.5 Ci $mmol^{-1}$), [^{32}P]-ATP (3000 Ci $mmol^{-1}$), [^{3}H]-*myo*-inositol (58.1 Ci $mmol^{-1}$) and [^{3}H]-inositol phosphate standards were obtained from Dupont-NEN (Boston, MA, U.S.A.).

Data analysis

Saturation binding data were analyzed by Scatchard plot. For competition binding data, the computer assisted (Inplot, GraphPad Software, San Diego, CA, U.S.A.) 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.mean. Statistal significance was determined by Students *t* test where a *P* value less than 0.05 was considered significant.

Results

Phorbol 12,13-dibutyrate and carbachol treatment increases protein kinase C activity

Since activation of PKC has been shown to be associated with the translocation of the enzyme from the cell cytosol to the plasma membrane (Kraft & Anderson, 1983), we evaluated the effects of PDBu and CCh on PKC subcellular distribution and activity in colonic smooth muscle. Basal, membrane-bound PKC activity was 7.3 pmol min^{-1} mg^{-1} protein which represented 29% of total cellular PKC activity, while the remaining 71% of activity (17.6 pmol min^{-1} mg^{-1}) was found in the cytosolic fraction. In the presence of PDBu (0.1 and 1 μM), the activity of membrane-bound PKC was increased to 191% and 224% of the control, respectively (Figure 1). As expected, these increases in membrane-bound PKC activity were accompanied by stimultaneous decreases in the amount of PKC present in the cytosol. Concentrations of the muscarinic receptor agonist CCh (0.1 to 10 μM) known to stimulate PI turnover (Zhang & Buxton, 1991) produced dose-dependent increases in PKC activity associated with the plasma membrane (Figure 1).

Phorbol 12,13-dibutyrate and forskolin treatment decreases phosphatidylinositol-turnover stimulated by carbachol

Treatment of colonic circular smooth muscle with FSK (1 nM→100 μM) for 10 min produced a dose-dependent increase in cyclic AMP (205 ± 18→670 ± 33 pmol mg^{-1} protein) with an EC_{50} of 1.9 μM. This change in cyclic AMP accumulation is associated with an inhibition of acetycholine-induced contractions (Smith *et al.*, 1992). Pretreatment of colonic circular smooth muscle with either PDBu (1 μM; 30 min) or FSK (1 μM; 10 min) did not modify the basal concentration of inositol phosphates (measured 30 min later in the presence of 10 mM Li^+), suggesting that neither PKC nor cyclic AMP-PK exert a tonic control over PI turnover in unstimulated tissues. As shown in Figure 2, after 30 min in

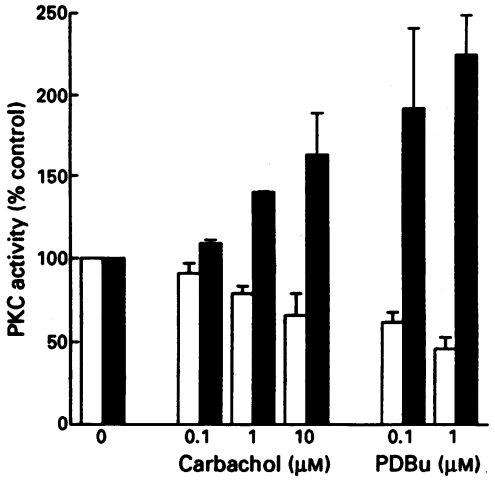


Figure 1 Effects of carbachol (CCh) and phorbol 12,13-dibutyrate (PDBu) on the subcellular distribution of protein kinase C (PKC) activity in canine colon circular smooth muscle. PKC activity in cytosolic and membrane fractions of colonic smooth muscle were assayed as described in Methods. Open column; cytosolic PKC; solid column: membrane-bound PKC. Enzyme activity is expressed as percentage increase over basal values determined in the absence of agonist or phorbol ester pretreatment (cytosolic control = 17.55 ± 8.3 pmol min^{-1} mg^{-1}; membrane bound activity = 7.3 ± 2.0 pmol min^{-1} mg^{-1}). Activation of the enzyme is associated with translocation of activity from soluble to particulate fractions. Data points are mean values ± range of two experiments performed in duplicate.

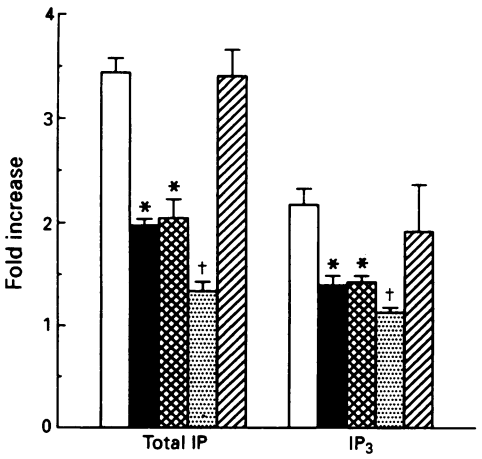


Figure 2 Effects of phorbol 12,13-dibutyrate (PDBu) on carbachol (CCh)-stimulated [^{3}H]-inositol phosphate accumulation. Circular smooth muscle tissues, pre-labelled with [^{3}H]-*myo*-inositol, were incubated with 100 μM CCh for 30 min in the presence of 10 mM LiCl (control). Open column: control; solid column: PDBu 1 μM for 30 min prior to addition of CCh; cross-hatched column: forskolin (FSK) 1 μM 10 min pretreatment; stippled column: PDBu + FSK; hatched column: 4α-phorbol 12,13-dideconoate (4α-PDD) 1 μM, 30 min pretreatment. Individual [^{3}H]-inositol phosphates were separated by SAX-h.p.l.c. as described under Methods. Data, determined as the area under the h.p.l.c. peak in c.p.m. for inositol 1,4,5-trisphosphate (ins-1,4,5-P_3) and each of the inositol phosphate isomers, are added together to generate total inositol phosphates. Values shown (mean ± s.e.mean, $n = 7$) are expressed as 100 μM CCh-induced fold increase of total InsP or Ins-1,4,5-P_3 over basal (total InsP and Ins-1,4,5-P_3: 505 ± 22 and 48 ± 4 c.p.m. mg^{-1} tissue wet weight).
*Significant difference from the control; $P<0.05$.
†Significant difference from PDBu or FSK alone; $P<0.05$.

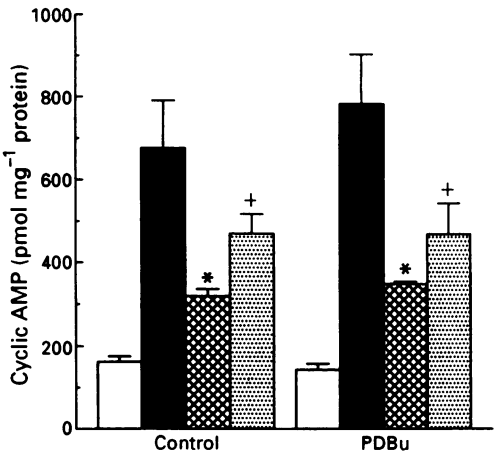


Figure 3 Effects of phorbol 12,13-dibutyrate (PDBu) on cyclic AMP accumulation in canine colon circular smooth muscle. Cyclic AMP was measured in colonic circular smooth muscle tissue before and after treatment of tissues with PDBu as described in Methods. Open column: basal; solid column: forskolin (FSK) 1 μM for 10 min; cross-hatched column: FSK 1 μM for 10 min in the presence of carbachol 10 μM; stippled column: isoprenaline 10 μM for 10 min. No effect of PDBu treatment on basal, on FSK stimulated, or on receptor-mediated stimulation or inhibition of adenylyl cyclase could be detected. Data are mean ± s.e.mean (vertical bars) of four experiments determined in duplicate.
*Significant difference from FSK alone; $P<0.05$.
†Significant difference from basal; $P<0.05$.

the presence of 10 mM Li^+, CCh (100 μM) produced a 2 fold increase in Ins-1,4,5-P_3 accumulation, a result mirrored by a 3.4 fold increase in total InsP accumulation. This CCh-induced InsP accumulation was significantly attenuated when tissues were preincubated for 30 min with 1 μM PDBu, a treatment which significantly increased membrane-bound PKC activity (see Figure 1). The effect of PDBu on CCh stimulated InsP accumulation is probably the result of PKC activation since the biologically inactive phorbol ester, 4α-phorbol 12,13-dideconoate (1 μM), was without effect (Figure 2). Pretreatment of tissues with 1 μM FSK for 10 min produced similar attenuation of InsP accumulation in response to CCh (Figure 2). The combined treatment of tissues with concentrations of both PDBu and FSK known to be maximal at the incubation times selected (data not shown) produced an even further reduction on CCh stimulated InsP accumulation, suggesting that activation of PKC and cyclic AMP-PK may act in disparate ways to produce inhibition of PLC activity.

Effects of phorbol 12,13-dibutyrate on cyclic AMP responses

In order to evaluate the specific effects of PDBu on PI turnover stimulated by CCh in colonic circular smooth muscle, we examined the effects of the phorbol ester on CCh-induced inhibition of FSK-stimulated cyclic AMP accumulation. The majority (80%) of muscarinic receptors present in colonic circular smooth muscle are of the M_2 subtype which mediate inhibition of agonist stimulated cyclic AMP accumulation *via* G_i (Zhang & Buxton, 1991). As shown in Figure 3, CCh (10 μM) reduced FSK-stimulated cyclic AMP accumulation by 50%. In contrast to its inhibitory effects on CCh-stimulated InsP accumulation, preincubation of the tissues with 1 μM PDBu for 30 min had no effect on either basal, on β adrenoceptor-mediated stimulation or on CCh-mediated inhibition of cyclic AMP accumulation, suggesting no effect of PKC on either the M_2 muscarinic receptor or the β-adrenoceptor. Moreover, these results demonstrate that in colonic smooth muscle, PDBu has no functional effect on either the G-proteins responsible for the dual regulation of adenylyl cyclase or adenylyl cyclase itself.

Regulation of muscarinic receptor antagonist binding by phorbol 12,13-dibutyrate and forskolin

Activation of PKC causes internalization of muscarinic receptors in neuroblastoma cells that requires the presence of functional cyclic AMP-PK (Scherer & Nathanson, 1990). To determine whether activation of PKC and/or cyclic AMP-PK could regulate cell surface muscarinic receptors in smooth muscle, freshly dispersed colonic smooth muscle cells were employed in muscarinic receptor antagonist radiogland binding. Cell surface, rather than total, muscarinic receptor number was determined by specific [^{3}H]-NMS binding. Under our assay conditions, the quaternary muscarinic receptor antagonist radioligand [^{3}H]-NMS labelled 22% fewer sites than [^{3}H]-QNB (Table 1). This suggests that muscarinic receptors are present at both cell surface and sequestered sites in colonic smooth muscle cells. Preincubation of colonic smooth muscle cells with 1 μM PDBu for 30 min at 32°C reduced subsequent maximal specific binding of [^{3}H]-NMS by 25% (Table 2) without altering the affinity of remaining cell surface receptors for [^{3}H]-NMS. In contrast, FSK (1 μM, 10 min) treatment significantly decreased the affinity of receptors for [^{3}H]-NMS (K_D: 0.2 nM→0.47 nM) without affecting maximal specific binding (Table 2).

In order to evaluate the effects of PDBu on M_2 and M_3 subtypes of the muscarinic receptor, we examined the ability of pirenzepine to compete for [^{3}H]-NMS binding to intact colonic myocytes. While muscarinic receptors of the M_1 subtype are thought to have a higher affinity for pirenzepine than M_3 receptors (Hulme *et al.*, 1990) our previous finding suggests that pirenzepine is also useful in studies of M_3 receptors. As we have described previously using [^{3}H]-QNB as the radiogland, (Zhang *et al.*, 1991), pirenzepine can discriminate the presence of both M_2 (low affinity for

Table 1 Comparison of [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) and [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) saturation binding in canine colonic circular smooth muscle cells

	B_{max} (sites/cell)	K_D (nM)
[^{3}H]-NMS	93,652 ± 5043*	0.38 ± 0.11
[^{3}H]-QNB	120,396 ± 5721	0.08 ± 0.02

Increasing concentrations (0.06–6 nM) of [^{3}H]-QNB and [^{3}H]-NMS were incubated with freshly isolated colonic circular smooth muscle cells at 32°C for 60 min. Specific binding was defined as the arithmetic difference between total binding and nonspecific binding in the presence of 10 μM atropine. Analysis of the specific binding data by nonlinear computer-based methods (fit to a rectangular hyperbola) confirmed that both [^{3}H]-QNB and [^{3}H]-NMS bound to a single class of binding sites in the cells. Data are means ± s.d. of two experiments determined in triplicate. *Statistically significant decrease compared to [^{3}H]-QNB binding ($P<0.05$).

Table 2 Effects of phorbol 12,13-dibutyrate (PDBu) and forskolin (FSK) on [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) binding in canine colonic circular smooth muscle cells

	Control	*PDBu*	*FSK*
K_D (nM)	0.20 ± 0.04	0.21 ± 0.07	*0.47 ± 0.06
B_{max} (sites/cell)	74,601 ± 6223	*56,336 ± 582	76,027 ± 4508

Saturation binding of [^{3}H]-NMS to intact colon circular smooth cells was measured in the absence (Control) or presence of PDBu (1 μM, 30 min) and FSK (1 μM, 10 min). K_D and B_{max} values were determined by Scatchard analysis. Data are means ± s.e.mean of three experiments determined in triplicate. Receptor density on control cells = 12 muscarinic receptors per μm^2 and is 60% of that determined using [^{3}H]-QNB (see discussion and data of Zhang *et al.*, 1991).
*Statistically significant changes compared to control ($P<0.05$).

pirenzepine; ~3.5 μM) and M_3 (high affinity for pirenzepine; ~25 nM) muscarinic receptors in the membrane homogenate of colonic circular smooth muscle. Pirenzepine competed completely for specific [^{3}H]-NMS binding in intact cells (Figure 4a). Under control conditions, competition data were best-fit when analyzed according to a two-site model ($F<0.01$). Thus, in control cells, pirenzepine interacted with an almost equal proportion of high- (M_3) and low-affinity (M_2) sites in a ratio of high to low affinities of 2:3 (Table 3). When the cells were pretreated with PDBu (1 μM, 30 min), however, there was a marked rightward shift and steepening in the pirenzepine competition curve (Figure 4a) associated with a 15% decrease in bound radioactivity. Analysis of these competition curves (Table 3) confirmed that pirenzepine bound preferentially to a single low-affinity site ($K_I = 270 \pm 30$ nM).

The loss of high-affinity binding sites for pirenzepine on the surface of PDBu-pretreated cells suggests that activation of PKC may cause preferential sequestration of M_3 muscarinic receptors. These sequestered or internalized receptor sites may not be accompanied by a significant change in the number of total receptor sites (Galper *et al.*, 1982; Feigenbaum & El-Fakahany, 1985), or such sites could be degraded or unavailable to radioligand and thus, total cellular receptor number decreased (Siman & Klein, 1979; Shifrin & Klein, 1980). To determine whether M_3 muscarinic receptors sequestered from the cell-surface following a short-term exposure to PDBu (30 min) were degraded intracellularly, we examined total cellular receptor number using the lipophilic muscarinic antagonist radioligand [^{3}H]-QNB. In untreated cells, pirenzepine competition curves were again best-fit

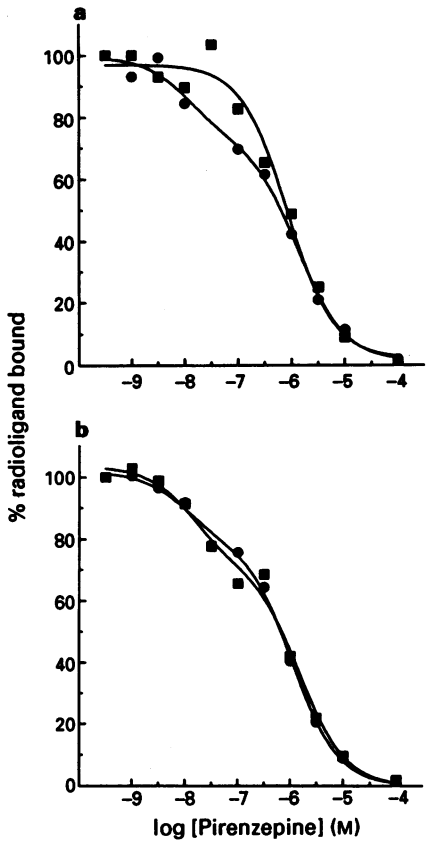


Figure 4 Effects of phorbol 12,13-dibutyrate (PDBu) on pirenzepine competition of [^{3}H]-N-methylscopolamine ([^{3}H]NMS) (a) and [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) (b) binding in intact colonic circular smooth muscle cells. Competition of [^{3}H]-NMS (0.2 nM) and [^{3}H]-QNB (0.1 nM) binding to intact smooth muscle cells by the M_3-more selective muscarinic antagonist pirenzepine was analyzed by nonlinear computer-based methods. (●) Control; (■) cells pretreated with 1 μM PDBu for 30 min prior to start of equilibrium binding. Data, normalized to binding in the absence of competitor (100%) and that occuring in the presence of 10 μM atropine (0%), are expressed as the percentage of maximal radioligand binding for each radioligand ([^{3}H]-NMS = 965 d.p.m. per 10^5 cells; [^{3}H]-QNB = 1280 d.p.m. per 10^5 cells). In the absence of PDBu pretreatment, competition curves are best fit, assuming the presence of two receptor binding sites. Competition of [^{3}H]-NMS binding in (a) following PDBu pretreatment of cells is significantly reduced (15%) and now best-fit assuming the presence of a single receptor site. Competition of [^{3}H]-QNB binding to cells (b) is unaltered by PDBu pretreatment. The binding parameters determined in several such experiments are summarized in Table 3.

assuming the presence of two receptor sites (Figure 4b; Table 3). The equilibrium dissociation constant determined for the high-affinity pirenzepine binding site was similar to that seen when [^{3}H]-NMS was employed. However, the binding was significantly greater (Table 3) and the relative contribution of high-affinity binding sites (M_3) to total binding sites ($M_3 + M_2$) was lower for pirenzepine competition with [^{3}H]-QNB (26%) compared to pirenzepine competition with [^{3}H]-NMS (42%; Table 3). Furthermore, pirenzepine competition was not altered in cells exposed first to PDBu, suggesting that sequestered receptors are not quickly degraded. These results show that the relative distribution of M_2 and M_3 muscarinic receptors on the cell surface is different from that inside the cell with the proportion of surface M_3 receptors being about equal to that of surface M_2 receptors.

Effects of phorbol 12,13-dibutyrate and forskolin treatment on agonist binding

One of the steps involved in protein kinase-regulated receptor response is the uncoupling of receptors from G-protein,

Table 3 Effects of phorbol 12,13-dibutyrate (PDBu) on pirenzepine binding to intact canine colonic circular smooth muscle cells

	K_Ih(nM)	K_Il(μM)	Rh (%)	Rl (%)
[^{3}H]-NMS				
Control	10.3 ± 3.0	0.54 ± 0.05	42 ± 7.8	58 ± 7.8
PDBu		0.27 ± 0.03		100
[^{3}H]-QNB				
Control	8.5 ± 1.6	1.34 ± 0.28	26 ± 3.5	74 ± 3.5
PDBu	11 ± 3.8	1.41 ± 0.39	30 ± 3.5	70 ± 3.5

Pirenzepine affinities (K_Ih, K_Il), and proportions of high- (Rh%) and low-affinity sites (Rl%) in antagonist competition binding for [^{3}H]-N-methylscopolamine ([^{3}H]-NMS, 0.18 nM) and [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB, 0.1 nM) binding in intact colonic circular smooth muscle cells (10^6 cells per tube) were determined by computer fit as described in the text. Cells were preincubated with PDBu (1 μM) at 32°C for 30 min. In the presence of PDBu, pirenzepine competition data were significantly (F-test) better-fit assuming the presence of a single low affinity site for [^{3}H]-NMS. Total specific binding (100%) in cells incubated with [^{3}H]-NMS at 0.2 nM was 3.7 ± 0.12 fmol per 10^5 cells, while [^{3}H]-QNB binding was 9.9 ± 0.47 fmol per 10^5 cells under similar conditions. Data are the mean ± s.e.mean of four experiments performed in triplicate.

Table 4 Effects of phorbol 12,13-dibutyrate (PDBu) and forskolin (FSK) on agonist binding to colonic circular smooth muscle muscarinic receptors

	−GTPγS			+GTPγS 100 μM		
	K_Ih(nM)	K_Il(μM)	Rl(%)	K_Ih(nM)	K_Il(nM)	Rl(%)
Control	24.1 ± 4.8	13.8 ± 3.2	43.2 ± 1.3	137 ± 79	85.6 ± 11	85.3 ± 0.1
FSK	24.9 ± 6.0	12.7 ± 4.0	40.7 ± 2.3	132 ± 69	75.6 ± 12	83.7 ± 6.1
PDBu	23.2 ± 5.3	10.0 ± 2.1	42.3 ± 1.5	73 ± 1	50.0 ± 4	86.7 ± 1.3
FSK + PDBu	34.6 ± 18	17.8 ± 4.9	43.9 ± 5.7	59 ± 24	63.0 ± 11	81.0 ± 8.0

Carbachol affinities (K_Ih, K_Il) and proportion of high- (Rh%) and low-affinity sites (R1%) in agonist competition for [^{3}H]-QNB (0.2 nM) binding in colonic membranes prepared as described in the text, were determined in the absence or presence of 1 μM PDBu and/or 1 μM FSK. Data are mean values ± s.e.mean of two to seven experiments determined in triplicate.

evidenced by the loss of high-affinity guanine nucleotide-sensitive agonist binding (Lefkowitz & Caron, 1988). Therefore, the effects of PDBu and FSK treatment on the ability of the muscarinic receptor agonist CCh to compete for [^{3}H]-QNB binding in the absence and presence of the stable GTP analogue GTPγS was tested in colonic circular smooth muscle. As might be expected in the untreated tissue, competition curves were shallow and best-fit by a two-site model (Figure 5). In the absence of GTPγS, the relative contribution of the high-and low-affinity agonist binding sites was about equal (Table 4). In the presence of a maximally effective concentration of GTPγS (100 μM), the CCh competition curve was significantly shifted to the right and the population of high-affinity agonist binding sites decreased from 57% to 15% of sites (Figure 5a; Table 4). Pretreatment of tissues with FSK (1 μM, 10 min) had no effect on either high- or low-affinity CCh binding sites or the effect of GTPγS on the agonist competition (Figure 5b; Table 4). Similar results were obtained with PDBu treatment or the combination of FSK and PDBu (Table 4).

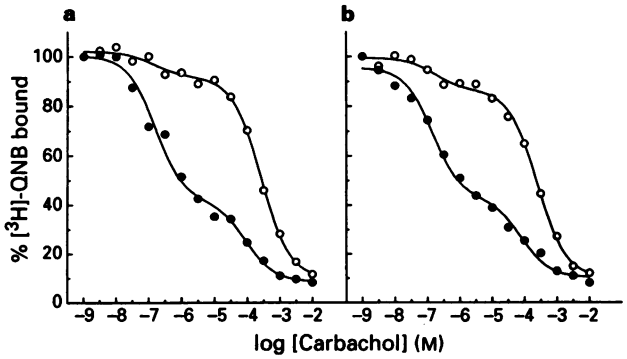


Figure 5 Effects of forskolin (FSK) on agonist competition of [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) binding in membranes of canine colon circular smooth muscle. Competition of [^{3}H-QNB (0.1 nM) binding by the muscarinic agonist carbachol was determined in control (a) and FSK (b)-treated membranes of colonic smooth muscle as described in Methods. Competition curves were generated in the absence (●) and presence (○) of a saturating concentration of GTPγS (100 μM). Data were analyzed by nonlinear computer-based methods which yielded best-fits assuming two components of agonist binding in all cases. Values, normalized to binding in the absence of competitor (100%) and that occurring in the presence of 10 μM atropine (0%), are expressed as the percentage of maximal [^{3}H]-QNB binding. Binding parameters determined in several such experiments and in the presence of PDBu and the combination of PDBu plus FSK are summarized in Table 4.

Discussion

We have examined the relationship between two second messengers, cyclic AMP and Ins-1,4,5-P_3, and the two protein kinases, PKC and cyclic AMP-PK, that are the consequence of elevation of these second messengers in canine colonic circular smooth muscle. Results presented have demonstrated that the activation of either PKC or cyclic AMP-PK partially blocks muscarinic receptor mediated PI hydrolysis. Although the combined effect of activation of PKC and cyclic AMP-PK was greater than activation of either kinase alone, the combined result is not strictly additive. Since we employ conditions for the activation of each kinase that we believe to be maximal, the data may suggest the involvement of distinct mechanisms underlying the inhibition of PLC activity.

Stimulation of PI turnover is a critical intracellular signalling pathway linked to activation of muscarinic receptors in many tissues and is thought to play an important role in the contraction of smooth muscle. We have recently demonstrated the presence of both M_2 and M_3 muscarinic receptor subtypes in the canine colonic circular smooth muscle (Zhang *et al.*, 1991). Activation of the M_3 muscarinic receptor mediates PI hydrolysis (Zhang & Buxton, 1991) and activates PKC. Activation of PKC results in phosphorylation of numerous protein substrates, and such action has been linked to a wide variety of biological responses, including negative feedback on PI turnover (El-Fakahany *et al.*, 1988). In the

present study, preincubation of colonic circular smooth muscle with PDBu resulted in inhibition of the PI response to CCh. The concentration of PDBu used for these effects elicted a significant increase of membrane-bound PKC activity. In addition, 4α-phorbol dideconoate, which does not activate PKC, was ineffective in blocking Ins-1,4,5,-P_3 accumulation. These results suggest that the observed effects of PDBu treatment are due to activation of PKC.

Although hormonal regulation of adenylyl cyclase is not affected by PKC, activation of adenylyl cyclase by FSK does affect hormonal regulation of PLC. It has been well documented that PLC-mediated hydrolysis of PIP_2 is affected by cyclic AMP-dependent phosphorylation. Elevations in cyclic AMP have been shown to inhibit agonist-dependent PI turnover in many cell types (Watson *et al.*, 1984; Neylon & Summers, 1988; Manolopoulos *et al.*, 1991) and to augment it in others (Blackmore & Exton, 1986; Olashaw & Pledger, 1988). While we measure no effect of cyclic AMP-PK activation on basal PLC activity in colon, such an effect has been measured in C_6Bul cultured cells (Ehlert & Tran, 1990).

Decreased Ins-1,4,5-P_3 formation following cyclic AMP elevation suggests that activation of cyclic AMP-PK may regulate PKC activity by decreasing the availability of DAG which would serve to assist translocation of the enzyme to the membrane. Indeed, McAtee & Dawson (1989) demonstrated that DAG was lowered substantially in NCB-20 cells treated with FSK and that this was associated with translocation of PKC into the cytosol.

Interestingly, the combined effects of forskolin and PDBu treatment on CCh stimulated Ins-1,4,5-P_3 formation in colonic smooth muscle were greater than their individual effects. This result may suggest the involvement of the distinct sites by which forskolin and PDBu inhibit PLC activity. Recently, it has been demonstrated that PKC and cyclic AMP-PK phosphorylate disparate PLC isozymes (Kim *et al.*, 1989; Ryu *et al.*, 1990). Treatment of C_6Bul cells with FSK increased serine phosphate in PLCγ, while the phosphate content of PLCβ and PLCδ were unchanged. In contrast, treatment of the cells with phorbol ester stimulated the phosphorylation of serine residues in PLCβ, while the phosphorylation state of PLCγ and PLCδ were unchanged. Reasoning that the absence of obvious membrane spanning domains in the known sequences of PLCβ, γ and δ isoforms of PLCs means that the enzyme must bind to the membrane prior to hydrolysis of its substrate, PIP_2; it is possible that this event is the target of PKC and cyclic AMP-PK phosphorylation.

It is clear that PLC is not the only target protein likely to be phosphorylated by PKC and cyclic AMP-PK. Phosphorylation has also been implicated in the regulation of muscarinic receptors. The purified M_1 receptor can be phosphorylated *in vitro* by both PKC and cyclic AMP-PK and the purified M_2 receptor can be phosphorylated by cyclic AMP-PK (Rosebaum *et al.*, 1987; Haga *et al.*, 1988). Phosphorylation of muscarinic receptors can be correlated with either a decrease in affinity or with sequestration of the receptor. In neuroblastoma cells, muscarinic receptors are rapidly sequestered following activation of PKC (Liles *et al.*, 1986) while increasing cellular cyclic AMP leads to no such effect (Scherer & Nathanson, 1990).

The present study demonstrates that activation of PKC results in a partial loss of muscarinic receptors from the surface of colonic circular smooth muscle cells within 30 min. [^{3}H]-NMS labelled 22% fewer sites than [^{3}H]-QNB in freshly isolated colonic smooth muscle cells (see Table 1). A similar result was obtained in freshly isolated intact chick heart cells (Brown & Goldstein, 1986). This suggests that muscarinic receptors are present at both cell surface and sequestered sites in colonic smooth muscle cells. Although it has been reported that in cultured human neuroblastoma cells all muscarinic receptors are accessible to [^{3}H]-NMS binding at 37°C (Fisher, 1988), this is not the case in freshly isolated smooth muscle cells. Pretreatment of colonic circular smooth muscle with PDBu decreased [^{3}H]-NMS maximal binding to cell surface receptors but did not affect their affinity for radioligand. We have determined that the relative distribution of M_2 and M_3 receptors between cell surface and cell interior is different. Our previous results with a lipophilic radioligand in tissue membranes (Zhang *et al.*, 1991) demonstrated that 20% of muscarinic receptors were of the M_3 subtype, but could not assign their location. The [^{3}H]-NMS binding in the intact smooth muscle cells shows that the relative density of M_3 muscarinic receptors is higher on the cell surface than the cell interior suggesting the relative importance of M_3 receptor signalling in the contractile response to acetylcholine *in vivo*. In fact, when the extent of [^{3}H]-QNB binding to cells determined to be M_3 sites is reconciled with the extent of [^{3}H]-NMS bound and predicted to be M_3 sites (Table 3), it is apparent that the absolute number of such sites is similar and thus, argues that most of the M_3 binding sites are on the cell surface. In accordance with published reports (Lai & El-Fakahany, 1986; Abdallah *et al.*, 1990; Lai *et al.*, 1990), our results demonstrate that activation of PKC selectively decreases high-affinity pirenzepine binding sites from the smooth muscle cell surface. Our reasoning that M_2 muscarinic receptors on colonic cells are not affected by PKC is supported by the observation that the purified M_2 muscarinic receptor from porcine atria is not phosphorylated by purified PKC (Rosenbaum *et al.*, 1987; Haga *et al.*, 1988). The M_3 receptors sequestered from the surface of the colonic smooth muscle cell surface following PDBu treatment are not rapidly degraded since [^{3}H]-QNB binding is not affected by PDBu. Such a result is consistent with the notion that cells modulate their responsiveness to activation of protein kinase C, acutely, by removing receptors from the extracellular environment.

In contrast to our findings in cells treated with PDBu, increasing cellular cyclic AMP content following FSK treatment decreased the affinity of cell surface receptors for [^{3}H]-NMS without affecting maximal binding (see Table 2). The effects of FSK treatment were most likely due to cyclic AMP-PK activation since incubation of FSK with cell membranes in the radioligand binding assay failed to affect binding. Non-specific affects of isobutyl methylxanthine (IBMX), the cyclic AMP-phosphodiesterase inhibitor used to ensure sustained elevation of cyclic AMP, were ruled out since it had no effect in the absence of FSK. Although we do not know the identity, M_2 *vs.* M_3, of the receptors whose affinity is altered by FSK treatment, it is known that M_2 receptors can be phosphorylated by cyclic AMP-PK *in vitro* and exist as a phosphoprotein in intact tissue (Rosenbaum *et al.*, 1987; Kwatra *et al.*, 1989).

Pretreatment of human neuroblastoma cells with phorbol ester converted multiple agonist binding sites to a single site with low affinity for agonist (Serra *et al.*, 1986). In colonic circular smooth muscle, despite effects of PDBu and FSK on antagonist radioligand binding and PLC activity, such treatment does not eliminate high-affinity agonist binding. Indeed, the lack of effects of PDBu and FSK added together on GTP-dependent agonist binding suggests that the receptor effects of activation of PKC and cyclic AMP-PK in this tissue do not occur at the level of guanine nucleotide binding protein:receptor interactions. These data are in agreement with findings in brain in which PDBu does not alter agonist binding (Abdallah *et al.*, 1990). In addition, Rosenbaum *et al.* (1987) demonstrated that phosphorylation of atrial M_2 receptors did not alter either agonist binding or the GTPase activity of G_i. These results, taken together with our own, suggest the notion that receptor phosphorylation serves to target receptors for events such as sequestration or internalization rather than regulating agonist binding. This notion is further supported by data showing that receptor phosphorylation is required for receptor internalization (Scherer & Nathanson, 1990). Our results also provide for the possibility that the effects of activation of PKC and cyclic AMP-PK contribute to altered interaction between the activated G-protein and PLC.

References

ABDALLAH, E.A.M., FORRAY, C. & EL-FAKAHANY, E.E. (1990). Relationship between the partial inhibition of muscarinic receptor-mediated phosphoinositide hydrolysis by phorbol esters and tetrodotoxin in rat cerebral cortex. *Mol. Brain Res.*, **8**, 1–7.

BLACKMORE, P.F. & EXTON, J.H. (1986). Studies on the hepatic calcium-mobilizing activity of aluminium fluoride and glucagon: Modulation by cAMP and phorbol myristate acetate. *J. Biol. Chem.*, **261**, 11056–11063.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

BROSTROM, M.A., LIN, X., CADE, C., GMITTER, D. & BROSTROM, C.O. (1989). Loss of a calcium requirement for protein synthesis in pituitary cells following thermal or chemical stress. *J. Biol. Chem.*, **264**, 1638–1643.

BROWN J.H. & GOLDSTEIN, D. (1986). Analysis of cardiac muscarinic receptors recognized selectively by nonquaternary but not by quaternary ligands. *J. Pharmacol. Exp. Ther.*, **238**, 580–586.

CANDELL, L.M., YUN, S.H., TRAN, L.L.P. & EHLERT, F.J. (1990). Differential coupling of subtypes of the muscarinic receptor to adenylate cyclase and phosphoinositide hydrolysis in the longitudinal muscle of the rat ileum. *Mol. Pharmacol.*, **38**, 689–697.

EHLERT, F.J. & TRAN, L.L.P. (1990). Regional distribution of M_1, M_2 and non-M_1, non-M_2 subtype of muscarinic binding sites in rat brain. *J. Pharmacol. Exp. Ther.*, **255**, 1148–1157.

EL-FAKAHANY, E.E., ALGER, B.E., LAI, W.S., PITLER, T.A., WORLEY, P.F. & BARABAN, J.M. (1988). Neuronal muscarinic responses: role of protein kinase C. *FASEB J.*, **2**, 2575–2583.

FEIGENBAUM, P. & EL-FAKAHANY, E.E. (1985). Regulation of muscarinic cholinergic receptor density of neuroblastoma cells by brief exposure to agonist: Possible involvement in densitization of receptor function. *J. Pharmacol. Exp. Ther.*, **233**, 134–140.

FERNANDES, L.B., FRYER, A.D. & HIRSHMAN, C.A. (1992). M_2 muscarinic receptors inhibit isoproterenol-induced relaxation of canine airway smooth muscle. *J. Pharmacol. Exp. Ther.*, **262**, 119–126.

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

GALPER, J.B., DZIEKAN, L.C., O'HARA, D.S. & SMITH, T.W. (1982). The biphasic response of muscarinic cholinergic receptors in cultured heart cells to agonists: Effects on receptor number and affinity in intact cells and homogenates. *J. Biol. Chem.*, **257**, 10344–10356.

GILMAN, A.G. (1970). A protein binding assay for adenosine 3':5'-cyclic monophosphate. *Proc. Natl. Acad. Sci. U.S.A.*, **67**, 305–312.

HAGA, T., HAGA, K., BERSTEIN, G., NISHIYAMA, T., UCHIYAMA, H. & ICHIYAMA, A. (1988). Molecular properties of muscarinic receptors. *Trends Pharmacol. Sci.*, **9** suppl, 12–18.

HU, J., WANG, S.-Z. & EL-FAKAHANY, E.E. (1991). Effects of agonist efficacy on desensitization of phosphoinositide hydrolysis mediated by m1 and m3 muscarinic receptors expressed in Chinese hamster ovary cells. *J. Pharmacol. Exp. Ther*, **257**, 938–945.

HULME, E.C., BIRDSALL, N.J.M. & BUCKLEY, N.J. (1990). Muscarinic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.*, **30**, 633–673.

IMAIZUMI, T., WATANABE, Y. & YOSHIDA, H. (1991). Phosphorylation of G_i protein by cyclic AMP-dependent protein kinase inhibits its dissociation into α-subunits and βγ-subunits by Mg^{2+} and GTPγS. *Eur. J. Pharmacol., Mol. Pharmacol.*, **207**, 189–194.

KIM, U.-H., KIM. J.W. & RHEE, S.G. (1989). Phosphorylation of phospholipase C-γ by cAMP-dependent protein kinase. *J. Biol. Chem.*, **264**, 20167–20170.

KRAFT, A.S. & ANDERSON, W.B. (1983). Phorbol esters increase the amount of Ca^{2+}, phospholipid-dependent protein kinase associated with plasma membrane. *Nature*, **301**, 621–623.

KWATRA, M.M., PTASIENSKI, J. & HOSEY, M.M. (1989). The porcine heart M_2 muscarinic receptor. Agonist-induced phosphorylation and comparison of properties with the chick heart receptor. *Mol. Pharmacol.*, **35**, 553–558.

LAI, W.S. & EL-FAKAHANY, E.E. (1986). A selective effect of protein kinase C activation on pirenzepine high-affinity muscarinic receptors in a neuronal clone. *Eur. J. Pharmacol.*, **129**, 201–202.

LAI, W.S. & EL-FAKAHANY, E.E. (1988). Regulation of [3H]phorbol-12,13-dibutyrate binding sites in mouse neuroblastoma cells: simultaneous down-regulation by phorbol esters and desensitization of their inhibition of muscarinic receptor function. *J. Pharmacol. Exp. Ther.*, **244**, 41–50.

LAI, W.S., ROGERS, T.B. & EL-FAKAHANY, E.E. (1990). Protein kinase C is involved in densensitization of muscarinic receptors induced by phorbol esters but not by receptor agonists. *Biochem. J.*, **267**, 23–29.

LEFKOWITZ, R.J. & CARON, M.G. (1988). Adrenergic receptors: Models for the study of receptors coupled to guanine nucleotide regulatory proteins. *J. Biol. Chem.*, **263**, 4993–4996.

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

MANOLOPOULOS, V.G., PIPILI-SYNETOS, E., DEN HERTOG, A. & NELEMANS, A. (1991). Inositol phosphates formed in rat aorta after α_1-adrenoceptor stimulation are inhibited by forskolin. *Eur. J. Pharmacol., Mol. Pharmacol.*, **207**, 29–36.

MCATEE, P. & DAWSON, G. (1989). Rapid dephosphorylation of protein kinase C substrates by protein kinase A activators results from inhibition of diacylglycerol release. *J. Biol. Chem.*, **264**, 11193–11199.

MCDANIEL, N.L., REMBOLD, C.M., RICHARD, H.M. & MURPHY, R.A. (1991). Cyclic AMP relaxes swine arterial smooth muscle predominantly by decreasing cell Ca^{2+} concentration. *J. Physiol.*, **439**, 147–160.

NEYLON, C.B. & SUMMERS, R.J. (1988). Inhibition by cAMP of the phosphoinositide response to α_1-adrenoceptor stimulation in rat kidney. *Eur. J. Pharmacol.*, **148**, 441–444.

NISHIZUKA, Y. (1986). Studies and perspectives of protein kinase C. *Science*, **233**, 305–312.

OLASHAW, N.E. & PLEDGER, W.J. (1988). Epidermal growth factor stimulates formation of inositol phosphates in BALB/c/3T3 cells pretreated with cholera toxin and isobutylmethylxanthine. *J. Biol. Chem.*, **263**, 1111–1114.

ORELLANA, S.A., SOLSKI, P.A. & BROWN, J.H. (1985). Phorbol ester inhibits phosphoinositide hydrolysis and calcium mobilization in cultured astrocytoma cells. *J. Biol. Chem.*, **260**, 5236–5239.

RHEE, S.G., SUH, P.-G., RYU, S.-H. & LEE, S.Y. (1989). Studies of inositol phospholipid-specific phospholipase C. *Science*, **244**, 546–550.

ROSENBAUM, L.C., MALENCIK, D.A., ANDERSON, S.R., TOTA, M.R. & SCHIMERLIK, M.I. (1987). Phosphorylation of the porcine atrial acetylcholine receptor by cyclic AMP dependent protein kinase. *Biochemistry*, **26**, 8183–8188.

RYU, S.H., KIM. U.-H., WAHL, M.I., BROWN, A.B., CARPENTER, G., HUANG, K.-P. & RHEE, S.G. (1990). Feedback regulation of phospholipase C-β by protein kinase *C.J. Biol. Chem.*, **265**, 17941–17945.

SCHERER, N.M. & NATHANSON, N.M. (1990). Differential regulation by agonist and phorbol ester of cloned m1 and m2 muscarinic acetylcholine receptors in mouse Y1 adrenal cells and in Y1 cells deficient in cAMP-dependent protein kinase. *Biochemistry*, **29**, 8475–8483.

SECREST, R.J., LUCAITES, V.L., MENDELSOHN, L.G. & COHEN, M.L. (1991). Protein kinase C translocation in rat stomach fundus: Effects of serotonin, carbamylcholine and phorbol dibutyrate. *J. Pharmacol. Exp. Ther.*, **256**, 103–109.

SERRA, M., SMITH, T.L. & YAMAMURA, H.I. (1986). Phorbol esters alter muscarinic receptor binding and inhibit polyphosphoinositide breakdown in human neuroblastoma (SH-Sy5Y) cells. *Biochem. Biophys. Res. Commun.*, **140**, 160–166.

SHIFRIN, G.S. & KLEIN, W.L. (1980). Regulation of muscarinic acetylcholine receptor concentration in cloned neuroblastoma cells. *J. Neurochem.*, **34**, 993–999.

SIMAN, R.G. & KLEIN, W.L. (1979). Cholinergic activity regulates muscarinic receptors in central nervous system cultures. *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4141–4145.

SMITH, T.K., REED, J.B. & SANDERS, K.M. (1987). Origin and propagation of electrical slow waves in circular muscle of canine proximal colon. *Am. J. Physiol.*, **252**, C215–C224.

SMITH, T.K., WARD, S.M., ZHANG, L., BUXTON, I.L.O., GERTHOFFER, W.T., SANDERS, K.M. & KEEF, K.D. (1992). β-Adrenergic inhibition of electrical and mechanical activity in canine colon: Role of cyclic AMP. *Am. J. Physiol.* (in press).

SOMLYO, A.P. & HIMPENS, B. (1989). Cell calcium and its regulation in smooth muscle. *FASEB J.*, **3**, 2266–2276.

WATSON, S.P., MCCONNELL, R.T. & LAPETINA, E.G. (1984). The rapid formation of inositol phosphates in human platelets by thrombin is inhibited by prostacyclin. *J. Biol. Chem.*, **259**, 13199–13203.

ZHANG, L. & BUXTON, I.L.O. (1991). Muscarinic receptors in canine colonic circular smooth muscle II. Signal transduction pathways coupled to the muscarinic receptors. *Mol. Pharmacol.*, **40**, 952–959.

ZHANG, L., HOROWITZ, B. & BUXTON, I.L.O. (1991). Muscarinic receptors in canine colonic circular smooth muscle I. Coexistence of M_2 and M_3 subtypes. *Mol. Pharmacol.*, **40**, 943–951.

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Evaluation of the relaxant effects of SCA40, a novel charybdotoxin-sensitive potassium channel opener, in guinea-pig isolated trachealis

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1 Experiments have been performed in order to analyse the mechanism whereby SCA40, a new imidazo[1,2-*a*]pyrazine derivative relaxes airway smooth muscle.

2 SCA40 (0.01–10 μM) caused a complete and concentration-dependent relaxation of guinea-pig isolated trachea contracted with 20 mM KCl but failed to inhibit completely the spasmogenic effects of 80 mM KCl.

3 Quinine (30 μM) antagonized the relaxant activity of SCA40 in 20 mM KCl-contracted guinea-pig isolated trachea. The ATP-sensitive K^+-channel blocker, glibenclamide (3 μM), did not antagonize the relaxant activity of SCA40 in either 20 mM KCl or 1 μM carbachol-contracted isolated trachea.

4 SCA40 (0.01–10 μM) and isoprenaline (0.1 nM–10 μM) caused a complete and concentration-dependent relaxation of guinea-pig isolated trachea contracted with carbachol 1 μM.

5 The large-conductance Ca^{2+}-activated K^+-channel blocker, charybdotoxin (60–180 nM), non-competitively antagonized the relaxant activity of isoprenaline on 1 μM carbachol-contracted trachea. The inhibition was characterized by rightward shifts of the isoprenaline concentration-relaxation curves with depression of their maxima.

6 The relaxant activity of SCA40 in 1 μM carbachol-contracted trachea was antagonized by charybdotoxin (60–600 nM) in an apparently competitive manner. The concentration-relaxation curves to SCA40 were shifted to the right with no significant alteration in the maximum response.

7 It is concluded that SCA40 is a novel potassium channel opener which is a potent relaxant of guinea-pig airway smooth muscle *in vitro*. The relaxant activity of SCA40 does not involve ATP-sensitive K^+-channels but rather large-conductance Ca^{2+}-activated K^+-channels or other charybdotoxin-sensitive K^+-channels.

Keywords: Guinea-pig trachealis; smooth muscle relaxation; SCA40; charybdotoxin; glibenclamide; isoprenaline; potassium channels

Introduction

We have previously described the pharmacological properties of imidazo[1,2-*a*]pyrazine derivatives (Sablayrolles *et al.*, 1984; Michel *et al.*, 1989; Bonnet *et al.*, 1992). These compounds exhibited theophylline-like properties, not blocked by β-adrenoceptor antagonists, potent smooth muscle relaxant activity *in vitro*, potent anti-bronchospasm activity *in vivo* and adenosine 3':5'-cyclic monophosphate (cyclic AMP) phosphodiesterase inhibitory activity.

Among the tested 8-alkylaminoimidazo[1,2-*a*]pyrazines, SCA40, 6-bromo-8-methylaminoimidazo[1,2-*a*]pyrazine-2-carbonitrile, exhibited the highest smooth muscle relaxant activities among the derivatives of the series. Indeed, SCA40 was shown to be 40 to 500 times more active than the reference drug theophylline *in vivo* and *in vitro*, respectively (Michel *et al.*, 1990). This potent smooth muscle relaxant activity, especially in guinea-pig tracheal tissue, could not be linked up to the SCA40 cyclic AMP phosphodiesterase inhibitory properties which are similar to those of theophylline (Bonnet *et al.*, 1992). Therefore other mechanism(s) must account for the potent relaxant activity of SCA40. Many studies have shown that an increase in intracellular cyclic AMP plays an important role in regulating tracheal smooth muscle contraction, but other cellular events may be associated with relaxation in airway smooth muscle. Recent studies have shown that K^+-channel agonists such as cromakalim and pinacidil induced hyperpolarization of the cell membrane which leads to tracheal smooth muscle relaxation (Allen *et al.*, 1986; Mellemkjær *et al.*, 1989). ATP-sensitive K^+-channels have been identified as the targets of these K^+-channel openers since the *in vitro* relaxant activity of cromakalim and pinacidil was blocked in an apparently competitive manner by the sulphonylurea, glibenclamide (Quast & Cook, 1989). ATP-sensitive K^+-channels are not the only type of K^+-channels involved in the regulation of trachealis cell polarization. Patch-clamp studies have shown that the plasmalemma of trachealis smooth muscle cells from ox (Green *et al.*, 1991), dog (McCann & Welsh, 1986) and pig (Huang *et al.*, 1987) is richly endowed with large-conductance, Ca^{2+}-dependent K^+-channels. Charybdotoxin (ChTX), a purified peptide toxin present in *Leiurus quinquestriatus* venom which has been found to block large conductance, Ca^{2+}-dependent K^+-channels in a variety of cells (Castle *et al.*, 1989) is able to block the tracheal relaxant activity of β-adrenoceptor agonists (Murray *et al.*, 1991). These observations imply that large conductance, Ca^{2+}-dependent K^+-channels may be involved in the tracheal relaxant activity of β-adrenoceptor agonists. These results show that hyperpolarization of tracheal smooth muscle induced by the opening of various type of K^+-channels may be involved in tracheal smooth muscle relaxation. As xanthines had been shown to possess K^+-channel opening properties (Jones *et al.*, 1990; Murray *et al.*, 1991), the aim of the present study was to investigate the potential K^+-channel opening properties of the purine-like derivative SCA40 in guinea-pig isolated trachea and to characterize the type of K^+-channel involved.

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Methods

Adult male Dunkin-Hartley guinea-pigs (Iffa Credo, Lyon, France), weighing 400–500 g, fed with UAR A114 diet and fasted 18 h prior the experiment were used. Guinea-pigs were killed by a blow to the head. Tracheae were excised, cleaned of adhering adipose and connective tissue and contractility of tracheal segments (4 tracheal rings in all cases) was measured by adapting the method previously described (Hooker *et al.*, 1977). Tissues were suspended in 20 ml organ baths containing a Chenoweth Koelle buffer. At the outset of each experiment, tissues were subjected to an imposed tension of 0.5 g and allowed to equilibrate for 20 to 30 min during which time they were washed every 4 min. Changes in tension were measured isometrically with a myograph transducer connected to a Physiograph Narco Bio-system.

Effects of SCA40 against tone induced by KCl

KCl (20 mM or 80 mM) induced contractions which reached a stable maximum within 5 min. Cumulative log concentration-response curves to SCA40 were determinated for trachea contracted with KCl 20 or 80 mM, taking the intensity of the initial contraction as 100%. Then, cumulative log concentration-response curves to SCA40 were determinated for trachea contracted with KCl 20 mM in the absence (control) or in the presence of glibenclamide (3 μM) or quinine (30 μM).

Effects of glibenclamide on the relaxant action of SCA in carbachol-contracted trachea

In these experiments, cumulative log concentration-response curves to SCA40 were determinated for trachea contracted with carbachol (1 μM) in the absence (control), or in the presence of 3 μM glibenclamide (20 min pretreatment).

Effects of charybdotoxin on the relaxant actions of SCA40 and isoprenaline

In a second set of experiments, cumulative log concentration-response curves to SCA40 and isoprenaline were determinated for trachea contracted with carbachol (1 μM) in the absence (control), or in the presence of 60, 180 or 600 nM ChTX (20 min pretreatment). In these experiments, tissues were suspended in 2 ml organ baths containing a Krebs buffer. In all the experiments, the cyclo-oxygenase inhibitor, indomethacin (1.4 μM) was added to the buffer in order to prevent spontaneous tone development due to released prostanoids. Half-$\log_{10}$ unit concentration increments were used for all log concentration-response curves. All relaxant responses are expressed as a percentage reduction in response to the appropriate spasmogen. For each relaxant drug, potency is expressed as the negative log EC_{50} where EC_{50} is the concentration producing 50% inhibition of the contraction. The EC_{50} values for control and treatment curves were calculated by linear regression analysis applied to the linear portion of each dose-response curve.

Statistical evaluation of results

Statistical evaluation of the results was assessed by use of a two-tailed, unpaired *t* test. The null hypothesis was rejected when $P<0.05$.

Drugs and solutions

The substances used were obtained from the following sources: Sigma Chemicals (U.S.A.): (±)-isoprenaline, glibenclamide, carbachol, quinine; Latoxan (France): purified charybdotoxin.

For *in vitro* experiments, 10^{-2} M stock solution of SCA40 and 10^{-3} M stock solution of glibenclamide were made up in ethanol. Further dilutions were made up in distilled water. KCl, carbachol, and quinine solutions were prepared in distilled water. Lyophilised ChTX has been reconstituted in saline solution (150 mM NaCl) and stored at −20°C until use. The Chenoweth-Koelle solution used had the following composition (mM): NaCl 120, KCl 5.6, $CaCl_2$ 2.4, $MgCl_2$ 2.2, $NaHCO_3$ 15 and glucose 10. The Krebs solution used had the following composition (mM): NaCl 120, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.6, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 10. These solutions were maintained at 37°C and gassed continuously with a mixture of 95% O_2, 5% CO_2.

Results

Effects of SCA40 against tone induced by KCl

Cumulative concentration-response curves to SCA40 on guinea-pig isolated trachea precontracted with KCl 20 and 80 mM are shown in Figure 1. SCA40 produced complete concentration-dependent inhibition of response to KCl 20 mM. During 80 mM KCl-induced contraction, SCA40 produced only partial relaxation, which corresponded to approximately 40% of the maximum relaxation that could be achieved against the 20 mM KCl-induced contraction. Moreover, the relaxation concentration-response curve to SCA40 against KCl 80 mM-induced contraction was shifted to the right approximately 1000 fold compared with SCA40 relaxant activity against KCl 20 mM ($-\log EC_{50} = 7.43 \pm 0.27$ and 4.44 ± 0.33 respectively). In the presence of glibenclamide 3 μM, the relaxation concentration-response curve to SCA40 against KCl 20 mM-induced contraction was not modified (Figure 2a). On the contrary, the relaxation concentration-response curve to SCA40 against KCl 20 mM-induced contraction in the presence of quinine (30 μM) was shifted to the right approximately 5 fold compared with SCA40 relaxant activity against KCl 20 mM alone ($-\log EC_{50} = 7.57 \pm 0.29$ and 6.84 ± 0.08 respectively) with no significant alteration in the maximum response (Figure 2b).

Effects of glibenclamide on the relaxant action of SCA40 in carbachol-contracted trachea

Cumulative concentration-response curves to SCA40 on guinea-pig isolated trachea precontracted with carbachol 1 μM in the presence or absence of glibenclamide 3 μM are shown in Figure 3. The tracheal contraction induced by carbachol

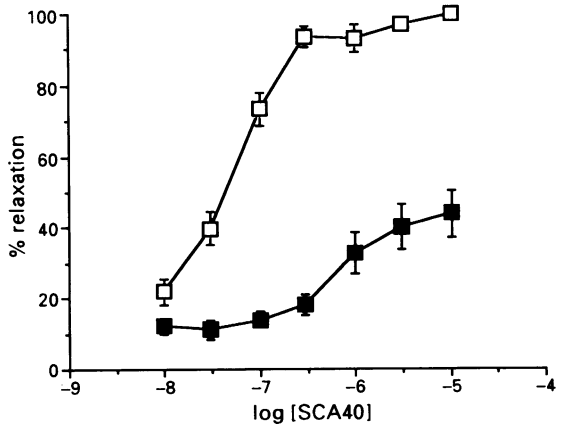


Figure 1 Guinea-pig isolated trachealis: relaxant activity of SCA40 against established contraction to KCl 20 mM (□) and KCl 80 mM (■). Abscissae: − log molar concentration of SCA40. Ordinate scale: percentage reduction in responses to KCl 20 mM or KCl 80 mM as appropriate. Each point is the mean derived from at least 6 experiments, vertical lines show sample s.e.mean values. All experiments were carried out in the presence of 1.4 μM indomethacin.

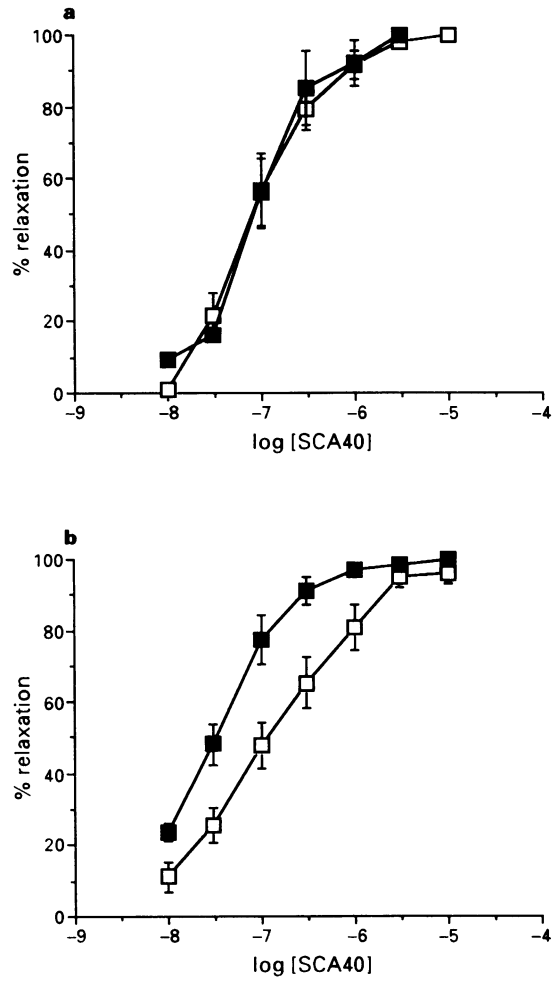


Figure 2 Guinea-pig isolated trachealis: relaxant activity of SCA40 against established contraction to KCl 20 mM in absence (■) or in presence of (a): glibenclamide 3 μM (□) and (b): quinine 30 μM (□). Abscissae: – log molar concentration of SCA40. Ordinate scale: percentage reduction in responses to KCl 20 mM. Each point is the mean derived from at least 6 experiments, vertical lines show sample s.e.mean values. All experiments were carried out in the presence of 1.4 μM indomethacin.

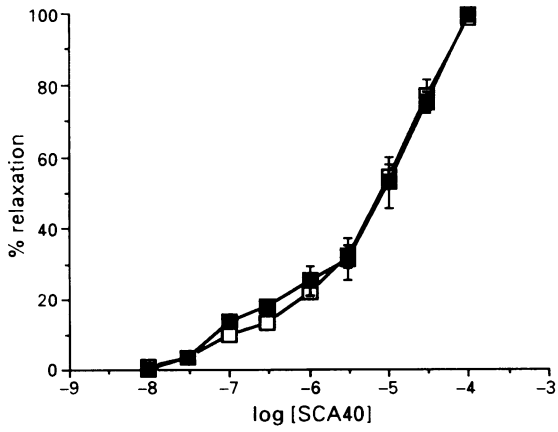


Figure 3 Guinea-pig isolated trachealis: the effects of glibenclamide (3 μM) on the relaxant action of SCA40 against established contraction to carbachol 1 μM. The log concentration-effect curves were obtained in the absence of glibenclamide (■) or in the presence of glibenclamide 3 μM (□). Abscissae: – log molar concentration of SCA40. Ordinate scale: percentage reduction in responses to carbachol 1 μM. Each point is the mean derived from at least 6 experiments, vertical lines show sample s.e.mean values. All experiments were carried out in the presence of 1.4 μM indomethacin.

1 μM represented 85 to 95% of the maximum contraction that could be induced by maximal concentrations of carbachol (10 μM). Under these conditions, SCA40 induced a complete and dose-dependent relaxation of carbachol-induced tracheal contraction. In the presence of glibenclamide 3 μM, the control concentration-response curves to SCA40 was not altered (Figure 3 and Table 1).

Effects of charybdotoxin on the relaxant actions of SCA40 and isoprenaline

Cumulative concentration-response curves to isoprenaline or SCA40 on isolated guinea-pig trachea precontracted with carbachol 1 μM in the presence or absence of ChTX 60, 180 or 600 nM are shown in Figure 4. In these conditions, ChTX 60, 180 and 600 nM did not produce any further increase in the carbachol-induced baseline tone and thus no functional antagonism of relaxation induced by ChTX should be expected. The non-selective β-adrenoceptor agonist, isoprenaline and SCA40 induced a complete relaxation of carbachol-induced tracheal contraction. The negative log EC_{50} for control isoprenaline and control SCA40 were 8.64 ± 0.10 and

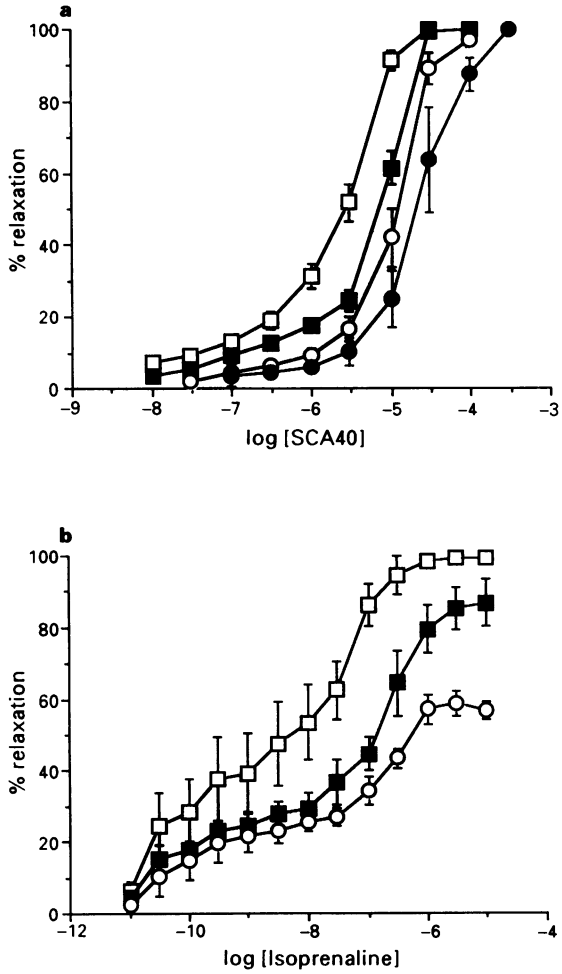


Figure 4 Guinea-pig isolated trachealis: the effects of charybdotoxin (ChTX) on the relaxant action of SCA40 (a) and isoprenaline (b) against established contraction to carbachol 1 μM. The log concentration-effect curves were obtained in the absence of ChTX (□) or in the presence of ChTX 60 mM (■), ChTX 180 mM (○) and ChTX 600 nM (●). The effects of ChTX 600 nM on the relaxant activity of isoprenaline were not tested. Abscissae: (a) – log molar concentration of SCA40, (b) – log molar concentration of isoprenaline. Ordinate scale: percentage reduction in responses to carbachol 1 μM. Each point is the mean derived from at least 6 experiments, vertical lines show sample s.e.mean values. All experiments were carried out in the presence of 1.4 μM indomethacin.

Table 1 Effects of charybdotoxin (ChTX) and glibenclamide on the relaxant activity of SCA40 and isoprenaline in guinea-pig isolated trachea contracted with 1 μM carbachol

Treatment	n	*− log EC_{50}*	*% maximum relaxation*
Isoprenaline			
Control	8	8.64 ± 0.10	99.4 ± 0.6
ChTX 60 nM	6	7.40 ± 0.76*	87.9 ± 7.1*
ChTX 180 nM	6	5.89 ± 0.21*	56.9 ± 2.5*
SCA40			
Control	8	5.92 ± 0.18	100.0 ± 0.0
ChTX 60 nM	6	5.51 ± 0.24	100.0 ± 0.0
ChTX 180 nM	6	5.18 ± 0.31*	97.1 ± 1.9
ChTX 600 nM	6	4.85 ± 0.19*	100.0 ± 0.0
SCA40			
Control	8	5.38 ± 0.12	100.0 ± 0.0
Glibenclamine 3 μM	6	5.40 ± 0.14	100.0 ± 0.0

Data are mean (± s.e.mean) values ($n = 6-8$) of pD_2 (− log EC_{50}) and percentage maximum relaxation.
*Indicates a significant difference from the corresponding values in control tissues (two-tailed unpaired t test). All experiments were carried out in the presence of 1 μM carbachol and 1.4 μM indomethacin.

5.92 ± 0.18 respectively. In the presence of ChTX 60 and 180 nM, the control concentration-response curves to isoprenaline were shifted to the right 17 and 563 fold, respectively, and the extent of relaxation to isoprenaline was reduced to 87.9 ± 0.6 and 56.9 ± 2.5% respectively of the control maximum relaxation (Figure 4b and Table 1). As ChTX 180 nM largely blocked the relaxant activity of isoprenaline, and considering the high cost of the toxin, the effects of ChTX 600 nM on the relaxant activity of isoprenaline have not been tested. In the same manner, the control concentration-response curves to SCA40 in the presence of ChTX 60, 180 and 600 nM were shifted to the right 2.6, 5.5 and 11.7 fold, respectively but the control maximum relaxation was unchanged (Figure 4a and Table 1).

Discussion

SCA40 is a newly synthesized imidazo[1,2-*a*]pyrazine derivative which exhibits potent smooth muscle relaxant properties *in vitro* (Michel *et al.*, 1990), potent anti-bronchospasm activity *in vivo* and a moderate cyclic AMP phosphodiesterase inhibitory activity (Bonnet *et al.*, 1992). It is therefore unlikely that an increase in cyclic AMP formation due to the inhibition of cyclic AMP phosphodiesterase could totally explain the potent SCA40 smooth muscle relaxant activity. Thus, other mechanisms of action have to be considered. In guinea-pig isolated trachea, SCA40 is able to inhibit completely the contractions induced by low concentrations of KCl (20 mM) as opposed to high concentrations (80 mM) of KCl. Such a pharmacological profile has been described for K^+-channel openers (Hamilton *et al.*, 1986; Robertson & Steinberg, 1990) which are able to block smooth muscle contractions induced by low K^+ concentrations (20 mM), but not high depolarizing K^+ concentrations (80 mM). With high depolarizing K^+ concentrations, potassium equilibrium potential and cell membrane potential are so close that hyperpolarization induced by K^+-channel opening is too weak to close voltage-operated Ca^{2+}-channels. Interestingly, SCA40, at high concentrations (10 μM) retained some relaxant activity against the spasm induced by 80 mM KCl (40% of the maximum relaxation that could be achieved against 20 mM KCl-induced contraction). This relaxant activity of SCA40 at high concentrations might be attributed to its cyclic AMP phosphodiesterase inhibitory properties.

In the presence of glibenclamide, a known ATP-sensitive K^+-channel blocker (Quast & Cook, 1989), the relaxant activity of SCA40 against 20 mM KCl-induced contraction was not altered. Similarly, glibenclamide failed to antagonize the tracheal relaxant activity of SCA40 against 1 μM carbachol-induced contraction. These results suggest that, as opposed to the known K^+-channels openers cromakalim, lemakalim or pinacidil, ATP-sensitive K^+-channels are not involved in the pharmacological activity of SCA40. On the contrary, the relaxant activity of SCA40 was antagonized in the presence of quinine, a non-selective blocker of Ca^{2+}-activated K^+-channels (Haylett & Jenkinson, 1990).

The relaxant activity of SCA40 and isoprenaline has been evaluated in presence of ChTX, an agent known to block the large-conductance Ca^{2+}-dependent K^+-channel (Cook & Quast, 1990), especially in guinea-pig isolated trachea (Murray *et al.*, 1991). As potassium is a potent blocker of ChTX binding in smooth muscle (Vasquez *et al.*, 1989), the smooth muscle relaxant activity of SCA40 and isoprenaline, in the presence and absence of ChTX, have not been evaluated in guinea-pig isolated trachea contracted with KCl but with 1 μM carbachol. ChTX (60 and 180 nM) induced a non-competitive inhibition of isoprenaline relaxant activity, i.e; a rightward shift in the concentration-response curves with decrease in the maximum response. These observations are in agreement with previous findings that ChTX was able to produce a non-competitive inhibition of isoprenaline or salbutamol-induced relaxation of guinea-pig isolated trachea (Jones *et al.*, 1990). In contrast, ChTX (60, 180, 600 nM) induced an apparently competitive inhibition of SCA40 relaxant activity (rightward shift in the concentration-response curves) with no significant alteration in the maximum response. These results indicate that ChTX-sensitive K^+-channels are involved in both SCA40 and isoprenaline relaxant activity in guinea-pig trachea.

SCA40 and isoprenaline could activate different ChTX-sensitive K^+-channels since ChTX has been reported to block not only the large conductance Ca^{2+}-dependent K^+-channel but also the intermediate Ca^{2+}-dependent K^+-channel (Haylett & Jenkinson, 1990), an n type voltage-dependent K^+-channel in human T lymphocytes (Attali *et al.*, 1992) and recently, a small conductance Ca^{2+}-dependent K^+-channel unaffected by apamin in rat aortic smooth muscle cells (Van Renterghem & Lazdunski, 1992). However, airway smooth muscle cell membranes from different species (McCann & Welsh, 1986; Huang *et al.*, 1987; Green *et al.*, 1991) are characterized by a dense distribution of large conductance Ca^{2+}-dependent K^+-channels and isoprenaline, a potent bronchodilator, has been reported to activate these K^+-channels in guinea-pig trachealis cell membranes (Jones *et al.*, 1990; Murray *et al.*, 1991). It is possible therefore that SCA40, which is also a potent bronchodilator, might activate the large Ca^{2+}-dependent K^+-channel in guinea-pig trachealis cell membranes.

The β-adrenoceptor agonist, isoprenaline and theophylline, a cyclic AMP phosphodiesterase inhibitor, both produce an elevation of intracellular cyclic AMP and are able to activate the large Ca^{2+}-dependent K^+-channel (Jones *et al.*, 1990; Murray *et al.*, 1991). Kume *et al.* (1989) have reported that isoprenaline opened large-conductance Ca^{2+}-dependent K^+-channel of rabbit trachealis cell membranes by activating protein kinase A and phosphorylation of some regulatory protein(s) on the cytoplasmic side of the membrane. In these conditions, it is easily understood that ChTX, which blocks the Ca^{2+}-dependent K^+-channel at the external pore of the membrane (Gimenez-Gallego *et al.*, 1988), non-competitively inhibited the relaxant activity of isoprenaline. In contrast, ChTX inhibited the relaxant activity of SCA40 in an apparently competitive manner which suggests a different mechanism of action. It could also be mentioned that, if the opening of K^+-channels is involved in the relaxant activity of isoprenaline or theophylline, it is not the only mechanism implicated since isoprenaline (Allen *et al.*, 1985) and theophylline (Small *et al.*, 1989) are able to relax completely guinea-pig trachea in a very K^+-rich (120 mM) medium. In contrast,

SCA40, even at high concentration (10 μM), induced only a partial relaxation in 80 mM KCl-contracted trachea. However, under these conditions the cyclic AMP phosphodiesterase activity of SCA40 seems to play a minor role in its relaxant activity.

All these results indicate that SCA40 may be the first known agent of a new class of K^+-channel openers, involving ChTX-sensitive K^+-channels. Among these ChTX-sensitive K^+-channels, only the large conductance Ca^{2+}-dependent K^+-channel has so far been described in trachealis cells and it might be thought that SCA40 relaxant activity involves this K^+-channel. Patch-clamp studies have to be performed in order to confirm this hypothesis. With the development of such compounds will come a greater understanding of the major role that K^+ channels play in the regulation of smooth muscle excitability. As airway smooth muscle cells are richly endowed with the large conductance Ca^{2+}-dependent K^+-channels, such compounds have potential interest as bronchodilators in the treatment of asthma.

References

ALLEN, S.L., BEECH, D.J, FOSTER, R.W., MORGAN, G.P. & SMALL, R.C. (1985). Electrophysiological and other aspects of the relaxant action of isoprenaline in guinea-pig isolated trachealis. *Br. J Pharmacol.*, **86**, 843–854.

ALLEN, S.L., BOYLE, J.P., CORTIJO, J., FOSTER, R.W., MORGAN, G.P. & SMALL, R.C. (1986). Electrical and mechanical effects of BRL-34915 in guinea-pig isolated trachealis. *Br. J. Pharmacol.*, **89**, 395–405.

ATTALI, B., ROMEY, G., HONORE, E., SCHMID-ALLIANA, A., MATTEI, M.G., LESAGE, F., RICARD, P., BARHANIN, J. & LAZDUNSKI, M. (1992). Cloning, functional expression, and regulation of two K^+ channels in human T lymphocyte. *J. Biol. Chem.*, **267**, 8650–8657.

BONNET, P.A., MICHEL, A., LAURENT, F., SABLAYROLLES, C., RECHENQ, E., MANI, J.C., BOUCARD, M. & CHAPAT, J.P. (1992). Synthesis and antibronchospastic activity of 8-alkoxy- and 8-alkylaminoimidazo[1,2-*a*]pyrazines. *J. Med. Chem.*, **35**, 3353–3358.

CASTLE, N.A., HAYLETT, D.J. & JENKINSON, D.H. (1989). Toxins in the characterization of potassium channels. *Trends Pharmacol. Sci.*, **12**, 59–65.

COOK, J.S. & QUAST, U. (1990). Potassium channel pharmacology. In *Potassium Channels: Structure, Classification, Function and Therapeutic Potential.* ed. Cook, N.S. pp. 181–231. Chichester: Ellis Horwood, Ltd.

GIMENEZ-GALLEGO, G., NAVIA, M.A., REUBEN, J.P., KATZ, G.M., KACZOROWSKY, G.J. & GARCIA, M.L. (1988). Purification, sequence, and model structure of charybdotoxin, a potent selective inhibitor of calcium-activated potassium channels. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 3329–3333.

GREEN, K.A., FOSTER, R.W. & SMALL, R.C. (1991). A patch-clamp study of K^+ channel activity in bovine isolated tracheal smooth muscle cells. *Br. J. Pharmacol.*, **102**, 871–878.

HAMILTON, T.C., WEIR, S.W. & WESTON, A.H. (1986). Comparison of the effects of BRL 34915 and verapamil on electrical and mechanical activity in rat portal vein. *Br. J. Pharmacol.*, **88**, 103–111.

HAYLETT, D.G. & JENKINSON, D.H. (1990). Calcium-activated potassium channels. In: *Potassium Channels: Structure, Classification, Function and Therapeutic Potential.* ed. Cook, N.S. pp. 70–95. Chichester: Ellis Horwood Limited.

HOOKER, C.S., CALKINS, P.Y. & FLEISH, J.H. (1977). On the measurement of vascular and respiratory smooth muscle in vitro. *Blood Vessels*, **14**, 1–11.

HUANG, H.M., DWYER, T.M. & FARLEY, J.M. (1987). Patch-clamp recording of single Ca^{2+}-activated K^+ channels in tracheal smooth muscle from swine. *Biophys. J.*, **51**, 50a.

JONES, T.J., CHARETTE, L., GARCIA, M.L. & KACZOROWSKI, G.J. (1990). Selective inhibition of guinea-pig trachea by charybdotoxin, a potent Ca^{2+}-activated K^+ channel inhibitor. *J. Pharmacol. Exp. Ther.*, **255**, 697–706.

KUME, H., TAKAI, A., TOKUNO, H. & TOMITA, T. (1989). Regulation of Ca^{2+}-dependent K^+-channel activity in tracheal myocytes by phosphorylation. *Nature*, **341**, 152–154.

MCCANN, J.D. & WELSH, M.J. (1986). Calcium-activated potassium channels in canine airways smooth muscle. *J. Physiol.*, **372**, 113–127.

MELLEMKJÆR, S., NIELSEN-KUDSK, J.E., NIELSEN, C.B. & SIGGAARD, C. (1989). A comparison of the relaxant effects of pinacidil in guinea-pig trachea, aorta and pulmonary artery. *Eur. J. Pharmacol.*, **167**, 275–280.

MICHEL, A., MONGOLD, J.J., BOUCARD, M., BONNET, P.A., SABLAYROLLES, C., RECHENQ, E. & CHAPAT, J.P. (1989). Spasmolytic activity of deaza-xanthines derivatives in the guinea-pig isolated ileum and trachea. *Fundam. Clin. Pharmacol.*, **3**, 451.

MICHEL, A., SABLAYROLLES, C., BONNET, P.A., RECHENQ, E., BOUCARD, M. & CHAPAT, J.P. (1990). Pharmacological properties of a new deazapurine derivative with potential spasmolytic activity. *Fundam. Clin. Pharmacol.*, **4**, 579–580.

MURRAY, M.A., BERRY, J.L., COOK, S.J., FOSTER, R.W., GREEN, K.A. & SMALL, R.C. (1991). Guinea-pig isolated trachealis: the effects of charybdotoxin on mechanical activity, membrane potential changes and the activity of plasmalemmal K^+-channels. *Br. J. Pharmacol.*, **103**, 1814–1818.

QUAST, U. & COOK, N.S. (1989). Moving together: K^+ channel openers and ATP-sensitive K^+ channels. *Trends Pharmacol. Sci.*, **10**, 431–435.

ROBERTSON, D.W. & STEINBERG, M.I. (1990). Potassium channel modulators: scientific applications and therapeutics promise. *J. Med. Chem.*, **33**, 1529–1541.

SABLAYROLLES, C., CROS, G., MILHAVET, J.C., RECHENQ, E., CHAPAT, J.P., BOUCARD, M., SERRANO, J.J. & MCNEILL, J.H. (1984). Synthesis of imidazo [1,2-*a*]pyrazine derivatives with uterin-relaxing, antibronchospastic, and cardiac-stimulating properties. *J. Med. Chem.*, **27**, 206–212.

SMALL, R.C., BOYLE, J.P., DUTY, S., ELLIOTT, K.R.F., FOSTER, R.W. & WATT, A.J. (1989). Analysis of the relaxant effects of AH 21-132 in guinea-pig isolated trachealis. *Br. J. Pharmacol.*, **97**, 1165–1173.

VAN RENTERGHEM, C. & LAZDUNSKI, M. (1992). A small-conductance charybdotoxin-sensitive, apamin-resistant Ca^{2+}-activated K^+-channel in aortic smooth muscle cells (A7r5 line and primary culture). *Plügers Arch. Eur. J. Physiol.*, **420**, 417–423.

VASQUEZ, J., FEIGENBAUM, P., KATZ, G., KING, V.F., REUBEN, J.P., ROY-CONTANCIN, L., SLAUGHTER, R.S., KACZOROWSKI, G.J. & GARCIA, M.L. (1989). Characterization of high affinity binding site for charybdotoxin in sarcolemmal membranes from bovine aortic smooth muscle. *J. Med. Chem.*, **264**, 20902–20909.

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Effects of direct renal arterial infusion of bufalin and ouabain in conscious sheep

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1 The presence of an endogenous digitalis-like factor (EDLF) in the plasma of both normal and volume expanded animals is well documented. In this study we have used ouabain and bufalin as pharmacological analogues to mimic the renal effects of EDLF and to investigate whether any interaction occurs between atrial natriuretic factor (ANF) and EDLF.

2 Conscious Na replete sheep with chronically indwelling catheters in the renal artery received renal arterial infusion of ouabain (1000 $\mu g\,h^{-1}$) or bufalin (500 $\mu g\,h^{-1}$) for 60 min. Renal arterial infusion of bufalin increased sodium excretion ($U_{Na}V$) from 120 ± 13 to 596 ± 161 $\mu mol\,min^{-1}$ after 45 min. Bufalin infusion did not alter glomerular filtration rate (GFR), effective renal plasma flow (ERPF), or lithium clearance. Ouabain infusion increased $U_{Na}V$ from 124 ± 57 to 764 ± 123 $\mu mol\,min^{-1}$ in the first hour after infusion.

3 ANF infusion increased $U_{Na}V$ from 159 ± 34 to 583 ± 134 $\mu mol\,min^{-1}$. When renal arterial bufalin infusion was followed by renal arterial ANF infusion (50 $\mu g\,h^{-1}$) $U_{Na}V$ was increased from 155 ± 31 to 795 ± 96 $\mu mol\,min^{-1}$. This increase in $U_{Na}V$ is approximately equal to the sum of the separate effects of bufalin and ANF.

4 The natriuretic effects of ouabain at pharmacological doses in sheep are confirmed by this study. The data presented here do not support the hypothesis that EDLF sensitizes the kidney to the natriuretic effects of ANF.

Keywords: Natriuretic hormone; natriuretic peptides, atrial; ouabain; bufanolides; sheep kidney

Introduction

The discovery of atrial natriuretic factor (ANF) by de Bold *et al.* (1981) and the subsequent explosion of research on ANF has provided new insight into the physiology of sodium homoeostasis. However, many findings remain unexplained and many workers would still argue that a second natriuretic endocrine system must exist (reviewed by de Wardener & Clarkson, 1985; Kramer *et al.*, 1990). Plasma from volume-expanded animals contains a substance which cross reacts with digoxin antibodies and which inhibits (Na^+K^+)-ATPase (Gruber *et al.*, 1980). Although a number of factors with natriuretic activity have been isolated, few have been chemically identified. Exceptions to this are the identification of linoleic (18:2) and oleic (18:1) acids (Tamura *et al.*, 1985) and lysophosphatidylcholine γ-stearol (Tamura *et al.*, 1987) and the recent isolation by Hamlyn *et al.* (1991) of a substance chemically indistinguishable from ouabain in human plasma.

Ouabain has been demonstrated to inhibit renal (Na^+K^+)-ATPase when administered *in vivo* (Toretti *et al.*, 1972), and the inhibition of renal (Na^+K^+)-ATPase has been shown to be correlated with natriuretic responses to ouabain (Hook, 1969). Present evidence suggests that it is highly likely that the endogenous digoxin-like substance (EDLF) is ouabain or a chemically similar substance (Hamlyn *et al.*, 1991).

Bufalin is a cardiodienolide isolated from toad skin (although not from any mammalian source) (Lichstein *et al.*, 1986) which has been demonstrated to have similar effects on the cardiovascular system to ouabain and other cardiac glycosides (Yamahara *et al.*, 1986). Recently Pamnani *et al.* (1991) have reported the renal effects of intravenous infusion of bufalin in the rat. Pamnani *et al.* (1991) found bufalin to be more potent than ouabain.

ANF has been demonstrated to have potent effects on the kidney and cardiovascular system and is generally believed to be an important part of the homeostatic system regulating sodium and fluid balance. It has become apparent however, that there is a discrepancy between the observed concentration of ANF in plasma and the level of exogenous ANF which is required to elicit a natriuretic response in the isovolaemic animal (Richards *et al.*, 1988; McDougall *et al.*, 1988). In circumstances of volume (McDougall *et al.*, 1991) or sodium loading (Yates *et al.*, 1990), the responsiveness of the kidney to ANF is observed to increase and in sodium depletion to decrease (Yates *et al.*, 1990). It is possible that the presence of EDLF in the plasma of volume-expanded subjects is responsible for the increased responsiveness to ANF during volume expansion. The natriuretic response to ANF may be synergistically enhanced by the presence of an endogenous inhibitor of (Na^+K^+)-ATPase during volume expansion. In addition, Morise *et al.* (1991) have reported a stimulation of ANF secretion and synthesis by ouabain and digoxin, indicating interaction between these substances.

This study examines the renal effects of ouabain and bufalin in conscious sheep. In addition, we examined the natriuretic effect of serial renal arterial infusions of bufalin and ANF to investigate any possible interaction between ANF and an EDLF.

Methods

Mature cross-bred merino ewes (body weight 28–42 kg) were prepared under general anaesthesia with a chronic indwelling cannula in the left renal artery, contralateral nephrectomy, left carotid artery loop and bilateral oophorectomy (Yates *et al.*, 1988).

On the day prior to experiment two polyethylene cannula were inserted into the right jugular vein, while the bladder was catheterized with a Foley self-retaining catheter.

Animals were offered 800 g lucerne-oaten chaff daily ([Na] 60–100 $mmol\,kg^{-1}$) and water *ad libitum*. On the day of experiment an 18 gauge needle was inserted into the exteriorised left carotid artery for direct measurement and

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on-line monitoring of blood pressure via a transducer (Scoggins *et al.*, 1985). Glomerular filtration rate (GFR), effective renal plasma flow (ERPF) and proximal tubular function were measured by established clearance procedures (Whitworth *et al.*, 1978; Yates *et al.*, 1988).

Each animal received an initial injection of [^{125}I]-pAH (4 μCi), [^{51}Cr]-EDTA (20 μCi) and Li (3 mmol) followed by continuous infusion of [^{125}I]-pAH (4 μCi h^{-1}), [^{51}Cr]-EDTA (20 μCi h^{-1}) and Li (1 mmol h^{-1}). After 60 min equilibration, a renal arterial infusion of 0.15 mol l^{-1} NaCl at 12 ml h^{-1} was started. At this time serial 15 min urine collections were commenced. A 5 ml blood sample was taken at the mid-point of each collection period. After 4 collection periods (60 min) the renal arterial infusion was changed to either bufalin (Sigma; 500 μg h^{-1}; $n = 5$) or ouabain (Sigma; 1000 μg h^{-1}; $n = 5$). After a further 4 collection periods (60 min) the bufalin or ouabain infusion was stopped and infusion of 0.15 mol l^{-1} NaCl only continued for 120 min. Urine and plasma samples were analysed for ^{125}I, ^{51}Cr (Cobra 5010 gamma counter, Packard), Na, K (System E2A Beckman), Ca (Autoanlyser IOI), and Li (SpectrAA Varian Techtron).

In a separate series of experiments a 60 min renal arterial bufalin infusion was followed by renal arterial infusion of synthetic human atrial natriuretic factor (99–126) (Peninsula) at 50 μg h^{-1} for 60 min ($n = 6$). Alternatively ANF only was infused after 120 min of 0.15 mol l^{-1} NaCl infusion ($n = 5$). ANF and bufalin were infused sequentially in an attempt to allow for the difference in time required for onset of effects, bufalin having no effect for 45 min, ANF having an observable effect within 15 min.

This study has not attempted to produce dose-response curves for the agents examined. It is impractical to administer multiple doses, of agents such as ouabain which have slow onset and turnoff of effect, on a single day. Also other agents such as ANF can produce tachyphlatic responses even after a short period of administration (Yates *et al.*, 1988).

Values are presented as mean ± standard error of mean (s.e.mean). Statistical analysis was performed using SPSS, by analysis of variance with the Student-Newman-Kuels procedure to identify changes from control at specific time points. Statistical significance was taken at $P < 0.05$.

Results

Sodium excretion ($U_{NA}V$) increased from a control level of 120 ± 13 to a mean level 485 ± 157 μmol min^{-1} in the 60 min period after bufalin infusion was stopped ($P < 0.05$). $U_{Na}V$ was not different to control in the second hour following bufalin infusion, i.e. 120 min after the start of bufalin infusion. This contrasts with the effect of ouabain which increased $U_{Na}V$ from a control of 124 ± 57 to 764 ± 123 μmol min^{-1} ($P < 0.05$) and remained elevated in the second hour post infusion (647 ± 135 μmol min^{-1}) (Figure 1).

Renal arterial infusion of bufalin increased the fractional excretion of Na (FE Na) from 2.9 ± 0.5% to 11.2 ± 3.4% in the first hour after bufalin infusion ($P < 0.05$). Ouabain infusion increased the FE Na from 2.9 ± 0.8% to 26 ± 4.4% in the first hour post infusion and to 25 ± 3.1% in the second hour post infusion ($P < 0.05$). The fractional clearance of lithium (C_{Li}/GFR) was not significantly changed following renal arterial infusion of bufalin, although it was increased by ouabain infusion from 35 ± 4% during the control period to 56 ± 5% in the first hour post infusion ($P < 0.05$). This increase was sustained during the second hour post-infusion.

Mean arterial pressure (MAP), heart rate (HR) and potassium excretion ($U_K V$) were unchanged by infusion of either bufalin or ouabain. Urine flow (U_V) was significantly increased ($P < 0.05$) by both bufalin and ouabain infusions following cessation of infusion (Table 1). The effect of bufalin on $U_{Ca}V$ was variable between animals although there was no statistically significant effect observed overall.

Bufalin infusion had no effect on glomerular infiltration (GFR) (control 32 ± 6 ml min^{-1}) or effective renal plasma flow (ERPF) (control: 248 ± 10 ml min^{-1}). Ouabain infusion

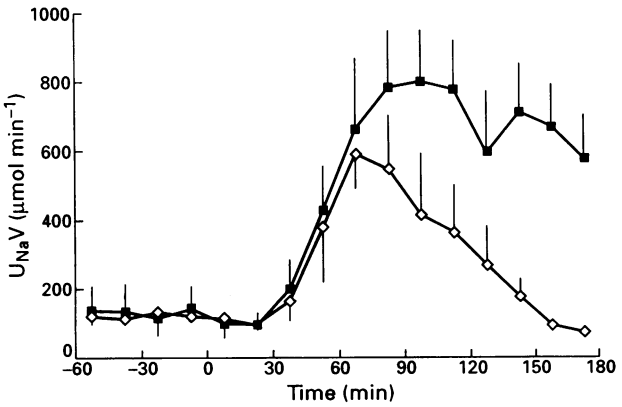


Figure 1 Effect of renal arterial infusion of bufalin (500 μg h^{-1}; $n = 5$) (◇) and ouabain (1000 μg h^{-1}; $n = 5$) (■) on sodium excretion in the conscious sheep. Infusion commenced at time 0 and ceased at 60 min.

Table 1 Effect of renal arterial infusion of ouabain (1000 μg h^{-1}) or bufalin (500 μg h^{-1})

		Control	*Infusion*	*1 h Post*	*2 h Post*
$U_{Na}V$	B	120 (13)	187 (49)	485 (157)*	163 (39)
(μmol min^{-1})	O	124 (57)	208 (57)	764 (123)*	647 (135)*
$U_K V$	B	114 (23)	102 (55)	111 (24)	105 (24)
(μmol min^{-1})	O	146 (54)	122 (34)	128 (17)	125 (7)
$U_{Ca}V$	B	0.38 (0.06)	0.76 (0.30)	2.87 (1.41)	0.93 (0.41)
(μmol min^{-1})	O	0.85 (0.38)	1.91 (1.26)	7.92 (2.47)*	5.16 (1.65)
GFR	B	32 (6)	33 (7)	30 (4)	29 (4)
(ml min^{-1})	O	27 (4)	27 (2)	21 (1)	18 (2)
ERPF	B	248 (10)	257 (32)	263 (27)	253 (28)
(ml min^{-1})	O	250 (29)	220 (9)	165 (9)*	205 (10)
C_{Li}/GFR	B	30 (2)	29 (2)	35 (5)	28 (3)
(%)	O	35 (4)	32 (4)	56 (5)*	57 (5)*
FE Na	B	2.9 (0.5)	4.4 (1.1)	11.2 (3.4)*	4.2 (0.1)
(%)	O	2.9 (0.8)	6.2 (1.7)	25.9 (4.4)*	25.0 (3.1)*
U_V	B	0.9 (0.1)	1.2 (0.2)	3.1 (1.0)*	1.2 (0.3)
(ml min^{-1})	O	1.0 (0.3)	1.5 (0.4)	6.2 (0.9)*	4.6 (0.9)*

*$P < 0.05$. Values are mean (s.e.mean). B: bufalin; O: ouabain; $U_{Na}V$: sodium excretion; $U_K V$: potassium excretion; $U_{Ca}V$: calcium excretion; U_V: urine flow rate; GFR: glomerular filtration rate; ERPF: effective renal plasma flow; FE Na: fractional sodium excretion; C_{Li}/GFR: fractional lithium clearance.

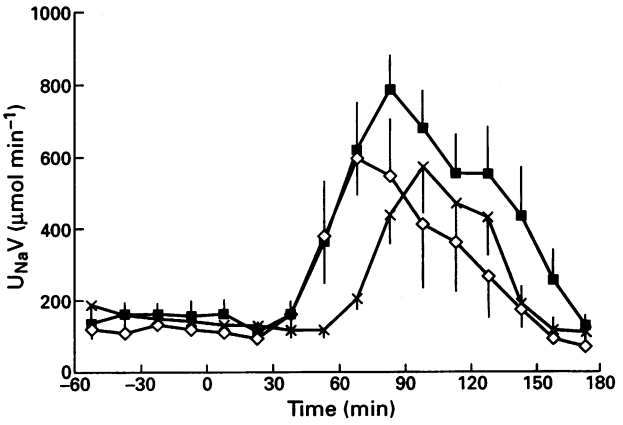


Figure 2 Comparison of the effects of renal arterial infusion of bufalin ($n = 5$) (◇), atrial natriuretic factor (ANF, $n = 5$) (×) and ANF + bufalin ($n = 6$) (■) on sodium excretion. Bufalin was infused between 0 and 60 min, ANF was infused between 60 min and 120 min.

however decreased ERPF ($P < 0.05$) in the first hour post infusion from a control of 250 ± 29 ml min^{-1} to 165 ± 9 ml min^{-1}. ERPF returned towards control levels in the second hour post infusion. GFR was not significantly altered by ouabain infusion.

Renal arterial infusion of bufalin did not produce any increase in $U_{Na}V$ in the first 45 min of infusion. In comparison, the natriuretic effects of ANF were observed within 15 min of infusion. The natriuretic effects of bufalin and ANF at the doses used in this study were of similar magnitude (maximum rate 596 ± 161 and 583 ± 134 μmol min^{-1} respectively). When bufalin and ANF infusions were, combined in the sequential manner described in the methods, a maximum rate of sodium excretion of 795 ± 96 μmol min^{-1} was reached (Figure 2).

Discussion

Comparison of the effects of approximately equimolar doses of bufalin and ouabain indicates that some differences may exist in their action on renal function and contrasts with the findings of Pamnani *et al.* (1991) who found bufalin to be the more potent agent. The difference in findings may reflect species variation, or perhaps be a consequence of the route of administration. Ouabain infusion produced larger and more sustained effects on $U_{Na}V$, $U_{Ca}V$, U_V, FE Na and C_{Li}/GFR than bufalin. In addition, ouabain infusion was associated with a significant decrease in ERPF which was not altered by bufalin infusion. Some similarities in effect were also apparent. In particular the time of onset before the renal effects were observed and the absence of any effect on potassium excretion. This suggests that both substances may be working through the same or similar mechanism(s), and that the difference in effect is primarily due to differences in potency. Alternatively, ouabain may be producing additional systemic effects which enhance the renal effects.

The long duration of action of ouabain is presumably due to the very slow rate of dissociation of ouabain from its binding sites on (Na^+K^+)ATPase (half-time approximately 150 min, Wallick *et al.*, 1980; Forbush, 1983). The much reduced duration of effect of bufalin may be due to its lack of sugar group, since Yoda *et al.* (1975) report that the presence and number of sugar groups on cardiac glycoside molecules determines their dissociation rate. Cardiac aglycones (e.g. ouabagenin) all have high dissociation rates compared to the cardiac glycosides. The shorter duration of effect of bufalin may make it preferable to ouabain as a pharmacological probe for investigation of the *in vivo* effects of EDLF.

The fractional clearance of Li is an indicator of proximal fractional fluid delivery (Koomans & Dorhout Mees, 1989; Leyssac, 1989). If bufalin or ouabain were inhibiting (Na^+K^+)-ATPase in the proximal tubule, they would be expected to cause an increase in the fractional clearance of Li. No significant effect of bufalin infusion was observed on this parameter despite a large increase in the fractional excretion of Na. Ouabain however did produce an increase in fractional Li clearance. Although bufalin had no significant effect on C_{Li}/GFR, when proximal Na reabsorption is calculated from the Li date (Fractional proximal Na reabsorption = $(GFR.P_{Li}-U_{Li}V)/(GFR.P_{Li})$) it is clear that the effect of bufalin on sodium excretion is mediated almost entirely through the proximal tubule. Similarly the natriuretic effect of ouabain is proximately mediated. Doucet & Barlet (1986) have reported binding sites for ouabain at two different sites on the renal tubule with different affinities. Binding sites on the collecting duct were found to have a higher affinity than those identified on the proximal tubule. The distal binding sites do not appear to contribute significantly to the natriuretic effect of ouabain or bufalin. The greater effect of ouabain on fractional Li clearance at a similar infusion rate suggests that either ouabain has a higher affinity for the proximal species of (Na^+K^+)-ATPase than does bufalin or else clearance of ouabain is sufficiently slow to allow a significantly higher plasma concentration of ouabain to accumulate. At higher rates of infusion bufalin might then be expected to cause a significant increase in fractional Li clearance.

Hamlyn *et al.* (1991) have reported the isolation of ouabain or an isomer of ouabain from human plasma. Further they have developed an immunoassay for ouabain with which this substance strongly cross reacts. Hamlyn *et al.* (1991) propose that EDLF is ouabain or an isomer of it. This finding is not easily reconcilable with the very low cross-reactivity of some digoxin antibodies which have detected 'EDLF' previously (Gruber *et al.*, 1980). The possibility does exist that Hamlym *et al.* (1991) have isolated a substance consisting of ouabagenin and a sugar such as L-fucose. The cross-reactivity of the EDLF isolated by Hamlyn *et al.* (1991) with digoxin antibodies has not been reported. Using this ouabain immunoassay they have measured plasma EDLF concentrations of 138 ± 43 pmol l^{-1} in man, and 80 ± 18 pmol l^{-1} in rats. Rats with mineralocorticoid induced hypertension had an elevated plasma concentration of 975 ± 79 pmol l^{-1}. The dose of ouabain used in the present study should produce a plasma concentration in the renal artery some 1000 times greater than the basal levels. Unpublished observations in the sheep revealed no natriuretic effect of ouabain at a dose 10 fold lower than the dose used here (Yates & McDougall, unpublished). It is unlikely that ouabain would circulate at sufficiently high concentrations to exert a direct natriuretic effect. If EDLF does have role in Na homeostasis, then the endogenous isomer must be significantly more active. Alternatively it may have an autocrine role within the kidney, or it is interacting with other physiological systems to increase greatly the magnitude of its effects. This does not mean that EDLF does not have direct effects on the vasculature or on other physiological systems and indeed de Wardener (1991) has suggested that EDLF is more important in cardiovascular control than in control of sodium homeostasis.

We have demonstrated unequivocally the alteration in renal response to ANF following alteration in sodium and/or volume status (Yates *et al.*, 1990; McDougall *et al.*, 1989; McDougall *et al.*, 1991) and by treatment with specific hormones (McDougall *et al.*, 1989). One possible mechanism to explain the alteration in renal sensitivity is that EDLF released during hypervolaemic or hypernatremic states was sensitizing the kidney to ANF. If this were the case it would have been expected that bufalin would markedly enhance the

natriuretic response to ANF. This was not observed and the additive nature of the combined response reinforces the different mechanisms of action of ANF and bufalin in the kidney.

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References

DE BOLD, A.J., BORENSTEIN, M.B., VERESS, A.T. & SONNENBERG, H. (1981). A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. *Life Sci.*, **28**, 89–94.

DE WARDENER, H.E. (1991). Kidney, salt intake and Na^+,K^+ATPase inhibitors in hypertension. *Hypertension*, **17**, 830–836.

DE WARDENER, H.E. & CLARKSON, E.M. (1985). Concept of natriuretic hormone. *Physiol, Rev.*, **65**, 658–759.

DOUCET, A. & BARLET, C. (1986). Evidence for differences in the sensitivity to ouabain of NaK-ATPase along the nephrons of rabbit kidney. *J. Biol. Chem.*, **261**, 993–995.

FORBUSH, B. III (1983). Cardiotonic steroid binding to Na,K-ATPase. *Current topic in Membranes and Transport*, **19**, 167–201.

GRUBER, K.A., WHITAKER, J.M. & BUCKALEW, V.M. Jr (1980). Endogenous digitalis-like substance in plasma of volume expanded dogs. *Nature*, **287**, 743–745.

HAMLYN, J.R., BLAUSTEIN, M.P., BOVA, S., DUCHARME, D.W., HARRIS, D.W., MANDEL, F., MATHEWS, W.R. & LUDENS, J.H. (1991). Identification and characterisation of a ouabain-like compound from human plasma. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 6259–6263.

HOOK, J.B. (1969). A positive correlation between natriuresis and inhibition of renal Na-K-adenosine triphosphatase by ouabain. *Proc. Soc. Exp. Biol. Med.*, **131**, 731–734.

KOOMANS, H.A. & DORHOUT MEES, E.J. (1989). Lithium in renal physiology: post conference discussion and consensus. *Kidney Int.*, **37** supplement 28, S78–79.

KRAMER, H.J., MEYER-LEHNERT, H., MICHEL, H. & PREDEL, H.-G. (1991). Endogenous natriuretic and ouabain-like factors: their roles in body fluid volume and blood pressure regulation. *Am. J. Hypertens.*, **4**, 81–89.

LEYSSAC, P.P. (1989). Validity of the lithium clearance concept assessed with micropuncture studies. *Kidney Int.*, **37** supplement 28, S17–21.

LICHSTEIN, D., KACHALASKY, S. & DEUTSCH, J. (1986). Identification of a ouabain-like compound in toad skin and plasma as a bufodienolide derivative. *Life Sci.*, **38**, 1261–1270.

McDOUGALL, J.G., COGHLAN, J.P., PARKES, D.G., SCOGGINS, B.A. & YATES, N.A. (1988). Hormonal and physiological modulation of the renal actions of atrial natriuretic factor. In *The Adrenal Gland: From Cloning to Clinic.* ed. Mantero, F., Biglieri, E.G., Funder, J.W. & Scoggins, B.A. pp. 145–159. New York: Raven Press.

McDOUGALL, J.G., YATES, N.A., COGHLAN, J.P., POTOCNIK, S.J. & SCOGGINS, B.A. (1991). Effect of volume expansion on the renal response to atrial natriuretic factor (ANF). In *Atrial and Brain Natriuretic Peptides* (Proceedings of the Kyoto Symposium on ANP, 1988). ed. Imura, H. & Matsuo, H. pp. 267–276. Tokyo: Kodansha Ltd.

MORISE, T., TAKEUCHI, Y., OKAMOTO, S. & TAKEDA, R. (1991). Stimulation of atrial natriuretic peptide secretion and synthesis by Na-K-ATPase inhibitors. *Biochem. Biophys. Res. Commun.*, **176**, 875–881.

PAMNANI, M.B., CHEN, S., BRYANT, H.J., SCHOOLEY, J.F. Jr, ELIADES, D.C., YUAN, C.M. & HADDY, F.J. (1991). Effects of three sodium-potassium adenosine triphosphatase inhibitors. *Hypertension*, **18**, 316–324.

RICHARDS, A.M., TONOLO, G., POLONIA, J. & MONTORSI, P. (1988). Contrasting plasma atrial natriuretic factor concentrations during comparable natriuresis with infusions of atrial natriuretic factor and saline in normal man. *Clin. Sci.*, **75**, 455–462.

SCOGGINS, B.A., ALLEN, K.J.S., COGHLAN, J.P., DENTON, D.A., FEI, D.T.W., TRESHAM, J.J., WANG, X. & WHITWORTH, J.A. (1985). Onset and dose-relationships of ACTH effects on blood pressure in sheep. *Hypertension*, **7**, 287–291.

TAMURA, M., INAGAMI, T., KINOSHITA, T. & KUWANO, H. (1987). A search for endogenous Na^+,K^+ATPase inhibitor in acutely volume-expanded hog plasma led to lysophosphatidylcholine γ-stearoyl. *J. Hypertens.*, **5**, 219–225.

TAMURA, M., KUWANO, H., KINOSHITA, T. & INAGAMI, T. (1985). Identification of linoleic and oleic acids as endogenous Na^+,K^+ATPase inhibitors from acute volume-expanded hog plasma. *J. Biol. Chem.*, **260**, 9672–9677.

TORETTI, J., HENDLER, E., WEINSTEIN, E., LONGNECKER, R.E. & EPSTEIN, F.H. (1972). Functional significance of Na-K-ATPase in the kidney: effects of ouabain inhibition. *Am. J. Physiol.*, **222**, 1398–1405.

WALLICK, E.T., PITTS, B.J.R., LANE, L.K. & SCHWARTZ, A. (1980). A kinetic comparison of cardiac glycoside interactions with Na^+K^+-ATPases from skeletal and cardiac muscle and from kidney. *Arch. Biochem. Biophys.*, **202**, 442–449.

WHITWORTH, J.A., COGHLAN, J.P., DENTON, D.A., FAN, J.S.K., McDOUGALL, J.G. & SCOGGINS, B.A. (1978). Exaggerated natriuresis in ACTH hypertensive sheep. *Nephron*, **22**, 473–478.

YAMAHARA, J., TANAKA, S., MATSUDA, H., SAVADA, T. & FUJIMURA, H. (1986). The mode of cardiac action of cardiotonic steroids isolated from Toad Cake in perfused working guinea-pig heart and the effect of cinobufagin on experimental heart failure. *Nippon Yakurigaku Zasshi*, **88**, 413–423.

YATES, N.A., COGHLAN, J.P., MURPHY, G.J., SCOGGINS, B.A. & McDOUGALL, J.G. (1990). Renal actions of atrial natriuretic factor: modulation of effect by changes in sodium status and aldosterone. *Am. J. Physiol.*, **258**, F684–689.

YATES, N.A., McDOUGALL, J.G., COGHLAN, J.P., GIBSON, A.P. & SCOGGINS, B.A. (1988). Renal effects of atrial natriuretic factor(99-126) in conscious sodium-replete sheep. *Clin. Exp. Pharmacol Physiol.*, **15**, 551–562.

YODA, S., SARRIF, A.M. & YODA, A. (1975). Structure-activity relationships of cardiotonic steroids for inhibition of sodium- and potassium-dependent adenosine triphosphatase. IV. Dissociation rate constants for complexes of the enzyme with cardiac oligodigitoxides. *Mol. Pharmacol.*, **11**, 647–652.

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β-Adrenoceptor agonist mediated relaxation of rat isolated resistance arteries: a role for the endothelium and nitric oxide

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1 Isoprenaline (10^{-9}–10^{-5} M) relaxed rat isolated mesenteric resistance arteries pre-contracted with K^+ (30–60 mM) (p EC_{50} (M) 8.03 ± 0.40; maximum relaxation 66.79 ± 2.43%, $n = 7$). This relaxation was partially attenuated by the nitric oxide (NO) synthase inibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M).

2 The β_2-adrenoceptor agonist, salbutamol (10^{-9}–10^{-5} M), produced a modest maximum relaxation (35.93 ± 2.93%), which was not sensitive to L-NAME.

3 The β_1-adrenoceptor agonist, dobutamine (10^{-9}–10^{-5} M), relaxed arteries precontracted with K^+. This relaxation was abolished by L-NAME (10^{-4} M) and also by propranolol (10^{-6} M), but not affected by D-NAME (10^{-4} M). The inhibition by L-NAME was partially reversed by L-arginine (10^{-3} M). Removal of the endothelium severely attenuated relaxation to dobutamine.

4 Contractile responses to depolarizing K^+ solutions were enhanced by the addition of L-NAME, and also by removal of the endothelium.

5 The above findings demonstrate that β_1-adrenoceptor activation causes relaxation via NO release from the endothelium of rat mesenteric resistance arteries. In addition, contraction to K^+ is modified by release of NO from the endothelium, possibly in response to tension development.

Keywords: β-Adrenoceptors; vascular smooth muscle; endothelium; nitric oxide; rat resistance artery

Introduction

Relaxation of vascular smooth muscle by β-adrenoceptor agonists is conventionally held to occur via activation of adenylate cyclase and an increase in intracellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) in the smooth muscle cell (Kukovetz *et al.*, 1981; Heeson & De Mey, 1990). Some reports, however, have suggested that the endothelium may play a facilitatory role in the relaxation to β-adrenoceptor agonists. These include the demonstration that removal of the endothelium from rat aorta (Kamata *et al.*, 1989) and canine coronary arteries (Rubanyi & Vanhoutte, 1985) attenuates isoprenaline-induced relaxation but does not affect the relaxation to forskolin (Kamata *et al.*, 1989). Not all are in agreement, however, as Moncada *et al.* (1991) found that isoprenaline-induced relaxation in rat aorta was neither endothelium-dependent, nor affected by inhibitors of nitric oxide (NO) synthase. Similarly, the absence of endothelium did not attenuate relaxation to isoprenaline in aorta and femoral artery of normotensive rats (Konishi & Su, 1983), although there was a slight reduction in the same arteries of spontaneously hypertensive rats. A study in the rat saphenous vein has also suggested that the endothelium is not involved in β-adrenoceptor agonist-induced relaxation in the venous circulation (De Mey & Vanhoutte, 1982).

Two studies have suggested more specifically that β-adrenoceptor-mediated relaxation may involve NO release from the endothelium of some preparations. In a preliminary report, Gray & Marshall (1991) found isoprenaline-induced relaxation to be endothelium-dependent in the rat isolated aorta, and the relaxation was attenuated by the nitric oxide (NO) synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME). L-NAME also partially inhibited the vasodilatation caused by the β_2-agonist, salbutamol, in the rat hindlimb *in vivo* (Gardiner *et al.*, 1991).

Other than the study by Gardiner *et al.* (1991) in the whole animal, all previous work has been carried out in isolated conduit arteries and no study has extensively investigated the role of the endothelium in β-adrenoceptor-agonist-mediated relaxation in resistance arteries. Since the β-adrenoceptors are largely involved in control of peripheral resistance by affecting tone in small arteries, this investigation has been carried out to determine the role of NO in β-adrenoceptor-mediated relaxation in resistance arteries. In a previous report (Heeson & De Mey, 1990), the non-selective β-agonist isoprenaline, and forskolin (a direct activator of adenylate cyclase), were found to relax rat mesenteric resistance arteries. In this study, using the same preparation, we have investigated the role of NO in the relaxation induced by isoprenaline, salbutamol (β_2-agonist), dobutamine (β_1-agonist), and forskolin, all generally held to relax vascular smooth muscle via cyclic AMP-dependent mechanisms. Endothelium-dependence of relaxation to dobutamine was also investigated by mechanical removal of the endothelium.

A preliminary account of part of this work has been presented to the British Pharmacological Society (Graves & Poston, 1992).

Methods

Mulvany-Halpern myograph

Resistance arteries were isolated from male Wistar-Kyoto rats (225–250 g, Bantin & Kingman, Hull, UK). After killing by cervical dislocation, mesenteric resistance arteries (fourth order branches) were dissected and mounted as a ring preparation on a myograph capable of measuring isometric tension (Mulvany & Halpern, 1977). The organ bath was filled with physiological salt solution (PSS) of the following compositon (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, EDTA 0.026 and glucose 5.5, pH 7.4, 37°C, bubbled with 5% CO_2 in O_2. The passive tension-internal circumference characteristics of each vessel

was determined and the internal diameter estimated (mean diameter 322 ± s.e.mean 3.1 μm, $n = 115$). The arteries were then set to an internal circumference equivalent to 90% of that which they would have when relaxed *in situ* under a transmural pressure of 100 mmHg. The maximum active tension for the minimum resting tension is developed at approximately this circumference (Mulvany & Halpern, 1977).

To assess their contractile response, the arteries were then maximally contracted for 2 min every 10 min on five occasions. The first, second and fifth contractions were to 5 μM noradrenaline (NA) in 125 mM potassium solution (KPSS, equimolar substitution of KCl by NaCl in PSS); (NAK). The third was to 5 μM NA in PSS, and the fourth to KPSS. Any artery failing to produce a maximum active tension equivalent to a pressure of 100 mmHg on the final contraction was rejected. After the third (5 μM NA) contraction, arteries were challenged with acetylcholine (ACh 5×10^{-6} M) to check endothelial function; all arteries relaxed by >85% of the initial contraction following this procedure. For the experiments involving endothelial removal this protocol was modified as described below.

Response to β-agonists and the effect of NO synthase inhibitors

Isoprenaline

A control contraction (13 min) to KPSS (K^+) was carried out with a concentration of potassium (30–60 mM) which led to approximately 50% of the tension developed with NAK. Any artery which did not maintain tension over this period was omitted from the study. The K^+ was then washed out and after 10 min the arteries again contracted with the same concentration of K^+. After 3 min of precontraction a cumulative concentration-response curve to isoprenaline (10^{-9} M–10^{-5} M, 2 min intervals) was then carried out. Following washout for 5 min, arteries were then incubated with L-NAME (10^{-4} M, 10 min) and a further concentration-response curve to isoprenaline constructed in the continued presence of L-NAME. In other experiments, the reproducibility of the isoprenaline relaxation was investigated by using the same protocol, but omitting L-NAME before and during the third contraction. The ability of the arteries to maintain reproducible contractions to K^+ over the length of the protocol was also studied by contracting arteries 3 times with K^+ (for 13 min) and preincubating with L-NAME (10^{-4} M) for the third contraction. The effect of isoprenaline on arteries pre-contracted by a receptor agonist rather than K^+ was investigated using the same protocol, but with substitution of K^+ with 3 μM NA. This dose of NA gave approximately 90% of the maximal contraction and produced a stable and sustained contraction. To confirm the involvement of β-adrenoceptors in isoprenaline-induced relaxation, the effect of propranolol, a non-selective β-antagonist, was investigated. Arteries were pre-contracted with K^+ as described above and two concentration-response curves to isoprenaline carried out, the second in the presence of propranolol (10^{-6} M).

Salbutamol

The effect of the β_2-agonist salbutamol (10^{-9} M–10^{-5} M) on K^+-induced contraction, and the effect of L-NAME upon salbutamol-induced relaxation were studied in the same arteries, following the protocol described above for isoprenaline.

Dobutamine

The effect of L-NAME upon relaxations elicited by dobutamine (β_1-agonist) were similarly examined. In control experiments, sequential relaxations to dobutamine were not reproducible, the second of two concentration-response curves demonstrating considerable tachyphylaxis. Concentration-response curves to dobutamine were therefore obtained and compared with those to dobutamine + L-NAME in another set of arteries. Arteries were submaximally precontracted with K^+ as described above and a concentration-response curve (10^{-9} M–10^{-5} M) to dobutamine performed with or without L-NAME (10^{-4} M). The ability of L-arginine to reverse blockade by L-NAME was investigated by incubating with L-arginine (10^{-3} M) for 20 min before addition of L-NAME (10^{-4} M) and the subsequent concentration-response curve to dobutamine was performed in the presence of L-NAME and L-arginine. The effect of D-NAME (10^{-4} M), the inactive enantiomer of L-NAME, was also investigated using an identical protocol to that described for L-NAME. The response to dobutamine in the presence of propranolol (10^{-6} M) was also studied.

Removal of the endothelium

To examine the effect of endothelium removal on relaxation to dobutamine the luminal surface of the artery was gently rubbed with a human forearm hair after the first contraction of the run-up procedure. The second, third and fourth contractions were to 5 μM NA and the arteries were challenged with ACh (5×10^{-6} M) after each contraction. Failure to relax more than 15% in 2 min was adopted as the criterion for efficient endothelium removal. If the arteries relaxed more than 15% further gentle rubbing was performed. Control experiments were performed which confirmed that repeated exposure to 10^{-5} M ACh did not impair the arteries ability to relax to ACh. Dobutamine-induced relaxation was investigated after removal of the endothelium, using the protocol described above.

Effect of L-NAME on forskolin-induced relaxation

Arteries were contracted with K^+ (30–60 mM) for 17 min. After washout (10 min), the arteries were again contracted to K^+ and a concentration-response curve to forskolin (10^{-8} M–10^{-5} M, 2 min intervals) carried out. Forskolin was first dissolved in ethanol and final concentrations of ethanol in the organ bath were less than 0.1%. In order to avoid the effects of tachyphylaxis, the above protocol was performed in a separate series of arteries, but with the addition of L-NAME (10^{-4} M) both for 10 min preceding and throughout the forskolin concentration-response curve.

Modification of K^+ contraction by NO

When studying agonist-induced relaxation it is important to produce similar tension in the test contracture to that in the control. In initial experiments using K^+ to preconstrict the arteries, considerable potentiation of the precontraction to K^+ occurred after incubation with L-NAME. This necessitated lowering the concentration of K^+ used in the presence of L-NAME to achieve similar tension. The potentiation by L-NAME suggested that NO release may attenuate K^+-induced tension development, and the effect of L-NAME upon K^+ concentration-response curves was therefore further investigated. A cumulative K^+ concentration-response curve was constructed and then repeated in the presence of L-NAME. Since a depolarizing potassium stimulus may lead to neurotransmitter release from adrenergic terminals (Vanhoutte *et al.*, 1981) the above protocol was repeated in the presence of phentolamine (10^{-5} M). The involvement of possible β-receptor stimulation was also investigated by the construction of two consecutive concentration-response curves to K^+ in the presence of phentolamine (10^{-5} M), and phentolamine plus propranolol (10^{-6} M) respectively. As a time control, two consecutive K^+ concentration-response curves were carried out in separate arteries. The effect of endothelial removal on the response to K^+ was also studied.

Statistical analysis

Relaxations to agonists are expressed as a percentage of the precontraction and are given as mean ± s.e.mean. Mean tensions for precontractions to K^+ and NA ($mN\,mm^{-1}$) are given for the initial isoprenaline experiments and were of similar magnitude for all subsequent protocols. Concentration-response curves for agonist-induced relaxation and contraction were fitted by the Graphpad package (GraphPAD Software Inc., San Diego, U.S.A.) to obtain maximum effect and pEC_{50} (− log of molar concentration producing 50% of maximum response). In the experiments in which K^+ concentration responses were investigated, results are expressed as percentages of the maximum response to NAK for each artery. Differences between means were assessed either by paired or unpaired *t* test as appropriate. For multiple comparisons ANOVA (Statview SE, Abaucus Concepts Inc.) followed by an unpaired *t* test was used.

Chemicals

The chemicals used in this study were acetylcholine chloride, L-NAME hydrochloride, L-arginine (free base), forskolin, and propranolol hydrochloride (Sigma, Poole, UK); D-NAME hydrochloride (Calbiochem-Novabiochem, Nottingham, UK); noradrenaline tartrate (Winthrop, Guildford, U.K.); isoprenaline hydrochloride (Pharmax, Bexley, U.K.); dobutamine hydrochloride (Eli Lilly & Co. Ltd, Basingstoke, U.K.); salbutamol sulphate (Allen & Hanburys Ltd, Greenford, U.K.); phentolamine mesylate (CIBA Laboratories, Horsham, U.K.). All other chemicals (Analar grade) were obtained from BDH, Poole, U.K.

Results

Response to β-agonists and the effect of NO synthase inhibitors

Isoprenaline

Isoprenaline relaxed rat mesenteric resistance arteries precontracted with K^+ (initial tension 2.18 ± 0.24 $nM\,mm^{-1}$) in a concentration-dependent manner (pEC_{50} (M) 8.03 ± 0.40; maximum relaxation 66.79 ± 2.43%, $n = 7$). Subsequent incubation with L-NAME (10^{-4} M) reduced the maximum relaxation to isoprenaline (initial tension 2.28 ± 0.06 mN mm^{-1}, maximum relaxation 27.64 ± 7.64%, $n = 7$, $P < 0.001$, paired *t* test). Two successive isoprenaline concentration-response curves, carried out as a control, showed no change in sensitivity (pEC_{50} 7.72 ± 0.2 v 7.63 ± 0.21, $n = 6$, $P = 0.42$ paired *t* test) but maximum relaxation in the second was less than the first (51.14 ± 4.68% v 60.96 ± 3.11%, $n = 6$, $P < 0.005$, paired *t* test). When results for this second isoprenaline concentration-response curve were compared to those for isoprenaline + L-NAME, the difference in the maximum response remained significant ($n = 7$, $P < 0.05$, unpaired *t* test) (Figure 1). The relaxation to isoprenaline of arteries pre-contracted with K^+ was completely inhibited by propranolol (10^{-6} M) (Figure 1). Isoprenaline also led to relaxation of arteries pre-contracted with NA, the addition of L-NAME again reducing the maximum relaxation to isoprenaline (73.50 ± 9.74%, control, v 40.89 ± 7.04%, L-NAME, $n = 6$, $P < 0.001$, paired *t* test). Time controls in which arteries were pre-contracted with K^+ without the addition of agonists showed 11.5 ± 4.8% maximum relaxation without L-NAME and 4.8 ± 5.5% with L-NAME ($n = 6$, $P = 0.38$, paired *t* test) (Figure 2).

Salbutamol

Salbutamol also relaxed arteries pre-contracted with K^+ (maximum relaxation 35.93 ± 2.93%), but L-NAME did not reduce the relaxation (maximum relaxation 33.05 ± 4.11%, $n = 6$, $P = 0.70$, paired *t* test) (Figure 2).

Dobutamine

The β_1-agonist dobutamine led to the relaxation of arteries pre-contracted with K^+ (maximum relaxation (10^{-5} M)

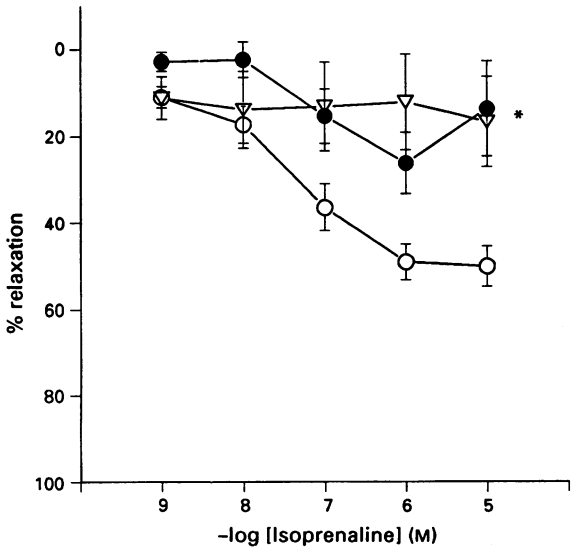


Figure 1 Relaxation of rat mesenteric resistance arteries to isoprenaline (10^{-9} M–10^{-5} M): control (○); propranolol 10^{-6} M (▽); N^{ω}-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M) (●). Results expressed as percentage relaxation (mean ± s.e.mean) of K^+ pre-contraction. Significant difference of maximum relaxation v control indicated by $^*P < 0.05$.

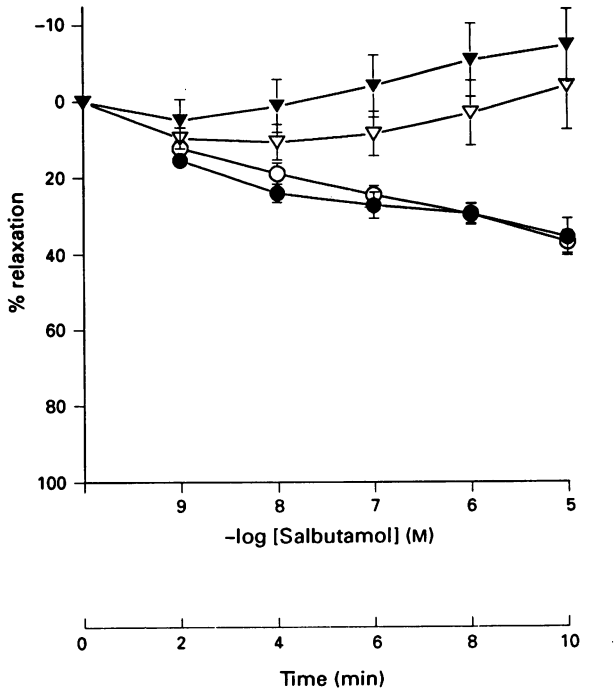


Figure 2 Relaxation of rat mesenteric resistance arteries to salbutamol (10^{-9} M–10^{-5} M): control (○) and N^{ω}-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M) (●). Data also shown for tension development to K^+ over the time course of the experiment, with (▼) and without (▽) L-NAME (10^{-4} M). Results expressed as percentage relaxation (mean ± s.e.mean) of K^+ pre-contraction.

65.1 ± 4.2%, $n = 9$). The maximum relaxation was completely inhibited by L-NAME (maximum relaxation 6.52 ± 3.2%, $n = 6$, $P < 0.001$ v control unpaired t test) but was not attenuated by D-NAME (10^{-4} M) (Figure 3). Preincubation with L-arginine (10^{-3} M) partially reversed the inhibition by L-NAME (Figure 3). Propranolol prevented relaxation to dobutamine and mechanical removal of the endothelium attenuated relaxation to dobutamine (Figure 4).

Effect of L-NAME on forskolin-induced relaxation

Forskolin relaxed arteries pre-contracted with K^+ in a concentration-dependent manner (pEC_{50} 7.32 ± 0.18; maximum relaxation 94.3 ± 2.1%, $n = 6$). L-NAME led to a slight increase in sensitivity to forskolin which did not reach significance (pEC_{50} 7.76 ± 0.08; $P = 0.06$ v control; maximum relaxation 98.6 ± 0.6%, $n = 6$, $P = 0.08$ v control, unpaired t test) (Figure 5).

Modification of K^+ contraction by NO

L-NAME increased both the sensitivity to K^+ (pEC_{50} 1.37 ± 0.01, control v 1.48 ± 0.03, L-NAME; $n = 6$, $P < 0.02$, paired t test) and the maximum response (65.82 ± 1.02% of contraction to NAK, control, v 78.44 ± 3.41%, L-NAME, $n = 6$, $P < 0.005$, paired t test). The maximum response between two successive concentration-response curves to K^+ in the absence of L-NAME was unaltered (75.1 ± 2.0% of NAK v 75.35 ± 4.6%, $n = 6$, $P = 0.72$, paired t test, $n = 6$). There was, however, a small but significant reduction in sensitivity to K^+ for the second curve (pEC_{50} 1.32 ± 0.03 v 1.27 ± 0.02, $n = 6$, $P < 0.05$ paired t test). Mechanical removal of the endothelium also increased the sensitivity to K^+ (pEC_{50} 1.37 ± 0.01; control, v 1.52 ± 0.03, without endothelium; $n = 6$, $P < 0.001$, unpaired t test) and the maximum response (65.8 ± 1.0% of NAK, control, v 90.4 ± 2.2% without endothelium, $n = 6$, $P < 0.001$, unpaired t test) (Figure 6a). The maximum tension development to NAK was 4.68 ± 0.17 mN mm^{-1} for the arteries used in the investigation of L-NAME, and 3.87 ± 0.17 mN mm^{-1} for those in which the endothelium was removed, the difference being attributable to slightly larger arteries being used for the L-NAME study (328 ± 9.1 μm v 289 ± 7.5 μm).

Phentolamine reduced K^+ induced tension (65.8 ± 1.0% of NAK, control v 31.1 ± 6.07% phentolamine, $n = 6$, $P < 0.001$ unpaired t test). Despite this α-adrenoceptor inhibition, L-NAME again led to potentiation of K^+-induced tension, affecting both the maximum response (31.1 ± 6.1% of NAK, control v 68.2 ± 3.73% L-NAME, $n = 6$, $P < 0.01$ paired t test) and the sensitivity (pEC_{50} 1.47 ± 0.06, control, v 1.56 ± 0.04, L-NAME, $n = 6$, $P < 0.05$ paired t test) (Figure 6b). β-Blockade with propranolol did not affect the maximum response to K^+ in the presence of phentolamine (20.2 ± 1.1% of NAK, control, v 23.14 ± 3.4%, propranolol, $n = 4$,

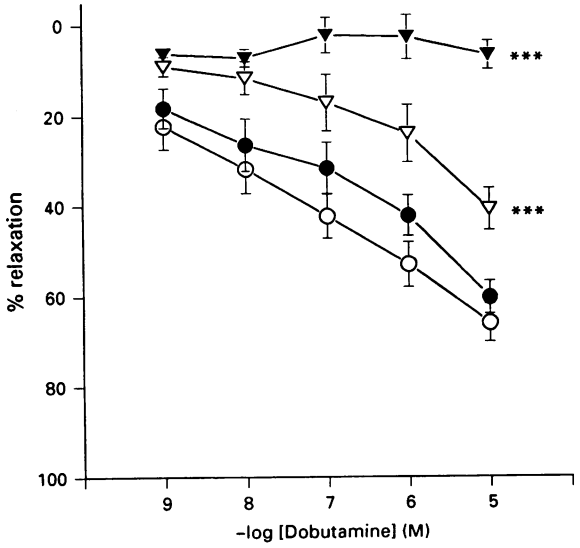


Figure 3 Relaxation of rat mesenteric resistance arteries to dobutamine (10^{-9} M–10^{-5} M): control (○); D^{ω}-nitro-L-arginine methyl ester (D-NAME, 10^{-4} M) (●); L-NAME (10^{-4} M) (▼); L-NAME (10^{-4} M) + L-arginine 10^{-3} M (▽). Results expressed as percentage relaxation (mean ± s.e.mean) of K^+ pre-contraction. Significant differences in maximum relaxation v control indicated by ***$P < 0.001$.

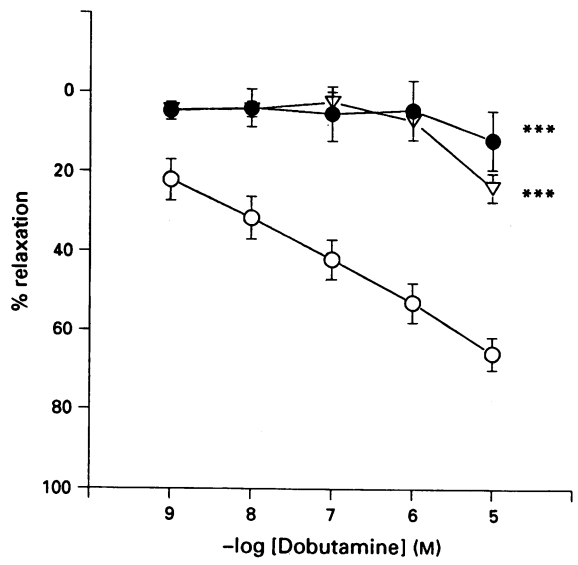


Figure 4 Relaxation of rat mesenteric resistance arteries by dobutamine (10^{-9} M–10^{-5} M): control (○); propranolol (10^{-6} M) (●); minus endothelium (▽). Results are expressed as percentage relaxation (mean ± s.e.mean) of K^+ pre-contraction. Significant difference of maximum relaxation v control indicated by ***$P < 0.001$.

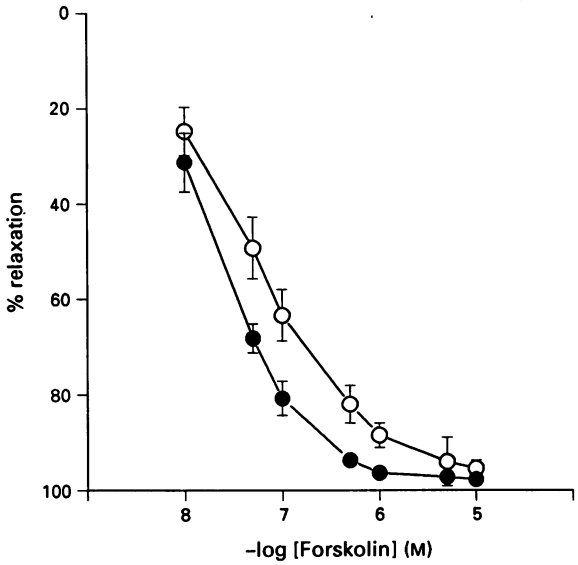


Figure 5 Relaxation of rat mesenteric resistance arteries to forskolin (10^{-8} M–10^{-5} M): control (○); N^{ω}-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M) (●). Results expressed as percentage relaxation (mean ± s.e.mean) of K^+ pre-contraction.

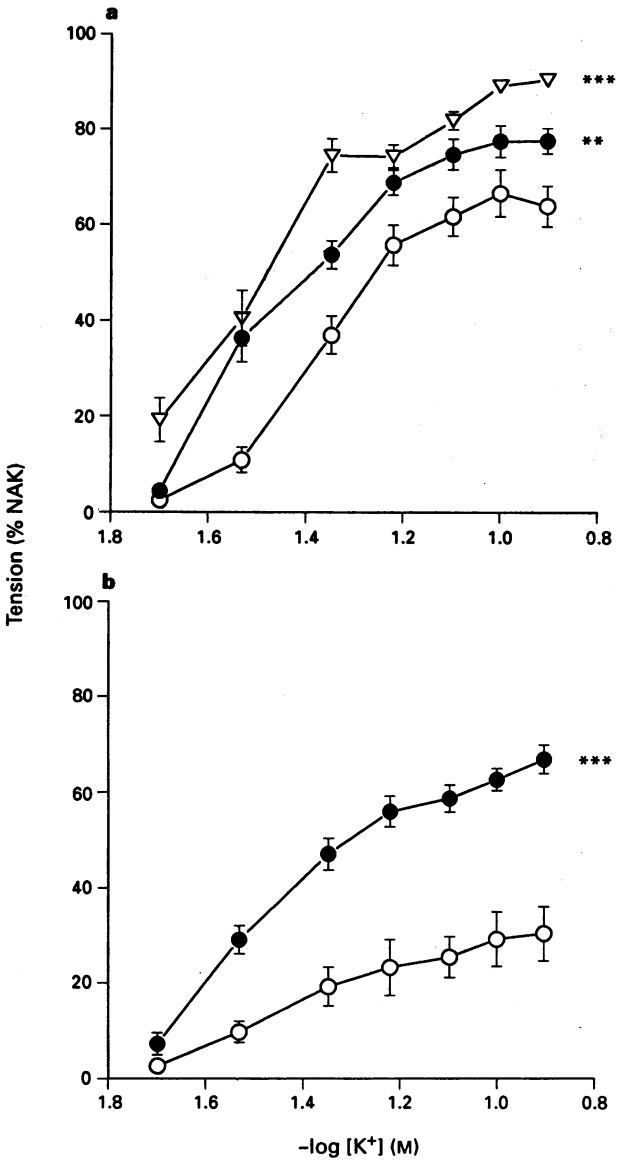


Figure 6 (a) Tension development to K^+ in rat mesenteric resistance arteries: control (○); N^{ω}-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M) (●), – endothelium (▽). (b) Tension development to K^+ in the presence of phentolamine (10^{-5} M) with (●) and without (○) L-NAME (10^{-4} M). Results expressed as percentage (mean ± s.e.mean) of contraction to NAK (5 μM NA; 125 mM K^+). Significant difference of maximum contraction v control indicated by $^{**}P<0.005$ and $^{***}P<0.001$.

$P<0.35$, paired t test), or significantly alter sensitivity (pEC_{50} 1.48 ± 0.01, control, v 1.39 ± 0.03, propranolol, $n=4$, $P=0.10$, paired t test).

Discussion

The isoprenaline-induced relaxation observed in rat mesenteric resistance arteries is in agreement with an earlier report by Heeson & De Mey (1990) in which this non-selective β-agonist was found to relax small arteries from the same circulation. In the present study, relaxation to isoprenaline was abolished by propranolol, confirming the involvement of β-adrenoceptors. Relaxation to isoprenaline was also inhibited, but not completely, by the NO synthase inhibitor, L-NAME, suggesting that in this preparation non-selective β-agonists may act partially through the release of NO. Isoprenaline-induced relaxation and the inhibitory effect of L-NAME were similar in arteries pre-contracted with NA or depolarizing potassium solution.

In order to investigate further the involvement of NO release by β-agonists, the effects of agonists specific to β_1- and β_2-adrenoceptors were determined. A relatively poor relaxation was found to the β_2-agonist salbutamol, which was unaffected by L-NAME. This modest relaxation to salbutamol was similar (as a percentage of the original tension) to the relaxation that Fujimoto *et al.* (1987) reported in strips of rat mesenteric artery pre-contracted with potassium. The lack of inhibition by L-NAME on β_2-agonist-induced relaxation agrees with the *in vivo* study of Gardiner *et al.* (1991) in which L-NAME did not attenuate the small vasodilatation to salbutamol observed in the rat mesenteric circulation. It is of interest that L-NAME reduced the hyperaemic vasodilatation to salbutamol in the rat hindlimb (Gardiner *et al.*, 1991) suggesting that the mechanism of relaxation to β-agonists may vary from circulation to circulation.

In contrast to salbutamol, the β_1-agonist dobutamine led to substantial relaxation of arteries pre-contracted with potassium. This relaxation was completely blocked by propranolol at all concentrations of dobutamine. This surprisingly effective inhibition is of interest as it suggests a very high affinity of propranolol for β-adrenoceptors in this preparation. Indeed, similarly profound inhibition of relaxation to a mixed β-agonist was found by Tsujimoto & Hoffman (1985) in the same circulation of the rat, but in arteries of larger diameter (3 mm). Whereas, L-NAME had no effect on β_2-induced relaxation, it effectively blocked the relaxation to dobutamine, suggesting that β_1-agonists led to relaxation in this circulation via the release of NO. The D-enantiomer of L-NAME (D-NAME) had no effect on dobutamine-induced relaxation, indicating that the inhibition of relaxation by L-NAME was due to a specific effect on NO synthase. The NO synthase substrate, L-arginine, only partially reversed the effects of L-NAME on dobutamine-induced relaxation. In larger arteries L-arginine completely reversed the effect of NO synthase inhibition (Palmer *et al.*, 1988; Rees *et al.*, 1989; Moore *et al.*, 1990), but this study agrees with others in resistance arteries which have also demonstrated partial reversal of nitric oxide inhibition. These include a study by Moore *et al.* (1990) using various NO synthase inhibitors in the perfused rat mesenteric bed, and another from our laboratory in human resistance arteries in which L-arginine only partially reversed the inhibitory effect of L-N^G monomethyl-arginine (L-NMMA) on ACh-induced relaxation (Woolfson & Poston, 1990). There is no obvious explanation for this observation, but there is increasing evidence that L-arginine and NO synthase inhibitors may not interact in an entirely competitive manner. This is supported by the frequent observation that greater concentrations of L-arginine than those calculated have often been required to achieve complete or even partial reversal of NO synthase inhibition (Amezcua *et al.*, 1989; Vallance *et al.*, 1989; Moore *et al.*, 1990; Woolfson & Poston, 1990).

The relaxation to dobutamine was also inhibited by mechanical removal of the endothelium, providing evidence that it is the endothelium which is the source of the NO released by β_1-activation. Little information is available concerning β-adrenoceptors in cells of the vascular endothelium, and the mechanism whereby β_1-adrenoceptor activation leads to NO release must be speculative. It could be suggested that nitric oxide is produced in response to β_1-induced elevation of cyclic AMP in the endothelial cell, but Kuhn *et al.* (1991) have shown that experimental elevation of cyclic AMP or cyclic GMP does not affect nitric oxide release from bovine cultured aortic endothelial cells. In vascular smooth muscle (Sadoshima *et al.*, 1988), β-adrenoceptor activation led to activation of calcium-activated potassium channels. The consequent hyperpolarization would lead to a fall in cell calcium and, in vascular smooth muscle, to relaxation, but in the endothelial cell hyperpolarization leads to an increase in cell calcium, the stimulus for NO release (Luckhoff & Busse,

1990). Whilst potassium channel activation and hyperpolarization would be an attractive hypothesis for endothelial cell NO release by β-agonists, it is an unlikely explanation for the present findings since β_1-induced relaxation occurred in arteries pre-contracted with a depolarizing potassium solution.

The relaxation of rat mesenteric resistance arteries to forskolin, a direct activator of adenylate cyclase, was not attenuated by L-NAME. In fact, there was a small but non-significant increase in sensitivity to forskolin in the presence of L-NAME. These experiments were caried out to determine whether L-NAME affected cyclic AMP dependent relaxation, since β-agonists are conventionally held to effect relaxation through this pathway in vascular smooth muscle. The results obviate any suggestion of a non-specific inhibitory effect of L-NAME on cyclic AMP-dependent relaxation, but cannot discount an effect of forskolin on the endothelium itself.

Further interesting observations to emerge from this study are the effects of L-NAME and endothelial removal on K^+-induced contraction, both of which increased the sensitivity and maximum response to K^+. This indicates that K^+-induced contraction is modified by release of NO. Modification by NO of NA-induced contraction has been previously reported in arteries from this vascular bed (Dohi *et al.*, 1990), and Martin *et al.* (1986) have come to similar conclusions from experiments in the rat aorta. They concluded that this was due to spontaneous release of NO and was not mediated via α-adrenoceptors. Because adrenergic nerve terminals may liberate noradrenaline when arteries are stimulated with potassium (Vanhoutte *et al.*, 1981) we investigated the effect of α-adrenoceptor blockade together with NO synthase inhibition. Indeed, phentolamine substantially attenuated K^+-induced contraction, but we found that L-NAME still potentiated the K^+ response in the presence of phentolamine. We may also conclude therefore that α-adrenoceptor-induced release of NO is not involved. Neither would it appear that β-adrenoceptor activation of NO is involved, as propranolol did not affect the response to potassium in the presence of phentolamine. It may therefore be the case that NO is released in direct response to tension development; indeed stretch activated calcium channels have been described in endothelial cells (Lansman *et al.*, 1987) and could provide a mechanism for release.

In conclusion, this is the first extensive study of β-adrenoceptor-mediated NO release in resistance arteries. As detailed in the introduction, several studies in conduit arteries have implied a facilitatory role for the endothelium and/or NO in β-adrenoceptor-mediated relaxation although not all are in agreement. This investigation demonstrates that β-agonist-induced relaxation of rat mesenteric resistance arteries can be mediated via endothelial release of NO and that, in this circulation, this is due to β_1-adrenoceptor stimulation. This study therefore suggests that β-adrenoceptors can produce relaxation via pathways other than those mediated via cyclic AMP.

References

AMEZCUA, J.L., PALMER, R.M.J., DE SOUZA, B.M. & MONCADA, S. (1989). Nitric oxide synthesized from L-arginine regulates vascular tone in the coronary circulation of the rabbit. *Br. J. Pharmacol.*, **96**, 418–424.

DE MEY, J.G. & VANHOUTTE, P.M. (1982). Heterogeneous behaviour of the canine arterial and venous wall: importance of the endothelium. *Circ. Res.*, **51**, 439–447.

DOHI, Y., THIEL, M.A., BUHLER, F.R. & LUSCHER, T.F. (1990). Activation of endothelial L-arginine pathway in resistance arteries. Effect of age and hypertension. *Hypertension*, **15**, 170–179.

FUJIMOTO, S., DOHI, Y., AOKI, K., ASANO, M. & MATSUDA, T. (1987). Diminished β-adrenoceptor-mediated relaxation of arteries from spontaneously hypertensive rats before and after development of hypertension. *Eur. J. Pharmacol.*, **136**, 317–324.

GARDINER, S.M., KEMP, P.A. & BENNETT, T. (1991). Effects of N^G-nitro-L-arginine methyl ester on vasodilator responses to acetylcholine, 5'-N-ethylcarboxyaminoadenosine or salbutamol in conscious rats. *Br. J. Pharmacol.*, **103**, 1725–1732.

GRAVES, J. & POSTON, L. (1992). Effect of inhibiting nitric oxide synthase on relaxations to isoprenaline in isolated rat mesenteric resistance arteries. *Br. J. Pharmacol.*, **106**, 23P.

GRAY, D.W. & MARSHALL, I. (1991). Isoprenaline relaxation of rat thoracic aorta is endothelium-dependent, releases nitric oxide and raises cyclic GMP and cyclic AMP. *Br. J. Pharmacol.*, **102**, 125P.

HEESON, B.-J & DE MEY, J.G.R. (1990). Effect of cyclic AMP-affecting agents on contractile reactivity of isolated mesenteric and renal resistance arteries of the rat. *Br. J. Pharmacol.*, **101**, 859–864.

KAMATA, K., MIYATA, N. & KASUYA, Y. (1989). Involvement of endothelial cells in relaxation and contraction responses to isoproterenol in naive and streptozotocin-induced diabetic rats. *J. Pharmacol. Exp. Ther.*, **249**, 890–894.

KONISHI, M. & SU, C. (1983). Role of the endothelium in dilator responses of spontaneously hypertensive rat arteries. *Hypertension*, **5**, 881–886.

KUHN, M., OTTEN, A., FROLICH, J.C. & FORSTERMANN, U. (1991). Endothelial cyclic GMP and cyclic AMP do not regulate the release of endothelium-derived relaxing factor/nitric oxide from bovine aortic endothelial cells. *J. Pharmacol. Exp. Ther.*, **256**, 677–682.

KUKOVETZ, W.R., POCH, G. & HOLZMANN, S. (1981). Cyclic nucleotides and relaxation of vascular smooth muscle. In *Vasodilatation*. eds. Vanhoutte, P.M. & Leuzen, I. pp. 339–353. New York: Raven Press.

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

LUCKHOFF, A. & BUSSE, R. (1990). Calcium influx into endothelial cells and formation of endothelium-derived relaxing factor is controlled by the membrane potential. *Pflügers Arch.*, **416**, 305–311.

MARTIN, W., FURCHGOTT, R.F., VILLANI, G.M. & JOTHIANDAN, D. (1986). Depression of contractile responses in rat aorta by spontaneously released endothelium-derived relaxing factor. *J. Pharmacol. Exp. Ther.*, **237**, 529–538.

MONCADA, S., REES, D.D., SCHULTZ, R. & PALMER, R.M.J. (1991). Development and mechanisms of a specific supersensitivity to nitrovasodilators after inhibition of vascular nitric oxide synthesis in vivo. *Proc. Natl. Acad. Sci. USA*, **88**, 2166–2170.

MOORE, P.K., AL-SWAYEH, O.A., CHONG, N.W.S., EVANS, R.A. & GIBSON, A. (1990). L-N^G-nitroarginine (L-NOARG), a novel, L-arginine reversible inhibitor of endothelium-dependent vasodilatation *in vitro*. *Br. J. Pharmacol.*, **99**, 408–412.

MULVANY, M.J. & HALPERN, W. (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.*, **41**, 19–26.

PALMER, R.M.J., REES, D.D., ASHTON, D.S. & MONCADA, S. (1988). L-arginine is the physiological precursor for the formation of nitric oxide in endothelium dependent relaxation. *Biochem. Biophys. Res. Commun.*, **153**, 1251–1256.

REES, D.D., PALMER, R.M.J., HODSOPN, H.F. & MONCADA, S. (1989). A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. *Br. J. Pharmacol.*, **96**, 418–424.

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

SADOSHIMA, J.-I., AKAIKE, N., KANAIDE, H. & NAKAMURA, M. (1988). Cyclic AMP modulates Ca-activated K channel in cultured smooth muscle cells of rat aortas. *Am. J. Physiol.*, **255**, H754–H759.

TSUJIMOTO, G. & HOFFMAN, B.B. (1985). Desensitization of β-adrenergic receptor-mediated vascular smooth muscle relaxation. *Mol. Pharmacol.*, **27**, 210–217.

VALLANCE, P., COLLIER, J. & MONCADA, S. (1989). Effects of endothelium-derived nitric oxide on peripheral arterial tone in man. *Lancet*, **ii**, 997–999.

VANHOUTTE, P.M., VERBEUREN, T.J. & WEBB, R.C. (1981). Local modulation of adrenergic neuroeffector interaction in the blood vessel wall. *Physiol. Rev.*, **61**, 151–247.

WOOLFSON, R.G. & POSTON, L. (1990). Effect of N^G-monomethyl-L-arginine on endothelium-dependent relaxation of human resistance arteries. *Clin. Sci.*, **79**, 273–278.

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Effects of ATP on cultured smooth muscle cells from rat aorta

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1 Membrane ionic currents provoked by externally applied ATP were studied by patch-clamp techniques in cultured aortic smooth muscle cells of the rat.

2 Using standard bath and pipette solutions and whole-cell voltage-clamp, ATP evoked an inward current when the cell membrane potential was held at − 50 mV and an outward current when the potential was held at 30 mV, with a reversal potential near − 10 mV.

3 Application of ATPγS gave results similar to those obtained with ATP, while adenosine, AMP and α,β-methylene ATP were ineffective. The ATP-activated current was inhibited by suramin, 100 μM.

4 ATP also induced a biphasic rise in internal free Ca levels as shown directly by Fura-2 measurements and by the increase in Ca-dependent K single-channel activity in cell-attached patches.

5 With outward current through K channels blocked by internal Cs and TEA, modification of the ionic composition of bath and pipette solutions revealed that the reversal potential for the ATP-induced whole-cell current closely followed E_{Cl}, the chloride equilibrium potential, and was insensitive to manipulations of the monovalent cation gradient.

6 These results indicate that in rat cultured aortic smooth muscle cells, ATP binding to P_2-purinoceptors produces increases of internal free Ca levels and subsequent activation of both Ca-dependent K and Cl currents.

Keywords: Vascular smooth muscle; ATP; purinoceptor; internal calcium; Ca-dependent K channel; Ca-dependent Cl channel

Introduction

Adenosine 5'-triphosphate (ATP) is considered to be one of the key physiological regulators of vascular tone (for review, see Burnstock & Kennedy, 1986). For example, ATP is an endothelium-dependent vasodilator but is also co-released with noradrenaline at sympathetic nerve terminals present in smooth muscle, thereby directly inducing vasoconstriction. Application of exogenous ATP causes membrane depolarization in many smooth muscle cells, for example, from ear artery (Suzuki, 1985; Benham *et al.*, 1987), vas deferens (Friel, 1988) and urinary bladder (Fuji, 1988). The patch-clamp technique has been used in a variety of smooth muscle preparations to investigate the membrane mechanism(s) underlying the ATP-induced depolarization. In smooth muscle cells from the rabbit ear artery and also in the rat vas deferens, ATP directly activates a Ca-permeable, non-selective cationic channel (Benham & Tsien, 1987a,b; Benham *et al.*, 1987; Nakazawa & Matsuki, 1987; Friel, 1988; Benham, 1989; Declerck *et al.*, 1989). Ca influx through this channel results in increases in internal free Ca levels in normal physiological solutions (Benham, 1989). On the other hand, in smooth muscle cells from the rat portal vein, ATP-induced depolarization was attributed to an increase in Na and Ca conductances together with the subsequent activation of Ca-dependent Cl channels (Karashima & Takata 1979; Xiong *et al.*, 1991). Furthermore, it has also been demonstrated in primary culture of rat aortic smooth muscle cells that ATP activates P_2-purinoceptors and induces the release of Ca from IP_3-sensitive intracellular stores (Phaneuf *et al.*, 1987; Tawada *et al.*, 1987). Similar actions of ATP were described in primary cultures of pig aortic smooth muscle cells, where cell membrane depolarization resulted from the activation of Ca-dependent Cl channels (Droogmans *et al.*, 1991).

Because of these apparent discrepancies concerning the effects of ATP on various types of vascular smooth muscular cells, we investigated the actions of exogenously applied ATP on membrane currents and on internal Ca levels in cultured aortic smooth muscle cells of the rat which retain some characteristics associated with a contractile or differentiated phenotype (Bodin *et al.*, 1987; 1991; Serebryakov & Takeda 1992).

Methods

Cell culture

Primary cultures of smooth muscle cells were prepared from aortae of 10 week-old male Wistar-Kyoto rats as previously described in detail (Bodin *et al.*, 1987; 1991; Serebryakov & Takeda, 1992). Briefly, following isolation and enzymatic digestion with collagenase and elastase of aortic tissue, dispersed cells were plated at 1500–2000 cells cm^{-2} onto collagen-coated glass coverslips in 35 mm Petri dishes in a defined serum-free culture medium (Eagle's MEM/Ham's F-10 (1:1 vol/vol; Eurobio), supplemented with 2% (vol/vol) synthetic serum substitutes Ultroser SF/Ultroser G (3:1 v/v; IBF), 100 u ml^{-1} penicillin G, 0.05 mM vitamin C and 2 mM glutamine) and kept in a 37°C incubator gassed with 5% CO_2/95% air.

Electrophysiology

Single cells were studied usually 7–15 days after plating by standard patch-clamp techniques (Hamill *et al.*, 1981). In some cases, cells from first passages were also used. Patch pipettes were made from thin-wall (Vitrex BRI/E) or thick-

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wall (Hilgenberg) borosilicate glass capillaries and had resistances of 3–8 MΩ when filled with normal internal KCl solution (Table 1). To minimize associated capacitance, pipettes were coated with beeswax and dipped in Sigmacote (Sigma). The bath (≈1.5 ml) was usually superfused with normal external NaCl solution (0.5–1.0 ml min^{-1}) to remove applied compounds and when different external solutions were used. ATP and other agonists (adenosine, ATPγS, α,β-methylene ATP, AMP; Sigma) were locally microperfused (5–10 s) by use of pressurized (≈10 kPa) puffer pipettes located about 50 μm downstream (relative to the bath superfusion) from the cell under study. Control application of external NaCl solution alone had no effect. Suramin (Bayer) was added directly to the bath. The ionic composition of the various external bath and pipette internal solutions used is given in Table 1. Data were recorded on FM or videotape and analysed after appropriate filtering and acquisition with a CED 1401 interface and software from CED and a laboratory personal computer (Goupil 386DX). Compensation for series resistance and capacitance was made using the inbuilt circuitry of the patch-clamp amplifier (List EPC-7). Voltage ramp commands (usually 1 s in duration) were used to obtain quasi steady-state current-voltage (*I-V*) curves with and without agonists. In some cases several responses were averaged, and net agonist *I-V* curves were obtained by subtraction of control *I-V*s from those in the presence of drug. Voltage-dependent Ca channel currents were recorded as previously described (Serebryakov & Takeda, 1992). Data are presented as mean ± s.e.mean for *n* cells where appropriate.

Measurement of intracellular calcium

The effects of ATP on internal free Ca levels were studied in Fura-2 AM loaded cells as previously described (André *et al.*, 1990). Briefly, confluent rat aortic myocytes plated on glass coverslips without collagen were washed of culture medium 3 times with 3 ml of Hank's balanced salt solution (HBSS; Gibco; composition in mM: NaCl 135, KCl 5, $CaCl_2$ 1.25, $MgCl_2$ 2, glucose 10, HEPES 20, pH 7.5). The cells were placed in a quartz spectrophotometric cuvette for 20 min at 37°C in Ca-free HBSS containing 5 μM Fura-2 AM (Molecular Probes). The cells were then washed 3 times with HBSS and left for an additional 10 min at 37°C to ensure complete hydrolysis of the Fura-2 AM. Internal Ca measurements were carried out at 37°C using a SPEX 1681 spectrofluorometer with excitation at 340 and 380 nm and emission measured at 510 nm as previously reported (André *et al.*, 1990). Calculation of internal Ca concentrations was made following Grynkiewicz *et al.* (1985), after calibration using 20 μM ionomycin (Calbiochem) and 5 mM Ca in HBSS followed by 20 mM EGTA.

Results

ATP-induced whole-cell current

The whole-cell membrane response to externally applied ATP (100 μM) is shown in Figure 1a from a single smooth muscle cell. The cell was voltage-clamped at a holding potential of − 50 mV and step depolarizations to 30 mV were given regularly. Standard external NaCl and pipette internal KCl solutions (Table 1) were used. After a latency of a few seconds, a transitory ATP-induced inward current developed at − 50 mV, and simultaneously, the outward current observed at 30 mV increased with much the same time course. Whole-cell, quasi steady-state *I-V* relationships in the absence and at the peak of the ATP-induced response were obtained by applying 1 s long voltage-ramp commands between − 50 and 180 mV (Figure 1b). The control *I-V* was fairly linear, while the *I-V* in the presence of ATP showed outward rectification at potentials more depolarized than 100 mV. The net ATP-activated current was obtained by subtraction of the

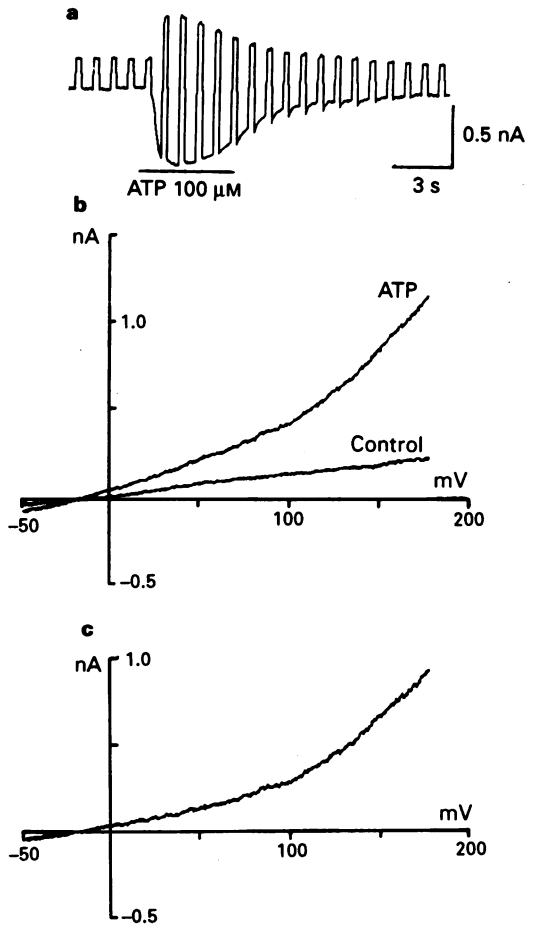


Figure 1 Responses of rat aortic smooth muscle cells under whole-cell voltage-clamp to microperfusion of ATP (100 μM). (a) ATP evoked an inward current at − 50 mV and an outward current at 30 mV. Holding potential was − 50 mV and 80 mV depolarizing voltage pulses 300 ms in duration were applied every 600 ms. (b) Current-voltage (*I-V*) relationships in control and with ATP (100 μM) were obtained using a 1 s voltage ramp from − 50 to 180 mV in another cell. (c) Net ATP-activated current obtained by subtraction of the control response from that measured in the presence of ATP for the cell in (b). The reversal potential of the ATP-activated current was − 18 mV in this cell. Note the outward rectification at positive potentials. (Standard NaCl bath and KCl pipette solutions).

control *I-V* curve from that observed in the presence of ATP (Figure 1c), and had a reversal potential of − 18 mV in this cell. The mean value of the reversal potential of the net ATP-induced current under these conditions was − 10.3 ± 10.1 mV ($n = 5$). Because this value is close to 0 mV, it may be that ATP activates either a non-selective cationic current or a Cl current, as the equilibrium potentials for both Cl and for monovalent cations is ≈0 mV with the solutions used. Furthermore, as ATP is known to cause increases in internal free Ca levels, it is very likely that at least part of the outward current at positive potentials results from activation of Ca-dependent K channels, as suggested from the observed outward rectification at positive potentials. Reversal potentials intermediate between 0 mV and E_K, which was usually − 85 mV, could be explained by simultaneous activation of several conductances.

When calcium was absent from the external solution (Na/0 Ca solution; Table 1) and when 10 mM BAPTA was used instead of 0.1 mM EGTA in the pipette internal KCl solution, ATP-activated currents were observed in 5 of 9 cells tested. In 2 of the 5 cells, responses similar to those in Figure 1 were observed, while in the 3 others, only outward currents at positive potentials were seen (results not shown). It was possible to obtain only a single response to ATP from each

Table 1 Ionic composition (in mM) of the solutions used

	External (bath) solutions							*Internal (pipette) solutions*			
	NaCl	Na/TEA	Na/0 Ca	Na/0 Cl	Low Na	20 Ba	Choline	KCl	Cs/TEA	Cs/Low Cl	Choline
NaCl	140	125	140	–	40	130	–	–	–	–	–
KCl	5	–	5	–	–	–	–	135	–	–	–
CsCl	–	–	–	–	20	–	–	–	140	20	–
Na glutamate	–	–	–	140	–	–	–	–	–	–	–
Cs glutamate	–	–	–	–	–	–	–	–	–	100	–
$CaCl_2$	2	2	–	2	2	–	2	–	–	–	–
$MgCl_2$	2	2	2	2	2	2	2	2	2	2	2
$BaCl_2$	–	–	–	–	–	20	–	–	–	–	–
TEA Cl	–	20	–	–	20	–	–	–	20	20	–
Choline Cl	–	–	–	–	65	–	140	–	–	–	140
Mg ATP	–		–	–	–	–	–	5	5	5	5
Li GTP	–	–	–	–	–	–	–	0.2	0.2	0.2	0.2
EGTA	–	–	–	–	–	–	–	0.1	0.1	0.1	0.1
Glucose	10	10	10	10	10	10	10	–	–	–	–
HEPES	10	10	10	10	10	10	10	10	10	10	10

The pH was adjusted to 7.3 with NaOH and KOH or CsOH for external and internal solutions respectively. In some internal solutions, 10 mM BAPTA was used instead of EGTA.

cell with these solutions. Note that this predominately outward current response was also observed for a few cells under the same experimental conditions as in Figure 1.

Several related nucleotides and adenosine were tested (all at 100 μM, in at least 4 cells) in order to characterize the purinoceptor type responsible for the whole-cell currents evoked by ATP (Figure 2a). With the same voltage-clamp protocol and solutions as in Figure 1a, only ATPγS, a non-hydrolysable ATP analogue, was as effective as ATP, while α,β-methylene ATP, AMP or adenosine had no marked action. The observed rank order of potency was ATP = ATPγS>> AMP = α,β-methylene ATP = adenosine = 0. When 100 μM suramin, a potent blocker of P_2 purinoceptors (Dunn & Blakeley, 1988; Nakazawa *et al.*, 1990a) was added to the bath, the currents activated by 100 μM ATPγS were inhibited partially after 15 min and totally after 20 min (Figure 2b; $n = 3$). A similar blocking action of suramin was found for currents induced by ATP ($n = 2$).

Effect of ATP on single-channel activity

When recordings from cell-attached patches were made from single cells bathed in standard external NaCl solution using a patch pipette containing standard internal KCl solution without MgATP, outward single-channel currents having an elementary conductance of 176 ± 10 pS were observed at pipette potentials depolarized with respect to the cell resting potential (Figure 3b; $n = 4$). These currents had an interpolated reversal potential close to 0 mV (assuming a cell resting potential of ≈ − 45 mV) and are thus probably due to the opening of large-conductance Ca-activated K channels (Bregestovski *et al.*, 1988; 1989). Upon application of 100 μM ATP to the cell, a large increase in the activity of this channel type in the patch was observed (Figure 3a). As many as 10 channels were simultaneously open at the peak of the response compared to basal levels, where 1 or rarely 2 channels were open on average. This increase in activity was transient, with a return to basal levels of channel openings ≈10 s after the end of ATP application (Figure 3c). The activation of Ca-sensitive K channels in the patch following ATP application to the cell exterior would be accounted for by an ATP-induced rise in cellular free Ca levels. The same effect was observed in 4 different cells.

Ionic nature of the ATP-activated current

In standard external NaCl and pipette internal KCl solutions, the reversal potential of ATP-activated current was close to 0 mV (Figure 1c). As indicated above, activation of either a Cl conductance or a non-selective cationic conductance would account for the ATP-induced current. In a first set of experiments, the contribution of monovalent cations was examined, firstly by substituting choline Cl for NaCl and KCl in the external and internal solutions respectively (Figure 4a–c), and secondly, by decreasing external NaCl by replacement with choline Cl and tetraethylammonium (TEA) Cl and by replacing internal KCl by CsCl and TEA Cl (Figure 4d–f). Under both of these conditions, the reversal potentials of the net ATP-activated current was close 0 mV, the expected value of E_{Cl}. When the internal solution contained Cs and TEA to block K currents and the Cl gradient was altered, either by decreasing external or internal Cl by substitution with glutamate (Figure 5), the reversal potentials of the ATP-induced currents followed the expected values of E_{Cl}. In symmetrical Cl solutions with K conductance blocked (Figure 5a), the reversal potential was somewhat closer to 0 mV (− 4.5 ± 4.8 mV; $n = 4$), than in Figure 1. When the external NaCl was replaced by Na glutamate, the reversal potential shifted rightwards to 63.0 ± 2.3 mV ($n = 3$), close to the calculated E_{Cl} of 73 mV (Figure 5b). When the internal Cl was decreased, the observed reversal potential was − 21.4 ± 1.8 mV ($n = 10$) and the calculated E_{Cl} was − 34 mV (Figure 5c). These data argue strongly that the ATP-induced current reversing at ≈0 mV in our normal external and internal solutions arises in large part from an increase in Cl conductance and not from an increase in a non-selective cationic conductance.

Effect of ATP on internal free calcium concentration

ATP (10 μM) produced biphasic increases in internal free calcium levels (Figure 6a), with an initial transient peak followed by a smaller maintained plateau of elevated Ca ($n = 8$ preparations). This second plateau phase depended on the presence of extracellular Ca (not shown), consistent with the commonly held view that the initial peak arises mostly from release of Ca from internal stores while the maintained phase requires Ca influx. Application of 100 μM Mn during the plateau phase caused a rapid decrease in Ca (Figure 6b; $n = 5$), presumably because of Mn permeation through an ATP-associated conductance (cf. Hallam *et al.*, 1988), thereupon resulting in a quenching of the intracellular Fura-2 Ca signal.

Effect of ATP on voltage-activated Ca current

As previously reported (Serebryakov & Takeda, 1992), depolarization-activated whole-cell inward Ba currents having a

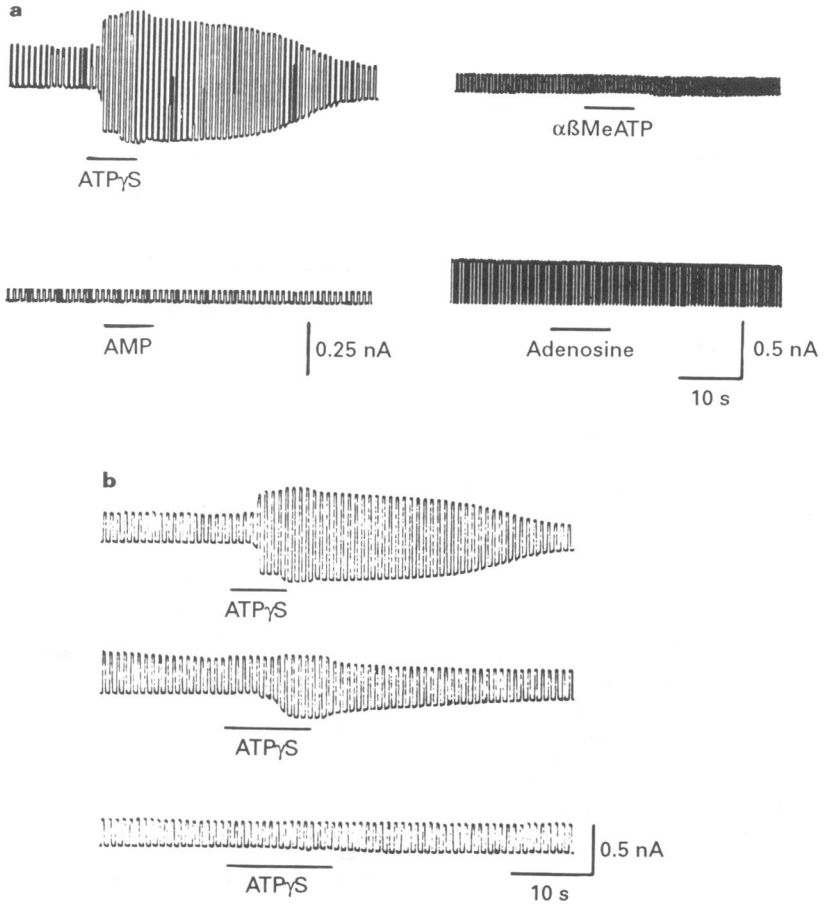
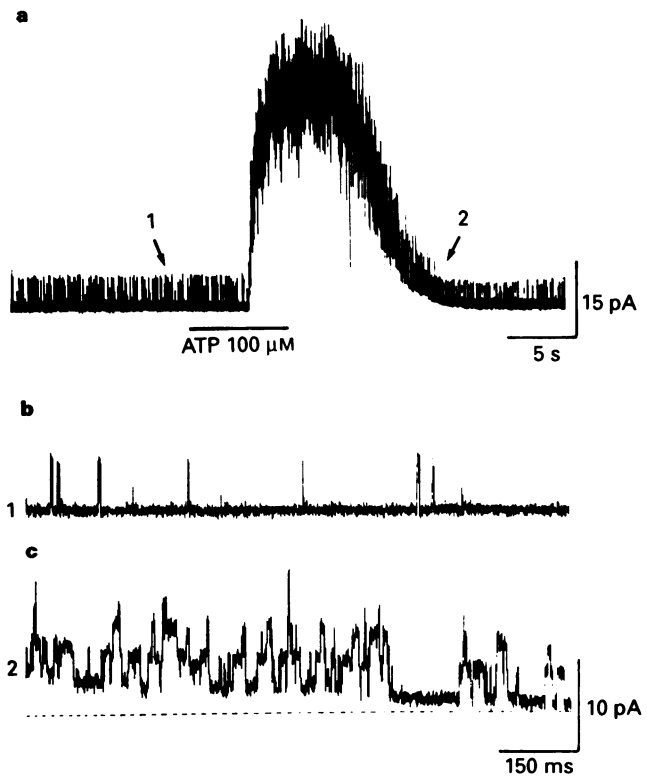


Figure 2 (a) Comparison of whole-cell current responses elicited by ATPγS, α,β-methylene ATP (αβMeATP), AMP and adenosine. Drugs were tested at 100 μM on different cells. Current scale is 0.5 nA except for AMP, where it is 0.25 nA. (b) ATPγS-activated current before (upper trace), 15 min (middle trace) and 20 min (lower trace) after bath application of suramin (100 μM). Voltage-clamp protocol as in Figure 1a. (Standard NaCl bath and KCl pipette solutions).



fast onset and a rapid time course of inactivation were observed with external 20 mM Ba bath and internal Cs/TEA pipette solutions. These currents have properties consistent with those of T-type or low-voltage-activated Ca channels: rapid inactivation during depolarizing steps, high sensitivity to block by Ni but not to Cd and steady-state half-activation and half-inactivation voltages of − 48 and − 41 mV, respectively (Serebryakov & Takeda, 1992). They were essentially unaffected by the dihydropyridine agonists (−)-Bay K 8644 and S(+)-202 791 at 1 μM, while the dihydropyridine antagonist nicardipine was effective at 5 and 10 μM, but not at 0.5 μM (Serebryakov & Takeda, 1992). Maximal Ca channel current amplitude occurred between − 10 and − 20 mV with a − 70 mV holding potential. When 100 μM ATP was applied by local microperfusion, peak Ca channel current was decreased with a rapid onset and in a reversible fashion (Figure 7a). The average decrease was 60.5 ± 13.0% with a recovery to 79.1 ± 12.3% of control values ($n = 5$). Figure 7b shows that 100 μM adenosine, which did not activate any current in our cells (cf. Figure 2a), had negligibly small effects on peak Ca channel current ($n = 3$).

Figure 3 (a) Single Ca-activated K channel activity from a cell-attached patch was greatly increased by microperfusion of the cell with ATP (100 μM). Pipette potential was clamped 60 mV positive to the cell resting potential. (b,c) Higher time resolution traces before and after ATP application for the indicated times in the recording in (a). (Standard NaCl bath solution; MgATP was absent from the KCl pipette solution).

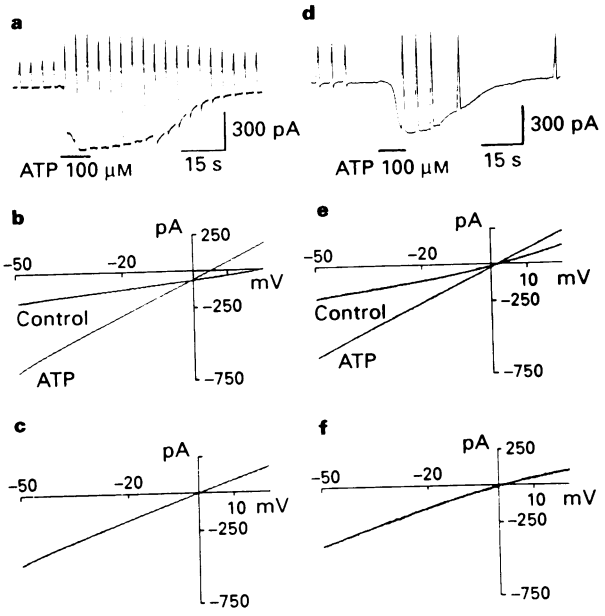


Figure 4 ATP-activated whole-cell current is not sensitive to changes in the monovalent cation gradient. (a–c) Currents evoked by ATP (100 μM) in symmetrical 140 mM choline Cl bath and pipette solutions. (d–f) Currents evoked by ATP (100 μM) in low monovalent cation bath solution and Cs/TEA pipette solution. (a,d) Voltage ramps 3 s long from − 50 to 20 mV were applied periodically. (b,e) *I-V* curves were obtained before and at peak of the ATP-activated current. (c,f) Net ATP-activated *I-V* relationships calculated by subtracting control responses from those in the presence of ATP had reversal potentials very close to 0 mV.

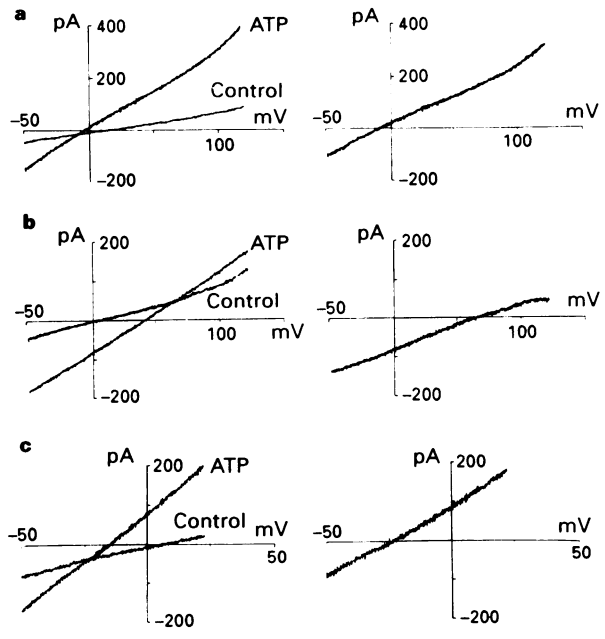


Figure 5 The reversal potential of the ATP-activated current is sensitive to the Cl gradient. Lefthand panels show *I-V* curves in control and in the presence of ATP while righthand panels illustrate the net ATP-activated current. (a) In symmetrical Cl solutions, the ATP-activated current reversed close to − 6 mV. (b) When external Cl was replaced by glutamate, the reversal potential shifted rightwards to 63 mV. (c) When the Cl concentration in pipette solution was decreased to 40 mM, the reversal potential shifted leftwards to − 25 mV. Voltage-clamp protocol as in Figure 4a; range was from − 50 to 120 mV (a,b) or to 20 mV (c). Cs/TEA was present in the pipette solutions to block K currents.

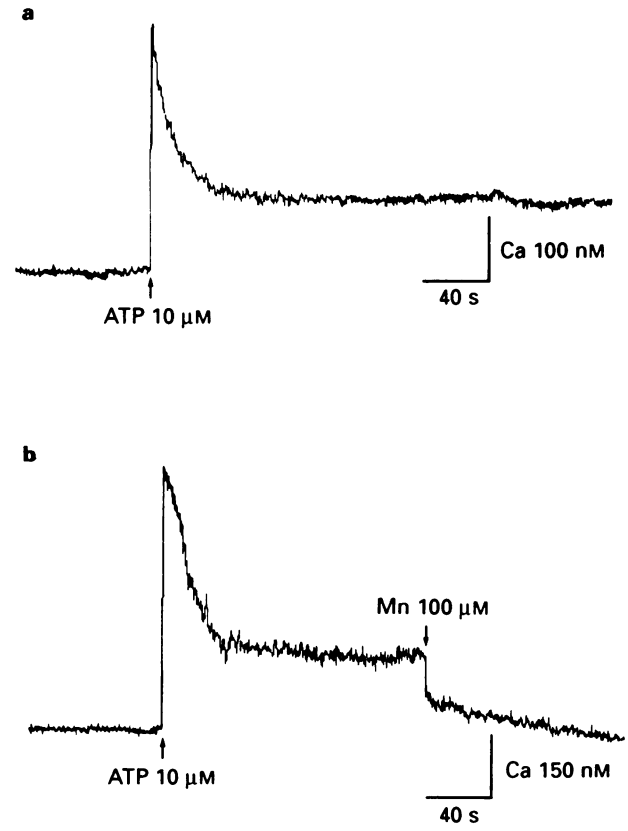


Figure 6 (a) ATP induced a biphasic increase in internal free Ca levels in Fura-2 loaded aortic smooth muscle cell monolayers. Basal Ca concentration initially was 105 nM. (b) The second plateau phase of elevated Ca was rapidly inhibited by Mn (100 μM). Basal Ca concentration initially was 179 nM.

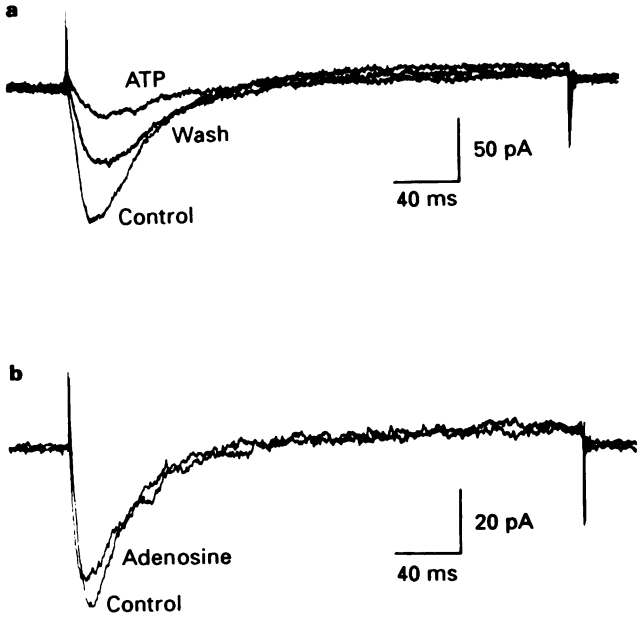


Figure 7 (a) Reversible inhibition of peak inward Ca channel current induced by ATP (100 μM). (b) Adenosine (100 μM) had negligibly small effects on peak Ca channel current. Bath solution contained *inter alia* 20 mM Ba and the pipette solution Cs/TEA. Holding potential − 70 mV, test potential − 10 mV. Note the different current amplitude scale bars in (a) and (b).

Discussion

In the present study, ATP-activated membrane currents were observed using whole-cell patch-clamp techniques in rat aortic myocytes that retain some aspects of a contractile or differentiated phenotype in culture (Serebryakov & Takeda, 1992). A similar action of ATP has already been reported in other mammalian smooth muscle cells (Karashima & Takata, 1979; Benham & Tsien 1987a,b; Benham *et al.*, 1987; Nakazawa & Matsuki, 1987; Friel, 1988; Declerck *et al.*, 1989; Droogmans *et al.*, 1991; Schneider *et al.*, 1991; Xiong *et al.*, 1991). Given that this reponse was elicited by ATP and ATPγS but not by AMP or adenosine, it may be inferred from the classification proposed by Burnstock & Kennedy (1985), that the P_2 subclass of purinoceptors mediates the responses described here. This is supported by the inhibition of the ATP-induced current caused by suramin, a P_2-receptor antagonist. The absence of effect of α,β-methylene ATP is therefore surprising, since it is generally considered to be a P_2-receptor agonist (cf. Burnstock & Kennedy, 1985; Benham & Tsien, 1987b). However, such a finding has already been described in smooth muscle cells from rat vas deferens (Friel, 1988). Similarly, in rat cultured aortic smooth muscle cells, α,β-methylene ATP produced only a relatively weak increase in internal free Ca compared to ATP (Tawada *et al.*, 1987). It was shown that the major part of the increase in internal free Ca was due to Ca release from IP_3-sensitive intracellular stores (Tawada *et al.*, 1987). In the present study, application of ATP caused a transient increase in internal free Ca levels in Fura-2 loaded aortic smooth muscle cells, followed by a sustained plateau phase which depended on the presence of extracellular Ca and which diminished rapidly following application of external Mn, presumably due to quenching of fluorescence following Mn entry (Figure 6). This ATP-induced rise in free Ca could also be demonstrated by the strong increase in activity of Ca-activated K channels recorded from cell-attached patches. The second plateau phase of elevated internal Ca levels is usually taken as evidence for an influx of Ca (e.g. Hallam *et al.*, 1988). Note that in non voltage-clamped cells, this Ca influx may arise secondarily to the opening of voltage-sensitive Ca channels produced by depolarizing ATP-activated current, as previously proposed (Droogmans *et al.*, 1991; Pacaud *et al.*, 1991).

In whole-cell recordings using our standard external and internal solutions, the ATP-activated current had a reversal potential around − 10 mV and approached 0 mV when K conductances were minimized. With Ca-activated K currents blocked by internal Cs and TEA, the remaining ATP-induced current could either be due to a non-selective cationic current or to a chloride current. In smooth muscle cells from rabbit ear artery (Benham *et al.*, 1987; Benham & Tsien, 1987a,b; Benham, 1989; Declerck *et al.*, 1989) and portal vein (Xiong *et al.*, 1991) and in rat vas deferens (Nakazawa & Matsuki, 1987; Friel, 1988), it was shown that ATP activated a non-selective cationic conductance. Furthermore, the ATP-induced inward current in rabbit ear artery cells was insensitive to changes in the Cl gradient and to Cl channel blockers (Benham *et al.*, 1987). However, here we were unable to demonstrate convincingly the involvement of such non-selective cationic channels, since neither replacement of monovalent cations by choline nor a decrease in external monovalent cation concentration diminished the ATP-induced current amplitude or changed its reversal potential. On the other hand, it may well be that there is activation of a non-selective cationic, Ca-permeable channel by ATP, but that both its single-channel conductance and the resulting whole-cell current are very small and thus masked by the activation of other currents. Such a non-selective cationic conductance would provide a pathway for Ca influx, as can be inferred from our Fura-2 experiments. Nevertheless, the effects of changing the Cl concentration gradient on the reversal potential of the ATP-activated current were clearly consistent with the idea that an increase in Cl conductance was produced by ATP. Our results are indeed compatible with those obtained in cultured smooth muscle cells from pig aorta where ATP induced a Ca-activated Cl current (Droogmans *et al.*, 1991). This current would produce membrane depolarization and presumably lead to a Ca entry via voltage-dependent Ca channels in non voltage-clamped cells (as used in the Fura-2 studies and *in situ*). We do not have any explanation as to why there is an apparent absence of ATP-activated non-selective cation currents and presence of Cl currents in our cells, other than the obvious differences between the tissue or species origins of the various smooth muscle cells studied.

Ca-activated Cl currents have been described in several other types of smooth muscle cells, including those from rat anococcygeus (Byrne & Large, 1987), rabbit (Byrne & Large, 1988) and rat (Pacaud *et al.*, 1989) portal vein, guinea-pig mesenteric artery (van Helden, 1988), and rabbit ear artery (Amédée *et al.*, 1990). In these smooth muscle cells, the Ca-activated Cl current was produced notably by adrenoceptor stimulation and consecutively, to a rise in internal free Ca levels. Similarly, in smooth muscle cells from porcine coronary and human mesenteric arteries, endothelin produced an increase in internal Ca and opening of Ca-activated Cl channels (Klöckner & Isenberg, 1991). This current probably contributes in a major fashion to agonist-associated membrane depolarization and cell contraction.

That ATP produces multiple types of current responses has been well-documented in many other cell types (for review see, Bean & Friel, 1990). For example, ATP activates non-selective cationic currents in rat sensory neurones (Krishtal *et al.*, 1983), in chick myotubes, (Hume & Honig, 1986), in PC12 cells, a phaeochromocytoma cell line (Nakazawa *et al.*, 1990a,b), and in cells from mouse (Sasaki & Gallacher, 1990) and rat (Vincent, 1992) lacrimal glands. Recently, ATP was reported to activate depolarizing Ca-dependent Cl currents in FRTL-5 cells, a rat thyroid cell line (Martin, 1992), in a small proportion of C3H101T1/2 cells, a mouse mesodermal stem cell line (Kubo, 1991), and in guinea-pig atrial cells (Matsuura & Ehara, 1992). In FRTL-5 cells, ATP acted via P_2-receptors, as adenosine and AMP were unable to mimic the responses produced by ATP. Based on the extracellular Ca-dependence of the ATP-induced currents, it was further concluded that in these cells, ATP also opens a Ca-influx pathway (Martin, 1992). Interestingly, in guinea-pig atrial cells, an atypical purinoceptor pharmacology was found, with ATP, ADP and adenosine being almost equieffective (Matsuura & Ehara, 1992). In bovine cultured aortic endothelial cells, ATP also produces rises in internal free Ca concomitant with activation of Ca-dependent K and Cl channels (Snetkov *et al.*, 1992).

A reversible decrease in voltage-dependent Ca channel current was caused by ATP but not adenosine. A similar decrease was produced with noradrenaline and adrenaline in the same cells (Serebryakov & Takeda, 1992) and in smooth muscle cells from rabbit ear artery (Droogmans *et al.*, 1987). In smooth muscle cells from the rabbit portal vein (Xiong *et al.*, 1991), a consistent inhibition of voltage-dependent Ca current was observed with 3 mM ATP, while a transient increase in Ca current was seen with 30 μM ATP. It was suggested that the action of ATP on voltage-sensitive Ca channels involved both GTP-binding proteins and a Ca-mediated inactivation process. A similar mechanism involving Ca-mediated inactivation was also proposed by Schneider *et al.* (1991) who described an ATP-induced inhibition of Ca current in myocytes of guinea-pig urinary bladder.

In summary, the data presented here demonstrate that in smooth muscle cells cultured from rat aorta, external ATP acting on P_2-purinoceptors causes increases in internal free Ca levels, leading to activation of Ca-dependent K and Cl channels. No convincing evidence was obtained for an ATP-induced non-selective cationic conductance in our cells. While it has been clearly shown that in other types of smooth muscle cells, a non-selective cationic current is activated by

ATP, it is not at all evident why there should be such heterogeneity especially between vascular myocytes obtained from large vessels.

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References

AMÉDÉE, T., BENHAM, C.D,. BOLTON, T.B., BYRNE, N.G. & LARGE, W.A. (1990). Potassium, chloride and non-selective cation conductances opened by noradrenaline in rabbit ear artery cells. *J. Physiol.*, **423**, 551–568.

ANDRE, P., SCHOTT, C., NEHLIG, H. & STOCLET, J.C. (1990). Aortic smooth muscle cells are able to convert angiotensin I to angiotensin II. *Biochem. Biophys. Res. Commun.*, **173**, 1137–1142.

BEAN, B.P. & FRIEL, D.D. (1990). ATP-activated channels in excitable cells. In *Ion Channels*. ed. Narahashi, T. Vol. 2, pp. 169–203. New York: Plenum.

BENHAM, C.D. (1989). ATP-activated channels gate calcium entry in single smooth muscle cells dissociated from rabbit ear artery. *J. Physiol.*, **419**, 689–701.

BENHAM, C.D., BOLTON, T.B., BYRNE, N.G. & LARGE, W.A. (1987). Action of externally applied adenosine triphosphate on single smooth muscle cells dispersed from rabbit ear artery. *J. Physiol.*, **387**, 473–488.

BENHAM, C.D. & TSIEN, R.W. (1987a). Receptor-operated, Ca^{2+}-permeable channels activated by ATP in arterial smooth muscle. *Nature*, **328**, 275–278.

BENHAM, C.D. & TSIEN, R.W. (1987b). Calcium-permeable channels in vascular smooth muscle: voltage-activated, receptor-operated and leak channels. In *Cell Calcium and the Control of Membrane Transport*. ed. Lazaro, J.M. & Eaton, D.C. *Soc. Gen. Physiol. Series*, Vol. 42, pp. 46–64. New York: Rockefeller University Press.

BODIN, P., RICHARD, S., TRAVO, C., BERTA, P., STOCLET, J.C., PAPIN, S. & TRAVO, P. (1991). Responses of subcultured rat aortic smooth muscle myocytes to vasoactive agents and KCl-induced depolarization. *Am. J. Physiol.*, **260**, C151–C158.

BODIN, P., STOCLET, J.C. & TRAVO, P. (1987). Differences in myocyte subpopulations from segments of the thoracic aorta and their modification with age and hypertension in the rat. *Cell. Tissue Res.*, **247**, 227–231.

BREGESTOVSKI, P.D., BOLOTINA, V.M. & SEREBRYAKOV, V.N. (1989). Fatty acid modifies Ca^{2+}-dependent potassium channel activity in smooth muscle cells from the human aorta. *Proc. R. Soc. B.*, **237**, 259–266.

BREGESTOVSKI, P.D., PRINTSEVA, O.YU., SEREBRYAKOV, V.N., STINNAKRE, J., TURMIN, A. & ZAMOYSKI, V.I. (1988). Comparison of Ca^{2+}-dependent K^+ channels in the membrane of smooth muscle cells isolated from adult and foetal aorta. *Pflügers Arch.*, **413**, 8–13.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptors? *Gen. Pharmacol.*, **16**, 433–440.

BURNSTOCK, G. & KENNEDY, C. (1986). A dual function for adenosine-5'-triphosphate in the regulation of vascular tone. Excitatory cotransmitter with noradrenaline from perivascular nerves and locally released inhibitory intravascular agent. *Circ. Res.*, **58**, 319–330.

BYRNE, N.G. & LARGE, W.A. (1987). Action of noradrenaline on single smooth muscle cells freshly dispersed from the rat anococcygeus muscle. *J. Physiol.*, **389**, 513–525.

BYRNE, N.G. & LARGE, W.A. (1988). Membrane ionic mechanisms activated by noradrenaline in cells isolated from the rabbit portal vein. *J. Physiol.*, **404**, 557–573.

DECLERCK, I., DROOGMANS, G. & CASTEELS, R. (1989). Excitatory agonists and Ca^{2+}-permeable channels in arterial smooth muscle cells. In *Essential Hypertension*, Vol. 2, ed. Aoki, K. pp. 45–56. Berlin: Springer Verlag.

DROOGMANS, G., CALLEWAERT, G., DECLERCK, I. & CASTEELS, R. (1991). ATP-induced Ca^{2+} release and Cl^- current in cultured smooth muscle cells from pig aorta. *J. Physiol.*, **440**, 623–634.

DROOGMANS, G., DECLERCK, I. & CASTEELS, R. (1987). Effect of adrenergic agonists on Ca^{2+}-channel currents in single vascular smooth muscle cells. *Pflügers Arch.*, **409**, 7–12.

DUNN, P.M. & BLAKELY, A.G.H. (1988). Suramin: a reversible P_2-purinoceptor antagonist in the mouse vas deferens. *Br. J. Pharmacol.*, **93**, 243–245.

FRIEL, D.D. (1988). An ATP-sensitve conductance in single smooth muscle cells from rat vas deferens. *J. Physiol.*, **401**, 361–380.

FUJI, K. (1988). Evidence for adenosine triphosphate as an excitatory transmitter in guinea-pig rabbit and pig urinary bladder. *J. Physiol.*, **404**, 39–52.

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

HALLAM, T.J., JACOB, R. & MERRITT, J.E. (1988). Evidence that agonists stimulate bivalent-cation influx into human endothelial cells. *Biochem. J,*. **255**, 179–184.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.*, **391**, 85–100.

HUME, R.I. & HONIG, M.G. (1986). Excitatory action of ATP on embryonic chick muscle. *J. Neurosci,*. **6**, 681–690.

KARASHIMA, T. & TAKATA, Y. (1979). The effect of ATP related compounds on the electrical activity of the rat portal vein. *Gen. Pharmacol.*, **10**, 477–487.

KLOCKNER, U. & ISENBERG, G. (1991). Endothelin depolarizes myocytes from porcine coronary and human mesenteric arteries through a Ca-activated chloride current. *Pflügers Arch.*, **418**, 168–175.

KRISHTAL, O.A., MARCHENKO, S.M. & PIDOPLICHKO, V.I. (1983). Receptor for ATP in the membrane of mammalian sensory neurones. *Neurosci. Lett.*, **35**, 41–45.

KUBO, Y. (1991). Properties of ionic currents induced by external ATP in a mouse mesodermal stem cell line. *J. Physiol.*, **442**, 691–710.

MARTIN, S.C. (1992). ATP activates a Ca^{2+}-dependent Cl^- current in the rat thyroid cell line, FRTL-5. *J. Membr. Biol,*. **125**, 243–253.

MATSUURA, H. & EHARA, T. (1992). Activation of chloride current by purinergic stimulation in guinea pig heart cells. *Circ. Res.*, **70**, 851–855.

NAKAZAWA, K., FUJIMORI, K., TAKANAKA, A. & INOUE, K. (1990a). Reversible and selective antagonism by suramin of ATP-activated inward current in PC12 phaeochromocytoma cells. *Br. J. Pharmacol.*, **101**, 224–226.

NAKAZAWA, K., FUJIMORI, K., TAKANAKA, A. & INOUE, K. (1990b). An ATP-activated conductance in pheochromocytoma cells and its suppression by extracellular calcium. *J. Physiol.*, **428**, 257–272.

NAKAZAWA, K. & MATSUKI, N. (1987). Adensoine triphosphate-activated inward current in isolated smooth muscle cells from rat vas deferens. *Pflügers Arch.*, **409**, 644–646.

PACAUD, P., LOIRAND, G., BARON, A., MIRONNEAU, C. & MIRONNEAU, J. (1991). Ca^{2+} channel activation and membrane depolarization mediated by Cl^- channels in response to noradrenaline in vascular myocytes. *Br. J. Pharmacol.*, **104**, 1000–1006.

PACAUD, P., LOIRAND, G., MIRONNEAU, C. & MIRONNEAU, J. (1989). Noradrenaline activates a calcium-activated chloride conductance and increases the voltage-dependent calcium current in cultured single cells of rat portal vein. *Br. J. Pharmacol.*, **97**, 139–146.

PHANEUF, B., BERTA, P., CASANOVA, J. & CAVADORE, J.C. (1987). ATP stimulates inositol phosphate accumulation and calcium mobilisation in primary culture of rat aortic myocytes. *Biochem. Biophys. Res. Commun.*, **143**, 454–460.

SASAKI, T. & GALLACHER, D.V. (1990). Extracellular ATP activates receptor-operated cation channels in mouse lacrimal acinar cell to promote calcium influx in the absence of phosphoinositide metabolism. *FEBS. Lett.*, **264**, 130–134.

SCHNEIDER, P., HOPP, H.H. & ISENBERG, G. (1991). Ca^{2+} influx through ATP-gated channels increments $[Ca^{2+}]i$ and inactivates I_{Ca} in myocytes from guinea-pig urinary bladder. *J. Physiol.*, **440**, 479–496.

SEREBRYAKOV, V. & TAKEDA, K. (1992). Voltage-dependent calcium current and the effects of adrenergic modulation in rat aortic smooth muscle cells. *Phil. Trans. R. Soc. B.*, **337**, 37–47.

SNETKOV, V.A., DUCHENE, A.D. & TAKEDA, K. (1992). Actions of external ATP on endothelial cells cultured from bovine aorta. *J. Physiol.*, abstr. (in press).

SUZUKI, H. (1985). Electrical responses of smooth muscle cells of the rabbit ear artery to adenosine triphosphate. *J. Physiol.*, **359**, 401–415.

TAWADA, Y., FURUKAWA, K.-I. & SHIGEKAWA, M. (1987). ATP-induced calcium transient in cultured aortic smooth muscle cells. *J. Biochem.*, **102**, 1499–1509.

VAN HELDEN, D.F. (1988). An alpha-adrenoceptopr-mediated chloride conductance in mesenteric veins of the guinea-pig. *J. Physiol.*, **401**, 489–501.

VINCENT, P. (1992). Cationic channels sensitive to extracellular ATP in rat lacrimal cells. *J. Physiol.*, **449**, 313–332.

XIONG, Z., KITAMURA, K. & KURIYAMA, H. (1991). ATP activates cationic currents and modulates the calcium current through GTP-binding protein in rabbit portal vein. *J. Physiol.*, **440**, 143–165.

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Ruthenium red, but not capsazepine reduces plasma extravasation by cigarette smoke in rat airways

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1 Cigarette smoke increases vascular permeability in rat airways by activating release of tachykinin from capsaicin-sensitive sensory nerves. However, the mechanism by which cigarette smoke induces secretion of sensory neuropeptides is unknown. Here we hypothesized that cigarette smoke activates sensory nerve endings via a mechanism similar to that of capsaicin.

2 We studied the effects of ruthenium red, an inorganic dye which blocks the cation influx promoted by capsaicin and of the capsaicin antagonist capsazepine on the increase in vascular permeability produced by cigarette smoke, capsaicin, hypertonic saline and substance P in the trachea of pentobarbitone anaesthetized rats. We also investigated the ability of cigarette smoke to desensitize sensory nerve fibres.

3 Ruthenium red (10 mM) by aerosol blocked the increase in vascular permeability induced by capsaicin (0.5 μM) and reduced the response to cigarette smoke (5 puffs) but did not affect responses evoked by hypertonic saline (7.2%) or by substance P (10 μM) (all given by aerosol). Aerosols of capsazepine (0.1 mM) prevented extravasation by capsaicin, but did not inhibit response to cigarette smoke, hypertonic saline or substance P. Finally, pre-exposure to a high dose of cigarette smoke (10 puffs) prevented the extravasation caused by cigarette smoke (5 puffs) itself and by intravenous capsaicin (150 $\mu g\,kg^{-1}$), but not that by intravenous substance P (10 $nmol\,kg^{-1}$).

4 The present results show that cigarette smoke: (a) increases vascular permeability in the rat airways by a mechanism that is not antagonized by capsazepine, and is partially sensitive to rutheniun red; (b) produces desensitization of capsaicin-sensitive sensory nerves. We propose that chemical(s) contained in or agent(s) produced by cigarette smoke in the airways share partially a common pathway with capsaicin to activate peptide release from capsaicin-sensitive sensory nerves, but do not bind to the putative 'capsaicin receptor'.

Keywords: Cigarette smoke; ruthenium red; capsazepine; capsaicin; tachykinins; substance P; plasma extravasation; trachea; sensory nerves; hypertonic saline

Introduction

Inhalation of cigarette smoke produced irritation of the airways and an inflammatory response which includes vascular extravasation. Because capsaicin desensitization (Lundberg & Saria, 1983) or pretreatment with different NK_1 receptor antagonists (Delay-Goyet & Lundberg, 1991; Piedimonte *et al.*, 1992) inhibits plasma exudation by cigarette smoke completely, it is concluded that this response is due to tachykinin release from capsaicin-sensitive nerve endings. Cigarette smoke includes particulate and vapour phases and contains a variety of irritants (Stedman, 1968). The ability of cigarette smoke to increase vascular permeability is apparently due to the constituents of the vapour phase, and not to particulate materials or nicotine (Lundberg *et al.*, 1983). There is indirect evidence that irritants contained in the vapour phase of cigarette smoke affect capsaicin-sensitive sensory neurones. For instance, the inhalation of acrolein reduces the content of substance P (SP) and calcitonin gene-related peptide in the sensory nerves of rat airways (Springall *et al.*, 1990). However, it is not known by which mechanism cigarette smoke promotes the release of neuropeptides from the peripheral endings of capsaicin-sensitive sensory neurones, and thus produces inflammatory responses in the airways.

Capsaicin exerts two selective actions on sensory nerves. First, it produces excitation coupled to Ca^{2+}-dependent release of neuropeptides by the opening of a channel which allows Ca^{2+} and Na^{+} to enter the nerve (Marsh *et al.*, 1987; Wood *et al.*, 1988). Second, exposure to high concentrations of capsaicin makes sensory nerves no longer excitable by capsaicin itself and by several other stimuli (capsaicin desensitization) (Holzer, 1991). Ruthenium red, an inorganic dye with Ca^{2+} entry blocking properties, inhibits the action of capsaicin on sensory nerves by blocking the cation channel opened by this drug (Wood *et al.*, 1988; Maggi *et al.*, 1989; Dray *et al.*, 1990; Amann & Maggi, 1991). Capsazepine, a newly developed analogue of capsaicin, inhibits responses to capsaicin without exhibiting any excitatory activity on sensory nerves (Bevan *et al.*, 1991; Dickenson & Dray, 1991; Urban & Dray, 1991). Capsazepine in the guinea-pig airways *in vitro* has been shown to behave as a non-competitive antagonist of capsaicin (Belvisi *et al.*, 1992).

Here we have investigated whether cigarette smoke promotes release of sensory neuropeptides by sharing the pathway activated by capsaicin. To test this hypothesis, we have studied the effect of ruthenium red and capsazepine on the plasma extravasation evoked by cigarette smoke, and have compared responses obtained with cigarette smoke with the effects resulting from administration of capsaicin. The effect of cigarette smoke was also compared with effects produced by hypertonic saline and SP. Hypertonic saline was chosen because it increases vascular permeability in rat airways by releasing tachykinins from sensory nerves (Umeno *et al.*, 1990) and because neuropeptide release by hypertonic saline is independent of the activation of the cation channel operated by capsaicin (Tramontana *et al.*, 1991; Del Bianco *et al.*,

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1992). SP was chosen because it increases vascular permeability by acting directly at the vascular level without the involvment of a neuronal mechanism. Finally we have tested the possibility that cigarette smoke produces desensitization of the nerve fibres.

Methods

General

We used pathogen-free F344 rats (10–12 wk of age) from Simonsen Laboratories, Inc., Gilroy, CA, U.S.A., weighing 200–300 g. On the day of the experiments, the rats were anaesthetized with sodium pentobarbitone (65 mg kg^{-1}, i.p.; Anatomy products Co., Arcadia, CA, U.S.A.). Vascular permeability was quantified by injection of the albumin tracer, Evans blue dye (3% (w/v) solution in 0.9% NaCl; Polysciences Inc., Warrington, PA, U.S.A.; 30 mg kg^{-1}, i.v. for 5 s) at the beginning of each challenge. Five min later, the chest was opened, the aorta cannulated through the left ventricle, and the circulation was perfused for 2 min with PBS at pH 5. In experiments in which 10 puffs of cigarette smoke were used, the vascular perfusion was performed at the end of the 10 min exposure. The trachea was dissected and opened along the ventral midline, blotted, weighed, and then incubated overnight at 60°C in 3 ml of formamide (Fisher Scientific, Santa Clara, CA, U.S.A.). The extravasation of the dye-labelled macromolecules was assessed by measuring the optical density of the formamide extracts at a wavelength of 620 nm with a spectrophotometer (UV160U, Shimatzu, Columbia, MD, U.S.A.). The amount of Evans blue dye extravasated in the tissue, expressed in ng mg^{-1} of wet weight, was interpolated from a standard curve of Evans blue dye concentrations.

Aerosol administration

Rats were ventilated artificially at a frequency of 65 breaths min^{-1} and at a tidal volume of 3 ml, using a constant-volume ventilator (Harvard Apparatus, Millis, MA, U.S.A.) connected to a tracheal cannula (Umeno *et al.*, 1990). The cannula having a conical tip with an inner diameter of 1 mm, was inserted for 1–3 mm between the cricoid cartilage and the first tracheal ring. Aerosols of drugs were generated by ultrasonic nebulizers (Pulmo-Sonic 25, De Vilbiss, Somerset, PA, U.S.A.) and delivered to the airways through the inspiratory limb of the ventilator. In the experiments with cigarette smoke, rats were exposed to the smoke of a reference cigarette (2R1 cigarettes prepared by the University of Kentucky Tobacco and Health Research) containing 32.9 mg tar and 2.19 mg nicotine. Each puff of smoke was collected into a syringe (1 puff = 30 ml) and then was expelled over 30 s into the tracheal cannula, followed by a 30 s interval of ventilation with room air (Dusser *et al.*, 1989).

To test the effect of ruthenium red on the extravasation produced by the different agents, the compound or its vehicle (0.9% saline) was given over 2 min, 20 min before the delivery of each stimulus. The aerosol of capsazepine or its vehicle (1% (v/v) ethanol in 0.9% saline) was administered over 2 min, 5 min before the challenge with the different stimuli. Aerosols of capsaicin (dissolved in ethanol to 1 mM concentration and then diluted in 0.9% saline), its vehicle (0.2% ethanol (v/v)), 7.2% NaCl or SP were delivered over 2 min.

Desensitization procedure

To study desensitization produced by cigarette smoke on the extravasation evoked by subsequently administered stimuli, rats received air (5 puffs) or cigarette smoke (5 or 10 puffs). Twenty min later the different stimuli were delivered. These consisted of cigarette smoke (10 puffs), capsaicin (150 μg kg^{-1}, i.v., dissolved in 0.9% saline containing 10% (v/v) ethanol, 10% (v/v) Tween 80 and further diluted 10 times in 0.9% saline) or SP (10 nmol kg^{-1}, i.v. diluted in 0.9% saline). It may be presumed that capsaicin and SP by i.v. administration allowed to obtain a more uniform drug distribution in the trachea than the aerosol administration. Therefore, this experimental condition was chosen to test the ability of cigarette smoke to desensitize sensory nerves completely in this tissue.

Drugs

All of the drugs used in this study were injected in a volume of 1 mg kg^{-1} of body weight. Substance P was purchased from Peninsula Lab. (Belmont, CA, U.S.A.). Ruthenium red and capsaicin were purchased from Sigma (St. Louis, MO, U.S.A.). Capsazepine was a kind gift of Dr S. Bevan (Sandoz Institute for Medical Research, London, U.K.).

Statistical analysis

Data are expressed as ng of Evans blue dye per mg of tissue and are the mean ± standard error of the mean (s.e.mean). Mean values of spectrophotometric measurements of Evans blue dye extravasation were analysed by one-way analysis of variance (ANOVA). Comparisons between means were performed by the Dunnett's multiple range test. In the case of comparisons between two groups, the statistical analysis was performed by means of the Student's t test. Differences having a P value <0.05 were considered significant.

Results

General

Exposure of the rat airways to room air for 5 min produced an extravasation of Evans blue dye that was 13 ± 2.5 ng mg^{-1} ($n = 4$). Exposure to cigarette smoke produced a dose-related increase in tracheal vascular extravasation which reached a maximum after 10 puffs (195 ± 15 ng mg^{-1}, $n = 5$). For the following experiments a dose of 5 puffs was chosen because this dose resulted in a submaximal effect (Figure 1).

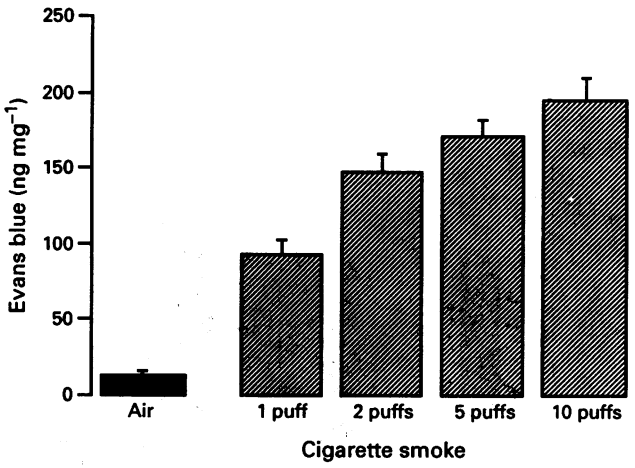


Figure 1 Effect of exposure to different numbers of puffs of cigarette smoke on the extravasation of Evans blue dye in the trachea of pentobarbitone anaesthetized rats. 2R1 cigarettes prepared by the University of Kentucky Tobacco and Health Research containing 32.9 mg tar and 2.19 mg nicotine were used. Each puff of smoke was collected into a syringe (1 puff = 30 ml) and then was expelled over 30 s into the tracheal cannula, followed by a 30 s interval of ventilation with room air. Each column is the mean ± s.e.mean (vertical bar) of at least 5 experiments.

Then, doses of capsaicin, hypertonic saline and SP that gave amounts of Evans blue dye extravasation comparable to that obtained with 5 puffs of cigarette smoke were selected. Ruthenium red aerosol given over 2 min, 20 min before the stimulus, reduced the dye extravasation evoked by aerosolized capsaicin (0.5 μM, for 2 min) in a dose-dependent manner, with almost complete inhibition at 10 mM (29 ± 5 ng mg^{-1}, $n = 5$) (Figure 2). Pretreatment with ruthenium red vehicle (0.9% saline, for 2 min, 20 min before) and challenge with 0.9% saline (for 2 min) gave an amount of Evans blue in the tissue that was 15.5 ± 3 ng mg^{-1} ($n = 5$).

Effect of ruthenium red and capsazepine

Next we compared the effects of 10 mM ruthenium red on the increase in vascular extravasation evoked by cigarette smoke, hypertonic saline and SP. Ruthenium red reduced extravasation evoked by cigarette smoke (5 puffs for 5 min) by 45%, and it did not affect the response to hypertonic saline (7.2% for 2 min) and slightly but significantly enhanced by 29% the effect of SP (10 μM for 2 min) (Figure 3).

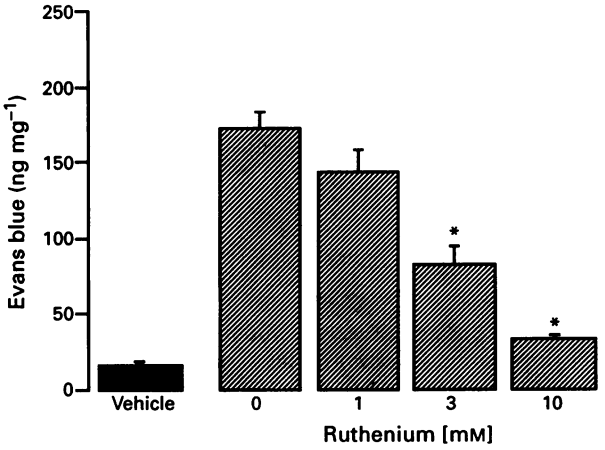


Figure 2 Effect of different concentrations of aerosolized ruthenium red (for 2 min, 20 min before capsaicin challenge) or 0.9% saline (0) on the Evans blue dye extravasation evoked by capsaicin aerosol (0.5 μM, for 2 min) in the trachea of pentobarbitone anaesthetized rats. Solid column (vehicle) indicates the extravasation produced by capsaicin vehicle delivered 20 min after 2 min pretreatment with 0.9% saline. Each column is the mean ± s.e.mean (vertical bars) of at least 5 experiments. $^*P < 0.01$.

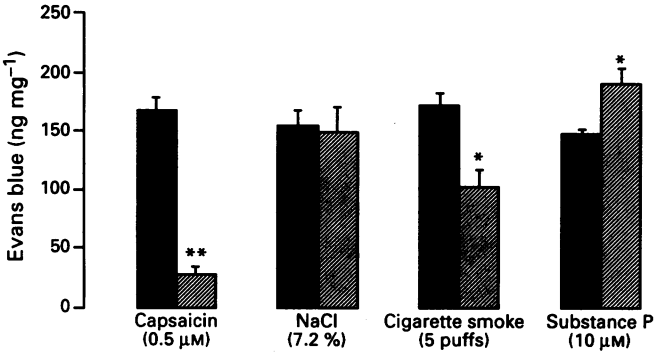


Figure 3 Effect of pretreatment with aerosolized ruthenium red (10 nM, for 2 min, 20 min before) (hatched columns) on the Evans blue dye extravasation evoked by aerosol administration of hypertonic saline (2 min) or substance P (2 min) and by delivery of cigarette smoke (5 puffs over 5 min) in the trachea of pentobarbitone anaesthetized rats. Solid columns = responses in absence of ruthenium red. Each column is the mean ± s.e.mean (vertical bars) of at least 5 experiments. $^*P < 0.01$, $^{**}P < 0.001$.

In another series of experiments, we found that capsazepine by aerosol (administered for 2 min, 5 min before the stimulus) inhibited the Evans blue dye extravasation evoked by aerosolized capsaicin (0.5 μM for 2 min) in a dose-dependent manner. The maximal inhibition was obtained with a concentration of 100 μM (Figure 4). Capsazepine aerosol (100 μM for 2 min) produced a slight increase in vascular extravasation (27 ± 5 ng mg^{-1}, $n = 4$) that was not significantly different as compared to the effect evoked by its vehicle (1% ethanol in 0.9% saline, administered for 2 min, 5 min before; 25 ± 4 ng mg^{-1}, $n = 4$). Then we tested the effect of this concentration of capsazepine on the responses produced by cigarette smoke and hypertonic saline: capsazepine did not affect the plasma extravasation induced by 5 puffs of cigarette smoke or by 7.2% NaCl (Figure 4).

Desensitization to cigarette smoke

Finally, we studied the effect of pre-exposure to cigarette smoke on the response to cigarette smoke itself and to intravenous capsaicin or SP. The response to cigarette smoke (5 puffs) was reduced by pre-exposure to 5 puffs of cigarette smoke (20 min before) by 58%. After pre-exposure to 10 puffs, the response to cigarette smoke was virtually abolished (2% of control). The response to capsaicin (150 μg kg^{-1}) was inhibited by pre-exposure to 5 puffs of cigarette smoke to 57% of that found in controls, and the response was markedly reduced by pre-exposure to 10 puffs of cigarette smoke (15% of control). The increase in Evans blue dye by SP (10 nmol kg^{-1}, i.v.) was not altered by pre-exposure to either 5 or 10 puffs of cigarette smoke (Figure 5).

Discussion

The present studies show that cigarette smoke-induced plasma extravasation is not sensitive to capsazepine, but it is partly reduced by ruthenium red. In the same series of experiments we found that ruthenium red or capsazepine did not affect Evans blue dye extravasation by hypertonic saline and ruthenium red had no influence on the response to SP. Finally, pre-exposure to cigarette smoke prevented the vascular extravasation evoked by capsaicin, but not that induced by SP.

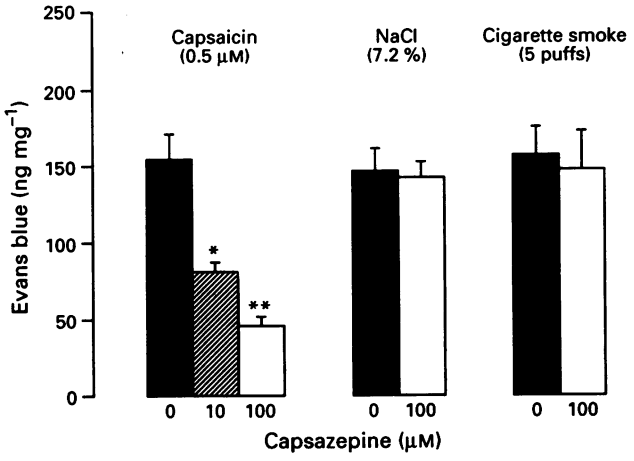


Figure 4 Effect of pretreatment with capsazepine aerosol (100 μM, open columns; 10 μM, hatched columns, for 2 min, 5 min before the challenge) or its vehicle (0.1% ethanol, for 2 min, 5 min before the challenge, solid columns) on the Evans blue dye extravasation evoked by aerosols of capsaicin (for 2 min) or hypertonic saline (for 2 min) and delivery of cigarette smoke (for 5 min) in the trachea of pentobarbitone anaesthetized rats. Each column is the mean ± s.e. mean (vertical bars) of at least 5 experiments. $^*P < 0.01$, $^{**}P < 0.001$.

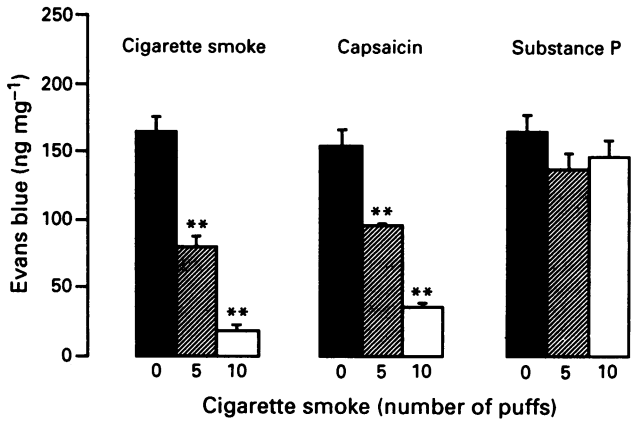


Figure 5 Effect of pretreatment with room air (over 5 min, solid columns), 5 puffs (over 5 min, hatched columns) or 10 puffs (over 10 min, open columns) of cigarette smoke on the Evans blue dye extravasation evoked by cigarette smoke (5 puffs over 5 min), capsaicin (150 $\mu g\,kg^{-1}$, i.v.) or substance P (10 $nmol\,kg^{-1}$, i.v.) in the trachea of pentobarbitone anaesthetized rats. Each column is the mean ± s.e.mean (vertical bars) of 6 experiments. $^{**}P<0.001$.

Ruthenium red is an effective functional antagonist of the action exerted by capsaicin on sensory nerves (Amann & Maggi, 1991). Ruthenium red prevents the release of sensory neuropeptides evoked by capsaicin but not by other stimuli such as high K^+ or bradykinin (Geppetti *et al.*, 1990; 1991). However, the use of ruthenium red for *in vivo* studies has been hampered by its toxicity (Amann *et al.*, 1990). We circumvented this problem by delivering the drug by aerosol. This mode of administration gave an effective local concentration in the airway without any apparent systemic toxic action; no rat died after the administration of this drug. Aerosol administration should minimize possible cardiovascular effects of ruthenium red, that theoretically could alter the response to the subsequent stimuli. However, cardiovascular effects by aerosolized ruthenium red, if any, does not seem to have a relevant action on subsequent plasma extravasation, because ruthenium red pretreatment selectively abolished dye extravasation by capsaicin, but not those by hypertonic saline or SP. These same considerations, discussed for ruthenium red, apply also to capsazepine (see below).

The observation that ruthenium red actually increases the response to SP shows that despite the high concentration of ruthenium red in the aerosol the amount of drug delivered over 2 min did not reduce the ability of postcapillary venules to respond to SP. Potentiation of bronchoconstriction by SP or by neurokinin A has been observed recently following subcutaneous administration of ruthenium red (Ballati *et al.*, 1992). These authors proposed that blockade of adrenergic outflow by ruthenium red could account for the observed potentiation. However, in the present study the reason why ruthenium red increases the response to SP remains to be defined.

Another drawback in the use of ruthenium red is that its selectivity for the capsaicin-activated channel is restricted to a narrow range of concentrations (Maggi *et al.*, 1988; 1989; Amann & Maggi, 1991). For this reason we compared the action of ruthenium red on the extravasation evoked by capsaicin with that exerted on the extravasation evoked by hypertonic saline. Hypertonic saline and capsaicin were both delivered by aerosol; both cause vascular extravasation by releasing tachykinins from sensory nerves (Umeno *et al.*, 1990; Piedimonte *et al.*, 1992). However, we found previously that hypertonic saline releases sensory neuropeptides by a mechanism that is insensitive to ruthenium red (Del Bianco *et al.*, 1992), in contrast with capsaicin. Because ruthenium red did not affect the dye extravasation induced by hypertonic saline but completely blocked the increase in vascular permeability evoked by capsaicin, it may be inferred that in the present experimental conditions its action is selectively directed at blocking the capsaicin-operated channel. As a consequence, the partial reduction produced by ruthenium red on cigarette smoke-evoked vascular extravasation suggests that cigarette smoke activates a mechanism, at least in part, similar to that stimulated by capsaicin.

Chemical(s) contained in cigarette smoke or agent(s) produced by contact of cigarette smoke with airway tissue could activate the capsaicin-operated mechanism by binding to the 'putative' receptor for capsaicin (Szallasi & Blunberg, 1990). To test this hypothesis, we used capsazepine, a recently developed compound which is devoid of any excitatory property and has been shown to block the action of capsaicin in different models, most probably by preventing the binding of capsaicin to its receptor (Bevan *et al.*, 1991; Dickenson & Dray, 1991; Urban & Dray, 1991). The finding that capsazepine blocked the increase in plasma extravasation evoked by capsaicin and did not affect the action of hypertonic saline indicates that capsazepine selectively antagonized the action of capsaicin. Because capsazepine did not reduce the response to cigarette smoke, it may be concluded that compound(s) contained in or produced by cigarette smoke, and which evokes release of sensory neuropeptides, exert this action without binding to the putative capsaicin receptor.

Exposure to a high dose of cigarette smoke prevented the action of a subsequent administration of cigarette smoke, thus indicating desensitization. This was not apparently due to an action of cigarette smoke at the vascular level because the response to intravenous SP was completely unaffected by such a treatment. However, protracted exposure to cigarette smoke impaired the ability of intravenous capsaicin to cause vascular extravasation in the airways, suggesting that the target of the desensitizing action of cigarette smoke is the sensory nerve. This finding is consistent with the observation that exposure to acrolein, an aldheyde contained in cigarette smoke, depleted the tissue content of sensory neuropeptides in the rat airways (Springall *et al.*, 1990). Several hypotheses on the modality by which capsaicin induces desensitization have been proposed: capsaicin may deplete the pool of releasable peptides from the sensory nerve endings (Hakanson *et al.*, 1987). It may, at low doses, exert selective desensitization. Selectivity is suggested by the observation that the nerve fibre no longer responds to capsaicin but is still excitable by other stimuli (Dray *et al.*, 1989). Finally, at higher doses, capsaicin exerts neurotoxic effects that lead to irreversible neuronal damage (Bevan & Szolcsanyi, 1990). Even though the present study clearly shows that cigarette smoke, like capsaicin, provokes sensory nerve desensitization, it does not provide information to indicate which of these different mechanisms proposed for explaining the desensitizing action of capsaicin is exerted by cigarette smoke.

Various endogenous agents have been reported to activate the 'efferent' function of capsaicin-sensitive sensory nerves by a ruthenium red-sensitive mechanism (Geppetti *et al.*, 1991; Mapp *et al.*, 1991). However, further studies are necessary to establish whether the compound(s) responsible for the ruthenium red-sensitive component of cigarette smoke action on sensory nerves is a chemical contained in cigarette smoke or an agent produced by cigarette smoke after contact with the airway tissue.

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References

AMANN, R., DONNERER, J., MAGGI, C.A., GIULIANI, S., DEL BIANCO, E., WEIHE, E. & LEMBECK, F. (1990). Capsaicin desensitization in vivo is inhibited by ruthenium red. *Eur. J. Pharmacol.*, **186**, 169–175.

AMANN, R. & MAGGI, C.A. (1991). Ruthenium red as a capsaicin antagonist. *Life Sci.*, **49**, 849–856.

BALLATI, L., MAGGI, C.A & EVANGELISTA, S. (1992). Effect of ruthenium red on the bronchoconstriction induced by capsaicin and by selective tachykinin receptor agonists in anaesthetized guinea-pig. *J. Auton. Pharmacol.*, **12**, 369–376.

BELVISI, M.G., MIRA, M., STRETTON, D. & BARNES, P.J. (1992). Capsazepine as a selective antagonist of capsaicin-induced activation of C-fibres in guinea-pig bronchi. *Eur. J. Pharmacol.*, **215**, 341–344.

BEVAN, S. & SZOLCSANYI, J. (1990). Sensory neuron-specific actions of capsaicin-mechanisms and applications. *Trends Pharmacol. Sci.*, **11**, 330–333.

BEVAN, S.J., JAMES, I.F., RANG, H.P., SHAH, K. & YEATS, J.C. (1991). The development of a capsaicin antagonist for the sensory neurone excitant capsaicin. *Br. J. Pharmacol.*, **102**, 77P.

DEL BIANCO, E., TRAMONTANA, M., BERTRAND, C. & GEPPETTI, P. (1992). Release of sensory CGRP by hypertonic NaCl is not blocked by tetrodotoxin, ω-conotoxin, nifedipine and ruthenium red. *Life Sci.-Pharmacol. Lett.*, **51**, 73–76.

DELAY-GOYET, P. & LUNDBERG, J.M. (1991). Cigarette smoke-induced airway oedema is blocked by the NK_1 antagonist, CP-96,345. *Eur. J. Pharmacol.*, **203**, 157–158.

DICKENSON, A.H. & DRAY, A. (1991). Selective antagonism of capsaicin by capsazepine – evidence for a spinal receptor site in capsaicin-induced antinociception. *Br. J. Pharmacol.*, **104**, 1045–1049.

DRAY, A., BETTANAY, J. & FOSTER, P. (1989). Capsaicin desensitization of peripheral nociceptor does not impair sensitivity to other noxious stimuli. *Neurosci. Lett.*, **99**, 50–54.

DRAY, A., FORBES, C.A. & BURGESS, G.M. (1990). Ruthenium red blocks the capsaicin-induced increase in intracellular calcium and activation of membrane currents in sensory neurones as well as the activation of peripheral nociceptors in vitro. *Neurosci. Lett.*, **110**, 52–59.

DUSSER, D.J., DJOKIC, T.D., BORSON, D.B. & NADEL, J.A. (1989). Cigarette smoke induces bronchoconstrictor hyperresponsiveness to substance P and inactivates airway neutral endopeptidase in the guinea pig. Possible role of free radicals. *J. Clin. Invest.*, **84**, 900–906.

GEPPETTI, P., DEL BIANCO, E., PATACCHINI, R., SANTICIOLI, P., MAGGI, C.A. & TRAMONTANA, M. (1991). Low pH-induced release of calcitonin gene-related peptide from capsaicin-sensitive sensory nerves: mechanism of action and biological response. *Neuroscience*, **41**, 295–301.

GEPPETTI, P., TRAMONTANA, M., SANTICIOLI, P., DEL BIANCO, E., GIULIANI, S. & MAGGI, C.A. (1990). Bradykinin-induced release of calcitonin gene-related peptide from capsaicin-sensitive nerves in guinea-pig atria: mechanism of action and calcium requirements. *Neuroscience*, **38**, 687–692.

HAKANSON, R., BEDING, B., EKMAN, R., HEILIG, M., WAHLESTEDT, C. & SUNDLER, F. (1987). Multiple tachykinin pools in sensory nerve fibres in the rabbit iris. *Neuroscience*, **21**, 943–950.

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

LUNBERG, J.M., MARTLING, C., SARIA, A., FOLKERS, K. & ROSELL, S. (1983). Cigarette smoke-induced airway oedema due to activation of capsaicin-sensitive vagal afferents and substance P release. *Neuroscience*, **10**, 1361–1368.

LUNDBERG, J.M. & SARIA, A. (1983). Capsaicin-induced desensitization to cigarette smoke, mechanical and chemical irritants. *Nature*, **302**, 251–253.

MAGGI, C.A., PATACCHINI, R., SANTICIOLI, P., GIULIANI, S., DEL BIANCO, E., GEPPETTI, P. & MELI, A. (1989). The 'efferent' function of capsaicin-sensitive nerves: ruthenium red discriminates between different mechanisms of activation. *Eur. J. Pharmacol.*, **170**, 167–177.

MAGGI, C.A., SANTICIOLI, P., GEPPETTI, P., PARLANI, M., ASTOLFI, M., PRADELLES, P., PATACCHINI, R. & MELI, A. (1988). The antagonism induced by ruthenium red of the actions of capsaicin on the peripheral terminals of sensory neurons: further studies. *Eur. J. Pharmacol.*, **154**, 1–10.

MAPP, C.E., BONIOTTI, A., GRAF, P.D., CHITANO, P., FABBRI, L.M. & NADEL, J.A. (1991). Bronchial smooth muscle responses evoked by toluene diisocyanate are inhibited by ruthenium red and by indomethacin. *Eur. J. Pharmacol.*, **200**, 73–76.

MARSH, S.J., STANSFELD, C.E., BROWN, D.A., DAVEY, R. & MCCARTHY, D. (1987). The mechanism of action of capsaicin on sensory C-type neurons and their axons in vitro. *Neuroscience*, **23**, 275–290.

PIEDIMONTE, G., BERTRAND, C., GEPPETTI, P., DESAI, M.C., SNIDER, R.M. & NADEL, J.A. (1992). Hypertonic saline-induced increase in airway vascular permeability is inhibited by NK_1 receptor antagonist. *Am. Rev. Resp. Dis.*, **145**, A683.

SPRINGALL, D.R., EDGINTON, J.A., PRICE, P.N., SWANSTON, D.W., NOEL, C., BLOOM, S.R. & POLAK, J.M. (1990). Acrolein depletes the neuropeptides CGRP and substance P in sensory nerves in rat respiratory tract. *Env. Health Persp.*, **85**, 151–157.

STEDMAN, R.L. (1968). The chemical composition of tobacco and tobacco smoke. *Chem. Rev.*, **68**, 153–207.

SZALLASI, A. & BLUMBERG, P.M. (1990). Specific binding of resiniferatoxin, an ultrapotent capsaicin analog, by dorsal root ganglion membranes. *Brain Res.*, **524**, 106–111.

TRAMONTANA, M., CECCONI, R., DEL BIANCO, E., SANTICIOLI, P., MAGGI, C.A., ALESSANDRI, M. & GEPPETTI, P. (1991). Hypertonic media produce Ca^{2+}-dependent release of calcitonin gene-related peptide from capsaicin-sensitive nerve fibres in the rat urinary bladder. *Neurosci. Lett.*, **124**, 79–82.

UMENO, E., MCDONALD, D.M. & NADEL, J.A. (1990). Hypertonic saline increases vascular permeability in the rat trachea by producing neurogenic inflammation. *J. Clin. Invest.*, **85**, 1905–1908.

URBAN, L. & DRAY, A. (1991). Capsazepine, a novel capsaicin antagonist, selectively antagonizes the effects of capsaicin in the mouse spinal cord in vitro. *Eur. J. Pharmacol.*, **134**, 9–11.

WOOD, J.N., WINTER, J., JAMES, I.F., RANG, H.P., YEATS, J. & BEVAN, S. (1988). Capsaicin-induced ion fluxes in dorsal root ganglion cells in culture. *J. Neurosci.*, **8**, 3208–3220.

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Chronic kinin receptor blockade induces hypertension in deoxycorticosterone-treated rats

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1 The contribution of endogenous kinins to the regulation of blood pressure and renal function of Wistar rats was evaluated by use of the new B_2-receptor antagonist, Hoe 140, (D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin).

2 Neither Hoe 140 (4 $\mu g\,h^{-1}$ s.c., for 6 weeks), nor vehicle altered systolic blood pressure (SBP, tail-cuff plethysmography) or renal function in rats, under normal conditions.

3 Chronic administration of deoxycorticosterone (DOC, 25 $mg\,kg^{-1}$ s.c., weekly) increased SBP slightly only after 6 weeks (from 124 ± 2 to 133 ± 3 mmHg, $P<0.05$). An earlier and greater rise in SBP ($P<0.01$) occurred when DOC was combined with chronic infusion of Hoe 140 (from 125 ± 1 to 154 ± 3, $P<0.01$). The hypertensive effect of Hoe 140 was confirmed by direct measurement of mean blood pressure (143 ± 2 vs 122 ± 2 mmHg in controls, $P<0.01$).

4 DOC caused an initial fall, followed by a transitory increase in urinary volume and sodium excretion; thereafter, both parameters returned to baseline. The initial antidiuretic and antinatriuretic effects were enhanced by Hoe 140 ($P<0.05$).

5 Urinary prostaglandin E_2 excretion was increased by DOC (from 106 ± 3 to 153 ± 4 ng $24\,h^{-1}$, $P<0.01$) and this effect was prevented by Hoe 140 (from 95 ± 3 to 104 ± 3 ng $24\,h^{-1}$, NS). By contrast, the high urinary vasopressin excretion and suppressed plasma renin activity found in DOC-treated rats were not altered by Hoe 140.

6 These data suggest that endogenous kinins play an important role in the regulation of arterial blood pressure under conditions of mineralocorticoid excess.

Keywords: Kinin antagonist; bradykinin; kallikrein; prostaglandins; renal function; blood pressure; mineralocorticoids

Introduction

The kallikrein-kinin system may play a role in the regulation of blood pressure and renal function. Kinins, derived from the enzymatic action of kallikrein on kininogen, are potent vasodilators which act as local hormones by stimulating the release of endothelium-derived relaxing factors and prostaglandins (Vanhoutte, 1989). In addition, they may modulate water and electrolyte homeostasis by acting in the vascular, interstitial and tubular compartments of the kidney (Carretero & Scicli, 1991). At least two subtypes of bradykinin receptors have been identified. While B_1-receptors are expressed during tissue injury, stress and other pathological conditions, B_2-receptors are responsible for most of the cardiovascular and renal effects of bradykinin and Lys-bradykinin in normal conditions (Rhaleb *et al.*, 1991).

Decreased renal synthesis and excretion of kallikrein have been reported in various models of genetic hypertension (Keiser *et al.*, 1976; Arbeit & Serra, 1985; Berry *et al.*, 1989; Pravanec *et al.*, 1991). Thus, defective generation of kinins, leading to predominance of vasoconstrictor tone and sodium retention, may contribute to the pathogenesis of arterial hypertension. By contrast, activation of the kallikrein-kinin system reportedly occurs in clinical forms of primary aldosteronism (Margolius *et al.*, 1972) as well as in rats given deoxycorticosterone (DOC) (Geller *et al.*, 1972; Keiser *et al.*, 1976; Holland *et al.*, 1980), an experimental model which mimics human hypermineralocorticoidism. Yet, the hypothesis that enhanced activity of the kallikrein-kinin system prevents chronic vasoconstriction induced by DOC is based mainly on indirect evidence (Majima *et al.*, 1991).

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Research on this issue has been hampered by the shortcoming of the first generation bradykinin receptor antagonists, namely their residual agonistic activity (Vavrek & Stewart, 1985; Mulinari *et al.*, 1987; Beierwaltes *et al.*, 1988; Rhaleb *et al.*, 1991) and susceptibility to fast enzymatic degradation (Griesbacher *et al.*, 1989), which precluded application of these compounds in chronic studies. The recent availability of a potent and long-acting bradykinin B_2-receptor antagonist, D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin (Hoe 140) (Lembeck *et al.*, 1991; Hock *et al.*, 1991; Wirth *et al.*, 1991) has offered the opportunity to evaluate the cardiovascular and renal effects of endogenous kinins in chronic conditions.

In an earlier study, we found that subcutaneous administration of Hoe 140 for one day did not alter blood pressure in DOC-treated rats, though it decreased urinary volume and urinary sodium excretion (Madeddu *et al.*, 1992). The present study was designed to evaluate the effects of chronic blockade of bradykinin B_2-receptors by Hoe 140 on blood pressure, renal function and hormonal parameters in rats. In particular, we examined whether endogenous kinins prevented development of the hypertensive effect of DOC.

Methods

Animals

Male Wistar rats (Morini, Como, Italy) weighing between 220 and 240 g were housed at a constant room temperature with a 12-h light-dark cycle and had free access to tap water and rat chow (Altromin MT, A Reiper S.p.A., Vandois, Italy).

Blood pressure measurement

Unrestrained rats were warmed for 10 to 15 min in a thermostatically controlled heating cabinet that supplied a continuous air flow at 35°C. Then, systolic blood pressure (SBP) and heart rate (HR) were measured by the tail-cuff plethysmography method using a W + W BP Recorder 8002 (Ugo Basile, Biological Research Apparatus, Comerio, Italy) with the rat gently wrapped in a cotton hand towel. Each pressure and heart rate value was obtained by averaging five to six individual readings.

Mean blood pressure (MBP) and HR were measured directly (see below) by use of a Statham transducer (Gould, Oxnard, CA, U.S.A.) connected to a four channel Quartet Recorder (Ugo Basile, Biological Research Apparatus, Comerio, Italy).

Experiment 1 Effects of Hoe 140 on blood pressure, heart rate and renal function in rats under normal conditions

Body weight, SBP, and HR were measured in the morning on two different occasions before starting the experimental period and then weekly.

Basal 24 h urine collections were obtained from rats in individual metabolic cages with free access to tap water but deprived of food. The same procedure was repeated on the 1st, 3rd, 5th and 7th day of the experimental period and then weekly for a further five weeks.

After completion of basal urine collections, rats were randomly allocated to two groups. Group 1 ($n = 12$) was treated with physiological saline and group 2 ($n = 16$) with Hoe 140 (Hoechst AG, Frankfurt, Germany). Either saline or Hoe 140 (4 $\mu g\ h^{-1}$) was infused subcutaneously for six weeks at the rate of 2.5 $\mu l\ h^{-1}$ by 2ML4 Alzet osmotic pumps (Alza Corporation, Palo Alto, CA, U.S.A.). Osmotic pumps were implanted under the skin between the scapulae with rats maintained under light ether anaesthesia. Pumps were replaced 28 days after implantation.

At the end of the experimental period, a PE-10 tubing (Clay-Adams, Parsippany, NJ, U.S.A.) catheter was inserted into the left femoral artery of rats under ether anaesthesia and advanced into the abdominal aorta; a PE-50 tubing catheter was inserted into the left carotid artery and advanced into the descending thoracic aorta; both catheters were tunneled under the skin and exteriorized at the back of the neck. Twenty-four hours later, MBP and HR of rat (free to move in their own cages) were measured for 30 min, via the femoral catheter. Then, the inhibitory activity of Hoe 140 was tested by comparing the vasodepressor effects of an intra-aortic bolus injection of bradykinin (Peninsula Laboratories, Belmont, CA, U.S.A.) 0.9 $\mu g\ kg^{-1}$ in 20 μl saline, via the carotid catheter, in groups given vehicle or antagonist.

Twenty-four hours later, rats were killed by decapitation and blood was collected from the trunk for 10 s into plastic tubes containing 20 μl EDTA (final concentration: 1 mg ml^{-1}) and centrifuged at 2,000 g for 10 min to separate plasma. Both kidneys were removed, blotted, weighed, minced, and homogenized in a glass homogenizer containing 0.1 M Tris-HCl buffer (pH 8.2). The homogenate was sonicated for 1 min with a Sonifier disintegrator (Branson Sonnic Power Co., Danbury, CO, U.S.A.) and centrifuged twice at 24,000 g for 30 min. All procedures were carried out at 4°C. Pooled supernatants were immediately used for measurement of kallikrein activity and protein concentration.

Experiment 2 Effects of Hoe 140 on blood pressure, heart rate and renal function in DOC-treated rats

Rats underwent an experimental protocol similar to experiment 1, except that from the first day of the experimental period both the vehicle- ($n = 18$) and the Hoe 140-groups ($n = 20$) received subcutaneous injections of deoxycorticosterone enantate, 25 mg kg^{-1} (Shering, Milan, Italy). This dose was repeated weekly for the duration of the study.

In preliminary experiments, we found that the plasma DOC concentration one week after the injection of 25 mg kg^{-1} body weight exceeded normal levels by six fold (603 ± 25 ng dl^{-1} vs 104 ± 10 ng dl^{-1} in controls).

Analytical procedures

Plasma renin activity (PRA) was measured by radioimmunoassay (Sorin, Saluggia, Italy) as the rate of angiotensin I generation at 37°C, pH 6.2, in the presence of angiotensinase inhibitors.

Urinary volume (UV) was determined gravimetrically. Urinary sodium (U_{Na}) was determined by flame photometry.

The urinary prostaglandin E_2 (PGE_2) concentration was measured by radioimmunoassay (Du Pont de Nemours, Dreieich, Germany) after acidification and extraction with Sep-Pak C18 columns (Water Associates, Inc., Milford, MA, U.S.A.). Extraction recovery of [^{3}H]-PGE_2 (5 pg) was 95 ± 2%.

The urinary vasopressin concentration was measured by radioimmunoassay (Buhlmann Laboratories AG, Basel, Switzerland) after acidification and extraction according to the method of Tausch *et al.* (1983). Recovery of standard arginine vasopressin (AVP, 10 pg) was 79 ± 4%.

Plasma osmolality was measured with a Fisker Osmometer and expressed as mosmol kg^{-1}.

Urinary and plasma creatinine were measured by an automatic analyzer (Hitachi 704). Glomerular filtration rate was calculated as the clearance of endogenous creatinine.

Kallikrein activity in urine and renal homogenates was measured by an end-point amidolytic method (Amundsen *et al.*, 1979) using the synthetic substrate H-D-Val-Leu-Arg-paranitroanilide (S2266, Kabi Diagnostica, Stockholm, Sweden) at a final concentration of 0.7 mM in 0.1 M Tris-HCl buffer (pH 8.2) containing soybean trypsin inhibitor (SBTI, Sigma Chemical Company, St. Louis, U.S.A.). Kallikrein activity was expressed as nmol *p*-nitroaniline released s^{-1} (nkat). Protein concentration was measured by the method of Lowry *et al.* (1951).

Statistical analysis

All data are expressed as mean ± s.e. mean. Multivariate repeated measures analysis of variance was performed to test for interaction between time and grouping factor. Then, univariate analysis of variance was used to test for differences among groups and over time. Differences within or between groups were determined by paired or unpaired t tests, respectively, with the Bonferroni multiple comparison adjustment. Mathematical and statistical analyses were performed with a Statview II package, on a Macintosh IICX computer.

Results

Body weight gain during the study was similar among groups (data not shown). As shown in Figure 1 (left panels), neither Hoe 140 nor its vehicle altered SBP and HR of rats, under normal conditions.

As shown in the right panels of Figure 1, chronic administration of DOC caused a slight increase in SBP only at the 6th week of the experimental period. By contrast, when DOC was given in Hoe 140-treated rats, a progressive rise in SBP was observed which was significantly greater than that observed in DOC-treated rats given vehicle ($P < 0.01$). HR remained unaltered in DOC-treated rats.

As shown in Table 1, the hypertensive effect found in DOC-treated rats given Hoe 140 was confirmed by direct measurement of MBP at the end of the study. Chronic infusion of Hoe 140 completely inhibited the depressor effect of bradykinin in rats with (0 ± 1 vs 22 ± 2 mmHg in con-

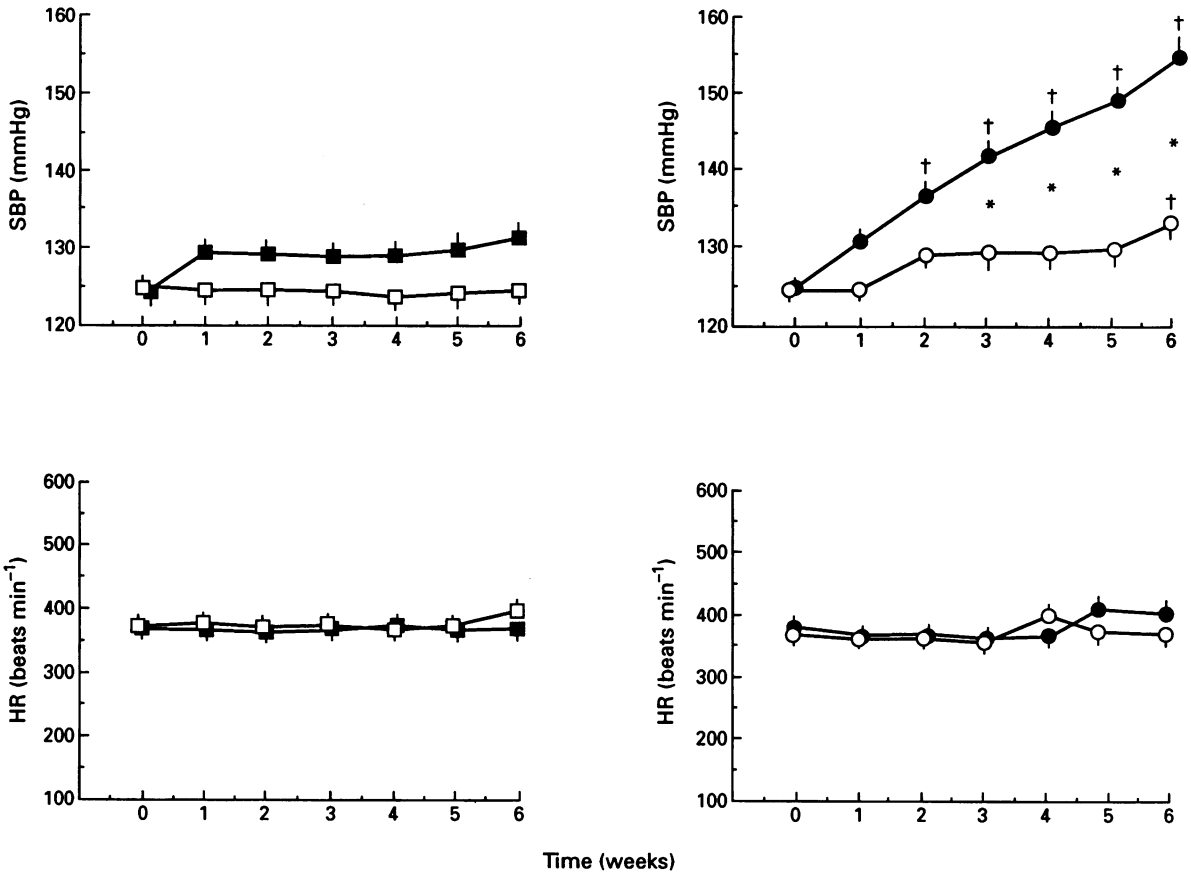


Figure 1 Line graphs showing the effects of the bradykinin antagonist Hoe 140 (4 μg h^{-1} s.c., ■, ●) or vehicle (□, ○) on systolic blood pressure (SBP) and heart rate (HR). Hoe 140 did not alter the cardiovascular parameters in rats under normal conditions (left panels), while it increased SBP when combined with deoxycorticosterone administration (right panels). SBP and HR were determined by tail-cuff plethysmography before starting the experimental period (time 0) and then weekly during the following 6 weeks. Values represent means ± s.e. mean (vertical lines). †$P<0.05$ vs baseline; *$P<0.05$ vs vehicle group.

Table 1 Cardiovascular parameters measured by direct method at the end of the study in rats with or without dexoycorticosterone (DOC) treatment

	Without DOC		*With DOC*	
Group	*Vehicle*	*Hoe 140*	*Vehicle*	*Hoe 140*
MBP (mmHg)	115 ± 2	113 ± 2	122 ± 2	143 ± 2*
HR (b min^{-1})	387 ± 7	401 ± 17	373 ± 9	380 ± 6

Values represent means ± s.e. mean. BP, mean blood pressure; HR, heart rate (beats min^{-1}).
*$P<0.05$ vs vehicle group.

trols) or without DOC treatment (0 ± 2 vs 15 ± 2 mmHg in controls).

As shown in Figure 2 (left panels), neither Hoe 140 nor its vehicle altered UV and $U_{Na}V$ in rats, under normal conditions. As shown in the right panels, DOC caused an initial fall followed by a transitory increase in urine volume and urinary sodium excretion. Then, both parameters returned to baseline. The initial antidiuretic and antinatriuretic effect was enhanced by Hoe 140 ($P<0.05$). During the following weeks, the response to DOC was not altered by Hoe 140, except that urine volume did not rise over the baseline levels. ANOVA detected a group difference in the urine volume and sodium changes from baseline at the first day of the experimental period ($P<0.05$).

Urinary kallikrein excretion increased in all groups during the study (Figure 3). This effect was proportional to body growth in rats under normal conditions, whereas it persisted even after normalization by body weight gain in DOC-treated rats (data not illustrated).

Kallikrein activity in renal homogenates was higher in DOC-treated rats (vehicle: 0.24 ± 0.01; Hoe 140: 0.22 ± 0.02 nkat g^{-1} protein) than in rats without DOC treatment (vehicle: 0.13 ± 0.01; Hoe 140: 0.13 ± 0.01 nkat g^{-1} protein). No significant difference in kidney weights was found among groups. As shown in the lower left panel of Figure 3, urinary PGE_2 excretion remained unchanged from baseline in rats under normal conditions. By contrast, urinary PGE_2 excretion was increased by DOC treatment (right panel) and this effect was prevented by Hoe 140 ($P<0.05$).

Urinary creatinine excretion increased similarly in all groups (Figure 4).

No significant difference in GFR, haematocrit and plasma osmolality was found among groups at the end of the study (Table 2). Urinary vasopressin excretion was significantly higher and PRA lower in DOC-treated rats. Hoe 140 did not alter the effect of DOC on vasopressin and PRA levels.

Discussion

The finding that chronic administration of Hoe 140, at a dose that abolished the vasodepressor effect of exogenous bradykinin, did not alter blood pressure and renal function in normotensive rats under normal conditions is consistent with previous studies in which inhibition of endogenous kinins was achieved for a shorter period of time by using the same antagonist (Madeddu *et al.*, 1992) or Fab fragments of kinin antibodies (Carretero & Scicli, 1991). Collectively, these results suggest that endogenous kinins do not play a major

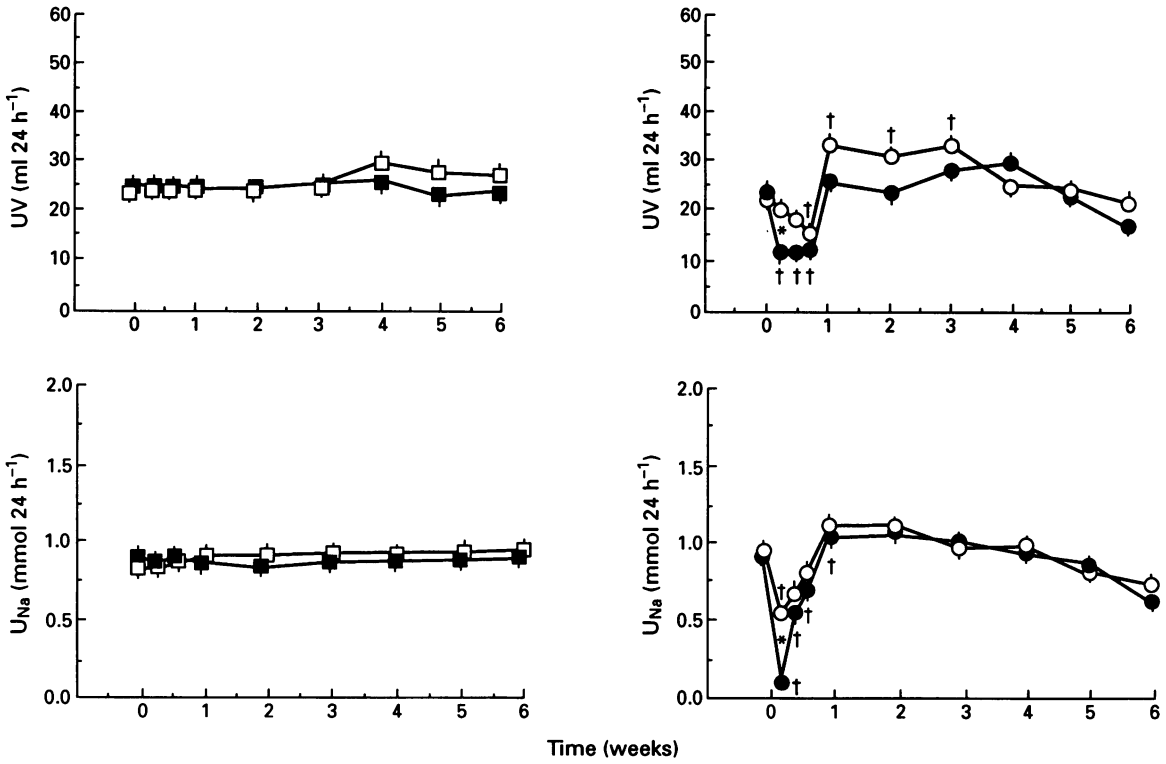


Figure 2 Line graphs showing the effects of the bradykinin antagonist, Hoe 140 (4 μg h^{-1} s.c., ■, ●) or vehicle (□, ○) on urinary volume (UV) and urinary sodium (UNa) excretion. Hoe 140 did not alter UV and UNa excretion in rats under normal conditions (left panels), while it enhanced the acute antidiuretic and antinatriuretic effect of deoxycorticosterone (right panels). Twenty-four hour urine collections were obtained before starting the experimental period (time 0). They were repeated on the 1st, 3rd, 5th and 7th days of the experiment and then weekly for the remaining weeks (wks). Values represent means ± s.e. mean (vertical lines). †$P<0.05$ vs baseline; *$P<0.05$ vs vehicle group.

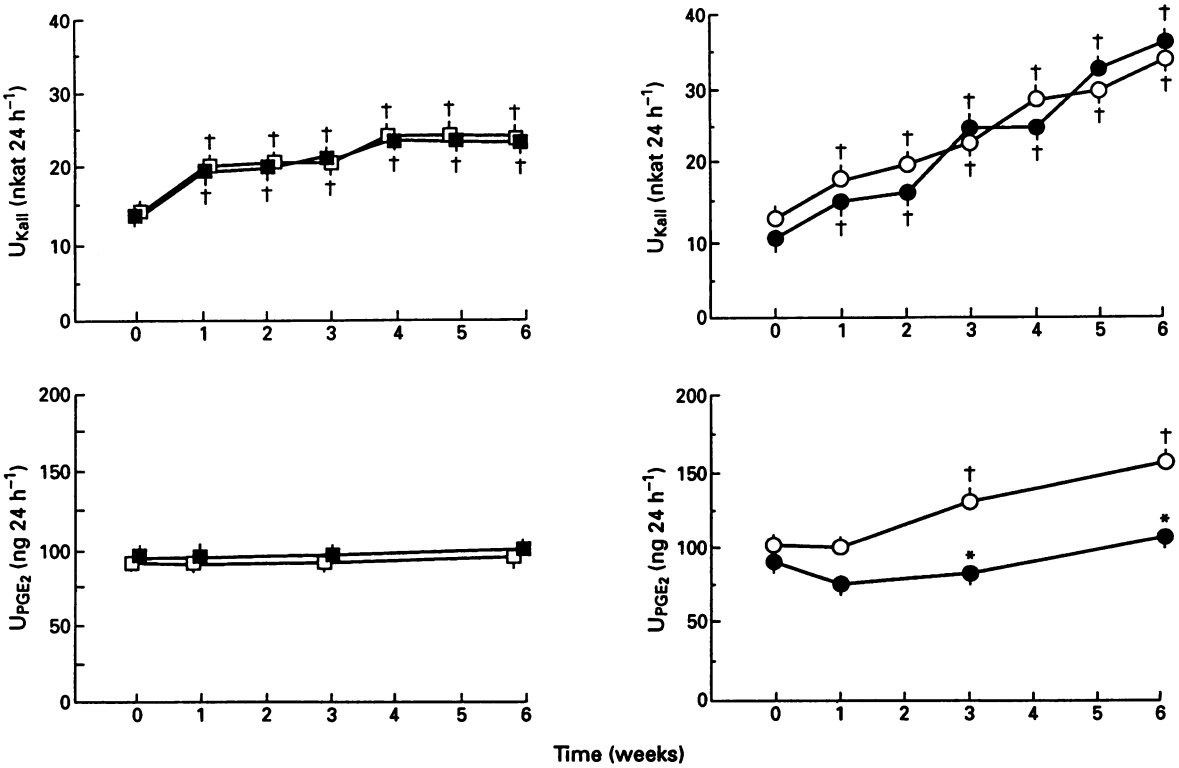


Figure 3 Line graphs showing the urinary excretion of kallikrein (U_{Kall}) and prostaglandin E_2 (U_{PGE_2}) in basal conditions and during the chronic infusion of the bradykinin antagonist Hoe 140 (4 μg h^{-1} s.c., ■, ●) or vehicle (□, ○). Hoe 140 did not alter the changes in U_{Kall} and U_{PGE_2} excretion in rats under normal conditions (left panels), while it prevented the increase in U_{PGE_2} induced by deoxycorticosterone (right panels). Values represent means ± s.e. mean (vertical lines). †$P<0.05$ vs baseline; *$P<0.05$ vs vehicle group.

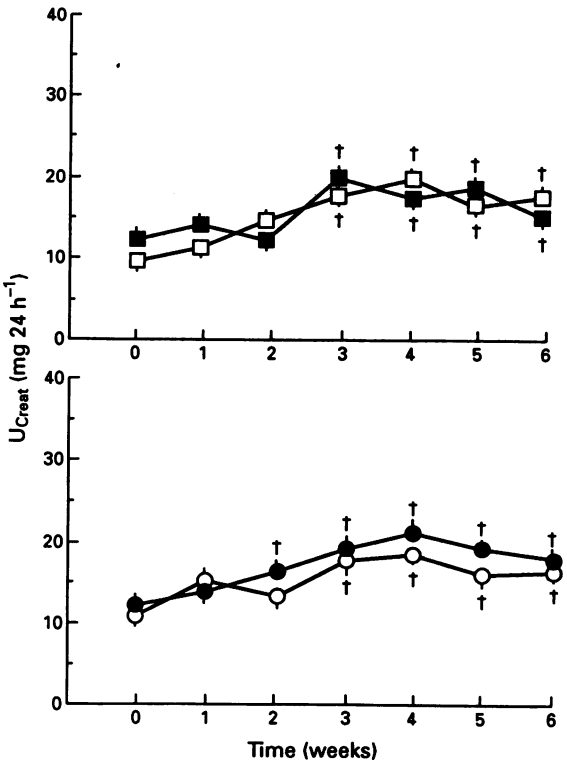


Figure 4 Line graphs showing the urinary creatinine (U_{Creat}) excretion before and during the chronic infusion of the bradykinin antagonist Hoe 140 (4 μg h^{-1} s.c., ■, ●) or vehicle (□, ○). The antagonist did not alter U_{Creat} excretion either in rats under normal conditions (upper panel) or during deoxycorticosterone treatment (lower panel). Values represent means ± s.e. mean (vertical lines). †$P<0.05$ vs baseline.

role in the regulation of blood pressure and renal function in normotensive rats.

It is known that chronic administration of DOC does not induce sustained hypertension unless combined with sodium loading and uninephrectomy. Activation of the renal kallikrein-kinin system, which is a typical feature in rats given chronic DOC administration (Geller *et al.*, 1972; Nakagawa *et al.*, 1991) as well as in human hypermineralocorticoidism (Margolius *et al.*, 1972) may represent an important compensatory response to counteract the vasoconstrictor and salt retaining effects of mineralocorticoids. This possibility is supported by our results which demonstrate for the first time that chronic blockade of bradykinin B_2-receptors causes hypertension in rats with an excess of mineralocorticoids.

Though a large body of evidence indicates that Hoe 140 is a selective B_2-receptor antagonist in the rat (Hock *et al.*, 1991; Lembeck *et al.*, 1991; Wirth *et al.*, 1991), a recent publication has suggested that it may be able to interact with B_1-receptors in a bovine aortic endothelial cell line (Weimer & Wirth, 1992). Therefore, caution is appropriate before attributing the hypertensive effect induced by Hoe 140 in DOC-treated rats to selective inhibition of B_2-receptors. Expression of B_1-receptors as well as enhanced formation of Ile-Ser-bradykinin (known also as T-kinin since it is released by trypsin incubated with plasma T-kininogen) reportedly occur in the rat after acute challenge, such as inflammation and tissue injury (Okamoto & Greenbaum, 1983; Mindroiu *et al.*, 1987). Surgical manipulation for osmotic pump implantation might have amplified the functional importance of B_1 receptors and stimulated the generation of T-kinin in our experimental conditions. This possibility has to be taken into account when interpreting the effects induced by Hoe 140 in DOC-treated rats. However, it should be pointed out that rats with or without DOC treatment underwent an identical experimental approach as far as surgical manipulation and osmotic pump implantation are concerned, but the antagonist caused hypertension in DOC-treated rats only. In addition, this hypertensive effect occurred at least two weeks after surgery. The observation that glandular kallikreins are unable to release kinins from T-kininogen (Okamoto & Greenbaum, 1983) speaks against the possibility that the increase in renal kallikrein content and excretion induced by DOC could enhance T-kinin formation.

It seems unlikely that nonspecific effects of the antagonist are responsible for the hypertensive effect found in DOC-treated rats. In contrast to the first generation kinin antagonists (Mulinari *et al.*, 1987; Beierwaltes *et al.*, 1988), Hoe 140 does not increase catecholamine (Bao *et al.*, 1992) or renin activity levels in plasma. In preliminary experiments, we also found that plasma and urinary catecholamine levels were not affected by chronic administration of Hoe 140 in DOC-treated rats. In addition, the antagonist proved to be specific since it did not alter the action of unrelated vasodilators (Madeddu *et al.*, 1992).

In the present study, the antidiuretic and antinatriuretic effect which occurred during the first days of DOC administration was enhanced by Hoe 140. It seems unlikely that these effects were due to changes in water intake since the latter parameter was not altered by the antagonist. Distribution of specific binding sites for bradykinin suggests that kinins may inhibit water and sodium reabsorption stimulated by mineralocorticoids or vasopressin at the level of the distal nephron (Tomita *et al.*, 1985).

Chronic administration of mineralocorticoids leads eventually to an increase in urinary excretion of water and sodium, known as the 'escape phenomenon'. In the present study, blockade of bradykinin-receptors by Hoe 140 did not alter the escape phenomenon observed in DOC-treated rats. At the end of the study, haematocrit and plasma osmolality levels were similar in rats given Hoe 140 or its vehicle. Collectively, these results suggest that endogenous kinins may counteract the early antidiuretic and antinatriuretic effect of DOC, whereas other compensatory mechanisms may be implicated in the escape phenomenon (Biglieri, 1991). We hypothesize that sodium retention may contribute to the

Table 2 Effect of Hoe 140 on renal function and hormonal parameters in Wistar rats with or without deoxycorticosterone (DOC) treatment

	Without DOC		*With DOC*	
Group	*Vehicle*	*Hoe 140*	*Vehicle*	*Hoe 140*
GRF (ml min^{-1})	1.6 ± 0.1	1.7 ± 0.1	2.0 ± 0.2	1.8 ± 0.1
U_{AVP} (pg 24 h^{-1})	60 ± 8	70 ± 9	348 ± 22	346 ± 25
PRA (ng ml^{-1} h^{-1})	0.85 ± 0.21	0.88 ± 0.10	0.09 ± 0.03	0.10 ± 0.03
Ht (%)	48 ± 1	46 ± 1	45 ± 2	48 ± 1
P_{osm} (mosmol kg^{-1})	284 ± 6	288 ± 6	272 ± 11	286 ± 7

Values represent means ± s.e. mean. GRF, glomerular filtration rate; U_{AVP}, urinary vasopressin excretion; PRA, plasma renin activity; Ht, haematocrit; P_{osm}, plasma osmolality.

initial rise of blood pressure, while different mechanisms could explain the development of sustained hypertension induced by Hoe 140.

The presence of locally synthesized kallikrein in rat vasculature supports the concept that endogenous kinins may regulate vascular resistance acting as paracrine hormones (Carretero & Scicli, 1991). Indeed, decreased generation of arterial kinins might be responsible, at least in part, for the increased peripheral resistance in one-kidney, one clip hypertension (Nolly *et al.*, 1990). Arterial hypertension induced by Hoe 140 in DOC-treated rats might be due to chronic inhibition of kinins generated within the vasculature leading to a predominance of vasopressor systems.

In the present study, the urinary excretion of vasopressin was increased in DOC-treated rats. Previous studies have shown that vasopressin can contribute in the development of DOC-induced hypertension either directly or by enhancing vascular sensitivity to other vasoconstrictors (Berecek *et al.*, 1982). Evidence for an interaction between kinins, prostaglandins, and vasopressin has been provided in the past: vasopressin is able to stimulate renal kallikrein excretion and, in turn, bradykinin antagonizes the effects of vasopressin either directly or by stimulating renal prostaglandin synthesis (Fejes-Toth *et al.*, 1980). In addition, prostaglandins and endothelium-derived relaxing factors contribute to the vasodilator action of bradykinin (Vanhoutte, 1989; Gardiner *et al.*, 1990). The finding that Hoe 140 prevents the increase in urinary PGE_2 induced by DOC indicates that kinins are responsible for the activation of renal synthesis and release of PGE_2 in the presence of an excess of mineralocorticoids. Enhanced vasopressin levels combined with reduced formation of vasodilator prostaglandins may contribute to the increase in blood pressure during chronic blockade of bradykinin receptors.

In conclusion, our results indicate that the kallikrein-kinin system plays an important role in the regulation of blood pressure in conditions of mineralocorticoid excess.

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References

AMUNDSEN, E., PUTTER, J., FRIBERGER, P., KNOS, M., LARSBROTEN, M. & CLAESON, G. (1979). Methods for the determination of glandular kallikrein by means of a chromogenic substrate. *Adv. Exp. Med. Biol.*, **120 A**, 83–95.

ARBEIT, L.A. & SERRA, S.R. (1985). Decreased total and active urinary kallikrein in normotensive Dahl salt susceptible rats. *Kidney Internat.*, **28**, 440–446.

BAO, G., GOHLKE, P., QADRI, F. & UNGER, T. (1992). Chronic kinin receptor blockade attenuates the antihypertensive effect of ramipril. *Hypertension*, **20**, 74–79.

BEIERWALTES, W.H., CARRETERO, O.A. & SCICLI, A.G. (1988). Renal hemodynamics in response to a kinin analogue antagonist. *Am. J. Physiol.*, **255**, F408–F414.

BERECEK, K.H., MURRAY, P.D., GROSS, F. & BRODY, M.J. (1982). Vasopressin and vascular reactivity in the development of DOCA Hypertension in rats with hereditary Diabetes Mellitus. *Hypertension*, **4**, 3–12.

BIGLIERI, E.G. (1991). Spectrum of mineralcorticoid hypertension. *Hypertension*, **17**, 251–261.

BERRY, T.D., HASSTEDT, S.J., HUNT, S.C., WU, L.L., SMITH, J.B., ASH, K.O. & WILLIAMS, R.R. (1989). A gene for high kallikrein may protect against hypertension in Utah kindreds. *Hypertension*, **13**, 3–8.

CARRETERO, O.A. & SCICLI, A.G. (1991). Local hormonal factors (intracrine, autocrine, and paracrine) in hypertension. *Hypertension*, **18** (Suppl I), I-58–I-69.

FEJES-TOTH, G., ZAHAJSKI, T. & FILEP, J. (1980). Effect of vasopressin on renal kallikrein excretion. *Am. J. Physiol.*, **8**, F388–F392.

GARDINER, S.M., COMPTON, A.M., KEMP, P.A. & BENNETT, T. (1990). Regional and cardiac haemodynamic responses to glyceryltrinitrate, acetylcholine, bradykinin and endothelin-1 in conscious rats; effects of N^G-nitro-L-arginine methyl ester. *Br. J. Pharmacol.*, **101**, 632–639.

GELLER, R.G., MARGOLIUS, H.S., PISANO, J.J. & KEISER, H.R. (1972). Effects of mineralcorticoids, altered sodium intake and adrenelectomy on urinary kallikrein in rats. *Circ. Res.*, **48**, 728–733.

GRIESBACHER, T., LEMBECK, F. & SARIA, A. (1989). Effects of the bradykinin antagonist B4310 on smooth muscles and blood pressure in the rats, and its enzymatic degradation. *Br. J. Pharmacol.*, **96**, 531–538.

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

HOLLAND, O.B., CHUD, J.M. & BRAUNSTEIN, H. (1980). Urinary kallikrein excretion in essential and mineralcorticoid hypertension. *J. Clin. Invest.*, **65**, 347–356.

KEISER, H.R., GELLER, R.G., MARGOLIUS, H.S. & PISANO, J.J. (1976). Urinary kallikrein in hypertensive animal models. *Fed. Proc.*, **35**, 199–202.

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

LOWRY, O.A., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 407–412.

MADEDDU, P., ANANIA, V., PINNA PARPAGLIA, P., DEMONTIS, M.P., VARONI, M.V., PISANU, G., TROFFA, C., TONOLO, G.C. & GLORIOSO, N. (1992). Effects of Hoe 140, a bradykinin B_2-receptor antagonist, on renal function in conscious normotensive rats. *Br. J. Pharmacol.*, **106**, 380–386.

MAJIMA, M., KATORI, M., HANAZUKA, M., MIZOGAMI, S., NAKANO, T., NAKAO, Y., MIKAMI, R., URYU, H., OKAMURA, R., MOHSIN, S.S.J. & OH-ISHI, S. (1991). Suppression of rat deoxycorticosterone-salt hypertension by kallikrein-kinin system. *Hypertension*, **17**, 806–813.

MARGOLIUS, H.S., GELLER, R., DE JONG, W., PISANO, J.J. & SJOERDSMA, A. (1972). Urinary kallikrein excretion in hypertension. *Circ. Res.*, **30**, 358–362.

MINDROIU, T., CARRETERO, O.A. & SCICLI, A.G. (1987). T-kininogen in rats plasma after nephrectomy and other surgical traumas. *Agents Actions*, **22**, 265–276.

MULINARI, A., BENETOS, A., GAVRAS, I. & GAVRAS, H. (1987). Interaction of bradykinin with catecholamines in blood pressure regulation. *Hypertension*, **10**, 374–377.

NAKAGAWA, M., STEWART, J.M., VAVREK, R.J. & NASJLETTI, A. (1990). Effects of a kinin antagonist on renal function in rats. *Am. J. Physiol.*, **258**, F643–F648.

NOLLY, H., CARRETERO, O.A., SCICLI, G., MADEDDU, P. & SCICLI, G. (1990). A kallikrein-like enzyme in blood vessels of one-kidney, one clip hypertensive rats. *Hypertension*, **16**, 436–440.

OKAMOTO, H. & GREENBAUM, L.M. (1983). Pharmacological properties of T-kinin (Isoleucyl-Seryl-Bradykinin) from rat plasma. *Biochem. Pharmacol.*, **32**, 2637–2643.

PRAVANEC, M., KREN, V., KUNES, J., SCICLI, A.G., CARRETERO, O.A., SIMONET, L. & KURTZ, T.W. (1991). Cosegregation of blood pressure with a kallikrein gene family polymorphism. *Hypertension*, **17**, 242–246.

RHALEB, N.E., TELEMAQUE, S., ROUSSI, N., DION, S., JUKIC, D., DRAPEAU, G. & REGOLI, D. (1991). Structure-activity studies of bradykinin and related peptides: B_2-receptor antagonists. *Hypertension*, **17**, 107–115.

TAUSCH, A., STEGNER, H., LEAKE, R.D., ARTMAN, H.G. & FISHER, D.A. (1983). Radioimmunoassay of arginine vasopressin in urine: development and application. *J. Clin. Endocr. Metab.*, **57**, 777–781.

TOMITA, K., PISANO, J.J. & KNEPPER, M.A. (1985). Control of sodium and potassium transport in the cortical collecting duct of the rat: effect of bradykinin, vasopressin, and deoxycorticosterone. *J. Clin. Invest.*, **76**, 132–136.

VANHOUTTE, P.M. (1989). Endothelium and control of vascular function: state of art lecture. *Hypertension*, **13**, 658–667.

VAVREK, R.J. & STEWART, J.W. (1985). Competitive antagonists of bradykinin. *Peptides*, **6**, 161–164.

WEIMER, G. & WIRTH, K. (1992). Production of cGMP via activation of B_1 and B_2 kinin receptors in cultured bovine aortic endothelial cells. *J. Pharmacol. Exp. Ther.*, **262**, 729–733.

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

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Potentiation of α-adrenoceptor-mediated responses following chronic β-adrenoceptor stimulation in the rat heart

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1 Noradrenaline and isoprenaline were infused subcutaneously in rats by use of Alzet osmotic minipumps. The effects of catecholamine infusion on ventricular α- and β-adrenoceptor density and also the responses of isolated cardiac tissues were compared with saline infusion.

2 Noradrenaline (1 mg kg^{-1}) or isoprenaline (40 μg kg^{-1}) infused for 3 days resulted in a desensitization of β-adrenoceptor-mediated responses of isolated left atria and papillary muscles. Concentration-response curves to isoprenaline were shifted to the right in left atria whilst maximum responses were reduced in papillary muscles. Right atrial rate responses were not affected by infusions of catecholamines.

3 Infusions of either noradrenaline or isoprenaline resulted in a supersensitivity of α-adrenoceptor-mediated responses in isolated papillary muscles with leftward displacements of concentration-response curves to phenylephrine.

4 The density of both ventricular [^{3}H]-dihydroalprenolol and [^{3}H]-prazosin binding sites was reduced following noradrenaline infusion. Isoprenaline infusion reduced only the density of [^{3}H]-dihydroalprenolol binding sites.

5 Noradrenaline infusion therefore 'down-regulates' both α- and β-adrenoceptors in the rat heart but at the same time ventricular α-adrenoceptor-mediated responses are enhanced. Isoprenaline similarly enhances responses to phenylephrine and possible mechanisms for this phenomenon are discussed.

Keywords: Cardiac adrenoceptors; β-adrenoceptor desensitization; osmotic minipumps; catecholamine infusion; supersensitivity; α-adrenoceptor regulation; cardiac sensitivity

Introduction

Both *in vitro* and *in vivo* studies have demonstrated that chronic β-adrenoceptor stimulation can result in the loss of cardiac β-adrenoceptor function with an uncoupling of the β-adrenoceptor from adenylate cyclase and a reduction in β-adrenoceptor density (Karliner *et al.*, 1986; Strasser *et al.*, 1985; 1988). Sympathomimetic amines can also elicit positive inotropic responses through the stimulation of cardiac α-adrenoceptor (Benfey, 1982; Chess-Williams & Broadley, 1987) and although there is little doubt that β-adrenoceptor desensitization plays a significant role in myocardial disease, the functional significance of cardiac α-adrenoceptors and the mechanisms involved in their regulation are less clearly understood.

In vitro experiments with isolated cells and tissues have suggested that noradrenaline desensitizes β-adrenoceptor-mediated responses without a concomitant change in cardiac α_1-adrenoceptor responsiveness (Chess-Williams, 1987; Reithmann & Werden, 1989). However, when β-adrenoceptor responsiveness is impaired by propranolol treatment, an increase in α_1-adrenoceptor density in the rat heart has been demonstrated (Mugge *et al.*, 1985). The time course of this effect has been studied and the effect found to be significant only after 2 days and maximal after 3 days (Steinkraus *et al.*, 1989). Changes in α-adrenoceptor responsiveness therefore, may not be as rapid as changes in β-adrenoceptor responsiveness where desensitization can occur after a matter of hours (Karliner *et al.*, 1986). The present study examines the α- and β-adrenoceptor-mediated inotropic responses of isolated left atria and papillary muscles removed from rats infused for 3 days with noradrenaline or the relatively pure β-adrenoceptor agonist, isoprenaline.

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Methods

Implantation of osmotic minipumps

Male Wistar rats (350–400 g) were anaesthetized with 0.5 ml kg^{-1} Hypnorm (0.315 mg ml^{-1} fentanyl citrate and 10 mg ml^{-1} fluanisone) and diazepam (5 mg kg^{-1}), and Alzet osmotic minipumps (model 2001) implanted subcutaneously between the shoulder blades. The animals were allowed to recover and then killed 3 days later. The minipumps delivered acidified saline (ascorbic acid 0.01 M in 0.09% NaCl), or drug (noradrenaline or isoprenaline) in acidified saline at a constant rate of 1 μl h^{-1}. Prior to implantation, minipumps were filled and left for 6 h at room temperature in saline solution. Animals were housed at 20°C on a 12 h light/dark cycle with food and water available *ad libitum*.

Isolated cardiac preparations

Three days after implantation, animals were killed by a blow to the head and exsanguinated. Left atria and left ventricular papillary muscles were rapidly removed and set up in a Krebs-bicarbonate solution (composition in mM: NaCl 118.4, KCl 4.7, $NaHCO_3$ 25.0, glucose 11.7, $MgSO_4$ 1.2, $KHPO_4$ 1.2 and $CaCl_2$ 1.9) gassed with 5% CO_2 in oxygen at 37°C. Tissues were suspended under 0.8 g tension and paced at 1 Hz by square-wave pulses (5 ms duration; threshold voltage +50%), delivered via bipolar electrodes, from Grass S48 stimulators. Isometric developed tension was recorded via Lectromed UF1 (57 g sensitivity range) force transducers, on a Lectromed MT6 chart recorder.

Tissues were equilibrated for 30 min with several changes of bathing medium. In each tissue, a cumulative concentration-response curve to isoprenaline was constructed, followed after washout, by one to phenylephrine and then calcium chloride which was added cumulatively from the starting concentration of 1.9 mM in the bath. All experiments were performed in the presence of metanephrine (10 μM) and

cocaine (10 μM) to inhibit extraneuronal and neuronal uptake respectively. The β-adrenoceptor antagonist, propranolol (1 μM) was also present when obtaining responses to phenylephrine.

Radioligand binding

After removing the papillary muscles, the remaining ventricular tissue was quickly immersed in ice-cold Tris buffer (50 mM) and a crude membrane fraction was prepared from the ventricular tissue by homogenizing the tissue in ice-cold Tris (50 mM) buffer for 15 s with an Ultra-Turrax homogenizer followed by 4 times 4 strokes of a Teflon homogenizer. The homogenate was centrifuged at 1,000 *g* for 10 min in an M.S.E. Chillspin centrifuge. The supernatant was then recentrifuged at 30,000 *g* for 20 min in an M.S.E. 18 centrifuge. The pellet was finally resuspended in 3 ml Tris buffer and used in a receptor binding assay. These procedures were carried out at 4°C.

The crude membrane fraction was assayed for α-adrenoceptor binding sites by use of [^{3}H]-prazosin and β-adrenoceptor binding sites with [^{3}H]-dihydoalprenolol ([^{3}H]-DHA). Samples (100 μl) of the membrane suspension were incubated at 37°C for 30 min with a range of radioligand concentrations (0.125–4.0 nM for [^{3}H]-prazosin and 0.5–16.0 nM for [^{3}H]-DHA) in a final volume of 250 μl. Non-specific binding was determined by displacement with 10 μM phentolamine in [^{3}H]-prazosin experiments or 200 μM isoprenaline in [^{3}H]-DHA experiments. Incubations were terminated by the addition of 1 ml ice-cold buffer, followed by rapid filtration through Whatman GF/B glass fibre filters. The filters were rapidly washed with 3 times 5 ml ice-cold buffer. Radioactivity remaining on the filters was measured by standard scintillation counting techniques. Specific binding as a percentage of the total binding amounted to approximately 60% and 80% for [^{3}H]-DHA and [^{3}H]-prazosin binding respectively. Protein content of the membrane fraction was determined by the method of Lowry *et al.* (1951).

Statistical analysis

Isolated tissues: increases in developed tension were plotted as a percentage of the maximum increase for each drug. Individual EC_{50} values (concentration to produce a half maximal response) were determined and geometric mean EC_{50} values with 95% confidence limits calculated. Mean maximum responses (± s.e.mean) were also calculated. ANOVA and Student's *t* test were performed on maximal responses and also logarithmic EC_{50} values to test for differences between groups of animals

Binding experiments: dissociation constants (K_d) and the density of specific binding sites (B_m) were calculated by Scatchard analysis of saturation curves performed in duplicate. Mean values (with s.e.mean) were calculated and unpaired Student's *t* test used to examine differences between groups. Where *P* values of less than 0.05 were obtained the change was taken as being statistically significant.

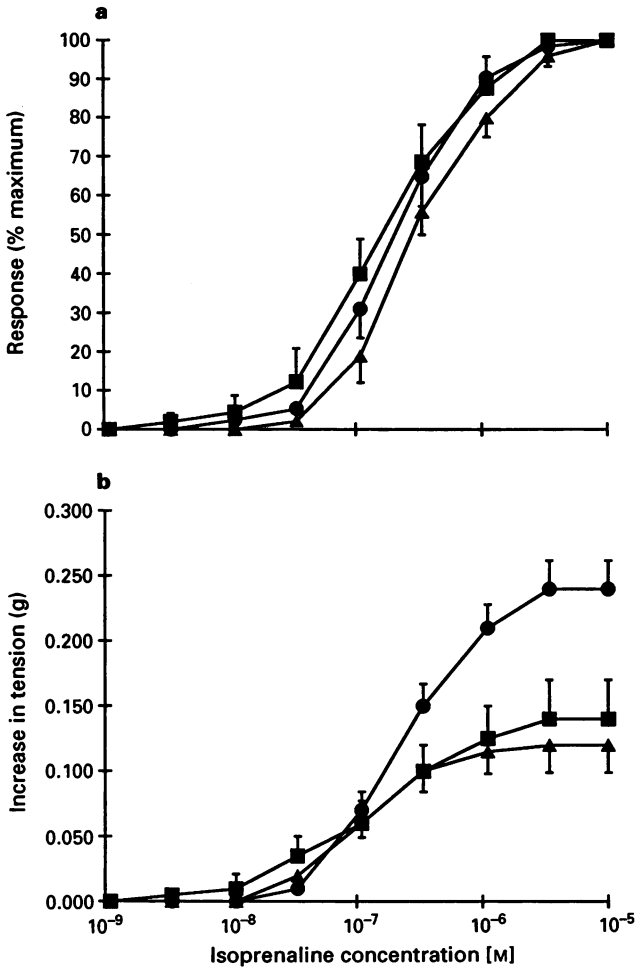


Figure 1 Cumulative concentration-response curves to isoprenaline of papillary muscles from rats infused with saline (●), noradrenaline (1 mg kg^{-1}, ■) or isoprenaline (40 μg kg^{-1}, ▲) for 3 days. Responses are plotted as (a) a percentage of the maximum response and (b) the increase in developed tension induced by isoprenaline.

Drugs used

[^{3}H]-dihydroalprenolol (110 Ci mmol^{-1}) and [^{3}H]-prazosin (23 Ci mmol^{-1}) were obtained from the Radiochemical Centre (Amersham, Bucks.). Phentolamine mesylate was kindly supplied as a gift by Ciba-Geigy. All other drugs were obtained as the hydrochloride salt from Sigma, Poole, Dorset. All reagents were of Analar grade.

Results

Isolated tissues

Paced papillary muscles: Noradrenaline infused for 3 days at a rate of 200 μg kg^{-1} h^{-1} had no significant effect on the *ex vivo* sensitivity of tissues to isoprenaline, phenylephrine or

Table 1 Geometric mean EC_{50} values (with 95% confidence limits) and maximum responses for isoprenaline and phenylephrine on papillary muscles from saline, noradrenaline and isoprenaline pretreated animals

	Isoprenaline			*Phenylephrine*		
Infusion	EC_{50} (nM)	*Max* (g)	n	EC_{50} (μM)	*Max* (g)	n
Saline	207.5 (117–368)	0.24 ± 0.02	10	20.4 (12.7–32.7)	0.09 ± 0.02	10
Noradrenaline	156 (49.6–491)	0.14 ± 0.02**	6	4.0 (3.0–5.3)*	0.13 ± 0.03	6
Isoprenaline	284 (172–469)	0.12 ± 0.02**	10	3.5 (1.5–8.0)**	0.10 ± 0.02	10

*$P<0.05$; **$P<0.01$

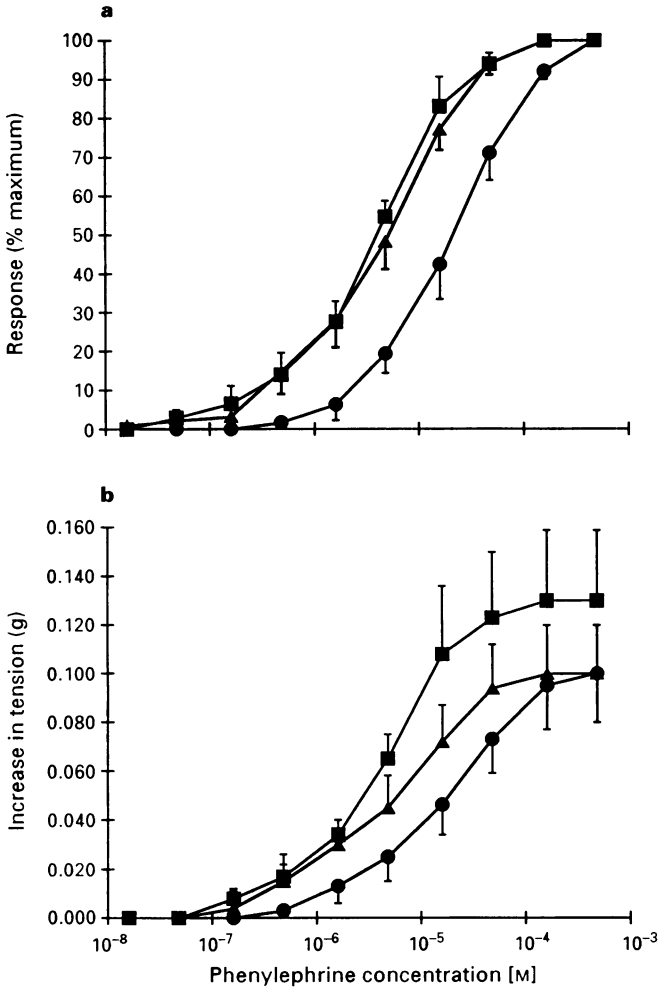


Figure 2 Cumulative concentration-response curves to phenylephrine of papillary muscles from rats infused with saline (●), noradrenaline (1 mg kg^{-1}, ■) or isoprenaline (40 μg kg^{-1}, ▲) for 3 days. Responses are plotted as (a) a percentage of the maximum response and (b) the increase in developed tension induced by isoprenaline.

calcium. When the rate of infusion was increased to 1 mg kg^{-1} h^{-1} for 3 days, β-adrenoceptor-mediated responses of papillary muscles to isoprenaline were depressed, the maximum increases in developed tension induced by isoprenaline being reduced compared with controls, but without a change in EC_{50} values (Figure 1, Table 1). Resting tensions developed by isolated papillary muscles from noradrenaline-infused animals (0.28 ± 0.04 g) were not significantly different from those following saline infusion (0.36 ± 0.04 g). Similar results were obtained when animals were infused with isoprenaline (40 μg kg^{-1} h^{-1}) for 3 days; the resting developed tension of 0.29 ± 0.04 g was similar to controls, but isoprenaline-induced increases in developed tension were depressed (Figure 1, Table 1).

Papillary muscle responses to the α-adrenoceptor agonist, phenylephrine, were enhanced following catecholamine infusions. The maximum changes in developed tension induced by phenylephrine were similar in papillary muscles from control and catecholamine-pretreated animals, but EC_{50} values to phenylephrine were significantly reduced by both noradrenaline and isoprenaline pretreatment (Figure 2, Table 1).

Paced left atria: left atrial responses to isoprenaline were also depressed following catecholamine infusion, the isoprenaline EC_{50} values being significantly increased compared with controls (Table 2). The initial resting tensions developed by isolated left atria before the addition of any drugs were similar for tissues from control (0.31 ± 0.04 g), noradrenaline-infused (0.36 ± 0.06 g) and isoprenaline-infused animals (0.25 ± 0.03 g), and maximum responses to isoprenaline were increased rather than depressed by noradrenaline infusion. Left atrial responses to phenylephrine were not altered by noradrenaline or isoprenaline pretreatment (Table 2).

Spontaneous right atria: right atrial rate responses to isoprenaline were not altered by infusion of any of the catecholamines. Isoprenaline EC_{50} values were 10.5 (6.2–18.8) nM for control tissues and 13.1 (8.2–21.1) nM and 13.7 (17.0–26.9) nM for tissues from noradrenaline- and isoprenaline-infused animals respectively ($n = 6$ for each group). Initial resting spontaneous rates (RAR) and maximum increases in rate (Max) induced by isoprenaline were also similar for tissues from control (RAR = 255 ± 11, Max = 185 ± 15 beats min^{-1}), noradrenaline-infused (RAR = 260 ± 10, Max = 190 ± 4 beats min^{-1}) and isoprenaline-infused animals (RAR = 278 ± 11, Max = 198 ± 6 beats min^{-1}). Phenylephrine in the presence of propranolol failed to induce any significant change in right atrial rate.

Table 2 Geometric mean EC_{50} values (with 95% confidence limits) and maximum responses for isoprenaline and phenylephrine on left atria from saline, noradrenaline and isoprenaline pretreated animals

	Isoprenaline			*Phenylephrine*		
Infusion	*EC_{50}* (nM)	*Max* (g)	n	*EC_{50}* (μM)	*Max* (g)	n
Saline	10.2 (5.7–18.0)	0.35 ± 0.07	7	6.2 (3.9–9.9)	0.15 ± 0.02	6
Noradrenaline	103.4 (55.5–192)*	0.75 ± 0.15*	6	12.9 (5.4–30.9)	0.17 ± 0.03	5
Isoprenaline	38.6 (27.3–53.9)*	0.59 ± 0.08*	6	7.6 (2.6–21.5)	0.13 ± 0.01	6

*$P < 0.05$

Table 3 Density of binding sites (B_{max}, fmol mg^{-1} protein) and dissociation constants (K_d, nM) for [^{3}H]-prazosin and [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) binding to ventricular membranes from saline and catecholamine pretreated rats

	[^{3}H]-prazosin			*[^{3}H]-DHA*		
Infusion	B_{max}	K_d	n	B_{max}	K_d	n
Saline	79.9 ± 3.8	0.06 ± 0.01	7	102.6 ± 7.5	0.52 ± 0.10	8
Noradrenaline	58.6 ± 2.7*	0.06 ± 0.01	5	66.8 ± 5.3*	0.43 ± 0.08	6
Isoprenaline	83.2 ± 5.2	0.04 ± 0.01	5	64.4 ± 5.0*	0.66 ± 0.07	7

*$P < 0.05$

Radioligand binding

Pretreatment of rats with noradrenaline, or isoprenaline for 3 days resulted in a 35–40% decrease in the density of ventricular [^{3}H]-DHA binding sites but the pretreatments did not alter the dissociation constants for the binding of [^{3}H]-DHA (Table 3). Noradrenaline pretreatment had a simliar effect on [^{3}H]-prazosin binding, reducing the density of binding sites without affecting the affinity of binding (Table 3). Ventricular [^{3}H]-prazosin binding characteristics were not affected by infusions of isoprenaline.

Discussion

The present results demonstrate a desensitization of β-adrenoceptor-mediated responses in cardiac tissues examined *ex vivo*, following chronic catecholamine infusion *in vivo*. The mechanisms involved in agonist-induced β-adrenoceptor desensitization have been extensively studied by other workers and have been shown to involve an uncoupling of the receptor from adenylate cyclase followed by an internalization of receptors (see Strasser *et al.*, 1988). In the present study, infusions of isoprenaline or noradrenaline induced a fall in the density of β-adrenoceptor binding sites as measured with [^{3}H]-DHA. It is interesting however, that the desensitization of inotropic β-adrenoceptor-mediated responses was expressed differently in the two cardiac tissues examined. In left atria, desensitization resulted in a rightward shift of concentration-response curves to isoprenaline, but in papillary muscles, maximum responses were depressed without a change in isoprenaline EC_{50} values. We have previously found a similar effect *in vitro* where incubation of tissues with noradrenaline results in a depression of maximum β-adrenoceptor-mediated responses in papillary muscles and a rightward shift of concentration-response curves to isoprenaline in left atria (Chess-Williams, 1987). A β-adrenoceptor reserve in rat atrium has previously been demonstrated following the irreversible inactivation of β-adrenoceptors with bromoacetylalprenololmenthane (BAAM) (Doggrell *et al.*, 1991). These results suggest that there are regional differences in the distribution of β-adrenoceptors in the rat heart which result in a receptor reserve in left atria but not papillary muscles. The density of β-adrenoceptor binding sites in rat atria however, is only half that found in the ventricle (Chess-Williams *et al.*, 1987) and suggests that the coupling of receptor occupation to the response is greater in the left atrium than in the ventricle. This would also explain the significantly lower ($P<0.01$) isoprenaline EC_{50} values obtained for left atria compared with those for papillary muscles.

The depression of tissue responsiveness appeared to be specific for the β-adrenoceptor, since responses to phenylephrine were unaffected by catecholamine infusion in left atria, whilst in papillary muscles, responses to phenylephrine were actually enhanced. The mechanism involved in the increase in cardiac α-adrenoceptor sensitivity is not clear but must be related to the β-adrenoceptor activity of noradrenaline since the selective β-adrenoceptor agonists, isoprenaline, induced similar changes in cardiac α-adrenoceptor sensitivity. An interaction between α- and β-adrenoceptor effects has previously been reported for rat cardiac myocytes (Danziger *et al.*, 1990).

Although ventricular α_1-adrenoceptor-mediated responses to phenylephrine were enhanced, the increase in tissue sensitivity was accompanied by a decrease in the density of [^{3}H]-prazosin binding sites. The mechanism involved in the ventricular α_1-adrenoceptor supersensitivity must therefore lie beyond the receptor. A number of situations (hypothermia, hypothyroidism) result in an inverse regulation of α- and β-adrenoceptor-mediated responses in the heart and an interaction between the second messenger systems for these receptors has been proposed (Kunos & Ishac, 1987). Cardiac β-adrenoceptor activation results in the generation of the second messenger adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Kaumann *et al.*, 1989), whilst α_1-adrenoceptor responses in the heart are mediated via phosphoinositols and protein kinase C (Kaku *et al.*, 1991). In several tissues, interactions between these two second messenger systems have been demonstrated. In platelets, increasing the levels of cyclic AMP inhibit the formation of inositol phosphates (Watson *et al.*, 1984) and also inhibit phosphorylation by protein kinase C (Takai *et al.*, 1982). Furthermore, elevating cyclic AMP levels inhibits the formation of inositol phosphates induced by α_1-adrenoceptor stimulation in rat aorta (Manolopoulos *et al.*, 1991) and kidney (Neylon & Summers, 1988). If such an interaction operates in the heart, a depressed β-adrenoceptor-cyclic AMP system might conceivably result in enhanced α_1-adrenoceptor-mediated responses. A balance between this enhancement of the second messenger system and a simultaneous 'down-regulation' of α-adrenoceptors (as evidenced by the reduction in [^{3}H]-prazosin binding) could explain the differences observed between atrial and ventricular tissues, although one might then have expected an enhancement of atrial α_1-adrenoceptor-mediated responses following isoprenaline infusion. Alternatively, chronic adrenoceptor stimulation may alter the coupling of adrenoceptors to second messenger systems. Treatment of cultured myocytes for two days with noradrenaline has been shown to cause an up-regulation in the levels of two pertussis toxin-sensitive G-proteins (Reithmann *et al.*, 1989). Whatever the mechanism involved in the α_1-adrenoceptor supersensitivity, the results support the hypothesis that myocardial α-adrenoceptors may act as a reserve mechanism to maintain myocardial responsiveness when β-adrenoceptor-mediated responses are depressed.

The present results therefore demonstrate a 'down-regulation' of both α_1- and β-adrenoceptors following chronic noradrenaline infusion in the rat. Although β-adrenoceptor-mediated responses are depressed by this treatment, ventricular α_1-adrenoceptor-mediated responses were enhanced. The mechanism(s) involved in this process require further investigation.

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References

BENFEY, B.G. (1982). Function of myocardial α-adrenoceptors. *Life Sci.*, **31**, 101–112.

CHESS-WILLIAMS, R. (1987). *In vitro* desensitization by noradrenaline of aortic but not myocardial α-adrenoceptor responses. *Br. J. Pharmacol.*, **91**, 328P.

CHESS-WILLIAMS, R.G. & BROADLEY, K.J. (1987). Examination of cardiac alpha-adrenoceptors from pharmacological responses and radioligand binding: comparison of rat and guinea-pig. *J. Pharmacol. Meth.*, **18**, 111–122.

CHESS-WILLIAMS, R., GRASSBY, P.F., BROADLEY, K.J. & SHERIDAN, D.J. (1987). Cardiac alpha- and beta-adrenoceptor sensitivity and binding characteristics after chronic reserpine pretreatment. *Naunyn-Schmiedeb. Arch. Pharmacol.*, **336**, 646–651.

DANZIGER, R.S., SAKAI, M., LAKATTA, E.G. & HANSFORD, R.G. (1990). Interactive α- and β-adrenergic actions of norepinephrine in rat cardiac myocytes. *J. Mol. Cell. Cardiol.*, **22**, 111–123.

DOGGRELL, S.A. (1991). The effects of bromoacetyalprenololmenthane (BAAM), an irreversible β-blocker, on the rat isolated atria and portal vein. In *Adrenoceptors: Structure, Mechanisms and Function. Advances in Pharmacological Sciences.* ed. Szabadi, E. & Bradshaw, C.M. p. 353. Basel: Birkhauser, Verlag.

KAKU, T., LAKATTA, E. & FILBURN, C. (1991). α-adrenergic regulation of phosphoinositide metabolism and protein kinase C in isolated cardiac myocytes. *Am. J. Physiol.*, **260**, C635–C642.

KARLINER, J.S., SIMPSON, P.C., HONBO, N. & WOLOSZYN, W. (1986). Mechanisms and time course of beta-adrenoceptor desensitisation in mammalian cardiac myocytes. *Cardiovasc. Res.*, **20**, 221–228.

KAUMANN, A.J., HALL, J.A., MURRAY, K.J., WELLS, F.C. & BROWN, M.J. (1989). A comparison of the effects of adrenaline and noradrenaline on human heart: the role of β_1- and β_2-adrenoceptors in the stimulation of adenylate cyclase and contractile force. *Eur. J. Pharmacol.*, **10** (suppl. B), 29–37.

KUNOS, G. & ISHAC, E.J.N. (1987). Mechanism of inverse regulation of alpha$_1$- and beta-adrenergic receptors. *Biochem. Pharmacol.*, **36**, 1185–1191.

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

MANOLOPOULOS, V.G., PIPILI-SYNETOS, E., DEN HERTOG, A. & NELEMANS, A. (1991). Inositol phosphates formed in rat aorta after α_1-adrenoceptor stimulation are inhibited by forskolin. *Eur. J. Pharmacol.*, **207**, 29–36.

MUGGE, A., REUPCKE, C. & SCHOLZ, H. (1985). Increased myocardial α_1-adrenoceptor density in rats chronically treated with propranolol. *Eur. J. Pharmacol.*, **112**, 245–252.

NEYLON, C.B. & SUMMERS, R.J. (1988). Inhibition by cAMP of phosphoinositide response to alpha$_1$-adrenoceptor stimulation in rat kidney. *Eur. J. Pharmacol.*, **148**, 441–447.

REITHMANN, C., GIERSCHIK, P., SIDIROPOULOS, D., WERDAN, K. & JACOBS, K.H. (1989). Mechanism of noradrenaline-induced heterologous desensitization of adenylate cyclase in rat heart muscle cells: increase in level of inhibitory G-protein α-subunit. *Eur. J. Pharmacol.*, **172**, 211–221.

REITHMAN, C. & WERDAN, K. (1989). Noradrenaline-induced desensitization in cultured heart cells as a model for the defects of the adenylate cyclase system in severe heart failure. *Naunyn Schmiedeb. Arch. Pharmacol.*, **339**, 138–142.

STEINKRAUS, V., NOSE, M., SCHOLZ, H. & THORMAHLEN, K. (1989). Time course and extent of adrenoceptor density changes in rat heart after β-adrenoceptor blockade. *Br. J. Pharmacol.*, **96**, 441–449.

STRASSER, R.H., CERIONE, J., CODINA, J., CARON, M.G. & LEFKOWITZ, R.J. (1985). Homologous desensitization of the β-adrenergic receptor: functional integrity of the desensitised receptor from mammalian lung. *Mol. Pharmacol.*, **28**, 237–245.

STRASSER, R.H., KRIMMER, J. & MARQUETANT, R. (1988). Regulation of β-adrenergic receptors: impaired desensitization in myocardial ischaemia. *J. Cardiovasc. Pharmacol.*, **12** (suppl. 1), S15.

TAKAI, Y., MINAKUCHI, R., KIKKAWA, U. *et al.* (1982). Membrane phospholipid turnover, receptor function and protein phosphorylation. *Prog. Brain Res.*, **56**, 287–301.

WATSON, S.P., MCCONNEL, R.T. & LAPENTINA, E.G. (1984). The rapid formation of inositol phosphates in human platelets by thrombin is inhibited by prostacyclin. *J. Biol. Chem.*, **259**, 13199–13203.

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Characterization of the effects of adenosine 5′-[β-thio]-diphosphate in rat liver

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1 In rat liver cells micromolar concentrations of adenosine 5′-[β-thio]diphosphate (ADPβS), activate glycogen phosphorylase by an adenosine 3′:5′-cyclic monophosphate (cyclic AMP)- independent mechanism.

2 As with adenosine 5′-triphosphate (ATP), ADPβS also inhibits the rise in cyclic AMP after glucagon.

3 Cytosolic Ca^{2+} measured in single cells is rapidly increased with a pattern similar for ADPβS and for ATP.

4 At variance with ATP, ADPβS hardly increases inositol 1,4,5-trisphosphate (IP_3) levels.

5 Phorbol myristic acetate, which inhibits only slightly the glycogenolytic effect of ATP, almost completely abolishes this effect of ADPβS.

6 With adenosine 5′-[β-[^{35}S]thio]diphosphate (ADPβ[^{35}S]) as radioligand, we detected specific purinoceptors on rat liver plasma membranes. Binding consists of a major binding component with $K_D = 0.7\ \mu M$ and $B_{max} = 51$ pmol mg^{-1} of protein, probably mediating the activation of glycogen phosphorylase, and a minor high affinity, low capacity binding component with no obvious function.

7 It is concluded that the differences in biological effects between ATP and ADPβS may involve different receptors and/or different transduction mechanisms and that ADPβ[^{35}S] can be used to detect the specific binding sites for ADPβS.

Keywords: Purinoceptors; rat liver; glycogenolysis; adenosine 5′-[β-thio]diphosphate

Introduction

In isolated hepatocytes of the rat, extracellular adenosine 5′-triphosphate (ATP) and adenosine 5′-diphosphate (ADP) are able to activate glycogen phosphorylase, which is associated with a rapid increase in inositol 1,4,5-trisphosphate (IP_3) levels and intracellular Ca^{2+}(Keppens & De Wulf, 1985; Charest *et al.*, 1985; Sistare *et al.*, 1985; Okajima *et al.*, 1987). The purinoceptor agonists ATP and ADP, like the other Ca^{2+}-dependent agonists (vasopressin, angiotensin II and the α_1-adrenoceptor agonists) also inhibit the glucagon (Keppens & De Wulf, 1985) and forskolin (Okajima *et al.*, 1987)-induced rise in adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels.

The rank order of potency of several ATP-analogues in activating glycogen phosphorylase (Keppens & De Wulf, 1985) pointed to the presence of P_{2Y}-purinoceptors according to the basic nomenclature of Burnstock & Kennedy (1985) (ATP = ADP > α,β-methylene-ATP, β,γ-methylene-ATP). Using adenosine 5′-[α-[^{35}S]thio]triphosphate (ATPα[^{35}S]) as a radioligand, we have characterized the purinoceptors on rat liver parenchymal cells and plasma membranes (Keppens & De Wulf, 1986), in human liver plasma membranes (Keppens *et al.*, 1989) and in plasma membranes of rabbit and guinea-pig liver (Keppens *et al.*, 1990) and shown that the detected binding site corresponds to a P_{2Y}-type of purinoceptor, mediating the glycogenolytic effect. Extracellular UTP induces glycogenolysis in rat hepatocytes in a manner very similar if not identical to ATP, suggesting that both agonists bind to the same receptor (Keppens *et al.*, 1992). On the other hand, important differences in biological effects between 2-methylthio adenosine triphosphate (2MeSATP), known to be the most potent P_{2Y}-agonist in several tissues, and ATP have been demonstrated in rat liver cells (Keppens & De Wulf, 1991). Although 2MeSATP is indeed more potent in activating glycogen phosphorylase than is ATP, thereby fitting the P_{2Y}-classification, the following results, at variance with those obtained with ATP, suggest that 2MeSATP probably binds to another type of P_2-purinoceptor: (a) IP_3-levels are hardly increased; (b) no anti-glucagon effect on cyclic AMP levels could be detected; (c) addition of phorbol myristic acetate totally blocked the activation of phosphorylase. Therefore rat liver, like other cell types (O'Connor *et al.*, 1991; O'Connor, 1992), may contain a heterogeneous 'mixed' receptor population with the following rank order of potency for glycogenolysis: 2MeSATP > ATP = ADP = UTP.

In turkey erythrocytes, adenosine 5′-[β-thio]diphosphate (ADPβs) activates a guanine nucleotide-sensitive phospholipase C with a severalfold increase in inositol phosphates (Boyer *et al.*, 1989; Martin & Harden, 1989; Berrie *et al.*, 1989). Cooper *et al.* (1989) used adenosine 5′-[β-[^{35}S]thio]-diphosphate (ADPβ[^{35}S]) as radioligand to study the P_{2Y}-receptors present on turkey erythrocyte plasma membranes.

This study was undertaken to evaluate the use of ADPβS and ADPβ[^{35}S] as suitable agonist and radioligand for the rat liver P_{2Y}-purinoceptors.

Methods

We used male Wistar-strain albino rats (200–250 g body weight) that were fed *ad libitum*.

Liver cells were isolated and incubated in a Krebs-Henseleit bicarbonate buffer equilibrated with O_2/CO_2 (19:1, v/v) as previously described (Vandenheede *et al.*, 1976) but without bacitracin. Liver plasma membranes were prepared according to the method of Pilkis *et al.* (1974) with slight modifications (Keppens & De Wulf, 1985). The plasma membranes were kept at −80°C until use.

Glycogen phosphorylase activity was determined as described by Vandeheede *et al.* (1976).

Cyclic AMP and IP_3 were measured with a competitive protein-binding technique, the former (Gilman, 1970) by

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using an assay kit from the Radiochemical Centre (Amersham, Bucks, U.K.) and the latter as described by Bredt *et al.* (1989) with slight modifications (Keppens *et al.*, 1992).

Intracellular Ca^{2+} was estimated by Fura-2 fluorescence. Cells were loaded with the acetoxymethylester of Fura-2 by incubation of hepatocytes, 0.5 to 1×10^6 cell ml^{-1}, in a solution containing (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 10, glutamate 4.5, pyruvate 5, HEPES 20 and $CaCl_2$ 2 pH 7.4 and supplemented with 1% bovine serum albumen (BSA), 0.05% pluronic acid, 10 μM Fura-2-AM and 0.5% dimethylsulphoxide at 30°C for about 20 to 30 min. The cells were then extensively washed and transferred into a Petri dish with a thin glass bottom, lightly coated with polylysine. The dish was mounted on a microscope (inverted Nikon Diaphot), equipped with a dual excitation microspectrofluorimetric system (Newcastle Photometric System). Fura-2 was alternately excited at 340 and 380 nm and emission fluorescence was collected at 510 nm. Measurements were performed at room temperature. $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence according to the equation $[Ca^{2+}] = K_D{*}B{*}(R-R_{min})/R_{max}\text{-}R)$ (Grynkiewicz *et al.*, 1985), with $K_D = 224$ nM, B the ratio of the fluorescence signals obtained by illuminating the cells at 380 nM taken at 0 (EGTA) or saturating Ca^{2+}-concentrations (ionomycin, 4-bromo A23187). R represents the ratio at any time whereas R_{max} and R_{min} are the ratios obtained at saturating, zero calcium respectively. The autofluorescence never exceeded 10% of the fluorescence of a Fura-loaded cell and was subtracted from the total fluorescence.

ADPβ[^{35}S] binding to purified liver plasma membranes was carried out according to the method described for ATPα[^{35}S] (Keppens & De Wulf, 1985). Briefly, ADPβ[^{35}S] was incubated at 37°C with liver plasma membranes (± 0.6 mg of protein ml^{-1}); bound and unbound ligand were separated by filtration on a Whatman GF/A filter. Non-specific binding was estimated in the presence of an excess of ADP and never exceeded 12% of the total binding. Analysis of the binding parameters was done with the computer programme 'Enzfitter' (Leatherbarrow, 1987) or with the fitting facilities of the graphic package Fig.P, used for drawing the figures.

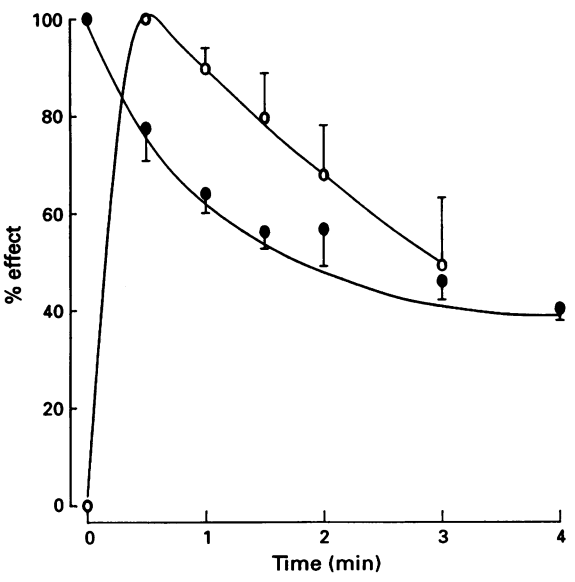


Figure 1 Time-dependent effect of adenosine 5'-[β-thio]diphosphate (ADPβS) on the activation of phosphorylase and of its inhibition of the cyclic AMP increase. Rat hepatocytes were preincubated at 37°C for 25 min with 10 mM glucose. For the activation of phosphorylase (○), 5 μM ADPβS was then added. Enzyme activity was determined at the indicated time points and is expressed as percentage of effect. Basal levels of phosphorylase *a* (10 ± 4 mu mg^{-1} of protein) were increased to 85 ± 5 mu mg^{-1} of protein (100%) after 30 s. For the inhibition of the cyclic AMP increase (●), cells were first challenged with 20 nM glucagon; 2 min later, 200 μM ADPβS was added and cyclic AMP levels were measured at the indicated time points. Basal values of cyclic AMP (4.1 ± 0.7 pmol mg^{-1} protein) were increased to 33 ± 3 pmol mg^{-1} protein (100%) 2 min after glucagon. Values are given as means ± s.e.mean ($n = 4$).

Materials

ADPβS (adenosine 5'-[β-thio]diphosphate) was from Boehringer, Mannheim (Germany), ADPβ[^{35}S] (adenosine 5'-[β-[^{35}S]thio]-diphosphate; specific activity 45.9 TBq $mmol^{-1}$, 1240 Ci $mmol^{-1}$) was purchased from New England Nuclear, Du Pont-NEN Research products, Brussels (Belgium). ATP, phorbol 12-myristic 13-acetate, ionomycin and collagenase type 1 were from Sigma Chemical Co., St. Louis, MO (U.S.A.). Fura 2 AM, pluronic acid and 4-bromo A23187 were from Molecular Probes Inc., Eugene, OR (U.S.A.). [^{3}H]-inositol-(1,4,5)-trisphosphate was from Amersham International, Amersham, Bucks. (U.K.). The sources of other chemicals have been given previously (Keppens *et al.*, 1989).

Results

We first compared some biological effects of ATP and ADPβS in rat hepatocytes in order to check whether the latter behaves as a P_2-purinceptor agonist.

Figure 1 shows the kinetics of ADPβS on the activation of glycogen phosphorylase and on the inhibition of the cyclic AMP rise after glucagon. Phosphorylase is already maximally activated after 30 s and declines afterwards. The anti-glucagon effect proceeds more slowly (maximum after about 2 min) but lasts longer. This is identical to what has been found for ATP (Keppens & De Wulf, 1985). The dose-dependent activation of phosphorylase (Figure 2a), estimated 30 s after the addition of increasing concentrations of ADPβS, reveals a K_a (half-maximal activation constant) value of ≈1 μM as was also obtained earlier with ATP (Figure 2a, dashed line, Keppens & De Wulf, 1985). The anti-glucagon effect of ADPβS is also similar to that of ATP (Figure 2b) with a K_i of about 20 μM, indicating that higher concentrations are needed to suppress the glucagon effect than to activate phosphorylase. Like ATP, ADPβS on its own does not influence cyclic AMP levels (not shown).

Since Ca^{2+} is proposed to be the main second messenger used by the P_2-purinoceptor agonists, we studied the rise in cytosolic Ca^{2+}. The experiments were performed on single hepatocytes to avoid the possibility that differences might be masked in whole cell suspensions. The cells were loaded with Fura 2 and superfused with 2 μM ADPβS or 2 μM ATP. A typical result is illustrated in Figure 3. The overall pattern of the Ca^{2+}-mobilization after the addition of both agonists is very similar. A rapid elevation in cytosolic Ca^{2+}can be detected, reaching a maximal value and then declining to a plateau, which persists as long as ATP or ADPβS is present. However, it should be noted that occasionally, some cells, clearly responsive to ATP, did not mobilize Ca^{2+} when superfused with ADPβS (not shown).

Next, we examined the generation of IP_3 by either agonist in the hepatocytes. Earlier studies (Charest *et al.*, 1985; Keppens *et al.*, 1992) revealed that higher concentrations of ATP are needed to increase IP_3 ($K_{IP} = 40$ μM) than to activate phosphorylase and that the IP_3-rise is very rapid (maximal after 5 s) and transient. However, when using ADPβS, it was found that only very high concentrations could augment IP_3-levels. Figure 4 shows the levels of IP_3, 5 s after 100 μM ATP or 100 μM ADPβS had been added. Clearly, ADPβS is far less able to generate the second messenger. It was checked that for ADPβS, a maximal response was also reached within 5 s. We checked that a 10 fold excess of ADPβS over ATP did not inhibit the rise of IP_3 normally observed after ATP (two independent experiments, data not shown).

To compare ATP and ADPβS further, we studied the effect of phorbol myristic acetate (PMA). PMA is known to in-

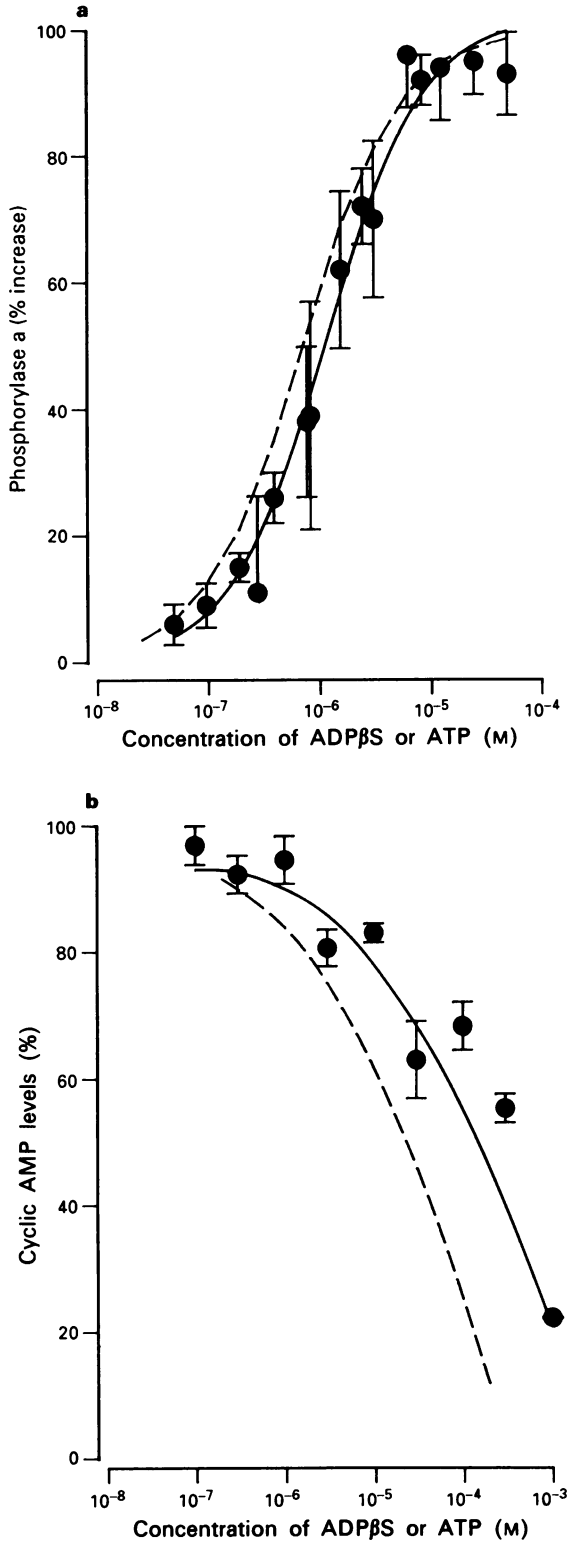


Figure 2 Dose-dependent effects of adenosine 5'-[β-thio]diphosphate (ADPβS) and ATP on the activation of glycogen phosphorylase and of the inhibition of the cyclic AMP increase. Rat hepatocytes were preincubated at 37°C for 25 min with 10 mM glucose. (a) They were then treated with increasing concentrations of ADPβS (●; solid line) for 30 s. The dashed line represents the theoretical curve for ATP with $K_a = 0.7\,\mu M$ (Keppens & De Wulf, 1985). (b) The cells were challenged for 2 min with glucagon (20 nM) and then for 2 min with ADPβS (●; solid line). The dashed line represents the curve for ATP with $K_i = 15\,\mu M$ (Keppens *et al.*, 1991). Results are expressed as percentage effect and are means ± s.e.mean. ($n = 4$). Lines drawn for the activation of phosphorylase are computer generated using the fitting facilities of the FigP programme or hand drawn for the inhibition of cyclic AMP increases.

crease the K_a value of phosphorylase activation after ATP (Keppens & De Wulf, 1991 and Figure 5b) about 2–3 fold. However, when used against ADPβS (Figure 5a), the activation of the enzyme was almost completely abolished.

In a further series of experiments, we examined the binding of ADPβ[^{35}S] to liver plasma membranes. Figure 6 illustrates the time-dependent association pattern for three different concentrations of ADPβ[^{35}S] and the dissociation of bound ligand induced by the addition of an excess of ADP. The association, fitting a first order equation, is rapid and an apparent equilibrium is reached in about 2 min. From the

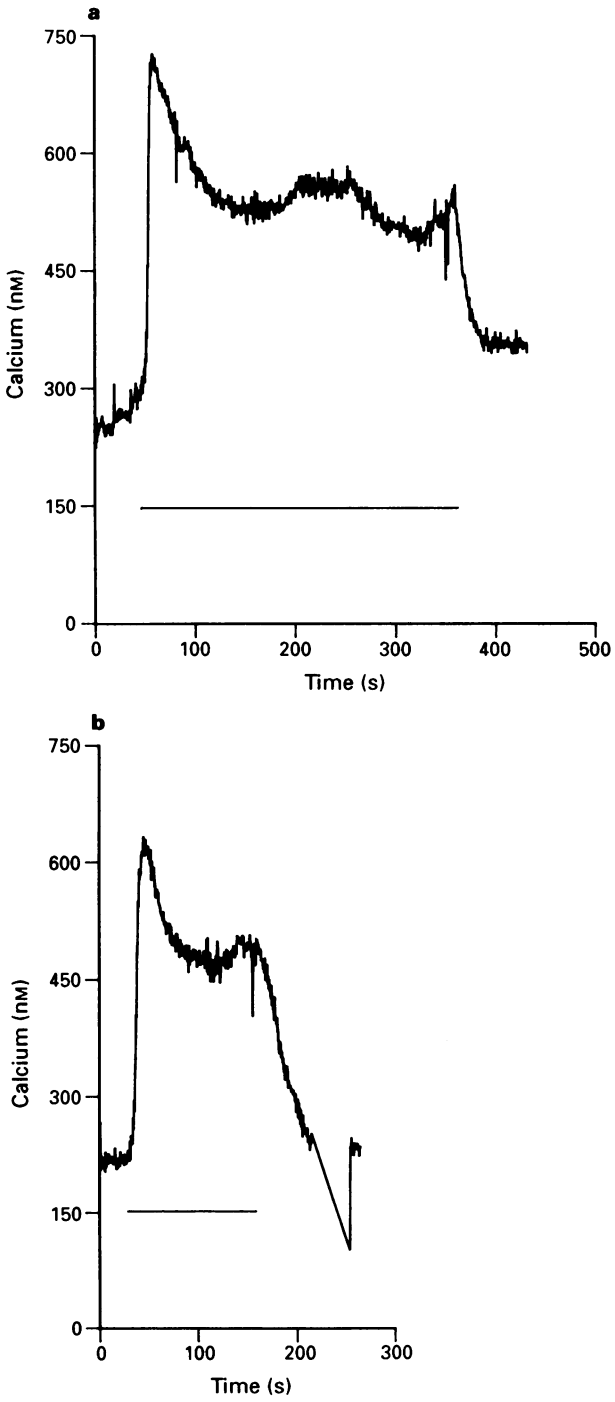


Figure 3 Increase of intracellular Ca^{2+}after adenosine 5'-[β-thio]diphosphate (ADPβS) and ATP in single hepatocytes loaded with Fura 2. A single hepatocyte was loaded with Fura 2 and superfused with buffer containing 2 μM ATP (a) or 2 μM ADPβS (b) during the time period indicated by the horizontal bar. Calcium concentrations were calculated as described in Methods.

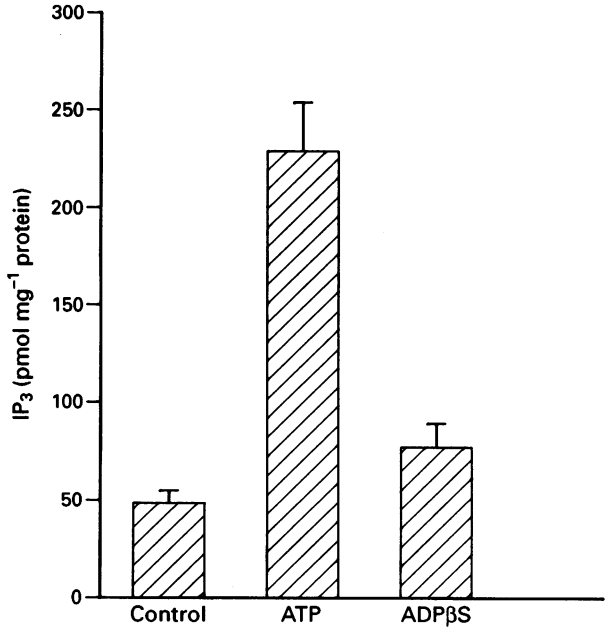


Figure 4 Effects of adenosine 5'-[β-thio]diphosphate (ADPβS) and ATP on inositol-(1,4,5)-trisphosphate (IP_3)-levels in hepatocytes. Hepatocytes were preincubated for 20 min at 37°C with 10 mM glucose. They were then challenged with 0.1 mM ADPβS or 0.1 mM ATP. Samples for IP_3-assay were taken 5 s later. Data shown are the means ± s.e.mean of three independent experiments, each done in triplicate. For ADPβS, the *t* test of independent data gave a *P* value of 0.05.

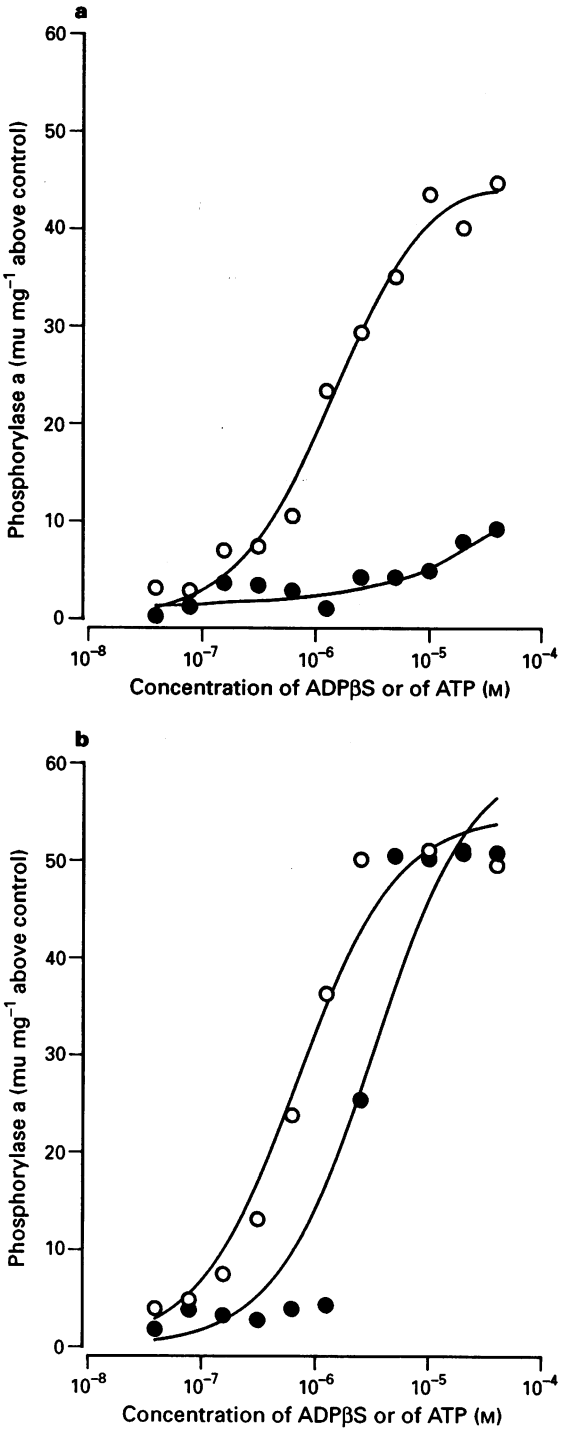


Figure 5 Effect of phorbol treatment of hepatocytes on the activation of glycogen phosphorylase by adenosine 5'-[β-thio]diphosphate (ADPβS) or ATP. Hepatocytes were first preincubated for 20 min at 37°C with 10 mM glucose and for another 10 min with either 1% dimethyl-sulphoxide (○) or phorbol myristic acetate (1.6 μM) dissolved in DMSO (●). Afterwards, increasing concentrations of ADPβS (a) or ATP (b) were added. Control values of phosphorylase were 9.64 ± 1.59 mu mg^{-1} of protein. Results shown are the mean of three independent experiments. Lines drawn are computer generated, using the fitting facilities of the FigP programme.

binding date, a k_{+1} (association rate constant) of 7.5 μM^{-1} min^{-1} has been calculated. The dissociation is also rapid with a k_{-1} (dissociation rate constant) of 3.5 min^{-1}. The ratio of both kinetic constants yields a dissociation constant K_D of about 0.5 μM. However, the dissociation is not complete and the reversibility of the binding but not the dissociation rate decreases with time. For the direct determination of the dissociation constant, different concentrations of the radioligand were incubated with plasma membranes for 1 min. We limited the incubation time to 1 min since at that time equilibrium is nearly obtained and reversibility is still more than 60% (Figure 6). Figure 7 shows the dose-dependent binding of ADPβ[^{35}S] to liver plasma membranes and the inset represents the Scatchard plot of the binding data. There is one major binding component with a B_{max} value of 51 pmol mg^{-1} of protein and with a dissociation constant K_D of 0.7 μM, a value close to the one deduced from the kinetic experiments. There is also a second but minor binding component with a B_{max} of 2.3 pmol mg^{-1} of protein and a dissociation constant in the nanomolar region (7 nM).

Discussion

ADPβS, the nonhydrolysable analogue of ADP, has been shown to elicit a P_{2Y}-purinoceptor-mediated response in plasma membranes of turkey erythrocytes. The agonist is even more powerful than ATP in stimulating inositol phosphate formation via a G-protein-dependent-phospholipase C (Boyer *et al.*, 1989; Berrie *et al.*, 1989) and could be used as a desensitizing agent for the enzyme (Martin & Harden, 1989). Moreover, the radioactive compound ADPβ[^{35}S] radiolabelled specifically the P_{2Y}-purinoceptor binding sites (Cooper *et al.*, 1989). It was therefore of interest to investigate the usefulness of ADPβS and ADPβ[^{35}S] as, respectively, a P_{2Y}-purinoceptor agent and radioligand for rat liver.

Firstly, the biological effects of ADPβS were compared with those of ATP. The results lead to distinct conclusions. The kinetics and dose-dependencies of the activation of glycogen phosphorylase (Figure 1 and Figure 2a) and of the inhibition of the increase in cyclic AMP levels after glucagon (Figure 1 and Figure 2b) as well as the increase in cytosolic Ca^{2+} (Figure 3) are almost identical for both agonists. These data could be interpreted to mean that binding occurs at the same P_{2Y}-type of purinoceptor. This conclusion is however

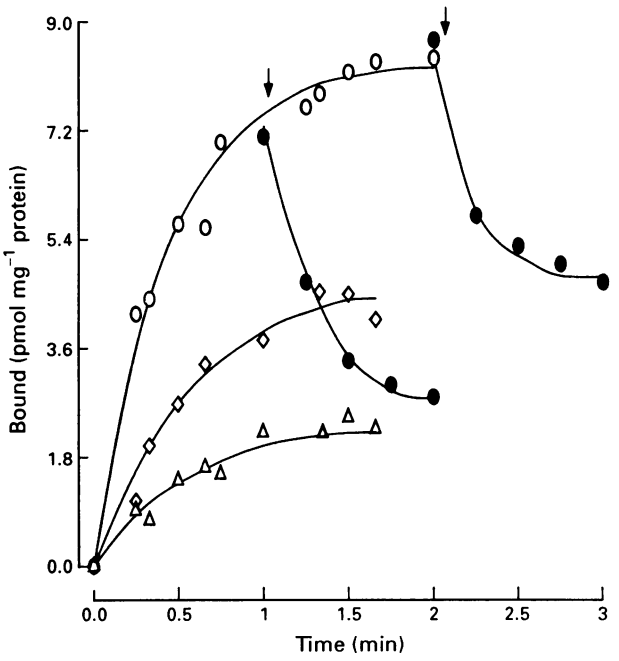


Figure 6 Association-dissociation pattern for the binding of adenosine 5'-[β-[^{35}S]thio]diphosphate (ADPβ[^{35}S]) to rat liver plasma membranes. Plasma membranes (0.6 mg of protein ml^{-1}) were incubated with 130 nM (○), 65 nM (◇) or 32 nM (△) ADPβ[^{35}S]. An excess of ADP (0.1 mM) was then added at the indicated times (arrows). Specific binding is plotted as a function of time. Values shown are means ($n = 3$ for the association and $n = 2$ for the dissociation).

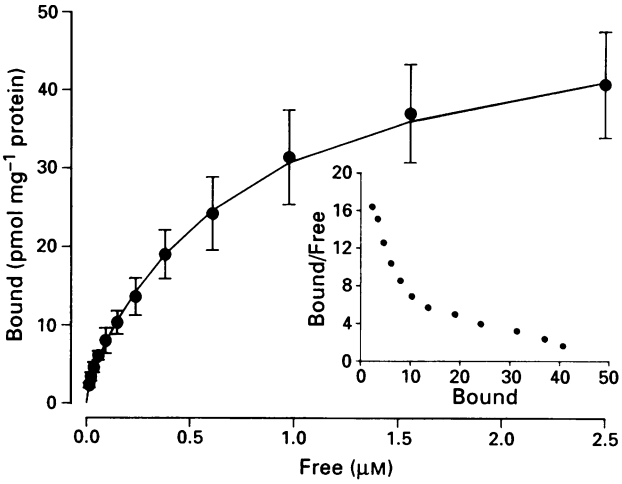


Figure 7 Dose-dependency of specific adenosine 5'-[β-[^{35}S]thio]-diphosphate (ADPβ[^{35}S]) binding to rat liver plasma membranes. Rat liver plasma membranes (0.6 mg of protein ml^{-1}) were incubated with the indicated concentrations of APDβ[^{35}S]. Specific binding was measured after 1 min; the values are given as means ± s.e.mean ($n = 5$). The inset shows the Scatchard plot of the binding data.

not consistent with other results. Indeed, in contrast to ATP but similar to 2MeSATP (Keppens & De Wulf, 1991; see also Introduction), which is also a powerful glycogenolytic agent, we found that ADPβS generated IP_3 only very moderately, even at very high concentrations, and that PMA almost completely counteracted its glycogenolytic effect. This either indicates that ADPβS interacts with poor affinity to the site linked to the activation of phospholipase C, or, alternatively, does interact with this site but produces only a partial effect. In both cases, a small increase (possibly sufficient for full activation of phosphorylase) is observed. In the latter case, and assuming that the affinity of ADPβS is about 0.7 μM (see later), one would expect ADPβS to inhibit the rise of IP_3 normally observed after ATP. We did not observe such an inhibition, suggesting that ADPβS, as also proposed for 2MeSATP (Keppens & De Wulf, 1991) binds to other P_2-purinoceptors, different from the classical P_{2Y}-receptor. It is however very unlikely that ADPβS and 2MeSATP activate the same binding site: indeed, in contrast to ADPβS (Figure 1b,2b), 2MeSATP is not able to suppress the cyclic AMP-rise after glucagon (Keppens & De Wulf, 1991).

The overall conclusion from our results with ADPβS is that this agonist may bind to a third type of P_2-receptor in rat liver parenchymal cells. This is in contrast with the situation in turkey erythrocytes in which ADPβS, like 2MeSATP, is a very powerful P_{2Y}-purinoceptor agonist (Boyer *et al.*, 1989; Berrie *et al.*, 1989; Martin & Harden, 1989). Apparently, the liver plasma membrane contains a complex mixture of P_2-purinoceptors with different affinities for different analogues. Alternatively, other transducing mechanisms could be triggered.

Okajima *et al.* (1987) demonstrated that for a series of purinoceptor agents, all activating phosphorylase and inhibiting the cyclic AMP-rise after forskolin treatment, two groups of agonists could be distinguished. One group, including GTP and ADP, exhibits similar abilities to that of ATP in raising inositolphosphate levels, whereas a second group, including α,β-methylene-ATP and β,γ-methylene-ATP affects only slightly inositol lipid metabolism. According to this classification, ADPβS would then belong to the second group of agonists.

The technique of recording Ca^{2+}-concentrations in single cells allows detection of differences at the cellular level. The fact that some cells, although sensitive to ATP, did not respond to ADPβS, could indicate that not all hepatocytes possess ADPβS-binding sites. Dixon *et al.* (1990) found differences between ATP and ADP in mobilizing intracellular Ca^{2+} in single cells. These authors obtained oscillations with clearly different frequencies for ATP and ADP and suggested the presence of two Ca^{2+}-mobilizing purinoceptors.

In the first part of this study, we have tentatively concluded that there are specific binding site(s) for ADPβS on rat hepatocytes. The question then arose whether ADPβ[^{35}S] could be used for further characterization of these site(s). From Figure 6 it is clear that although the binding is not fully reversible, the radioligand rapidly and specifically interacts with the liver plasma membrane receptor(s). A dissociation constant of about 0.7 μM could be derived from the kinetic experiments as well as from the equilibrium binding data (Figure 7). This binding component exhibits a K_D-value very close to the half-maximal activation of phosphorylase ($K_a = 1$ μM) by ADPβS and may therefore mediate the glycogenolytic effect of the agonist. The role for the minor binding component with a K_D of 7 nM, derived from the equilibrium binding study and representing about 5% of the total binding, is not obvious since no biological effect of ADPβS is observed at or below that concentration.

ADPβ[^{35}S] thus seems to be a suitable radioligand for detecting the binding sites mediating the biological effects of ADPβS in rat liver cells, bearing in mind that these sites differ from those used by ATP as well as by 2MeSATP.

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References

BERRIE, C.P., HAWKINS, P.T., STEPHENS, L.R., HARDEN, T.K. & DOWNES, C.P. (1989). Phosphatidylinositol 4,5-bisphosphate hydrolysis in turkey erythrocytes is regulated by P_{2Y}-purinoceptors. *Mol. Pharmacol.*, **35**, 526–532.

BOYER, J.L., DOWNES, C.P. & HARDEN, T.K. (1989). Kinetics of activation of phospholipase C by P_{2Y} purinergic receptor agonists and guanine nucleotides. *J. Biol. Chem.*, **264**, 884–890.

BREDT, D.S., MOUREY, R.J. & SNYDER, S.H. (1989). A simple, sensitive and specific radioreceptor assay for inositol 1,4,5-trisphosphate in biological tissues. *Biochem. Biophys. Res. Commun.*, **159**, 976–982.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen. Pharmacol.*, **16**, 433–440.

CHAREST, R., BLACKMORE, P.F. & EXTON, J.H. (1985). Characterization of responses of isolated hepatocytes to ATP and ADP. *J. Biol. Chem.*, **260**, 15789–15794.

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

DIXON, C.J., WOODS, N.M., CUTHBERTSON, K.S.R. & COBBOLD, P.H. (1990). Evidence for two Ca^{2+}-mobilizing purinoceptors on rat hepatocytes. *Biochem. J.*, **269**, 499–502.

GILMAN, A.G. (1970). A protein binding assay for adenosine 3':5'-cyclic monophosphate. *Proc. Natl. Acad. Sci. U.S.A.*, **67**, 305–312.

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

KEPPENS, S. & DE WULF, H. (1985). P_2-purinergic control of the liver glycogenolysis. *Biochem. J.*, **231**, 797–799.

KEPPENS, S. & DE WULF, H. (1986). Characterization of the liver P_2-purinoceptor involved in the activation of glycogen phosphorylase. *Biochem. J.*, **240**, 367–371.

KEPPENS, S. & DE WULF, H. (1991). Characterization of the biological effects of 2-methylthio-ATP on rat hepatocytes: clear-cut differences with ATP. *Br. J. Pharmacol.*, **104**, 301–304.

KEPPENS, S., VANDEKERCKHOVE, A. & DE WULF, H. (1989). Characterization of purinoceptors present on human liver plasma membranes. *FEBS Lett.*, **248**, 137–140.

KEPPENS, S., VANDEKERCKHOVE, A. & DE WULF, H. (1990). Characterization of the purinoceptors present in rabbit and guinea pig liver. *Eur. J. Pharmacol.*, **182**, 149–153.

KEPPENS, S., VANDEKERCKHOVE, A. & DE WULF, H. (1992). Extracellular ATP and UTP exert similar effects on rat isolated hepatocytes. *Br. J. Pharmacol.*, **105**, 475–479.

LEATHERBARROW, R.J. (1987). *Enzfitter, a Non Linear Regression Data Analysis Program for the IBM PC.* Amsterdam, The Netherlands: Elsevier Science Publications BV.

MARTIN, M.W. & HARDEN, T.K. (1989). Agonist-induced desensitization of a P_{2Y}-purinergic receptor-regulated phospholipase C. *J. Biol. Chem.*, **264**, 19535–19539.

O'CONNOR, S.E., DAINTY, I.A. & LEFF, P. (1991). Further subclassification of ATP receptors based on agonist studies. *Trends Pharmacol. Sci.*, **12**, 137–141.

O'CONNOR, S.E. (1992). Recent developments in the classification and functional significance of receptors for ATP and UTP, evidence for nucleotide receptors. *Life Sci.*, **50**, 1657–1664.

OKAJIMA, F., TOKOMITSU, Y., KONDO, Y. & UI, M. (1987). P_2-purinergic receptors are coupled to two signal transduction systems leading to inhibition of cyclic AMP generation and to production of inositol trisphosphate in rat hepatocytes. *J. Biol. Chem.*, **262**, 13483–13490.

PILKIS, S.J., EXTON, J.H., JOHNSON, R.A. & PARK, C.R. (1974). Effects of glucagon on cyclic AMP and carbohydrate metabolism in livers from diabetic rats. *Biochim. Biophys. Acta*, **343**, 250–267.

SISTARE, F.D., PICKING, R.A. & HAYNES, R.C. Jr. (1985). Sensitivity of the response of cytosolic calcium in Quin-2-loaded rat hepatocytes to glucagon, adenine nucleosides and adenine nucleotides. *J. Biol. Chem.*, **260**, 12744–12747.

VANDENHEEDE, J.R., KEPPENS, S. & DE WULF, H. (1976). The activation of phosphorylase *b* kinase by glucagon. *FEBS Lett.*, **61**, 213–217.

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Mediation of the antidepressant-like effect of 8-OH-DPAT in mice by postsynaptic 5-HT_{1A} receptors

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1 The 5-hydroxytryptamine $(5\text{-HT})_{1A}$ agonist 8-hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT) has been evaluated in a mouse model for detecting potential antidepressants (Porsolt test). The effects of various receptor antagonists, lesions of brain monoaminergic neurones and chronic drug treatments on this 8-OH-DPAT-induced response have also been determined.

2 8-OH-DPAT (0.3–10.0 mg kg^{-1}, s.c.) dose-dependently increased the mobility of mice in the Porsolt test. Other selective 5-HT_{1A} receptor ligands (0.3–30 mg kg^{-1}, s.c.) either mimicked the 8-OH-DPAT response (ipsapirone, at 10 and 30 mg kg^{-1}, s.c.) or were inactive (buspirone and gepirone). However, each of these compounds ($\leqslant$ 100 mg kg^{-1}, p.o.) inhibited the response to 8-OH-DPAT (3 mg kg^{-1}, s.c.) when given concurrently.

3 The putative 5-HT_{1A} antagonists, spiroxatrine (1–30 mg kg^{-1}, p.o.), (±)-pindolol (30 mg kg^{-1}, p.o.) and methiothepin (3–10 mg kg^{-1}, p.o.), each attenuated the 8-OH-DPAT (3 mg kg^{-1}, s.c.)-induced increase in mobility.

4 The dopamine D_1 receptor antagonist, SCH 23390 (3–10 mg kg^{-1}, p.o.), weakly reversed the 8-OH-DPAT response. Antagonists at 5-HT_{1C}/5-HT_2 receptors (ketanserin; 0.1–3.0 mg kg^{-1}, p.o.), 5-HT_3 receptors (ondansetron; 0.03–10 mg kg^{-1}, p.o.), α_1-adrenoceptors (prazosin; 1–3 mg kg^{-1}, p.o.), α_2-adrenoceptors (idazoxan; 3–30 mg kg^{-1}, p.o.), β_1-adrenoceptors (metoprolol; 1–30 mg kg^{-1}, p.o.), β_2-adrenoceptors (ICI 118,551; 1–30 mg kg^{-1}, p.o.), dopamine D_2 receptors (sulpiride; 10–300 mg kg^{-1}, p.o.) and opiate receptors (naloxone; 3–100 mg kg^{-1}, p.o.) had no effect on the 8-OH-DPAT response.

5 Selective destruction of 5-HT neurones with 5,7-dihydroxytryptamine or inhibition of 5-HT synthesis with *p*-chlorophenylalanine did not change the 8-OH-DPAT response in the Porsolt test. This response was also unaltered by pretreatment with the noradrenergic neurotoxin, DSP-4.

6 Administration of 8-OH-DPAT (3 mg kg^{-1}, s.c.) twice-daily for 10 days attenuated the hypothermia, but not the increased mobility, induced by 8-OH-DPAT (3 mg kg^{-1}, s.c.). Similarly, repeated administration of amitriptyline (3–30 mg kg^{-1}), desipramine (3–30 mg kg^{-1}) or dothiepin (10–100 mg kg^{-1}) also attenuated the former, but not the latter, response.

7 We conclude that 8-OH-DPAT produces an antidepressant-like effect in the Porsolt test which is mediated via postsynaptic 5-HT_{1A} receptors.

Keywords: 8-Hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT); antidepressant; 5-HT_{1A} receptors; Porsolt test

Introduction

8-Hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT) is a selective agonist at the 5-HT_{1A} receptor subtype (Hjorth *et al.*, 1982; Peroutka, 1988). It induces a number of behavioural and physiological changes in rodents. For example, hypothermia has been observed in both mice and rats (Goodwin *et al.*, 1985a,b; Hjorth, 1985). Other behavioural effects include induction of a weak '5-HT syndrome' in rats (Hjorth *et al.*, 1982; Tricklebank *et al.*, 1984), but not in C57/Bl/6 mice (Goodwin & Green, 1985; Heal *et al.*, 1989a), and alterations in rat feeding behaviour (Dourish *et al.*, 1985; Hutson *et al.*, 1988). Furthermore, this compound has been shown to exert cardiovascular effects which are centrally mediated and may involve α_2-adrenoceptors (Fozard *et al.*, 1987).

Recently, it has also been reported that 8-OH-DPAT has an antidepressant-like effect in several rat models of depression (Cervo & Samanin, 1987; Kennett *et al.*, 1987; Giral *et al.*, 1988; Wieland & Lucki, 1990). Other 5-HT_{1A} ligands, buspirone, gepirone and ipsapirone, are also active in these models (Kennett *et al.*, 1987; Giral *et al.*, 1988). This finding is in agreement with recent reports of the antidepressant efficacy of these compounds in clinical trials (Beneke *et al.*, 1988; Cott *et al.*, 1988; Fabre, 1990; Robinson *et al.*, 1990), in addition to their more well-established anxiolytic activity (Robinson, 1987; Beneke *et al.*, 1988; Cott *et al.*, 1988; Robinson *et al.*, 1990).

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The effects of 8-OH-DPAT in a mouse paradigm capable of identifying antidepressant drugs (Porsolt *et al.*, 1977; Porsolt, 1981; Buckett *et al.*, 1982) have now been studied. Since 8-OH-DPAT had an antidepressant-like action in this test of increasing the mobility of mice, we have characterized this response using a range of receptor antagonists and determined whether the antidepressant-like effect is mediated by pre- or postsynaptic 5-HT_{1A} receptors by administration of the 5-HT neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT) or the 5-HT synthesis inhibitor, *p*-chlorophenylalanine (PCPA). We have also investigated whether noradrenergic neurones modulate the 8-OH-DPAT response by selectively lesioning central noradrenergic neurones with DSP-4 (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine). Having characterized the 8-OH-DPAT response, the effects of repeated administration of 8-OH-DPAT or antidepressants on 8-OH-DPAT-induced mobility in the Porsolt test have been determined.

Some of the data have previously appeared in abstract form (Luscombe *et al.*, 1988; 1989a,b).

Methods

Animals

Male CD1 mice (20–26 g; Charles River) were used in all studies. They were housed in a temperature-controlled room

(21 ± 2°C) on a 12 h light-dark cycle, with food and water *ad libitum*. Approximately 6 h before the test, the animals were divided into groups of five (4 g weight range within the test), food was removed and they were transferred to the temperature-controlled test room (21 ± 2°C).

Experimental procedures

Porsolt test (Porsolt, 1981); measurement of antidepressant activity Drugs were administered either subcutaneously or orally as indicated below; where two treatments were administered the oral dose was given immediately prior to subcutaneous injection. Details of chronic drug treatments and pretreatments given to lesion brain monoamine systems are given below. One hour after administration of the final treatment the behaviour of the mice was observed immediately prior to placing them in the test apparatus. The mobility of individual mice was measured by a Doppler radar system (Buckett *et al.*, 1982) between 2 min and 6 min after placing them in the test apparatus (Porsolt *et al.*, 1977). All tests were conducted between 14 h 00 min and 16 h 30 min.

Measurement of body temperature Body temperature was measured by insertion of a thermistor probe into the oesophagus. The temperature of mice was determined 0.5 h before subcutaneous administration of either 8-OH-DPAT (3 mg kg^{-1}) or saline, and again 0.5 h after treatment. A further 0.5 h later these mice were evaluated in the Porsolt test. A high dose of 3 mg kg^{-1} was used so that the hypothermic and antidepressant-like response to 8-OH-DPAT could both be determined in the same mouse.

Biochemical assessment of central neuronal lesions Brains were removed from mice immediately after completion of the Porsolt test. A group of mice not exposed to the Porsolt test were used to control for any effects of the experiment on brain monoamine levels. Following homogenization of the whole brain in 0.1 M perchloric acid (BDH) containing 0.4 M sodium metabisulphite (Sigma), concentrations of 5-HT, noradrenaline and dopamine were determined by high performance liquid chromatography (h.p.l.c.) and electrochemical detection. A 25 cm × 4.6 mm Spherisorb 5 μm ODS2 column was used and the mobile phase had the following composition: 0.1 M sodium dihydrogen phosphate (Fisons), ethylenediaminetetraacetic acid (EDTA; BDH), methanol 20% (Rathburn Chemicals) at pH 3.4. A BAS amperometric detector was used and the potential of the working electrode set at 0.65 V against an Ag/AgCl reference electrode.

Drug treatments

5-HT_{1A} agonists alone 8-OH-DPAT, ipsapirone, buspirone and gepirone were administered acutely either orally or subcutaneously. The effects of 14 day oral administration of buspirone (18 mg kg^{-1}) and gepirone (25 mg kg^{-1}) were also investigated.

Receptor antagonists versus 8-OH-DPAT Receptor antagonists were orally administered immediately prior to subcutaneous injection of 3 mg kg^{-1} 8-OH-DPAT, a dose giving a reproducible increase in mobility.

These tests therefore consisted of four treatment groups:- the absolute control (vehicle p.o. + saline s.c.), 8-OH-DPAT (vehicle p.o. + 8-OH-DPAT s.c.), antagonist alone (drug p.o. + saline s.c.) and the combined treatment (drug p.o. + 8-OH-DPAT s.c.). The antagonist alone group showed its effect on the mobility of mice in the test situation.

Brain monoamine depletion and lesion studies The 5-HT synthesis inhibitor PCPA (600 mg kg^{-1}, i.p.) or saline (10 ml kg^{-1}, i.p.) were injected 120 h, 72 h and 24 h prior to evaluation in the Porsolt test. In a separate group of mice, the 5-HT neurotoxin, 5,7-DHT (75 μg in 4 μl) or saline-ascorbate (4 μl) were injected by the intracerebroventricular (i.c.v.) route into halothane-anaesthetized mice, pretreated with desipramine (5 mg kg^{-1}, i.p., 0.5 h previously) to protect noradrenergic neurones, by the method of Heal (1984). Mice were then housed singly for the 14 days prior to evaluation in the Porsolt test.

The noradrenergic neurotoxin, DSP-4 (100 mg kg^{-1}, i.p.), or saline (10 ml kg^{-1}, i.p.) were administered to mice pretreated with zimeldine (5 mg kg^{-1}, i.p., 0.5 h previously) to protect 5-HT neurones, on two separate occasions seven days apart. Three days after the final treatment mice were evaluated in the Porsolt test.

Chronic administration of 8-OH-DPAT or tricyclic antidepressants 8-OH-DPAT or saline were administered subcutaneously twice-daily for 10 days. Amitriptyline, desipramine, dothiepin or deionised water were administered orally at a range of doses once daily for 10 days. The choice of doses was determined by experiments in this strain of mice which had shown that at least the top dose of each antidepressant attenuated 8-OH-DPAT-induced hypothermia after 10 days of treatment (unpublished data). The higher range of doses used for dothiepin reflect its relatively rapid metabolism in rodents (Glass, personal communication). The last dose was administered 24 h before either 8-OH-DPAT (3 mg kg^{-1}, s.c.) or saline.

These tests consisted of four treatment groups:- the absolute control (10 day vehicle + saline s.c.), 8-OH-DPAT (10 day vehicle + 8-OH-DPAT s.c.), chronic treatment alone (10 day pretreatment + saline s.c.) and the combined treatments (10 day pretreatment + 8-OH-DPAT s.c.). The chronic treatment alone group showed whether the pretreatment altered the mobility of mice in the test situation.

Drugs

8-OH-DPAT HBr (Research Biochemicals), buspirone HCl (Sigma), gepirone HCl and ipsapirone 2HCl (synthesized by Boots Pharmaceuticals Research Department) were dissolved in either deionised water for oral administration or saline for subcutaneous injection. Spiroxatrine (Research Biochemicals), (±)-pindolol (Sigma), ondansetron HCl (synthesized by Boots Pharmaceuticals Research Department), prazosin HCl (Sigma), idazoxan HCl (Reckitt & Colman), (±)-metroprolol (+)-tartrate (Sigma), ICI 118,551 (erythro-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol HCl; ICI), naloxone HCl (Endo), amitriptyline HCl (Sigma), desipramine HCl (Sigma) and dothiepin HCl (Boots Pharmaceuticals) were dissolved in deionised water prior to oral administration. (±)-Sulpiride (Sigma) was dissolved in deionised water minimally acidified with acetic acid. Methiothepin maleate (Hoffmann-La Roche), ketanserin tartrate (Janssen) and SCH 23390 maleate (synthesized by Boots Pharmaceuticals Research Department) were given orally as suspensions in cellosize (0.25% solution of hydroxyethylcellulose in deionised water).

For lesioning or neurotransmitter depletion experiments, DSP-4 HCl (Research Biochemicals), PCPA methyl ester HCl (Sigma), desipramine HCl and zimeldine HCl (Astra) were dissolved in saline prior to systemic administration. Compounds were administered at 10 ml kg^{-1} by the oral, subcutaneous and intraperitoneal routes. 5,7-DHT (Sigma) was dissolved in 0.9% saline containing 0.4 mg ml^{-1} ascorbic acid (Fisons) prior to i.c.v. injection. Reagents used in biochemical assays were of analytical grade purity and obtained from the indicated suppliers.

Statistics

A multiple comparison test (Williams' test; Williams, 1972) was used to compare the effect on mobility of a range of oral or subcutaneous doses of drug relative to vehicle-treated

control mice. The Williams' test was also employed to assess the effect on mobility of a range of doses of receptor antagonist or antidepressant combined with 8-OH-DPAT compared with the effect of 8-OH-DPAT alone, that is, 8-OH-DPAT + antagonist or antidepressant versus 8-OH-DPAT. The Sidak multiple t test was used to compare the effect on mobility of 8-OH-DPAT or saline administration to antagonist or antidepressant-pretreated mice at each dose of antagonist or antidepressant. The latter test was also employed to evaluate the effect of other pretreatments (for example, lesions, PCPA, repeated administration of 8-OH-DPAT, or appropriate controls) on either mobility, brain monoamine levels or body temperature following 8-OH-DPAT or saline treatment.

Results

Effect of 5-HT$_{1A}$ agonists on mobility

8-OH-DPAT (0.3–10 mg kg^{-1}) dose-dependently increased the mobility of mice in the Porsolt test following subcutaneous administration, an antidepressant-like effect (Figure 1). The marked effect caused by 1–10 mg kg^{-1} 8-OH-DPAT was not accompanied by overt behavioural stimulation such as facets of the '5-HT syndrome'; indeed higher doses of 8-OH-DPAT sedated the mice. Oral administration of 8-OH-DPAT (0.03–10 mg kg^{-1}) had no significant effect on mobility.

Ipsapirone also dose-dependently increased the mobility of mice after injection by the subcutaneous route with a lowest effective dose of 10 mg kg^{-1} (Figure 2a); the mice were sedated following administration of 30 mg kg^{-1}. However, oral ipsapirone (1–100 mg kg^{-1}) had no effect in the Porsolt test (Figure 2d, open column). Subcutaneous injection of buspirone (0.3–30 mg kg^{-1}) did not increase mobility in the Porsolt test and actually decreased the response at a dose of 30 mg kg^{-1} (Figure 2b) which induced some sedation in the mice. Oral administration of buspirone (1–100 mg kg^{-1}) did not alter the mobility of mice (Figure 2e, open column). Gepirone did not alter mobility after either subcutaneous (0.3–30 mg kg^{-1}) or oral (1–100 mg kg^{-1}) administration (Figures 2c and 2f, open column, respectively); a degree of sedation was observed in the mice treated with the highest dose by each route.

An oral ED_{50} was determined for the antagonism of 8-OH-DPAT by buspirone (18 mg kg^{-1}) and gepirone (25 mg kg^{-1}) (see below; Figures 2e and 2f, respectively). Since, buspirone is effective in man only after repeated administration (Goldberg & Finnerty, 1979; Robinson *et al.*, 1990), buspirone and gepirone were orally administered to mice at these doses for 14 days with the final dose 1 h before analysis in the Porsolt test. An antidepressant-like effect of increased mobility was still not present (mean ± s.e.mean mobility: deionised water 236 ± 41; buspirone (18 mg kg^{-1}) 251 ± 49; gepirone (25 mg kg^{-1}) 295 ± 28 (n = 10 mice per treatment)).

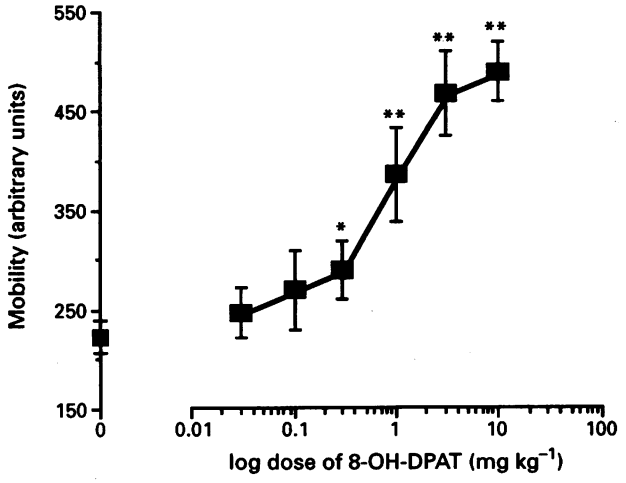


Figure 1 Effect of subcutaneous administration of 8-hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT) on the mobility of mice in the Porsolt test. $^{**}P<0.01$; $^{*}P<0.05$ vs vehicle control (Williams' test). n = at least 10 mice per treatment group.

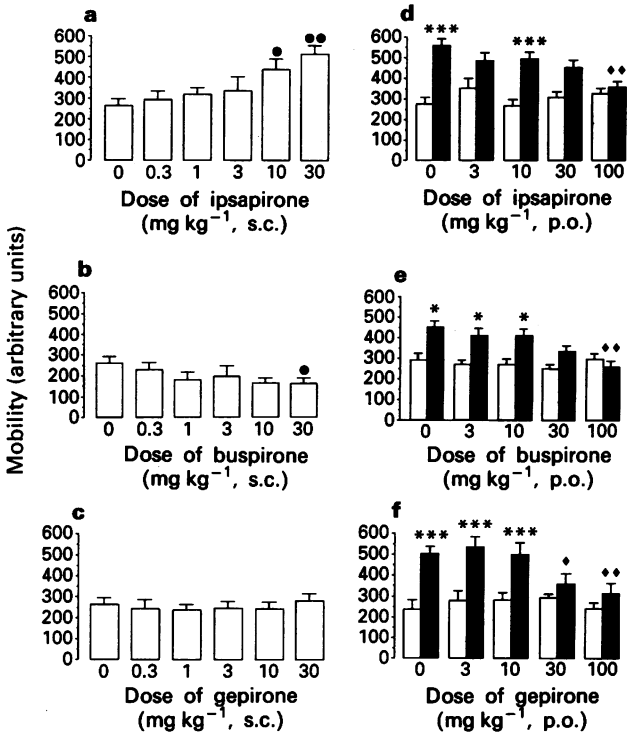


Figure 2 Effect on the mobility of mice in the Porsolt test of administration of ipsapirone, buspirone and gepirone either alone (a–c) or in combination with 8-hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT, 3 mg kg^{-1}, s.c.) (d–f). Each column represents the mean mobility of at least 10 mice per treatment group, the positive s.e.mean is also shown. (d–f): Open columns administered saline (s.c.); solid columns administered 3 mg kg^{-1} 8-OH-DPAT (s.c.). All mice also administered either vehicle (0 mg kg^{-1}) or oral dose of drug as indicated. $^{\bullet\bullet}P<0.01$; $^{\bullet}P<0.05$, drug alone vs vehicle control (Williams' test). $^{***}P<0.001$; $^{*}P<0.05$, drug + 8-OH-DPAT vs drug + saline (Williams' test). $^{\diamond\diamond}P<0.01$; $^{\diamond}P<0.05$, drug + 8-OH-DPAT vs deionised water + 8-OH-DPAT (Sidak multiple t test).

Effects of receptor antagonists on the 8-OH-DPAT response

A subcutaneous dose of 3 mg kg^{-1} 8-OH-DPAT reproducibly evoked increased mobility in the Porsolt test (Figures 2–4) and was therefore used in all antagonist studies.

The 5-HT$_{1A}$ partial agonists ipsapirone, buspirone and gepirone (3–100 mg kg^{-1}, p.o. immediately prior to 8-OH-DPAT) each dose-dependently inhibited 8-OH-DPAT-induced mobility with lowest effective doses of 100, 100 and 30 mg kg^{-1}, respectively (Figures 2d–f). The antagonism was pharmacological rather than non-specific sedation because the inhibitory doses did not decrease the mobility of mice treated with saline (Figures 2d–f). The 5-HT$_{1A}$ antagonist, spiroxatrine (1–30 mg kg^{-1}, p.o. immediately prior to 8-OH-DPAT) also dose-dependently attenuated the 8-OH-DPAT response (Figure 3a) with a total reversal at doses of 10 and 30 mg kg^{-1}. Mice given spiroxatrine plus saline showed similar mobility to the control group (Figure 3a). (±)-Pindolol, a putative 5-HT$_{1A}$ antagonist, also inhibited the 8-OH-DPAT-evoked response although only after pretreatment with 30 mg kg^{-1} (Figure 3b); (±)-pindolol did not itself effect the mobility of mice (Figure 3b).

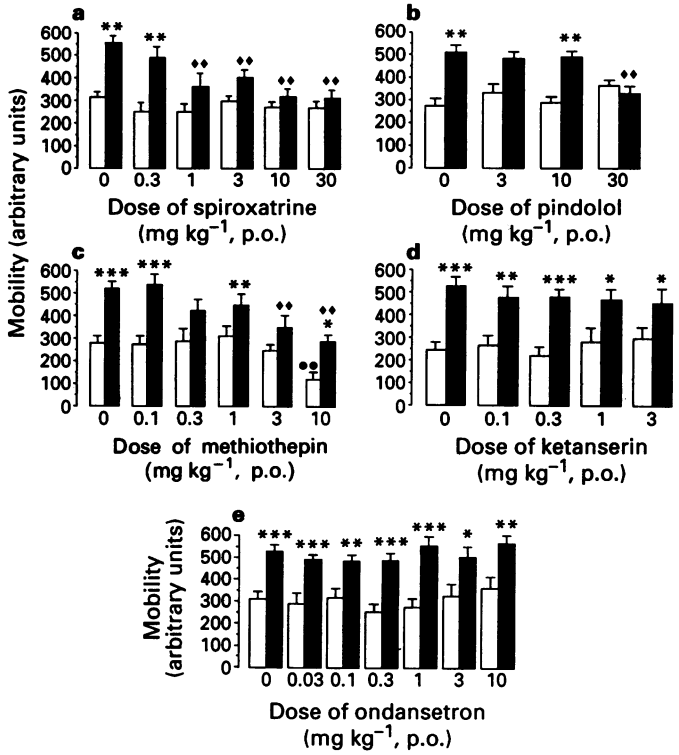


Figure 3 Effect on the mobility of saline- or 8-hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT)-treated mice in the Porsolt test of administration of antagonists at 5-HT receptors: (a) spiroxatrine, (b) (±)-pindolol, (c) methiothepin, (d) ketanserin and (e) ondansetron. Each column represents the mean mobility of at least 10 mice per treatment group, the positive s.e.mean is also shown. Open columns administered saline (s.c.); solid columns administered 3 mg kg^{-1} 8-OH-DPAT (s.c.). All mice also administered either vehicle (0 mg kg^{-1}) or oral dose of antagonist as indicated. $^{\bullet\bullet}P<0.01$, drug alone vs vehicle control (Williams' test). $^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$, antagonist + 8-OH-DPAT vs antagonist + saline (Williams' test). $^{\bullet\bullet}P<0.01$, antagonist + 8-OH-DPAT vs vehicle + 8-OH-DPAT (Sidak multiple t test).

Table 1 Effects of adrenoceptor and dopamine receptor antagonists on 8-hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT)-induced mobility in the Porsolt test

Antagonist	*Oral dose* (mg kg^{-1})	*Mobility of mice: Saline-treated*	*8-OH-DPAT-treated*
Prazosin	0	273 ± 24	525 ± 31**
	1	257 ± 38	511 ± 34**
	3	307 ± 35	499 ± 42*
Idazoxan	0	257 ± 20	465 ± 30**
	3	259 ± 26	405 ± 37*
	10	267 ± 27	487 ± 31***
	30	375 ± 22	464 ± 33
Metoprolol	0	256 ± 16	495 ± 27***
	1	254 ± 29	467 ± 14***
	3	279 ± 32	488 ± 37**
	10	252 ± 25	498 ± 40***
	30	256 ± 38	472 ± 25***
ICI 118,551	0	256 ± 16	495 ± 27***
	1	268 ± 20	473 ± 32***
	3	267 ± 28	460 ± 29***
	10	264 ± 31	473 ± 50**
	30	240 ± 38	516 ± 50***
SCH 23390	0	295 ± 26	541 ± 46***
	0.1	241 ± 39	510 ± 58**
	0.3	205 ± 34	449 ± 47***
	1	223 ± 36	495 ± 37***
	3	264 ± 34	410 ± 28*††
	10	197 ± 26	302 ± 36††
Sulpiride	0	198 ± 24	440 ± 56***
	10	199 ± 22	390 ± 38***
	30	221 ± 47	493 ± 41***
	100	182 ± 36	461 ± 52***
	300	190 ± 33	444 ± 50***

Mean ± s.e.mean mobility values (arbitrary units) obtained from at least 10 mice per treatment group.
$^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$, antagonist or vehicle + 8-OH-DPAT vs antagonist or vehicle + saline (Williams' test).
$^{††}P<0.01$, antagonist + 8-OH-DPAT vs vehicle + 8-OH-DPAT (Sidak multiple t test).

The mixed 5-HT$_{1/2}$ antagonist, methiothepin (3–10 mg kg^{-1}, p.o.), produced dose-dependent inhibition of the 8-OH-DPAT-induced increase in mobility (Figure 3c). The antagonism by 10 mg kg^{-1} methiothepin was, however, probably due in part to a generalized decrease in mobility which was also observed in control mice (Figure 3c) which were sedated. This was not the case for the inhibition of 8-OH-DPAT obtained with 3 mg kg^{-1} methiothepin (Figure 3c). The 5-HT$_{1C}$/5-HT$_2$ antagonist, ketanserin (0.1–3.0 mg kg^{-1}, p.o.) neither inhibited the response to 8-OH-DPAT, nor itself altered the mobility of mice in the Porsolt test (Figure 3d). The 5-HT$_3$ antagonist, ondansetron (0.03–10 mg kg^{-1}, p.o.), was similarly ineffective alone and against the 8-OH-DPAT response (Figure 3e).

Prazosin (1–3 mg kg^{-1}, p.o.) and idazoxan (3–30 mg kg^{-1}, p.o.), antagonists at α_1- and α_2-adrenoceptors respectively, neither altered the increased mobility produced by 8-OH-DPAT nor themselves changed the mobility of mice in the Porsolt test (Table 1). Similarly, antagonists at β_1- (metoprolol; 1–30 mg kg^{-1}, p.o.) and β_2-adrenoceptors (ICI 118,551; 1–30 mg kg^{-1}, p.o.) did not modify the 8-OH-DPAT response or themselves alter mouse mobility in this test (Table 1).

The dopamine D$_1$ antagonist, SCH 23390 (0.3–10 mg kg^{-1}, p.o.) caused some attenuation of the 8-OH-DPAT response at each dose (Table 1), but at 0.3 and 1 mg kg^{-1} this effect was probably non-specific because the mobility of saline-treated mice was reduced to a similar degree (Table 1). Thus, only the higher doses of 3 and 10 mg kg^{-1} SCH 23390 clearly antagonized the increased mobility evoked by 8-OH-DPAT. The D$_2$ antagonist, sulpiride (10–300 mg kg^{-1}, p.o.) neither inhibited the 8-OH-DPAT response nor itself altered mobility (Table 1).

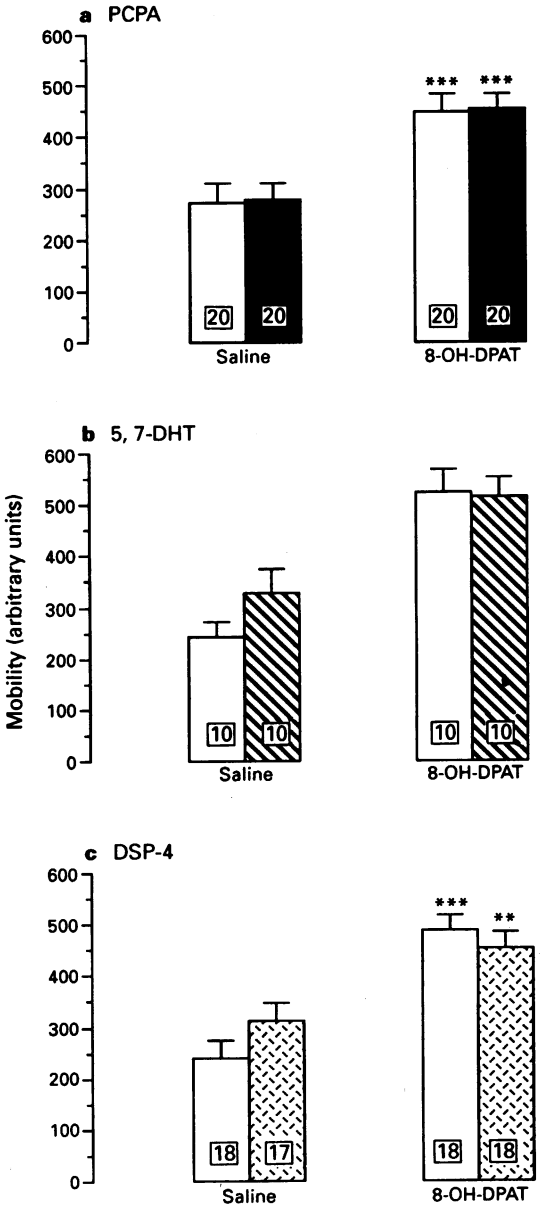
The opiate receptor antagonist, naloxone (3–100 mg kg^{-1}, p.o.) did not affect the mobility of mice subsequently administered either 8-OH-DPAT or saline (data not shown).

Effects of central monoamine depletion and selective neuronal lesions

Depletion of 5-HT neuronal stores by repeated administration of PCPA, did not change the mobility of 8-OH-DPAT-treated mice in the Porsolt test (Figure 4). In addition, the mobility of mice in this test was not changed by PCPA alone. PCPA decreased cerebral 5-HT content by 65% (Table 2). Brain levels of noradrenaline were marginally reduced (13%) by PCPA, but dopamine levels were unaffected (Table 2).

Lesioning of the 5-HT system by i.c.v. injection of 5,7-DHT did not significantly alter the response to 8-OH-DPAT (3 mg kg^{-1}, s.c.) in the Porsolt test (Figure 4). 5,7-DHT decreased brain 5-HT levels by 57%, but did not alter noradrenaline or dopamine levels (Table 2).

Pretreatment with DSP-4 neither altered the increased mobility evoked by 8-OH-DPAT in the Porsolt test, nor the mobility of saline-treated mice (Figure 4). There was a 62% reduction in brain noradrenaline levels following DSP-4 treatment (Table 2). Dopamine levels were slightly lowered (9%), but 5-HT levels were unaffected (Table 2).



Chronic administration of 8-OH-DPAT or tricyclic antidepressants

The increased mobility produced by 8-OH-DPAT (3 mg kg^{-1}) was not altered by pretreatment of the mice twice-daily for 10 days with 8-OH-DPAT (3 mg kg^{-1}, s.c.) (Table 3). In contrast, the marked hypothermia induced by a single dose of 8-OH-DPAT (3 mg kg^{-1}, s.c.) was attenuated by 10 days twice-daily pretreatment with 8-OH-DPAT (3 mg kg^{-1}, s.c.) (Table 3).

Pretreatment for 10 days with a range of oral doses of the tricyclic antidepressants, desipramine, amitriptyline (both 3–30 mg kg^{-1}) and dothiepin (10–100 mg kg^{-1}), did not alter the increased mobility response to 8-OH-DPAT (3 mg kg^{-1}, s.c.) (Table 4). In each case, the top doses of these antidepressants had been previously shown to attenuate 8-OH-DPAT-induced hypothermia following 10 day administration to this strain of mice (unpublished data).

Discussion

The data are discussed in relation to the pharmacological nature and then the synaptic location of the receptors mediating the antidepressant-like effect of 8-OH-DPAT.

Is 8-OH-DPAT-induced mobility in the Porsolt test mediated by 5-HT_{1A} receptors?

The 5-HT_{1A} receptor agonist, 8-OH-DPAT, increased the mobility of mice in the Porsolt test complementing the observation that, in rats, 8-OH-DPAT demonstrates antidepress-

Figure 4 The effect of lesions and 5-hydroxytryptamine (5-HT) synthesis inhibition on the mobility of 8-hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT)- and saline-treated mice in the Porsolt test. Each column represents the mean mobility of the indicated number of mice per treatment group (see inset); the positive s.e.mean is also shown. Open columns pretreated with appropriate vehicle; filled columns pretreated with PCPA (a), 5,7-DHT (b) or DSP-4 (c) as indicated. All mice also acutely administered either saline or 8-OH-DPAT (3 mg kg^{-1}). ***$P<0.001$; **$P<0.01$ vs appropriate saline-treated mice (Sidak multiple t test). See Methods for full treatment schedules for 5,7-dihydroxytryptamine (5,7-DHT), *p*-chlorophenylalanine (PCPA) and N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4).

Table 2 The effect of lesions and 5-hydroxytryptamine (5-HT) synthesis inhibition on mouse brain 5-HT, noradrenaline (NA) and dopamine (DA) levels

Pretreatment + Test	n	*5-HT*	*NA*	*DA*
(A) *Effect of 5,7-DHT*				
Vehicle + Saline	10	584 ± 36	313 ± 21	938 ± 52
Vehicle + 8-OH-DPAT	10	627 ± 35	274 ± 12	893 ± 25
5,7-DHT + Saline	10	248 ± 52***	281 ± 10	925 ± 22
5,7-DHT + 8-OH-DPAT	10	161 ± 17***	260 ± 9	987 ± 59
(B) *Effect of PCPA*				
Vehicle + Saline	20	553 ± 25	354 ± 11	866 ± 20
Vehicle + 8-OH-DPAT	20	603 ± 24	330 ± 16	822 ± 21
PCPA + Saline	20	191 ± 10***	308 ± 12*	789 ± 36
PCPA + 8-OH-DPAT	20	206 ± 13***	305 ± 9*	799 ± 20
(C) *Effect of DSP-4*				
Vehicle + Saline	18	446 ± 15	348 ± 9	799 ± 17
Vehicle + 8-OH-DPAT	18	525 ± 14	327 ± 9	791 ± 18
DSP-4 + Saline	17	437 ± 12	133 ± 10***	725 ± 25**
DSP-4 + 8-OH-DPAT	18	521 ± 11	114 ± 6***	703 ± 24**

Values are mean ± s.e.mean monoamine levels in ng g^{-1} wet weight tissue.
***$P<0.001$; **$P<0.01$; *$P<0.05$ vs appropriate vehicle-treated control (Sidak multiple t test).
Vehicle represents the appropriate control treatment for the agent used, as described in the Methods. The treatment schedules for 5,7-dihydroxytryptamine (5,7-DHT), *p*-chlorophenylalanine (PCPA) and N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) are also given in the Methods.

Table 3 The effect of repeated twice-daily administration of 8-hydroxy-2-(dipropylaminotetralin) (8-OH-DPAT, 3 mg kg^{-1}, s.c.) or saline (1 ml kg^{-1}, s.c.) for 10 days on 8-OH-DPAT (3 mg kg^{-1}, s.c.)-induced hypothermia and on the mobility of mice in the Porsolt test

	Temperature		*Mobility*
Pretreatment + Test	− 0.5 h	+ 0.5 h	+ 1 h
Saline + Saline	38.47 ± 0.10	38.05 ± 0.12	338 ± 35
8-OH-DPAT + Saline	38.16 ± 0.13	38.17 ± 0.15	250 ± 25*
Saline + 8-OH-DPAT	38.21 ± 0.15	36.79 ± 0.22***	464 ± 31**
8-OH-DPAT + 8-OH-DPAT	38.14 ± 0.13	37.98 ± 0.12†	448 ± 39**

Values are mean ± s.e.mean temperature (°C) or mobility (arbitrary units) of 16–20 mice.
Statistical analysis:
(i) Temperature; ***$P<0.001$ vs saline + saline group at + 0.5 h; †$P<0.05$ vs saline + 8-OH-DPAT pretreatment group at + 0.5 h.
(ii) Mobility; **$P<0.01$; *$P<0.05$ vs saline + saline; (Sidak multiple t test).

Table 4 Effects of 10-day oral administration of tricyclic antidepressants on 8-hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT)-induced mobility in the Porsolt test

	Oral dose	*Mobility of mice:*	
Antidepressant	(mg kg^{-1})	*Saline-treated*	*8-OH-DPAT-treated*
Desipramine	0	246 ± 27	377 ± 38*
	3	206 ± 30	418 ± 48**
	10	220 ± 36	438 ± 41**
	30	232 ± 26	464 ± 32**
Amitriptyline	0	217 ± 39	459 ± 52**
	3	306 ± 39	412 ± 55
	10	302 ± 54	486 ± 53*
	30	273 ± 55	478 ± 55*
Dothiepin	0	222 ± 37	392 ± 39**
	10	260 ± 34	450 ± 45*
	30	274 ± 42	410 ± 40*
	100	252 ± 43	386 ± 67*

Mean ± s.e.mean mobility values (arbitrary units) obtained from at least 10 mice per treatment group.
**$P<0.01$; *$P<0.05$, antidepressant or deionised water + 8-OH-DPAT vs antidepressant or deionised water + saline (Williams' test).

ant-like properties (Cervo & Samanin, 1987; Kennett *et al.*, 1987; Giral *et al.*, 1988; Wieland & Lucki, 1990). Although 8-OH-DPAT increases locomotor activity and induces facets of the '5-HT-syndrome' in the rat (Hjorth *et al.*, 1982; Tricklebank *et al.*, 1984), neither of these behavioural responses is observed after 8-OH-DPAT administration to mice (Goodwin & Green, 1985; Heal *et al.*, 1989a), unless high doses are administered intravenously (Yamada *et al.*, 1988), a finding we have been unable to replicate in our mice. Indeed, in agreement with the reports on C57/Bl/6Ola mice, we found that subcutaneous administration of 8-OH-DPAT produced a mild sedative effect in male CD1 mice. Therefore, the antidepressant-like effect of 8-OH-DPAT in this model is not a reflection of increased locomotor activity.

This effect of 8-OH-DPAT was mimicked by ipsapirone, but not by buspirone or gepirone; despite these drugs having all been claimed as selective 5-HT$_{1A}$ receptor agonists (Gilbert & Dourish, 1987; Sprouse & Aghajanian, 1987) and all have been reported to possess antidepressant activity in man (Beneke *et al.*, 1988; Cott *et al.*, 1988; Fabre, 1990; Robinson *et al.*, 1990). These three compounds also do not show a consistent effect in the forced swim test in rats. Thus, Wieland & Lucki (1990) reported that ipsapirone, buspirone and gepirone each induced an antidepressant-like effect, although only after subcutaneous administration of relatively high doses (20 mg kg^{-1} 1 h, 5 h and 24 h prior to the test). In contrast, Przegalinski and colleagues (1990) reported that ipsapirone and buspirone were both ineffective after either one or three doses, although they did find a relatively potent antidepressant-like effect with gepirone with both treatment schedules.

When administered in combination with 8-OH-DPAT, ipsapirone, buspirone and gepirone each caused a dose-dependent inhibition of the mobility response to 8-OH-DPAT in mice. This finding suggests that the drugs are acting as 5-HT$_{1A}$ receptor antagonists in these experiments and is in agreement with previous data reporting their inhibition of 8-OH-DPAT-induced behavioural responses in the rat (Reynolds *et al.*, 1986; Smith & Peroutka, 1986).

The involvement of 5-HT$_{1A}$ receptors in the increased mobility effect of 8-OH-DPAT was confirmed by investigating the effects of antagonists at 5-HT$_{1A}$ and other monoamine receptors. Thus the response was dose-dependently inhibited by the 5-HT antagonists, spiroxatrine (5-HT$_{1A}$; Nelson & Taylor, 1986), (±)-pindolol (5-HT$_{1A}$; Hamon *et al.*, 1986) and methiothepin (non-selective; Leysen *et al.*, 1981; Hibert & Middlemiss, 1986) but not ketanserin (5-HT$_{1C}$/5-HT$_2$; Leysen *et al.*, 1981; Hoyer *et al.*, 1985). Although spiroxatrine and ketanserin both have affinity for dopamine D$_2$ (Niemegeers *et al.*, 1964; Leysen *et al.*, 1977; Costall & Naylor, 1978) and the former is also a potent opioid receptor ligand (Niemegeers *et al.*, 1964), inhibition by an effect of spiroxatrine at these receptors can be discounted because of the lack of inhibition by sulpiride and naloxone.

The involvement of catecholamine-containing neurones in the increased mobility effect of 8-OH-DPAT was also determined. In these studies, 8-OH-DPAT-induced mobility in the Porsolt test was not inhibited by the α- and β-adrenoceptor antagonists, prazosin (α_1; Massingham *et al.*, 1981), idazoxan (α_2; Doxey *et al.*, 1983), metoprolol (β_1; Ablad *et al.*, 1975) and ICI 118,551 (β_2; Bilski *et al.*, 1983). Furthermore, extensive lesioning of noradrenergic neurones with the selective neurotoxin, DSP-4, also had no influence on the increased mobility induced by 8-OH-DPAT. Thus, the data consistently argue against a role for adrenergic neurones in this effect of 8-OH-DPAT which contrasts with their involvement in other central 5-HT$_{1A}$ receptor-mediated responses, including the 5-HT syndrome (Goodwin *et al.*, 1987), hypothermia (Green *et al.*, 1986) and 5-HT release in the suprachiasmatic nucleus (Marsden & Martin, 1986).

The possible involvement of dopaminergic neurones is less clear. In this study, the D$_2$ antagonist, sulpiride, did not attenuate the 8-OH-DPAT-induced increase in mobility in mice. Cervo & Samanin (1987), however, have previously reported that sulpiride at the high dose of 100 mg kg^{-1} i.p. did inhibit the antidepressant-like effect of 8-OH-DPAT in the rat. Similarly, although high doses of the D$_1$ antagonist, SCH 23390, weakly reversed this 8-OH-DPAT response in mice, this finding does not necessarily implicate dopamine receptors because with a K_i of 339 nM (Cheetham, personal communication) SCH 23390 may have sufficient affinity for 5-HT$_{1A}$ receptors to inhibit the response directly.

Overall, the results strongly argue that the increase in mobility in the Porsolt test induced by 8-OH-DPAT is a specific 5-HT$_{1A}$ receptor-mediated effect and, furthermore, that noradrenergic neurones are not involved in its expression. The potential role of dopaminergic neurones in this 8-OH-DPAT-induced effect has not been fully clarified.

What is the synaptic location of 5-HT$_{1A}$ receptors mediating 8-OH-DPAT-induced mobility in the Porsolt test?

The synaptic location of the 5-HT$_{1A}$ receptors was determined by use of selective lesions or depletion of 5-HT containing neurones. Treatment with 5,7-DHT or PCPA had no effect on the 8-OH-DPAT response despite the levels of 5-HT depletion being in excess of those producing a markedly enhanced 5-HT-mediated head-twitch response in mice (Heal *et al.*, 1985). This indicates that the 5-HT$_{1A}$ receptors are located on sites which are postsynaptic to 5-hydroxytryptaminergic neurones. This hypothesis is also supported by previous studies by ourselves and others with 5,7-DHT and PCPA which clearly showed that the 5-HT$_{1A}$ receptors mediating 8-OH-DPAT-induced hypothermia in mice are autoreceptors located on 5-HT-containing neurones (Goodwin *et al.*, 1985a; Heal *et al.*, 1989b).

Two further pieces of indirect evidence also support the proposal that the 5-HT$_{1A}$ receptors mediating the increased mobility produced by 8-OH-DPAT are postsynaptic.

Firstly, repeated administration of 8-OH-DPAT does not modify the ability of 8-OH-DPAT to increase the mobility of mice in the Porsolt test, but rapidly down-regulates the hypothermic response to 8-OH-DPAT which is mediated by presynaptic 5-HT$_{1A}$ receptors (De Souza *et al.*, 1986; Luscombe *et al.*, 1989b; Martin & Heal, 1991). In addition, the 8-OH-DPAT-induced mobility response in mice is not changed by 10 day pretreatment with tricyclic antidepressants whereas repeated antidepressant treatment does attenuate 8-OH-DPAT-induced hypothermia in mice (Goodwin *et al.*, 1985b; Martin & Heal, 1991). The lack of effect of the antidepressants on the former 8-OH-DPAT response does not appear to be due to the length of time (24 h) between the final dose of antidepressant and administration of 8-OH-DPAT, since it has previously been reported that 8-OH-DPAT-induced hypothermia in mice is attenuated for up to 72 h following cessation of chronic antidepressant treatment (Goodwin *et al.*, 1985b). These data further suggest that the increased mobility induced in mice by 8-OH-DPAT is not mediated by presynaptic 5-HT$_{1A}$ receptors.

Secondly, electrophysiological studies have shown that whilst buspirone, gepirone and ipsapirone are full agonists at the somatodendritic 5-HT$_{1A}$ autoreceptor (Sprouse & Aghajanian, 1987), they are partial agonists at the postsynaptic 5-HT$_{1A}$ receptor (Martin & Mason, 1987). In the present paradigm, these compounds all antagonized the increased mobility response to 8-OH-DPAT which indicates that the somatodendritic 5-HT$_{1A}$ autoreceptor is not involved in the response. An additional indication against involvement of this receptor is the observation that doses (10 μg kg^{-1}, i.v.) of 8-OH-DPAT much lower than the 3 mg kg^{-1} used in the present study, maximally inhibit raphe neurone firing (Crespi *et al.*, 1990). The fact that ipsapirone displayed both agonist and antagonist properties whilst buspirone and gepirone exerted only antagonist activity, suggests that ipsapirone may have greater efficacy than buspirone and gepirone at the postsynaptic 5-HT$_{1A}$ receptor.

In the rat, the evidence pertaining to the synaptic location of the 5-HT$_{1A}$ receptors mediating the 8-OH-DPAT effect is much more contradictory. Thus, Cervo & Samanin (1987) reported that 5,7-DHT attenuated the 8-OH-DPAT antidepressant-like effect and concluded that it was acting at presynaptic 5-HT$_{1A}$ receptors in the dorsal raphe nuclei since application of 8-OH-DPAT to this area increased the swimming response (Cervo *et al.*, 1988). In contrast, PCPA did not attenuate the antidepressant-like effects of either 8-OH-DPAT (Wieland & Lucki, 1990) or gepirone (Chojnacka-Wójcik *et al.*, 1991), causing these authors to conclude that the antidepressant response to 5-HT$_{1A}$ agonists is mediated by postsynaptic 5-HT$_{1A}$ receptors.

A similar divergence between studies using mice and rats has previously emerged with another 5-HT$_{1A}$-mediated response, viz hypothermia. Here, evidence that this effect is mediated by 5-HT$_{1A}$ autoreceptors is clear for the mouse (Goodwin *et al.*, 1985b; Martin & Heal, 1991), but not for the rat (Hjorth, 1985; Hutson *et al.*, 1987; Goodwin *et al.*, 1987). While it is unclear why studies in the rat should produce conflicting results, it does suggest that the mouse is the better species for development of the Porsolt test and hypothermia models to study 5-HT$_{1A}$ receptor function.

In conclusion, therefore, this investigation has clearly demonstrated that 8-OH-DPAT produces an antidepressant-like effect in mice and that this effect is mediated through the selective activation of 5-HT$_{1A}$ receptors. In addition, evidence from direct and indirect studies has consistently indicated that the 5-HT$_{1A}$ receptors involved are postsynaptic to 5-HT-containing neurones.

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References

ABLAD, B., BORG, K.O., CARLSSON, E., EK, L., JOHNSSON, G., MALMFORS, T & REGARDH, C.G. (1975). A survey of the pharmacological properties of metoprolol in animals and man. *Acta Pharmacol. Toxicol.*, **36** (Suppl. V), 7–23.

BENEKE, M., KÜMMEL, B., ROED, I.S. & SPECHTMEYER, H. (1988). Treatment of anxiety neurosis with ipsapirone. *Psychopharmacology*, **96** (Suppl.), S353.

BILSKI, A.J., HALLIDAY, S.E., FITZGERALD, J.D. & WALE, J.L. (1983). The pharmacology of a β_2-selective adrenoceptor antagonist (ICI 118,551). *J. Cardiovasc. Pharmacol.*, **5**, 430–437.

BUCKETT, W.R., FLETCHER, J., HOPCROFT, R.H. & THOMAS, P.C. (1982). Automated apparatus for behavioural testing of typical and atypical antidepressants in mice. *Br. J. Pharmacol.*, **75**, 170P.

CERVO, L., GRIGNASCHI, G. & SAMANIN, R. (1988). 8-Hydroxy-2-(di-*n*-propylamino)tetralin, a selective serotonin$_{1A}$ receptor agonist, reduces the immobility of rats in the forced swimming test by acting on the nucleus raphe dorsalis. *Eur. J. Pharmacol.*, **158**, 53–59.

CERVO, L. & SAMANIN, R. (1987). Potential antidepressant properties of 8-hydroxy-2-(di-n-propylamino)tetralin, a selective serotonin$_{1A}$ receptor agonist. *Eur. J. Pharmacol.*, **144**, 223–229.

CHOJNACKA-WÓJCIK, E., TATARCZYNSKA, E., GOLEMBIOWSKA, K. & PRZEGALINSKI, E. (1991). Involvement of 5-HT$_{1A}$ receptors in the antidepressant-like activity of gepirone in the forced swimming test in rats. *Neuropharmacology*, **30**, 711–717.

COSTALL, B. & NAYLOR, R.J. (1978). Dopamine antagonistic effects of a series of analogues of oxiperomide and spiroxatrine measured behaviourally in the rodent. *J. Pharm. Pharmacol.*, **30**, 693–698.

COTT, J.M., KURTZ, N.M., ROBINSON, D.S., LANCASTER, S.P. & COPP, J.E. (1988). A 5-HT$_{1A}$ ligand with both antidepressant and anxiolytic properties. *Psychopharmacol. Bull.*, **24**, 164–167.

CRESPI, F., GARRATT, J.C., SLEIGHT, A.J. & MARSDEN, C.A. (1990). *In vivo* evidence that 5-hydroxytryptamine (5-HT) neuronal firing and release are not necessarily correlated with 5-HT metabolism. *Neuroscience*, **35**, 139–144.

DE SOUZA, R.J., GOODWIN, G.M., GREEN, A.R. & HEAL, D.J. (1986). Effect of chronic treatment with 5-HT$_1$ agonist (8-OH-DPAT and RU 24969) and antagonist (isapirone) drugs on the behavioural responses of mice to 5-HT$_1$ and 5-HT$_2$ agonists. *Br. J. Pharmacol.*, **89**, 377–384.

DOURISH, C.T., HUTSON, P.H. & CURZON, G. (1985). Low doses of the putative serotonin agonist 8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT) elicit feeding in the rat. *Psychopharmacology*, **86**, 197–204.

DOXEY, J.C., ROACH, A.G. & SMITH, C.F.C. (1983). Studies on RX781094: a selective, potent and specific antagonist of α_2-adrenoceptors. *Br. J. Pharmacol.*, **78**, 489–505.

FABRE, L.F. (1990). Buspirone in the management of major depression: a placebo-controlled comparison. *J. Clin. Psychiatry*, **51** (Suppl.), 55–61.

FOZARD, J.R., MIR, A.K. & MIDDLEMISS, D.N. (1987). Cardiovascular response to 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) in the rat: site of action and pharmacological analysis. *J. Cardiovasc. Pharmacol.*, **9**, 328–347.

GILBERT, F. & DOURISH, C.T. (1987). Effects of the novel anxiolytics gepirone, buspirone and ipsapirone on free feeding and on feeding induced by 8-OH-DPAT. *Psychopharmacology*, **93**, 349–352.

GIRAL, P., MARTIN, P., SOUBRIÉ, P. & SIMON, P. (1988). Reversal of helpless behavior in rats by putative 5-HT_{1A} agonists. *Biol. Psychiatry*, **23**, 237–242.

GOLDBERG, H.L. & FINNERTY, R.J. (1979). The comparative efficacy of buspirone and diazepam in the treatment of anxiety. *Am. J. Psychiatry*, **136**, 1184–1187.

GOODWIN, G.M., DE SOUZA, R.J. & GREEN, A.R. (1985a). The pharmacology of the hypothermic response in mice to 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT). A model of presynaptic 5-HT_1 function. *Neuropharmacology*, **24**, 1187–1194.

GOODWIN, G.M., DE SOUZA, R.J. & GREEN, A.R. (1985b). Presynaptic serotonin receptor-mediated response in mice attenuated by antidepressant drugs and electroconvulsive shock. *Nature*, **317**, 531–533.

GOODWIN, G.M., DE SOUZA, R.J., GREEN, A.R. & HEAL, D.J. (1987). The pharmacology of the behavioural and hypothermic responses of rats to 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT). *Psychopharmacology*, **91**, 506–511.

GOODWIN, G.M. & GREEN, A.R. (1985). A behavioural and biochemical study in mice and rats of putative agonists and antagonists for 5-HT_1 and 5-HT_2 receptors. *Br. J. Pharmacol.*, **84**, 743–753.

GREEN, A.R., GOODWIN, G.M., DE SOUZA, R.J. & HEAL, D.J. (1986). The β_2-adrenoceptor agonists, clenbuterol and salbutamol, enhance the hypothermic action of 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) in mice by a central mechanism. *Neuropharmacology*, **25**, 21–24.

HAMON, M., COSSERY, J.-M., SPAMPINATO, U. & GOZLAN, H. (1986). Are there selective ligands for 5-HT_{1A} and 5-HT_{1B} receptor binding sites in brain? *Trends Pharmacol. Sci.*, **7**, 336–338.

HEAL, D.J. (1984). Phenylephrine-induced activity in mice as a model of central α_1-adrenoceptor function; effects of acute repeated administration of antidepressant drugs and electroconvulsive shock. *Neuropharmacology*, **23**, 1241–1251.

HEAL, D.J., PHILPOT, J., MOLYNEUX, S.G. & METZ, A. (1985). Intracerebroventricular administration of 5,7-dihydroxytryptamine to mice increases both head-twitch response and the number of cortical 5-HT_2 receptors. *Neuropharmacology*, **24**, 1201–1205.

HEAL, D.J., PROW, M.R., MARTIN, K.F. & BUCKETT, W.R. (1989a). The interaction of 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) with α_2-adrenoceptor mediated behavioural responses. *Br. J. Pharmacol.*, **96**, 306P.

HEAL, D.J., PROW, M.R., MARTIN, K.F. & BUCKETT, W.R. (1989b). Effects of lesioning central 5-HT and noradrenaline containing neurones on 8-OH-DPAT hypothermia, mydriasis and hypoactivity. *Br. J. Pharmacol.*, **97**, 406P.

HIBERT, M. & MIDDLEMISS, D.N. (1986). Stereoselective blockade at the 5-HT autoreceptor and inhibition of radioligand binding to central 5-HT recognition sites by the optical isomers of methiothepin. *Neuropharmacology*, **25**, 1–4.

HJORTH, J. (1985). Hypothermia in the rat induced by the potent serotoninergic agent 8-OH-DPAT. *J. Neural Transm.*, **61**, 131–135.

HJORTH, S., CARLSSON, A., LINDBERG, P., SANCHEZ, D., WIKSTROM, H., ARVIDSSON, L.-E., HACKSELL, U. & NILSSON, J.L.G. (1982). 8-Hydroxy-2-(di-*n*-propylamino)-tetralin, 8-OH-DPAT, a potent and selective simplified ergot congener with central 5-HT-receptor stimulating activity. *J. Neural Transm.*, **55**, 169–188.

HOYER, D., ENGEL, G. & KALKMAN, H.O. (1985). Molecular pharmacology of 5-HT_1 and 5-HT_2 recognition sites in rat and pig brain membranes: radioligand binding studies with [^{3}H]5-HT, [^{3}H]8-OH-DPAT, (−)[^{125}I]iodocyanopindolol, [^{3}H]mesulergine and [^{3}H]ketanserin. *Eur. J. Pharmacol.*, **118**, 13–23.

HUTSON, P.H., DOURISH, C.T. & CURZON, G. (1988). Evidence that the hyperphagic response to 8-OH-DPAT is mediated by 5-HT_{1A} receptors. *Eur. J. Pharmacol.*, **150**, 361–366.

KENNETT, G.A., DOURISH, C.T. & CURZON, G. (1987). Antidepressant-like action of 5-HT_{1A} agonists and conventional antidepressants in an animal model of depression. *Eur. J. Pharmacol.*, **134**, 265–274.

LEYSEN, J.E., AWOUTERS, F., KENNIS, L., LADURON, P.M., VANDENBERK, J. & JANSSEN, P.A.J. (1981). Receptor binding profile of R 41 468, a novel antagonist at 5-HT_2 receptors. *Life Sci.*, **28**, 1015–1022.

LEYSEN, J.E., TOLLENAERE, J.P., KOCH, M.H.J. & LADURON, P. (1977). Differentiation of opiate and neuroleptic receptor binding in rat brain. *Eur. J. Pharmacol.*, **43**, 253–267.

LUSCOMBE, G.P., HUTCHINS, L.J. & BUCKETT, W.R. (1989a). Repeated administration of tricyclic antidepressants does not alter the antidepressant-like effect of 8-OH-DPAT in mice. *Br. J. Pharmacol.*, **97**, 400P.

LUSCOMBE, G.P., MARTIN, K.F., HUTCHINS, L.J. & BUCKETT, W.R. (1989b). Attenuation of the hypothermia but not the antidepressant-like effect of 8-OH-DPAT following its repeated administration to mice. *Br. J. Pharmacol.*, **96**, 307P.

LUSCOMBE, G.P., MARTIN, K.F., HUTCHINS, L.J., GOSDEN, J. & BUCKETT, W.R. (1988). Involvement of 5-HT_{1A} receptors in the antidepressant-like effect of 8-OH-DPAT in a putative model of depression in mice. *Br. J. Pharmacol.*, **95**, 784P.

MARSDEN, C.A. & MARTIN, K.F. (1986). Involvement of 5-HT_{1A}- and α_2-receptors in the decreased 5-hydroxytryptamine release and metabolism in rat suprachiasmatic nucleus after intravenous 8-hydroxy-2-(*n*-dipropylamino)tetralin. *Br. J. Pharmacol.*, **89**, 277–286.

MARTIN, K.F. & HEAL, D.J. (1991). 8-OH-DPAT-induced hypothermia in rodents. A specific model of 5-HT_{1A} autoreceptor function? In *Serotonin: Molecular Biology, Receptors and Functional Effects*. ed. Fozard, J.R. & Saxena, P.R. pp. 483–490. Basel: Birkhäuser.

MARTIN, K.F. & MASON, R. (1987). Isapirone is a partial agonist at 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}) receptors in the rat hippocampus: electrophysiological evidence. *Eur. J. Pharmacol.*, **141**, 479–483.

MASSINGHAM, R., DUBOCOVICH, M.L., SHEPPERSON, N.B. & LANGER, S.Z. (1981). In vivo selectivity of prazosin but not WB 4101 for postsynaptic α_1-adrenoceptors. *J. Pharmacol. Exp. Ther.*, **217**, 467–474.

NELSON, D.L. & TAYLOR, E.W. (1986). Spiroxatrine: a selective serotonin$_{1A}$ receptor antagonist. *Eur. J. Pharmacol.*, **124**, 207–208.

NIEMEGEERS, C.J.E., VERBRUGGEN, F.J., VAN NEUTEN, J.M. & JANSSEN, P.A.J. (1964). Spiroxamide (R 5188): a new compound producing morphine-like and chlorpromazine-like effects in animals. *Int. J. Neuropharmacol.*, **2**, 349–354.

PEROUTKA, S.J. (1988). 5-Hydroxytryptamine receptor subtypes. *Annu. Rev. Neurosci.*, **11**, 45–60.

PORSOLT, R.D. (1981). Behavioral despair. In *Antidepressants: Neurochemical, Behavioral and Clinical Perspectives*. ed. Enna, S.J., Malick, J.B. & Richalson, E. pp. 121–139. New York: Raven Press.

PORSOLT, R.D., BERTIN, A. & JALFRE, M. (1977). Behavioural despair in mice: a primary screening test for antidepressants. *Arch. Int. Pharmacodyn. Ther.*, **229**, 327–336.

PRZEGALINSKI, E., TATARCZYNSKA, E. & CHOJNACKA-WÓJCIK, E. (1990). Antidepressant-like activity of ipsapirone, buspirone and gepirone in the forced swimming test in rats pretreated with proadifen. *J. Psychopharmacol.*, **4**, 204–209.

REYNOLDS, L.S., SEYMOUR, P.A. & HEYM, J. (1986). Inhibition of the behavioral effects of 8-OH-DPAT by the novel anxiolytics buspirone, gepirone and isapirone. *Soc. Neurosci. Abstr.*, **12**, 132.1

ROBINSON, C.P. (1987). Buspirone hydrochloride (Bespar®; Buspar®)–a new anxiolytic agent. *Drugs of Today*, **23**, 311–319.

ROBINSON, D.S., RICKELS, K., FEIGHNER, J., FABRE, L.F., GAMMANS, R.E., SHROTRIYA, R.C., ALMS, D.R., ANDARY, J.J. & MESSINA, M.E. (1990). Clinical effects of the 5-HT_{1A} partial agonists in depression: a composite analysis of buspirone in the treatment of depression. *J. Clin. Psychopharmacol.*, **10** (Suppl.), 67S–76S.

SMITH, L.S. & PEROUTKA, S.J. (1986). Differential effects of 5-hydroxytryptamine$_{1A}$ selective drugs on the 5-HT behavioral syndrome. *Pharmacol. Biochem. Behav.*, **24**, 1513–1519.

SPROUSE, J.S. & AGHAJANIAN, G.K. (1987). Electrophysiological responses of serotonergic dorsal raphe neurons to 5HT_{1A} and 5HT_{1B} agonists. *Synapse*, **1**, 3–9.

TRICKLEBANK, M.D., FORLER, C. & FOZARD, J.R. (1984). The involvement of subtypes of the 5-HT_1 receptor and of catecholaminergic systems in the behavioural response to 8-hydroxy-2-(di-n-propylamino)tetralin in the rat. *Eur. J. Pharmacol.*, **106**, 271–282.

WIELAND, S. & LUCKI, I. (1990). Antidepressant-like activity of 5-HT_{1A} agonists measured with the forced swim test. *Psychopharmacology*, **101**, 497–504.

WILLIAMS, D.A. (1972). The comparison of several dose levels with a zero dose control. *Biometrics*, **28**, 519–531.

YAMADA, J., SUGIMOTO, Y. & HORISAKA, K. (1988). The behavioural effects of 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) in mice. *Eur. J. Pharmacol.*, **154**, 299–304.

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Modulation of α_1-adrenoceptors and functional consequences in the bisected rat vas deferens following chronic inhibition of neuronal noradrenaline uptake

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1 The adaptational changes induced after chronic inhibition of neuronal noradrenaline uptake on both functional responsiveness of α_1-adrenoceptor activation and [^{3}H]-prazosin binding were investigated in prostatic and epididymal portions of the rat vas deferens.

2 Contractile concentration-response curves to phenylephrine and saturation isotherms of [^{3}H]-prazosin binding to homogenates of each of the portions of the bisected rat vas deferens were determined 48 h after the last injection of desipramine, nomifensine or nisoxetine (10 mg kg^{-1}; i.p. for 14 days).

3 Treatment with both nomifensine and nisoxetine decreased the potency (pD_2) of phenylephrine by about 10 and 8 fold respectively in the epididymal portion. However, administration of desipramine only reduced the potency of the α_1-adrenoceptor agonist by about 1.8 fold. None of the treatments modified the maximal effect (E_{max}) elicited by phenylephrine in this portion of the vas deferens. In the prostatic portion only the treatment with nomifensine (1.4 fold) and nisoxetine (1.8 fold) decreased the potency of phenylephrine; the maximal contraction elicited by the agonist after the treatments was also reduced.

4 Chronic treatment with either nomifensine or nisoxetine did not change the K_D for [^{3}H]-prazosin binding in either epididymal or prostatic membranes. However, these two treatments resulted in a significant decrease in the [^{3}H]-prazosin B_{max} in membranes in both portions of rat vas deferens. The reduction in density of α_1-adrenoceptors was higher in the epididymal than the prostatic half. Desipramine reduced the B_{max} only in the epididymal portion.

5 These results indicate that differential regulation of α_1-adrenoceptors in either portion of the rat vas deferens could result from a greater degree of activation of these receptors in the epididymal half after chronic inhibition of neuronal noradrenaline uptake. The different functional consequences of the loss of α_1-adrenoceptors in each portion seems to be explained on the basis of a different relationship between the occupancy of the receptor and the response elicited.

Keywords: α_1-Adrenoceptors; [^{3}H]-prazosin binding; inhibitors of neuronal noradrenaline uptake; rat vas deferens

Introduction

Several studies seem to indicate that the prostatic and epididymal portions of the rat vas deferens differ not only in their motor transmission (McGrath, 1978; Brown *et al.*, 1983) but in their postsynaptic responsiveness to α-adrenoceptor agonists (Vardolov & Pennefather, 1976; Kasuya & Suzuki, 1979; MacDonald & McGrath, 1980; Moore & Griffiths, 1982; Badia & Sallés, 1989; Sallés & Badia, 1991). These differences in sensitivity to α-adrenoceptor agonists are likely to involve more efficient α_1-adrenoceptor coupling to stimulus-response mechanisms in the epididymal portion (Sallés & Badia, 1991). In addition, the prostatic portion of the rat vas deferens has a more dense noradrenergic innervation (Anton *et al.*, 1977) and a larger endogenous noradrenaline content (Zieher & Jaim-Etcheverri, 1971; Sjöstrand, 1981) compared to the epididymal half.

Drugs which inhibit neuronal noradrenaline uptake make more neurotransmitter available at postsynaptic α_1-adrenoceptors and may therefore lead to compensatory down-regulation of these receptors. However, the loss of postsynaptic receptors, by such a mechanism, will depend, among other factors, on the density and symmetry of innervation and the width of the synaptic gap. Likewise, the functional consequences induced by such a compensatory mechanism rely on the efficiency of the stimulus-response coupling in the tissue under study. In order to gain more insight into the characteristics and regulation of postsynaptic α_1-adrenoceptors of both portions of rat vas deferens, it was decided to study the influence of long-term inhibition of neuronal noradrenaline uptake on these receptor sites. Three potent inhibitors of neuronal noradrenaline uptake, desipramine, nomifensine and nisoxetine were selected (Richelson & Pfenning, 1984). The effect of these chronic treatments on the number and functional responsiveness of α_1-adrenoceptors in the epididymal and prostatic portions of rat vas deferens was analyzed. A preliminary account of this work has been presented to the XIth International Congress of Pharmacology, Amsterdam, June 1990, (Badia & Sallés, 1990).

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Methods

Male Wistar rats (140–200 g) with free access to food and water and maintained on a 12 h light/dark schedule were injected i.p. daily for 14 days with either saline or 10 mg kg^{-1} of nisoxetine, nomifensine or desipramine. Animals were killed by decapitation 48 h after 1 or 14 days of treatment. Both vasa deferentia were removed, carefully cleaned and bisected into prostatic and epididymal portions and used for functional or binding studies.

Functional studies

For the measurement of contractile responses induced by phenylephrine or noradrenaline, the tissues were suspended in isolated organ baths containing 20 ml of a physiological salt solution of the following composition (in mM): NaCl 112.0, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.1, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 11.1. The solution was maintained at 32 ± 0.5°C and gassed with 95% O_2 and 5% CO_2. Propra-

nolol (1 μM), desipramine (0.1 μM) and normetanephrine (1 μM) were present continuously in the medium throughout the experiments to block β-adrenoceptors, neuronal and extraneuronal noradrenaline uptake respectively (Minneman *et al.*, 1983). The experimental conditions were thus carefully controlled to obtain accurate estimates of the potency of agonists on the α_1-adrenoceptors. To assess the influence of neuronal noradrenaline uptake mechanisms on the concentration-response curves to noradrenaline and phenylephrine in each portion of the vas deferens of control animals, some experiments were carried out in the presence or absence of desipramine alone (0.1 μM) in the physiological salt solution.

At least 45 min were allowed to elapse before starting the experiment. After this equilibration period at a resting tension of 0.5 g, the tissues were exposed twice to a maximal concentration of noradrenaline and thoroughly washed. Contractions were registered by means of an isometric PIODEN (UF-1) transducer attached on a OmniScribe pen-recorder. Agonist-induced increases in isometric tension were determined with a non-cumulative dosage schedule where each concentration was washed out before the next concentration was added, using 6 min intervals between concentrations. Responses were measured as the peak tension developed while the preparation was exposed to the agonist and are expressed as a percentage of the maximal contraction induced by noradrenaline or phenylephrine. Data from concentration-response experiments for phenylephrine or noradrenaline were analyzed by nonlinear regression analysis, using the GraphPAD computer programme (ISI, Philadelphia, PA, USA), which gave estimates of the pD_2 ($-\log EC_{50}$, negative $\log_{10}$ of molar concentration of agonist producing 50% of its maximal effect), the maximal effect (E_{max}) and slope of the curve.

Radioligand binding studies

For binding assays, the epididymal and prostatic portions from several animals were pooled separately and placed in 10 ml of ice cold 50 mM Tris/HCl buffer pH 7.5, and homogenized with a Polytron type homogenizer (Rafer[10/20], setting 6, 2 × 15 s). Homogenates were filtered through a double layer of surgical gauze and centrifuged at 49,000 *g* for 20 min. The resultant pellet was washed twice by resuspension and centrifugation under the same conditions. Final pellets were resuspended in the same buffer at 2 mg protein ml^{-1} approximately. For saturation experiments aliquots (100 μg protein) of membrane suspensions were incubated in triplicate with seven concentrations (ranging from 0.05 to 4 nM) of [^{3}H]-prazosin in 0.25 ml (final volume) of 50 mM Tris/HCl buffer pH 7.5 at 25°C for 45 min. Incubations were stopped by rapid filtration through Whatman GF/C glass fibre filters that were washed 3 times with 5 ml of the same buffer. Radioactivity retained in the filters was determined at 48–50% efficiency. Nonspecific binding was defined as that insensitive to 10 μM phentolamine. Protein was estimated as described by Lowry *et al.* (1951) with bovine serum albumin used as standard.

Individual saturation isotherms for [^{3}H]-prazosin specific binding were analyzed by non-linear regression analysis, using the EBDA programme adapted for microcomputers as described by McPherson (1983). Parameters estimated were K_D, B_{max} and the Hill coefficient.

Statistical analysis

All the results are expressed as the means ± s.e.mean. Statistical significance of the observed differences was assessed by two tailed Student's *t* test. The statistical significance of the differences between the Hill coefficients and unity was tested with Student's *t* test, under a null hypothesis (Hill coefficient = 1).

Drugs

Drugs used and their sources were: noradrenaline bitartrate, phenylephrine hydrochloride, desipramine hydrochloride and propranolol hydrochloride (Sigma), normetanephrine hydrochloride (Calbiochem), phentolamine methansulphonate (Ciba-Geigy), nomifensine hydrogen tartrate (Hoechst), nisoxetine hydrochloride (Lilly) and [^{3}H]-prazosin (82 Ci $mmol^{-1}$, New England Nuclear).

Results

Functional studies

In order to determine the influence of neuronal uptake mechanisms on the potency of α-adrenoceptor agonists in each portion of rat vas deferens, some experiments were designed to evaluate the influence of desipramine on the concentration-response curves to noradrenaline and phenylephrine. In the presence of 0.1 μM desipramine, only the maxima of the concentration-response curves to noradrenaline were markedly enhanced in both portions of rat vas deferens. This effect was pronounced in the epididymal than in the prostatic portions, with approximately 100% and 40% increases respectively (Table 1). An increase (5 fold) in the potency of noradrenaline was observed only in the epididymal half (Table 1).

Two days after the administration of a single dose (10 mg kg^{-1}; i.p.) of nisoxetine, nomifensine or desipramine neither the pD_2 nor the E_{max} values for phenylephrine were significantly modified in either half of the vas deferens. After 14 days of treatment with each compound the concentration-response curves to phenylephrine were shifted to the right in the epididymal portion (Figure 1b). The treatments with nisoxetine and nomifensine reduced the potency of phenylephrine by about 8 fold ($P<0.01$) and 10 fold ($P<0.01$) respectively. However, desipramine treatment only reduced the potency of the agonist by about 1.8 fold ($P<0.05$) (Table 2). It should be noted that the three treatments did not modify the maxima of the concentration-response curves to phenylephrine in the epididymal portion (Figure 1b and Table 2). In contrast, the administration of nisoxetine and nomifensine for 14 days resulted in a marked reduction (32% and 45% respectively) of the maximal contraction elicited by phenylphrine in the prostatic half of rat vas deferens (Figure 1a and Table 2). In addition, these two treatments only induced small changes in the potency of phenylephrine in this portion (Table 2). The treatment with desipramine did not modify the concentration-response curve of phenylephrine in the prostatic half of the rat vas deferens (Table 2).

Binding studies

Specific binding of [^{3}H]-prazosin was saturable and Scatchard analysis of saturation isotherms resulted in straight lines in both control and treated groups (Figure 2). The Hill coefficients for the saturation isotherms were not significantly different from unity and the specific binding determined by 10 μM phentolamine was approximately 70% of the total binding at a concentration of [^{3}H]-prazosin close to the K_D value. As shown in Table 3, 2 weeks of treatment with nisoxetine, nomifensine or desipramine did not affect the α_1-adrenoceptor affinity for [^{3}H]-prazosin in either epididymal or prostatic membranes. However, the long-term treatment with nisoxetine and nomifensine resulted in a significant decrease in the maximal number of α_1-adrenoceptor binding sites (B_{max}) in both portions. In the epididymal half a 40% or 30% decrease was observed with nisoxetine and nomifensine respectively. In the prostatic half the treatments with nisoxetine and nomifensine resulted in smaller decreases in the density of α_1-adrenoceptors of 23% and 15% respectively. In

Table 1 Responses of the bisected rat vas deferens to noradrenaline and phenylephrine in the absence and presence of 0.1 μM desipramine in the physiological salt solution

	Control		*Desipramine* (0.1 μM)	
	pD_2	E_{max}	pD_2	E_{max}
Prostatic portion				
Noradrenaline	5.30 ± 0.06	450 ± 35	5.31 ± 0.05	630 ± 25**
Phenylephrine	5.02 ± 0.04	420 ± 15	5.03 ± 0.02	413 ± 15
Epididymal portion				
Noradrenaline	5.60 ± 0.05	1162 ± 30	6.31 ± 0.04**	2037 ± 45**
Phenylephrine	5.84 ± 0.01	2063 ± 28	5.86 ± 0.02	2103 ± 40

Data are expressed as means ± s.e.mean of 10 experiments. E_{max} is the maximal muscle contraction to agonist expressed as mg of tension.
**$P < 0.01$ when compared to the values obtained in the absence of 0.1 μM of desipramine.

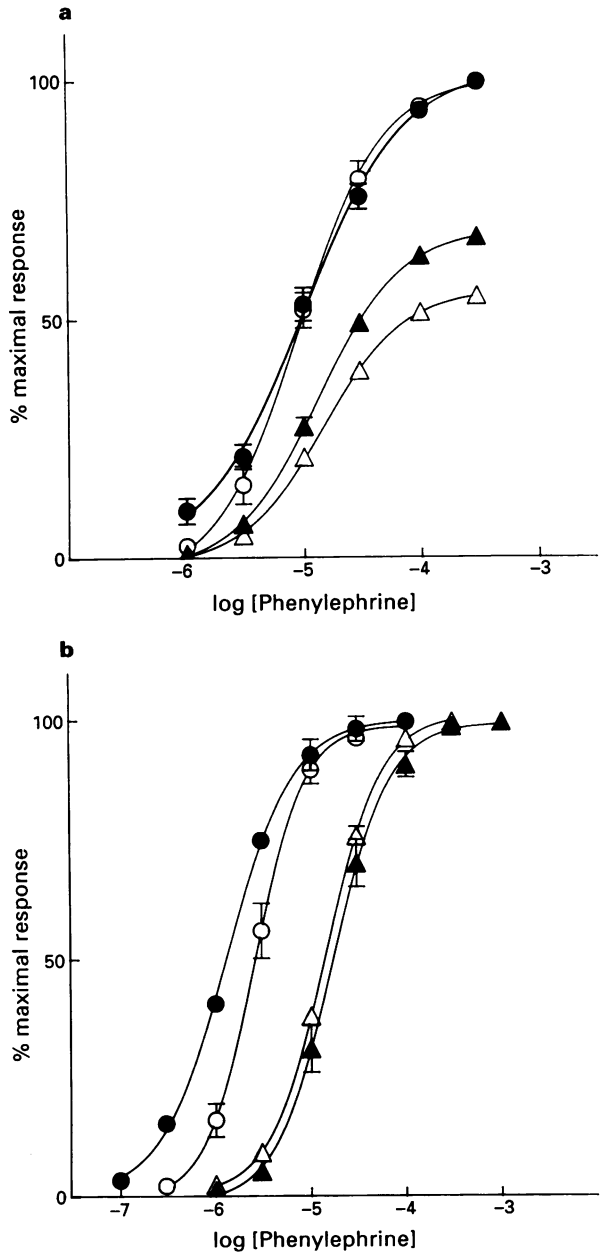


Figure 1 Effects of chronic treatment with several inhibitors of neuronal noradrenaline uptake on the concentration-response curves to phenylephrine in the prostatic (a) and the epididymal (b) portions of rat vas deferens. Responses to phenylephrine from vehicle- (●), and desipramine- (○), nomifensine- (▲), and nisoxetine-treated animals (△), are calculated as a percentage of the averaged maximum response in control tissues. Each point represents the mean ± s.e.mean (vertical bars) of six experiments on separate preparations.

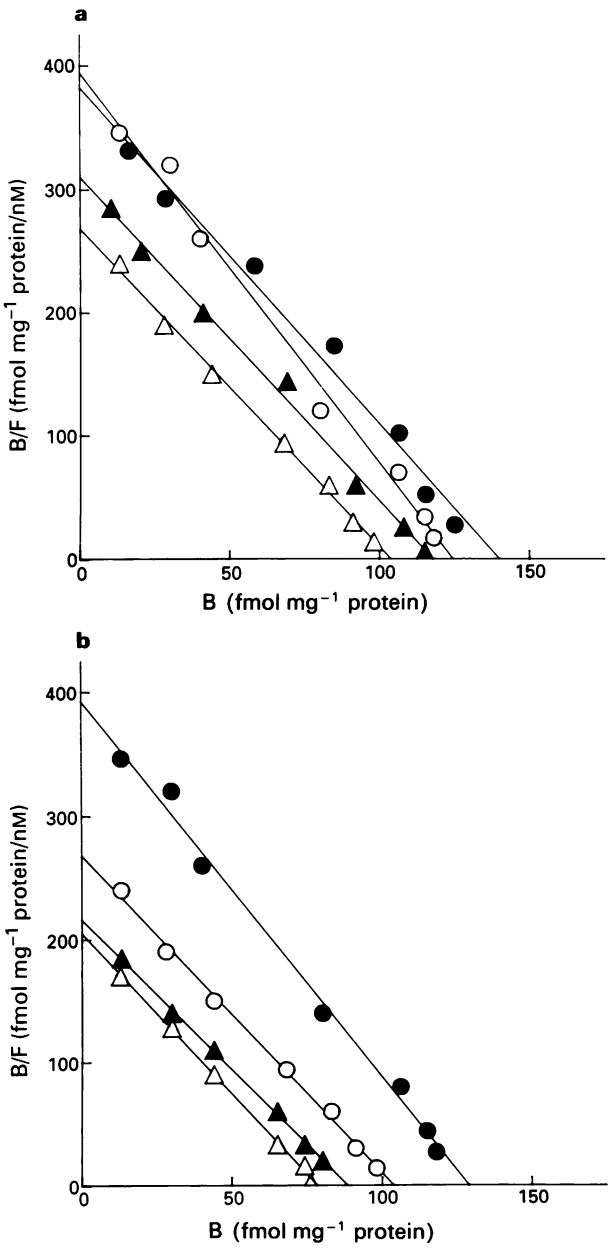


Figure 2 Scatchard plots for [^{3}H]-prazosin binding to membranes from (a) prostatic and (b) epididymal portions of rat vas deferens. Vehicle- (●), desipramine- (○), nomifensine- (▲), and nisoxetine-treated animals (△). The values on the y-axis were determined by dividing the amount bound (B, fmol mg^{-1} protein) by the concentration of free radioligand (F, nM). The results shown are from a representative experiment carried out in triplicate.

Table 2 Effects of chronic treatment (10 mg kg^{-1}; i.p.; 14 days) with several inhibitors of neuronal noradrenaline uptake on the agonist activity of phenylephrine at postsynaptic α_1-adrenoceptors in the bisected rat vas deferens

	pD_2	Δ	E_{max}	$\%E_{max}$
Prostatic portion				
Control	5.02 ± 0.04	–	400.0 ± 50.2	–
Nisoxetine	4.77 ± 0.03**	1.8	219.2 ± 49.3**	55
Nomifensine	4.89 ± 0.02*	1.4	272.5 ± 26.7*	68
Desipramine	4.99 ± 0.05	–	517.5 ± 51.2	–
Epididymal portion				
Control	5.86 ± 0.01	–	2012.8 ± 113.2	–
Nisoxetine	4.99 ± 0.06**	8	2004.5 ± 30.4	–
Nomifensine	4.89 ± 0.02**	10	1841.6 ± 40.7	–
Desipramine	5.59 ± 0.04*	1.8	1983.7 ± 69.8	–

Data are expressed as mean ± s.e.mean of 6 experiments. E_{max} is the maximal muscle contraction to phenylephrine expressed as mg of developed tension. $\%E_{max}$ is the percentage of the ratio E_{max} treated/E_{max} control and Δ indicates the potency changes calculated as antilog (pD_2 control - pD_2 treated) where these are significantly different from control.
**$P<0.01$; *$P<0.05$ *vs* control value.

contrast, treatment with desipramine only reduced the number of α_1-adrenoceptors (by about 19%) in the epididymal half.

Discussion

It has been established that an increase in availability of noradrenaline in the synaptic cleft by long-term inhibition of its neuronal uptake results in a reduction in the number of central and peripheral α_2-adrenoceptors and in a reduction in agonist potency (Svensson & Usdin, 1978; Smith *et al.*, 1982; García-Sevilla & Zubieta, 1986; Vila *et al.*, 1990). However, much less is known about the desensitization of responses mediated by α_1-adrenoceptors following their chronic activation, or of the involvement of changes in receptor density in such modifications. The present study was designed to examine the regulation of both density and responsiveness of α_1-adrenoceptors, in two tissue samples with a different efficiency of coupling between the occupancy of these receptors and the responses elicited (Sallés & Badia, 1991), after chronic stimulation resulting from the long-term inhibition of neuronal noradrenaline uptake by antidepressant drugs.

The results clearly show that the long-term treatment with inhibitors of neuronal noradrenaline uptake leads to α_1-adrenoceptor down-regulation in both portions of rat vas deferens, probably due to an increased level of noradrenaline in the vicinity of its receptors. In this context, it should be borne in mind that the motor transmission in the rat vas deferens, independently of the adrenergic or non-adrenergic nature of the nerve terminals (Sneddon *et al.*, 1982), is modulated by presynaptic α_2-adrenoceptors, stimulation of which inhibits the release of the motor transmitter (Drew, 1977). Furthermore, these receptors become desensitized by chronic treatment with antidepressant drugs (García-Sevilla & Zubieta, 1986). Therefore, a reduced sensitivity of presynaptic α_2-adrenoceptors to noradrenaline should result in an increased transmission in these nerve terminals, an effect that could also contribute to the development of postsynaptic α_1-adrenoceptor down-regulation.

The long-term treatment with nomifensine and nisoxetine decreased the maximal number of binding sites labelled with [^{3}H]-prazosin in both portions of rat vas deferens, but the decreases were higher in the epididymal than in the prostatic portions (Table 3). In contrast, long-term treatment with desipramine, that is also a potent inhibitor of noradrenaline uptake (Richelson & Pfenning, 1984), only decreased the number of [^{3}H]-prazosin binding sites in the epididymal half. Desipramine, in contrast to the other two compounds, has a significant affinity for α_1-adrenoceptors (Richelson & Nelson, 1984) and displays α_1-adrenoceptor antagonist activity in the rat vas deferens (Bradley & Doggrell, 1985). Thus, α_1-adrenoceptor antagonism by desipramine could oppose the effects due to the increase of synaptic noradrenaline that result from neuronal uptake inhibition.

The greater reduction in α_1-adrenoceptor density achieved in the epididymal portion may be explained on the basis of the results shown in Table 1. The maximal contractile responses induced by noradrenaline in the epididymal half were potentiated to a greater extent than those in the prostatic half following inhibition of neuronal uptake by desipramine. Similar results have been reported with desipramine (Gonçalves & Guimaraes, 1991), or cocaine (Werneck de Avellar *et al.*, 1990). Pharmacologically, the magnitude of the potentiation can reflect the importance of the uptake process as a modulator of agonist concentrations in the receptor compartment and it has been shown that the epididymal portion has a greater capacity for noradrenaline accumulation than the prostatic portion (Werneck de Avellar *et al.*, 1990). Therefore, all these results could reflect a greater influence of the removal process, and consequently of its chronic inhibition, in the epididymal portion of rat vas deferens. The greater reduction in α_1-adrenoceptor density in the epididymal versus the prostatic portion could result from a greater degree of receptor activation in this half after inhibition of neuronal noradrenaline uptake and it has been suggested that postjunctional α_1-adrenoceptors are located closer to the varicosities in the epididymal than in the prostatic portion (Gonçalves & Guimaraes, 1991). Although a good correlation has been established between the potentiation of the responses to noradrenaline with the neuromuscular cleft width, but not with density of noradrenergic innervation (Verity, 1971; Trendelenburg, 1972), it should be noted that neuromuscular distance in some tissues may be exceeded in importance by assymetry of innervation (Kenakin, 1984). Thus, the denser noradrenergic innervation in the prostatic portion (Anton *et al.*, 1977) does not necessarily imply that the α_1-adrenoceptors are confined to the synaptic region and evidence for extrasynaptic localization of α_1-adrenoceptors

Table 3 Effects of chronic treatment with several inhibitors of neuronal noradrenaline uptake on binding of [^{3}H]-prazosin to α_1-adrenoceptors of prostatic and epididymal portions of rat vas deferens

	Prostatic portion			*Epididymal portion*		
	K_D	B_{max}	Δ	K_D	B_{max}	Δ
Control	0.36 ± 0.03	137.5 ± 12.3		0.34 ± 0.03	129.4 ± 8.9	
Nisoxetine	0.30 ± 0.02	105.1 ± 6.7*	23	0.29 ± 0.02	78.4 ± 1.1**	40
Nomifensine	0.42 ± 0.02	117.1 ± 4.1*	15	0.41 ± 0.05	88.2 ± 5.1**	30
Desipramine	0.38 ± 0.05	120.4 ± 10.1	—	0.41 ± 0.03	105.5 ± 2.1*	19

Data are expressed as means ± s.e.mean of 6 independent experiments. K_D is the affinity constant for [^{3}H]-prazosin in nM, and B_{max} maximal number of binding sites in fmol mg^{-1} protein. Δ% of decrease of B_{max} control where these are significantly different from control.
**$P<0,01$; *$P<0.05$ versus control value.

has been reported in the guinea-pig mesenteric and rat tail arteries (Itoh *et al.*, 1983).

Although a reduction in α_1-adrenoceptor density has been obtained in the whole rat vas deferens after chronic treatment with desipramine (20% reduction, as determined by [^{3}H]-WB-4101 binding) (Wetzel *et al.*, 1981), this has not been consistently reproduced in other tissues, including the central nervous system (Vetulani *et al.*, 1984; Kendall & Nahorski, 1985; Newman & Lerer, 1988; Sallés *et al.*, 1989). Various factors affecting the concentration of noradrenaline in the synaptic cleft may account for these differences between tissues, and the smooth muscle of the rat vas deferens has the densest innervation and probably the narrower synaptic cleft (10–30 nm) (Furness & Iwajama, 1971), as compared to other noradrenergically innervated tissues. A high affinity binding site for [^{3}H]-desipramine, apparently associated with the neuronal noradrenaline uptake complex, is present in both peripheral and central tissues possessing noradrenergic innervation (Raisman *et al.*, 1982) and it has been demonstrated that there is a 16 fold higher binding capacity for this ligand in the rat vas deferens compared to the rat cerebral cortex (1680 versus 101 fmol mg^{-1} protein), data that are in parallel with the endogenous noradrenaline content determined in both tissues (12756 versus 120 ng g^{-1} tissue) (Langer *et al.*, 1984). Thus, the increase in noradrenaline concentration in the synaptic cleft following inhibition of neuronal noradrenaline uptake is likely to be greater in the rat vas deferens than in other tissues.

In the epididymal portion of the vas deferens, the three treatments decreased the potency of phenylephrine. In contrast, in the prostatic half nomifensine and nisoxetine not only reduced the potency but also the maximal contractions elicited by phenylephrine while treatment with desipramine did not modify the responses. It can be assumed that the α_1-adrenoceptor subsensitivity observed after chronic treatment with the three compounds is not due to a direct antagonism of receptors by residual drug present in the tissue, because of the relatively low affinity of these compounds for the α_1-adrenoceptors (Richelson & Nelson, 1984).

Recently, it has been demonstrated that there is a non-linear relationship between response and occupancy of α_1-adrenoceptors in both halves of rat vas deferens, the occupancy-response coupling being more efficient in the epididymal than in the prostatic portion (Sallés & Badia, 1991). In fact, in the epididymal half the maximal effect could be obtained when only 30% of the α_1-adrenoceptors were occupied. An initial decrease in receptor density will increase the fraction of receptors that need to be occupied to produce a response, resulting in a parallel rightward shift of the concentration-response curve. In contrast, in the prostatic portion all the α_1-adrenoceptors need to be occupied to induce a maximal effect and a decrease in receptor density will decrease the maximal response with only slight modification to the potency of the agonist. This pattern of functional changes with the loss of receptors agrees with the data obtained in our study in both portions of rat vas deferens.

In conclusion, our results indicate that the distinct regulation of α_1-adrenoceptors in both portions of the rat vas deferens could result from a greater activation of these receptors in the epididymal as compared to the prostatic halves after chronic inhibition of neuronal noradrenaline uptake. Our results could support the hypothesis that, in the prostatic portion, postjunctional α_1-adrenoceptors are less influenced by noradrenaline released from sympathetic nerve terminals, because they are probably more distant from the varicosities. The functional consequences observed in each portion of rat vas deferens after the decrease of the density of α_1-adrenoceptors could be explained as a result of the existence of non-linearity of the relationship between agonist occupancy and response in these tissues.

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References

ANTON, P.G., DUNCAN, M.E. & MCGRATH, J.C. (1977). An analysis of the anatomical basis for the mechanical response to motor nerve stimulation of the rat vas deferens. *J. Physiol.*, **273**, 23–43.

BADIA, A. & SALLES, J. (1989). Effects of St-587 on the α-adrenoceptors in the bisected rat vas deferens. *J. Pharm. Pharmacol.*, **41**, 612–616.

BADIA, A. & SALLES, J. (1990). Adaptational changes of α_1-adrenoceptors in the bisected rat vas deferens after chronic inhibition of neuronal uptake. *Eur. J. Pharmacol.*, **183**, 739.

BRADLEY, L. & DOGGRELL, S.A. (1985). Modification by desipramine of the effects of α-adrenoceptor antagonists on the contractile responses of the trisected rat vas deferens. *Gen. Pharmacol.*, **16**, 475–482.

BROWN, D.A., DOCHERTY, J.R., FRENCH, A.M., MACDONALD, A., MCGRATH, J.C. & SCOTT, N.C. (1983). Separation of adrenergic and non-adrenergic contractions to field stimulation in the rat vas deferens. *Br. J. Pharmacol.*, **79**, 379–393.

DREW, G.M. (1977). Pharmacological characterization of the presynaptic α_2-adrenoceptors in the rat vas deferens. *Eur. J. Pharmacol.*, **42**, 123–130.

FURNESS, J.B. & IWAJAMA, T. (1971). Terminal axons ensheated in smooth muscle cells of the vas deferens. *Z. Zellforsch*, **113**, 259–270.

GARCIA-SEVILLA, J.A. & ZUBIETA, J.K. (1986). Activation and desensitization of presynaptic α_2-adrenoceptors after inhibition of neuronal uptake by antidepressant drugs in the rat vas deferens. *Br. J. Pharmacol,*. **89**, 673–683.

GONÇALVES, J. & GUIMARAES, S. (1991). Influence of neuronal uptake on pre- and postjunctional effects of α-adrenoceptor agonists in tissues with noradrenaline-ATP cotransmission. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **344**, 532–537.

ITOH, T., KITAMURA, K. & KURIYAMA, H. (1983). Roles of extrajunctional receptors in the response of guinea-pig mesenteric and rat tail arteries to adrenergic nerves. *J. Physiol.*, **345**, 409–422.

KASUYA, Y. & SUZUKI, N. (1979). Variation of postjunctional natures along the length of the rat vas deferens as a cause of regional difference in the sensitivity to norepinephrine. *Arch. Int. Pharmacodyn.*, **241**, 24–31.

KENAKIN, T.P. (1984). The classification of drugs and drug-receptors in isolated tissues. *Pharmacol. Rev.*, **36**, 165–222.

KENDALL, D.A. & NAHORSKI, S.R. (1985). 5-Hydroxytryptamine-stimulated inositol phospholipid hydrolysis in rat cerebral cortex slices: pharmacological characterization and effects of antidepressants. *J. Pharmacol. Exp. Ther.*, **233**, 473–479.

LANGER, S.Z., TAHRAOUI, L., RAISMAN, R., ARBILLA, S., NAJAR, M. & DEDEK, J. (1984). [^{3}H]desipramine labels a site associated with the neuronal uptake of noradrenaline in the peripheral and central nervous system. In *Neuronal and Extraneuronal Events in Autonomic Pharmacology*. ed. Fleming, W.W., Langer, S.Z., Graefe, K.-H. & Weiner, N. pp. 37–49. New York: Raven Press.

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

MACDONALD, A. & MCGRATH, J.C. (1980). The distribution of adrenoceptors and other drug receptors between the two ends of the rat vas deferens as revealed by selective agonists and antagonists. *Br. J. Pharmacol.*, **71**, 445–458.

MCGRATH, J.C. (1978). Adrenergic and non-adrenergic components in the contractile response of the rat vas deferens to a single indirect stimulus. *J. Physiol.*, **283**, 23–39.

MCPHERSON, G.A. (1983). A practical computer-based approach to the analysis of radioligand binding experiments. *Computer Progr. Biomed,*. **17**, 107–113.

MINNEMAN, K.P., FOX, A.W. & ABEL, P.W. (1983). Occupancy of α_1-adrenergic receptors and contraction of rat vas deferens. *Mol. Pharmacol.*, **23**, 359–368.

MOORE, P.K. & GRIFFITHS, R.J. (1982). Pre-synaptic and post-synaptic effects of xylazine and naphazoline on the bisected rat vas deferens. *Arch. Int. Pharmacodyn.*, **260**, 70–77.

NEWMAN, M.E. & LERER, B. (1988). Effects of lithium and desipramine administration on agonist-stimulated inositol phosphate accumulation in rat cerebral cortex. *Biochem. Pharmacol,*. **37**, 1991–1995

RAISMAN, R., SETTE, M., PIMOULE, C., BRILEY, M. & LANGER, S.Z. (1982). High-affinity [^{3}H]desipramine binding in the peripheral and central nervous system: a specific site associated with the neuronal uptake of noradrenaline. *Eur. J. Pharmacol.*, **78**, 345–351.

RICHELSON, E. & NELSON, A. (1984). Antagonism by antidepressants of neurotransmitter receptors of normal human brain in vitro. *J. Pharmacol. Exp. Ther.*, **230**, 94–112.

RICHELSON, E. & PFENNING, M. (1984). Blockade by antidepressants and related compounds of biogenic amine uptake into rat brain synaptosomes: most antidepressants selectively block norepinephrine uptake. *Eur. J. Pharmacol.*, **104**, 277–286.

SALLES, J. & BADIA, A. (1991). Mechanisms underlying the differential sensitivity to α_1-adrenoceptor activation in the bisected rat vas deferens. *Br. J. Pharmacol,*. **102**, 439–445.

SALLES, J. & CLARO, E., GARCIA, A., PICATOSTE, F. & BADIA, A. (1989). Differential effects of chronic treatment with mianserin and protryptiline on rat brain cortical α_1-adrenoceptors. *Brain Res,*. **498**, 366–370.

SJOSTRAND, N.O. (1981). Smooth muscles of vas deferens and other organs in the male reproductive tract. In *Smooth Muscles* ed. Bülbring, E., Brading, A., Jones, A. & Tomita, T. pp. 367–376. London: Edward Arnold.

SMITH, C.B., GARCIA-SEVILLA, J.A. & HOLLINGSWORTH, P.J. (1982). Alpha$_2$-adrenoceptors in rat brain are decreased after long-term tricyclic antidepressant drug treatment. *Brain Res.*, **210**, 413–418.

SNEDDON, P., WESTFALL, D.P. & FEDAN, J.S. (1982). Cotransmitters in the motor nerves of the guinea-pig vas deferens: electrophysiological evidence. *Science*, **218**, 693–695.

SVENSSON, T.H. & USDIN, T. (1978). Feed-back inhibition of brain noradrenaline neurons by tricyclic antidepressants: α-receptor mediation. *Science*, **202**, 1089–1091.

TRENDELENBURG, U. (1972). Factors influencing the concentration of catecholamines at the receptors. In *Catecholamines, Handbook of Experimental Pharmacology*, vol XXXIII. ed. Blaschko, H. & Muscholl, E. pp. 726–761. New York: Springer-Verlag.

VARDOLOV, L. & PENNEFATHER, J.N. (1976). Regional variation in the distribution of α-adrenoceptors in the vas deferens of the rat. *Arch. Int. Pharmacodyn.*, **221**, 212–222.

VERITY, M.A. (1971). Morphologic-studies of vascular neuroeffector apparatus. In *Physiology and Pharmacology of Vascular Neuroeffector Systems*. ed. Bevan, J.A., Furchgott, R.F., Maxwell, R.A., Somlyo, A.P. pp. 2–12. Basel: Karger.

VETULANI, J., ANTKIEWICZ-MICHALUK, L. & ROKOSZ-PELC, A. (1984). Chronic administration of antidepressant drugs increases the density of cortical [^{3}H]prazosin binding sites in the rat. *Brain Res.*, **310**, 360–362.

VILA, E., SALLES, J. & BADIA, A. (1990). Effects of chronic antidepressant treatment on α_1- and α_2-adrenoceptors in the rat anococcygeus muscle. *J. Neural Trans,*. **82**, 205–212.

WERNECK DE AVELLAR, M.C., KOBASHI, Y.L. & MARKUS, R.P. (1990). Age-related changes in neuronal uptake of noradrenaline. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **341**, 295–300.

WETZEL, H.W., BRILEY, M.S. & LANGER, S.Z. (1981). [^{3}H]WB-4101 binding in the rat vas deferens. Effects of chronic treatments with desipramine and prazosin. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **317**, 187–192.

ZIEHER, L.M. & JAIM-ETCHEVERRI, G. (1971). Regional variations in the distribution of noradrenaline along the rat vas deferens. *J. Pharmacol. Exp. Ther.*, **23**, 61–63.

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Comparison of the regional haemodynamic effects of the AT_1-receptor antagonists, losartan and EXP 3174, in water-deprived Brattleboro rats

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1 Two separate groups ($n = 8$) of Brattleboro rats, chronically instrumented for the measurement of regional haemodynamics, underwent a 2-stage experimental protocol. Initially, animals had their drinking water removed and cardiovascular recordings were made every 30 min for the following 6 h. Then animals received either the AT_1-receptor antagonist, losartan (3 mg kg^{-1}, i.v., $n = 8$), or an initially equi-hypotensive dose of its active metabolite, EXP 3174 (1 mg kg^{-1}, i.v., $n = 8$), and the resultant cardiovascular changes were monitored for a further 2 h. A third group of Brattleboro rats ($n = 8$) was water-deprived for 8 h to serve as a time control.

2 In all 3 groups of animals, mesenteric and hindquarters vasoconstriction (beginning approximately 30 and 120 min, respectively, after water was removed) occurred much earlier than changes in blood pressure, since increases in blood pressure were significant only after 5–6 h of water deprivation; renal perfusion was largely unchanged. The observation of a differential onset of haemodynamic changes (i.e. mesenteric > hindquarters >> renal) extends our previous findings, in which measurements were made only at the end of a 14 h period of water-deprivation.

3 When given after 6 h of water deprivation, losartan and EXP 3174 produced directionally similar, but temporally disparate, haemodynamic effects. EXP 3174 caused a depressor response associated with marked renal, mesenteric and hindquarters vasodilatations which were well maintained over 2 h. Losartan evoked similar changes to EXP 3174 in the first 5 min, but in contrast to EXP 3174, blood pressure showed some recovery and all 3 vascular conductance values returned to baseline (i.e. pre-drug) levels over the following 10–20 min. Thereafter, hypotension and renal, mesenteric and hindquarters vasodilatation again occurred, and these changes were maintained for the rest of the 2 h. However, compared with losartan, EXP 3174 caused significantly greater mesenteric and hindquarters vasodilatation, even at times when both compounds lowered blood pressure to the same extent.

4 The biphasic cardiovascular response caused by losartan is consistent with the conversion of the parent compound to EXP 3174. Whether or not the enhanced vasodilator effect of EXP 3174 over losartan is related to pharmacodynamic differences (i.e., noncompetitive versus competitive antagonism, respectively), and/or to differences in the amount of EXP 3174 generated from losartan is not known at present.

Keywords: Losartan; EXP 3174; haemodynamic effects; water deprivation; Brattleboro rats

Introduction

It is now well established that there are at least 2 subtypes of angiotensin II (AII) receptors which have a widespread central and peripheral distribution (Timmermans *et al.*, 1991; Steckeling *et al.*, 1992). The AT_1-receptors are defined by their sensitivity to losartan (DuP 753), whereas AT_2-receptors are those that are sensitive to PD 123177 and related analogues (Bumpus *et al.*, 1991).

AT_1-receptors in the peripheral vasculature mediate vasoconstriction (Wong *et al.*, 1990a; Chiu *et al.*, 1990), and, as a consequence, losartan lowers blood pressure in spontaneously hypertensive rats (SHR) (Wong *et al.*, 1990d), in rats with hypertension due to renal artery ligation (Wong *et al.*, 1990a,c), in water-deprived Brattleboro rats (Batin *et al.*, 1991a,b) and in frusemide-treated, normotensive rats (Wong *et al.*, 1990b). In contrast, losartan is ineffective in rats with DOCA-salt hypertension and in normotensive rats, both of which are models with normal or low renin states (Wong *et al.*, 1990b,c). In our previous studies (Batin *et al.*, 1991a, b), the cardiac and regional haemodynamic effects of losartan were assessed in water-deprived Brattleboro rats, a 15 min incremental dosing schedule being employed as used by Wong *et al.* (1990b,c). We noted that the cardiovascular effects of low doses of losartan (up to 3 mg kg^{-1}) were transient, and this is consistent with the effects of losartan in other studies (Wong *et al.*, 1990b,c,d).

While our previous experiments were in progress, it was reported that in rats, losartan has an active metabolite, EXP 3174, which was more potent that the parent compound in a variety of assays (Wong *et al.*, 1990e). Additionally, unlike losartan, EXP 3174 behaved as a noncompetitive AT_1-receptor antagonist (Wong *et al.*, 1990e).

In our earlier studies, the possible contribution of the effect of EXP 3174 to the action of losartan could not be directly assessed because losartan was given in an incremental dosing schedule, and EXP 3174 was not available for testing at that time. Therefore, the major aim of the present study was to compare the regional haemodynamic effects of initially equi-hypotensive doses of losartan and EXP 3174 in conscious, water-deprived Brattleboro rats.

Previous studies from our laboratory have shown that interference with the renin-angiotensin system (RAS) has profound effects on cardiovascular status in water-deprived Brattloboro rats, but not in water-replete Brattleboro rats (Gardiner & Bennett, 1985). Water deprivation elevates plasma AII levels (Bennett & Gardiner, 1987) and blood pressure (Gardiner & Bennett, 1985; Batin *et al.*, 1991a,b), and these changes are associated with marked mesenteric and

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hindquarters vasonconstriction and a decrease in cardiac output (Gardiner *et al.*, 1988b; Muller *et al.*, 1991; Batin *et al.*, 1991a,b). However, while these effects have been reported after 14 h water deprivation, the time course of their onset has not been previously documented. Therefore, a secondary aim of the present study was to delineate the time course of regional haemodynamic changes in Brattleboro rats during water deprivation.

Methods

Male, Brattleboro rats (350 to 400 g) were anaesthetized (sodium methohexitone, 60 mg kg^{-1} i.p., supplemented as required) and had pulsed Doppler flow probes implanted to monitor changes in renal, mesenteric and hindquarters blood flows (Gardiner *et al.*, 1990b). At least 7 days later, animals were briefly anaesthetized (sodium methohexitone, 40 mg kg^{-1} i.p.) and 3 separate catheters were implanted in the right jugular vein and a catheter inserted into the distal abdominal aorta, via the caudal artery. Animals were returned to their home cages and left to recover for at least 48 h before experiments were begun.

Continuous recordings were made of mean and phasic intra-arterial blood pressures and instantaneous heart rate, and mean and phasic renal, mesenteric and hindquarters Doppler shift signals using a modified (Gardiner *et al.*, 1990a) pulsed Doppler system (Crystal Biotech, Holliston, U.S.A.). It has been shown that % change in the Doppler shift signal is a reliable index of change in blood flow (Haywood *et al.*, 1981). Regional vascular conductances were calculated by dividing the appropriate mean Doppler shift by mean arterial blood pressure (Gardiner *et al.*, 1990b).

Three separate groups of Brattleboro rats (each $n = 8$) were used in these studies. Baseline values for the cardiovascular variables were recorded for at least 30 min prior to the removal of drinking water, and the cardiovascular variables were monitored every 30 min for the following 6 h. After that time, one group of Brattleboro rats received losartan in a dose of 3 mg kg^{-1} i.v. Previous studies had shown that the dose of 3 mg kg^{-1} was the highest dose of losartan which caused transient effects (Wong *et al.*, 1990b,c,d; Batin *et al.*, 1991a,b) although in those studies cardiovascular changes were monitored for 15 min only. Therefore, we chose this dose for the present experiments, but monitored its effect over 2 h.

A second group of rats received EXP 3174 at a dose of 1 mg kg^{-1} i.v., 6 h after water deprivation and the effects of this compound were monitored for a further 2 h. This dose of EXP 3174 was chosen (on the basis of pilot experiments) to match the initial hypotensive effect of losartan. At these concentrations, losartan and EXP 3174 caused almost complete inhibition of the cardiovascular responses to exogenous AII for at least 3 and 6 h, respectively (Wong *et al.*, 1990b,c; Widdop *et al.*, 1992).

A third group of Brattleboro rats served as a time control for the drug treatment groups, and so these animals were deprived of water for a total of 8 h, but with no drug interventions.

Data analysis

In each group, the effect of water deprivation, relative to baseline status, was assessed by one-way analysis of variance (ANOVA) with repeated measures. The effects of losartan and EXP 3174 were assessed in a similar manner, but the drug-induced changes given in the Results are relative to the immediate, preceding (i.e. after 6 h water deprivation) baseline. For clarity, the group standard errors are given in the figures, and these were calculated by use of the equation $\sqrt{EMS/n}$, where EMS is the error mean square from the ANOVA and n is the number of animals in the group. In addition, to allow analysis of the cardiovascular effects of losartan and EXP 3174 over the 2 h observation period, the areas over (for mean arterial blood pressure (MAP)) or under (for all other haemodynamic variables) the curves following administration of either compound were quantified for each individual animal on the basis of change relative to baseline (using a Fortran programme written in our Department). These values (in arbitrary units), as well as the end-point values (i.e. after 120 min), were compared by the nonpaired Student's t test. A P value <0.05 was taken as significant.

Drugs

Losartan (DuP 753; 2-n-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl)-biphenyl-4-yl)methyl] imidazoline, potassium salt) was dissolved in isotonic saline, while EXP 3174 ((2-n-buytl-4-chloro-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl) methyl] imidazole-5-carboxylic acid) was dissolved in equal parts of 5% $NaHCO_3$ and 5% dextrose. Losartan and EXP 3174 were obtained from Dr R. Smith (DuPont, U.S.A.).

Results

Resting cardiovascular variables

The cardiovascular variables in the resting state, i.e. prior to removal of water, were not significantly different in the 3 groups of Brattleboro rats (Table 1).

Effect of water deprivation (Figure 1)

There were no significant differences in the cardiovascular changes between the 3 groups of Brattleboro rats in response to 6 h of water deprivation ($P>0.05$, ANOVA). There were small increases (6 to 12 mmHg) in MAP which were significant ($P<0.05$) by 5 h in all 3 groups of Brattleboro rats, and heart rate tended to decrease but this was significant ($P<0.05$) only in the group subsequently given losartan. Renal flow did not change significantly in any group, although there were small decreases (12 to 18%) in renal conductance which were significant ($P<0.05$) by 5–6 h in all 3 groups of Brattleboro rats. In contrast, there were significant reductions ($P<0.01$) in mesenteric flow (14 to 16%) and conductance (13 to 15%) as soon as 30 min after drinking water was removed, and these changes reached a

Table 1 Resting cardiovascular variable in the 3 groups of Brattleboro rats ($n = 8$)

	Control group	*Losartan group*	*EXP 3174 group*
Heart rate (beats min^{-1})	318 ± 9	321 ± 5	337 ± 10
Mean arterial blood pressure (mmHg)	114 ± 2	110 ± 2	110 ± 2
Renal flow (kHz)	8.2 ± 1.0	6.4 ± 1.2	7.9 ± 0.7
Mesenteric flow (kHz)	8.5 ± 1.2	8.9 ± 1.1	11.2 ± 0.8
Hindquarters flow (kHz)	4.5 ± 0.4	5.6 ± 0.7	4.4 ± 0.3
Renal conductance ([kHz $mmHg^{-1}$]10^3)	71 ± 8	58 ± 11	72 ± 6
Mesenteric conductance ([kHz $mmHg^{-1}$]10^3)	75 ± 11	81 ± 10	102 ± 7
Hindquarters conductance ([kHz $mmHg^{-1}$]10^3)	40 ± 4	51 ± 7	40 ± 3

Values are mean ± s.e.mean.

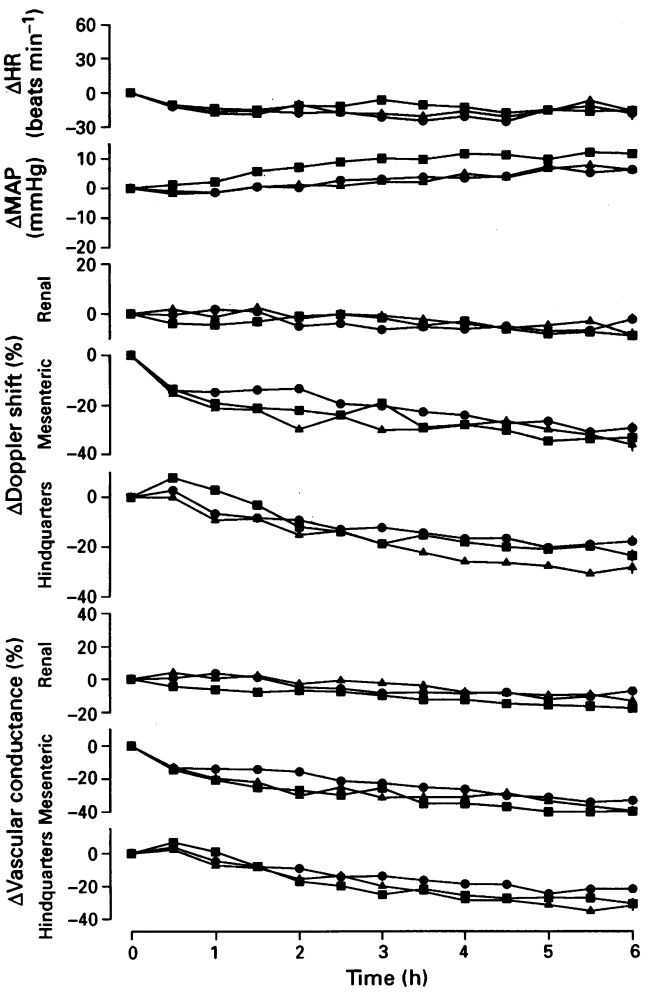


Figure 1 Changes in cardiovascular responses during 6 h water deprivation in a control group of Brattleboro rats (●, $n = 8$) and in 2 other groups subsequently given either losartan (▲, $n = 8$) or EXP 3174 (■, $n = 8$). Values are mean with group s.e.mean shown by vertical lines at 6 h (where there is no vertical line, this is contained within the symbol) (see text for details).

nadir (30 to 40% reductions) 5–6 h after water deprivation. Significant ($P<0.01$) decreases in hindquarters flow (9 to 15%) and conductance (9 to 17%) were not seen until 2 h after water was removed; as in the mesenteric vascular bed these changes reached a nadir (20 to 35% reductions) 5–6 h after deprivation (see Figure 1).

In the group of rats that were deprived of water for a further 2 h, the aforementioned cardiovascular changes were well maintained over this time, indicating there was a steady state between 6–8 h (Figure 2).

Effect of losartan (Figure 2)

Losartan (3 mg kg^{-1} i.v.), given to Brattleboro rats 6 h after water deprivation, caused a biphasic haemodynamic response; there was an immediate reduction in MAP of 20 ± 2 mmHg. However, although the reduction in MAP was significant ($P<0.01$) throughout the 2 h observation period, there was a partial recovery of MAP between 5 and 25 min after giving losartan. The initial depressor response to losartan was associated with tachycardia (1–10 min, maximum = 60 ± 11 beats min^{-1}, $P<0.01$), as well as transient increases in renal (1–5 min, maximum = 24 ± 4%, $P<0.01$), and mesenteric (1–4 min, maximum = 32 ± 4%, $P<0.01$) blood flows; the transient increase in hindquarters flow failed to achieve significance. However, there were significant increases in calculated vascular conductances in the renal, mesenteric and hindquarters vascular beds (all $P<0.01$) over the first 5 min (maximum = 49 ± 8%), 10 min (maximum = 50 ± 5%) and 5 min (maximum = 37 ± 8%) min, respectively, following administration of losartan. However, these values returned to baseline levels (i.e. those recorded immediately prior to giving losartan) between 10–20 min (renal conductance), 15–25 min (mesenteric conductance) or 10–60 min (hindquarters conductance) after giving losartan. Thereafter, renal, mesenteric and hindquarters flows and vascular conductances showed secondary increases ($P<0.01$) for the rest of the observation period (Figure 2).

Effect of EXP 3174 (Figure 2)

EXP 3174 (1 mg kg^{-1}, i.v.), given to a separate group of Brattleboro rats, 6 h after water deprivation, caused an immediate and sustained reduction in blood pressure and sustained increases in renal and mesenteric flows and conductances over the subsequent 2 h observation period (all variables $P<0.01$). Hindquarters flow and conductance were also significantly increased ($P<0.01$), although the former variable did not increase significantly until 15 min after giving EXP 3174 and thereafter was elevated from 50–120 min ($P<0.01$) (Figure 2).

For comparison, the changes for each cardiovascular variable are listed 120 min after either losartan or EXP 3174

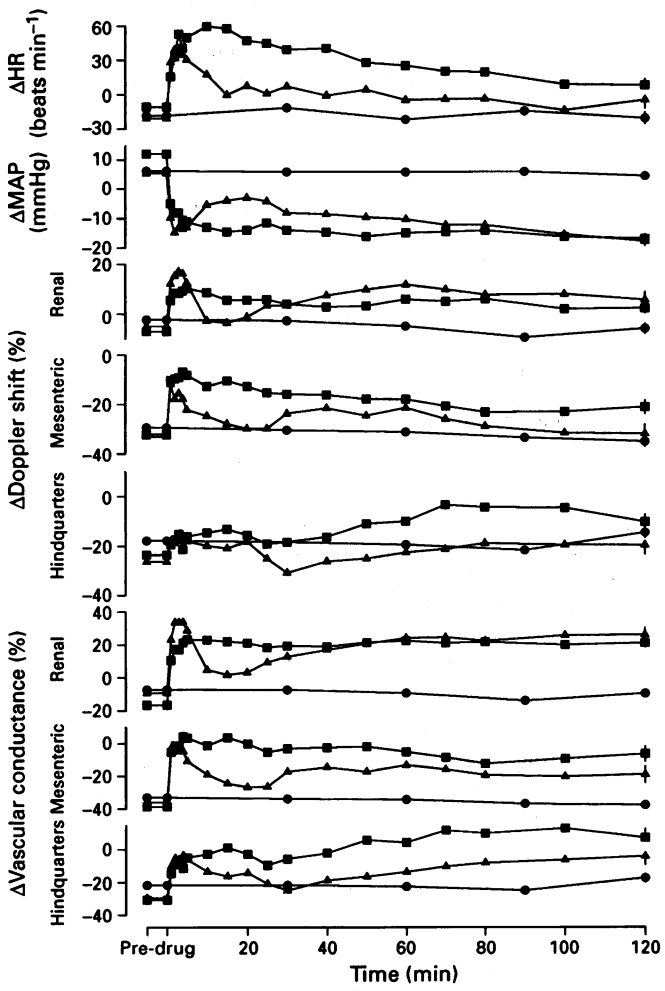


Figure 2 Changes in cardiovascular variables during 6–8 h water deprivation in a control group of Brattleboro rats (●, $n = 8$), and the effect of either 3 mg kg^{-1} losartan i.v. (▲, $n = 8$) or 1 mg kg^{-1} EXP 3174 i.v. (■, $n = 8$) administered 6 h after water deprivation (i.e. pre-drug). Values are plotted as changes from the original (pre-water deprivation) baseline. Values are mean with group s.e. mean shown by vertical lines at 120 min (where there is no vertical line, this is contained within the symbol) (see text for details).

Table 2 Changes (from pre-drug baseline) in cardiovascular variables 120 min after losartan (3 mg kg^{-1}, i.v.) or EXP 3174 (1 mg kg^{-1}, i.v.) and area over or under curve (AOC or AUC) for changes over 2 h after administration of either compound ($n = 8$)

	Losartan		*EXP 3174*	
	Responses at 120 min	*Integrated response* (AOC or AUC)	*Responses at 120 min*	*Integrated response* (AOC or AUC)
Δ Heart rate (beats min^{-1})	16 ± 10	3004 ± 815	19 ± 4	4625 ± 707
Δ Mean arterial blood pressure (mmHg)	−23 ± 2	1950 ± 264	−29 ± 2	3177 ± 258**
Renal flow (%Δ)	11 ± 6	1687 ± 401	11 ± 3	1671 ± 431
Mesenteric flow (%Δ)	1 ± 5	1430 ± 312	16 ± 5	2477 ± 304*
Hindquarters flow (%Δ)	10 ± 5	1049 ± 336	18 ± 4	2210 ± 297*
Renal conductance (%Δ)	39 ± 8	3960 ± 791	46 ± 4	5416 ± 483
Mesenteric conductance (%Δ)	26 ± 5	3404 ± 388	52 ± 7**	6521 ± 570**
Hindquarters conductance (%Δ)	37 ± 7	3037 ± 465	55 ± 4*	6184 ± 578**

Values are mean ± s.e.mean
*$P<0.05$; **$P<0.01$ versus corresponding value for losartan.

(Table 2). In addition, the overall cardiovascular response to both compounds was assessed by calculating the area over curve (for MAP) or area under curve (for all other variables) over 2 h (Table 2). These data indicate that EXP 3174 exerted significantly greater mesenteric and hindquarters vasodilator effects than losartan, although there were similar reductions in MAP with both compounds.

Discussion

In the present study, we compared the regional haemodynamic effects of losartan and EXP 3174 in water-deprived Brattleboro rats. The fact that both compounds caused marked hypotension confirms the dependence of cardiovascular status on the RAS in this model (Gardiner & Bennett, 1985). Six to 8 h of water deprivation caused marked mesenteric and hindquarters, but less renal, vasoconstriction. This order of effect, i.e. mesenteric > hindquarters >> renal, is very similar to that seen in previous studies in which measurements were made after 14 h of water deprivation (Gardiner *et al.*, 1988b; Muller *et al.*, 1990; Batin *et al.*, 1991a). In agreement with those studies, we found that blood pressure increased only slightly, presumably because the marked peripheral vasoconstriction was largely offset by a decrease in cardiac output (Batin *et al.*, 1991b), consequent upon a reduction in plasma volume (Gardiner & Bennett, 1985). Moreover, we also found that differential haemodynamic changes occurred as early as 30 min (for mesenteric) and 2 h (for hindquarters) after drinking water was removed, at a time when blood pressure was unchanged. Thus, it would seem that the mesenteric and hindquarters vasoconstrictions were responsible for the disproportionately greater decrease in total peripheral conductance than cardiac output (Batin *et al.*, 1991b). Renal perfusion was largely unchanged throughout the 6–8 h period of water deprivation; as pointed out previously (Batin *et al.*, 1991b), this is notable because Brattleboro rats show marked antidiuresis during water deprivation (Bennett & Gardiner, 1987).

The administration of losartan or EXP 3174 after 6 h water deprivation caused directionally similar, but temporally disparate, haemodynamic effects. While EXP 3174 caused marked hypotension and renal, mesenteric and hindquarters vasodilatations which were sustained over 2 h, losartan caused maximal changes in the first 5 min. These changes wāned over the following 10–20 min, to the extent that all 3 vascular conductance values were not significantly different from the baseline values recorded immediately prior to giving losartan, although MAP was still significantly decreased. However, thereafter, maximal hypotension and near-maximal vasodilatations re-developed in all three vascular beds for the rest of the observation period. The initial, transient, vasodilator response to losartan (3 mg kg^{-1}) was essentially identical to that seen previously by us (Batin *et al.*, 1991a,b). It is unlikely that the transience of the effects of losartan was due to a waning of drug action, since the active metabolite, EXP 3174, caused immediate and sustained cardiovascular effects. Rather, it is more likely that the resultant hypotension triggered additional stimulation of the RAS which then offset the competitive action of losartan (Batin *et al.*, 1991b). Indeed, this proposal is supported by the findings that losartan caused a compensatory increase in plasma AII levels, even after a single administration (Bunkenburg *et al.*, 1991; Christen *et al.*, 1991; Mizuno *et al.*, 1992). The fact that EXP 3174 had sustained vasodilator effects is consistent with the inability of raised AII levels to surmount the noncompetitive blockade exerted by EXP 3174. Interestingly, EXP 3174 also caused a significantly greater vasodilator response, compared with losartan, even at times when the hypotensive effects of both compounds were essentially identical. Previously, we showed that losartan increased cardiac output (Batin *et al.*, 1991b). Therefore, in order for blood pressure to be reduced to comparable levels with both AT_1-receptor antagonists, it follows that EXP 3174 must have caused a greater increase in cardiac output than losartan, and this partly offset the larger vasodilatation evoked by EXP 3174. Such an increase in cardiac output would most likely have been a consequence of the reduction in afterload as well as being reflexly mediated (Batin *et al.*, 1991b).

Whether or not the enhanced vasodilator action of EXP 3174 over losartan is related to pharmacodynamic differences (i.e., noncompetitive versus competitive antagonism, respectively), and/or to differences in the amount of EXP 3174 generated from losartan is not known. Surprisingly, there is a lack of published pharmacokinetic data on losartan, although it has been reported that similar amounts of losartan and EXP 3174 are found in plasma after oral dosing with losartan in rats (Wong *et al.*, 1990e) (although this is not the case in dogs, see Wong *et al.*, 1991a). Thus, it is likely that the secondary phase of the cardiovascular response to losartan was due to its conversion to EXP 3174.

Sustained cardiovascular changes, similar to those found here with losartan or EXP 3174 have been seen with three different angiotensin converting enzyme (ACE) inhibitors in the same experimental model (Gardiner *et al.*, 1988b; Tomlinson *et al.*, 1990; Miller *et al.*, 1990). The present study thus confirms that those findings were most likely due to inhibition of AII production, rather than to accumulation of vasodilator peptides (e.g. bradykinin) which are metabolized by the enzyme (Muller *et al.*, 1990). However, ACE inhibitors, and the AT_1-receptor antagonists studied here exerted effects on the hindquarters vasculature which are at variance with the systemic effects of exogenous AII, since this peptide causes marked renal and mesenteric vasoconstriction, but has minimal effect in the hindquarters vasculature (Gardiner *et al.*, 1988a; Widdop *et al.*, 1992). In this context, it has recently been reported that the prejunctional AII recep-

tor mediating the facilitation of noradrenaline release from sympathetic nerve terminals is of the AT_1-receptor subtype (Wong *et al.*, 1991b; 1992). Therefore, one possible explanation for the hindquarters vasodilatation caused by losartan and EXP 3174 is that these compounds inhibited the prejunctional AT_1-receptors in the hindquarters and thereby suppressed AII-induced facilitation of neurally-mediated vasoconstriction.

In conclusion, we have shown that, while EXP 3174 caused sustained hypotension and vasodilatation, a dose of losartan with an initial equi-hypotensive effect caused a biphasic cardiovascular response, with secondary vasodilatation in the mesenteric and hindquarters vascular beds which was significantly less than that seen with EXP 3174. These data are consistent with the conversion of losartan to EXP 3174 in conscious Brattleboro rats.

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References

BATIN, P., GARDINER, S.M., COMPTON, A.M. & BENNETT, T. (1991a). Differential regional haemodynamic effects of the nonpeptide angiotensin II antagonist, DuP 753, in conscious, water-deprived, Brattleboro rats. *Life Sci.*, **48**, 733–739.

BATIN, P., GARDINER, S.M., COMPTON, A.M., KEMP, P.A. & BENNETT, T. (1991b). Cardiac haemodynamic effects of the nonpeptide angiotensin II receptor antagonist, DuP 753, in conscious Long Evans and Brattleboro rats. *Br. J. Pharmacol.*, **103**, 1585–1591.

BENNETT, T. & GARDINER, S.M. (1987). Water deprivation: Effects on fluid and electrolyte handling and plasma biochemistry in Long Evans and Brattleboro rats. *J. Physiol.*, **385**, 35–48.

BUMPUS, F.M., CAT, K.J., CHIU, A.T., DE GASPARO, M., GOODFRIEND, T., HUSAIN, A., PEACH, M.J., TAYLOR, D.G. & TIMMERMAN,S P.B.M.W.M. (1991). Nomenclature for angiotensin receptors. *Hypertension*, **17**, 720–721.

BUNKENBURG, B., SCHNELL, C., BAUM, H.-P., CUMIN, F. & WOOD, J.M. (1991). Prolonged angiotensin II antagonism in spontaneous hypertensive rats. *Hypertension*, **18**, 278–288.

CHIU, A.T., MCCALL, D.E., PRICE, W.A., WONG, P.C., CARINI, D.J., DUNCIA, J.V., WEXLER, R.R., YOO, S.E., JOHNSON, A.L. & TIMMERMANS, P.B.M.W.M. (1990). Non-peptide angiotensin II receptor antagonists. VII. Cellular and biochemical pharmacology of DuP 753, an orally active antihypertensive agent. *J. Pharmacol. Exp. Ther.*, **252**, 711–718.

CHRISTEN, Y., WAEBER, B., NUSSBERGER, J., PORCHET, M., BORLAND, R.M., LEE, R.J., MAGGON, K., SHUM, L., TIMMERMANS, P.B.M.W.M. & BRUNNER, H.R. (1991). Oral administration of DuP 753, a specific angiotensin II receptor antagonist, to normal male volunteers. *Circulation*, **83**, 1333–1342.

GARDINER, S.M. & BENNETT, T. (1985). Interactions between neural mechanisms, the renin-angiotensin system and vasopressin in the maintenance of blood pressure during water deprivation; studies in Long Evans and Brattleboro rats. *Clin. Sci.*, **69**, 647–657.

GARDINER, S.M., BENNETT, T. & COMPTON, A.M. (1988a). Regional haemodynamic effects of neuropeptide Y, vasopressin and angiotensin II in conscious, unrestrained, Long Evans and Brattleboro rats. *J. Auton. Nerv. Syst.*, **24**, 15–27.

GARDINER, S.M., COMPTON, A.M. & BENNETT, T. (1988b). Regional haemodynamic effects of atrial natriuretic peptide or captopril in Brattleboro rats. *Am. J. Physiol.*, **255**, R737–R743.

GARDINER, S.M., COMPTON, A.M., BENNETT, T. & HARTLEY, C.J. (1990a). Can pulsed Doppler technique measure changes in aortic flow in conscious rats? *Am. J. Physiol.*, **259**, H448–H456.

GARDINER, S.M., COMPTON, A.M., KEMP, P.A. & BENNETT, T. (1990b). Regional and cardiac haemodynamic effects of N^G-nitro-L-arginine methyl ester in conscious, Long Evans rats. *Br. J. Pharmacol.*, **101**, 625–631.

HAYWOOD, J.R., SHAFFER, R.A., FASTENOW, C., FINK, G.D. & BRODY, M.J. (1981). Regional blood flow measurement with pulsed Doppler flowmeter in conscious rat. *Am. J. Physiol.*, **241**, H273–H278.

MIZUNO, K., NIIMURA, S., TANI, M., HAYA, H., COMIBUCHI, T., SANADA, H. & FUKUCHI, S. (1992). Antihypertensive and hormonal activity of MK 954 in spontaneously hypertensive rats. *Eur. J. Pharmacol.*, **215**, 305–308.

MULLER, A.F., GARDINER, S.M., COMPTON, A.M. & BENNETT, T. (1990). Regional haemodynamic effects of captopril, enalaprilat and lisnopril in conscious water-replete and water-deprived Brattleboro rats. *Clin Sci.*, **79**, 393–401.

STECKELING, V.M., BOTTARI, S.P. & UNGER, T. (1992). Angiotensin receptor subtypes in the brain. *Trends Pharmacol. Sci.*, **13**, 365–368.

TIMMERMANS, P.B.M.W.M., WONG, P.C., CHIU, A.T. & HERBLIN, W.F. (1991). Non-peptide angiotensin II receptor antagonists. *Trends Pharmacol. Sci.*, **12**, 55–62.

TOMLINSON, K.C., GARDINER, S.M. & BENNETT, T. (1990). Hypotensive effects of angiotensin II analogues and angiotensin converting enzyme inhibitors in water-deprived Brattleboro rats. *J. Cardiovasc. Pharmacol.*, **15**, 562–568.

WIDDOP, R.E., GARDINER, S.M., KEMP, P.A., & BENNETT, T. (1992). Inhibition of the haemodynamic effects of angiotensin II in conscious rats by AT_2-receptor antagonists given after the AT_1-receptor antagonist, EXP 3174. *Br. J. Pharmacol.*, (in press).

WONG, P.C., BERNARD, R. & TIMMERMANS, P.B.M.W.M. (1992). Effect of blocking angiotensin II receptor subtype on rat sympathetic nerve function. *Hypertension*, **19**, 663–667.

WONG, P.C., HART, S.D., DUNCIA, J.V. & TIMMERMANS, P.B.M.W.M. (1991a). Nonpeptide angiotensin II receptor antagonists. Studies with DuP 753 and EXP 3174 in dogs. *Eur. J. Pharmacol.*, **202**, 323–330.

WONG, P.C., HART, S.D. & TIMMERMANS, P.B.M.W.M. (1991b). Effect of angiotensin II antagonism on canine renal sympathetic nerve function. *Hypertension*, **17**, 1127–1134.

WONG, P.C., HART., S.D., ZASPEL, A.M., CHIU, A.T., ARDECKY, R.J., SMITH. R.D. & TIMMERMANS, P.B.M.W.M. (1990a). Functional studies of nonpeptide angiotensin II receptor subtype-specific ligands: DuP 753 (All-1) and PD 123177 (All-2). *J. Pharmacol. Exp. Ther.*, **255**, 584–592.

WONG, P.C., PRICE, W.A., CHIU, A.T., DUNCIA, J.V., CARINI, D.J., WEXLER, R.R., JOHNSON, A.L. & TIMMERMANS, P.B.M.W.M. (1990b). Non-peptide angiotensin II receptor antagonists. VIII. Characterization of functional antagonism displayed by DuP 753, an orally active antihypertensive agent. *J. Pharmacol. Exp. Ther.*, **252**, 719–725.

WONG, P.C., PRICE, W.A., CHIU, A.T., DUNCIA, J.V., CARINI, D.J., WEXLER, R.R., JOHNSON, A.L. & TIMMERMANS, P.B.M.W.M. (1990c). Non-peptide angiotensin II receptor antagonists. IX. Antihypertensive activity in rats of DuP 753, an orally active antihypertensive agent. *J. Pharmacol. Exp. Ther.*, **252**, 726–732.

WONG, P.C., PRICE, W.A., CHIU, A.T., DUNCIA, J.V., CARINI, D.J., WEXLER, R.R., JOHNSON, A.L. & TIMMERMANS, P.B.W.M.W. (1990d). Hypotensive action of DuP 753, an angiotensin II antagonist, in spontaneously hypertensive rats. *Hypertension*, **15**, 459–468.

WONG, P.C., PRICE, W.A., CHIU, A.T., DUNCIA, J.V., CARINI, D.J., WEXLER, R.R., JOHNSON, A.L. & TIMMERMANS, P.B.M.W.M. (1990e). Nonpeptide angiotensin II receptor antagonists. XI. Pharmacology of EXP 3174: an active metabolite of DuP 753, an orally active antihypertensive agent. *J. Pharmacol. Exp. Ther.*, **255**, 211–217.

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The regulation of aortic endothelial cells by purines and pyrimidines involves co-existing P_{2y}-purinoceptors and nucleotide receptors linked to phospholipase C

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1 We have examined the phospholipase C responses in bovine aortic endothelial cells to purines (ATP, ADP and analogues) and the pyrimidine, uridine triphosphate (UTP).

2 The cells responded to purines in a manner consistent with the presence of P_{2y} purinoceptors; both 2-methylthioadenosine 5'-triphosphate (2MeSATP) and adenosine 5'-0-(2-thiodiphosphate) (ADPβS) were potent agonists (EC_{50} 0.41 μM and 0.85 μM respectively) while β, γ-methylene ATP at 300 μM was not.

3 The cells also responded to UTP. The maximal response to UTP was less than that for either 2MeSATP and ADPβS while adenosine 5'-0-(3-thiotriphosphate) (ATPγS) gave the largest maximal response.

4 The concentration-effect curve to UTP was additive in the presence of either 2MeSATP or ADPβS. However, the concentration-effect curves to ATPγS reached the same maximum in the presence or absence of UTP.

5 Suramin, at concentrations between 10 μM and 100 μM was a competitive antagonist for the response to ADPβS and 2MeSATP but not the response to UTP.

6 The results show that there are two separate, co-existing, receptor populations: P_{2y}-purinoceptors (responding to purines) and nucleotide receptors (responding to both purines and pyrimidines). We conclude that purines such as ATP/ADP may regulate aortic endothelial cells by interacting with two phospholipase C-linked receptors.

Keywords: Endothelial cells; P_2-purinoceptors; P_{2Y}; nucleotide receptors

Introduction

The role of endothelial cells in a variety of processes, such as vascular permeability, smooth muscle contraction and thrombotic events, is under the control of several receptors, including those responding to adenosine 5'-triphosphate (ATP) and adenosine 5'-diphosphate (ADP) (the P_2-purinoceptors). Lacking selective antagonists, the subclassification of these P_2 receptors has been based upon rank order of agonist potencies. The order α,β-methylene ATP = β,γ-methylene-ATP > ATP = 2-methylthioadenosine 5'-triphosphate (2MeSATP) is characteristic of P_{2x} receptors, while 2MeSATP > ATP > α,β-methylene ATP = β,γ-methylene ATP is taken to indicate the presence of P_{2Y} receptors (Burnstock & Kennedy, 1985). Aortic endothelial cells have been shown to respond with relaxation of associated smooth muscle by action of ATP analogues at P_{2Y} receptors (Martin *et al.*, 1985; Houston *et al.*, 1987) which are linked to phospholipase C (Pirotton *et al.*, 1987; Allsup & Boarder, 1990). Phospholipase D (Martin & Michaelis, 1989; Pirotton *et al.*, 1990; Purkiss *et al.*, 1992; Purkiss & Boarder, 1992) is also activated by ATP in these cells. The consequent regulation of intracellular Ca^{2+} and protein kinases is considered to be the mechanism whereby these cells control their various functions, such as production of endothelium-derived relaxing factor and prostacyclin.

There have been various additions to this classification of P_2-purinoceptors, such as the proposal for P_{2T} and P_{2Z} subtypes located on platelets and mast cells respectively (Gordon, 1986). It has recently become apparent that many responses to 5'-nucleotides do not fall into the above classification, including those that are insensitive to both 2MeSATP and β,γ-methylene ATP (e.g. Allsup & Boarder, 1990; Fine *et al.*, 1989). In some cases these non-P_{2X} and non-P_{2Y} responses can also be elicited by uridine triphosphate (UTP) (Demolle *et al.*, 1988; Fine *et al.*, 1989; Pfeilschifter, 1990; Davidson *et al.*, 1990; Brown *et al.*, 1991; Murrin & Boarder, 1992). While it has been argued that some responses are mediated by separate pyrimidinoceptors (for UTP) and purinoceptors (for ATP and ADP) (Seifert & Schultz, 1989), there is now compelling evidence for a nucleotide receptor which responds to both UTP and the purines (O'Connor *et al.*, 1991). In this paper we have re-examined the phospholipase C response in bovine cultured aortic endothelial cells and show that these cells are regulated by UTP as well as ATP and analogues, and that these responses reflect the presence of both nucleotide receptors and P_{2Y}-purinoceptors.

Methods

Cell culture

Bovine aortic endothelial cells (BAEC's) were prepared from fresh aortae by digestion with 2 to 5 mg ml^{-1} collagenase (Booyse *et al.*, 1975). Cells were grown in 175 cm^2 flasks (Nunc) in Minimal Essential Medium D-valine, with 10% foetal calf serum, 10% newborn calf serum, 25 i.v. ml^{-1} penicillin, 25 μg ml^{-1} streptomycin, 10 mg ml^{-1} gentamycin, 250 μg ml^{-1} fungizone and 27 mg ml^{-1} glutamine, and maintained in water saturated 95% air, 5% CO_2 atmosphere at 37°C. Cells were passaged into 24 well plates for experimental use. The cell culture formed a confluent monolayer of homogeneous cells positive for Factor VIII immunofluourescence.

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Agonist-stimulated total inositol phosphate production

In order to study total [^{3}H]-inositol phosphates, confluent monolayers were loaded for 48 h with *myo*-[2-^{3}H]-inositol at 0.074 MBq ml^{-1} in a volume of 0.5 ml medium comprising medium M199, penicillin (25 i.v. ml^{-1}), streptomycin (25 μg ml^{-1}), glutamine (27 mg ml^{-1}) and fungizone (250 μg ml^{-1}). Before use the loading medium was removed and the cells washed with balanced salt solution (BSS) comprising (mM): NaCl 125, KCl 5.4, $NaHCO_3$ 16.2, MgCl 0.8, glucose 5.5, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 30, NaH_2PO_4 1, LiCl 10, $CaCl_2$, 1.8, buffered to pH 7.4 with NaOH, and gassed with 95% O_2, 5% CO_2. In experiments performed with nominally Ca^{2+}-free media $CaCl_2$ was omitted and the solution made up in plastic beakers. Lithium was present for 10 min before agonist addition and during the stimulation in a volume of 0.5 ml. The reaction was terminated by the addition of 0.5 ml ice cold methanol and the cells scraped and extracted into chloroform. The aqueous phase was taken and total [^{3}H]-inositol phosphates were batch purified on Dowex-1 (Cl^-) before counting (Rooney & Nahorski, 1986). EC_{50} values for the concentration-response curves were determined with the Graph-Pad programme.

Materials

Myo-[2-^{3}H]-inositol (17 Ci mmol^{-1}) was purchased from NEN Dupont (UK) Ltd., (Herts). 2MeSATP was from Research Biochemicals, Semat, (Herts), other chemicals were from Sigma (Poole, Dorset) or Fisons (Loughborough, Leicestershire). Suramin was a gift from ICI (Alderley Park, Cheshire).

Results

Characteristics of [^{3}H]-inositol phosphate production by 5'-nucleotide analogues

Incubation of bovine aortic endothelial cells with 30 μM concentrations of 2MeSATP, adenosine 5'-0-(3-thiotriphosphate) (ATPγS), adenosine 5'-0-(2-thiodiphosphate) (ADPβS), uridine triphosphate (UTP), ATP and β,γ-methylene ATP, in the presence of 10 mM LiCl, resulted in a rapid accumulation of total [^{3}H]-inositol polyphosphates (Figure 1). This accumulation was roughly linear for up to 20 min for each agonist. The continued accumulation produced by ADPβS and ATPγS compared with 2MeSATP, ATP and UTP may be explained by their being less susceptible to degradation by ectonucleotidases. The selective P_{2x} agonist, β,γ-methylene ATP, failed to produce any significant increase in inositol phosphates up to 60 min.

In order to characterize further the agonist-induced response in bovine aortic endothelial cells, a series of agonist concentration-effect (E/[A]) curves were carried out between 0.03 and 300 μM. Preliminary experiments showed that in the presence of 1.8 mM $CaCl_2$ a plateau in the response to each agonist was difficult to obtain. This may be as a result of non-specific permeabilization of the cell membrane, and the influx of extracellular Ca^{2+}, by the charged ionic forms of ATP. However, carrying out these experiments in nominally Ca^{2+}-free media led to routinely obtainable plateaus in the responses and hence the assessment of EC_{50} values. As can be seen in Figure 2, the E/[A] curves produced by ATPγS were unaffected by the omission of calcium from the medium except for formation of plateaus between 100 and 300 μM. This was typical of the other agonists tested (data not shown) and all E/[A] curves constructed were with nominally Ca^{2+}-free media.

Figure 3 shows E/[A] curves to 2MeSATP, ADPβS, ATPγS, UTP and β,γ-methylene ATP. They produced the following rank order of agonist potency: 2MeSATP (0.42 ± 0.08)> ADPβS (0.85 ± 0.4)> ATPγS (11.65 ± 2.9)> UTP

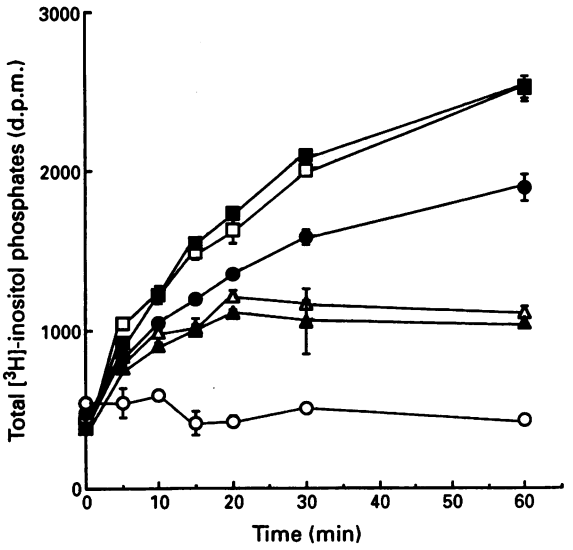


Figure 1 Time course of total inositol phosphate accumulation in bovine aortic endothelial cells. [^{3}H]-inositol-labelled cells were incubated for the times indicated in BSS (with 1.8 mM $CaCl_2$) containing 10 mM LiCl at 37°C. All agonists were added at a concentration of 30 μM; (■) ATPγS; (□) ADPβS; (●) 2MeSATP; (Δ) UTP; (▲) ATP; (○) β,γ-MeATP. Total [^{3}H]-inositol phosphates were measured, as described in the Methods. The data are mean ± s.e.mean of assays performed in triplicate and are representative of at least two similar experiments for each agonist. For abbreviations in this and subsequent figure legends, see text.

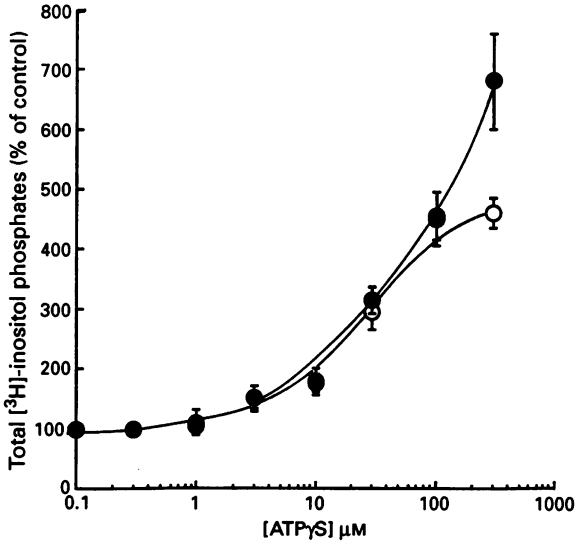


Figure 2 Agonist concentration-effect curves for [^{3}H]-inositol phosphate accumulation in response to ATPγS in bovine aortic endothelial cells, comparing results obtained with cells incubated in BSS + LiCl (10 mM) containing 1.8 mM $CaCl_2$ (●) with those stimulated with nominally Ca^{2+}-free media (○). [^{3}H]-inositol-labelled cells were stimulated with the agonist concentrations shown for 30 min at 37°C. Total [^{3}H]-inositol phosphates were separated as described in the Methods. Results are the mean ± s.e.mean (vertical bars) of triplicate assays and are representative of three similar experiments.

(32.8 ± 9.4)>> β,γ-methylene ATP (numbers in parentheses are EC_{50} in μM ± s.e.mean derived from at least three separate experiments each carried out in triplicate; 2MeSATP and ADPβS were significantly different from ATPγS, $P<0.01$ by Student's *t* test). The P_{2Y} receptor agonists, 2MeSATP and ADPβS, were the most potent tested, ADPβS produced a slightly larger response than 2MeSATP, which may be due to its resistance to breakdown. The response to ATPγS (an agonist at both P_{2Y}-purinoceptors and nucleotide receptors)

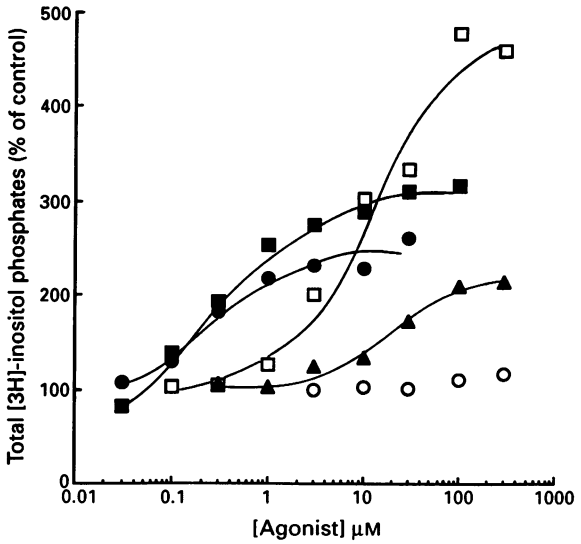


Figure 3 Agonist concentration-effect curves for stimulation of [^{3}H]-inositol phosphate production in bovine aortic endothelial cells by a number of 5'-nucleotide analogues. Cells were loaded with [^{3}H]-inositol and incubated with the above agonist concentrations for 30 min at 37°C in nominally Ca^{2+} free BSS with LiCl (10 mM). Total [^{3}H]-inositol phosphates were collected on Dowex-1 (Cl^-) as detailed in the experimental procedures. Each curve represents the mean for 3 to 6 separate experiments performed in triplicate and are expressed as a percentage of the control value for each curve (error bars omitted for clarity). (●) 2MeSATP; (■) ADPβS; (□) ATPγS; (▲) UTP; (○) β,γ-MeATP.

was larger than to the other agonists tested (significantly different from the response to 2MeSATP at $P<0.01$) and approximately equal in size to the combined responses of ADPβS and UTP. In all E/[A] curves carried out, β,γ-methylene ATP failed to produce a significant stimulation.

Additivity of concentration-effect curves

To determine whether there were two separate receptor populations for 5'-nucleotides in BAEC's, E/[A] curves were constructed to ATPγS and UTP either alone or in the presence of 100 μM UTP, 2MeSATP or ADPβS.

Figure 4 shows E/[A] curves to UTP in the presence or absence of 2MeSATP (Figure 4a) or ADPβS (Figure 4b). Neither 2MeSATP nor ADPβS produced any detectable shift in the UTP E/[A] curve. However, there was a significant increase in inositol phosphate production equal in magnitude to that produced by 100 μM concentrations of 2MeSATP or ADPβS.

Figure 5 shows E/[A] curves to ATPγS in the presence and absence of UTP. In the presence of 100 μM UTP the maximal response to ATPγS was unchanged (i.e. the responses were not additive) although the basal value was increased due to the stimulation produced by UTP.

Inhibition by suramin

Suramin has been shown to antagonize many biological responses dependent on ATP binding, including those at both P_{2X} and P_{2Y} purinoceptors (Hoyle *et al.*, 1990; Leff *et al.*, 1990). Here we asked whether this generally non-specific competitive antagonist would have a differential effect on responses to purine and pyrimidine agonists. Figures 6a and 6b show the effect of increasing concentrations of suramin on 2MeSATP and ADPβS E/[A] curves. The incremental shift to the right shows that suramin does act as a competitive antagonist at P_{2Y} receptors. Schild analysis of these curves produced pK_B s of 5.65 ± 0.75 and 5.6 ± 0.4 with slopes of 1.14 ± 0.08 and 1.0 ± 0.05 for ADPβS and 2MeSATP respectively; each value was the mean from three separate experiments each carried out in triplicate. In contrast, when similar

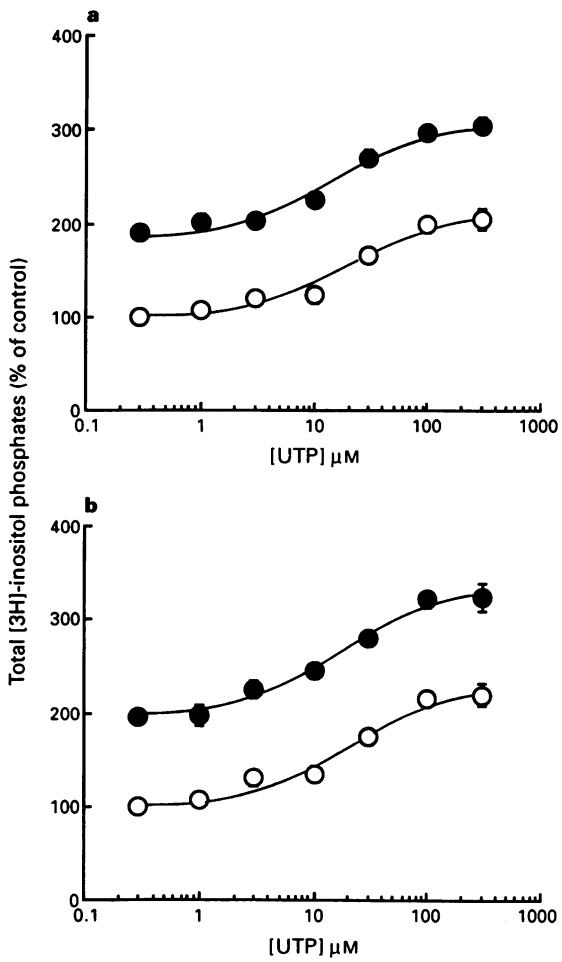


Figure 4 Concentration-effect curves for UTP in the presence (●) or absence (○) of 100 μM 2MeSATP (a) or 100 μM ADPβS (b). Stimulations were for 15 min in nominally Ca^{2+}-free BSS with LiCl (10 mM). The response is formation of total [^{3}H]-inositol phosphates. Each set of curves is the mean ± s.e.mean of 5 to 7 paired experiments.

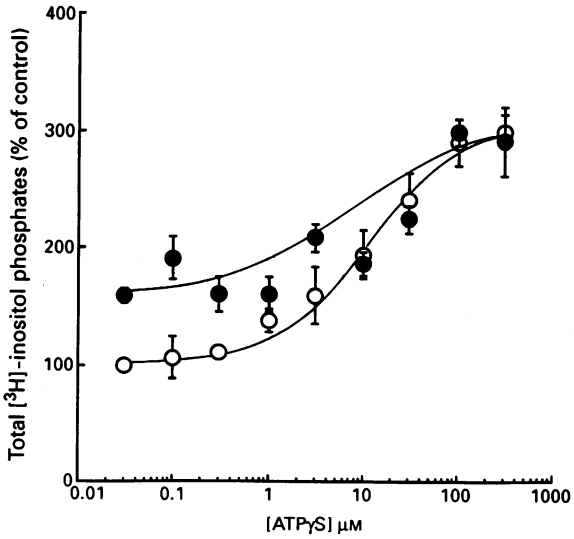


Figure 5 Concentration-effect curves for ATPγS in the presence (●) or absence (○) of 100 μM UTP. Stimulations were for 15 min in nominally Ca^{2+}-free BSS with LiCl (10 mM). The response is formation of total [^{3}H]-inositol phosphates. Each set of curves is the mean ± s.e.mean of 5 to 7 paired experiments.

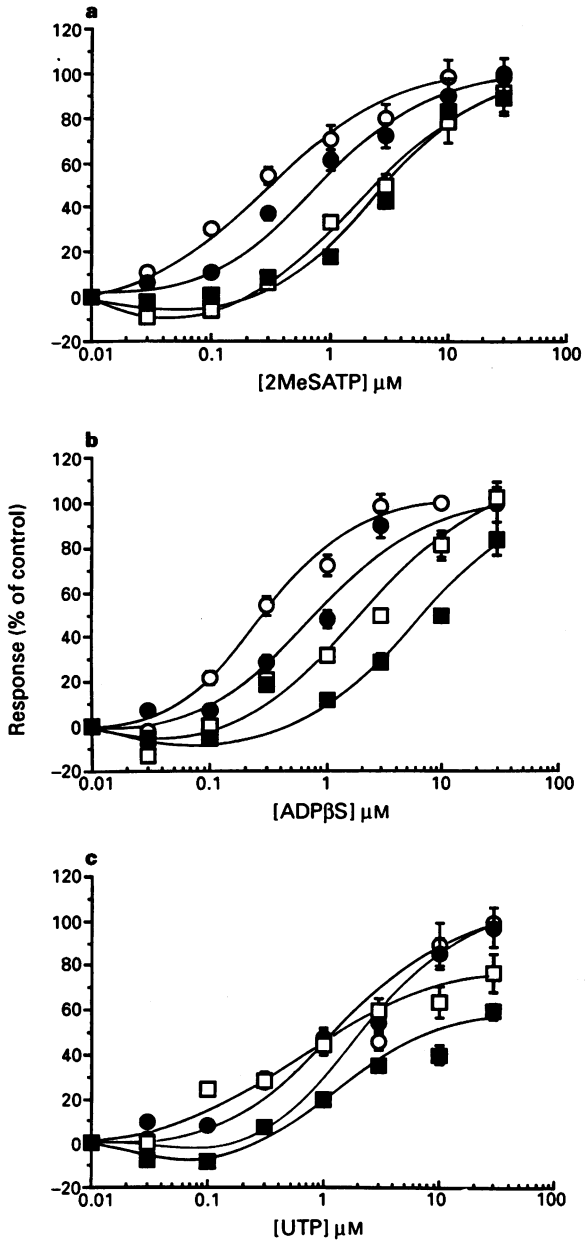


Figure 6 Concentration-effect curves for 2MeSATP (a), ADPβS (b) and UTP (c) in the presence of zero (○); 10 μM (●); 30 μM (□) and 100 μM (■) suramin. Stimulations were for 30 min in nominally Ca^{2+}-free BSS with LiCl (10 mM). The response is formation of total [^{3}H]-inositol phosphates. Each set of curves is representative of three separate experiments, each performed in triplicate and values are ± s.e.mean expressed as a percentage of the maximum control value.

experiments were carried out with UTP stimulation, Figure 6c, there was only a small rightward shift in the E/[A] curve in the presence of 100 μM suramin and a depression in the maximum response achieved with the concentration of UTP used. Schild analysis could not be performed as there was no shift in the E/[A] curve at lower doses of suramin. Suramin, therefore, has a differential effect on the response to P_{2Y} and UTP stimulation.

Discussion

In this study we have used the agonist stimulated formation of total [^{3}H]-inositol (poly)phosphates in cells prelabelled with [^{3}H]-inositol as an index of receptor-regulated phospholipase C. We have previously characterized bovine aortic endothelial cells with respect to this procedure and shown that the response to purine analogues has the characteristics of a P_{2Y} purinoceptor (Allsup & Boarder, 1990). In the same study we found that other cells, derived from the bovine adrenal medulla, had responses which were neither P_{2X}- nor P_{2Y}-purinoceptor mediated. Subsequent work of our own (unpublished) has shown that these cells also respond to UTP and work recently undertaken by us and others (Demolle *et al.*, 1988; Fine *et al.*, 1989; Pfeilschifter, 1990; Davidson, 1990; Murrin & Boarder, 1992; Brown *et al.*, 1992) provides a body of evidence indicating widespread occurrence in other cell types of a 'nucleotide' receptor which is responsive to both UTP and ATP. This work may suggest that the nucleotide receptor has ATP as its natural agonist in most circumstances. In the vascular endothelial cells of the major blood vessels the principal source of endogenous 5'-nucleotide agonists is likely to be ATP/ADP from platelets. ATP released from the endothelial cells themselves may also play a role. However, platelets are also known to release UTP (Seifert & Schultz, 1989), albeit at a much lower concentration. We therefore set out to investigate phospholipase C-linked responses to UTP, and their relationship to receptors responding to ATP, in cultured bovine aortic endothelial cells.

The results reported here are consistent with the earlier observations that aortic endothelial cells have responses expected of a P_2-purinoceptor (e.g. they respond sensitively to 2MeSATP, ADPβS and ATP but not to β,γ-methylene ATP). However, we show that they also respond to UTP. This response to UTP could be at a pyrimidinoceptor population separate from the purinoceptors, or at nucleotide receptors which respond to both. The results described here show that throughout its full dose-response range, UTP was additive with both 2MeSATP and ADPβS. Furthermore, the EC_{50} for UTP was unaffected by the presence of these agonists. This demonstrates that the P_{2Y}-specific agonists (2MeSATP and ADPβS) act at different receptors from UTP.

The conclusion that the P_{2Y} receptors are not responsible for the response to UTP is confirmed by the results with suramin, which competitively attenuates the response to P_{2Y}-specific agonists but has little effect on the response to UTP. Having established that there are two distinct receptor populations, we then need to determine whether the receptor mediating the UTP response can also be activated by purines. If they can then the response is at a nucleotide receptor, if not, then the response is at a pyrimidinoceptor. This is an important point, because if it is a nucleotide receptor then the aortic endothelial cells are regulated by ATP/ADP through two distinct receptor populations. We have described here concentration-effect data to ATPγS in the presence and absence of UTP which shows that ATPγS does act at the same receptor population as UTP. This shows that the aortic endothelial cells have a nucleotide receptor as defined in recent publications (e.g. Davidson *et al.*, 1990; O'Connor *et al.*, 1991; Murrin & Boarder, 1992; Brown *et al.*, 1992).

These previous studies have established a rank order of agonist potency characteristic of nucleotide receptors in which UTP, ATP and ATPγS are effective agonists while 2MeSATP, α,β-methylene ATP and β,γ-methylene ATP are not. In the present study we were unable to use such a rank order to suggest the presence of a nucleotide receptor because of the co-existence of P_{2Y} receptors. A further potential problem with the interpretation of data is the use of long incubation times with agonists which are liable to breakdown. While it seems likely that the response to agonists shown in Figure 1 is affected by differential breakdown, this time course data also indicates that the use of 15 min incubation in the additivity experiments, combined with the use of agonists which are relatively resistant to breakdown (ATPγS, ADPβS, β,γ-methylene ATP), means that the essential conclusions of this study are not compromised.

In summary these results show that in bovine aortic endothelial cells there are two receptor types which will mediate the response to purines such as ATP/ADP. These are

the P_{2Y}-purinoceptors and the nucleotide receptors. The latter receptors may also be responsible for responses to UTP, such as that originating from aggregating platelets. Definitive characterization of these two receptors will await selective antagonists and purification and cloning of receptors (e.g. Murphy & Toffany, 1990). In the meantime we have shown that suramin can distinguish between the two receptor populations in bovine aortic endothelial cells. However, it is intriguing to note that while suramin was less effective at the nucleotide receptors in these cells, it is an effective competitive antagonist at neuronal nucleotide receptors found on PC12 cells (Murrin & Boarder, 1992). One possible explanation is that there are subtypes of nucleotide receptors.

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References

ALLSUP, D.J. & BOARDER, M.R. (1990). Comparison of P_2 purinergic receptors of aortic endothelial cells with those of adrenal medulla: Evidence for heterogeneity of receptor subtype and of inositol phosphate response. *Mol. Pharmacol.*, **38**, 84–91.

BOOYSE, F.M., SEDLAK, B.J. & RAFELSON, M.E. (1975). Culture of arterial endothelial cells: characterisation and growth of bovine aortic cells. *Thromb. Diath. Haemorrh.*, **34**, 825–839.

BROWN, H.A., LAZAROWSKI, E.R., BOUCHER, R.C. & HARDEN, T.K. (1991). Evidence that UTP and ATP regulate phospholipase C through a common extracellular 5'-nucleotide receptor in human airway epithelial cells. *Mol. Pharmacol.*, **40**, 648–655.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen. Pharmacol.*, **16**, 433–440.

DAVIDSON, J.S., WAKEFIELD, I.K., SOHNIUS, U., VAN DER MERWE, P.A. & MILLAR, R.P. (1990). A novel extracellular nucleotide receptor coupled to phosphoinositide C in pituitary cells. *Endocrinology*, **126**, 80–87.

DEMOLLE, D., LAGNEAU, C. & BOEYNAEMS, J.M. (1988). Stimulation of prostacyclin release from aortic smooth muscle cells by purine and pyrimidine nucleotides. *Eur. J. Pharmacol.*, **155**, 339–343.

FINE, J., COLE, P. & DAVIDSON, J.S. (1989). Extracellular nucleotides stimulate receptor mediated calcium mobilization and inositol phosphate production in human fibroblasts. *Biochem. J.*, **263**, 371–376.

GORDON, J.L. (1986). Extracellular ATP: Effects, source and fate. *J. Biochem.*, **233**, 309–319.

HOUSTON, D.A., BURNSTOCK, G. & VANHOUTTE, P.M. (1987). Different P_2-purinergic subtypes of endothelium and smooth muscle in canine blood vessels. *J. Pharmacol. Exp. Ther.*, **241**, 501–509.

HOYLE, C.H.V., KNIGHT, G.E. & BURNSTOCK, G. (1990). Suramin antagonises responses to P_2-purinoceptor agonists and purinergic nerve stimulation in guinea-pig urinary bladder and taenia coli. *Br. J. Pharmacol.*, **99**, 617–621.

LEFF, P., WOOD, B.E. & O'CONNOR, S.E. (1990). Suramin is a slowly-equilibrating but competitive antagonist at P_{2X}-receptors in the rabbit isolated ear artery. *Br. J. Pharmacol.*, **101**, 645–649.

MARTIN, W., CUSACK, N.J., CARLETON, J.S. & GORDON, J.L. (1985). Specificity of P_2-purinoceptor that mediates endothelium dependent relaxation of the pig aorta. *Eur. J. Pharmacol.*, **108**, 295–299.

MARTIN, T.W. & MICHAELIS, K.C. (1989). P_2-purinergic agonists stimulate phosphodiesteratic cleavage of phosphatidylcholine in endothelial cells. *J. Biol. Chem.*, **264**, 8847–8856.

MURPHY, P.M. & TOFFANY, H.L. (1990). Characterisation of phagocyte P_2 nucleotide receptors expressed in xenopus oocytes. *J. Biol. Chem.*, **265**, 11615–11621.

MURRIN, R.J.A. & BOARDER, M.R. (1992). Neuronal 'nucleotide' receptor linked to phospholipase C and phospholipase D? Stimulation of PC12 cells by ATP analogues and UTP. *Mol. Pharmacol.*, **41**, 561–568.

O'CONNOR, S.E., DAINTY, I.A. & LEFF, P. (1991). Classification of P_2 purinoceptors: Trends emerging from recent agonist-based studies. *Trends Pharmacol. Sci.*, **12**, 137–141.

PFEILSCHIFTER, J. (1990). Comparison of extracellular ATP and UTP signalling in rat renal mesengial cells. No indications for the involvement of separate purino- and pyrimidino- ceptors. *Biochem. J.*, **272**, 469–472.

PIROTTON, S., RABAYE, B., LAGNEAU, C. & BOEYNAEMS, J.-M. (1990). Adenine nucleotides modulate phosphatidylcholine metabolism in aortic endothelial cells. *J. Cell Physiol.*, **142**, 449–457.

PIROTTON, S., RASPE, E., DEMOLLE, D., ERNEAUX, C. & BOEYNAEMS, J.-M. (1987). Involvement of inositol 1,4,5-trisphosphate and calcium in the action of adenine nucleotides on aortic endothelial cells. *J. Biol. Chem.*, **262**, 17461–17466.

PURKISS, J.R. & BOARDER, M.R. (1992). Stimulation of phosphatidate synthesis in endothelial cells in response to P_2-receptor activation: evidence for phospholipase C and phospholipase D involvement, phosphatidate and diacylglycerol interconversion and the role of protein kinase C. *Biochem. J.*, **287**, 31–36.

PURKISS, J.R., OWEN, P.J., JONES, J.A. & BOARDER, M.R. (1992). Stimulation of phosphatidic acid synthesis in bovine aortic endothelial cells in response to activation of P_2-purinergic receptors. *Biochem. Pharmacol.*, **43**, 1235–1242.

ROONEY, T.A. & NAHORSKI, S.R. (1987). Regional characterisation of agonist and depolarisation induced phosphoinositide hydrolysis in rat brain. *J. Pharmacol. Exp. Ther.*, **239**, 873–879.

SEIFERT, R. & SCHULTZ, G. (1989). Involvement of pyrimidinoceptors in the regulation of cell functions by uridine and uracil nucleotides. *Trends Pharmacol. Sci.*, **10**, 365–369.

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Effects of interactions of naturally-occurring neuropeptides on blood flow in the rat knee joint

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1 Changes of blood flow in the rat knee joint, measured by laser Doppler flowmetry, were produced by topical application of naturally-occurring neuropeptides to the joint capsule.

2 Substance P (SP), neurokinin A (NKA), and neurokinin B (NKB) all produced dose-dependent transient vasodilatation of the rat knee joint microvasculature. NKB showed significantly smaller vasodilator responses compared to SP and NKA which were similar in their potencies.

3 Calcitonin gene-related peptide (CGRP) produced dose-dependent vasodilatation which was more pronounced than that produced by the neurokinins. The rank order of potency was: CGRP>SP = NKA>NKB. The vasodilator effect of CGRP was also more prolonged and this extended phase was abolished by co-administration of SP.

4 Cross-tachyphylaxis was not observed with the different neurokinins, but SP and NKA showed novel antagonistic effects on NKB-induced vasodilatation.

5 Co-administration of 1 nmol of the specific NK_1 receptor antagonist, CP-96345, with 1 nmol of each of the neurokinins produced significant inhibition of the vasodilator response to SP but did not affect vasodilator responses to NKA and NKB. Co-administration of CP-96345 with the neurokinins plus superfusion of the rat knee joint with a solution containing 0.1 mM CP-96345 further reduced the vasodilator responses to SP but again the vasodilator responses to NKA and NKB were not significanly altered.

6 The results suggest that multiple neurokinin receptor types may be present in the rat knee joint which could mediate the vasodilator responses of the different neurokinins. Co-release of neuropeptides from sensory nerve endings in the rat knee joint may have inter-regulatory actions on their individual responses on the microvasculature.

Keywords: Neuropeptides; vasodilatation; neurokinins; substance P; neurokinin A; neurokinin B; calcitonin gene-related peptide; joint inflammation; CP-96345

Introduction

Substance P (SP) is a neuropeptide localized in central and peripheral terminals of primary sensory neurones (Hokfelt *et al.*, 1975) and has been considered as the putative transmitter (Otsuka & Konishi, 1976) involved in the mediation of pain (Randic & Miletic, 1977). When administered into the synovial cavity of rat knee, SP produced marked plasma extravasation (Lam & Ferrell, 1989a,b) and when applied on exposed rat knee joint blood vessels, SP produced transient but pronounced vasodilatation (Grice *et al.*, 1990). Antidromic electrical stimulation of articular C-fibres has been shown to produce plasma extravasation into the synovial cavity of the cat knee (Ferrell & Russell, 1986). Prior intra-articular administration of the substance P antagonist, D-Pro^4, D-Trp^{7,9,10} SP $_{(4-11)}$ totally inhibited this response (Ferrell & Russell, 1986). Thus, the available evidence suggests that apart from a neurotransmitter role in conveying nociceptive information from the periphery to the central nervous system, SP may also have an important role in mediating neurogenic inflammation.

In recent years a number of investigators have suggested the possibility of multiple receptor types for tachykinins (Falconeri-Erspamer *et al.*, 1980; Lee *et al.*, 1982). Within a relatively short period since then, two new mammalian tachykinins sharing similar structures to SP, neurokinin A (NKA) and neurokinin B (NBK) were isolated, purified and sequenced (Kangawa *et al.*, 1983; Kimura *et al.*, 1983). The discovery of these tachykinins, or more appropriately called neurokinins since they are synthesized and stored in nervous structures and act as neurotransmitters, permitted the identification of neurokinin receptor types according to their respective endogenous ligands. At present, three distinct neurokinin receptor types in various mammalian tissues have been confirmed (Beaujouan *et al.*, 1984; Buck *et al.*, 1984; Regoli *et al.*, 1985; 1987a,b); the neurokinin-1 (NK_1), neurokinin-2 (NK_2), and neurokinin-3 (NK_3) receptors, at which SP, NKA, and NKB are the preferential endogenous agonists, respectively. Plasma extravasation in rat skin (Andrews *et al.*, 1989) and in the rat knee joint (Lam & Ferrell, 1991) has been shown to be mediated by the NK_1 receptor subtype.

Sensory neurones commonly contain more than one type of neuropeptide, with SP and calcitonin gene-related (CGRP) often co-localized in many types of nociceptive afferent fibres (Fischer *et al.*, 1985). CGRP has been shown to be a potent vasodilator in several species including man (Brain *et al.*, 1985). In the rabbit, it can potentiate oedema induced by other mediators of increased microvascular permeability such as platelet-activating factor (PAF), C5a des Arg, N-formyl-methionyl-leucyl-phenylalanine (FMLP) and leukotriene B_4 (LTB_4) (Brain & Williams, 1985). In rat skin, CGRP can also potentiate SP-induced oedema (Brain & Williams, 1985). Thus, the effect of neuropeptides on microvascular tone could have an important influence on the final manifestations of inflammatory processes. The present study investigates the effects of naturally-occurring neuropeptides on blood flow in the rat knee joint. Possible interactions of these neuropeptides were also studied.

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Methods

Experiments were performed on male Wistar rats (300–400 g) deeply anaesthetized by intraperitoneal injection of a mixture of urethane (470 mg kg^{-1}) and diazepam (1 mg kg^{-1}). Blood pressure was monitored continuously via a cannula inserted into the carotid artery. Relative changes in blood flow were monitored by laser Doppler flowmetry (LDF) using a single channel flowmeter (Moor Instruments MBF3) with an near infra-red laser (720 nm) and recorded with a time constant of 3 s. LDF is based on the principle that coherent light scattered by moving erythrocytes experiences a frequency (Doppler) shift that is proportional to the velocity of erythrocytes flowing through the volume of tissue illuminated by the laser radiation (Nilsson *et al.*, 1980a,b). Although this technique provides a continuous and non-invasive signal related to blood flow, it cannot measure absolute flow but can be usefully employed to assess relative changes in blood flow.

The anterior region of the knee was exposed and a fibre-optic probe containing two fibres (each of diameter 100 μm; one fibre emitting laser radiation whilst the other collects the backscattered photons) was placed just above the surface which was continuously superfused with physiological saline at a rate of 0.1 ml min^{-1} by a peristaltic pump (Watson-Marlow 101U). The tip of the probe was placed 1–2 mm central to the medial edge of the patellar ligament (Figure 1), over an area of joint capsule which we have confirmed histologically to consist only of synovial tissues. It has been shown that a laser Doppler flowmeter using a near infra-red laser source samples a larger volume of tissue than an instrument using a shorter wavelength laser (Obeid *et al.*, 1990). In preliminary experiments we have shown that the instrument used in the present study is able to sample blood flow in deeper synovial vessels as vasodilatation is still detected with flowmeter probe placed over the exposed surface of the capsule when the synovial cavity is perfused with CGRP.

Drugs were administered as a bolus applied to the surface in a volume of 0.1 ml. Data are presented as maximum change in the laser flux signal from the preceding basal level. The basel reading may vary in different animals depending on probe position, thus necessitating expression of the vasodilator response as a percentage change from the control level which was normalised to 0%. Analysis of Variance (ANOVA) was used to analyse differences between dose-response curves. Differences were considered significant if the P values were 5% or less. Values in the histograms represent means ± s.e.mean. An unpaired one-tailed t test was used to analyse the difference between means.

Human α-CGRP, SP, and CP-96345 ((2S,3S)-cis-2-(diphenylmethyl)-N-((2-methoxyphenyl)methyl)-1-azabicyclo[2.2.2]oct-an-3-amine) were kindly donated by Celltech Ltd (Berkshire), ICI Pharmaceuticals, and Pfizer Ltd, respectively. All other drugs were purchased from Cambridge Research Biochemicals Ltd. Neurokinin B was dissolved in 10% ammonium hydroxide and subsequent dilutions were made in physiological saline. In all other cases physiological saline was used as the solvent.

Results

Vasodilatation of rat knee joint blood vessels induced by naturally-occurring neuropeptides

The four neuropeptides SP, NKA, NKB, and CGRP all produced an increase in the basal laser flux signal, indicating that they were all effective in eliciting vasodilatation of rat knee joint blood vessels. With the exception of NKB, the neuropeptides produced a drop in blood pressure when applied to the knee joint at doses above 1 nmol. Thus, higher doses were not used as the hypotension they induce would tend to offset their vasodilator effects. With the doses used in the present study, blood pressure was found to remain constant throughout the experiment as long as the animal remained deeply anaesthetized. The vasodilator effects of these neuropeptides were tachyphylactic as was observed in our previous study on specific neurokinin receptor agonists (Grice *et al.*, 1990). Thus, dose-response curves to the neurokinins were achieved by allowing 15 min to elapse between each drug application to avoid tachyphylaxis. Figure 2 shows that all the neurokinins were effective in producing dose-dependent vasodilatation in the rat knee joint. The maximum vasodilator responses achieved at 1 nmol were 55.8 ± 3.4%, 68.5 ± 10.2%, and 47.6 ± 5.6% increase from basal perfusion levels for SP, NKA and NKB, respectively. Statistical ana-

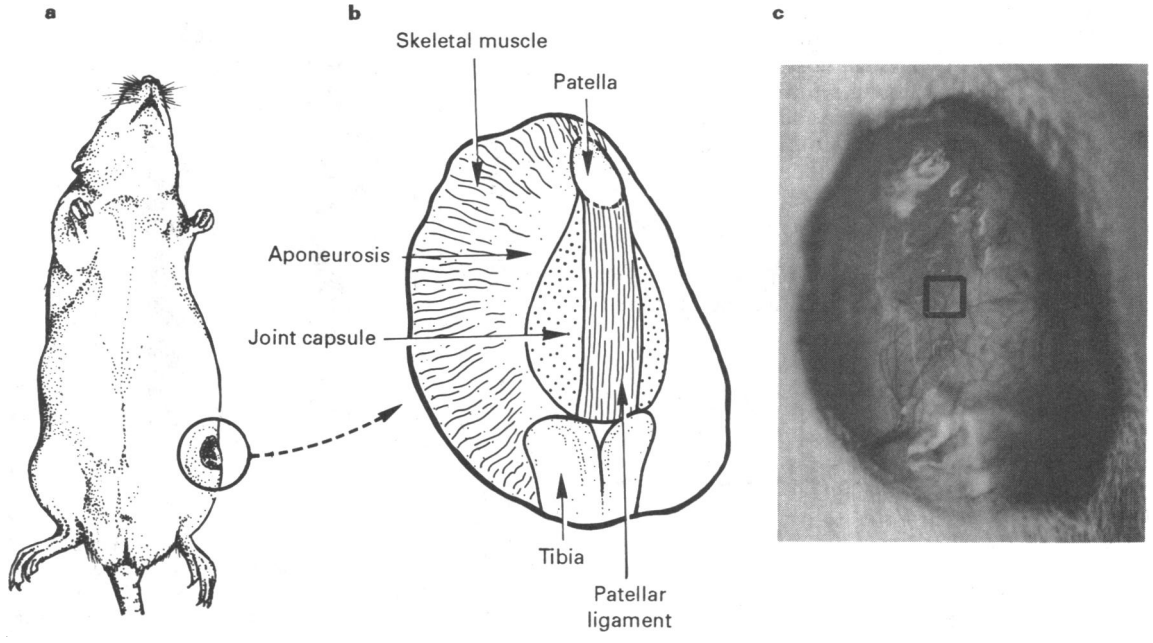


Figure 1 Photographic illustration of an exposed rat knee prepared for assessment of knee joint perfusion by a laser Doppler flowmeter (LDF). (a) Drawing of a rat indicating the exposed medial aspect of the knee joint which is photographically illustrated in (c). The principal anatomical features are shown diagrammatically in (b). The black rectangle in (c) outlines areas which were suitable for LDF probe placement.

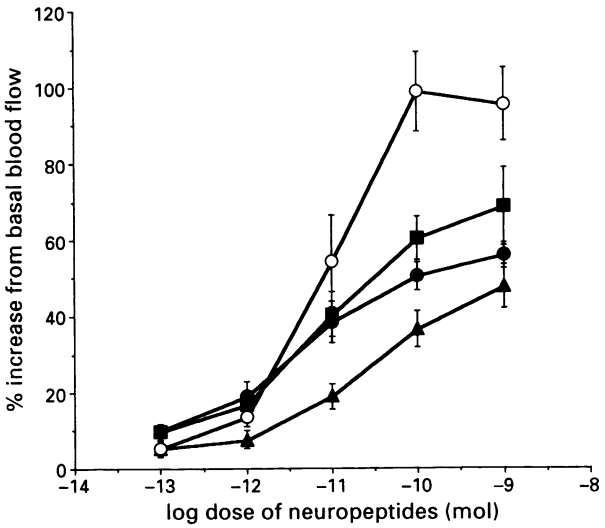


Figure 2 Effect of calcitonin gene-related peptide (○, $n = 7$), substance P (●, $n = 7$), neurokinin A (■, $n = 7$), and neurokinin B (▲, $n = 7$) on articular blood flow of the rat knee joint capsule. Drugs were administered as a bolus applied to the rat knee joint surface in a volume of 0.1 ml. Data are shown as means ± s.e.mean (shown by vertical bars) expressed as a percentage change from control (normalised to 0%).

lysis showed no difference between the SP and NKA dose-response curves ($P = 0.394$), whereas the dose-response curve to NKB differed significantly from that of SP ($P = 0.005$) and NKA ($P = 0.003$) indicating the lesser potency of this agent.

Application of CGRP to the rat knee joint produced substantially greater vasodilator responses than those induced by the neurokinins. CGRP produced a maximum vasodilatation of 95.3 ± 9.5% at 1 nmol and its dose-response curve was significantly different from those of SP ($P = 0.013$) and NKB ($P = 0.0005$), but not significantly different from that of NKA ($P = 0.157$). However, there were significant differences when their responses at the two highest concentrations were compared ($P = 0.034$ at 0.1 nmol and $P = 0.040$ at 1 nmol). The characteristic vasodilator response to CGRP was also unique as it took longer to peak (92 ± 9.2 s; $n = 7$) and often remained visible more than 30 min after administration, whereas the neurokinins showed a transient vasodilator effect with a peak at 40 ± 5.35 s ($n = 7$) and returned to the control level in 111.43 ± 8.07 s ($n = 7$) after administration. The durations of the vasodilator responses to SP and CGRP are shown in Figure 3.

Effects of different combinations of neurokinins

Possible interactions of the neurokinins on rat knee joint blood flow were studied by investigating the effects of the different agents after an initial dose of another neurokinin. As illustrated in Figure 4, the vasodilator effect of 1 nmol SP (60.5 ± 7.0%) was not significantly different 5 min after pretreatment with 1 nmol NKA (51.7 ± 7.9%) or NKB (57.6 ± 7.4%). Similarly, the vasodilator effect of NKA (49.4 ± 6.8%) at the same concentration, was essentially the same after pretreatment with SP (56.5 ± 6.3%) or NKB (47.1 ± 2.5%). However, the vasodilator effect of 1 nmol NKB (48.5 ± 4.2%) was substantially reduced after pretreatment with SP (6.1 ± 2.9%; $P < 0.001$) or NKA (5.5 ± 1.9%; $P < 0.001$).

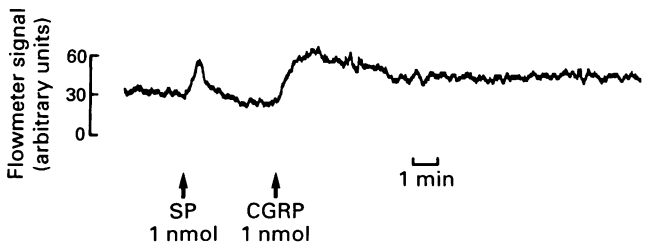


Figure 3 Trace showing the effect on the laser Doppler flux signal of topical application (0.1 ml) of substance P (SP) and calcitonin gene-related peptide (CGRP) to the rat knee joint capsule.

Effects of a specific NK_1 receptor antagonist on neurokinin-induced vasodilatation

The effects of a non-peptide specific NK_1 antagonist, CP-96345 (Snider *et al.*, 1991), on the vasodilator responses to the different neurokinins are shown in Figure 5. Co-administration of 1 nmol CP-96345 with 1 nmol of the neurokinins resulted in a reduction of the vasodilator response to SP, but the responses to NKA and NKB were not affected. Co-administration of 1 nmol CP-96345 and 1 nmol neurokinins plus superfusion of the rat knee joint with a solution containing 0.1 mM of CP-96345 produced a further reduction of the vasodilator response to SP but the vasodilator responses to NKA and NKB were again unaffected when compared statistically to the control response. Control experiments with CP-96345 alone showed no alteration of the basal blood flow in the presence of the antagonist.

Effects of SP on CGRP-induced vasodilatation

Application of 1 nmol CGRP to the rat knee joint produced a long lasting vasodilator response with 51.1 ± 10.6% of the maximum response still persisting 30 min after drug adminis-

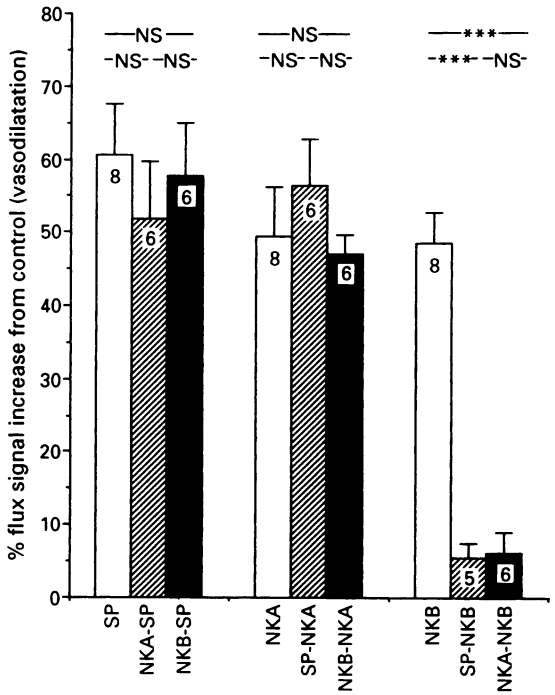


Figure 4 Effects of substance P (SP), neurokinin A (NKA), and neurokinin B (NKB) alone, and after pretreatment with either substance P (SP-), neurokinin A (NKA-), or neurokinin B (NKB-) on the laser Doppler signal obtained from the rat knee joint. All drugs were adminstered as a bolus of 1 nmol applied to the rat knee joint surface in a volume of 0.1 ml. In experiments where two applications of neurokinins were applied, a 5 min interval was allowed between the applications. Data are shown as means ± s.e.mean (vertical bars). NS = no significant difference; *** = $P < 0.001$.

tration. However, when the same dose of CGRP was co-administered with 1 nmol SP, the duration of the vasodilator response was substantially reduced. Only 11.6 ± 5.6% of the maximum response was observed 5 min after co-administration. The vasodilator response was no longer visible after 30 min. These results are shown in Figure 6.

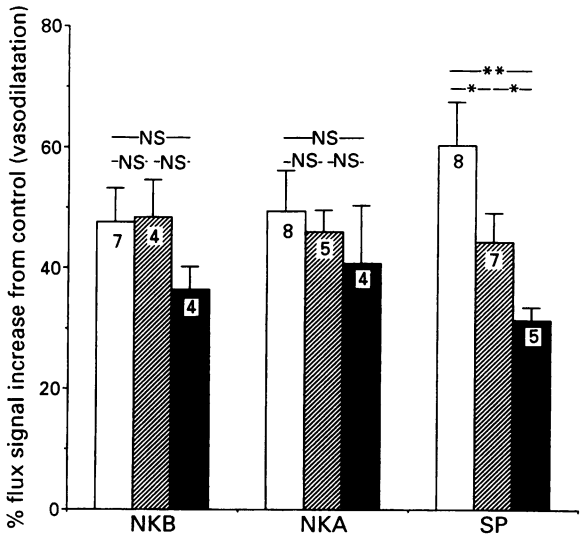


Figure 5 Effects of the specific NK_1 receptor antagonist, CP-96345, on vasodilator responses to the neurokinins NKB, NKA, and substance P (SP). The open columns illustrate control response to 1 nmol doses of the neurokinins; cross-hatched columns illustate co-administration of 1 nmol CP-96345 with 1 nmol neurokinins applied directly onto the rat knee joint in a volume of 0.1 ml; solid columns illustrate co-administration of the antagonist with neurokinins plus superfusion of the rat knee joint with 0.1 mM CP-96345. Data are shown as means ± s.e.mean (vertical bars). NS = no significant difference; * = $P<0.05$; ** = $P<0.01$.

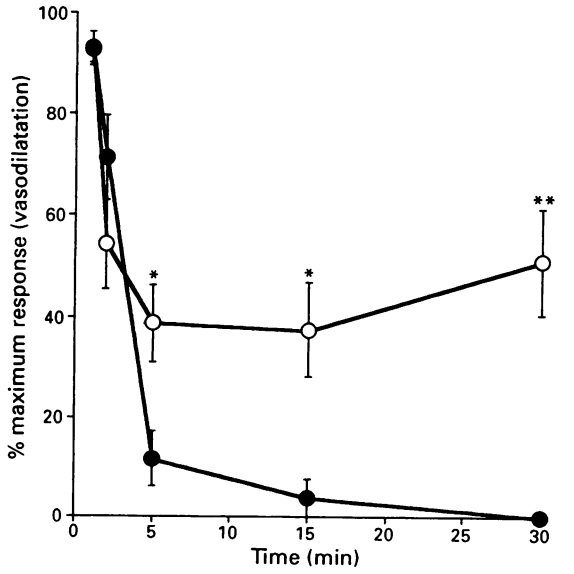


Figure 6 Effects of substance P (SP) on the time course of calcitonin gene-related peptide (CGRP)-induced vasodilatation in the rat knee joint. The open symbols (○; $n = 14$) represent the time course of the vasodilator response to 1 nmol CGRP applied as a bolus onto the rat knee joint surface in a 0.1 ml volume. The closed symbols (●; $n = 10$) illustrate the time course of the vasodilator response with co-administration of 1 nmol CGRP and 1 nmol SP to the rat knee joint. Data are shown as percentage of the maximum response (means ± s.e.mean, vertical bars). *$P<0.05$; **$P<0.01$.

Discussion

Laser-Doppler flowmetry is a convenient and simple way to measure continuously changes in microvascular blood flow. The technique does not measure blood flow in units of velocity but rather measures relative blood flow changes. It has mainly been employed for the measurement of blood flow changes in skin (Nilsson *et al.*, 1980a,b; Andrews & Helme, 1989; Hornyak *et al.*, 1990). The present study extends this method for the measurements of microvascular blood flow changes in the rat knee joint.

The results show that naturally-occurring neuropeptides can significantly influence rat knee joint microvascular tone. All the neuropeptides tested in the present study were effective vasodilators. Andrews & Helme (1989) have shown that the neurokinins were equipotent in producing vasodilator responses in rat skin and the duration of these responses was no more than 5 min. Our results confirmed the transient nature of neurokinin-induced vasodilatation but showed NKB to be less potent than SP and NKA which were similar in their responses in the rat knee joint. Although plasma extravasation in the rat knee joint has been shown to be mediated predominately by the NK_1 receptor subtype (Lam & Ferrell, 1991), the present study shows that the specific NK_1 receptor antagonist, CP-96345, selectively inhibited SP-induced vasodilatation whilst leaving the vasodilator responses to NKA and NKB unaltered. This suggests that unlike plasma extravasation, blood flow changes in the rat knee joint could be mediated by multiple neurokinin receptor types.

It has been shown that CP-96345 has higher affinity for NK_1 receptors found in man, guinea-pig and rabbit, than for NK_1 receptors found in rat, mouse, and chicken (Gitter *et al.*, 1991). The fact that CP-96345 produced incomplete inhibition of the SP response even at high concentrations (0.1 mM) in the present studies may reflect this low affinity characteristic of CP-96345 in rats. Alternatively, the incomplete inhibition by CP-96345 could suggest only partial involvement of NK_1 receptors in mediating the vasodilator response to SP. The use of higher concentrations of CP-96345 to achieve total blockade of the SP responses was prevented due to possible non-specific effects of CP-96345 at higher concentrations. Recently, a new non-peptide NK_1 receptor antagonist (RP-67580) has been developed and this has been shown to possess higher affinity than CP-96345 in rats and mice (Fardin *et al.*, 1992; Boyce *et al.*, 1992). Further experiments with RP-67580 may provide evidence to support or discard the possibilities of total or partial involvement of NK_1 receptors in mediating the SP vasodilator response.

Our experiments on combination effects of the different neurokinins provided further evidence for the involvement of multiple receptor types. The results show that the vasodilator response to SP was not affected by pretreatment with NKA, or NKB. Neither was the vasodilator effect produced by NKA affected by pretreatment with SP, or NKB. Had the vasodilator effects of the different neurokinins been mediated by the same receptor type, cross-tachyphylaxis would have been observed as was found to be the case in our previous study between SP and the specific NK_1 receptor agonist, [Sar^9, $Met(O_2)^{11}$]-substance P (Grice *et al.*, 1990). There is speculation that the equipotency of these neurokinins in producing vasodilatation in rat skin might indicate the existence of a fourth neurokinin receptor subtype (Andrews & Helme, 1989), but there is limited evidence in support of this view. Furthermore, the neurokinins do not appear to be equipotent in the rat knee joint. It seems more reasonable at this stage to assume that there are multiple neurokinin receptor types mediating the vasodilator responses of neurokinins in the rat knee joint.

An interesting finding was that although NKB did not show cross-tachyphylaxis on the vasodilator responses to SP or NKA, pretreatment of the rat knee joint with SP or NKA

resulted in potent inhibition of the vasodilator response to NKB. As this inhibition of the NKB response is unlikely to have resulted from cross-tachyphylaxis, this suggests that SP and NKA could have novel antagonistic effects on the NK_3 receptor subtype. Furthermore, it is interesting to note that the inhibitory effect was observed 5 min after application of SP or NKA when their vasodilator effects had subsided. In future studies it would be worthwhile to repeat these experiments with longer time intervals to determine the duration of the inhibition. The significance of this novel antagonistic action, and whether the antagonism is specific to joint blood vessels or occurs in other microvascular beds, remains to be elucidated.

The vasodilator effect of CGRP on the rat knee was found to be more potent and longer in duration than those produced by the neurokinins. The long lasting effect of CGRP was also observed in rabbit (Brain & Williams, 1985), rat (Brain & Williams, 1989), and human skin (Brain *et al.*, 1985). CGRP has also been shown to be a more potent vasodilator than SP or NKA in the human (Franco-Cereceda & Rudehill, 1989) and pig (Franco-Cereceda *et al.*, 1987) precontracted coronary artery. In the rat skin, it has been shown that co-administration of SP with CGRP abolished the prolonged vasodilator effect of CGRP (Brain & Williams, 1989). This was attributed to the SP-induced release of proteases from skin mast cells which degrade CGRP and thus terminated its vasodilator activity. Our results are in support of this view as co-administration of SP with CGRP also inhibited the prolonged vasodilator effects of CGRP in the rat knee joint. Nerves containing SP and CGRP have been identified in the rat synovium (Hukkanen *et al.*, 1991). These findings may suggest that SP has a regulatory role on the vasodilator responses of CGRP, especially as the two neuropeptides may be co-released from the same nerve endings in the rat synovium.

In conclusion, the present study has shown that blood flow measurements can be made in rat knee joint by laser Doppler flowmetry. All four naturally-occuring neuropeptides tested were effective vasodilators on the rat knee joint and showed a rank order of potency of CGRP > SP = NKA > NKB. The lack of cross-tachyphylaxis by the different neurokinins and more importantly, the selective inhibition of CP-96345 on SP-induced vasodilatation suggest different neurokinin receptor types may be involved in mediating vasodilator responses to neurokinins. SP may be a possible regulator of the vasodilator actions of CGRP in the rat knee joint. The present study also indicates a possible novel antagonistic activity of SP and NKA on NKB-induced vasodilatation.

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References

ANDREWS, P.V. & HELME, R.D. (1989). Tachykinin-induced vasodilatation in rat skin measured with a laser-Doppler flowmeter: evidence for receptor-mediated effects. *Regul. Pept.*, **25**, 267–275.

ANDREWS, P.V., HELME, R.D. & THOMAS, K.L. (1989). NK_1 receptor mediation of neurogenic plasma extravasation in rat skin. *Br. J. Pharmacol.*, **97**, 1232–1238.

BEAUJOUAN, J.L., TORRENS, Y., VIGER, A. & GLOWINSKI, J. (1984). A new type of tachykinin binding site in the rat brain characterised by specific binding of a labelled eledoisin derivative. *Mol. Pharmacol.*, **26**, 248–254.

BOYCE, S., RUPNIAK, N.M.J., YOUNG, L., RYCROFT, W., BRISTOW, L., IVERSON, S.D. & HILL, R.G. (1992). Antinociceptive effects of racemic RP-67580, but not racemic CP-96345, on acetic acid-induced writhing and formalin paw in mice. *Neuropeptides*, **22**, 10.

BRAIN, S.D. & WILLIAMS, T.J. (1985). Inflammatory oedema induced by synergism between calcitonin gene-related peptide (CGRP) and mediators of increased vascular permeability. *Br. J. Pharmacol.*, **86**, 655–660.

BRAIN, S.D. & WILLIAMS, T.J. (1989). Interactions between the tachykinins and calcitonin gene-related peptide lead to the modulation of oedema formation and blood flow in rat skin. *Br. J. Pharmacol.*, **97**, 77–82.

BRAIN, S.D., WILLIAMS, T.J., TIPPINS, J.R., MORRIS, H.R. & MACINTYRE, I. (1985). Calcitonin gene-related peptide is a potent vasodilator. *Nature*, **313**, 54–56.

BUCK, S.H., BURCHER, E., SCHULTZ, C.W., LOVENBERG, W. & O'DONAHUE, T.L. (1984). Novel pharmacology of substance K-binding sites: a third type of tachykinin receptor. *Science*, **226**, 987–989.

FALCONERI-ERSPAMER, G., ERSPAMER, V. & PICCINELLI, D. (1980). Parallel bioassay of physalaemin and kassinin, a tachykinin dodecapeptide from the skin of the African frog *Kassina senegalensis*. *Naunyn-Schmeideb. Arch. Pharmacol.*, **311**, 61–65.

FARDIN, V., FLAMAND, O., FOUCAULT, F., JOLLY, A., BOCK, M.D. & GARRETT, C. (1992). RP 67580, a non-peptide substance P antagonist: comparison with other antagonists and affinities in different animal species. *Neuropeptides*, **22**, 22.

FERRELL, W.R. & RUSSELL, N.J. (1986). Extravasation in the knee induced by antidromic stimulation of artcular C fibre afferents of the anaesthetised cat. *J. Physiol.*, **379**, 407–416.

FISCHER, J., FORSSMAN, W.G., HOKFELT, T., LUNDBERG, T.M., REINECKE, M., TSCHOPP, F.A. & WIESENFELD-HALLIN, Z. (1985). Immunoreactive calcitonin gene-related peptide and substance P: co-existence in sensory neurones and behavioural interactions after intrathecal administration in the rat. *J. Physiol.*, **362**, 29P.

FRANCO-CERECEDA, A. & RUDEHILL, A. (1989). Capsaicin-induced vasodilatation of human coronary arteries in vitro is mediated by calcitonin gene-related peptide rather than substance P or neurokinin A. *Acta Physiol. Scand.*, **136**, 575–580.

FRANCO-CERECEDA, A., RUDEHILL, A. & LUNDBERG, J.M. (1987). Calcitonin gene-related peptide but not substance P mimics capsaicin-induced coronary vasodilatation in pig. *Eur. J. Pharmacol.*, **142**, 235–243.

GITTER, B.D., WATERS, D.C., BRUNS, R.F., MASON, N.R., NIXON, J.A. & HOWBERT, J.J. (1991). Species differences in affinities of non-peptide antagonists for substance P receptors. *Eur. J. Pharmacol.*, **197**, 237–238.

GRICE, C.A., LAM, F.Y. & FERRELL, W.R. (1990). Response of knee joint blood vessels to neurokinin receptor agonists in anaesthetised rats. *J. Physiol.*, **429**, 75P.

HOKFELT, T., KELLERTH, J.O., NILSSON, G. & PERNOW, B. (1975). Experimental immunohistochemical studies on the localization and distribution of substance P in the cat primary sensory neurones. *Brain Res.*, **100**, 235–252.

HORNYAK, M.E., NAVER, H.K., RYDENHAG, B. & WALLIN, B.G. (1990). Sympathetic activity influences the vascular axon reflex in the skin. *Acta Physiol. Scand.*, **139**, 77–84.

HUKKANEN, M., GRÖNBLAD, M., REES, R., KOTTINEN, Y.T., GIBSON, S.J., HIETANEN, J., POLAK, J.M. & BREWERTON, N.A. (1991). Regional distribution of mast cells and peptide containing nerves in normal and adjuvant arthritis rat synovium. *J. Rheumatol.*, **18**, 177–183.

KANGAWA, K., MINAMINO, N., FUKUDA, A. & MATSUO, H. (1983). Neuromedin K: A novel mammalian tachykinin identified in porcine spinal cord. *Biochem. Biophys. Res. Commun.*, **114**, 533–540.

KIMURA, S., OKADA, M., SUGITA, Y., KANAZAWA, I. & MUNEKATO, E. (1983). Novel neuropeptides, neurokinin α and β, isolated from porcine spinal cord. *Proc. Jpn. Acad. (B)*, **59**, 101–104.

LAM, F.Y. & FERRELL, W.R. (1989a). Inhibition of carrageenan-induced inflammation in the rat knee joint by substance P antagonist. *Ann. Rheum. Dis.*, **48**, 928–932.

LAM, F.Y. & FERRELL, W.R. (1989b). Capsaicin suppresses substance P-induced joint inflammation in the rat. *Neurosci. Lett.*, **105**, 155–158.

LAM, F.Y. & FERRELL, W.R. (1991). Specific neurokinin receptors mediate plasma extravasation in the rat knee joint. *Br. J. Pharmacol.*, **103**, 1263–1267.

LEE, C.M., IVERSEN, L.L., HANLEY, M.R. & SANDBERG, B.E.B. (1982). Possible existence of multiple receptors for substance P. *Naunyn-Schmiedeb. Arch. Pharmacol.*, **318**, 281–287.

NILSSON, G.E., TENLAND, T. & OBERG, P.A. (1980a). A new instrument for continuous measurement of tissue blood flow by light beating spectroscopy. *IEEE Trans. Biomed. Eng. BME.*, **27**, 12–19.

NILSSON, G.E., TENLAND, T. & OBERG, P.A. (1980b). Evaluation of a laser Doppler flowmeter for measurement of tissue blood flow. *IEEE Trans. Biomed. Eng. BME.*, **27**, 597–604.

OBEID, A.N., DOUGHERTY, G. & PETTINGER, S. (1990). *In vivo* comparison of a twin wavelength laser Doppler flowmeter using He-Ne and laser diode sources. *J. Med. Eng. Tech.*, **14**, 102–110.

OTSUKA, M. & KONISHI, S. (1976). Substance P and excitatory transmitter of primary sensory neurones. *Cold Spring Harbor Symp. Quant. Biol.*, **40**, 135–143.

RANDIC, M. & MILETIC, V. (1977). Effect of substance P in cat dorsal horn neurones activated by noxious stimuli. *Brain Res.*, **128**, 164–169.

REGOLI, D., DRAPEAU, G., DION, S. & D'ORLEANS-JUSTE, P. (1987a). Mini review. Pharmacological receptors for substance P and neurokinins. *Life Sci.*, **40**, 107–117.

REGOLI, D., DRAPEAU, G., DION, S. & D'ORLEANS-JUSTE, P. (1987b). Receptors for neurokinins in peripheral organs. In *Substance P and Neurokinins*, Montreal '86. ed. Henry, J.L., Couture, R., Cuello, A.C., Pelletier, G., Quirion, R. & Regoli, D. pp. 99–107. New York: Springer-Verlag.

REGOLI, D., MIZRALI, J., D'ORLEANS-JUSTE, P., DION, S., DRAPEAU, G. & ESCHER, E. (1985). Substance P antagonists showing some selectivity for different-receptor types. *Eur. J. Pharmacol.*, **109**, 121–125.

SNIDER, R.M., CONSTANTINE, J.W., LOWE, J.A., LONGO, K.P., LEBEL, W.S., WOODY, H.A., DROZDA, S.E., DESAI, M.C., VINICK, F.J., SPENCER, R.W. & HESS, H.-J. (1991). A potent nonpeptide antagonist of the substance P (NK_1) receptor. *Science*, **251**, 435–437.

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Role of leukotrienes in airway hyperresponsiveness in guinea-pigs

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1 Repeated aerosolization of leukotriene C_4 (LTC_4) to guinea-pigs produced leftward shift in their pulmonary resistance (R_L) dose-response curves to inhaled acetylcholine (ACh) without increasing the maximum responses.

2 Repeated LTC_4 aerosolization did not increase airway eosinophils.

3 The 5-lipoxygenase-activating protein (FLAP) inhibitor, MK-886, prevented the leftward shift in R_L dose-response curves to ACh following repeated antigen challenge in guinea-pigs.

4 MK-886 did not inhibit the increased maximal R_L produced by repeated antigen challenge, nor inhibit the airway eosinophilia induced by repeated antigen challenge.

5 Our findings suggest that leukotrienes may account for the leftward shift in pulmonary resistance responses caused by antigen but do not cause the airway eosinophilia nor enhanced maximum bronchoconstrictor response to antigen.

Keywords: Airway hyperresponsiveness; asthma; leukotrienes; eosinophilia

Introduction

The sulphidopeptide leukotrienes, LTC_4, LTD_4, and LTE_4 have been demonstrated to be produced from asthmatic lung tissue *in vitro* (Dahlen *et al.*, 1983) and *in vivo* (Casale *et al.*, 1987; Chan-Yeung *et al.*, 1989) and to cause human bronchial smooth muscle contraction *in vitro* (Dahlen *et al.*, 1980; Hanna *et al.*, 1981; Jones *et al.*, 1982) and bronchoconstriction *in vivo* (Holroyde *et al.*, 1981; Griffin *et al.*, 1983; Barnes *et al.*, 1984). Although there is evidence for these leukotrienes playing a significant role in both acute and late phase asthmatic responses, their importance as mediators of non-specific airway hyperreactivity remains unknown.

We have previously established a guinea-pig model which demonstrated severe acute bronchoconstriction following antigen challenge of previously sensitized animals, profound airway eosinophilia and hyperresponsiveness to acetylcholine (Ishida *et al.*, 1989). Tracheal smooth muscle from these hyperresponsive guinea-pigs also demonstrated increased shortening and force generation compared with controls (Ishida *et al.*, 1990a), findings compatible with human asthmatic bronchial smooth muscle responses (Schellenberg & Foster, 1984; DeJongste *et al.*, 1987; Bai, 1990).

The present study aimed to evaluate the importance of sulphidopeptide leukotrienes in this model of antigen-induced hyperresponsiveness by employing a drug that inhibits leukotriene synthesis by acting on the 5-lipoxygenase activating protein (FLAP) (Rouzer *et al.*, 1990; Miller *et al.*, 1990; Dixon *et al.*, 1990) as well as evaluate the effect of repeated LTC_4 inhalation alone on airway responsiveness and eosinophilia.

Methods

Protocol A (Repeated aerosolized LTC_4 challenge)

Thirteen Cam-Hartley female guinea-pigs (250 g to 300 g at the start of the experiment) were challenged in a large chamber with an aerosol of either 10^{-6} M LTC_4 (Merck-Frosst Lab. Pte. Claire, PQ, Canada), dissolved in 0.05% guinea-pig albumin or vehicle alone for 10 min twice a week for four to six weeks. Three days after the last challenge, acetylcholine (ACh) dose-response characteristics were evaluated by the method described below.

Protocol B (Effect of a leukotriene biosynthesis inhibitor upon repeated antigen challenge)

In order to evaluate the effect of an inhibitor of 5-lipoxygenase activating protein (FLAP), we chose to evaluate MK-886 (3-[1-(p-chlorophenyl)-5-isopropyl-3-tert-butythio-1H-indol-2-yl]-2,2-dimethylpropanoic acid; Merck-Frosst Lab, Pte. Claire, PQ, Canada), a well characterized compound (Rouzer *et al.*, 1990; Miller *et al.*, 1990; Dixon *et al.*, 1990), on antigen-induced airway hyperresponsiveness. We adapted the protocol described in detail previously (Ishida *et al.*, 1989). Briefly, 17 Cam-Hartley female guinea-pigs (250–300 g) were sensitized to ovalbumin (OA) by inhalation of an aerosolized solution of 1% ovalbumin (OA) (Sigma Chemical Co, St. Louis, MO, U.S.A.) dissolved in normal saline containing 4% heat-killed *Bordatella pertussis* vaccine (Connaught Laboratories, Toronto, Ont., Canada).

Antigen challenges began one week after the sensitization. Two h before each challenge, the leukotriene biosynthesis inhibitor treatment group ($n = 7$) received MK-886 3 mg kg^{-1} orally using a temporary stomach tube (PE-90) under halothane anaesthesia. Animals receiving OA alone ($n = 7$) received a sham treatment. Diphenhydramine (0.2 ml of 40 mg ml^{-1} saline solution) was given i.p. 1 h before each OA challenge to protect the animals from anaphylactic death. Animals were placed in the exposure chamber in which they inhaled nebulized OA 0.5% solution (DeVilbiss ultrasonic nebuliser) until each animal showed a change in breathing pattern (increased respiratory rate or indrawing) and cyanosis. The maximal exposure time was 10 min. Antigen challenge was repeated twice a week for 4 to 6 weeks. The control group ($n = 12$) was sensitized and challenged with saline aerosol using an identical protocol.

Pulmonary resistance measurement

Guinea-pigs were anaesthetized with α-chloralose (Sigma Chemical Co., St. Louis, MO, U.S.A.) 80 mg kg^{-1} i.p., tracheostomized, paralyzed with succinylcholine (0.5 mg kg^{-1}, i.m.), placed in a pressure-sensitive plethysmograph containing a copper wire mesh heat sink and ventilated with

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a Harvard animal ventilator (Model 608; Harvard Apparatus Co., South Natick, MA, U.S.A.) delivering a tidal volume of 3 ml at a frequency of 60 breaths per min. Volume signals were obtained from a sensitive pressure transducer (MP45-2, ± 2 cmH_2O; Validyne Corp., Northridge, CA, U.S.A.) which measured changes in box pressure. The plethysmograph was calibrated with a 10 ml syringe, a 20 ml volume change resulting in 2 cmH_2O pressure within the box. The pressure response was linear to a volume change of 20 ml. The volume signal was electronically differentiated to determine flow. Transpulmonary pressure was determined using a pressure transducer (Model 267BC; Sanborn Co., Waltham, MA, U.S.A.), by comparing pressure at the tracheal opening with esophageal pressure measured from a saline-filled PE-90 tube in the distal esophagus. The pressure signal was calibrated using a water-filled manometer. The response was linear over the range ± 50 cmH_2O. The volume, flow, and pressure signals were recorded (General Scanning Model RS-5P recorder), and pulmonary resistance (R_L) was calculated from these signals using the method of von Neergaard & Wirz (1927) by a computer programme developed in this laboratory (Hulbert *et al.*, 1985). After the baseline R_L values were stabilized within 5%, guinea-pigs were given saline aerosol followed by increasing doses of aerosolized ACh solution, and the changes in R_L and heart rate were measured.

Inhalational acetylcholine dose-response measurements

ACh dose-response measurements were made 3 days following the last antigen challenge. Acetylcholine chloride (Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in normal saline to produce a stock solution of 150 mg ml^{-1}. Serial dilutions were made to produce solutions of 50, 15, 5, 1.5, 0.5 mg ml^{-1}. Aerosols were produced by placing 5 ml of saline or ACh solution into a Hudson blue nebuliser driven by compressed air at 5 l min^{-1} and connected by a T-piece into the airway chamber. Six puffs of 3 ml tidal breath for each dose were used for normal saline, followed by increasing ACh concentrations (0.5, 1.5, 5, 15, 50, 150 mg ml^{-1}). By observing the pressure tracing immediately after delivery of the aerosol, the peak response was determined for each dose. R_L and heart rate were measured at this point and the average of the three highest R_L values was taken for the response to each ACh concentration. The next dose was delivered after the R_L response had returned to the baseline except following the 50 mg ml^{-1} dose where we accepted a return to within 50% of the original baseline prior to delivery of the final dose.

In four separate guinea-pigs, two receiving MK-886 3 mg kg^{-1}, p.o. 2 h prior to ACh, ACh dose-responses were not different.

Histological examination

Four guinea-pigs from the LTC_4 group, 4 from the Control group, 5 from the OA + MK-886 group, 6 from the OA alone group and 11 from the Control group were randomly selected for morphometric analysis. Anaesthetized animals were killed by exsanguination and the lungs and trachea inflated to 15 cmH_2O pressure with glutaraldehyde 2.5% in buffer and then immersed in the fixative. Tissue samples were taken from the trachea, left and right main bronchi, left upper and lower bronchi, right middle and lower bronchi, and the parenchyma of the same lobes. The tissue was then dehydrated with a graded ethanol series and embedded in glycol methacrylate (JB-4, Polysciences, Warrington, PA, U.S.A.). Thin (3 μm) sections were cut and mounted on glass slides and stained with either Toluidine Blue O or Congo Red and Harris' haematoxylin. Using a digitizer, the internal perimeter (Pi) of the airway, marked by the epithelial luminal surface, and the luminal surface of the smooth muscle were measured for determination of epithelial and submucosal connective tissue areas. Airway size was determined by Pi as outlined by James *et al.* (1988). Cell counts were determined for epithelial and connective tissue compartments.

Data analysis

The mean response at each dose for R_L was plotted against the dose of ACh on a semi-log scale. R_L at each ACh concentration was compared between groups. Statistical analysis was performed by multivariate repeated measures analysis of variance (Fleiss, 1985). Comparison of morphometric parameters between OA challenged and control groups was performed for various airway sizes by random effects linear regression analysis (Feldman, 1988). Analysis of the data obtained from both control and OA groups revealed no difference compared with these identical groups from previous studies and therefore the cumulative data for these were used, resulting in $n = 16$ for control and $n = 32$ for OA-challenged groups.

Results

Airway responses to acetylcholine

Repeated aerosol challenge with LTC_4 did not alter baseline R_L (Table 1) but produced a significant leftward shift in the ACh dose-response curve as shown in Figure 1. This increased responsiveness was clearly demonstrated when evaluating the provocative concentrations of ACh required to produce 5 fold (PC_5), 10 fold (PC_{10}) and 20 fold (PC_{20}) increased in R_L from baseline (Table 1). Despite this leftward

Table 1 Baseline pulmonary resistance (R_L) and provocative concentrations (PC) of acetylcholine for control and treatment groups

	Baseline R_L (cmH_2O ml^{-1} s^{-1})	PC_5 (mg ml^{-1})	PC_{10} (mg ml^{-1})	PC_{20} (mg ml^{-1})	PC_{30} (mg ml^{-1})
Control	0.123 ± 0.006	13.3	17.5	24.5	31.3
($n = 16$)		(12.2,14.4)	(16.4,18.6)	(23.0,26.1)	(29.2,33.6)
LTC_4	0.156 ± 0.018	5.24**	7.09**	10.5**	16.1**
($n = 7$)		(4.58,6.00)	(6.31,7.97)	(9.30,11.9)	(13.0,20.1)
OA + MK-886	0.165 ± 0.017	13.6	17.9	25.6	33.2†
($n = 5$)		(11.7,15.8)	(16.0,20.1)	(23.1,28.5)	(29.5,37.3)
OA	0.141 ± 0.008	10.0	12.6*	16.6**	20.3**
($n = 32$)		(9.10,10.9)	(11.5,13.8)	(15.3,18.1)	(18.6,22.2)

Baseline R_L values are means ± s.e.mean. PC values are geometric means with 95% confidence intervals shown in the parentheses below the mean values for five fold (PC_5), ten fold (PC_{10}), 20 fold (PC_{20}) and 30 fold (PC_{30}) increases from baseline R_L.
LTC_4 = repeated leukotriene C_4 aerosol challenge.
OA = repeated ovalbumin aerosol challenge without (OA) or with (OA + MK-886) leukotriene synthesis inhibitor MK-886 pretreatment.
*$P < 0.05$; **$P < 0.01$ different from control group; †$P < 0.05$ different from OA group.

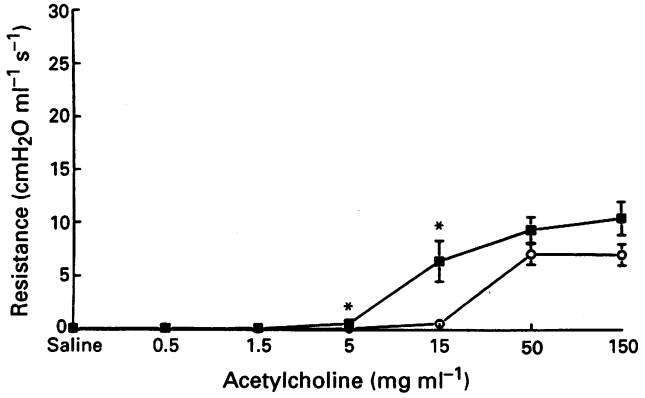


Figure 1 Pulmonary resistance changes with increasing acetylcholine concentrations (mg ml^{-1}) in guinea-pigs repeatedly challenged with aeorosolized leukotriene C_4 (LTC_4) ($n = 7$) or saline ($n = 6$). See text for detailed methods. Data are expressed as the means ± s.e.mean (vertical bars). LTC_4 challenged animals (■) showed a significant leftward shift in acetylcholine dose-response curve without changing the maximal response compared with saline challenged controls (○). *$P < 0.01$; significantly different from control

shift in the ACh response, repeated LTC_4 challenge did not increase the maximum R_L produced by 150 mg ml^{-1} ACh (Figure 1).

As previously demonstrated (Ishida *et al.*, 1989), repeated challenge with the antigen ovalbumin (OA) caused both a leftward shift in the ACh dose-response curve and a greater maximal response (Figure 2). PC values were significantly reduced for the OA challenged group (Table 1). Pretreatment prior to each of the multiple OA challenges with the leukotriene biosynthesis inhibitor MK-886 inhibited the OA-induced leftward shift in the ACh dose-response curve but did not reduce the greater maximal response to ACh (Figure 2, Table 1).

Airway eosinophilia

Repeated LTC_4 inhalations did not change the number of eosinophils in either the epithelium or submucosa. Quantitation of eosinophils within the epithelium of airways of all sizes are shown in Figure 3a: which shows that there was

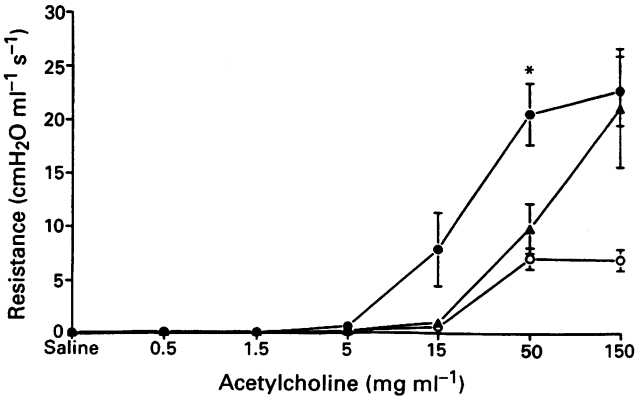


Figure 2 Pulmonary resistance changes with increasing acetylcholine concentrations (mg ml^{-1}) in guinea-pigs sensitized and repeatedly challenged with ovalbumin (OA) with pretreatment of leukotriene biosynthesis inhibitor MK-886 (▲, $n = 5$) or without pretreatment (●, $n = 7$), or sensitized and repeatedly challenged with saline (○, $n = 12$). See text for detailed methods. Data are expressed as the means ± s.e.mean (vertical bars). OA group showed leftward and upward shift in the acetylcholine (ACh) dose-response curve compared with the control group ($P < 0.05$ for ACh 5–50 mg ml^{-1} doses). MK-886 pretreatment inhibited the leftward shift induced by repeated antigen challenge without changing the maximal responses. (*$P < 0.05$ compared with MK-886 pretreated)

complete overlap in the 95% confidence intervals of the regression lines for eosinophils per mm^2 epithelium versus airway size for the guinea-pigs receiving LTC_4 versus vehicle alone, with the analysis of difference overlapping the zero difference line (Figure 3b).

As noted in our previous study, repeated OA challenge caused a significant increase in epithelial eosinophils of airways of all sizes (Figure 4a). Pretreatment with MK-886 did not change the eosinophil numbers infiltrating the airway epithelium of guinea-pigs when compared with those from animals receiving OA alone, with eosinophil numbers remaining significantly greater than controls (Figures 4a and b).

Discussion

Our results suggest that sulphidopeptide leukotrienes may contribute to antigen-induced airway hyperresponsiveness, but alone are insufficient to produce this phenomenon. The changes in the ACh dose-response curve produced by the leukotriene biosynthesis inhibitor MK-886 treatment prior to repeated antigen inhalation were completely compatible with the changes produced by repeated LTC_4 inhalations alone. Thus, the leukotriene biosynthesis inhibitor prevented the leftward shift in the dose-response produced by antigen without affecting the increased maximal R_L, whereas LTC_4 inhalations caused a shift to the left without increasing the maximal R_L. We have not previously been able to isolate these two components of the ACh dose-response curve in

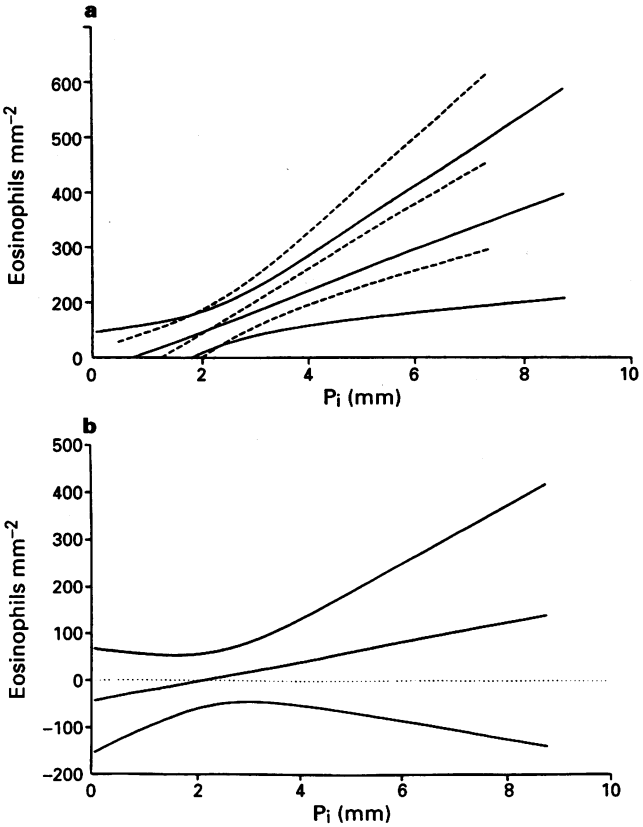


Figure 3 (a) Eosinophils per mm^2 epithelium of airways from saline challenged controls ($n = 4$) and from leukotriene C_4 (LTC_4) challenged guinea-pigs ($n = 4$) 3 days following the last aerosolized challenge. Regression lines and 95% confidence intervals are shown for LTC_4 challenged (– – –) and control (—) guinea-pigs. Pi = internal airway perimeter measured as the luminal border. Airway sizes included small airways (Pi = 0.7–3.4 mm), lobar bronchi (Pi = 3.7–6.3), main bronchi (5.8–6.9 mm) and tracheae (6.2–8.7 mm). (b) Analysis of difference between eosinophils per mm^2 epithelium for the LTC_4 vs control groups, demonstrating no difference. Pi values are as in (a)

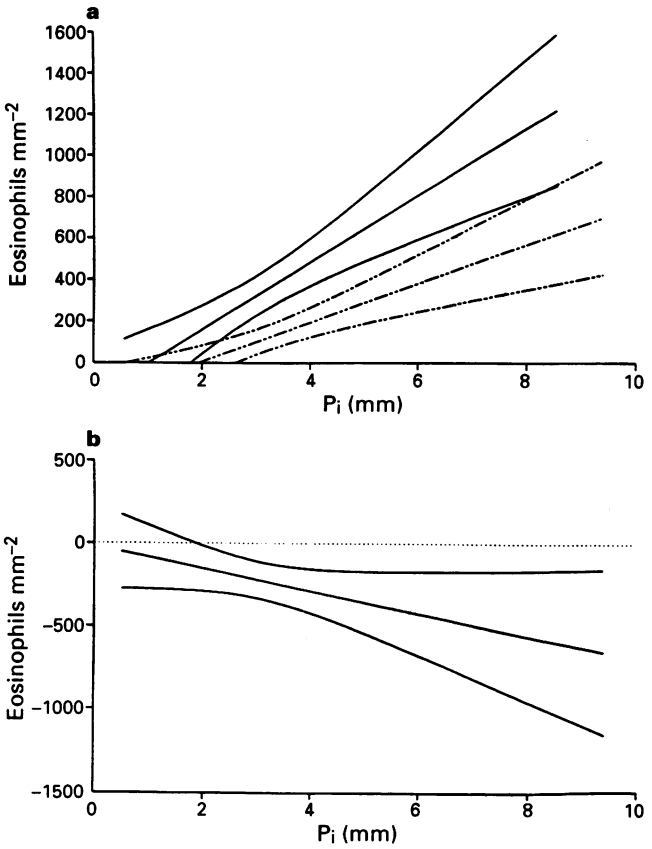


Figure 4 (a) Eosinophils per mm^2 in epithelium of airways from saline challenged controls (— - - - —), $n = 11$, and from ovalbumin (OA) challenged guinea-pigs following pretreatment with the leukotriene synthesis inhibitor MK-886 (—), $n = 5$, 3 days following the last aerosolized challenge. Regression lines and 95% confidence intervals are not shown for the data from 6 OA challenged guinea-pigs which did not differ from that of OA plus MK-886. Pi = internal airway perimeter as in Figure 3. (b) Analysis of difference between eosinophils per mm^2 epithelium for the groups demonstrates significantly increased number of eosinophils in airway epithelium in OA challenged animals (both OA and OA + NK-886 groups) compared with the control group at all but the smallest airway sizes (Pi < 2 mm). Pi values include small airways (0.7–3.4 mm), lobar bronchi (3.7–6.3 mm), main bronchi (5.8–6.9 mm) and tracheae (6.2–8.7 mm). There was no significant difference in eosinophil numbers in airway epithelium between OA and OA + MK-886 groups.

that interventions have altered both components to a comparable degree (Ishida *et al.*, 1990b; Matsuse *et al.*, 1991), although our assessment of nedocromil sodium pretreatment (Schellenberg *et al.*, 1991) did not extend the ACh dose-response far enough to evaluate this adequately. In retrospect, this is a limitation of our study in that Sont *et al.* (1992) have subsequently shown in asthmatics that nedocromil sodium inhibits the sensitivity, but not the maximal response, to spasmogens.

The leftward shift can be considered increased sensitivity since both the threshold concentration and the concentration of ACh producing a half-maximal response are reduced. Increased sensitivity of *in vitro* smooth muscle preparations is usually considered to reflect changes in agonist receptor interaction although it could theoretically be produced by changes in any component of early signal transduction such as phospholipase C activation, IP_3 formation, intracellular Ca^{2+} mobilization, etc. It is impossible to address these alternatives in an *in vivo* preparation such as ours, but we do not believe that an increase in ACh receptor number is the reason since the increased airway responsiveness is not agonist specific and no data exist to suggest that leukotrienes alter ACh receptor numbers.

An increased sensitivity without increased maximal response to ACh is not compatible with a number of other potential mechanical causes of airway hyperresponsiveness. These include both an increase in amount of airway smooth muscle or an increase in wall thickness of the airway. Our previous morphometric analysis failed to find an increase in wall thickness (Ishida *et al.*, 1989) or amount of smooth muscle (Ishida *et al.*, 1990a) in airways from guinea-pigs demonstrating increased hyperresponsiveness following repeated antigen challenge. We would also predict that a decrease in load against which the smooth muscle must contract would cause an increased maximal response.

Increased sensitivity without increased maximal bronchoconstriction could also be produced by an increase in epithelial permeability allowing a higher concentration of ACh to reach the smooth muscle. Although leukotrienes have been demonstrated to increase vascular permeability (Dahlen *et al.*, 1981), we believe this cannot account for our findings for the following reasons. Firstly, the ACh responses were assessed 72 h after the last challenge with either LTC_4 or antigen, a time chosen in order to avoid more transient changes such as permeability changes to a mediator. Secondly, evidence is lacking that leukotrienes alter epithelial permability. Since the ACh was given by aerosol rather than intravenously, epithelial permeability is the critical factor. By 72 h post-antigen or post-LTC_4 the epithelium shows no evidence of damage morphologically. Lastly, inhalation of ACh did not alter heart rate in either group of guinea-pigs, thereby differing from the responses seen shortly after smoke inhalation which we attributted to increased permeability (James *et al.*, 1987).

The present study has not addressed which of the sulphidopeptide leukotrienes cause increased sensitivity. We chose to administer LTC_4 because of the rationale that the airways would be exposed to it as well as the metabolites LTD_4 and LTE_4. Receptors for LTC_4 (Pong *et al.*, 1983) and LTD_4 (Pong & DeHaven, 1983) have been identified in lung tissue and it has been suggested that there may be an LTE_4-specific receptor (Lee *et al.*, 1984) based on its ability to potentiate effects of other agonists upon airway smooth muscle *in vitro*. This effect is modest and unlikely to account for *in vivo* changes noted three days after the last leukotriene challenge. Our study has no means of differentiating a leukotriene effect directly involving smooth muscle versus an effect upon a modulatory component such as a neural or cellular factor potentiating smooth muscle responses.

The lack of airway eosinophilia following LTC_4 challenge was not surprising as this leukotriene is not a chemotactic factor. However, the failure of MK-886 treatment to prevent antigen-induced airway eosinophilia argues against LTB_4 being an important mediator of airway eosinophilia in our model. This differs from the finding of Taylor *et al.* (1989) where lavage eosinophils following a single antigen challenge were reduced by LTB_4 antagonists. We postulate that the repeated antigen challenges in our protocol generate chemotactic factors other than, or in addition to, LTB_4.

The relevance of our findings to human asthma remains to be determined. Our guinea-pig model shares a number of features with human asthma including acute bronchoconstriction to aerosolized antigen, airway eosinophilia and hyperresponsiveness, plus enhanced airway smooth muscle shortening *in vitro*. However, it employs high antigen exposures over a relatively short time frame to induce these changes which appear to be reversible within one month after antigen exposures are discontinued. The present evaluation of leukotriene synthesis inhibitors and antagonists in asthma will determine the utility of the model for predicting drug efficacy.

In summary, our data support a role for sulphidopeptide leukotrienes in causing increased sensitivity of airways to contractile agonists without altering the maximal contractile response or airway eosinophilia.

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References

BAI, T.R. (1990). Abnormalities in airway smooth muscle in fatal asthma. *Am. Rev. Respir. Dis.*, **141**, 552–552.

BARNES, N.C., PIPER, P.J. & COSTELLO, J.F. (1984). Comparative effects of inhaled leukotriene D_4 and histamine in normal human subjects. *Thorax*, **39**, 500–504.

CASALE, T.B., WOOD, D., RICHERSON, H.B., ZEHR, B., ZAVALA, D.C. & HUNNINGHAKE, G.W. (1987). Direct evidence of a role for mast cells in the pathogenesis of allergen-induced bronchoconstriction. *J. Clin. Invest.*, **80**, 1507–1511.

CHAN-YEUNG, M., CHAN, H., TSE, K.S., SALARI, H. & LAM, S. (1989). Histamine and leukotrienes release in bronchoalveolar fluid during plicatic acid-induced bronchoconstriction. *J. Allergy Clin. Immunol.*, **84**, 762–768.

DAHLEN, S.-E., HANSSON, G., HEDQVIST, P., BJORCK, T., GRANSTROM, E. & DAHLEN, B. (1983). Allergen challenge of lung tissue from asthmatics elicits bronchial contraction that correlates with the release of leukotrienes C_4, D_4 and E_4. *Proc. Natl. Acad. Sci. U.S.A.*, **8**, 1712–1716.

DAHLEN, S.-E., BJORK, J., HEDQVIST, P., ARFORS, K.E., HAMMARSTROM, S., LINDGREN, J.A. & SAMUELSSON, B. (1981). Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: *in vitro* effects with relevance to the acute inflammatory response. *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 3887–3891.

DAHLEN, S.-E., HEDQVIST, P., HAMMARSTROM, S. & SAMUELSSON, B. (1980). Leukotrienes are potent constrictors of human bronchi. *Nature*, **288**, 484–486.

DEJONGSTE, J.C., MONS, H., BONTA, I.L. & KERREBIJN, K.F. (1987). *In vitro* responses of airways from an asthmatic patient. *Eur. J. Resp. Dis.*, **71**, 23–29.

DIXON, R.A.F., DIEHL, R.E., OPAS, E., RANDS, E., VICKARS, P.J., EVANS, J.F., GILLARD, J.W. & MILLER, D.K. (1990). Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. *Nature*, **343**, 282–284.

FELDMAN, H.A. (1988). Families of lines: random effects in linear regression analysis. *J. Appl. Physiol.*, **64**, 1721–1732.

FLEISS, J.L. (1985). *The Design and Analysis of Clinical Experiments*. New York: John Wiley & Sons.

GRIFFIN, M., WEISS, J.W., LEITCH, A.G., MCFADDEN, E.R., COREY, E.J., AUSTEN, K.F. & DRAZEN, J.M. (1983). Effects of leukotriene D on the airways in asthma. *N. Engl. J. Med.*, **308**, 436–439.

HANNA, C.J., BACH, M.K., PARÉ, P.D. & SCHELLENBERG, R.R. (1981). Slow-reacting substances (leukotrienes) contract human airway and pulmonary vascular smooth muscle. *Nature*, **290**, 343–344.

HOLROYDE, M.C., ALTOUNYAN, R.E.C., COLE, M., DIXON, M. & ELLIOTT, E.V. (1981). Bronchoconstriction produced in man by leukotrienes C and D. *Lancet*, **ii**, 17–18.

HULBERT, W.C., MCLEAN, T., WIGGS, B., PARÉ, P.D. & HOGG, J.C. (1985). Histamine dose-response curves in guinea pigs. *J. Appl. Physiol.*, **58**, 625–634.

ISHIDA, I., KELLY, L.J., THOMSON, R.J., BEATTIE, L.L. & SCHELLENBERG, R.R. (1989). Repeated antigen challenge induces airway hyperresponsiveness with tissue eosinophilia in guinea pigs. *J. Appl. Physiol.*, **67**, 1133–1139.

ISHIDA, K., PARÉ, P.D., THOMSON, R.J. & SCHELLENBERG, R.R. (1990a). Increased *in vitro* responses of tracheal smooth muscle from hyperresponsive guinea pigs. *J. Appl. Physiol.*, **68**, 1316–1320.

ISHIDA, K., THOMSON, R.J., BEATTIE, L.L., WIGGS, B. & SCHELLENBERG, R.R. (1990b). Inhibition of antigen-induced airway hyperresponsiveness, but not acute hypoxia or airway eosinophilia, by an antagonist of platelet-activating factor. *J. Immunol.*, **144**, 3907–3911.

JAMES, A.L., DIRKS, P., OHTAKA, H., SCHELLENBERG, R.R. & HOGG, J.C. (1987). Airway hyperresponsiveness to intraveneous and inhaled acetylcholine in the guinea pig after cigarette smoke exposure. *Am. Rev. Respir. Dis.*, **136**, 1158–1162.

JAMES, A.L., PARÉ, P.D. & HOGG, J.C. (1988). Effects of lung volume, bronchoconstriction and cigarette smoke on morphometric airway dimensions. *J. Appl. Physiol.*, **64**, 913–919.

JONES, T.R., DAVIS, C. & DANIEL, E.E. (1982). Pharmacological study of contractile activity of leukotriene C_4 and D_4 on isolated human airway smooth muscle. *Can. J. Physiol. Pharmacol.*, **60**, 638–643.

LEE, T.H., AUSTEN, K.F., COREY, E.J. & DRAZEN, J.M. (1984). Leukotriene E_4-induced airway hyperresponsiveness of guinea pig tracheal smooth muscle to histamine and evidence for three separate sulfidopeptide leukotriene receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 4922–4925.

MATSUSE, T., THOMSON, R.J., CHEN, X.-R., SALARI, H. & SCHELLENBERG, R.R. (1991). Capsaicin inhibits airway hyperresponsiveness but not lipoxygenase activity or eosinophilia after repeated aerosolised antigen in guinea pigs. *Am. Rev. Respir. Dis.*, **144**, 368–372.

MILLER, D.K., GILLARD, J.W., VICKER, P.J., SADOWSKI, S., LEVEILLE, C., MANCINI, J.A., CHARLESON, P., DIXON, R.A.F., FORD-HUTCHINSON, A.W., FORTIN, R., GAUTHER, J.V., RODKEY, J., ROSEN, R., ROUZER, C., SIGAL, I.S., STRADER, C.D. & EVANS, J.F. (1990). Identification and isolation of a membrane protein necessary for leukotriene production. *Nature*, **343**, 278–281.

PONG, S.S. & DEHAVEN, R.N. (1983). Characterization of a leukotriene D_4 receptor in guinea pig lung. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 7415–7419.

PONG, S.S., DEHAVEN, R.N., KUEHL, F.A. Jr. & EGAN, R.W. (1983). Leukotriene C_4 binding to rat lung membranes. *J. Biol. Chem.*, **258**, 9616–9619.

ROUZER, C.A., FORD-HUTCHINSON, A.W., MORTON, H.E. & GILLARD, J.W. (1990). MK-886, a potent and selective leukotriene biosynthesis inhibitor blocks and reverses the membrane association of 5-lipoxygenase in ionophore-challenged leukocytes. *J. Biol. Chem.*, **265**, 1436–1442.

SCHELLENBERG, R.R. & FOSTER, A. (1984). *In vitro* responses of human asthmatic airway and pulmonary vascular smooth muscle. *Int. Arch. Allergy Appl. Immunol.*, **75**, 237–241.

SCHELLENBERG, R.R., ISHIDA, K. & THOMSON, R.J. (1991). Nedocromil sodium inhibits airway hyperresponsiveness and eosinophilic infiltration induced by repeated antigen challenge in guinea-pigs. *Br. J. Pharmacol.*, **103**, 1842–1846.

SONT, J.K., BEL, E.H., DIJKMAN, J.H. & STERK, P.J. (1992). The long-term effect of nedocromil sodium on the maximal degree of airway narrowing to methacholine in atopic asthmatic subjects. *Clin. Exp. Allergy*, **22**, 554–560.

TAYLOR, B.M., CRITTENDEN, N.J., RICHARDS, I.M. & SUN, F.F. (1989). The effect of the leukotriene B_4 (LTB_4) antagonists U-75,302 and U-75,485 on guinea pig eosinophil chemotaxis and activation *in vitro* and *in vivo*. *Am. Rev. Respir. Dis.*, **139**, A913. Abstract 3992.

VON NEERGAARD, K. & WIRZ, K. (1927). Die Messung der Stromung-swiderstande in den Atemwegen des Menschen, insbesondere bei Asthma und Emphysem. *Z. Klin. Med.*, **105**, 51–82.

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The modulation of inflammatory oedema by calcitonin gene-related peptide

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1 The effect of calcitonin gene-related peptide (CGRP) when given with or as a pretreatment to oedema inducing agents was investigated in the skin and paws of male Wistar and Sprague Dawley rats.

2 Oedema formation at intradermally-injected sites in the skin was measured by a ^{125}I-labelled human serum albumin accumulation technique and paw oedema was measured by a weight displacement technique.

3 CGRP (30 pmol) when given with, or as a 20 min pretreatment, markedly potentiated oedema formation induced by substance P (100 pmol) in rat skin. In comparison, CGRP had little effect on 5-hydroxytryptamine (5-HT, 0.1–3 nmol)-induced oedema when given as a co-injection but significantly potentiated 5-HT-induced oedema when given as a 20 min pretreatment in the skin. Similar results were obtained in both Wistar and Sprague Dawley rats.

4 Pretreatment with CGRP (30 pmol) had little modulatory effect on oedema induced by substance P (100 pmol) in the presence of the vasodilator prostanoid, prostaglandin E_1 (PGE_1, 850 pmol) in the skin of Wistar rats.

5 Pretreatment with CGRP (30 pmol) caused a non-significant increase in carrageenin (300 μg)-induced oedema in the hind paw of Wistar rats. Capsaicin (100 nmol) given as a pretreatment had little effect on carrageenin-induced oedema.

6 CGRP (30 pmol), given as a pretreatment, had little modulatory effect on 5-HT (3 nmol)-induced oedema in the paw of Wistar rats but a non-significant decrease in paw oedema was observed in Sprague Dawley rats.

7 The results suggest that CGRP, either given together with or as a pretreatment to mediators of increased microvascular permeability, depending on circumstance, can act in a synergistic manner to increase oedema formation. Little evidence was obtained for an anti-inflammatory effect of CGRP in either the rat skin or paw.

Keywords: Calcitonin gene-related peptide; oedema; inflammation

Introduction

The activation of the efferent function of sensory neurones results in an inflammatory response involving vasodilatation and increased vascular permeability (Jancso *et al.*, 1967). The stimulated sensory nerves release vasoactive neuropeptides the best described of which are substance P (SP) and calcitonin gene-related peptide, CGRP (see Holzer, 1988, for review).

Substance P and the related neurokinins are potent mediators of increased microvascular permeability in many tissues that include human and rat skin (Hagermark *et al.*, 1977; Devillier *et al.*, 1986; Brain & Williams, 1989). SP can act directly via NK_1 receptors on endothelial cells to induce plasma protein leakage (Andrews *et al.*, 1989) or by activating mast cells to release histamine and, in rodents, 5-hydroxytryptamine (5-HT) (Foreman *et al.*, 1983; Brain & Williams, 1989). CGRP is a potent vasodilator with a long duration of action (Brain *et al.*, 1985), yet when given alone has little effect on vascular permeability (Brain & Williams, 1985). Previous studies in rabbit and rat skin have shown that CGRP, given intradermally together with mediators of increased microvascular permeability, can act to increase local blood flow and consequently potentiate oedema formation (Brain & Williams, 1985; Gamse & Saria, 1985). More recent studies show that CGRP can also potentiate neutrophil accumulation induced by inflammatory mediators that include interleukin-1 and C5a (Buckley *et al.*, 1991a,b).

Capsaicin, the pungent extract of peppers, selectively activates a subset of primary sensory neurones to release neuropeptides such as CGRP and SP, resulting in vasodilatation and increased microvascular permeability. The nerves become desensitized after the initial depolarization of the sensory nerve terminals and further stimulation has little excitatory effects (see Maggi & Meli, 1988; Holzer, 1991). In addition in high concentrations and when administered in neonates, capsaicin can have a toxic effect on sensory nerves, leading to sensory nerve degeneration (Jancso *et al.*, 1967). Capsaicin pretreatment has been used in many inflammatory models to investigate for a neurogenic inflammatory component. It is proposed that capsaicin desensitizes or destroys the sensory nerves and hence removes the neurogenic components of inflammation. Use of capsaicin pretreatment protocols has led to results which suggest the involvement of a sensory neurogenic component in carrageenin-induced inflammation and in adjuvant arthritis in the rat (Colpaert *et al.*, 1983; Raychaudhuri *et al.*, 1991) as well as in human skin after allergen challenge in atopic subjects (Lunblad *et al.*, 1985; McCusker *et al.*, 1989). It has also been shown in guinea-pigs that capsaicin pretreatment inhibits antigen-induced airways hyper-responsiveness (Matsuse *et al.*, 1991) and in rabbits inhibits platelet activating factor-induced hyper-responsiveness (Spina *et al.*, 1991).

A recent study (Raud *et al.*, 1991) has suggested that CGRP, in addition to capsaicin, possesses anti-inflammatory properties when given as a pretreatment in inflammatory models. In the hamster cheek pouch preparation, pretreatment with CGRP or capsaicin resulted in an inhibition of the plasma leakage response to histamine and leukotriene B_4

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(LTB_4). In the rat paw, pretreatment with CGRP resulted in a significant inhibition of 5-HT-induced oedema and also in human skin pretreatment with capsaicin or CGRP produced an inhibition of the histamine-induced wheal. Raud and co-workers suggest that in these studies CGRP is active via anti-inflammatory mechanisms and that capsaicin is acting to release endogenous anti-inflammatory CGRP.

These results together with our previous results, suggest that CGRP can act to modulate inflammatory events via more than one mechanism. In this study we have compared the inflammatory effect of CGRP when given with, or as a pretreatment to, oedema-inducing agents in rat dorsal skin and paw.

Methods

Measurement of oedema formation in rat dorsal skin

Oedema in response to intradermally (i.d.) injected test agents was determined by use of an ^{125}I-labelled serum albumin accumulation technique previously described (Brain & Williams, 1985). Male Wistar and Sprague Dawley rats (200–350 g) were anaesthetized with sodium pentobarbitone (Sagatal 45 mg kg^{-1} intraperitoneally, i.p.). The dorsal skin was shaved and Evan's blue (0.5 ml kg^{-1} of 2.5% w/v) containing ^{125}I human serum albumin (0.1 MBq) injected intravenously (i.v.) via the tail vein. Test agents were made up in Tyrode solution and injected i.d. into previously randomized sites with two replicates for each agent. After 30 min a 5 ml blood sample was taken by cardiac puncture. This was centrifuged at 8000 *g* for 5 min to obtain a plasma sample. The rat was decapitated and the skin removed. Test sites were punched out (diameter 16 mm) and the radioactivity counted in a gamma counter for each skin site and the plasma sample. The volume of oedema at each site was determined from the ^{125}I activity of 1 ml of plasma.

CGRP was either injected with the oedema producing agents in 0.1 ml volumes or given as a pretreatment 20 min prior to the injection of oedema producing agents in 0.05 ml volumes. When CGRP was given as a pretreatment, control sites received Tyrode solution (0.05 ml).

Measurement of oedema formation in the rat paw

Paw oedema in male Wistar and Sprague Dawley rats was measured by a method of weight displacement (Winter *et al.*, 1963). Rats were anaesthetized with sodium pentobarbitone (45 mg kg^{-1}, i.p.) or given light ether anaesthesia for the purpose of injections. The hind paws were randomly assigned as 'control' or 'test'. Prior to treatment, baseline paw weights were measured for each hind paw. Each hind paw was dipped, up to the distinct line where hair is observed (the hairline), into a beaker containing 75 ml water. The beaker, sited on a Mettler PC 100 top pan balance, was calibrated before each reading to zero weight. Agents were injected subplantar in 0.1 ml volumes into the hind paws with one paw acting as test and the contralateral paw acting as control. CGRP, capsaicin or vehicle were each injected as required 20 min before injection of 5-HT or carrageenin. Each paw was reweighed at 0.5 and 1.0 h after injection of 5-HT and also at 1.5 h after injection of carrageenin. The amount of oedema was calculated by subtraction of paw weight from the baseline paw weight at each time point.

Materials

5-HT creatinine sulphate complex (Sigma, U.K.) was dissolved in Tyrode solution (composition in mM: NaCl 137, KCl 2.7, $MgCl_2$ 0.5, NaH_2PO_4 0.4, $NaHCO_3$ 11.9 and glucose 5.6) or saline (0.9%). PGE_1 (Sigma, U.K.) was dissolved in ethanol and for use was diluted with Tyrode. Substance P and Compound 48/80 (Sigma, U.K.) and human α CGRP (a gift from Celltech, U.K.) were dissolved in saline (0.9%) and stored in 0.1 ml aliquots at −20°C. They were then diluted with Tyrode solution immediately prior to use (or also saline (0.9%) in the case of CGRP). Capsaicin was dissolved in ethanol, polyoxyethylene sorbitan monooleate (Tween 80) and saline (0.9%) in a ratio of 2:1:7. This was further diluted with saline (0.9%). Carrageenin (Sigma, U.K.) was dissolved in saline (0.9%) or Tyrode solution.

Statistics

For the assessment of results obtained in the skin oedema experiments Bonferroni's modified *t* test was employed which uses the s.e. estimate for the analysis of variance to account for the use of multiple tests being performed. For analysis of paw oedema experiments Student's *t* test was performed ($*P<0.05$ and $**P<0.01$ for both tests).

Results

Effect of CGRP on oedema formation in rat skin

SP (100 pmol), induced a significant ($P<0.001$) increase in skin oedema, whilst a vasodilator dose of CGRP (30 pmol) had no significant oedema-inducing effect compared to control, Tyrode-injected sites. When CGRP (30 pmol) was given together with SP (100 pmol) there was a significant potentiation ($P<0.001$) of substance P-induced oedema (Figure 1a). CGRP (30 pmol), given as a pretreatment 20 min before SP, also produced a significant potentiation in SP-induced oedema ($P<0.01$, compared to pretreatment with Tyrode prior to injecting SP, Figure 1b). Similar results were obtained in Sprague Dawley rats. SP induced an increase in

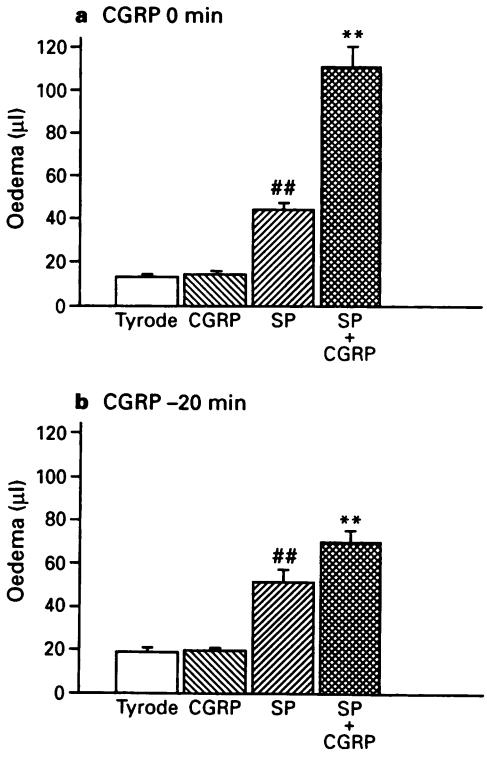


Figure 1 Effect of calcitonin gene-related peptide (CGRP, 30 pmol) given as a co-injection (a) and as a 20 min pretreatment (b) on substance P (SP, 100 pmol)-induced oedema in the skin of Wistar rats. In (a) CGRP was injected i.d. together with SP (cross hatched columns) in 0.1 ml volumes at time zero. In (b) sites were either pretreated with Tyrode or CGRP in 0.05 ml volumes 20 min before the injection of SP (also given in 0.05 ml volume). Oedema was measured 30 min from time zero. Values given are mean ± s.e.mean (vertical bars) for $n = 8$ rats. $\#\#P<0.01$ compared to tyrode control. $**P<0.01$ compared to SP.

oedema, 42.2 ± 4.3 l, mean ± s.e.mean, $n = 5$ which was singificantly potentiated by either a co-injection 99.4 ± 5.9 l ($P<0.001$) or a 20 min pretreatment injection 67.1 ± 7.8 l ($P<0.01$), of CGRP (30 pmol).

5-HT (0.1–3 nmol) produced a dose-dependent increase in oedema formation in the skin of Wistar rats but CGRP (30 pmol) when co-injected with each dose of 5-HT had no significant effect on oedema formation (Figure 2a). Similar results were obtained with Sprague Dawley rats (Figure 2c). When CGRP was given as a pretreatment to Wistar rats, 20 min before injection of 5-HT, oedema formation was potentiated at all doses of 5-HT ($P<0.05$–0.001) (Figure 2b). However when CGRP was given as a 20 min pretreatment to 5-HT in Sprague Dawley rats, a significant potentiating effect of 5-HT-induced oedema was only observed with the highest dose of 5-HT (Figure 2d) ($P<0.01$).

The mast cell degranulating agent Compound 48/80 (0.5 μg), was also used to induce mast cell-dependent oedema. Compound 48/80 induced an oedema of 52.6 ± 4.7 μl ($n = 3$) when given after site pretreatment with Tyrode. After site pretreatment with CGRP (30 pmol) the oedema was significantly increased ($P<0.01$) to 61.9 ± 5.49 μl ($n = 3$). Thus after pretreatment with CGRP there was, as seen with SP and 5-HT, a tendency for potentiation of oedema in the skin of rats.

Effects of pretreatment with CGRP in the presence of the vasodilator, PGE_1

To try and determine if the vasodilator effect of CGRP was masking anti-inflammatory activity, experiments with SP were carried out in the presence of a near-maximal vasodilator dose of the prostanoid, PGE_1. Therefore after giving CGRP (or Tyrode, for control) as a pretreatment, substance P was injected with the vasodilator PGE_1. PGE_1 (850 pmol) potentiated oedema induced by substance P and pretreatment with CGRP had little further modulatory effect (Figure 3).

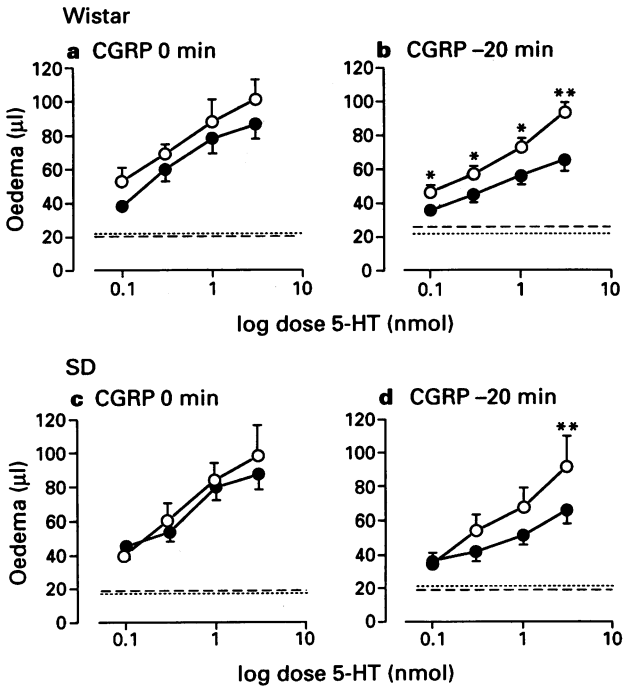


Figure 2 Effect of calcitonin gene-related peptide (CGRP, 30 pmol) on 5-hydroxytryptamine (5-HT)-induced oedema in the skin of Wistar or Sprague Dawley (SD) rats. The effects of co-injection and pretreatment of CGRP in the skin of Wistar rats (a) and (b) and Sprague Dawley rats (c) and (d) is shown. In (a) and (c) CGRP was injected with 5-HT in 0.1 ml volumes i.d. at time zero. In (b) and (d) sites were either pretreated with Tyrode or CGRP in 0.05 ml volumes 20 min before injection of 5-HT (also given in 0.05 ml volume). In all graphs, (●) represent control 5-HT-induced oedema; (○) represent CGRP effects on 5-HT-induced oedema which was measured 30 min from time zero. Dashed lines represent plasma protein extravasation at Tyrode control sites and dotted lines CGRP sites. Values given are mean ± s.e.mean for $n = 6$–12 rats. $^*P<0.05$; $^{**}P<0.01$ compared with 5-HT alone.

Effect of pretreatment with CGRP on oedema inducing agents in the rat hind paw

5-HT (3 nmol) induced an increase in paw volume as observed by Raud *et al.* (1991), however, in our experiments

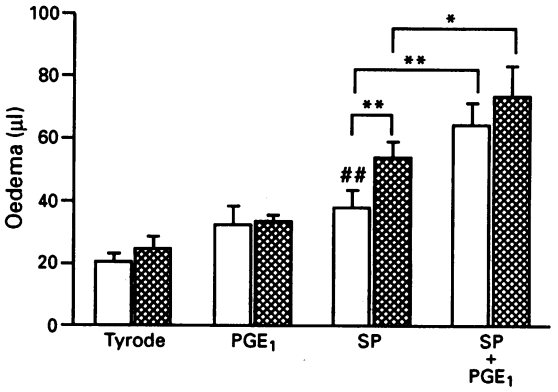


Figure 3 Effect of pretreatment with CGRP (30 pmol) on oedema induced by substance P (100 pmol) in the presence of the vasodilator PGE_1 (850 pmol). Sites were pretreated with Tyrode (open bars) or CGRP (cross-hatched bars) in 0.05 ml volumes i.d., 20 min prior to the injection of test agents (also given as a 0.05 ml volume). Oedema was measured 30 min from time zero. Values given are mean ± s.e.mean for $n = 4$ or 5 rats. $\#\#P<0.01$ compared to Tyrode control. $^*P<0.05$; $^{**}P<0.01$ as illustrated.

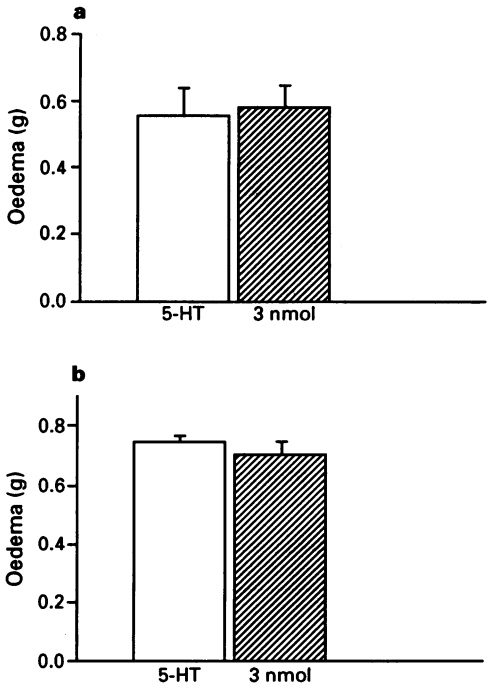


Figure 4 Effect of pretreatment with calcitonin gene-related peptide (CGRP, 30 pmol) on 5-hydroxytryptamine (5-HT, 3 nmol)-induced oedema in the hind paw of Wistar rats with Tyrode (a) or saline (b) used as the vehicle. Open columns represent pretreatment with vehicle and hatched columns represent pretreatment with CGRP. CGRP or vehicle was injected subplantar into the hind paw 20 min before giving 5-HT. In both (a) and (b) oedema formation was measured 30 min from time zero. Values given are mean ± s.e.mean for $n = 4$ rats.

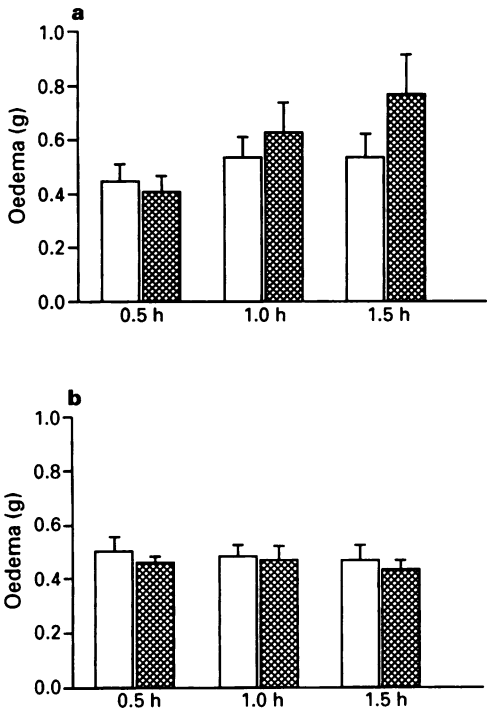


Figure 5 Effect of pretreatment with calcitonin gene-related peptide (CGRP, 30 pmol) (a) and capsaicin (100 nmol) (b) on carrageenin (300 μg)-induced oedema in the hind paw of Wistar rats. Paws were injected subplantar with saline (open columns), CGRP or capsaicin (hatched columns) in 0.1 ml volumes 20 min before injection of carrageenin (also added in 0.1 ml volume). Oedema was measured at 0.5, 1.0 and 1.5 h from time zero. Values given are mean ± s.e.mean (vertical bars) for $n = 4$ rats.

using Wistar rats, pretreatment with CGRP had no effect on paw oedema irrespective of whether the experiment was carried out using a buffered medium (Tyrode) or saline (as in protocols of Raud and co-workers), see Figure 4.

Carrageenin (300 μg) produced an increase in oedema in the rat hind paw. Pretreatment of the rat hind paw with CGRP (30 pmol) resulted in an initial, but insignificant reduction in carrageenin-induced oedema at 0.5 h compared to pretreatment with vehicle. At 1 and 1.5 h though there was a trend towards an increase (although statistically non-significant) in oedema in the paws of rats pretreated with CGRP compared to paws pretreated with vehicle (Figure 5a). Pretreatment with capsaicin (100 nmol) had little effect on carrageenin paw volume (Figure 5b).

Importance of rat strain on ability of CGRP to modulate oedema in the rat hind paw

In further experiments Sprague Dawley rats were used to repeat more exactly the protocol of Raud *et al.* (1991). In paws pretreated with CGRP (30 pmol) there was a mean reduction in 5-HT-induced oedema compared to vehicle pretreated paws (Table 1) but this did not reach significance and in two rats CGRP pretreatment produced an increase in 5-HT-induced oedema. A non-significant effect was also seen in rats anaesthetized for injections with ether (as used by Raud *et al.*, 1991).

Discussion

Our results from experiments carried out in the skin of both Wistar and Sprague Dawley rats show that CGRP does not inhibit oedema formation when administered either with 5-HT and SP, or as a pretreatment. Indeed, as previously shown (Brain & Williams, 1985; Gamse & Saria, 1985), CGRP potentiates oedema formation induced by SP. CGRP also potentiated oedema formation induced by 5-HT in this study, but only when skin sites were pretreated with CGRP prior to 5-HT injection. When CGRP was co-injected with 5-HT there was no effect on 5-HT-induced oedema. The lack of potentiation of 5-HT-induced oedema by CGRP, when co-injected, may be related to vasoconstrictor activity of 5-HT. This was not possible to measure for technical reasons, but is suggested by the observation of pallor at some 5-HT-injected sites. Thus an anti-inflammatory effect of 5-HT was not observed within skin and this result was supported by a lack of modulatory effect of CGRP on oedema induced by compound 48/80 which stimulates mast cell activation and thus releases endogenous 5-HT (together with other mast cell-derived mediators). In addition, results with the prostanoid vasodilator suggest that an anti-inflammatory effect of CGRP is not revealed when experiments are carried out in a dilated microvascular bed, where further activity of CGRP as a vasodilator is minimal.

It has previously been shown that CGRP potentiates the oedema-inducing actions of a range of mediators of increased microvascular permeability, including neutrophil-dependent mediators in rabbit skin (Brain & Williams, 1985; Buckley *et al.*, 1991a,b). Evidence suggests that potent microvascular vasodilators such as CGRP and the prostanoids PGE_1, PGE_2 and PGI_2 potentiate oedema formation in skin by increasing blood flow at the arteriolar level, which then leads to an increased intravenular post capillary pressure and in turn increased plasma protein extravasation from postcapillary venules, made leaky by mediators of increased microvascular permeability (see Williams & Peck, 1977).

Little inhibition of 5-HT- or carrageenin-induced oedema was observed in the hind paw of the Wistar rat, when CGRP was given as a pretreatment. Carrageenin induces oedema in the rat hind paw in a biphasic manner. The release of 5-HT and histamine is largely responsible for the initial phase of oedema. Later a delayed response occurs when polymorphonuclear cells migrate to the site (Sedgwick & Willoughby, 1989) and probably contribute to the oedema. CGRP showed no evidence of inhibiting this reaction when given as a pretreatment in the rat paw but rather a trend towards a

Table 1 Effect of calcitonin gene-related peptide (CGRP) pretreatment on 5-hydroxytryptamine (5-HT)-induced oedema in the hind paw of Sprague Dawley and Wistar rats under different anaesthetics

Strain and anaesthetic	*Pretreatment*	*Oedema* (g) mean (s.e.mean) 0.5 h	1.0 h	n
Wistar and pentobarbitone	Saline	0.75 (0.02)	0.81 (0.02)	4
	CGRP	0.71 (0.04)	0.82 (0.01)	
Sprague Dawley pentobarbitone	Saline	0.62 (0.08)	0.61 (0.09)	11
	CGRP	0.50 (0.07)	0.52 (0.08)	
Sprague Dawley ether	Saline	0.26 (0.04)	0.19 (0.04)	6
	CGRP	0.18 (0.02)	0.11 (0.03)	

Rat hind paws were preinjected subplantar with saline or CGRP (30 pmol) in 0.1 ml volumes 20 min prior to injection of 5-HT (3 nmol) also given as a 0.1 ml volume. Oedema was measured 30 and 60 min from time zero.

potentiation of carrageenin-induced oedema at later time points was observed. Pretreatment with capsaicin had no effect on carrageenin paw oedema. This is in contrast to other studies such as where carrageenin-induced pleural cavity oedema in the rat has been attenuated following high dose capsaicin pretreatment (50–100 mg kg^{-1} s.c. daily for 1 week; Raychaudhurie *et al.*, 1991). However in our study a lower dose was used, in keeping with the suggestion of Raud and co-workers that capsaicin releases an anti-inflammatory activity.

Raud and co-workers (1991) used Sprague Dawley rats, with ether anaesthesia (over the injection period), to exhibit a clear CGRP-induced inhibition of paw oedema. In experiments carried out in our laboratories, following the exact protocol, a trend towards an inhibition of 5-HT-induced oedema was observed but significance was not reached. We cannot therefore support the findings of Raud *et al.* (1991). Our inability to reproduce the results of Raud and co-workers in experiments in the rat paw is not understood. However it is suggested that the Sprague Dawley rat is more susceptible, when compared with the Wistar rat, to an anti-inflammatory effect of CGRP (J. Raud, personal communication). It is obvious that results from the study of Raud and co-workers (1991) demonstrate that CGRP and capsaicin pretreatment in the hamster cheek pouch inhibits plasma protein extravasation induced by histamine and leukotriene B_4. They suggest that capsaicin acts via the release and thus actions of endogenous CGRP. This is an interesting concept which deserves further study, although our results in the rat skin and paw do not support the concept that CGRP generally acts in an anti-inflammatory manner.

The mechanisms by which CGRP could exert anti-inflammatory effects are unknown. In the microcirculation evidence suggests that CGRP can act to increase blood flow directly via a $CGRP_1$-like receptor, that is inhibited by the CGRP antagonist $CGRP_{8-37}$ (Han *et al.*, 1990; Hughes & Brain, 1991). However, little is known about CGRP receptors in rat skin where $CGRP_{8-37}$ has pro-inflammatory effects and appears not to inhibit CGRP-induced activity, thus negating its use for mechanistic studies (Brain *et al.*, 1992). It is possible that CGRP exerts its anti-inflammatory effects via $CGRP_1$ receptor/cyclic AMP-dependent mechanisms; anti-inflammatory effects of PGE_1 have been suggested to occur via cyclic AMP-dependent mechanisms (Raud *et al.*, 1988; 1989).

In conclusion and in support of our previous studies CGRP, as a consequence of its vasodilator activity, can act in a synergistic manner with mediators of increased microvascular permeability or neutrophil accumulation to promote the inflammatory response (Brain *et al.*, 1985; Buckley *et al.*, 1991a,b; Cambridge & Brain, 1992). Evidence also suggests that CGRP pretreatment can lead to an inhibition of inflammatory oedema which is clearly observed in the hamster cheek pouch (Raud *et al.*, 1991). In the rat, the oedema potentiating effects of CGRP appear to depend on the mediators involved in inducing oedema. Clearly when SP is used to induce oedema in the skin of rats both pretreatment and co-injection of CGRP results in a significant potentiation of SP-induced oedema. However when 5-HT is used, CGRP has little modulatory effect when given as a co-injection although when given as a pretreatment a potentiation of oedema can be observed in the skin. In our experiments, such a pretreatment in the rat paw results in no significant modulatory effect, whereas Raud *et al.* (1991) have demonstrated that pretreatment with CGRP in the rat hind paw results in an anti-inflammatory action against 5-HT-induced oedema. Hence in the rat the effects of CGRP appear to be variable, since CGRP exhibited no significant inhibitory effect on rat paw or skin oedema in this study, but has previously been shown to inhibit rat paw oedema (Raud *et al.*, 1991).

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References

ANDREWS, P.A., HELME, R.D. & THOMAS, K.L. (1989). NK-1 receptor mediation of neurogenic plasma extravasation in rat skin. *Br. J. Pharmacol.*, **97**, 1232–1238.

BRAIN, S.D., CAMBRIDGE, H., HUGHES, S.R. & WILSONCROFT, P. (1992). Evidence that calcitonin gene-related peptide contributes to inflammation in the skin and joint. *Proc. Natl. Acad. Sci. U.S.A.*, **657**, 412–419.

BRAIN, S.D. & WILLIAMS, T.J. (1985). Inflammatory oedema induced by synergism between calcitonin gene-related peptide (CGRP) and mediators of increased vascular permeability. *Br. J. Pharmacol.*, **86**, 855–860.

BRAIN, S.D. & WILLIAMS, T.J. (1989). Interactions between the tachykinins and calcitonin gene-related peptide lead to the modulation of oedema formation and blood flow in rat skin. *Br. J. Pharmacol.*, **97**, 77–82.

BRAIN, S.D., WILLIAMS, T.J., TIPPINS, J.R., MORRIS, H.R. & MACINTYRE, I. (1985). Calcitonin gene-related peptide is a potent vasodilator. *Nature*, **313**, 54–56.

BUCKLEY, T.L., BRAIN, S.D., COLLINS, P.D. & WILLIAMS, T.J. (1991a). Inflammatory edema induced by interactions between IL-1 and the neuropeptide calcitonin gene-related peptide. *J. Immunol.*, **146**, 3424–3430.

BUCKLEY, T.L., BRAIN, S.D., RAMPART, M. & WILLIAMS, T.J. (1991b). Time-dependent synergistic interactions between the vasodilator neuropeptide, calcitonin gene-related peptide (CGRP) and mediators of inflammation. *Br. J. Pharmacol.*, **103**, 1515–1519.

CAMBRIDGE, H. & BRAIN, S.D. (1992). Calcitonin gene-related peptide increases blood flow and potentiates plasma protein extravasation in the rat knee joint. *Br. J. Pharmacol.*, **106**, 746–750.

COLPAERT, F.C., DONNERER, J. & LEMBECK, F. (1983). Effects of capsaicin on inflammation and on the substance P content of nervous tissues in rats with adjuvant arthritis. *Life Sci.*, **32**, 1827–1834.

DEVILLIER, P., REGOLI, D., ASSERAF, A., DESCURS, B., MARSAC, J. & RENOUX, M. (1986). Histamine release and local responses of rat and human skin to substance P and other mammalian tachykinins. *Pharmacology*, **32**, 340–347.

FOREMAN, J.C., JORDAN, C.C., OEHME, P. & RENNER, H. (1983). Structure-activity relationships for some substance P-related peptides that cause wheal and flare reactions in human skin. *J. Physiol.*, **335**, 449–465.

GAMSE, R. & SARIA, A. (1985). Potentiation of tachykinin-induced plasma protein extravasation by calcitonin gene-related peptide. *Eur. J. Pharmacol.*, **114**, 61–66.

HAGERMARK, O., HOKFELT, T. & PERNOW, B. (1977). Flare and itch induced by substance P in human skin. *J. Invest. Derm.*, **71**, 233–235.

HAN, S.-P., NAES, L. & WESTFALL, T.C. (1990). Inhibition of periarterial nerve stimulation-induced vasodilatation of the mesenteric arterial bed by CGRP (8-37) and CGRP receptor desensitization. *Biochem. Biophys. Res. Commun.*, **168**, 786–791.

HOLZER, P. (1988). Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. *Neurosci.*, **24**, 739–768.

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

HUGHES, S.R. & BRAIN, S.D. (1991). A calcitonin gene-related peptide (CGRP) antagonist (CGRP8-37) inhibits microvascular response induced by CGRP and capsaicin in skin. *Br. J. Pharmacol.*, **104**, 738–742.

JANCSO, N., JANCSO-GABOR, A. & SZOLCSANYI, J. (1967). Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin. *Br. J. Pharmacol.*, **31**, 138–151.

LUNDBLAD, L., LUNDBERG, J.M., ANGGARD, A. & ZETTERSTROM, O. (1985). Capsaicin pretreatment inhibits the flare component of the cutaneous allergic reaction in man. *Eur. J. Pharmacol.*, **113**, 461–462.

MAGGI, C.A. & MELI, A. (1988). The sensory-efferent function of capsaicin-sensitive sensory neurons. *Gen. Pharmacol.*, **19**, 1–43.

MATSUSE, T., THOMSON, R.J., CHEN, X.-R., HASSAN, S. & SCHELLENBERG, R.R. (1991). Capsaicin inhibits airway hyperresponsiveness but not lipoxygenase activity or eosinophilia after repeated aerosolized antigen in guinea pigs. *Am. Rev. Respir. Dis.*, **144**, 368–372.

MCCUSKER, M.T., CHUNG, K.F., ROBERTS, N.M. & BARNES, P.J. (1989). Effect of topical capsaicin on the cutaneous responses to inflammatory mediators and to antigen in man. *J. Allergy Clin. Immunol.*, **83**, 1118–1124.

RAUD, J., DAHLEN, S.-E., SYDBOM, A., LINBOM, L. & HEDQVIST, P. (1988). Enhancement of acute allergic inflammation by indomethacin is reversed by prostaglandin E_2: apparent correlation with in vivo modulation of mediator release. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 2315–2319.

RAUD, J., LUNDEBERG, T., BRODDA-JANSEN, G., THEODORSSON, E. & HEDQVIST, P. (1991). Potent anti-inflammatory action of calcitonin gene-related peptide. *Biochem. Biophys. Res. Commun.*, **180**, 1429–1435.

RAUD, J., SYDBOM, A., DAHLEN, S.-E. & HEDQVIST, P. (1989). Prostaglandin E_2 prevents diclofenac-induced enhancement of histamine release and inflammation evoked by in vivo challenge with compound 48/80 in the hamster cheek pouch. *Agents Action*, **28**, 108–114.

RAYCHAUDHURI, A., COLOMBO, C., PASTOR, G., WONG, M. & JENG, A.Y. (1991). Effect of capsaicin on carrageenan-induced inflammation in rat pleurisy and exudate substance P level. *Agents Action*, **34**, 251–253.

SEDGWICK, A.D. & WILLOUGHBY, D.A. (1989). Animal models for testing drugs on inflammatory and hypersensitivity reactions. In *Textbook of Immunopharmacology*. ed. Dale, M.M. & Foreman, J.C. pp. 253–261. London: Blackwell Scientific Publications.

SPINA, D., MCKENNIFF, M.G., COYLE, A.J., SEEDS, E.A.M., TRAMONTANA, M., PERRETTI, F., MANZINI, S. & PAGE, C.P. (1991). Effect of capsaicin on PAF-induced bronchial hyperresponsiveness and pulmonary cell accumulation in the rabbit. *Br. J. Pharmacol.*, **103**, 1268–1274.

WILLIAMS, T.J. & PECK, M.J. (1977). Role of prostaglandin-mediated vasodilatation in inflammation. *Nature*, **270**, 530–532.

WINTER, C.A., RISLEY, E.A. & NUSS, G.W. (1963). Anti-inflammatory and antipyretic activities of indomethacin, 1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-indole-3-acetic acid. *J. Pharmacol. Exp. Ther.*, **141**, 369–376.

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The influence of the γ_{2L} subunit on the modulation of responses to $GABA_A$ receptor activation

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1 Whole-cell patch clamp recordings were made from L-cells transfected with 2 combinations of subunits of the $GABA_A$ receptor. Log concentration-response curves were constructed to γ-aminobutyric acid (GABA) on $\alpha_1,\beta_1,\gamma_{2L}$ containing cells and compared to those from α_1,β_1 containing cells. The effects of flunitrazepam, pentobarbitone and alphaxalone on the concentration-response relationships were also examined.

2 From the log concentration-response curves, GABA had a mean (± s.e.mean) $pEC_{50} = 5.2 \pm 0.09$ and slope factor = 1.7 ± 0.02 on $\alpha_1,\beta_1,\gamma_{2L}$ cells which were significantly different from the values obtained from α_1,β_1 cells where the $pEC_{50} = 5.6 \pm 0.02$ and the slope = 1.5 ± 0.02.

3 Flunitrazepam produced a parallel leftward shift of GABA concentration-response curves on $\alpha_1,\beta_1,\gamma_{2L}$ cells. The EC_{50} for flunitrazepam = 6.3 ± 2.7 nM. No increase in the maxima of the GABA concentration-response curves was found in the presence of flunitrazepam. Flunitrazepam did not potentiate responses from α_1,β_1 cells.

4 The log concentration-response curves from both populations of cells were shifted to the left by equal amounts by pentobarbitone. A significant increase in the maximal response to GABA was also produced by pentobarbitone. This occurred at lower concentrations of pentobarbitone on α_1,β_1 cells.

5 Alphaxalone produced leftward shifts of GABA log concentration-response curves of similar magnitudes in both populations of cells. Significant increases in the maxima were found at 100 nM in α_1,β_1 cells but not up to 1 μM in $\alpha_1,\beta_1,\gamma_{2L}$ cells.

6 These results provide further evidence of the modulatory role of the γ_{2L} subunit of the $GABA_A$ receptor containing α_1 and β_1 subunits. As well as influencing the apparent affinity of GABA and conferring benzodiazepine modulation, it also appeared to regulate the increase in maximal response produced in the presence of barbiturates and steroids. This latter effect may imply that barbiturates and steroids increase the channel open-state probability in the presence of GABA and that this effect is diminished by the presence of the γ_{2L} subunit.

Keywords: Concentration-response relationship; $GABA_A$ receptor; recombinant receptor; benzodiazepine; flunitrazepam; alphaxalone; pentobarbitone

Introduction

Potentiation of responses mediated by $GABA_A$ receptors has been observed with a number of compounds such as barbiturates (Nicoll, 1975), benzodiazepines (Choi *et al.*, 1977) and steroids (Harrison & Simmonds, 1984). These modulators selectively potentiate responses at this receptor, which has since been shown to be composed of a number of subunits that have been classified as members of α, β, γ etc. families (Schofield *et al.*, 1987; Pritchett *et al.*, 1989; see Luddens & Wisden, 1991 for review). Functional expression of γ-aminobutyric acid (GABA) receptors has provided a powerful tool to assess the pharmacological consequences and likely physiological occurrences of known combinations of these subunits (Schofield *et al.*, 1987; Pritchett *et al.*, 1989; Malherbe *et al.*, 1990; Puia *et al.*, 1990; 1991; Knoflach *et al.*, 1992).

Much of the preceding work on the modulation of responses of recombinant GABA receptors has involved measuring the %-potentiation of the response to a single concentration of agonist. Potentiation of individual responses produces a leftward shift of log concentration-response curves that has been well documented in intact preparations and isolated neurones (Barker & Ransom, 1978; Simmonds, 1981; Little, 1984). However, modulation of $GABA_A$ receptors by benzodiazepines, barbiturates and steroids is thought to involve separate sites on the receptor complex and is probably brought about by different mechanisms. Therefore, to investigate the underlying causes of $GABA_A$ receptor potentiation we have examined the effects of flunitrazepam, pentobarbitone and alphaxalone on the properties of the GABA log concentration-response curves produced in clonal cell lines stably transfected with known combinations of subunits. Furthermore, to investigate the effects of the γ_{2L} subunit we have compared the modulation of responses to GABA in cells transfected with $\alpha_1,\beta_1,\gamma_{2L}$ subunits (Hadingham *et al.*, 1992; Horne *et al.*, 1992) with those transfected with only α_1,β_1 subunits.

Methods

Mouse fibroblasts were maintained and transfected as described previously (Whiting *et al.*, 1991; Hadingham *et al.*, 1992). Cell lines containing α_1,β_1 or $\alpha_1,\beta_1,\gamma_{2L}$ (Whiting *et al.*, 1990) were plated onto coverslips 5 days prior to recording and expression was induced with 100 nM dexamethasone. During electrophysiological recordings, cells were bathed in a solution comprising (mM): NaCl 137, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 5, D-glucose 11 and sucrose 13, the pH was adjusted to 7.2 with NaOH. Patch pipettes contained (mM): CsCl 130, $MgCl_2$ 1, HEPES 10 and EGTA 11. The pH was adjusted to 7.3 with HCl (~13 mM) and intracellular and extracellular solutions were approximately isotonic (~310

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mOsm). Membrane currents were monitored with a List LM-EPC7 amplifier at a holding potential of −20 mV. Series resistance was not monitored or compensated for, as we previously found no evidence to suggest that significant clamp errors occurred during experiments similar to those described here (Horne et al., 1992).

Log concentration-response curves were constructed to incremental concentrations of GABA applied rapidly from a double barrelled pipette (Horne et al., 1992). GABA was applied for 2–3 s with a 1 min wash period allowed before the next application to avoid any possible problems caused by desensitization at the higher concentrations. Responses were measured at peak height. Concentration-response curves were redetermined in the presence of modulator and both curves were fitted to the logistic equation:

$$y = R_{max}/(1 + (EC_{50}/x)^H),$$

where, R_{max} = maximal response, EC_{50} = concentration producing half maximal response, H = slope factor.

Concentration-ratios were calculated from the quotient of the EC_{50}s obtained from the fitted curves in the absence and presence of modulator.

All bulk materials were obtained from BDH or FSA. GABA, flunitrazepam, EGTA, HEPES and pentobarbitone Na were obtained from Sigma. Flunitrazepam and alphaxalone were initially dissolved in ethanol at 10 mM, EGTA was initially dissolved in the minimum required CsOH. All other compounds dissolved into the bathing medium. All values are given as mean ± s.e.mean, and statistical analyses were by two-tailed Student's paired t test unless otherwise stated.

Results

GABA concentration-response curves

$\alpha_1,\beta_1,\gamma_{2L}$ GABA concentration-response curves were well fitted by the logistic equation (Figure 1) as reported previously for this cell line (Horne et al., 1992). From the values taken from individual curves a mean $pEC_{50} = 5.2 \pm 0.09$ and a mean slope factor = 1.66 ± 0.019 ($n = 96$) were obtained. A poorer fit to the data was obtained when the concentration-response curves were compared to a model of binding to 5 identical, independent sites (Figure 1) which, in turn, was a better fit than assuming any lower number of binding sites. When a second concentration-response curve was constructed on 5 cells, although a consistent reduction in maxima was obtained, there was no significant difference between the maxima ($P = 0.46$) or pEC_{50} ($P = 0.25$) of the initial and repeated curve (Table 1).

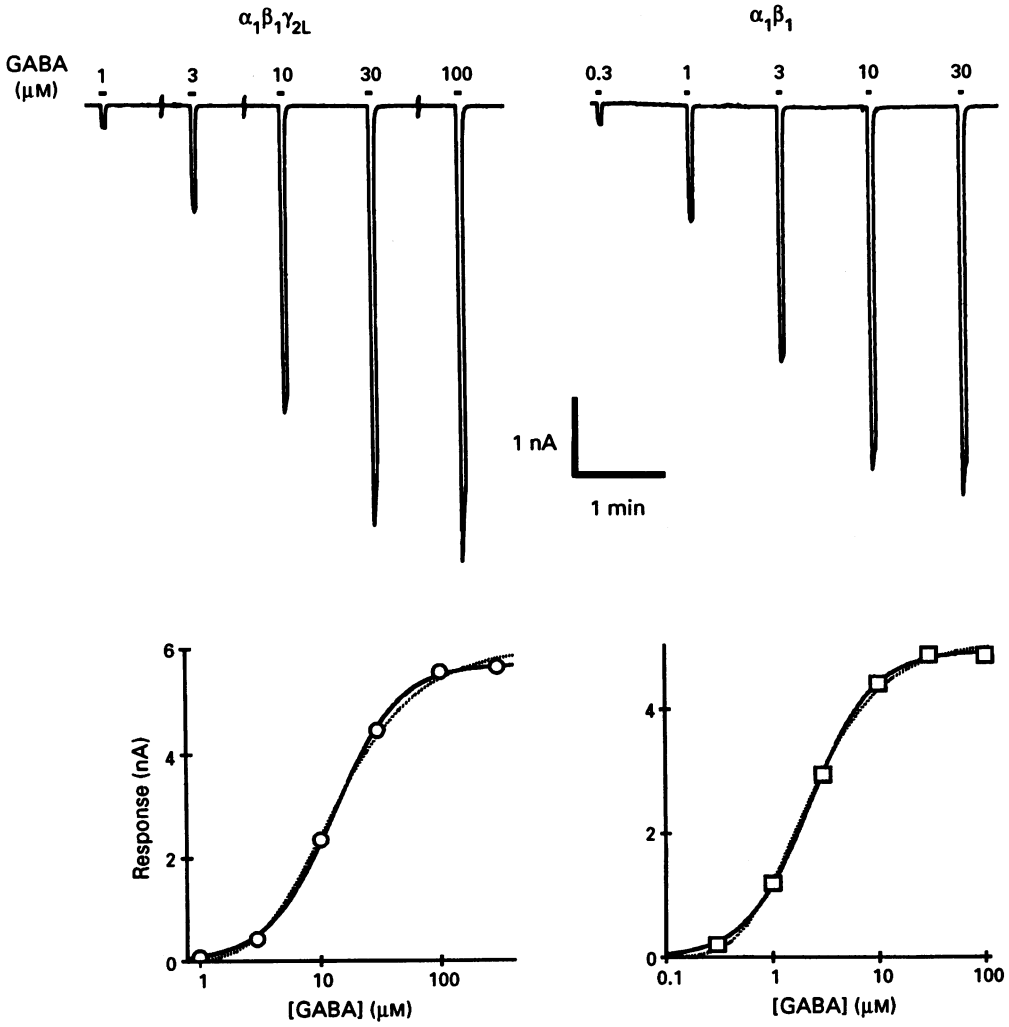


Figure 1 Log concentration-response curves to γ-aminobutyric acid (GABA). Top traces show continuous chart records of responses to incremental concentrations of GABA for receptors composed of $\alpha_1,\beta_1,\gamma_{2L}$ (left) and α_1,β_1 (right) subunits of the $GABA_A$ receptor. Responses were measured at peak height and plotted against log concentration of GABA as shown on the lower graphs which were taken from cells different from those illustrated above. These curves were well fitted by the logistic equation (see Methods, unbroken line). The broken line is an iterative fit to the data of the equation $y = R_{max}/(1 + EC_{50}/x)^5$ as a model of activation of 5 identical independent sites per receptor.

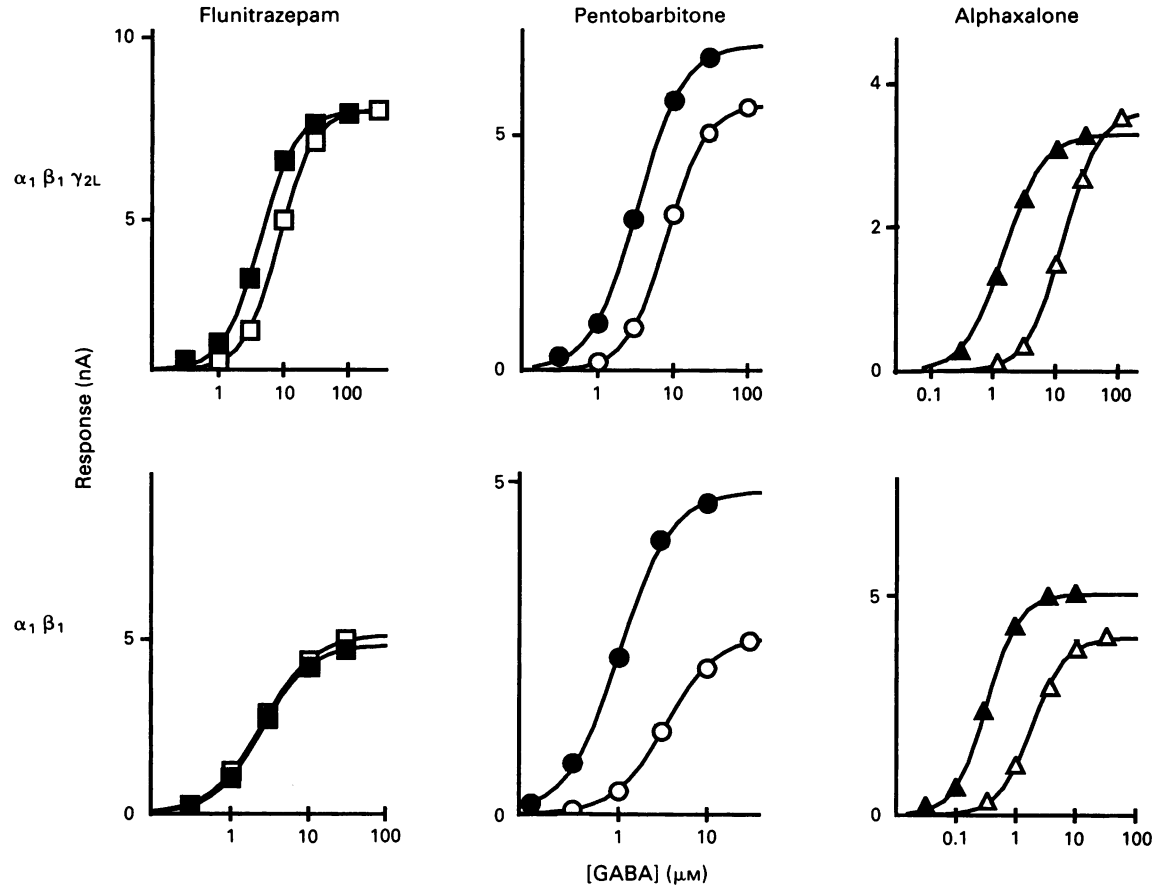


Figure 2 Modulation of γ-aminobutyric acid (GABA) concentration-response relationships. Each plot shows a control curve (open symbols) and one repeated in the presence of either 300 nM flunitrazepam, 30 μM pentobarbitone or 1 μM alphaxalone (filled symbols) for both combinations of subunits. All are fitted to the logistic equation. Each pair shows data taken from the same cell. Potentiation by flunitrazepam was only seen when the γ_{2L} subunit was present. With pentobarbitone and alphaxalone, potentiation was associated with an increase in maxima of the concentration-response curve on α_1,β_1 receptors. This increase in maxima was only seen with $\alpha_1,\beta_1,\gamma_{2L}$ in the presence of 30 μM pentobarbitone and not at all in alphaxalone up to 1 μM.

Table 1 Changes in the maxima of γ-aminobutyric acid (GABA) log concentration-response curves produced by flunitrazepam, pentobarbitone and alphaxalone

	$\alpha_1,\beta_1,\gamma_{2L}$		α_1,β_1	
Treatment	*Δmax*	n	*Δmax*	n
Control	−4.3 ± 4.2	5	−13.7 ± 2.9	7
Flunitrazepam				
300 nM	−5.2 ± 2.6	4	−13.5 ± 4.6	4
Pentobarbitone				
3 μM	−8.3 ± 2.7	3	+10.3 ± 4.3	4
10 μM	+0.9 ± 2.0	3	+35.1 ± 10.0*	3
30 μM	+12.6 ± 4.0*	4	+77.9 ± 18.8*	4
Alphaxalone				
1 nM	–	–	−7.7 ± 2.6	3
10 nM	+1.3 ± 5.0	4	−10.4 ± 4.7	3
30 nM	+0.2 ± 1.9	3	–	–
100 nM	−7.6 ± 5.1	3	+32.1 ± 15.3*	5
300 nM	−8.3 ± 1.1	3	–	–
1000 nM	−11.8 ± 1.8	3	+18.2 ± 7.9*	4

Log concentration-response curves were produced in cells transfected with the indicated combinations of subunits of the GABA$_A$ receptor. A second curve was constructed either in the absence (control) or presence of the stated concentration of modulator. Changes in the maxima (Δmax) are indicated as a mean percentage increase (+) or decrease (−) compared to the initial curve. Statistical analyses were performed on the untransformed maximal values.
*Statistically significant increase.

α_1,β_1 Log concentration-response curves for α_1,β_1 were also better fitted by the logistic equation than one describing up to 5 independent sites (Figure 1). A mean pEC_{50} of 5.6 ± 0.02 and a slope factor = 1.45 ± 0.019 (n = 58) were obtained. The probability of these mean values being identical to those from $\alpha_1,\beta_1,\gamma_{2L}$ cells was very low ($P<0.001$ for both EC_{50} and slope).

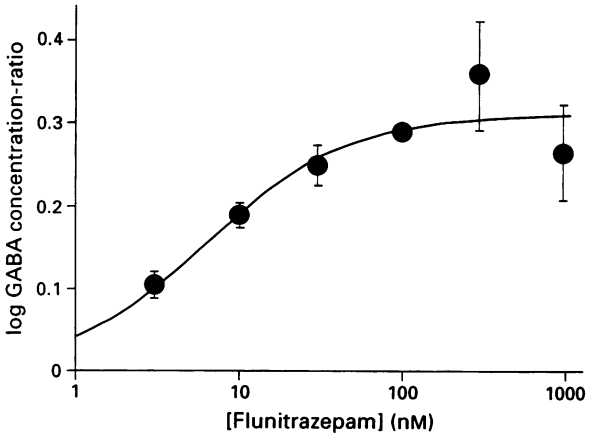


Figure 3 Log concentration-response curve to flunitrazepam. γ-Aminobutyric acid (GABA) concentration-ratios were calculated and plotted against the corresponding concentration of flunitrazepam. Each value is the mean ± s.e.mean (vertical bars) of 4 individual shifts.

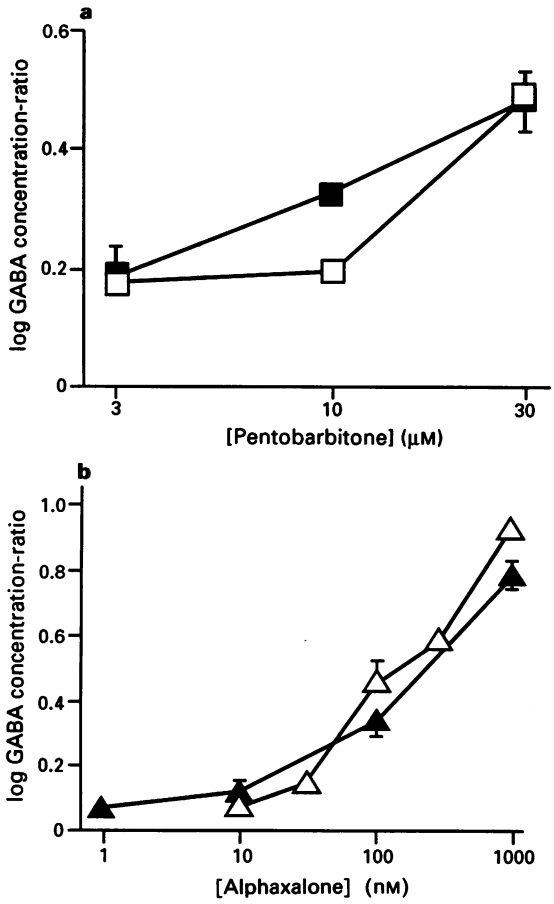


Figure 4 Log concentration-response curves to pentobarbitone and alphaxalone. γ-Aminobutyric acid (GABA) concentration-ratios were calculated in the presence of pentobarbitone (a) or alphaxalone (b) for both $\alpha_1,\beta_1,\gamma_{2L}$ (open symbols) and α_1,β_1 (filled symbols) combinations of subunits.

When a second concentration-response curve was produced on 7 of these cells a consistent and significantly different maximum ($P = 0.023$) was obtained (Table 1) but no significant difference in pEC_{50} was found between the initial and repeated curve.

Flunitrazepam

$\alpha_1,\beta_1,\gamma_{2L}$ Flunitrazepam (3–1000 nM) produced parallel shifts to the left of the log concentration-response curve to GABA (Figure 2) with no significant increase in maximum (Table 1). Individual log concentration-ratios were plotted against log flunitrazepam concentration and the logistic equation was fitted (Figure 3). From this plot, an $EC_{50} = 6.4 \pm 2.7$ nM, slope factor $= 0.99 \pm 0.49$ and maximum log concentration-ratio $= 0.31 \pm 0.03$ were obtained.

α_1,β_1 Flunitrazepam (300 nM) produced no significant change in either the EC_{50} or the maximum of the concentration-response curve to GABA (Figure 2, Table 1).

Pentobarbitone

$\alpha_1,\beta_1,\gamma_{2L}$ Pentobarbitone (3–30 μM) produced parallel shifts of the GABA concentration-response curves (Figure 2). GABA concentration-ratios were calculated at the level of the EC_{50} and plotted against log pentobarbitone concentration (Figure 4). Pentobarbitone (30 μM) produced a significant increase in the maximum ($P = 0.041$; Table 1) and a significant decrease in the slope factor (1.41 ± 0.08 compared to 1.64 ± 0.11 in controls: $P = 0.04$) of the GABA concentration-response curve. No significant difference in the maxima of the curves was produced by lower concentrations of pentobarbitone.

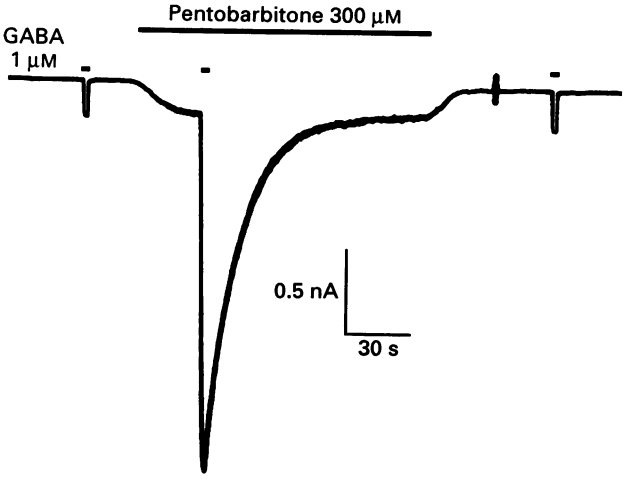


Figure 5 Direct activation of the γ-aminobutyric acid (GABA) receptor by pentobarbitone. The trace shows a response to GABA before, during and after the addition of 300 μM pentobarbitone from a cell transfected with α_1,β_1 subunits of the $GABA_A$ receptor.

At higher concentrations pentobarbitone has been shown to evoke an inward current in these cells (Horne *et al.*, 1992). Pentobarbitone (300 μM) evoked an inward current 0.2 ± 0.06 times that of 3 μM GABA and produced a potentiation $= 282 \pm 66.8\%$ ($n = 4$) of this response.

α_1,β_1 Leftward shifts of the log concentration-response curves were produced by pentobarbitone (Figure 2). Concentration-ratios were calculated from the GABA EC_{50}s and these are compared to those from $\alpha_1,\beta_1,\gamma_{2L}$ cells in Figure 4. These shifts were non-parallel, producing increases in the maxima of these curves (Table 1) that were significantly different at 10 μM ($P = 0.0001$) and 30 μM ($P = 0.033$). The slope factor was also significantly different in pentobarbitone (e.g. control $= 1.49 \pm 0.04$ compared to 1.32 ± 0.05 in 30 μM pentobarbitone: $P = 0.04$).

Direct effects were also observed with 300 μM pentobarbitone on these cells (Figure 5). A current $= 0.98 \pm 0.16$ times that to 1 μM GABA, a concentration equi-effective to 3 μM on $\alpha_1,\beta_1,\gamma_{2L}$ cells, was evoked and at this concentration the response to 1 μM GABA was potentiated by $737 \pm 261\%$ ($n = 4$).

Alphaxalone

$\alpha_1,\beta_1,\gamma_{2L}$ Alphaxalone (10–1000 nM) produced parallel shifts of the log concentration-response curve with no change in the maximum or slope factor (Figure 2, Table 1). As for the other modulators, log concentration-ratio was plotted against log alphaxalone concentration (Figure 4).

α_1,β_1 Alphaxalone produced non-parallel leftward shifts of the concentration-response curves producing significant increases in the maxima at 0.1 and 1 μM (Figure 2, Table 1). Concentration-ratios are plotted in Figure 4. No significant difference in the slope factor was produced.

Discussion and conclusions

The sigmoidal form of log concentration-response curves has long been successfully modelled assuming a dynamic reversible association with a finite number of receptor sites (Hill, 1909; see Mackay, 1977 for overview). This approach continues to provide strong evidence that the response is essentially

a function of the proportion of the available receptors that are occupied by the agonist, obtaining a maximum when, at least in voltage-clamped single cells, all receptors are activated. From this consideration the EC_{50} of an agonist should represent the concentration at which half the receptor population is activated and thus provide an estimate of the affinity of the agonist for the receptor. Whilst this estimate may, for a number of reasons, prove to be inaccurate (e.g. Colquhoun, 1987), we have assumed that the EC_{50} provided our best measure for comparison when changes in affinity occur.

Modelling the log concentration-response curves obtained from both combinations of receptors using the logistic equation provided good fits to the experimental data. As reported earlier for $\alpha_1,\beta_1,\gamma_{2L}$ receptors (Horne *et al.*, 1992) the concentration-response curves to α_1,β_1 receptors were also no better fitted by assuming up to 5 identical, independent binding sites (Colquhoun & Ogden, 1988; Bean, 1990). Thus, for both combinations the possibility remains that cooperativity of binding occurs within the receptor complex under our experimental conditions and, therefore, this property appears not to be dependent upon the γ_{2L} subunit. As with the remainder of this discussion our data provide no evidence as to whether or not any effect of the γ_{2L} subunit is dependent upon the 8 amino acid insert that produces the long variant (Whiting *et al.*, 1990).

The γ_{2L} subunit produced a significantly steeper slope factor when it was incorporated into the receptor complex. As suggested by Verdoorn *et al.* (1990) this may have resulted from the inclusion of an extra binding site for GABA within the complex. As those workers also recorded responses to GABA from receptors comprising each of the subunits alone, it seems improbable that the γ_{2L} subunit replaced another that does not have a binding site for GABA. An alternative explanation could be that the number of subunits per receptor was increased when the γ_{2L} was included, with this subunit acting as an insert to the α, β complex, although this would seem unlikely. Perhaps a more plausible explanation is that the γ_{2L} may increase the effectiveness of the proposed cooperative process. Whilst these possibilities are not easy to investigate by this approach, it does seem clear that the γ_{2L} decreased the potency of GABA at the receptor. Whether this represented a real decrease in affinity for the recognition site or merely resulted from the inclusion of an additional site is unclear.

Concentration-response curves proved to be reasonably reproducible when repeated on the same cell. A significantly different (lower) maximal response was found on the second curve in α_1,β_1 cells. When the γ_{2L} subunit was present this decrease was also a common but not significantly different observation. The pEC_{50}s were not significantly different for the control and repeated curve for both populations of cells. Thus, these findings probably reflect a slight 'run-down' of responses over the timecourse of the experiments but this did not appear to compromise the results obtained.

The concentration-response curves were shifted to the left when the GABA response was potentiated. In the case of the α_1,β_1 combination the shift was often non-parallel with an increase in the maximum response. Therefore, the EC_{50} was always used as a measure for comparison as outlined above. When the two receptor combinations were compared, there was little difference in the magnitude of the shift of the concentration-response curve produced by alphaxalone over the concentration-ranges tested (Puia *et al.*, 1990). Similarly both receptors were sensitive to pentobarbitone both for potentiation of GABA and direct receptor activation. There was, however, a difference in the magnitude of the potentiation produced by the 10 μM concentration of pentobarbitone. It is difficult to explain this finding. It may represent differences in the apparent affinity for pentobarbitone at the two receptors, although this would seem unlikely as concentrations immediately above and below 10 μM did not produce different effects. This was difficult to investigate further as higher concentrations of pentobarbitone directly activated the receptor. In contrast to alphaxalone, the slope factor of the GABA concentration-response curve was significantly different (lower) in the presence of pentobarbitone. This occurred whether the γ_{2L} subunit was present or not. Whilst it is not easy to interpret this change in slope factor it presumably emphasized that whilst the modulation of GABA by barbiturates and steroids has been found to be similar mechanistically, their loci of action may be different.

As reported by Pritchett *et al.* (1989), the inclusion of the γ_{2L} subunit was required before flunitrazepam modulated the GABA response. No significant effect was found on α_1,β_1 receptors whilst concentration-dependent shifts in $\alpha_1,\beta_1,\gamma_{2L}$ concentration-response curves were produced. From these shifts an EC_{50} was calculated for flunitrazepam of about 6 nM. Whilst this is slightly smaller than the value we obtained earlier (Horne *et al.*, 1992) for flunitrazepam ($\sim$18 nM) the values fall within each others 95% confidence limits and so are unlikely to represent a significant difference.

An increase in the maximum of the GABA concentration-response curve was produced by pentobarbitone on both receptors and by alphaxalone on α_1,β_1 receptors. Flunitrazepam, by contrast, produced shifts in $\alpha_1,\beta_1,\gamma_{2L}$ curves with no increase in maximum. It may be that the different mechanisms by which these compounds modulate GABA underlie the differences on the maxima. Thus, whilst flunitrazepam has been shown to increase the frequency of openings, both pentobarbitone and alphaxalone-like steroids have been shown to produce an increase in the mean open time of the activated channel (Study & Barker, 1981; Callachan *et al.*, 1987). That the increase in maxima produced by these compounds may be due to a reduction of desensitization seems unlikely as pentobarbitone increased rather than decreased the fade of the response seen with high concentrations of GABA. The increase in frequency produced by flunitrazepam would therefore presumably be limited to submaximal concentrations, and then only when the γ_{2L} subunit was present. These data are consistent with benzodiazepines exerting their effects through an increase in the affinity of GABA for its recognition sites.

If the increase in maxima produced by pentobarbitone and alphaxalone was caused by an increase in channel open time, then the γ_{2L} subunit appears to influence this effect as well. Of the concentrations of pentobarbitone tested, only the highest (30 μM) produced a significant increase in the maxima when the γ_{2L} subunit was present whereas 10 μM was effective, in this respect, on α_1,β_1 receptors. Similarly, whilst alphaxalone up to 1 μM produced no increase in the maxima of concentration-response curves in $\alpha_1,\beta_1,\gamma_{2L}$ cells, 100 nM was sufficient to produce this effect when the γ_{2L} subunit was omitted from the combination of subunits. These results may imply that either the γ_{2L} subunit limits the maximal increase in channel open time produced by the modulator or that, in the absence of a modulator, the probability of channel opening during agonist occupation is limited when this subunit is not present. Therefore, in the presence of a modulator there would be a greater opportunity for an increase in channel open time and, thus an increase in the maximal response. This would also indicate that the probability of channel activation following the binding of GABA is increased by the inclusion of the γ_{2L} subunit.

In summary, our data provide further evidence that the γ_{2L} subunit regulates the pharmacology of the GABA$_A$ receptor. As previously reported by Pritchett *et al.* (1989), this subunit was required before α_1,β_1 containing receptors were modulated by flunitrazepam. Although we obtained no evidence for a γ_{2L} regulation of the change in GABA affinity produced by pentobarbitone and alphaxalone, this subunit did alter the increase in the maximum potentiation of the GABA response produced by these modulators.

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References

BARKER, J.L. & RANSOM, B. (1978). Pentobarbitone pharmacology of mammalian central neurones grown in tissue culture. *J. Physiol.*, **280**, 355–372.

BEAN, B.P. (1990). ATP-activated channels in rat and bullfrog sensory neurones: concentration-dependence and kinetics. *J. Neurosci.*, **10**, 1–10.

CALLACHAN, H., COTTRELL, N.Y., HATHER, N.Y., LAMBERT, J.J., NOONEY, J.M. & PETERS, J.A. (1987). Modulation of the $GABA_A$ receptor by progesterone metabolites. *Proc. R. Soc. B*, **231**, 359–369.

CHOI, D.W., FARB, D.H. & FISHBACH, G.D. (1977). Chlordiazepoxide selectivity augments GABA action in spinal cord cell cultures. *Nature*, **269**, 342–344.

COLQUHOUN, D. (1987). Affinity, efficacy, and receptor classification: is the classical theory still useful? In *Perspectives on Receptor Classification*. ed. Black, J.W., Jenkinson, D.H. & Gerskowitch, V.P. pp. 103–114. New York: Alan R. Liss.

COLQUHOUN, D. & OGDEN, D.C. (1988). Activation of ion channels in the frog endplate by high concentrations of acetylcholine. *J. Physiol.*, **395**, 131–159.

HADINGHAM, K.L., HARNESS, P.C., MCKERNAN, R.M., QUIRK, K., LE BOURDELLES, B., HORNE, A.L., KEMP, J.A., BARNARD, E.A., RAGAN, C.I. & WHITING, P.J. (1992). Stable expression of mammalian type A γ-aminobutyric acid receptors in mouse cells: demonstration of functional assembly of benzodiazepine-responsive sites. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 6378–6382.

HARRISON, N.L. & SIMMONDS, M.A. (1984). Modulation of the GABA receptor complex by a steroid anaesthetic. *Brain Res.*, **323**, 287–292.

HILL, A.V. (1909). The mode of action of nicotine and curari, determined from the form of the contraction curve and the method of temperature coefficients. *J. Physiol.*, **39**, 361–373.

HORNE, A.L., HADINGHAM, K.L., MACAULAY, A.J., WHITING, P. & KEMP, J.A. (1992). The pharmacology of recombinant $GABA_A$ receptors containing bovine α_1, β_1, γ_{2L} subunits stably transfected into mouse fibroblast L-cells. *Br. J. Pharmacol.*, **107**, 732–737.

KNOFLACH, F., BACKUS, K.H., GILLER, T., MALHERBE, P., FLIMLIN, P., MOHLER, H. & TRUBE, G. (1992). Pharmacological and electrophysiological properties of recombinant $GABA_A$ receptors comprising the α_3,β_1 and γ_2 subunits. *Eur. J. Neurosci.*, **4**, 1–9.

LITTLE, H.J. (1984). The effects of benzodiazepine agonists inverse agonists and Ro15-1788 on the responses of the superior cervical ganglion to GABA *in vitro*. *Br. J. Pharmacol.*, **83**, 57–68.

LUDDENS, H. & WISDEN, W. (1991). Function and pharmacology of multiple $GABA_A$ receptors. *Trends Pharmacol. Sci.*, **12**, 49–51.

MACKAY, D. (1977). A critical survey of receptor theories of drug action. In *Kinetics of Drug Action*. ed. van Rossum, J.M. Berlin: Springer-Verlag.

MALHERBE, P., SIGEL, E., BAUR, R., PERSOHN, E., RICHARDS, J.G. & MOHLER, H. (1990). Functional characteristics and sites of gene expression of the α_1,β_1,γ_2-isoform of the rat $GABA_A$ receptor. *J. Neurosci.*, **10**, 2330–2337.

NICOLL, R.A. (1975). Pentobarbital: action on frog motoneurons. *Brain Res.*, **96**, 119–123.

PRITCHETT, D.B., SONTHEIMER, H., SHIVERS, B.D., YMER, S., KETTENMANN, H., SCHOFIELD, P.R. & SEEBURG, P.H. (1989). Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. *Nature*, **338**, 582–585.

PUIA, G., SANTI, M., VICINI, S., PRITCHETT, D.B., PURDY, R.H., PAUL, S.M., SEEBURG, P.H. & COSTA, E. (1990). Neurosteroids act on recombinant human $GABA_A$ receptors. *Neuron.*, **4**, 759–765.

PUIA, G., VICINI, S., SEEBURG, P.H. & COSTA, E. (1991). Influence of recombinant γ-aminobutyric acid-$_A$ receptor subunit composition on the action of allosteric modulators of γ-aminobutyric acid gated Cl^- currents. *Mol. Pharmacol.*, **39**, 691–696.

SCHOFIELD, P.R., DARLISON, M.G., FUJITA, N., BURT, D.R., STEPHENSON, F.A., RODRIGUEZ, H., RHEE, L.M., RAMACHANDRAN, J., REALE, V., GLENCORSE, T.A., SEEBURG, P.H. & BARNARD, E.A. (1987). Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor super-family. *Nature*, **328**, 221–227.

SIMMONDS, M.A. (1981). Distinction between the effects of barbiturates, benzodiazepines and phenytoin on responses to γ-aminobutyric acid receptor activation and antagonism by bicuculline and picrotoxin. *Br. J. Pharmacol.*, **73**, 739–747.

STUDY, R.E. & BARKER, J.L. (1981). Diazepam and (−)pentobarbital: fluctuation analysis reveals different mechanisms for potentiation of γ-aminobutyric acid responses in cultured central neurons. *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 7180–7184.

VERDOORN, T.A., DRAGUHN, A., YMER, S., SEEBURG, P.H. & SAKMANN, B. (1990). Functional properties of recombinant rat $GABA_A$ receptors depend upon subunit composition. *Neuron*, **4**, 911–928.

WHITING, P., MCKERNAN, R.M. & IVERSEN, L.L. (1990). Another mechanism for creating diversity in γ-aminobutyrate type A receptors: RNA splicing directs expression of two forms of the γ2 subunit, one of which contains a protein kinase C phosphorylation site. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 9966–9970.

WHITING, P., SCHOEPFER, R., LINDSTROM, J. & PRIESTLEY, T. (1991). Structural and pharmacological characterization of the major brain nicotinic acetylcholine receptor subtype stably expressed in mouse fibroblasts. *Mol. Pharmacol.*, **40**, 463–472.

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Reversal by suramin of neuromuscular block produced by pancuronium in the anaesthetized rat

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1 Rats were anaesthetized with sodium pentobarbitone and maximal twitches of a tibialis anterior muscle were evoked by stimulation of the motor nerve.

2 Suramin, injected intravenously in a series of cumulative bolus doses, each 15 mg kg^{-1}, completely reversed a 90% depression of twitches maintained by a continuous intravenous infusion of pancuronium. The cumulated dose necessary to restore twitches to 50% of their control amplitude was 35 mg kg^{-1}. Suramin did not modify a similar degree of block produced by suxamethonium, nor did it affect the amplitude of control maximal twitches, even in cumulative doses up to 150 mg kg^{-1}.

3 The effects of bolus doses of suramin (85 mg kg^{-1}), neostigmine (0.03 mg kg^{-1}) and 4-aminopyridine (1.2 mg kg^{-1}), calculated to restore pancuronium-blocked twitches to 95% of control amplitude, were compared. Suramin produced the most rapid reversal (1.1 ± 0.5 min), but its duration of action was the shortest (9.4 ± 1.6 min). Suramin was without effect on heart rate or blood pressure in the doses used.

4 The results showed that suramin reversed neuromuscular block produced by nondepolarizing blocking drug, pancuronium, but was without effect on a block produced by the depolarizing blocking drug, suxamethonium. Its short duration of action suggests that suramin would probably not be of value clinically as a reversal agent. However, it is possible that it might serve as a starter compound for the synthesis and development of a new class of reversal agents for use in anaesthetic practice.

Keywords: 4-Aminopyridine; neostigmine; neuromuscular blockade reversal agents; pancuronium; suxamethonium, suramin

Introduction

Suramin is a dye stuff with trypanocidal activity, and as such has been in clinical use against African sleeping sickness since 1920. The drug has been shown to be a reversible competitive antagonist of P_2-purinoceptors (Dunn & Blakely, 1988; Den Hertog *et al.*, 1989; Leff *et al.*, 1990) and of nucleotide receptors of skeletal myotubes (Henning *et al.*, 1992a). Recently we have shown suramin to antagonize the neuromuscular effects of a number of non-depolarizing muscle relaxants *in vitro* (Henning *et al.*, 1992b). In that study we also observed that the mechanism of the antagonistic action of suramin is different from those of the cholinesterase inhibitors and 4-aminopyridine (4-AP) and that it is primarily due to a postsynaptic effect. The potential clinical relevance of the above observations prompted us to undertake this study with the object of investigating the ability of suramin to reverse neuromuscular block produced by pancuronium in the anaesthetized animal.

Methods

After institutional approval, 18 male Wistar rats of 250–300 g body weight were studied. The animals were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) The trachea was intubated and artificial ventilation maintained with room air delivered by a Braun type respirator at a frequency of 90 breaths per min and a tidal volume of 20 ml kg^{-1}. Arterial pressure was monitored (Statham P23Db transducer) via a cannula inserted into the carotid artery. The mean arterial pressure was maintained greater than 100 mmHg throughout the experiments by infusion of a NaCl/glucose solution (0.45%/2.5% w/v) at a rate of 2.5 ml kg^{-1} h^{-1}. Both external jugular veins were cannulated and used either for the injection of the test drugs or administration of sodium pentobarbitone 24 mg kg^{-1} h^{-1} for the maintenance of anaesthesia.

Maximal isometric twitches of the left tibialis anterior muscle were evoked by supramaximal square wave stimuli of 0.2 ms duration applied to the sciatic nerve at a frequency of 0.1 Hz. The tendon of insertion was attached to a force displacement transducer (Maryland Instruments Ltd.) and the twitches were recorded on a polygraph (Astro-Med). Rectal and muscle temperatures were maintained between 37 and 38°C.

Experimental procedure

In the first part of the study in control experiments ($n = 3$) the neuromuscular effects of suramin alone were investigated. Doses of 15 mg kg^{-1} suramin were administered repeatedly 2 min apart until a total dose of 150 mg kg^{-1} was reached and the effects on the indirectly evoked twitch tension were observed.

In the second dose-finding part of the study, the potency of suramin in reversing pancuronium block was determined. Pancuronium ($n = 3$) or suxamethonium ($n = 3$) was administered intravenously by continuous infusion to achieve and maintain 90% depression of twitch tension for at least 15 min. At this point (with the infusion still running) doses of suramin (15 mg kg^{-1}) were administered and the recovery of the twitch tension observed. Before the next incremental dose was given, the twitch response to the previous dose was allowed to stabilize as judged by recording of three consecutive twitches of equal tension. The administration of incremental doses was continued during pancuronium block until recovery of the twitched tension to greater than 95% of its control value was produced. In the absence of any antagonistic effects (i.e. in the case of suxamethonium block) 10 incremental doses of suramin were administered 2 min apart.

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In the third part of the study the antagonistic effects of suramin ($n = 3$) were compared with those of neostigmine ($n = 3$) and 4-AP ($n = 3$). In these experiments a steady state 90% depression of the twitch tension was induced and maintained with pancuronium as described above. The reversal agents were then given as intravenous bolus doses–suramin 80 mg kg^{-1}, 4-AP 1.2 mg kg^{-1} and neostigmine 0.03 mg kg^{-1}–at the time when the 90% depression of the twitch tension had been constant for at least 15 min. The time from antagonist administration to its peak effect (onset), the magnitude of the antagonism, and the time during which the twitch response was greater than 50% of control twice tension (duration) were determined. Only one dose of antagonist was studied in each animal.

Statistical analysis

Results in the text, figures and tables are presented as mean ± s.e.mean. Statistical analysis was by paired or unpaired Student's t-test for differences between individual drugs, $P < 0.05$ being regarded as significant. Mean arterial blood pressure was calculated as (2 × diastolic pressure + systolic pressure)/3.

Drugs

Pancuronium was obtained from Organon Teknika, Boxtel (NL), suramin from Bayer, Leverkusen (Germany) and suxamethonium from Sigma, St. Louis (U.S.A.). Neostigmine methylsulphate and 4-AP HCl were obtained from the Hospital Pharmacy, Groningen (NL).

Results

Suramin, when given alone up to a total dose of 150 mg kg^{-1}, had no effect on the amplitude of maximal twitches in the anaesthetized rat (Figure 1a).

The infusion rates required to maintain a steady state 90% depression of the twitch tension were 0.64 ± 0.1 mg kg^{-1} h^{-1} and 16.9 ± 1.7 mg kg^{-1} h^{-1} with pancuronium and suxamethonium, respectively. The steady state 90% block maintained with pancuronium was completely reversed after the administration of six incremental doses of suramin (Figure 1b). In contrast, the block maintained with suxamethonium remained unaltered, even after ten incremental doses of suramin (Figure 1c). The dose of suramin necessary to produce a 95% antagonism of the pancuronium depressed twitch tension derived from the log dose-effect curve was about 85 mg kg^{-1} (Figure 2).

Data regarding the comparative antagonistic properties of suramin, neostigmine and 4-AP are summarized in Table 1. There was no difference in the magnitude of the steady state pancuronium block nor in the magnitude of reversal after the antagonists between the three experimental groups. Consequently, the antagonistic effects of neostigmine, 4-AP and suramin are comparable after the doses employed in this study. The onset of suramin-induced reversal was significantly faster and its duration significantly shorter ($P < 0.01$) than those after neostigmine and 4-AP. 4-AP produced the slowest onset and longest duration of the reversal, whereas neostigmine occupied an intermediate position. Heart rate and blood pressure remained unchanged in all experiments after suramin (Figure 3). Neostigmine (0.03 mg kg^{-1}) produced a transiently elevated blood pressure of about 13%. 4-AP (1.2 mg kg^{-1}) caused sustained increases of about 23% of both heart rate and blood pressure (Figure 3).

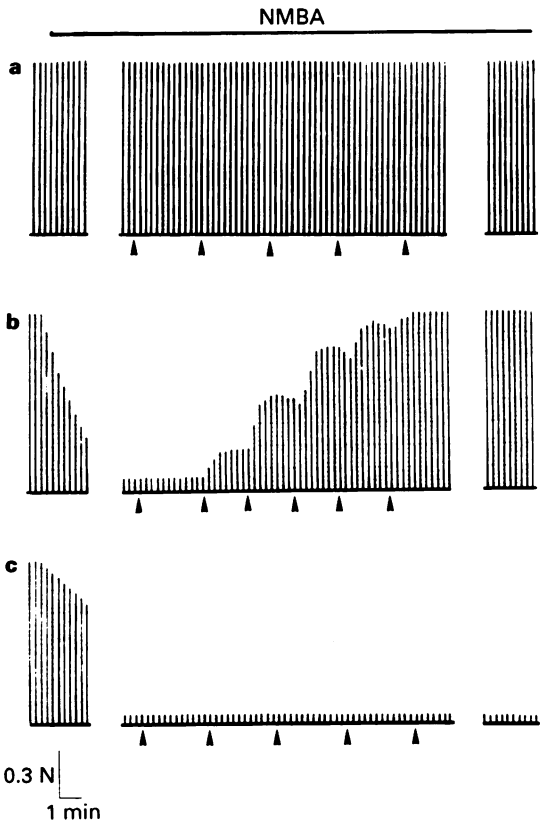


Figure 1 The action of suramin on the contraction of indirectly stimulated rat tibialis anterior muscle paralysed by different neuromuscular blocking agents (NMBA). Pen recorder traces show the initial twitch amplitude (left panel), at steady 90% blockade of twitch for 15 min, followed by doses of suramin (15 mg kg^{-1}; a; middle panel) and a total dose of 150 mg kg^{-1} (right panel). If suramin did not display an effect, incremental doses were applied every 2 min, otherwise after three consecutive twitches of equal tension. (a) The lack of effect of suramin on a control preparation. (b) The reversal action of suramin on a paralysis induced by pancuronium. Note the short duration of the incremental doses. (c) The lack of effect of suramin on a paralysis induced by suxamethonium.

Table 1 Reversal properties of suramin as compared to equipotent doses of neostigmine and 4-aminopyridine (4-AP)

	Neostigmine ($n = 3$)	*4-AP* ($n = 3$)	*Suramin* ($n = 3$)
Dose (mg kg^{-1})	0.03	1.2	85
Initial block (%)	93 ± 4	92 ± 6	92 ± 3
Antagonism (%)	98 ± 1	95 ± 4	98 ± 1
Onset time (min)	2.0 ± 0.2[a]	6.8 ± 1.1[a,b]	1.1 ± 0.5[b]
Duration 50% (min)	19.0 ± 3.0[c]	42.1 ± 6.0[c]	9.4 ± 1.6[c]

A bolus dose of the reversal agent was applied during an infusion of pancuronium producing a steady 90% block for 15 min. The antagonism is expressed as the maximum percentage of the initial twitch height. Onset was calculated as the time from antagonist administration to peak effect and duration as the time during which the twitch response was greater than 50% of control twitch tension

[a,b,c]Significant differences between similarly marked groups, $P < 0.01$

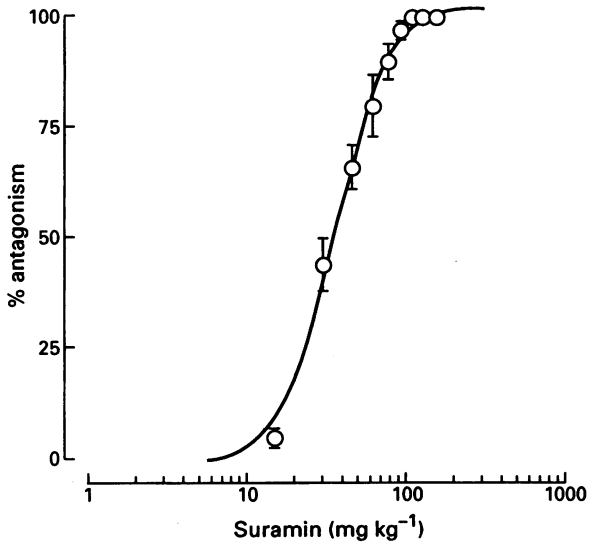


Figure 2 Log dose-effect curve of the reversal effect of suramin on the paralysis maintained by the non-depolarizing relaxant pancuronium. Increments of suramin (15 mg kg^{-1}) were added after three consecutive twitches of equal tension in rat tibialis anterior muscle preparation after a steady block (90%) had been obtained for 15 min. The antagonism (± s.e.mean, vertical bars) is expressed as percentage of the difference between the initial twitch height and the twitch at steady block ($n = 3$). The data were fitted to the logistic function $E = a[D]^b/([X]^b + [D]^b)$, in which a, [X] and b are the asymptote, location and slope parameters, respectively.

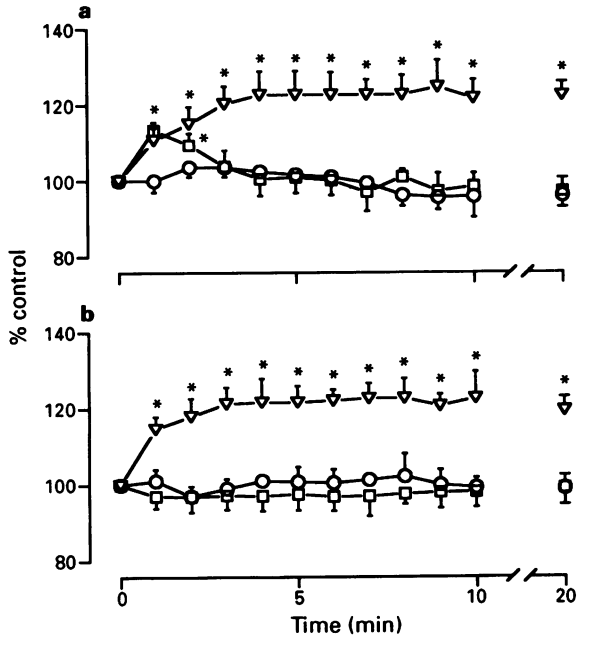


Figure 3 The cardiovascular effects of the reversal agents suramin, neostigmine and 4-aminopyridine (4-AP). Equipotent doses calculated to produce 95% restoration of twitch tension (suramin, ○, 85 mg kg^{-1}, $n = 3$; neostigmine, □, 0.03 mg kg^{-1}, $n = 3$; 4-AP ▽, 1.2 mg kg^{-1}, $n = 3$) were applied as bolus doses during a continuous infusion by pancuronium that produced a steady 90% block. (a) The effects of mean arterial blood pressure, (b) the effect on heart rate. Values (± s.e.mean, vertical bars) are expressed as the percentage of initial values. *$P < 0.01$ as compared to initial values.

Discussion

The results of the present study support our earlier findings *in vitro* (Henning *et al.*, 1992b) which showed that suramin is capable of reversing block produced by non-depolarizing neuromuscular blocking agents, but is without apparent effect on block produced by suxamethonium. Suramin had no effect on maximal twitches evoked by indirect stimulation in the absence of neuromuscular blocking drugs. In comparison with equipotent doses of neostigmine and 4-AP (Table 1) suramin possessed the shortest onset and shortest duration of action. Suramin was the least potent of the three antagonist drugs. On a weight basis, there was a 70 fold and 2800 fold difference between the antagonistic potencies of suramin and 4-AP, and suramin and neostigmine, respectively. Suramin is a large molecule, and on a molar basis the ratio of potencies of suramin and 4-AP, for example, reduces to 4.6:1.

More important, however, is the relationship between the antagonistic dose in the rat and the therapeutic trypanocidal dose recommendation for suramin in man (15 mg kg^{-1} weekly). In clinical trials involving patients with renal or adrenal adenocarcinoma the therapeutic plasma concentrations after repeated weekly doses each 12 mg kg^{-1}, ranged between 200 and 300 μg ml^{-1} (Stein *et al.*, 1989). Assuming a limited distribution of suramin to the extracellular fluid and taking account of its long elimination half-life of 50 days (Stein *et al.*, 1989), the above concentrations would correspond to those after a bolus dose of 60–90 mg kg^{-1}, which is close to the antagonistic doses of suramin used in the present study. Consequently, anaesthesiologists should be aware that the plasma concentrations of suramin in patients on treatment with this compound might be high enough to antagonize the effects of non-depolarizing agents during surgery.

Suramin is a P_2-purinoceptor antagonist on several cell types (Dunn & Blakely, 1988; Den Hertog *et al.*, 1989; Leff *et al.*, 1990; Henning *et al.*, 1992a). P_2-purinoceptors are also present both on vascular smooth muscle (Burnstock & Kennedy, 1986; Fleetwood & Gordon, 1987; Schlicker *et al.*, 1989; Leff *et al.*, 1990) and on cardiac cells (Fleetwood & Gordon, 1987; Pelleg *et al.*, 1990). However, no appreciable changes in the heart rate and mean blood pressure were observed after the administration of suramin in this study. Higher doses (130 mg kg^{-1}) of suramin than those used in this study produced a sustained increase of diastolic blood pressure (about 16%) in the pithed rat (Schlicker *et al.*, 1989). Consequently, the possibility of cardiovascular effects in man cannot be entirely excluded. In contrast with 4-AP (Bowman *et al.*, 1981; Biessels *et al.*, 1984) suramin appeared to be without central nervous system effects at antagonistic dose levels.

One may speculate that the reversal property of suramin on neuromuscular block by pancuronium may be related to its P_2-purinoceptor antagonism. The agonist, ATP, for P_2-purinoceptors is co-released with acetylcholine at the neuromuscular junction (Silinsky, 1975; Unsworth & Johnson, 1990). In rat isolated diaphragm, the reversal action of suramin is probably due to a postsynaptic effect (Henning *et al.*, 1992b). P_2-purinoceptors have been shown on skeletal myotubes (Kolb & Wakelam, 1983; Henning *et al.*, 1992a); however, their presence on innervated, adult skeletal muscle has not yet been shown. Therefore, whether the reversal effect is caused by the interaction of suramin with postsynaptic P_2-purinoceptors, the binding to nicotinic receptors as suggested previously (Henning *et al.*, 1992b), inhibition of ATP-dependent enzymes (Fortes *et al.*, 1973; Mahoney *et al.*, 1990), or by some other mechanism remains to be established.

Although a fast onset of antagonistic action is a desirable clinical property, a short duration of reversal is undesirable because of possible re-establishment of the neuromuscular blockade. Therefore it is unlikely that suramin itself would ever be used as a reversal agent in anaesthetic practice. However, suramin may serve as a model for the development of a novel type of reversal agent after the mechanism of its antagonistic effect at the neuromuscular junction has been completely elucidated.

References

BIESSELS, P.T.M., AGOSTON, S. & HORN, A. (1984). Comparison of the pharmacological actions of some new 4-aminopyridine deriyatives. *Eur. J. Pharmacol.*, **106**, 319–325.

BOWMAN, W.C., MARSHALL, R.J., RODGER, I.W. & SAVAGE, A.O. (1981). Actions of 4-aminopyridine on the cardiovascular systems of anaesthetized cats and dogs. *Br. J. Anaesth.*, **53**, 555–565.

BURNSTOCK, G. & KENNEDY, C. (1986). A dual function for adenosine-5'-triphosphate in the regulation of vascular tone. *Cir. Res.*, **58**, 319–330.

DEN HERTOG, A., NELEMANS, A. & VAN DEN AKKER, J. (1989). The inhibitory action of suramin on the P_2-purinoceptor response in smooth muscle cells of guinea-pig taenia caeci. *Eur. J. Pharmacol.*, **166**, 531–534.

DUNN, P.M. & BLAKELY, A.G. (1988). Suramin: a reversible P_2-purinoceptor antagonist in mouse vas deferens. *Br. J. Pharmacol.*, **93**, 243–245.

FLEETWOOD, G. & GORDON, J.L. (1987). Purinoceptors in the rat heart. *Br. J. Pharmacol.*, **90**, 219–227.

FORTES, P.A.G., ELLORY, J.C. & LEW, V.L. (1973). Suramin: a potent ATPase inhibitor which interacts on the inside surface of the sodium pump. *Biochem. Biophys. Acta.*, **318**, 262–272.

HENNING, R.H., NELEMANS, S.A., VAN DEN AKKER, J. & DEN HERTOG, A. (1992a). The nucleotide receptors on mouse C2C12 myotubes. *Br. J. Pharmacol.*, **106**, 853–858.

HENNING, R.H., NELEMANS, S.A., SCAF, A.H.J., VAN EEKEREN, J., AGOSTON, S. & DEN HERTOG, A. (1992b). Suramin reverses non-depolarizing neuromuscular blockade in rat diaphragm. *Eur. J. Pharmacol.*, **216**, 73–79.

KOLB, H.A. & WAKELAM, M.J.O. (1983). Transmitter-like action of ATP on patched membranes of cultured myoblasts. *Nature*, **303**, 621–623.

LEFF, P., WOOD, B.E. & O'CONNOR, S.E. (1990). Suramin is a slowly-equilibrating but competitive antagonist at P_{2x}-receptors in the rabbit isolated ear artery. *Br. J. Pharmacol.*, **101**, 645–649.

MAHONEY, C.W., AZZI, A. & HUANG, K.P. (1990). Effect of suramin, an anti-human immunodeficiency virus reserve transcriptase agent, on protein kinase C. *J. Biol. Chem.*, **265**, 5424–5428.

PELLEG, A., HURT, C.M. & MICHELSON, E.L. (1990). Cardiac effects of adenosine and ATP. *Ann. N.Y. Acad. Sci.*, **603**, 19–31.

SCHLICKER, E., URBANEK, E. & GÖTHERT, M. (1989). ATP, α,β-methylene ATP and suramin as tools for characterization of vascular P_{2x} receptors in the pithed rat. *J. Auton. Pharmacol.*, **9**, 371–380.

SILINSKY, E.M. (1975). On the association between transmitter secretion and the release of adenine nucleotides from mammalian motor nerve terminals. *J. Physiol.*, **247**, 145–162.

STEIN, C.A., LAROCCA, R.V., THOMAS, R., MCATEE, N. & MYERS, C.E. (1989). Suramin: an anticancer drug with a unique mechanism of action. *J. Clin. Oncology*, **7**, 499–508.

UNSWORTH, C.D. & JOHNSON, R.G. (1990). ATP compartmentation in neuroendocrine secretory vesicles. *Ann. N.Y. Acad. Sci.*, **603**, 353–363.

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Effects of propafenone on calcium current in guinea-pig ventricular myocytes

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1 The modulation of L-type voltage-sensitive calcium channels in guinea-pig isolated ventricular myocytes by propafenone was examined by the whole cell voltage-clamp technique.

2 Propafenone, $10^{-7}-5\times10^{-5}$ M, produced a concentration-dependent inhibition of Ca current (I_{Ca}) without any significant change in the current-voltage relation. Half-blocking concentration (IC_{50}) of propafenone for the peak I_{Ca} at +10 mV was 5×10^{-6} M.

3 The voltage-dependence of I_{Ca} inactivation was shifted in the hyperpolarizing direction in the presence of 5×10^{-6} M propafenone.

4 A frequency-dependent relative block by propafenone was observed after repetitive depolarizing test pulses at a frequency of 0.5 and 1 Hz. The recovery of I_{Ca} from inactivation was prolonged by propafenone and the reactivation exhibited an additional exponential component attributed to the slow release from drug block of Ca channels.

5 These results suggest that propafenone, at therapeutic concentrations exhibits Ca channel blocking properties that may be involved in its antiarrhythmic mechanism.

Keywords: Propafenone; calcium current; patch-clamp; ventricular myocytes

Introduction

Propafenone hydrochloride is an antiarrhythmic drug widely used for the management of supraventricular and ventricular arrhythmias (Harron & Brogden, 1987; Funck-Brentano *et al.*, 1990). The drug has demonstrated a high affinity for binding to the inactivated state of the Na channel with a kinetic profile similar to flecainide and encainide (Kohlhardt *et al.*, 1983; Honjo *et al.*, 1989; Valenzuela *et al.*, 1989). In addition, propafenone has special properties that set it apart from the other class I antiarrhythmic agents. Thus, propafenone exhibits a consistent β-adrenoceptor blocking action *in vitro* (Hapke & Prigge, 1976; Delgado *et al.*, 1985; Kroemer *et al.*, 1989) and *in vivo* (Müller-Pelzer *et al.*, 1983) as well as possible calcium-channel blocking properties. As a matter of fact, propafenone exerts a clear negative inotropic effect (Dukes & Vaughan Williams, 1984; Delgado *et al.*, 1985) and at therapeutic concentrations depresses the slow action potentials elicited in fibres partially depolarized with K^+ (Ledda *et al.*, 1981; Delgado *et al.*, 1985). Moreover, using the voltage-clamp technique some authors have reported that propafenone also reduces the slow inward Ca current in rabbit sino-atrial cells (Satoh & Hashimoto, 1984) and ventricular cardiac muscle fibres (Kohlhardt, 1977). Furthermore, experiments on rat isolated aortae have demonstrated that propafenone inhibits Ca entry through voltage- and receptor-operated channels as well as Ca release from intracellular stores, which again suggests that propafenone may exert Ca channel blocking properties in vascular smooth muscle (Carron *et al.*, 1991).

All these results suggest that propafenone inhibits the slow inward current (I_{Ca}) in cardiac cells; however, detailed studies of the inhibitory effect of propafenone on I_{Ca} have not yet been performed in cardiac cells. Therefore, the present study was undertaken to elucidate the possible Ca channel blocking effect of propafenone by the use of single cardiac cells isolated from guinea-pig ventricles.

Our results show that propafenone blocks I_{Ca} with both concentration- and frequency-dependent characteristics, and that these effects are related to the high affinity of the drug for binding to the inactivated state of the channel.

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Methods

Cell isolation

Single ventricular myocytes of the guinea-pig heart were obtained by enzymatic dissociation following the procedure described by Isenberg & Klöckner (1982). Hearts were removed from previously heparinized (500 u, i.p.) guinea-pig weighing 400–600 g, and mounted in a Langendorff perfusion system for retrograde perfusion of the coronary vascular bed. The heart was perfused at 10 ml min^{-1} with the following oxygenated solutions at 37°C: (1) HEPES Tyrode solution for 4 min; (2) nominally calcium-free solution for 10 min; (3) nominally calcium-free solution containing 0.4 mg ml^{-1} collagenase (Type II, Worthington) for 10 min and (4) modified 'KB' or 'Kraftbrühe' medium (Isenberg & Klöckner, 1982) for another 10 min period. The hearts were removed from the Langendorff column, and the ventricles dissected and cut into small pieces which were placed in a beaker containing 'KB' solution and gently shaken to disperse the isolated cells. The resulting cell suspension was filtered through a 250 μm nylon mesh and centrifuged at low speed (20 *g*). Finally, the cell pellet was stored in 'KB' medium for 1–2 h. An aliquot of this cell suspension was placed in a recording chamber 0.5 ml in volume attached on the stage an inverted microscope (Olympus IMT-2) and after an additional period of 20 min, the bath superfusate was replaced with the Ca-containing HEPES-Tyrode solution. Experiments were performed at room temperature (22–26°C) on Ca-tolerant healthy ventricular myocytes identified by their rod-like, striated appearance.

Recording techniques

Voltage-clamp experiments were performed with the single pipette in the whole-cell configuration of the patch-clamp

technique (Hamill *et al.*, 1981) by using an Axopatch-1D patch-clamp system (Axon Instruments). Suction pipettes were made from 1.5–1.6 mm o.d. soft glass capillary tubing with a microprocessor-based patch pipette puller (P.80/PC, Sutter Instruments). The pipettes had a tip resistance of 1.5–3 Megaohms after backfilling with the experimental internal solution. During the experiments, membrane potential and current data were displayed on a storage oscilloscope (Tecktronix 2214). Series resistance was compensated after the seal was broken to provide the fastest capacity transient possible without ringing of the amplifier. With maximal current (I) not exceeding 1 nA, the voltage drop error resulting from the residual series resistance (Rs) estimated by I.Rs (Halliwell *et al.*, 1987), was less than 4 mV. Currents were filtered with an eight-pole low-pass Bessel filter at a cutoff frequency of 2 kHz, digitized, stored and analyzed on an compaq IBM AT 386/25 computer system using the pClamp software package version 5.1 (Axon Instruments, Burlingame, California, U.S.A.). Selected records were printed out with a laser printer (Hewlett Packard Laser Jet printer series II). The data were compensated for the liquid junction potential between internal and external solutions (about 3 mV). Each pulse protocol was applied at intervals longer than 30 s to avoid the frequency-dependent effect of propafenone.

Solutions and drugs

Composition of the HEPES-Tyrode solution was as follows (mM): NaCl 150, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHCO_3$ 5.8, NaH_2PO_4 0.4, glucose 5.5 and HEPES 5.0 (pH adjusted at 7.4 with NaOH), and the solution was equilibrated with 100% O_2. The nominally calcium-free solution had the same composition as that of HEPES-Tyrode without $CaCl_2$. The 'KB' medium containing (mM): KCl 30, KH_2PO_4 10, $MgCl_2$. $6H_2O$ 5, taurine 15, glutamic acid 70, ethylene glycol *bis*-(beta-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) 0.5, ATP 5 and KOH 30 (pH = 7.3). Potassium currents were eliminated by inclusion of Cs^+ in the pipette solution and by removal of K^+ in the external solution after the whole-cell configuration was achieved. In addition, 15–30 μM tetrodotoxin (TTX) was included in the external solution to block sodium current. The pipette internal solution consisted of (mM): CsCl 140, $MgCl_2$ 2, HEPES 10, $CaCl_2$ 1, EGTA 11, ATP 5 (pH adjusted to 7.2 with CsOH). By use of these solutions Ca currents were effectively isolated from Na and K currents. In some experiments neither Cs^+ nor TTX was used and the composition of the internal filling solution was as follows (mM): KCl 150, $MgCl_2$ 1.0, HEPES 5, EGTA 5, ATP 5 (pH 7.2 with KOH).

Propafenone hydrochloride (Knoll Ibérica) as a powder was initially dissolved in deionized water to make a stock solution (10^{-2}–10^{-3} M), and desired drug concentration was then obtained by adding a small amount of stock solution to the perfusate.

Statistics

Results are expressed as mean ± s.e.mean and Student's t test was used to determine the significance of differences between test and control values.

Results

Blocking action of propafenone on I_{Ca}

In order to determine the effects of propafenone on inward calcium current (I_{Ca}), ventricular cells were clamped at −40 mV to inactivate the sodium current and 150 ms depolarizing test pulses to +10 mV were applied at a basal rate of 0.03 Hz. The peak I_{Ca} was related to the zero current level. Figure 1a illustrates the time course of the onset of the effect of propafenone 5×10^{-6} M. Propafenone decreased the I_{Ca} within 1–2 min after beginning of the superfusion and its effects reached a steady state after 7 min. At this time the cell was perfused with drug-free solution which was followed by a progressive recovery of current toward control, so that after 5 min the peak I_{Ca} reached approximately 80% of the control values (619 pA vs 710 pA). The differences observed at this time may occur under control conditions due to the 'run-down' of I_{Ca} which contributes in part to mask the recovery from blockade during washing.

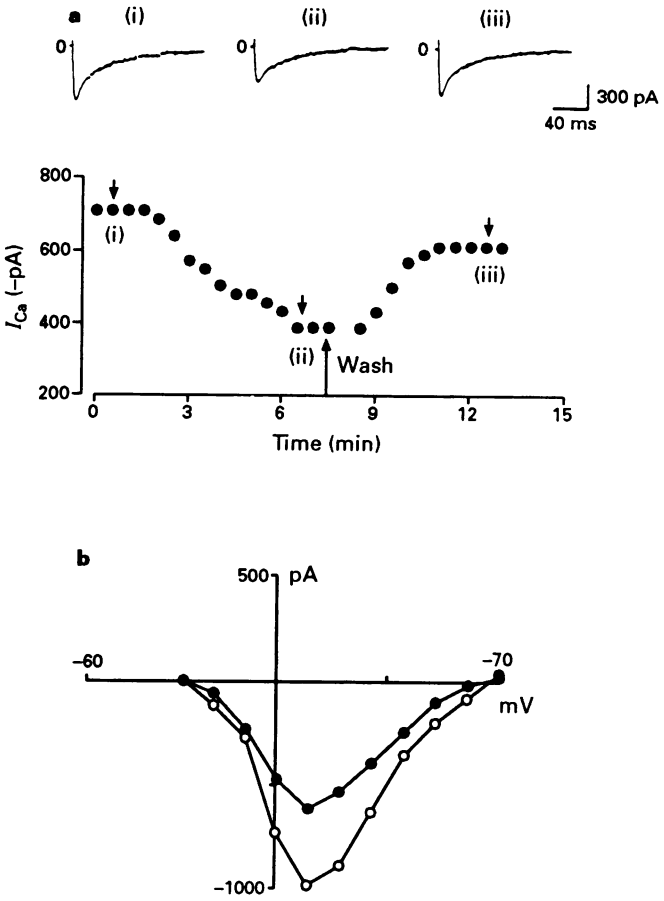


Figure 1 (a) Time course of the onset of the effects of propafenone (5×10^{-6} M) on the inward current (I_{Ca}) elicited by depolarization from −40 to +10 mV at 0.03 Hz. The external solution was zero K. Patch pipette internal solution contained CsCl. At the top of the figure, I_{Ca} values in control (trace i), in the presence of propafenone (trace ii) and after washing (trace iii) are shown. The time course of the drug effects is shown below after the application in (ii) and after washout in (iii). (b) I-V relationship of I_{Ca} obtained under control conditions (○) and 7 min after application of propafenone (5×10^{-6} M) (●). Depolarizing pulses of 150 ms in duration were applied from a holding potential of −40 mV to membrane potentials between −30 and +70 mV.

To determine the effects of propafenone on current-voltage relationships (I-V) of I_{Ca}, 150 ms depolarizing pulses were applied at a basal rate of 0.03 Hz from a holding potential of −40 mV to membrane potentials between −30 and +70 mV. Peak amplitude of I_{Ca} was taken as the difference between the peak of the inward current and the current at the end of 150 ms. Figure 1b shows a typical I-V relationship obtained under control conditions and 7 min after application of 5×10^{-6} M propafenone. In the absence of propafenone, the maximum peak inward current was obtained near +10 mV and the averaged peak amplitude of I_{Ca} at this potential was 813 ± 53 pA ($n = 30$). Propafenone decreased the peak I_{Ca} (from 978 pA to 606 pA) but it had no effect on the threshold potential (−30 mV), the potential at which I_{Ca} was maximum and the apparent reversal potential (about

+65 mV), i.e., it had no effect on the shape of the relationship. Similar effects were obtained at all concentrations of propafenone tested.

Figure 2 shows the concentration-response plot for a wide range of propafenone concentrations. In these experiments peak I_{Ca} was obtained on depolarizations from −40 mV to +10 mV at 0.03 Hz and the peak I_{Ca} in control was taken as 1.0 and compared with the peak I_{Ca} measured 7 min after the beginning of drug superfusion. The average of I_{Ca} decrease produced by propafenone, 10^{-6} M, 5×10^{-6} M, 10^{-5} M and 5×10^{-5} M was 30.4 ± 5.9% ($n = 6$), 52.8 ± 4.1% ($n = 13$), 62.5 ± 8.5% ($n = 3$) and 93.7 ± 5.7% ($n = 7$) respectively. Due to the very few experimental data points obtained, we could only approximate the IC_{50} value by interpolating the regression function obtained by least square analysis on experimental data with the relative I_{Ca} value = 0.5. These results led to an approximate IC_{50} value of 5×10^{-6} M. At concentrations up to 10^{-6} M the washout of the cell with drug-free solution resulted in recovery of current toward control, while at 5×10^{-5} M no recovery was observed. Because of the estimated IC_{50} value, in the following experiments we selected the concentration of 5×10^{-6} M to analyze the effects of propafenone on I_{Ca}.

Tonic and frequency-dependent effects of propafenone

A standardized protocol was used to evaluate the relative contribution of tonic blockade of I_{Ca} by propafenone. I_{Ca} was elicited by 150 ms depolarizing test pulses to +10 mV at 0.05 Hz from a holding potential of −40 mV. After steady-state amplitude of I_{Ca} was obtained, depolarizing pulses were discontinued, drug was applied and, after a 3 min quiescent period, depolarizing test pulses were resumed. Tonic blockade was assessed as the difference in peak I_{Ca} in the control and the first pulse after drug exposure. In the experiment shown in Figure 3, propafenone produced very little inhibition of Ca current of the first clamp pulse after the quiescent period (from 560 pA to 537 pA) but blockade increased with repeated depolarizations. In six cells the averaged tonic block induced by propafenone 5×10^{-6} M was 6.9 ± 1.6%. These results suggest that propafenone shows a weak affinity for binding to the resting state of the calcium channel.

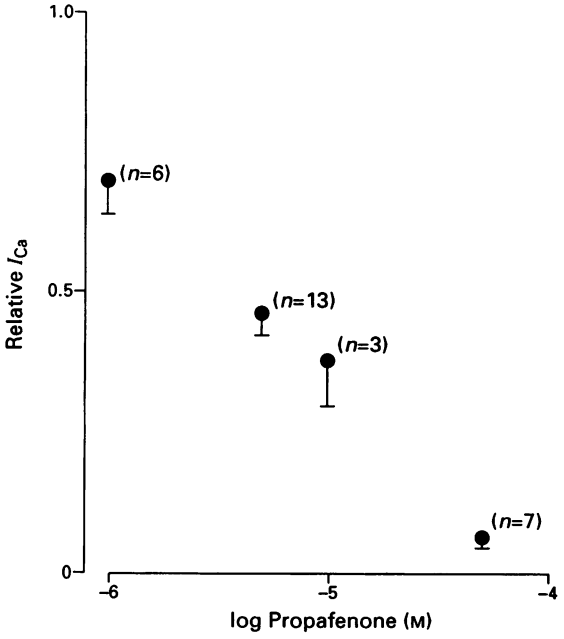


Figure 2 Dose-response plot of propafenone in single ventricular cells. I_{Ca} was elicited by depolarization from −40 to +10 mV at 0.03 Hz. The peak I_{Ca} in control was taken as 1.0 and compared with the peak I_{Ca} measured 7 min after beginning the drug superfusion. The symbols and vertical bars indicate the mean and s.e.mean, respectively. The number of observations for each concentration is indicated in parentheses. The approximate IC_{50} was 5×10^{-6} M.

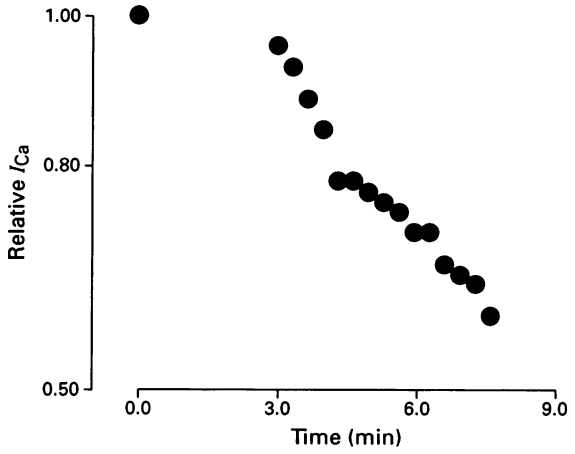


Figure 3 Relative contribution of tonic blockade of I_{Ca} by propafenone (5×10^{-6} M). I_{Ca} was elicited by 150 ms depolarizing test pulses to +10 mV at 0.05 Hz from a holding potential of −40 mV until the peak I_{Ca} reached steady-state. At that moment depolarizing pulses were discontinued and drug was applied. After 3 min of quiescence, depolarizing test pulses were resumed. Peak I_{Ca} in the control was taken as 1.0 and compared with the peak I_{Ca} recorded after 3 min of quiescence.

As previously described with other Ca channel blocking drugs (Ehara & Kaufmann, 1978; McDonald *et al.*, 1980; Trautwein *et al.*, 1981; Pelzer *et al.*, 1982; Lee & Tsien, 1983; Uehara & Hume, 1985) the inhibitory effect on I_{Ca} induced by propafenone was greatly enhanced by repetitive membrane depolarization, which indicated a large degree of frequency-dependent block. An example of such an experiment is illustrated in Figure 4, where the peak I_{Ca} associated with each pulse is plotted as a function of pulse number. In this cell 15 consecutive voltage-clamp pulses of 150 ms in duration, from a holding potential of −40 mV to a test potential of +10 mV were applied at 0.5 and 1 Hz (5 min between trains) in the absence and presence of 5×10^{-6} M propafenone. Frequency-dependent blockade was measured as the difference between peak I_{Ca} for the first and 15th pulses in control and after drug exposure. The results plotted in Figure 4 show that under control conditions the magnitude of the peak I_{Ca} declined after 15 pulses by 7% and 20% for 0.5 and 1 Hz, respectively, probably because of an incomplete recovery of I_{Ca} from inactivation. However, in the presence of 5×10^{-6} M propafenone there was a dramatic decline in the peak I_{Ca} during the train of 15 depolarizing pulses (57.1% and 82.8% for 0.5 and 1 Hz respectively). In 3 cells driven at 0.5 and 1 Hz propafenone decreased the I_{Ca} by 57.2 ± 5.5 ($P < 0.01$) and 82.4 ± 0.2 ($P < 0.01$), respectively.

Effect of 5×10^{-6} M propafenone on the voltage-dependence of I_{Ca} inactivation

Because the I_{Ca} blockade by Ca channel blocking drugs can be modulated by membrane potential (McDonald *et al.*, 1980; Hondeghem & Katzung, 1984; Sanguinetti & Kass, 1984), the interaction between propafenone block and calcium channel availability over a range of potentials was analyzed. For kinetic analysis of I_{Ca}, all experiments were performed in zero K^+ external solution and in the presence of 15 μM TTX and patch pipette contained CsCl. The voltage-dependence of I_{Ca} availability was examined by use of a double-pulse protocol: 2 s conditioning pulses of variable amplitude (−55 to +10 mV) which produced steady state

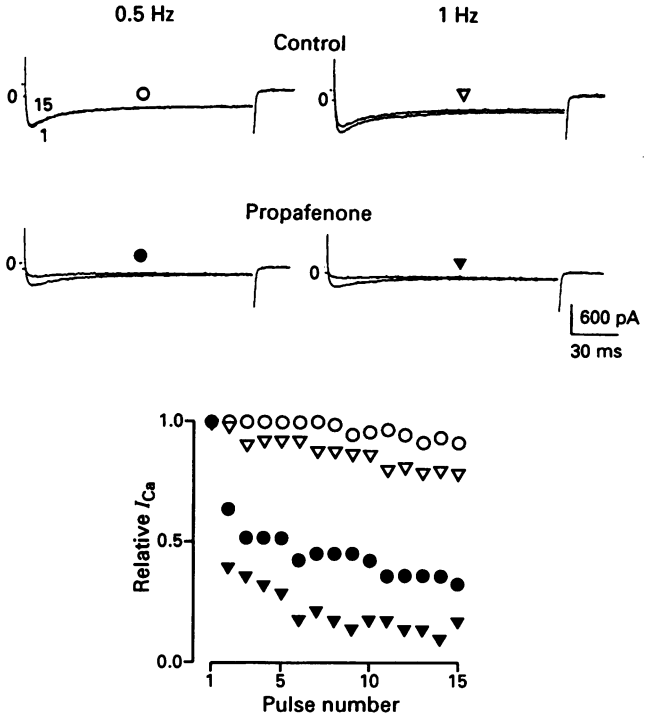


Figure 4 Effects of repetitive pulses at 0.5 and 1 Hz on the modulation of I_{Ca} by propafenone (5×10^{-6} M). Fifteen depolarizing pulses of 150 ms duration were applied to +10 mV from a holding potential of −40 mV at the frequency of 0.5 (circles) and 1 Hz (triangles) during control (open symbols) and after drug superfusion (closed symbols). Upper panel shows current records for pulse 1 and 15 with and without the drug. Bottom panel summarizes the results of plotting normalized I_{Ca} values for individual pulse numbers. The external solution was normal Tyrode.

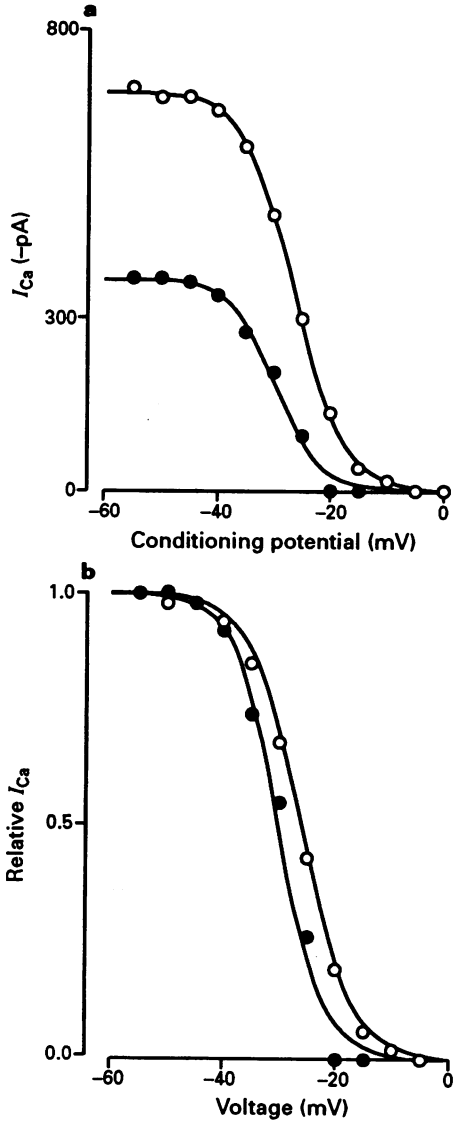


Figure 5 Effects of propafenone on the steady-state inactivation curve for I_{Ca}. The membrane potential was held at −40 mV. After the application of 2 s conditioning pulses to various levels ranging from −55 to +10 mV in 5 mV steps, the membrane was depolarized to +10 mV for 150 ms. (a) Shows the I_{Ca} values at individual conditioning potentials in control (○) and after applying propafenone (5×10^{-6} M) (●). In (b), the steady-state inactivation curve of I_{Ca} was constructed by normalizing the I_{Ca} values. The line drawn through the data points represents the equation:

$$I = I_{max}/(1 + \exp[(V_m - V_h)/K])$$

Where V_h is the potential of half inactivation, V_m is the conditioning potential and K is the slope factor of the curve. Under control conditions, $V_h = -26.0$ mV and $K = 4.5$. After exposure to propafenone (5×10^{-6} M), $V_h = -29.7$ mV and $K = 4.0$.

inactivation of I_{Ca} (Yatani *et al.*, 1986) under control conditions were followed by a 10 ms return to the holding potential and a 150 ms test pulse to +10 mV. Figure 5a shows a representative steady-state inactivation curve for I_{Ca}. The data were fitted with a nonlinear least-squares regression analysis to a Boltzmann distribution (Hodgkin & Huxley, 1952):

$$I = I_{max}/(1 + \exp[(V_m - V_h)/K])$$

where V_h is the potential of half inactivation, V_m is the prepulse potential and K is the slope factor of the curve. The curve was S-shaped, with conditioning pulses to more negative potentials being associated with greater I_{Ca} values. Propafenone (5×10^{-6} M) decreased I_{Ca} at −55 mV from 697 to 368 pA as expected from the concentration-response curve. Figure 5b shows the resultant steady-state inactivation curves obtained after individual tail currents (I_{test}) were normalized to the tail currents elicited by conditioning pulse to −55 mV (I_{max}) and plotted against the conditioning potential. In this individual cell, propafenone shifted the V_h value from −26.0 mV to −29.7 mV, while the slope factor was almost similar to the control value (4.5 vs 4.0, respectively). The mean control value for V_h in 5 cells was -25.3 ± 0.8 mV and in the presence of 5×10^{-6} M propafenone the curve shifted significantly in the hyperpolarizing direction with a mean value for V_h of -32.7 ± 1.7 mV ($P < 0.01$). It is worth noting that no change in the slope factor of the curve under the two conditions was observed (3.7 ± 0.3 during control and 3.9 ± 0.6, in the presence of propafenone, $n = 5$).

The results suggest that propafenone possesses a high affinity for the inactivated state of the Ca channel. However, it is of interest to analyze the possibility as to whether propafenone can block calcium channels in the open state. In the simplest case, in which the inactivated state is assumed to be absorbing, the open channel block can be measured by comparing the waveform of the currents during depolarizing voltage pulses before and after exposure to propafenone (Yantani *et al.*, 1986). Drugs that bind to the open state of the channel have been shown to increase the inactivation rate of current through the channels during depolarizing voltage pulses after applying the drug (Lee & Tsien, 1983; Colquhoun & Hawkes, 1983). Figure 6a shows the time course of I_{Ca} before and after the application of propafenone (5×10^{-6} M). The Ca currents recorded with a faster time scale (Figure 6b) revealed that propafenone not only reduced the

peak magnitude of the I_{Ca} but also hastens its decay. The relaxation process was well described by a double exponential fit. The fast time constant was similar in the presence and absence of propafenone (5.1 ± 0.3 and 5.5 ± 0.1 ms, respectively, $n = 6$) but the slow time constant was shortened by the drug (41.3 ± 1.6 ms in control and 26.9 ± 1.9 ms in the presence of propafenone, $n = 6$, $P < 0.001$).

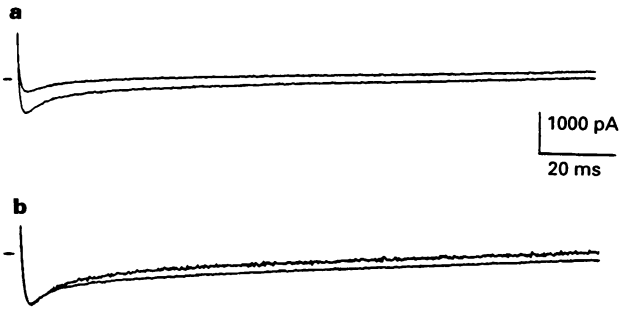


Figure 6 Effects of propafenone (5×10^{-6} M) on the rate of inactivation of I_{Ca} during depolarizing voltage pulse. (a) Current records in response to 100 ms clamp step to +10 mV from a holding of −40 mV at a rate of 0.03 Hz, control (○) and after applying propafenone (●) are superimposed. (b) The current traces before (○) and after applying propafenone (●) were scaled and were superimposed to compare the inactivation time course of I_{Ca}. The relaxation process was well described by a double exponential fit. The fast time constant was similar in the presence and absence of propafenone (5.1 vs 5.0 ms) but the slow time constant was shortened by propafenone (42.8 vs 25.6 ms).

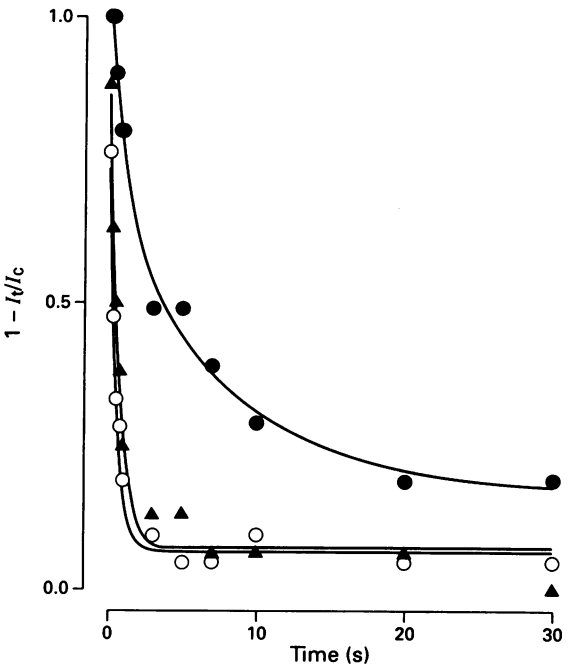


Figure 7 Effects of propafenone (5×10^{-6} M) on the recovery of I_{Ca}. A 2 s conditioning pulse which produced apparent inactivation at +10 mV was followed by a test pulse of 150 ms duration from a holding potential of −40 mV to +10 mV at various intervals (from 100 ms to 30 s). The peak current for each test pulse (I_t) was normalized to that for the control pulse applied 30 s before the conditioning pulse (I_c). The data were fitted by a least squares method of a sum of two exponentials: $y = A_1e^{-t/\tau 1} + A_2e^{-t/\tau 2}$, where y is the ratio of unrecovered current ($1-I_t/I_c$). In the absence of propafenone (○) the time course was best fitted by one exponential with a recovery time constant of 503 ms. With propafenone (●), the recovery of I_{Ca} was best fitted by two exponentials, 1100 ms and 8 s for the fast and slow components, respectively. After washout (▲), the time course of recovery was again fitted by a single exponential, with a recovery time constant similar to the control (658 ms).

Effects of propafenone on I_{Ca} reactivation

The frequency-dependent blocking action of I_{Ca} observed in the presence of the drug suggested that the recovery from inactivation after depolarizing test pulse may also be slowed by propafenone. In order to examine this possibility the time course of recovery from inactivation of I_{Ca} was investigated by use of a double-pulse protocol. A 2 s conditioning prepulse which produced apparent inactivation at +10 mV was followed by a test pulse of 150 ms duration from a holding potential of −40 mV to +10 mV at various intervals (from 100 ms to 30 s). The peak current for each test pulse was normalized to that for the control pulse applied 30 s before the conditioning pulse and is plotted as a function of the recovery period in Figure 7. In the absence of propafenone, the time course of recovery was best fitted by a single exponential function with a time constant of recovery (τ) of 503 ms while in the presence of 5×10^{-6} M propafenone the recovery process was markedly slowed being better described by two exponential functions (time constant of 1100 ms and 8 s for the fast and slow component respectively). The figure also shows that when the same experimental protocol was repeated after perfusing the cell with drug-free solution, the time course of recovery was again fitted by a single exponential which reached values similar to the control ($\tau = 658$ ms). Similar findings were observed in another two cells. Then, it may be suggested that the slow component of the Ca current recovery with propafenone reflects the time course of drug dissociation from the inactivated Ca channel, whereas the fast component seems to reflect, at least in part, the recovery of drug-unbound channels from inactivation. The slow recovery of propafenone-bound channels from inactivation is consistent with the observation that propafenone produced a profound frequency-dependent I_{Ca} block even at 0.5 Hz.

Discussion

The results of the present investigation provide evidence that L-type Ca channels of guinea-pig isolated ventricular myocytes are blocked reversibly by propafenone. This conclusion is based on the finding that this drug blocks in a concentration-dependent manner the calcium inward current activated from potentials positive to −40 mV, the half-maximum inhibition (IC_{50}) occurring at a concentration of about 5×10^{-6} M. At the holding potential used in this study most T-type Ca channels are inactivated (Bean, 1985; Mitra & Morad, 1986), and thus the contribution of I_{Ca} through these channels can be ruled out. Propafenone reduced the peak amplitude of I_{Ca} without affecting the voltage-dependence of Ca channel activation or the slope factor of the inactivation curve. These results suggested that propafenone reduced the maximum conductance of I_{Ca}. The finding that propafenone blocks I_{Ca} agrees well with previous studies on cardiac tissues in which at the same range of concentrations, propafenone produced concentration-dependent decrease of the V_{max} of the slow action potentials, a valid index of the I_{Ca} (Malecot & Trautwein, 1987), both in guinea-pig ventricular muscle fibres (Delgado *et al.*, 1985) and in dog Purkinje fibres (Malfatto *et al.*, 1988). Moreover, voltage-clamp experiments on rabbit sinus node cells (Satoh & Hashimoto, 1984) have demonstrated that propafenone inhibited the I_{Ca}, but only at concentrations higher than 10^{-5} M. The discrepancy in the concentrations of propafenone required to inhibit the I_{Ca} can probably be attributed to the different methodology used (voltage-clamp vs patch-clamp and experimental protocols), even though species and/or tissue differences can also account for this discrepancy.

Furthermore, propafenone exerted voltage- and frequency-

dependent blockade of I_{Ca}. The inhibitory effects of a variety of Ca channel antagonists on myocardial Ca channels may be explained according to the modulated receptor hypothesis (Hondeghem & Katzung, 1977; Hille, 1977a,b; Uehara & Hume, 1985) which postulated that drug affinity for the Ca channel is modulated by channel state (i.e. rested, activated, inactivated). In the present experiments, propafenone produced little or no tonic I_{Ca} block, i.e. block of the Ca channels in the absence of stimulation, which indicated that it exhibits a low affinity for the resting state of the channels. In addition, the block of I_{Ca} by propafenone was markedly dependent on the frequency of stimulation. Use or frequency-dependence block of I_{Ca} has been hypothesized to occur because depolarizing pulses cause channel to cycle between resting, open, and inactivated states. If the affinity of a drug is much greater for open and/or inactivated channels than resting channels, then significant drug binding may occur during depolarizing steps even though little drug was bound under resting conditions. If during a train of depolarizing pulses the time available between pulses is not enough to allow drug unbinding from the inactivated channels, then block will accumulate until a steady-state level is reached. In the present experiments propafenone shifted the steady-state Ca inactivation curve toward more negative potentials, which indicated that the block of I_{Ca} by propafenone is also modulated by membrane potential. This shift suggested that propafenone-induced blockade of I_{Ca} is more pronounced when the channels are in the inactivated state and thus, that it exhibits a higher affinity for the inactivated state than for the resting state of the Ca-channel. According to Bean *et al.* (1983), the shift in the middle point of the steady state availability curve (V_h) is related to the drug concentration and dissociation constants for binding to inactivated (K_I) and rested state channels (K_R) as follows:

$$V_h = K \ln [(1 + [B]/K_R/(1 + [B]/K_I)]$$

where K is the slope factor of the inactivation curve ($K = 4.0$ in Figure 5). Plugging in the shift (ΔV_h) of -7 mV produced by 5×10^{-6} M propafenone in Figure 5, along with the value for K_R from Figure 2, gives a value of $K_I = 5 \times 10^{-7}$ M for 'binding' to the inactivated Ca channels.

The frequency-dependent block of I_{Ca} by propafenone can also be explained by a decrease in the recovery of inactivation. Several organic Ca antagonists have been shown to prolong the recovery from inactivation (Sanguinetti & Kass, 1984; Uehara & Hume, 1985). In the present study, the recovery process was slowed by propafenone being better described by a double exponential, the slower phase possibly indicating a slow dissociation of drug molecules from the inactivated Ca channels (Hondeghem & Katzung, 1984; Uehara & Hume, 1985). This finding can explain the marked use-dependent block of I_{Ca} induced by propafenone at slow frequencies of stimulation (0.5 and 1 Hz). However, it cannot explain by itself the shift of the inactivation curve to more negative potentials as the slope factor remains unchanged. On the other hand, within the context of a modulated-receptor hypothesis, the kinetics of I_{Ca} recovery for drugs that are predominantly charged at physiological pH are slower than for drugs that are predominantly neutral (Hille, 1977b; Uehara & Hume, 1985). The apparent pK_a of propafenone has been calculated as 9.0 (Courtney *et al.*, 1987) and is similar to that of D-600 ($pK_a = 8.6$) (Uehara & Hume, 1985) indicating that both drugs are predominantly charged at physiological pH. In addition, this property suggests that propafenone must cross the cell membrane to gain access to its site of action (Hescheler *et al.*, 1982). Further experiments will be necessary to prove this possibility.

Finally, the concentration of propafenone used in this study (10^{-6} M–5×10^{-5} M) included not only the therapeutic plasma levels (482 $\mu g\,l^{-1}$–1812 $\mu g\,l^{-1}$: 1.3×10^{-6} M–5.3×10^{-6} M; Keller *et al.*, 1978; Karaguezian *et al.*, 1983; Connolly *et al.*, 1983) but also the toxic plasma level of drug. Therefore, the observed I_{Ca} block produced by propafenone may be responsible for some of its cardiodepressant effects as well as for its antiarrhythmic effectiveness on supraventricular arrthythmias. Propafenone can depress cardiac automaticity (Tamargo & Delgado, 1985) and marked bradycardia, sino-atrial block and sinus arrest have been reported after the administration of propafenone (Connolly *et al.*, 1983; Schlepper, 1987). On the other hand, Honjo *et al.* (1989) found that propafenone at concentrations $>10^{-6}$ M shortened action potential duration in single ventricular myocytes from guinea-pig and this shortening was accompanied by an attenuation of sarcomere shortening during twitch contraction suggesting that the Ca-antagonist effect of propafenone could be involved. Furthermore at high concentrations *in vitro*, propafenone exerted a negative inotropic effect in isolated atrium or ventricle compatible with calcium-channel blockade (Dukes & Vaughan Williams, 1984; von Philipsborn *et al.*, 1984). Moreover, since the I_{Ca} plays a major role in both conduction and automaticity of the A-V node, the marked frequency-dependent I_{Ca} block appears also to be important to explain the clinical effectiveness of propafenone against paroxysmal and non-paroxysmal A-V nodal reentrant tachycardia (Funk-Brentano *et al.*, 1990).

In conclusion, the present data demonstrate that in guinea-pig ventricular myocytes propafenone produces a concentration-dependent inhibition of I_{Ca} which can be involved in its antiarrhythmic mechanism.

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References

BEAN, B.P. (1985). Two kinds of calcium channels in canine atrial cells. *J. Gen. Physiol.*, **86**, 1–30.

BEAN, B.P., COHEN, C.J. & TSIEN, R.W. (1983). Lidocaine block of cardiac sodium channels. *J. Gen. Physiol.*, **81**, 613–642.

CARRON, R., PEREZ-VIZCAINO, F., DELPON, E. & TAMARGO, J. (1991). Effects of propafenone on ^{45}Ca movements and contractile responses in vascular smooth muscle. *Br. J. Pharmacol.*, **103**, 1453–1457.

COLQUOHOUN, D. & HAWKES, A.G. (1983). The principles of the stochastic interpretation of ion-channel mechanisms. In *Single-Channel Recording*. ed. Sakmann, B. & Neher, E. pp. 135–175. New York: Plenum Press.

CONOLLY, S., KATES, R., LEBSACK, C., HARRISON, D. & WINKLE, R. (1983). Clinical pharmacology of propafenone. *Circulation*, **68**, 589–596.

COURTNEY, K.R. (1987). Review: quantitative structure/activity relations based on used-dependent block and repriming kinetics in myocardium. *J. Mol. Cell. Cardiol.*, **19**, 319–330.

DELGADO, C., TAMARGO, J. & TEJERINA, T. (1985). Electrophysiological effects of propafenone in untreated and propafenone pretreated guinea-pig atrial and ventricular muscle fibres. *Br. J. Pharmacol.*, **86**, 765–775.

DUKE, I. & VAUGHAN WILLIAMS, E. (1984). The multiple modes of action of propafenone. *Eur. J. Cardiol.*, **5**, 115–125.

EHARA, T. & KAUFMAN, R. (1978). The voltage and time-dependent effects of (−) Verapamil on the slow inward current in isolated cat ventricular myocardium. *J. Pharmacol. Exp. Ther.*, **207**, 49–55.

FUNCK-BRENTANO, C., KROEMER, H.K., LEE, J.T. & RODEN, D.M. (1990). Propafenone. *N. Engl. J. Med.*, **22**, 518–525.

HALLIWELL, J.V., PLANT, T.D. & STANDEN, N.B. (1987). Voltage clamp techniques. In *Microelectrode Techniques*. ed. Standen, N.B., Gray, P.T. & Whittaker, M.J. pp. 13–28. Cambridge: Company of Biologists.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.*, **391**, 85–100.

HAPKE, H.J. & PRIGGE, E. (1976). Zur Pharmakologie von 2'-[Hydroxy-3-(propylamino)-propoxy]-3-phenyl-propiophenon (Propafenon, SA 79)-hydrochlorid. *Arzneimittel Forschung*, **26**, 1849–1857.

HARRON, W.G. & BROGDEN, N. (1987). Propafenone. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in the treatment of arrhythmias. *Drugs*, **34**, 617–647.

HESCHELER, J., PELZER, D., TRUBE, G. & TRAUTWEIN, W. (1982). Does the organic calcium channel blocker D-600 act from inside or outside on the cardiac cell membrane? *Pflügers Arch.*, **303**, 287–291.

HILLE, B. (1977a). The pH-dependent rate of action of local anesthetics on the node of Ranvier. *J. Gen. Physiol.*, **69**, 475–496.

HILLE, B. (1977b). Local anesthetic: hydrophilic and hydrophobic pathways for drug-receptor reaction. *J. Gen. Physiol.*, **69**, 497–515.

HODGKIN, A.L. & HUXLEY, A.F. (1952). A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol.*, **117**, 500–544.

HONDEGHEM, L.M. & KATZUNG, B.G. (1977). Time- and voltage-dependent interactions of antiarrhythmic drugs with cardiac sodium channels. *Biochim. Biophys. Acta*, **472**, 373–398.

HONDEGHEM, L.M. & KATZUNG, B.G. (1984). Antiarrhythmic agents: the modulated receptor mechanism of action of sodium and calcium channel-blocking drugs. *Annu. Rev. Pharmacol. Toxicol.*, **24**, 387–423.

HONJO, H., WATANABE, T., KAMIYA, K., KODAMA, I. & TOYAMA, J. (1989). Effects of propafenone on electrical and mechanical activites of single ventricular myocytes isolated from guinea-pig hearts. *Br. J. Pharmacol.*, **97**, 731–738.

ISENBERG, G. & KLÖCKNER, U. (1982). Calcium tolerant ventricular myocytes prepared by preincubation in a 'KB medium'. *Pflügers Arch.*, **395**, 6–18.

KARAGUEUZIAN, H., FUJIMOTO, T., KOTOH, T., MCKULLEN, A. & MANDEL, W. (1983). Suppression of ventricular arrhythmias by propafenone, a new antiarrhythmic agent, during acute myocardial infarction in the conscious dogs. A comparative study with lidocaine. *Circulation*, **66**, 1190–1198.

KELLER, R., MEYER-ESTORF, G., BECK, O. & HOCHREIN, H. (1978). Correlation between serum concentration and pharmacological effects on atrio-ventricular conduction time of the antiarrhythmic drug propafenone. *Eur. J. Clin. Pharmacol.*, **13**, 17–20.

KOHLHARDT, M. (1977). Der Einfluss von Propafenon auf den transmembranören Na^+ und Ca^{++} Strom der Warblütter-Myokardfasermembran. In *Forschritte in der Pharmakotherapie von Herzhythmusstörungen*. ed. Hochrein, H., Hapke, H.J. & Beck, O. pp. 35–58. Stuttgard: Fischer.

KOHLHARDT, M., SEIFERT, C. & HONDEGHEM, L.M. (1983). Tonic and phasic I_{Na} blockade by antiarrhythmics: different properties of drug binding to fast sodium channels as judged from V_{max} studies with propafenone and derivatives in mammalian ventricular myocardium. *Pflügers Arch.*, **396**, 199–209.

KROEMER, H.K., FUNCK-BRENTANO, C., SILBERSTEIN, D.J., WOOD, A.J., EICHELBAUM, M., WOOSLEY, R.L. & RODEN, D.M. (1989). Stereoselective disposition and pharmacologic activity of propafenone enantiomers. *Circulation*, **79**, 1068–1076.

LEDDA, F., MANTELLI, L., MANZINI, S., AMERINI, S. & MUGELLI, A. (1981). Electrophysiological and antiarrhythmic properties of propafenone in isolated cardiac preparations. *J. Cardiovasc. Pharmacol.*, **315**, 55–62.

LEE, K.S. & TSIEN, R.W. (1983). Mechanism of calcium channel blockade by verapamil, D-600, diltiazem and nitrendipine in single dialysed heart cells. *Nature*, **302**, 790–794.

MALECOT, C.O. & TRAUTWEIN, W. (1987). On the relationship between Vmax of slow responses and Ca-current availability in whole-cell clamped guinea-pig heart cells. *Pflügers Arch.*, **410**, 15–22.

MALFATTO, G., ZAZA, A., FOSTER, M., SODOWICK, B., DANIOL, P. JR. & ROSEN, M.R. (1988). Electrophysiologic, inotropic and antiarrhythmic effects of propafenone, 5-hydroxypropafenone and N-depropylpropafenone. *J. Pharmacol. Exp. Ther.*, **246**, 419–426.

MCDONALD, T.F., PELZER, D. & TRAUTWEIN, W. (1980). On the mechanism of slow calcium channel block in heart. *Pflügers Arch.*, **385**, 175–179.

MITRA, R. & MORAD, M. (1986). Two types of calcium channels in guinea-pig ventricular myocytes. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 5340–5344.

MÜLLER-PELZER, H., GREGER, G., NEUGEBAUER, G. & HOLLMANN, M. (1983). Beta-blocking and electrophysiological effects of propafenone in volunteers. *Eur. J. Clin. Pharmacol.*, **25**, 831–833.

PELZER, D.W., TRAUTWEIN, W. & MCDONALD, T.F. (1982). Calcium channel block and recovery from block in mammalian ventricular muscle treated with organic channel inhibitors. *Pflügers Arch.*, **394**, 97–105.

PHILIPSBORN, G.V., GRIES, J., HOFMANN, H.P., KREISKOTT, H., KRETZSCHMAR, R. & MÜLLER, C.D. (1984). Pharmacological studies on propafenone and its main metabolite 5-hydroxypropafenone. *Arzneim. Forsch.*, **34**, 1489–1497.

SANGUINETTI, M.C. & KASS, R.S. (1984). Voltage-dependent block of calcium channel current in the calf cardiac Purkinje fiber by dihydropyridine calcium channel antagonists. *Circ. Res.*, **55**, 336–348.

SATOH, H. & HASHIMOTO, K. (1984). Effect of propafenone on the membrane currents of rabbit sino-atrial node cells. *Eur. J. Pharmacol.*, **99**, 185–191.

SCHLEPPER, M. (1987). Propafenone, a review of its profile. *Eur. Heart. J.*, **8**, Suppl A, 27–32.

TAMARGO, J. & DELGADO, C. (1985). Electrophysiological effects of propafenone on isolated guinea-pig ventricular muscle and sheep Purkinje fibres. *Eur. J. Pharmacol.*, **118**, 331–340.

TRAUTWEIN, W., PELZER, D., MCDONALD, T.F. & OSTERRIEDER, W. (1981). AQA 39, a new bradycardic agent which blocks myocardial calcium (Ca) channels in a frequency- and voltage-dependent manner. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **317**, 228–232.

UEHARA, A. & HUME, J.R. (1985). Interactions of organic calcium channel antagonists with calcium channels in single frog atrial cells. *J. Gen. Physiol.*, **85**, 621–647.

VALENZUELA, C., DELPON, E. & TAMARGO, J. (1989). Electrophysiological interactions between mexiletine and propafenone in guinea-pig papillary muscles. *J. Cardiovasc. Pharmacol.*, **14**, 351–357.

YATANI, A., BROWN, A.M. & SCHWARTZ, A. (1986). Bepridil block of cardiac calcium and sodium channels. *J. Pharmacol. Exp. Ther.*, **237**, 9–17.

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Effects of analogues of adenine nucleotides on increases in intracellular calcium mediated by P_{2T}-purinoceptors on human blood platelets

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1 By use of a number of analogues of adenine nucleotides, the structure-activity relationships of the human platelet receptor for adenosine 5'-diphosphate (ADP) mediating increases in intracellular calcium were investigated, and compared with the known structure-activity relationships for induction by ADP of platelet aggregation.

2 ADP, 2-methylthioadenosine 5'-diphosphate (2-methylthio-ADP), adenosine 5'-O-(1-thiodiphosphate) (ADP-α-S) and adenosine 5'-O-(2-thiodiphosphate) (ADP-β-S) each induced increases in intracellular calcium in a manner similar to their reported ability to induce human platelet aggregation. The effects of these agonists were antagonized by ATP, with a pA_2 value in each case consistent with the inhibition by ATP of ADP-induced aggregation. In the case of ADP, the inhibition by ATP of increases in intracellular calcium was shown to be competitive by Schild analysis.

3 Of the analogues tested as inhibitors of the effect of ADP on intracellular calcium, 2-chloroadenosine 5'-triphosphate (2-chloro-ATP), adenosine 5'-O-(1-thiotriphosphate) (ATP-α-S), P_1, P_5-diadenosine pentaphosphate (Ap_5A) and adenylyl 5'-(β,γ-methylene)diphosphonate (AMPPCP) were apparently competitive antagonists, although only one concentration of each antagonist was used. There was a good correlation between the pA_2 values found here for these antagonists including ATP, and their pA_2 values reported for inhibition of ADP-induced aggregation. Adenosine 5'-(α,β-methylene)triphosphonate (AMPCPP) and uridine 5'-triphosphate (UTP) (100 μM) were only very weak inhibitors of the effect of ADP on intracellular calcium, and this is consistent with their weak actions as inhibitors of aggregation. 2-Methylthioadenosine 5'-triphosphate (2-methylthio-ATP) (50 μM) non-competitively inhibited the effect of ADP on intracellular calcium, in a very similar way to its inhibition of ADP-induced aggregation.

4 The good correspondence found for these analogues between their effect on intracellular calcium and on aggregation confirms that there is a causal relationship between these actions of ADP, and that they are mediated by the same receptor on platelets. These findings cast further doubt on the use of the affinity reagent 5'-fluorosulphonylbenzoyladenosine (FSBA) as an antagonist and label for the ADP receptor, as this compound has been reported to inhibit aggregation but not ADP-induced increases in intracellular calcium.

Keywords: ADP; platelets; purinoceptors; adenine nucleotides; intracellular calcium

Introduction

Extracellular adenine nucleotides are known to have a number of biological effects on a wide range of tissues via specific cell surface receptors known as P_2-purinoceptors. These receptors have been subdivided into at least four classes, P_{2X} and P_{2Y} which exist on smooth muscle and many other cell types such as hepatocytes, P_{2Z} which exist on mast cells and other immune cells and P_{2T} which are found on platelets (for reviews see Gordon, 1986; Kennedy, 1990). The P_{2T} receptor is unique in that adenosine 5'-diphosphate (ADP) is the endogenous agonist and adenosine 5'-triphosphate (ATP) is a competitive antagonist rather than an agonist. The structure-activity relationships for a number of synthetic analogues of ADP and ATP at the P_{2T} receptor also differ from those of the other P_2 subtypes (Cusack & Hourani, 1990).

Human platelets respond to ADP by a change in shape and the exposure of fibrinogen receptors which results in aggregation, and these responses are thought to be due to an increase in intracellular calcium levels. The major effect of ADP appears to be the opening of a receptor-operated cation channel to cause the rapid influx of extracellular calcium, and there is also evidence for mobilization of calcium from internal stores although ADP has not been consistently reported to induce changes in phosphoinositide metabolism (Sage & Rink, 1987; Drummond & MacIntyre, 1987; Sage *et al.*, 1989; 1990; Mahaut-Smith *et al.*, 1990; Vickers *et al.*, 1990). ADP also inhibits adenylate cyclase in platelets, but the relationship between this effect and the other effects of ADP is not clear. Inhibition of adenylate cyclase in platelets by compounds acting directly on the cyclase, such as 2',5'-dideoxyadenosine, does not induce or enhance aggregation (Haslam *et al.*, 1978), but the inhibition of adenylate cyclase by ADP receptor agonists may promote aggregation in the presence of inhibitory agonists which act by stimulating adenylate cyclase (Cusack & Hourani, 1982a). ADP itself is roughly equipotent as an aggregating agent and as an inhibitor of adenylate cyclase, but some 2-substituted analogues, such as 2-methylthioadenosine 5'-diphosphate (2-methylthio-ADP) are considerably more potent as inhibitors of adenylate cyclase than they are as aggregating agents (Macfarlane *et al.*, 1982; 1983; Cusack & Hourani, 1982d). In contrast, although the phosphate-modifed analogues, adenosine 5'-O-(1-thiodiphosphate) (ADP-α-S) and adenosine 5'-O-(2-thiodiphosphate) (ADP-β-S), both act as partial agonists of aggregation, only ADP-β-S has any agonist action for inhibition of adenylate cyclase, with ADP-α-S acting as a full antagonist of this response (Cusack & Hourani, 1981a,b).

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The lack of correlation for agonists between these two effects of ADP, aggregation and the inhibition of adenylate cyclase, does imply that they are not causally related, and it has been suggested that they are mediated by different receptors (Macfarlane *et al.*, 1982; 1983). However, a good correlation was found for inhibition of these two effects by a series of antagonists which were shown to be competitive by Schild analysis, which is good evidence that only one receptor is involved (Cusack & Hourani, 1982c). An affinity reagent which has been used extensively as an irreversible ADP antagonist, 5'-fluorosulphonylbenzoyladenosine (FSBA), has been shown to inhibit shape change and aggregation induced by ADP but not the effect of ADP on adenylate cyclase and this has been taken as evidence for two ADP receptors on platelets mediating these two responses. A 100 kDa protein labelled by FSBA in intact platelets has been called 'aggregin' and has been claimed to be the ADP receptor mediating aggregation, and a model has been proposed in which it is closely associated with the fibrinogen receptor complex and sterically hinders its assembly until this hindrance is relieved by ADP binding or proteolytic cleavage (for review see Colman, 1990). However, doubts remain as to the specificity of FSBA, as it has been reported also to inhibit aggregation induced by prostaglandin endoperoxides, collagen, thrombin and adrenaline, which act at their own distinct platelet receptors. This has been taken to imply a central role for released ADP in aggregation induced by these agents (Colman, 1986), but could also reflect the nonspecific nature of the inhibition caused by FSBA (see Macfarlane, 1987; Hourani & Cusack, 1991 for discussion). A complicating factor in the acceptance of FSBA as an ADP antagonist is the report that FSBA does not inhibit the ability of ADP to increase intracellular calcium levels in platelets, with the suggestion that this effect of ADP may also be mediated by a separate receptor from that causing platelet shape change (Rao & Kowalski, 1987).

The structure-activity relationships of the ADP receptor causing increases in intracellular calcium have not been investigated, although it has been briefly stated that AMP and adenosine do not mimic this effect and that ATP acts as an antagonist as expected (Drummond & MacIntyre, 1987). In this study we have therefore used a number of analogues of ADP and of ATP, the actions of which on ADP-induced aggregation and the inhibition of adenylate cyclase are known, and have measured their effect on intracellular calcium levels in an attempt to investigate the relationship between these actions of ADP on platelets.

Methods

Intracellular calcium measurements

Venous blood was taken from healthy human volunteers who had not taken any aspirin for 10 days, collected into one-sixth of the volume of acid-citrate-dextrose (25 g l^{-1} trisodium citrate, 15 g l^{-1} citric acid, 20 g l^{-1} glucose), and centrifuged at 260 *g* for 20 min. The supernatant platelet-rich plasma was removed, and incubated with fura-2-AM (4 μM) at 37°C for 45 min. The fura-2-loaded platelets were then collected by centrifugation at 680 *g* for 20 min in the presence of prostacyclin (1 μM), and resuspended at 10^8 ml^{-1} in HEPES-saline-(composition mM: NaCl 145, KCl 5, N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) 10, glucose 10, bovine serum albumin 2 mg ml^{-1}, adjusted to pH 7.4 with NaOH (1 M)). Aliquots (700 μl) were incubated in siliconized glass tubes at 37°C for 3 min in the presence of 1 mM $CaCl_2$, and transferred to a Perkin-Elmer LS50 Luminescence Spectrometer for estimation of intracellular calcium. The stirred platelet suspension was excited alternately at 340 nm and 380nm, with emitted light measured at 510 nm, and intracellular calcium concentrations were calculated from the ratio of intensities at 340 nm and 380 nm (Grynkiewicz *et al.*, 1985). The maximal intensity ratio was determined following lysis of the platelets with 0.1% Triton X-100, and the minimum intensity ratio was determined by adding 20 mM tris(hydroxymethyl)aminomethane (Tris) followed by 10 mM [ethylene-*bis*(oxyethylenenitrilo)]tetraacetic acid (EGTA), and these measurements were used to calibrate the fluorescence signal by the Perkin Elmer Intracellular Biochemistry programme. Agonists were added in 14 μl of water, and antagonists where used were added simultaneously with the agonist. Responses were quantified as the maximal change in intracellular calcium levels following addition of the agonist, log concentration-response curves were obtained at least three times with blood from different donors, and the results were pooled. EC_{50} values were obtained by regression analysis of the linear parts of the concentration-response curves, and dose-ratios for antagonists were calculated from the EC_{50} values in the absence and presence of antagonist. $K/_D$ values were obtained from these dose-ratios using the formula K_D = concentration of antagonist/(dose-ratio − 1), and pA_2 values were calculated as $-\log_{10}K_D$.

Materials

Prostacyclin, ADP, ATP, adenosine 5'-(α,β-methylene)triphosphonate (AMPCPP), adenylyl 5'-(β,γ-methylene)diphosphonate (AMPPCP), P_1, P_5-diadenosine pentaphosphate (Ap_5A), uridine 5'-triphosphate (UTP), fura-2-AM, BSA, EGTA, Tris, diethylaminoethyl (DEAE)-Sephadex A25 and myosin (rabbit muscle) were obtained from Sigma UK. ADP-β-S and adenosine 5'-O-(1-thiotriphosphate) (ATP-α-S) (S_P isomer) were from Boehringer Mannheim, and 2-chloroaden

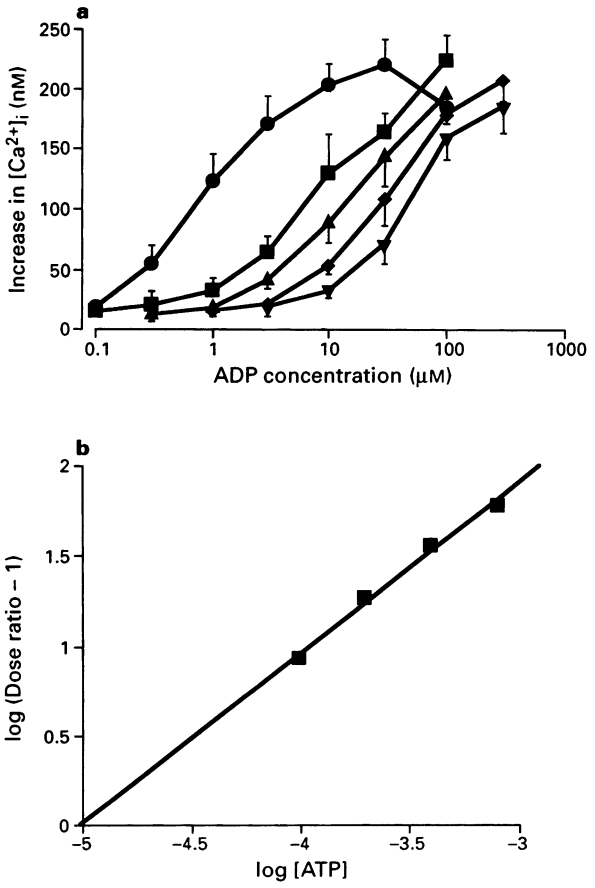


Figure 1 Effect of ATP on increases in intracellular calcium ($[Ca^{2+}]_i$) in human platelets induced by ADP. (a) Effect of ADP alone (●) or in the presence of ATP at 100 μM (■), 200 μM (▲), 400 μM (◆) or 800 μM (▼). Each point is the mean of at least 3 determinations, and the vertical bars show s.e.mean. (b) Schild plot of the data presented in (a).

osine 5'-triphosphate (2-chloro-ATP), 2-methylthioadenosine 5'-triphosphate (2-methylthio-ATP) and 2-methylthio-ADP were obtained from Research Biochemicals Incorporated. ADP-α-S (S_P isomer) was synthesized by enzymatic dephosphorylation of ATP-α-S using myosin as described by Eckstein & Goody (1976). All other reagents were analytical grade from BDH. The purity of all nucleotides was checked before use by high performance liquid chromatography, and compounds were purified where necessary by ion exchange chromatography on DEAE-Sephadex eluted with a linear gradient of 1 to 800 mM triethylammonium bicarbonate.

Results

ADP dose-dependently induced increases in intracellular calcium concentration, with an EC_{50} value of 0.8 μM. This effect of ADP was inhibited by ATP, which caused parallel shifts to the right of the log concentration-response curve to ADP (Figure 1a). Schild analysis of this inhibition by ATP resulted in a slope of 0.95 ± 0.07 (not significantly different from 1, $P > 0.5$) and a pA_2 value of 5.01 (K_D 9.8 μM) (Figure 1b). The effect of ADP was also inhibited in an apparently competitive manner by 2-chloro-ATP (30 μM), ATP-α-S (10 μM),

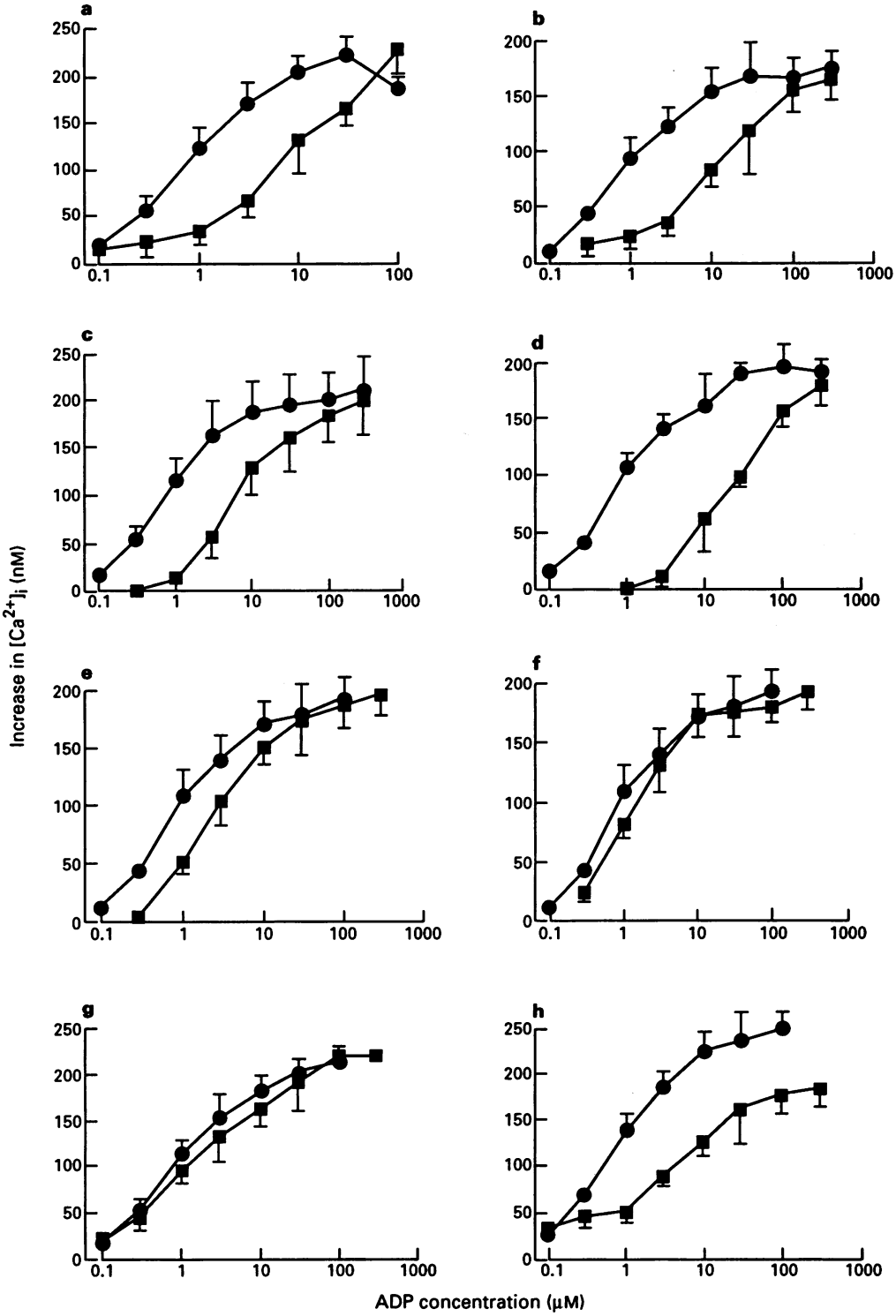


Figure 2 Increases in $[Ca^{2+}]_i$ in human platelets induced by ADP alone (●) or in the presence (■) of (a) ATP (100 μM), (b) 2-chloro-ATP (30 μM), (c) ATP-α-S (10 μM), (d) Ap_5A (200 μM), (e) AMPPCP (100 μM), (f) AMPCPP (100 μM), (g) UTP (100 μM) or (h) 2-methylthio-ATP (50 μM). Each point is the mean of at least three determinations, and the vertical bars show s.e.mean. For abbreviations, see text.

Ap_5A (200 μM) and AMPPCP (100 μM) (Figure 2a–e), but little inhibition was observed in the presence of AMPCPP (100 μM) (Figure 2f) or of UTP (100 μM) (Figure 2g). The pA_2 values for the antagonists derived from the dose-ratios were 5.60 for 2-chloro-ATP, 5.92 for ATP-α-S, 5.10 for Ap_5A and 4.27 for AMPPCP, while those for AMPCPP and for UTP were both less than 3.82. 2-Methylthio-ATP (50 μM) also inhibited the effect of ADP, but this inhibition was not competitive and it could not be overcome by high concentrations of ADP (300 μM) (Figure 2h).

2-Methylthio-ADP also induced increases in intracellular calcium concentrations and was about 20 times more potent than ADP, with an EC_{50} value of 41 nM. The effect of 2-methylthio-ADP was inhibited by ATP (100 μM) in an apparently competitive manner, giving a pA_2 for ATP of 4.66 (Figure 3). ADP-α-S and ADP-β-S each caused increases in intracellular calcium concentrations, but in each case achieved only around 60% of the maximal effect of ADP even at high concentrations (100 or 300 μM) (Figure 4a and b). The effects of ADP-α-S and ADP-β-S were inhibited by ATP (100 μM) in an apparently competitive manner, giving pA_2 values for ATP of 5.55 and 5.32 respectively (Figure 4c).

Discussion

These results represent the first detailed investigation into the structure-activity relationships of the ADP receptor which mediates increases in intracellular calcium in platelets. As expected the log concentration-response curve to ADP itself, with the EC_{50} value of 0.8 μM, is similar to that usually found for induction of aggregation, in washed platelets or in platelet-rich plasma, and it is also similar to that found in previous studies of the concentration-dependence of the effect of ADP on intracellular calcium (Drummond & MacIntyre, 1987; Sage & Rink, 1987). The EC_{50} value for 2-methylthio-ADP (41 nM) is somewhat lower than that reported for induction of aggregation by this agonist (approximately 200 nM), but is higher than that reported for inhibition of adenylate cyclase (approximately 5 nM) (Cusack & Hourani, 1982d; Macfarlane *et al.*, 1983). The effect of 2-methylthio-ADP on intracellular calcium, like that on aggregation and on adenylate cyclase (Cusack & Hourani, 1982d), was competitively inhibited by ATP with a pA_2 value similar to the inhibition by ATP of the effects of ADP, indicating that 2-methylthio-ADP is acting via the ADP receptor. The phosphorothioate analogues of ADP, ADP-α-S and ADP-β-S each achieved lower maximal increases in intracellular calcium than did ADP, with ADP-α-S being more potent than ADP-β-S, and their effects were also competitively inhibited by ATP with an appropriate pA_2 value, indicating that these compounds are also acting via the ADP receptor, presumably as partial agonists. Their effects on intracellular calcium therefore mimic exactly their effects on aggregation, where they are partial agonists with ADP-α-S again being more potent than ADP-β-S, but in the case of ADP-α-S at least differ from their effects on adenylate cyclase, where ADP-α-S acts as an antagonist (Cusack & Hourani, 1981a,b). Overall therefore the ability of agonists to cause increases in intracellular calcium seems to agree more closely with their ability to induce aggregation than to inhibit adenylate cyclase, which supports the idea that intracellular calcium is the most important signal for ADP-induced aggregation.

Of the analogues tested as antagonists, ATP, 2-chloro-ATP, ATP-α-S, Ap_5A and AMPPCP caused apparently competitive inhibition of the effect of ADP on intracellular calcium, although apart from ATP, only one concentration of each antagonist was tested. AMPCPP and UTP were essentially inactive as inhibitors and 2-methylthio-ATP was a non-competitive inhibitor. The weak action of AMPCPP is in agreement with its reported weak inhibitory effect on ADP-induced aggregation (Lips *et al.*, 1980), and confirms the sensitivity of the ADP receptor to modifications of the diphosphate chain. The lack of inhibitory effect of UTP is also not unexpected, as although UTP itself has not been tested as an inhibitor of ADP-induced aggregation, the corresponding uridine diphosphate, UDP, is 500–1000 times less potent than ADP as an aggregating agent (Chambers *et al.*,

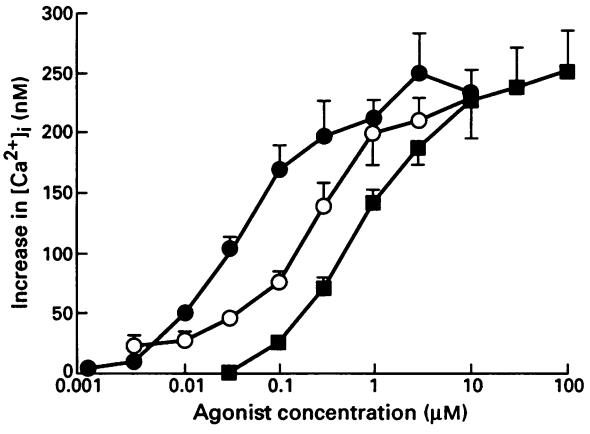


Figure 3 Increases in $[Ca^{2+}]_i$ in human platelets induced by ADP (■), 2-methylthio-ADP (●) or 2-methylthio-ADP in the presence of ATP (100 μM) (○). Each point is the mean of at least three determinations, and the vertical bars show s.e.mean. For abbreviations, see text.

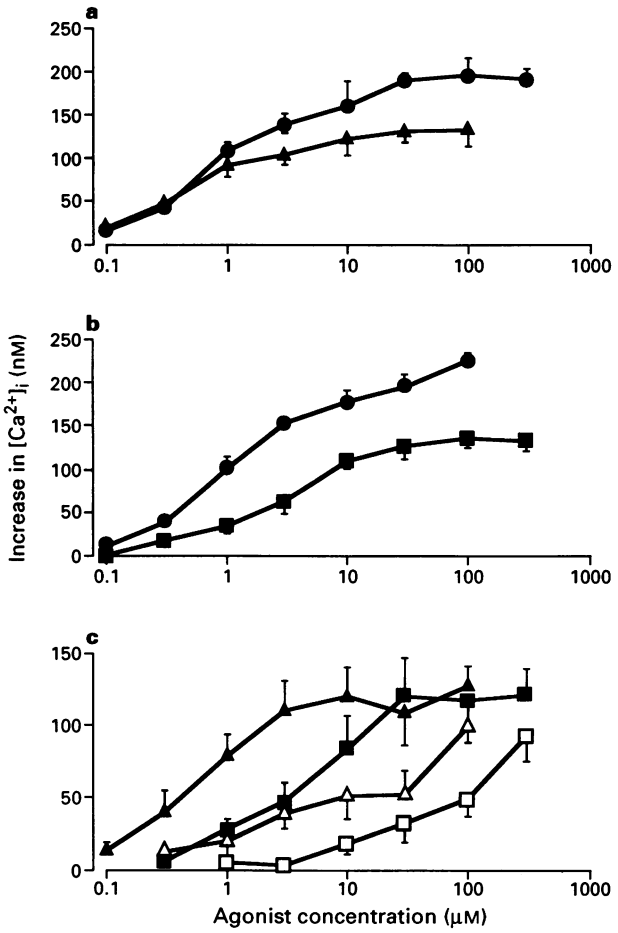


Figure 4 Increases in $[Ca^{2+}]_i$ in human platelets induced by ADP (●) or by (a) ADP-α-S (▲) or (b) ADP-β-S (■). (c) Effects of ADP-α-S (▲,△) or of ADP-β-S (■,□) alone (closed symbols) or in the presence of ATP (100 μM) (open symbols). Each point is the mean of at least three determinations, and the vertical bars show s.e.mean. For abbreviations, see text.

1968), and the phosphorothioate analogue of UTP, UTP-α-S, is much less potent than ATP-α-S as an inhibitor of ADP-induced shape change and aggregation, with 700–800 μM required to inhibit aggregation induced by 5 μM ADP (Greco *et al.*, 1992). UTP-α-S was reported in the same study to compete with [^{3}H]-ADP for two binding sites on formaldehyde-fixed human platelets with K_i values of 27.5 nM and 4.8 μM (Greco *et al.*, 1992), but in view of the discrepancy between these binding affinities and the weak inhibitory action of UTP-α-S these binding sites may not represent functional ADP receptors. Although there is increasing interest at the moment in the idea that there may be nucleotide receptors which recognize both ATP and UTP (O'Connor *et al.*, 1991), and there has even been a suggestion that there may be specific pyrimidoceptors (Siefert & Schultz, 1989), the platelet ADP receptor appears to be a true purinoceptor, with a high degree of specificity towards the adenine base (see Haslam & Cusack, 1981; Hourani & Cusack, 1991 for review).

2-Methylthio-ATP inhibited ADP-induced increases in intracellular calcium but in a non-competitive manner, and this is very similar to its effects on aggregation, as in each case the concentration-response curve to ADP in the presence of the inhibitor reached a plateau at about 60–70% of the normal maximal effect. Several 2-alkylthio analogues of ATP and of AMP, including 2-methylthio-ATP, have been shown to be non-competitive but highly specific inhibitors of ADP-induced aggregation, with an unknown mechanism of action (Cusack & Hourani, 1982b; Hourani *et al.*, 1986). Again, however, the correspondence between the effect of 2-methylthio-ATP on ADP-induced aggregation and increases in intracellular calcium supports the idea that calcium is the trigger for ADP-induced aggregation.

For the five competitive antagonists, the pA_2 values obtained here were in good agreement with the pA_2 values reported by Cusack & Hourani (1982c) for inhibition of ADP-induced aggregation (correlation coefficient 0.97, $P < 0.05$). This confirms the conclusion above that the two events are causally related and that they are mediated by the same receptor. The reported failure of FSBA, which inhibits aggregation, also to inhibit ADP-induced increases in intracellular calcium (Rao & Kowalski, 1987) must cast serious doubt on the claim that it is an ADP antagonist, and therefore that the 100 kDa protein ('aggregin') which it labels is the ADP receptor (Colman, 1986; 1990).

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References

CHAMBERS, D.A., SALZMAN, E.W. & NERI, L.L. (1968). Platelet nucleotide metabolism and platelet aggregation. *Exp. Biol. Med.*, **3**, 62–70.

COLMAN, R.W. (1986). Platelet activation: role of an ADP receptor. *Seminars in Haematol.*, **23**, 119–128.

COLMAN, R.W. (1990). Aggregin: a platelet ADP receptor mediating activation. *FASEB J.*, **4**, 1425–1435.

CUSACK, N.J. & HOURANI, S.M.O. (1981a). Partial agonist behavior of adenosine 5'-O-(2-thiodiphosphate) on human platelets. *Br. J. Pharmacol.*, **73**, 405–408.

CUSACK, N.J. & HOURANI, S.M.O. (1981b). Effects of R_P and S_P diastereoisomers of adenosine 5'-O-(1-thiodiphosphate) on human platelets. *Br. J. Pharmacol.*, **73**, 409–412.

CUSACK, N.J. & HOURANI, S.M.O. (1982a). Differential inhibition by adenosine or by prostaglandin E_1 of human platelet aggregation induced by adenosine 5'-O-(1-thiodisphosphate) and adenosine 5'-O-(2-thiodiphosphate). *Br. J. Pharmacol.*, **75**, 257–259.

CUSACK, N.J. & HOURANI, S.M.O. (1982b). Specific but non-competitive inhibition by 2-alkylthio analogues of adenosine 5'-monophosphate and adenosine 5'-triphosphate of human platelet aggregation induced by adenosine 5'-diphosphate. *Br. J. Pharmacol.*, **75**, 397–400.

CUSACK, N.J. & HOURANI, S.M.O. (1982c). Adenosine 5'-diphosphate antagonists and human platelets: no evidence that aggregation and inhibition of stimulated adenylate cyclase are mediated by different receptors. *Br. J. Pharmacol.*, **76**, 221–227.

CUSACK, N.J. & HOURANI, S.M.O. (1982d). Competitive inhibition by adenosine 5'-triphosphate of the actions on human platelets of 2-chloroadenosine 5'-diphosphate, 2-azidoadenosine 5'-diphosphate and 2-methylthioadenosine 5'-diphosphate. *Br. J. Pharmacol.*, **77**, 329–333.

CUSACK, N.J. & HOURANI, S.M.O. (1990). Subtypes of P_2-purinoceptors. Studies using analogues of ATP. *Ann. N.Y. Acad. Sci.*, **603**, 172–181.

DRUMMOND, A.H. & MACINTYRE, D.E. (1987). Platelet inositol lipid metabolism and calcium flux. In *Platelets in Biology and Pathology III.* ed. MacIntyre, D.E. & Gordon, J.L. pp. 373–431. Amsterdam: Elsevier.

ECKSTEIN, F. & GOODY, R.S. (1976). Synthesis and properties of diastereoisomers of adenosine 5'-(O-1-thiotriphosphate) and adenosine 5'-(O-2-thiotriphosphate). *Biochemistry*, **15**, 1685–1691.

GORDON, J.L. (1986). Extracellular ATP: effects, sources and fate. *Biochem. J.*, **233**, 309–319.

GRECO, N.J., TANDON, N.N., JACKSON, B.W. & JAMIESON, G.A. (1992). Low structural specificity for nucleoside triphosphates as antagonists of ADP-induced platelet activation. *J. Biol. Chem.*, **267**, 2966–2970.

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

HASLAM, R.J. & CUSACK, N.J. (1981). Blood platelet receptors for ADP and for adenosine. In *Purinergic Receptors: Receptors and Recognition*, Series B, Volume 12. ed. Burnstock, G. pp. 221–285. London: Chapman & Hall.

HASLAM, R.J., DAVIDSON, M.M.L. & DESJARDINS, J.V. (1978). Inhibition of adenylate cyclase by adenosine analogues in preparations of broken and intact human platelets. *Biochem. J.*, **176**, 83–95.

HOURANI, S.M.O. & CUSACK, N.J. (1991). Pharmacological receptors on blood platelets. *Pharmacol. Rev.*, **43**, 243–298.

HOURANI, S.M.O., WELFORD, L.A. & CUSACK, N.J. (1986). 2-MeS-AMP-PCP and human platelets: implications for the role of adenylate cyclase in ADP-induced aggregation. *Br. J. Pharmacol.*, **87**, 84P.

KENNEDY, C. (1990). P_1- and P_2-purinoceptor subtypes – an update. *Arch. Int. Pharmacodyn.*, **303**, 30–50.

LIPS, J.P.M., SIXMA, J.J. & SCHIPHORST, M.E. (1980). Binding of adenosine diphosphate to human blood platelets and to isolated blood platelet membranes. *Biochim. Biophys. Acta*, **628**, 451–467.

MACFARLANE, D.E. (1987). Agonists and receptors: adenosine diphosphate. In *Platelet Responses and Metabolism, Vol. II. Receptors and Metabolism.* ed. Holmsen, H. pp. 19–35. Florida: CRC Press.

MACFARLANE, D.E., MILLS, D.C.B. & SRIVASTAVA, P.C. (1982). Binding of 2-azidoadenosine [β-^{32}P]-diphosphate to the receptor on intact human platelets which inhibits adenylate cyclase. *Biochemistry*, **21**, 544–549.

MACFARLANE, D.E., SRIVASTAVA, P.C. & MILLS, D.C.B. (1983). 2-Methylthioadenosine [β-^{32}P]-diphosphate. An agonist and radioligand for the receptor that inhibits the accumulation of cyclic AMP in intact blood platelets. *J. Clin. Invest.*, **71**, 420–428.

MAHAUT-SMITH, M., SAGE, S.O. & RINK, T.J. (1990). Receptor-operated single channels in intact human platelets. *J. Biol. Chem.*, **265**, 10479–10483.

O'CONNOR, S.E., DAINTY, I.A. & LEFF, P. (1991). Further subclassification of ATP receptors based on agonist studies. *Trends Pharmacol. Sci.*, **12**, 137–141.

RAO, A.K. & KOWALSKI, M.A. (1987). ADP-induced platelet shape change and mobilization of cytoplasmic ionised calcium are mediated by distinct binding sites on platelets: 5'-p-fluorosulfonylbenzoyladenosine is a weak platelet agonist. *Blood*, **70**, 751–756.

SAGE, S.O., MERRITT, J.E., HALLAM, T.J. & RINK, T.J. (1989). Receptor-mediated calcium entry in fura-2-loaded human platelets stimulated with ADP and thrombin. *Biochem. J.*, **258**, 923–926.

SAGE, S.O., REAST, R., & RINK, T.J. (1990). ADP evokes biphasic Ca^{2+} influx in fura-2-loaded human platelets. Evidence for Ca^{2+}entry regulated by the intracellular Ca^{2+}store. *Biochem. J.*, **265**, 675–680.

SAGE, S.O. & RINK, T.J. (1987). The kinetics of changes in intracellular calcium concentration in Fura-2-loaded human platelets. *J. Biol. Chem.*, **262**, 16364–16369.

SEIFERT, R. & SCHULTZ, G. (1989). Involvement of pyrimidoceptors in the regulation of cell functions by uridine and by uracil nucleotides. *Trends Pharmacol. Sci.*, **10**, 365–369.

VICKERS, J.D., KINLOUGH-RATHBONE, R.L., PACKHAM, M.A. & MUSTARD, J.F. (1990). Inositol phospholipid metabolism in human platelets stimulated by ADP. *Eur. J. Biochem.*, **191**, 521–528.

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2-Naphthalenesulphonyl L-aspartyl-(2-phenethyl)amide (2-NAP)–a selective cholecystokinin CCK_A-receptor antagonist

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1 The *in vitro* pharmacological characterization of the sodium salt of 2-naphthalenesulphonyl l-aspartyl-(2-phenethyl)amide [2-NAP], a hydrophilic compound derived from the C-terminal aspartate-phenylalanine dipeptide of cholecystokinin (CCK), is described.

2 2-NAP behaved as a competitive antagonist of sulphated cholecystokinin octapeptide (CCK-8) at CCK_A-receptors in both intact tissue bioassays (guinea-pig gall bladder, pancreas and ileum, human and rabbit gall bladder) and a radioligand displacement assay (guinea-pig pancreatic cells). The mean pK_B, over assays, was 6.5.

3 Compared to the other assays, the rabbit gall bladder assay gave a significantly higher pK_B estimate [7.0] for 2-NAP and a significantly lower estimate [8.9] for devazepide (formerly L-364,718 and MK-329), a well-characterized CCK_A-receptor antagonist; these anomalous results suggest that a different class of CCK_A-receptors may be involved.

4 2-NAP, was found to be highly selective, having at least 300 fold greater affinity for CCK_A-receptors than for 50 other pharmacological loci, including gastrin/CCK_B, as estimated by bioassay or radioligand displacement.

Keywords: Receptor, cholecystokinin; muscle, smooth, gall bladder, ileum; pancreas; cerebral cortex; stomach; 2-NAP

Introduction

The principle, that the properties of hormone efficacy and affinity are chemically-separable in hormone analogues, has been exploited in the discovery of antagonists for, *inter alia*, histamine H_2-receptors (Black *et al.*, 1972), β-adrenoceptors (Black & Stephenson, 1962) and angiotensin II-receptors (Marshall *et al.*, 1970). Thus, the retention and subsequent enhancement of the receptor recognition features of a hormone and the emasculation of its efficacy has resulted in the identification of highly specific, competitive antagonists. In common with other groups (Morley *et al.*, 1965; Spanarkel *et al.*, 1983; Horwell *et al.*, 1990) we have been trying to apply this principle to the cholecystokinin (CCK) family of hormones.

Two main classes of CCK/gastrin receptors have been identified: CCK_A and CCK_B/gastrin (see Woodruff & Hughes, 1991). We have used guinea-pig gall bladder contraction (Bishop *et al.*, 1992a) to assay CCK_A-receptor interactions and acid secretion in the isolated, lumen-perfused, stomach of the mouse (Black & Shankley, 1985) to assay CCK_B/gastrin receptor interactions. Using these assays to evaluate analogues of CCK fragments we found that the dipeptide, N-tert-butyloxycarbonyl-aspartyl-phenylalanin-amide (Boc-Asp-Phe-NH_2), representing the two amino acid-fragment C-terminus common to both CCK and gastrin, was a low affinity partial agonist at CCK_B/gastrin receptors (log $[A_{50}]$ -5.58 ± 0.15, max. effect $64.5 \pm 10.4\%$ of max. effect to pentagastrin, $n = 5$, unpublished observations) but inactive at CCK_A-receptors (100 μM, in both guinea-pig gallbladder and pancreatic fragments, $n = 4$, unpublished observations). The latter result accords with the finding of Jensen *et al.* (1983) who reported that 300 μM Boc-Asp-Phe-NH_2 was required for significant CCK_A-receptor antagonism in guinea-pig pancreatic tissue. The selective activity we found was abolished by removal of the C-terminal amide of Boc-Asp-Phe-NH_2. Replacement of the N-tert-butyloxycarbonyl group in this compound with an analogue, the 2-naphthalene sulphonyl group, to give 2-naphthalenesulphonyl l-aspartyl-(2-phenethyl)amide (2-NAP, Figure 1) now produced significant affinity for CCK_A-receptors without the expression of efficacy. The efficacy at CCK_B/gastrin receptors was restored and the affinity for CCK_A-receptors was lost by introduction of the C-terminal-amide into 2-NAP.

The interesting symmetry of these and other structure-activity relations is being reported elsewhere. The *in vitro* pharmacological characterization of 2-NAP, as a selective, competitive antagonist of CCK_A-receptors is described here.

Methods

Bioassays based on measuring changes in contractility are often complicated by the responses 'fading', by the development of 'spontaneous' contractions or by large 'amplitude' variation between preparations. Fade and spontaneity can often be controlled by varying temperature and Ca^{2+} concentration; amplitude variation can be offset by normalising to an independent maximal stimulus. An attempt was made to optimise each of the bioassays by modifying experimental conditions and protocols to improve response stability and reproducibility both within and between preparations.

CCK_A-receptor bioassays

Guinea-pig gall bladder The assay was performed as described by Bishop *et al.* (1992a) which uses low Ca^{2+} Krebs-

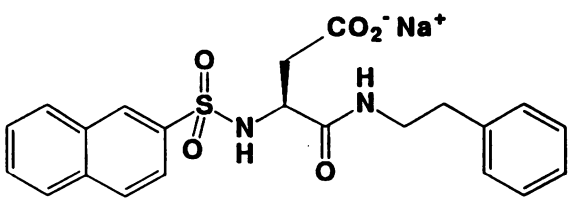


Figure 1 Structure of 2-NAP (2-naphthalenesulphonyl l-aspartyl-(2-phenethyl)amide).

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Henseleit solution at 29°C with the modification that contractile responses were normalized to a maximal muscarinic response produced by 10 μM 5-methylfurmethide (5-MeF).

Rabbit gall bladder The assay was performed as for the guinea-pig gall bladder on tissues from male, New Zealand white rabbits (1.5–2 kg). 5-MeF did not contract this preparation and a maximal response to CCK-8 could not be readily reversed by washout, therefore no normalization procedure was used.

Human gall bladder Gall bladders were obtained from four patients undergoing gallbladder surgery. The assay was performed as for the guinea-pig gall bladder assay except that the $[Ca^{2+}]$ was normal (2.5 mM) and the temperature 25°C. Muscle strips (~3 × 10 mm) were cut from the lumenal portion of the gall bladder. Following preliminary studies, which showed that addition of 2-NAP to a stable, CCK-8-evoked contraction produced a relaxation, a single-shift, competitive interaction study was conducted on gall bladder tissue from a 71 year old male with pancreatitis diagnosed 2 weeks prior to surgery.

Guinea-pig ileum Longitudinal strips of guinea-pig ileum were set up for isotonic recording of muscle contraction under a 0.35 g load in Krebs-Henseleit buffer. In contrast to the gallbladder, CCK contracts guinea-pig ileum indirectly, by releasing acetylcholine (Vizi *et al.*, 1972). Therefore, sensitivity to CCK_A-receptor stimulation was increased by adding physostigmine (0.2 μM) to the bath. The resulting increase in spontaneous activity was reduced by lowering the temperature to 23°C. As CCK_B/gastrin receptors are also present on the vagal nerve terminals (Lucaites *et al.*, 1991) a selective concentration of the CCK_B/gastrin antagonist L-365,260 (0.2 μM, pK_i at CCK_B/gastrin receptors ~8.8, pK_B at CCK_A receptors ~6.5; Lotti & Chang, 1989) was added to avoid confounding effects. Preparations were washed twice during an initial stabilization period of 20 min, and then exposed to three 20 min cycles of 5-MeF (1 μM incubated for 10 min followed by washout).

Guinea-pig pancreas fragments–amylase release assay

The assay was based on the method described for the rat pancreas by Scarpignato *et al.* (1989). For each assay the pancreas was removed from three guinea-pigs (Dunkin Hartley 200–300 g), washed in buffer and the glandular tissue cut into fragments (~1 mm^3); 0.3 ml of tissue was placed in a flow cell (made from 2 ml polythene syringes), superfused with the buffer at 37°C (1.5 ml min^{-1}) and gassed with carbogen. Amylase in the superfusate was measured with the Phadebas Amylase Kit (Pharmacia Diagnostics).

[^{125}I]-Bolton Hunter-CCK-8 displacement from guinea-pig pancreas

The pancreas was removed from two male guinea-pigs (Dunkin Hartley, 200–300 g) and homogenized in 20 volumes of ice-cold HEPES buffer (10 mM; brought to pH 7.2 with ~0.5 ml 10 M NaOH l^{-1} and containing 130 mM NaCl, 4.7 mM KCl, 1 mM EGTA and 0.25 g l^{-1} bacitracin) in a Polytron (Brinkmann, PT10, setting 7) for 6s. The homogenate was centrifuged at 39 800 *g* for 15 min at 4°C. The supernatant was discarded and the pellet resuspended in 20 volumes of buffer with a Teflon-in-glass homogenizer and recentrifuged as above. The final pellet was resuspended in the Teflon-in-glass homogeniser to a tissue concentration of 1 mg wet weight of original tissue ml^{-1} and filtered sequentially through 500 μM and 200 μM (poresize) Nytex mesh. Membrane homogenates (400 μl) were incubated for 150 min at 21°C, in triplicate, with nominally 20 pM [^{125}I]-Bolton Hunter-CCK-8 ([^{125}I]-BH-CCK-8) and unlabelled ligands to a final volume of 0.5 ml buffer. The buffer also contained 0.3 μM CI-988 (also known as PD134308) to inhibit binding to any CCK_B gastrin receptors (pK_B ~8.8, Hughes *et al.*, 1990) which may be present (Da-Hong *et al.*, 1987). The exact concentration of the radioligand was calculated for each experimental run by counting the gamma emission of an aliquot of the diluted radiolabel and convert-ing this to a concentration on the basis that the specific activity was 2200 Ci $mmol^{-1}$. Non-specific binding was that obtained in the presence of a selective concentration (10 nM) of the CCK_A-receptor antagonist devazepide (formerly L-364, 718 and MK-329) (pK_i ~10; Chang & Lotti, 1986). Following incubation, free ligand was removed by rapid vacuum-filtration (Brandell Cell Harvester) through Whatman GF/B filters, pre-soaked in ice-cold 50 mM Tris HCl (pH 7.4). The filters were washed immediately, three times, with 3 ml ice-cold 50 mM Tris HCl (pH 7.4) and bound radioactivity determined by counting in an LKB Clinigamma counter.

The assay was validated by use of the CCK_A-receptor antagonists devazepide and loxiglumide which yielded pK_i values of 10.05 (95% c.l. 9.76, 10.35) and 7.00 (95% c.l. 6.82, 7.19), respectively, in accord with previously published results (Chang & Lotti, 1986; Setnikar *et al.*, 1987; Bishop *et al.*, 1992a).

Selectivity studies

Selectivity of 2-NAP at CCK_A- over CCK_B/gastrin receptors was assessed. CCK_B/gastrin receptor activity was measured by use of the isolated, lumen-perfused, stomach of the mouse; guinea-pig stomach fundic muscle strip and mouse cerebral cortex CCK_B radioligand binding assays. Selectivity of 2-NAP over several non-CCK-receptor assays was measured with both bioassays and radioligand binding assays (for details see Table 2).

Experimental protocols

A single agonist concentration-effect (E/[A]) curve was obtained in each assay in the absence or presence of antagonist pre-incubated for 60 min. Where CCK-8 produced sustained responses, single cumulative E/[A] curves were obtained. In the guinea-pig ileum, CCK-8 produced spike and fade responses. Therefore sequential curves were obtained using a 15 min cycle; the tissues were exposed to CCK-8 for ~30s, followed by washout and re-application of 2-NAP. Cumulative CCK-8 E/[A] curves were obtained on the pancreas fragments assay with a 16 min time cycle in which, following addition of CCK-8 to the reservoir, a 12 min collection period was allowed (void perfusate) and the following 4 min of superfusate collected for amylase analysis.

Data analysis

Logistic curve-fitting E/[A] data were fitted to a three parameter logistic function to provide estimates of the midpoint slope parameter index (p), midpoint location ($\log[A_{50}]$) and upper asymptote (α) of the curves. These parameters, expressed as mean ± s.e.mean were used for subsequent analysis (Student's *t* test and one-way analysis of variance, ANOVA, as appropriate) and display of the data (Black & Shankley, 1985). On the guinea-pig pancreas fragment assay it was not possible to fit E/[A] data to the logistic function because the curves were bell-shaped and asymmetric about the log $[A_{50}]$. Therefore, ANOVA tests were not performed on the slopes and asymptotes of the curves and log $[A_{50}]$ values were measured graphically from plots of the E/[A] data. These values were subsequently fitted to the model of competitive antagonism.

Competitive analysis Competitive analysis was performed as described previously (Black *et al.*, 1985b). Antagonist equili-

brium dissociation constants were estimated as $-\log K_B$ (i.e. pK_B). Where only a single antagonist concentration was examined, simple competition was assumed provided there was a parallel shift of the CCK-8 E/log[A] curve with no significant difference in α. The ability of compounds to inhibit competitively the specific binding of [^{125}I]-BH-CCK-8 to CCK_A sites was analysed by the programme ALLFIT which provided estimates of the IC_{50} values for a test ligand. The pK_i values of test compounds were calculated from the Cheng-Prussof equation. The pK_i values are quoted with the s.e.mean. The 95% confidence limits are calculated using $(n-1)$ d.f., where n is the number of experiments (i.e. pK_i determinations) performed.

Combined dose-ratio analysis This analysis was performed according to a procedure (Shankley *et al.*, 1988) based on the method of Paton & Rang (1965). In brief, when two antagonists act at the same site, then their combined dose-ratio is given by:

$$r_{(B+C)} = r_B + r_C - 1,$$

were r_B and r_C are the dose ratios obtained independently in the presence of the antagonists B and C, respectively. When two antagonists act independently their combined dose-ratio is given by:

$$r_{(B+C)} = r_B . r_C$$

Two reference CCK_A-receptor antagonists have been used: the non-peptide, benzodiazepine-related devazepide and 4-CTP which is a derivative based on the C-terminal tetrapeptide sequence of CCK and gastrin, (i.e. N-tert-butyloxycarbonyl-l-tryptophyl-l-leucyl-l-aspartyl-2(4-chlorophenyl)ethylamide).

Compounds

The sulphated C-terminal octapeptide of cholecystokinin (CCK-8) and pentagastrin were obtained from Cambridge Research Biochemicals Ltd. CCK-8 was dissolved in 10% absolute ethanol to provide a final stock solution of 2 mM concentration. Pentagastrin was dissolved in 100% dimethylformamide (DMF) to give a 100 mM stock. [^{125}I]-BH-CCK-8 was purchased from DuPont, Stevenage, Herts. Some of the experiments used the free acid formulation of 2-NAP which behaved quantitatively in an identical manner to the sodium salt. 2-NAP was dissolved in 100% DMF to give a 20 mM stock.

Devazepide, L-365,260 (3R(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-N'-(3-methylphenyl) urea), CI-988 ([[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo[3.3.1.1^{3,7}]dec-2-yloxy)carbonyl]amino]propyl]amino]-1-phenyl-ethyl]amino]-4-oxobutanoic acid) and 4-CTP, loxiglumide (all prepared by James Black Foundation chemists) were dissolved in DMF to give 2 mM stock concentrations. Caerulein, bacitracin, atropine, (+)-tubocurarine, cyproheptidine, methysergide, (−)-propranolol, (−)-salbutamol, (−)-isoprenaline, histamine, acetylcholine and 5-hydroxytryptamine were purchased from Sigma. Angiotensin II was purchased from Cambridge Research Biochemicals. The following compounds were obtained as gifts: 5-methylfurmethide iodide (Wellcome Research Plc), mepy-ramine (May and Baker), tiotidine (ICI Pharmaceuticals plc), DuPont 753 (1-(2'-(5-tetrazoyl)phenyl)benzyl 2-butyl-4-chloro-5-methanol-imidazole, K^+ salt; DuPont-Merck Inc.). All compounds were subsequently diluted in distilled water. The maximum volume of solvents added to any one 20 ml organ bath was 20 µl ethanol, 20 µl DMF or 500 µl distilled water. Neither the vehicles nor the antagonists were found to produce significant effects on baseline activity.

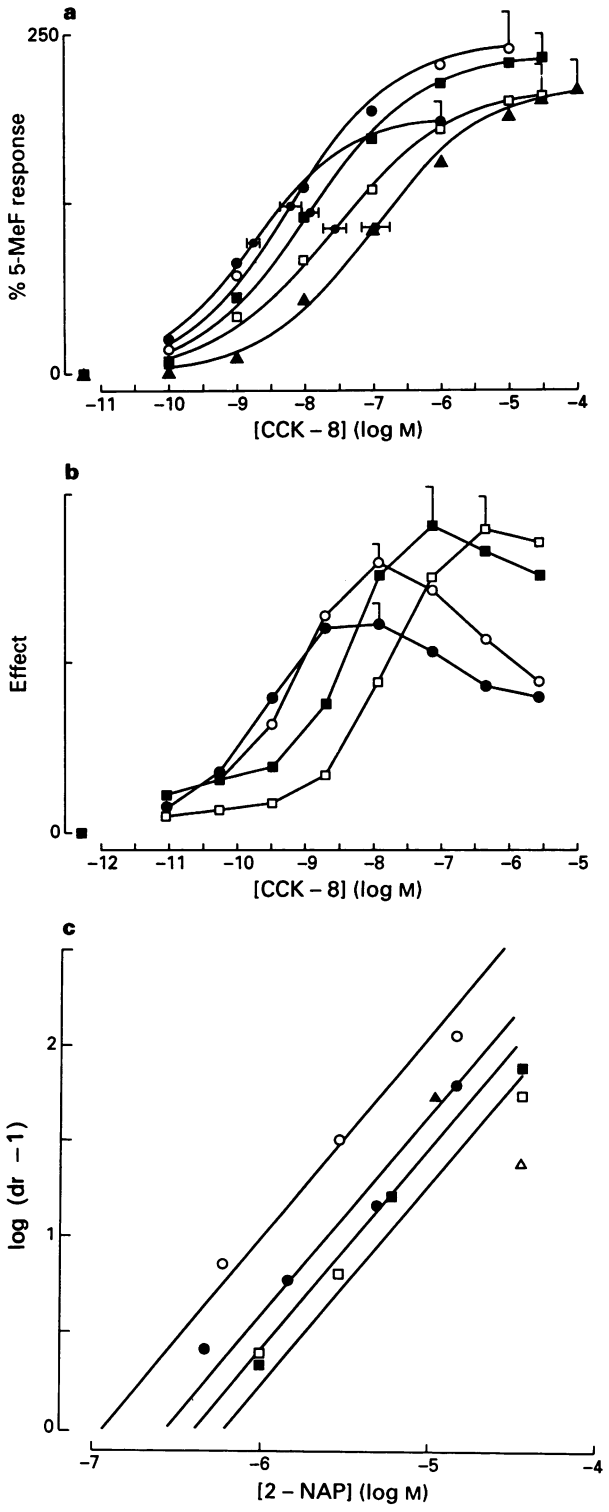


Figure 2 Analysis of competitive antagonism (a) CCK-8 E/log[A] curves ($n = 6/8 \pm$ s.e.mean) obtained on the guinea-pig gall bladder assay in the absence (●) and presence of (○) 0.5, (■) 1.5, (□) 5, (▲) 15 µM 2-NAP. Ordinate scale: contraction as a percentage of response to 5-methylfurmethide (5-MeF, 10 µM). (b) CCK-8 E/log[A] relationships ($n = 4/8 \pm$ s.e.mean) obtained on the guinea-pig pancreas assay in the absence (●) and presence of (○) 1, (■) 9, (□), 36 µM 2-NAP. Ordinate scale: Effect as amylase release in arbitary units. (c) Schild plots for the interaction between CCK-8 and 2-NAP in (●) guinea-pig gall bladder, (○) rabbit gall bladder, (■) guinea-pig pancreas, (□) guinea-pig ileum. For comparison the Schild plot values for the interaction between caerulein and 2-NAP (10 µM) in the guinea-pig gall bladder. (▲) and for CCK-8 and 2-NAP (30 µM) in the human gall bladder (△) are also shown. dr:dose ratio.

Results

CCK_A-receptor antagonism by 2-NAP

Guinea-pig tissue assays We have used devazepide as a standard reference CCK_A-receptor antagonist on the assays. The pK_B estimates were as follows: guinea-pig gall bladder 9.98 (95% c.l. 9.77, 10.17) (Bishop *et al.*, 1992a), pancreas 9.86 (95% c.l. 9.62, 10.10) and ileum 10.0 (95% c.l. 9.46, 10.54). A pK_i value of 10.05 (95% c.l. 9.76, 10.35) was estimated on the pancreatic radioligand binding assay.

2-NAP behaved as a simple competitive antagonist of CCK-8 on the guinea-pig gall bladder, pancreas (Figure 2) and ileum bioassays and on the pancreatic radioligand binding assay. The dissociation constant values estimated were not significantly different from each other and ranged from 6.21 to 6.65 (Figure 3 and Table 1). As interpreted by combined dose-ratio analysis (Table 2), 2-NAP acted syntopically with CCK-8 and the benzodiazepine derivative, devazepide, and with CCK-8 and the peptide analogue 4-CTP. Antagonism was independent of the agonist. Thus, on the guinea-pig gall bladder assay, a single concentration of 2-NAP (10 μM) produced a parallel rightward shift of caerulein E/[A] curves and a pK_B value of 6.65 (95% c.l. 6.37, 6.93) was estimated (Table 1).

Rabbit gall bladder In the rabbit gall bladder, although 2-NAP behaved as a simple competitive antagonist, the pK_B value estimated was 6.95 (95% c.l. 6.52, 7.38) (Table 1). A pK_B value of 8.87 (95% c.l. 8.48, 9.25) was estimated for devazepide.

Human gall bladder In the human gall bladder preparation (8 tissues from one tissue sample), a parallel rightward shift of the CCK-8 E/log[A] curve of 1.32 ± 0.39 log units was observed in the presence of 2-NAP (30 mM) equivalent to a pK_B value of 5.82 (95% c.l. 4.58, 7.06) (Table 1).

Analysis of 2-NAP antagonism between CCK_A-receptor assays

We wanted to know whether the estimated dissociation constants for 2-NAP given in Table 1 formed a homogeneous group. Conventionally, one-way ANOVA would be used;

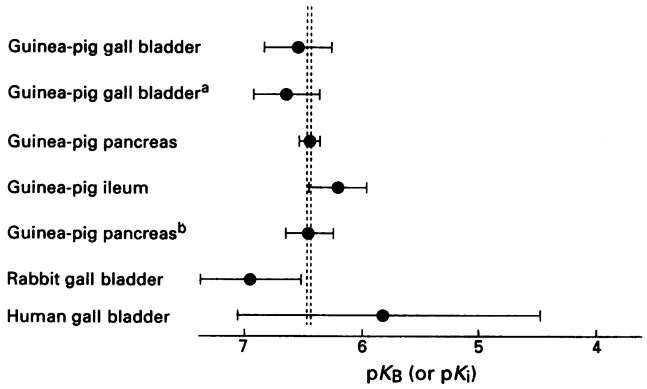


Figure 3 Graphical representation of the estimates of the 2-NAP pK_B (or pK_i), together with 95% confidence limits, from the CCK_A-receptor assays listed in Table 1. The broken lines show the range of the weighted and unweighted global mean values of the complete data sets.
[a] vs. caerulein; [b] pK_i

Table 2 Combined dose-ratio analysis

B:	Devazepide (3 nM)	4-CTP (30 μM)
C:	2-NAP (10 μM)	2-NAP (10 μM)
Observed log r_B	1.14 ± 0.28	1.10 ± 0.38
Observed log r_C	1.41 ± 0.24	1.65 ± 0.32
Multiplication:		
Expected log $r_{(B+C)}$	2.55	2.76
Addition:		
Expected log $r_{(B+C)}$	1.58	1.75
Observed log $r_{(B+C)}$	1.69 ± 0.09	1.50 ± 0.27
Test Statistics:		
Devazepide ± 2-NAP		
S_M ± s.e.mean	− 0.86 ± 0.37* (95% c.l. − 1.64, − 0.08)	
S_A ± s.e.mean	0.11 ± 0.20 (95% c.l. −0.31, 0.53)	
4-CTP + 2-NAP		
S_M ± s.e.mean	− 1.26 ± 0.47* (95% c.l. − 2.15 − 0.27)	
S_A ± s.e.mean	− 0.25 ± 0.25 (95% c.l. − 0.78, 0.28)	

*Significantly different from zero, $P<0.05$.

Table 1 Analysis of CCK-8/caerulein v 2-NAP interactions

A Intact tissue bioassays

Assay	*b* (s.e.mean)	pK_B (s.e.mean)	*N*	*95% c.l.*
Guinea-pig:				
gall bladder	0.94 (0.12)	6.55 (0.14)	29	6.26, 6.84
gall bladder[a]	ND	0.65 (0.13)	11	6.37, 6.93
pancreas	0.94 (0.06)	6.45 (0.04)	24	6.36, 6.54
ileum	0.84 (0.08)	6.21 (0.12)	28	5.96, 6.46
Rabbit				
gall bladder	0.84 (0.20)	6.95 (0.21)	22	6.52, 7.38
Human				
gall bladder	ND	5.82 (0.39)	8	4.58, 706

B Radioligand displacement assay

Assay	*b* (s.e.mean)	pK_i (s.e.mean)	*N*	*95% c.l.*
Guinea-pig pancreatic cells	0.83 (0.06)	6.45 (0.07)	5	6.24, 6.65

pK_B and pK_i values obtained from CCK_A-receptor assays. N represents the number of agonist dose-response curves used in the experiment or, in the case of radioligand displacement assay, the number of repeated experiments (where each experiment used triplicate samples) performed to obtain a pIC_{50}.
b is the Schild plot slope parameter determined in the functional tissue assays and the mid-point slope of the 2-NAP concentration-displacement curve of specific [^{125}I]-BH-CCK-8 binding in the radioligand studies.
[a]Caerulein as agonist.
ND: not determined.

however, this is not applicable in this case as there are clear differences in the size of the variances associated with the pK_B/pK_i estimates. Consequently, we have used the method of Cochran (1954) based on a weighted F-test which indicated that the data are significantly ($P<0.05$) non-homogeneous. That this is due apparently to the high pK_B estimate from the rabbit gall bladder assay is shown from the following analyses. Firstly, a global mean was calculated from all the pK_B (and pK_i) estimates in Table 1 and then each assay pK_B value was tested against this value using the individual 95% confidence limits. The global mean was calculated in three ways: the unweighted arithmetic mean of the estimates, a weighted mean using the number of experiments (N in Table 1) as a weighting factor and a weighted mean using $1/se^2$ as a weighting factor (Cochran, 1954). These gave very similar values of 6.44, 6.48 and 6.46, respectively. Only the 95% confidence intervals of the rabbit gall bladder pK_B value excluded these global mean values. Secondly, the weighted F-test was not significant when performed without the data from the rabbit gall bladder experiment. The global average pK_B values without the rabbit gall bladder result was 6.36, 6.39 and 6.46, respectively, when calculated by the methods above.

Selectivity of 2-NAP

CCK_B/gastrin-receptor selectivity 2-NAP (100 μM) had no significant effect on pentagastrin-stimulation of gastric acid secretion from the mouse stomach and smooth muscle contraction of the guinea-pig fundus muscle assays. In the mouse cortex CCK_B-radioligand binding assay, a pK_i value of 4.16 was estimated (Table 3).

Non-CCK/gastrin-receptor selectivity 2-NAP was inactive at 100 μM in several functional bioassays unrelated to the CCK/gastrin family of receptors with the exception of an antihistaminic action observed on the guinea-pig ileum equivalent to a pK_B value of ~4.4 (Table 3).

In addition, 37 radioligand binding assays 2-NAP had no significant activity.

Discussion

2-NAP behaved as a simple competitive antagonist of CCK-8 in a number of CCK_A-receptor assays (pK_B~6.5). Previously, combined dose-ratio analyses, *inter alia*, have been used to establish that loxiglumide, lorglumide and devazepide interact with CCK-8 at a homogeneous population of receptors in the guinea-pig gall bladder assay (Bishop *et al.*, 1992a). This conclusion is supported by the finding here that 2-NAP behaved competitively and syntopically at CCK_A-receptors with two different chemical classes of CCK_A-receptor antagonist (devazepide, a benzodiazepine-based non-peptide and the tetrapeptide-derived compound, 4-CTP). Additionally, on the guinea-pig gall bladder, the antagonism was shown to be independent of the choice of CCK_A-receptor agonist because 2-NAP had similar affinity against both CCK-8 and caerulein. In preliminary studies in rat pancreas fragments, prepared in the same way as the guinea-pig pancreas fragments, 2-NAP had no agonist action and antagonized the secretory effect of CCK-8 giving an estimated pK_B value of ~6.5 (data not shown).

Interestingly, the variance about the pK_B estimate made on the guinea-pig pancreatic fragment assay was considerably smaller than the others (Table 1). The inability to fit objectively the individual E/[A] curves from this assay was compensated by a high degree of reproducibility between individual assay replicates as judged by the log [A_{50}] estimates.

In the ileum assay the presence of CCK_B/gastrin-receptors coupled to smooth muscle contraction necessitated the inclusion of a selective CCK_B/gastrin-receptor antagonist (L-365,260) in the bathing solution. The concentration chosen was slightly less than the reported equilibrium dissociation constant for CCK_A-receptors (~300 nM, Lotti & Chang, 1989). According to the principle of combined dose-ratios (Paton & Rang, 1965), if the concentration used was equal to the K_B value for CCK_A-receptor blockade the error in the estimation of the affinity of 2-NAP would be expected to be 0.3 log units, which may not be experimentally-detectable. Notwithstanding this, the ileum pK_B estimate was 0.34 log unit lower than the value obtained on the guinea-pig gall bladder assay, although this difference was not significant.

Analysis of the equilibrium dissociation constants for all the CCK_A-receptor assays indicated that the rabbit gall bladder pK_B estimate was atypical. This preliminary suggestion of receptor heterogeneity was supported by the finding that the devazepide pK_B estimate (8.87) in this assay appears to be low; most other estimates of the equilibrium dissociation constant made in assays containing CCK_A receptors are approximately 0.1 nM (pK_B 9.98, guinea-pig gall-bladder, Bishop *et al.*, 1992a; pK_B 10.09, guinea-pig ileum, D'Amato *et al.*, 1991; pA_2 9.89 and 9.93, guinea-pig ileum and colon respectively, Chang & Lotti, 1986; pA_2 9.6, rat pancreatic acini amylase secretion, Louie *et al.*, 1988) although lower values have been reported (pK_d 9.28, rat solubilized pancreatic CCK receptors, Chang *et al.*, 1987; pK_i 8.4, guinea-pig pancreatic acini, Huang *et al.*, 1989). The fact that the

Table 3 Summary of selectivity studies with 2-NAP

Receptor	*Assay*	*Agonist*	*Result*
Gastrin	mouse stomach[a]	pentagastrin	IA 100 μM
Gastrin	g-p fundus[b]	pentagastrin	IA 100 μM
$5\text{-HT}_{1C/1D/3}$	rat fundus[c]	5-HT	IA 100 μM
5-HT_2	rabbit aorta[d]	5-HT	IA 100 μM
Angiotensin II	rabbit aorta[e]	angiotensin II	IA 100 μM
Histamine H_1	g-p ileum[f]	histamine	pK_B~4.4
Histamine H_2	g-p right atrium[g]	histamine	IA 100 μM
β_1-Adrenoceptors	g-p right atrium[h]	isoprenaline	IA 100 μM
β_2-Adrenoceptors	g-p trachea[i]	salbutamol	IA 100 μM
Muscarinic-ACh	g-p trachea[j]	5-mef	IA 100 μM
Nicotinic-ACh	frog rectus[k]	ACh	IA 100 μM
α_1-Adrenoceptor	rabbit aorta[l]	Noradrenaline	IA 100 μM
EDRF-release	rat aorta[m]	5-mef	IA 100 μM
Chronotropy	g-p right atrium[n]		IA 100 μM
CCK_B	g-p cerebral cortex[o]		$pK_i = 4.16 \pm 0.11$

The basic methods used were, as far as possible, based on the following references: [a]Black & Shankley, 1985; [b]Bishop *et al.*, 1992b; [c]Frankhuijzen & Bonta, 1974; [d]Black & Shankley, 1990; [e]Liu *et al.*, 1992; [f]Kenakin, 1980; [g]Shankley *et al.*, 1988; [h]Black *et al.*, 1985a; [i]Kenakin, 1980–using rings of trachea; [j]Shankley *et al.*, 1988; [k]Edinburgh Staff, 1970; [l]Beckeringh *et al.*, 1984; [m]Berg *et al.*, 1992; [n]Black *et al.*, 1986; [o]Clark *et al.*, 1986 and Chang & Lotti, 1986.
IA: inactive.

affinity estimate of 2-NAP was higher and that of devazepide lower, than expected implies that the differences did not arise from differences in the ligand concentration, for example, due to loss of the antagonist from the domain of the receptors or saturable CCK-8 uptake.

Although the pK_B estimate for 2-NAP on the human gall bladder was low, the importance of this finding warrants further study. The limited number of experiments from a single tissue sample was associated with a large variance and consequently the pK_B estimate was not different from the global mean values. However, classification of the human gall bladder receptors mediating the contraction to CCK is unclear; for example, in one report, devazepide was found to be weakly active as an antagonist ($pK_B < 6$) of CCK-8-induced contractions (D'Amato *et al.*, 1991) while another study found that devazepide potently ($IC_{50} = 1$ nM) displaced [^{125}I]-D-Tyr-Gly[Nle^{28,31},pNO_2-Phe^{33}]CCK-26-33 binding to human gall bladder CCK binding sites (Schjoldager *et al.*, 1989).

The high selectivity of 2-NAP (> 300 fold) for CCK_A- over the hormone-family-related gastrin/CCK_B-receptors was shown, in functionally-intact tissues, by the failure of 2-NAP to antagonize the effects of pentagastrin in the guinea-pig fundus and mouse stomach assays. This conclusion was reinforced by the radioligand displacement studies (CCK_B, cerebral cortex:CCK_A, pancreas) which gave an affinity ratio of 180 fold in comparison to other CCK_A-receptor antagonists, for example, lorglumide (100 fold) (Lotti & Chang, 1989), devazepide (3000 fold) (Chang & Lotti, 1986) and loxiglumide (30 fold) (Setnikar *et al.*, 1987). Studies using an extensive range of bioassays confirmed the high specificity of 2-NAP with respect to other pharmacological sites of action (Table 3).

The partition coefficient of 2-NAP (log P − 0.9) measured between chloroform and Krebs-Henseleit was found to be 100 000 fold and 23 fold respectively lower than the other CCK_A-receptor antagonists, devazepide (log P 4.1) and loxiglumide (log P 0.5). The lipophilicity of drugs is recognised as an important determinant of the rate at which they cross biological membranes such as the blood-brain barrier (Rall *et al.*, 1959). Therefore, we expect that the actions of 2-NAP will be predominantly confined *in vivo* to peripheral CCK_A-receptors. Therefore, 2-NAP might be a useful tool for future investigations of the physiology and pharmacology of CCK_A-receptors.

References

D'AMATO, M., STAMFORD, I.F. & BENNETT, A. (1991). Studies of three non-peptide cholecystokinin antagonists (devazepide, lorglumide, and loxiglumide) in human isolated alimentary muscle and guinea-pig ileum. *Br. J. Pharmacol.*, **102**, 391–395.

BECKERINGH, J.J., THOOLEN, J.M.C., DE JONGE, A., WILFFERT, R., TIMMERMANS, P.B.M.W.M. & VAN ZWIETEN, P.A. (1984). Differential effects of the calcium entry blocker D600 on contractions of rat and guinea-pig aortas, elicited by various alpha-1 adrenoceptor agonists. *J. Pharmacol. Exp. Ther.*, **229**, 515–521.

BERG, T., SCHOYEN, H., WASSDAL, I., HULL, R.A.D., GERSKOWITCH, P. & TOFT, K. (1992). Characterization of a new kallikrein-like enzyme (KLP-S3) of the rat submandibular gland. *Biochem. J.*, **281**, 819–828.

BISHOP, L.A., GERSKOWITCH, V.P., HULL, R.A.D., SHANKLEY, N.P. & BLACK, J.W. (1992a). Combined dose-ratio analysis of cholecystokinin receptor antagonists, devazepide, lorglumide and loxiglumide in the guinea-pig gall bladder. *Br. J. Pharmacol.*, **106**, 61–66.

BISHOP, L.A., GERSKOWITCH, V.P., HULL, R.A.D., SHANKLEY, N.P. & BLACK, J.W. (1992b). Characterisation of the CCK_A and gastrin receptors coupled to muscle contraction in guinea-pig fundus using receptor desensitisation. *Br. J. Pharmacol.*, **107**, 36P.

BLACK, J.W., DUNCAN, W.A.M., DURANT, C.J., GANELLIN, C.R. & PARSONS, E.M. (1972). Definition and antagonism of histamine H_2-receptors. *Nature*, **236**, 385–390.

BLACK, J.W., GERSKOWITCH, V.P., LEFF, P. & SHANKLEY, N.P. (1985a). Pharmacological analysis of β-adrenoceptor-mediated agonism in the guinea-pig, isolated, right atrium. *Br. J. Pharmacol.*, **84**, 779–785.

BLACK, J.W., GERSKOWITCH, V.P., LEFF, P. & SHANKLEY, N.P. (1986). Analysis of competitive antagonism when this property occurs as part of a pharmacological resultant. *Br. J. Pharmacol.*, **89**, 547–555.

BLACK, J.W., LEFF, P. & SHANKLEY, N.P. (1985b). Further analysis of anomalous pK_B values for histamine H_2-receptor antagonists on the isolated mouse stomach assay. *Br. J. Pharmacol.*, **86**, 581–587.

BLACK, J.W. & SHANKLEY, N.P. (1985). The isolated stomach preparation of the mouse: a physiological unit for pharmacological analysis. *Br. J. Pharmacol.*, **86**, 571–579.

BLACK, J.W. & SHANKLEY, N.P. (1990). Interpretation of agonist affinity estimations: the question of distributed receptor states. *Proc. R. Soc. B.*, **240**, 503–518.

BLACK, J.W. & STEPHENSON, J.S. (1962). Pharmacology of a new adrenergic beta-receptor blocking compound (nethalide). *Lancet*, **II**, 311–314.

CHANG, R.S.L. & LOTTI, V.J. (1986). Biochemical and pharmacological characterization of an extremely potent and selective nonpeptide cholecystokinin antagonist. *Proc. Natl. Acad. Sci., U.S.A.*, **83**, 4923–4926.

CHANG, R.S.L., LOTTI, V.J. & CHEN, T.B. (1987). Characterization of [^{3}H](±)L-364, 718 binding to solubilized cholecystokinin (CCK) receptors of rat pancreas. *Biochem. Pharmacol.*, **36**, 1709–1714.

CLARK, C.R., DAUM, P. & HUGHES, J. (1986). A study of the cerebral cortex cholecystokinin receptors using two radioligand probes: evidence for a common CCK-8 and CCK-4 cholecystokinin receptor binding site. *J. Neurochem.*, **46**, 1094–1101.

COCHRAN, W.G. (1954). The combination of estimates from different experiments. *Biometrics*, **10**, 101–129.

DA-HONG, Y., NOGUCHI, M., ZHI-CHAO, Z., VILLANUEVA, M.L., GARDNER, J.D. & JENSEN, R.T. (1987). Characterisation of gastrin receptors on guinea pig pancreatic acini. *Am. J. Physiol.*, **253**, G793–G801.

EDINBURGH STAFF (1970). In *Pharmacological Experiments on Isolated Preparations*. 2nd Edition. Edinburgh: Livingstone,

FRANKHUIJZEN, A.L. & BONTA, I.L. (1974). Receptors involved in the action of 5-HT and tryptamine on the isolated rat stomach fundus preparation. *Eur. J. Pharmacol.*, **26**, 220–230.

HORWELL, D.C., BIRCHMORE, B., BODEN, P.R., HIGGINBOTTOM, M., PING HO, Y., HUGHES, J., HUNTER, J.C. & RICHARDSON, R.S. (1990). α-Methyl tryptophanylphenylalanines and their arylethylamine 'dipeptoid' analogues of the tetrapeptide cholecystokinin (30–33). *Eur. J. Med. Chem.*, **25**, 53–60.

HUANG, S.C., ZHANG, L., CHIANG, H.-C.V., WANK, S.A., MATON, P.N., GARDNER, J.D. & JENSEN, R.T. (1989). Benzodiazepine analogues L365,260 and L364,718 as gastrin and pancreatic CCK receptor antagonists. *Am. J. Physiol.*, **257**, G169–G174.

HUGHES, J., BODEN, P., COSTALL, B., DOMENEY, A., KELLY, E., HORWELL, D.C., HUNTER, J.C., PINNOCK, R.D. & WOODRUFF, G.N. (1990). Development of a class of selective cholecystokinin type B receptor antagonists having potent anxiolytic activity *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 6728–6732.

JENSEN, R.T., JONES, S.W. & GARDNER, J.D. (1983). Carboxy-terminal fragments of cholecystokinin. A new class of cholecystokinin receptor antagonists. *Biochim. Biophys. Acta*, **757**, 250–258.

KENAKIN, T.P. (1980). Effect of equilibration time on the attainment of equilibrium between antagonists and drug receptors. *Eur. J. Pharmacol.*, **66**, 295–306.

LIU, Y.J., SHANKLEY, N.P., WELSH, N.J. & BLACK, J.W. (1992). Evidence that the apparent complexity of receptor antagonism by angiotensin-II analogues is due to a reversible and syntopic action. *Br. J. Pharmacol.*, **106**, 233–241.

LOTTI, V.J. & CHANG, R.S.L. (1989). A new potent and selective non-peptide gastrin antagonist and brain cholecystokinin receptor (CCK-B) ligand: L365260. *Eur. J. Pharmacol.*, **162**, 273–280.

LOUIE, D.S., LIANG, J.P. & OWYANG, C. (1988). Characterization of a new CCK antagonist, L364,718: in vitro and in vivo studies. *Am. J. Physiol.*, **255**, G261–G266.

LUCAITES, V.L., MENDELSOHN, L.G., MASON, N.R. & COHEN, M.L. (1991). CCK-8, CCK-4 and gastrin-induced contractions in guinea pig ileum: evidence for differential release of acetylcholine and substance P by CCK-A and CCK-B receptors. *J. Pharmacol. Exp. Ther.*, **256**, 695–703.

MARSHALL, G.R., VINE, W. & NEEDLEMAN, P. (1970). Specific competitive inhibitor of angiotensin II. *Proc. Natl. Acad. Sci. U.S.A.*, **67**, 1624–1630.

MORLEY, J.S., TRACY, H.J. & GREGORY, R.A. (1965). Structure-function relationships in the active C-terminal tetrapeptide sequence of gastrin. *Nature*, **207**, 1356–1359.

PATON, W.D.M. & RANG, H.P. (1965). The uptake of atropine and related drugs by intestinal smooth muscle of the guinea-pig in relation to acetylcholine receptors. *Proc. R. Soc. B.*, **163**, 1–44.

RALL, D.P., STABENAU, J.R. & ZUBROD, C.G. (1959). Distribution of drugs between blood and cerebrospinal fluid: General methodology and effects of pH gradients. *J. Pharmacol. Exp. Ther.*, **125**, 185–193.

SCARPIGNATO, C., VARGA, G., DOBRONYI, I. & PAPP, M. (1989). Effect of a new potent CCK antagonist lorglumide, on caerulein- and bombesin-induced pancreatic secretion and growth in the rat. *Br. J. Pharmacol.*, **96**, 661–669.

SCHJOLDAGER, B., MOLERO, X. & MILLER, L.J. (1989). Functional and Biochemical Characterization of the human gallbladder muscularis cholecystokinin receptor. *Gastroenterology*, **96**, 1119–1125.

SETNIKAR, I., BANI, M., CEREDA, R., CHISTE, R., MAKOVEC, F., PACINI, M.A., REVEL, L., ROVATI, L.C. & ROVATI, L.A. (1987). Pharmacological characterisation of a new potent and specific nonpolypeptide cholecystokinin antagonist. *Arz.-Forsch.*, **37**, 703–707.

SHANKLEY, N.P., BLACK, J.W., GANELLIN, C.R. & MITCHELL, R.C. (1988). Correlation between log $P_{OCT/H20}$ and pK_B estimates for a series of muscarinic and histamine H_2-receptor antagonists. *Br. J. Pharmacol.*, **94**, 264–274.

SPANARKEL, M., MARTINEZ, J., BRIET, C., JENSEN, R.T. & GARDNER, J.D. (1983). Cholecystokinin-27-32-amide a member of a new class of cholecystokinin receptor antagonists. *J. Biol. Chem.*, **258**, 6746–6749.

VIZI, E.S., BERTACCINI, G.M. IMPICCIATORE, M. & KNOLL, J. (1972). Acetylcholine-releasing effect of gastrin and related peptides. *Eur. J. Pharmacol.*, **17**, 175–178.

WOODRUFF, G.N. & HUGHES, J. (1991). Cholecystokinin antagonists. *Annu. Rev. Pharmacol. Toxicol.*, **31**, 469–501.

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Mechanism of staurosporine-induced decrease in acetylcholine receptor recovery from desensitization

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1 Previously, we showed in voltage-clamped snake twitch muscle fibres that the extent of recovery of the nicotinic acetylcholine (ACh) receptor from carbachol-induced desensitization is reduced by pretreatment with the protein kinase inhibitor staurosporine. The present studies were undertaken to determine the mechanism underlying the staurosporine-induced inhibition of recovery.

2 Pretreatment with 0.5 μM staurosporine significantly decreased the extent of recovery of spontaneous miniature endplate current (m.e.p.c.) amplitudes in preparations exposed to 540 μM carbachol. The decrease in recovery of m.e.p.c. amplitude by staurosporine was dependent on the duration of carbachol exposure. No significant decrease in m.e.p.c. amplitude was observed with a 1 min exposure to agonist, whereas a significant decrease in recovery was seen with agonist exposures between 5–10 min. Further, the effect of staurosporine pretreatment on ACh receptor recovery was long-lasting such that m.e.p.c. amplitude remained decreased for at least 60 min.

3 Estimation of mean channel conductance by noise analysis during local perfusion of 20 μM carbachol demonstrated a decrease in conductance from 52 pS to 23 pS in staurosporine-treated preparations following recovery from desensitization. Staurosporine treatment in the absence of desensitization did not alter the mean channel conductance.

4 A single population of ACh-activated single channel currents with a conductance of 45–49 pS was recorded in cell-attached patches from enzymatically cleaned endplates in control and staurosporine-treated preparations not exposed to carbachol.

5 At staurosporine-treated endplates exposed to carbachol and then allowed to recover, a population of small conductance (23 pS) channels was observed. These channels were not normally seen in control preparations which had undergone carbachol-induced desensitization and recovery.

6 We suggest that the decrease in m.e.p.c. amplitude observed following recovery from desensitization in staurosporine-treated endplates results from the activation of a mixture of small and large conductance ACh receptor channels.

Keywords: Nicotinic acetylcholine receptors; staurosporine; desensitization; protein phosphorylation; neuromuscular junction; single channels; m.e.p.c.

Introduction

Desensitization of the nicotinic acetylcholine (ACh) receptor (nicotinic cholinoceptor) is the gradual loss of receptor response produced during prolonged exposure to cholinoceptor agonists. Following removal of the agonist, endplate sensitivity returns as ACh receptors are converted to an activatable state (Katz & Thesleff, 1957). Recently, we demonstrated that pretreatment with staurosporine, a potent inhibitor of several protein kinases (Tamaoki *et al.*, 1986), reduced the extent of recovery from carbachol-induced desensitization (Hardwick *et al.*, 1991). Based on these observations, we proposed that phosphorylation of the ACh receptor or an associated protein may be required for complete recovery from desensitization. Other investigations had previously demonstrated that the ACh receptor is a substrate for phosphorylation by at least three different protein kinases and that phosphorylation of the receptor is one of the mechanisms regulating the rate of desensitization (Huganir & Greengard, 1990).

In our initial studies, we did not establish the mechanism responsible for the staurosporine-induced decrease in the extent of recovery. Further, we did not test whether the inhibitory effect of staurosporine on recovery required that most ACh receptors be fully desensitized or whether the decrease in recovery would occur equally well following short exposures to agonist which do not produce substantial desensitization. Therefore, the experiments described in this report were designed to (1) determine the mechanism underlying the staurosporine-induced decrease in recovery from desensitization and (2) test whether this inhibitory action of staurosporine requires that the agonist exposure be sufficiently long to desensitize completely most of the activatable ACh receptors.

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Methods

General methods

All experiments were done on potassium-depolarized twitch fibre endplates of costocutaneous muscles isolated from garter snakes (*Thamnophis*) following procedures described previously (Connor *et al.*, 1984; Fiekers *et al.*, 1987; Hardwick *et al.*, 1991). Snakes were killed by rapid decapitation and the muscles, with portions of the rib and skin, were pinned out on sylgard-coated Petri dishes in a control physiological solution containing in mM: NaCl 159, KCl 2.5, $MgCl_2$ 4.2, $CaCl_2$ 1.0, HEPES 1.0, pH 7.3 at 20–22°C (Dionne & Parsons, 1981). The muscle preparations were then bathed for 20–30 min in an isotonic potassium propionate solution (in mM: potassium propionate 161, $MgCl_2$ 4.2, $CaCl_2$ 1.0, $CsCl_2$ 5.0, HEPES 1.0, pH 7.3 at 20–22°C) before the experiments were started. The potassium-depolarized muscle preparation was used for several reasons. First, no depolarization of the muscle fibres occurred when agonist was applied. Therefore, membrane voltage was constant during both desensitization onset and recovery. Second, the frequency of spontaneous miniature endplate currents (m.e.p.cs) was increased in the high potassium solution such that an adequate

number of m.e.p.cs could be recorded in a short time period to allow the use of the mean m.e.p.c. amplitude to estimate endplate sensitivity (Hardwick *et al.*, 1991). Lastly, the endplate region could be voltage clamped over a wide range of membrane voltages with only one species of monovalent cation (K^+) contributing significantly to m.e.p.cs or ACh-induced single channel currents. Carbachol (540 μM, Sigma Chemical Co., St. Louis, MO, U.S.A.) and staurosporine (0.5 μM in 0.05% dimethyl sulphoxide (DMSO), Calbiochem, San Diego, CA, U.S.A.) were added directly to the isotonic potassium solution bathing the muscle preparations. In the case of staurosporine, the preparations were exposed for 10–15 min to the drug and then washed several times with drug-free isotonic potassium solution prior to initiating the experiments. The concentration of 0.5 μM was chosen on the basis of our previous studies (Hardwick *et al.*, 1991).

Electrophysiological methods

For standard voltage clamp experiments, the endplate region of an individual twitch fibre was voltage-clamped by a two microelectrode system similar to that employed in previous studies (Dionne & Parsons, 1981; Connor *et al.*, 1984; Fiekers *et al.*, 1987). M.e.p.cs were recorded from fibres voltage-clamped between − 165 mV and + 50 mV and a 20–30 s sample was stored on a FM tape recorder for later analysis. M.e.p.c. frequence was approximately 10 events s^{-1} and showed no apparent change following any of the treatments. Mean m.e.p.c. amplitude and decay time constant were determined from an average of 50–100 m.e.p.cs by use of the programme SCAN, provided by Dr John Dempster (University of Strathclyde).

For noise analysis, recordings were obtained from endplates voltage-clamped to − 100 mV which were initially perfused with the potassium propionate solution to establish the background noise and then perfused with the same solution containing 20 μM carbachol. Mean channel conductance was determined by a computer programme, SPAN, written by Dr John Dempster (University of Strathclyde). Mean channel conductance was determined for control and staurosporine-treated preparations before and after exposure to 540 μM carbachol for 5–10 min. M.e.p.cs were recorded from several different fibres in the same preparations and averaged.

Cell-attached patch clamp techniques were used for single channel recording (Hamill *et al.*, 1981). Patch pipettes were fabricated from borosilicate glass (Corning 7052, o.d. 1.50 mm, i.d. 1.10 mm), coated with wax to reduce capacitance and connected to an Axopatch-1C patch clamp amplifier. The patch pipette contained the isotonic potassium propionate solution with the magnesium chloride concentration raised to 8.2 mM to facilitate seal formation and 1 μM ACh to activate the ACh receptors. The current output signal was low-pass filtered at 2 kHz (8-pole Bessel) and stored on a videocassette recorder for later analysis. The current signal was sampled at 33 kHz for analysis using the programme pClamp (version 5.5.1, Axon Instruments).

To patch clamp the endplate region, the nerve terminal was removed by enzymatic dissociation (Betz & Sakmann, 1973). Muscle preparations were incubated for 2 h at room temperature in the control sodium-containing solution containing 0.2% collagenase (Type 1A, Sigma). The muscles were then rinsed with the isotonic potassium solution (to allow the muscles to contract and relax) before starting an incubation for 30–45 min in the potassium propionate solution containing 0.02% protease (Type XXV, Sigma). The nerve was then gently pulled off and the endplates exposed for patch clamping.

Statistical analyses

All results are expressed as the mean ± s.e.mean obtained in a number of different fibres. In addition, each series of experiments was done using at least three different animals. Statistical significance was determined by Student's *t* test or analysis of variance (ANOVA) with $P<0.05$ considered significant.

Results

The staurosporine-induced inhibition of recovery is dependent on the duration of carbachol exposure and is long-lasting

In the present study, we have used mean m.e.p.c. amplitude to estimate the recovery from carbachol-induced desensitization. For the initial series of experiments, m.e.p.cs were recorded from fibres voltage-clamped to − 100 mV. Recordings were obtained from different fibres in the same muscle preparations. The control preparations were kept in the isotonic potassium solution and the test preparations were pretreated with isotonic potassium containing 0.5 μM staurosporine for 10–15 min. Some of the control and test preparations were then exposed to 540 μM carbachol for 1–10 min followed by a recovery period of 5–15 min with drug-free isotonic potassium solution before the m.e.p.cs were recorded. The results of this initial series of experiments showed that the staurosporine-induced decrease in m.e.p.c. amplitude following exposure to carbachol depended on the duration of agonist exposure (Figure 1). The mean m.e.p.c. amplitude following recovery from the carbachol application was significantly less in staurosporine-treated preparations when the agonist was present for 5 or 10 min, but not in preparations exposed to carbachol for only 1 min. Note also that the mean m.e.p.c. amplitude recorded from control fibres following the recovery from carbachol decreased as a function of the duration of exposure to agonist.

A second series of experiments was completed to establish how long the staurosporine-induced decrease in extent of recovery lasted. For these experiments, the recovery of m.e.p.c. amplitude was estimated in fibres voltage-clamped to − 100 mV after an initial 5 min exposure to 540 μM carbachol followed by either a 5–15 min recovery period or a 60 min recovery period. The results, summarized in Table 1,

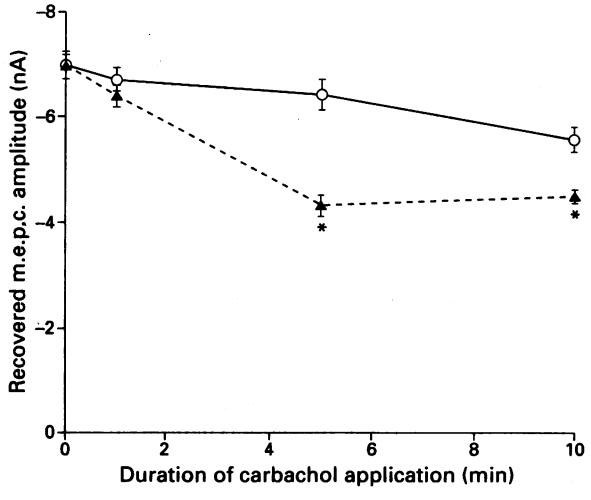


Figure 1 The inhibition of the extent of recovery by staurosporine is dependent on the duration of carbachol application. Control (○) or staurosporine (0.5 μM)-treated preparations (▲) were incubated in isotonic potassium solution containing 540 μM carbachol for 1, 5, or 10 min and then rinsed with agonist-free isotonic potassium solution. M.e.p.cs were recorded from endplates voltage-clamped to − 100 mV 5–15 min after the start of the wash. The average m.e.p.c. amplitude versus the duration of carbachol application is shown. Pretreatment with staurosporine caused a significant decrease in m.e.p.c. amplitude versus control preparations (*$P<0.05$ by Student's *t* test) with 5 and 10 min carbachol applications only. The points represent the mean ± s.e.mean for 10 or more individual fibres.

Table 1 Long-lasting inhibition of the extent of recovery from desensitization by staurosporine

Wash time:	5–15 min		>60 min	
	m.e.p.c. amplitude (nA)	τ (ms)	*m.e.p.c. amplitude* (nA)	τ (ms)
Control	− 6.43 ± 0.25 (18)	1.73 ± 0.08 (18)	− 6.64 ± 0.40 (13)	1.60 ± 0.08 (13)
Staurosporine	− 4.32 ± 0.20** (24)	1.67 ± 0.06^{NS} (24)	− 5.18 ± 0.30* (9)	1.39 ± 0.07^{NS} (9)

Control and staurosporine (0.5 μM)-treated preparations were incubated with 540 μM carbachol for 5 min and then washed with control potassium propionate solution. M.e.p.cs were recorded from endplates voltage-clamped to − 100 mV at either 5–15 min or 60 min following the start of the wash. Values represent the mean m.e.p.c. amplitude ± s.e.mean of *n* fibres (shown in parentheses). There was no significant change in m.e.p.c. amplitude in control preparations after recovery versus pre-carbachol amplitudes. Pretreatment with staurosporine produced a significant decrease in m.e.p.c. amplitude verus control values at both early and late recovery times with no significant change in the decay time constant.

**$P < 0.001$ vs control by Student's *t* test

*$P < 0.02$ vs control by Student's *t* test

^{NS}not significantly different from control.

indicate that there was no significant difference in the mean m.e.p.c. amplitude or decay time constant following exposure to carbachol in the control preparations after recovery times of 5–15 min or 60 min. Previously, we showed that the m.e.p.c. amplitude returned to its maximum value within a few minutes (Hardwick *et al.*, 1991). The present results show that in preparations which were pretreated with 0.5 μM staurosporine, the mean m.e.p.c. amplitude remained significantly smaller than that at control endplates after 60 min of recovery. Although the m.e.p.c. amplitude was decreased, the decay time constant remained unchanged. The long duration of action was fortuitous because it insured that we could use the same experimental protocol in the patch clamp studies to investigate potential mechanisms underlying the staurosporine-induced decrease in extent of recovery from desensitization.

The reversal potential of m.e.p.cs was not different at control and staurosporine treated endplates following recovery from carbachol exposure

A difference in mean m.e.p.c. amplitude between control and staurosporine-treated fibres would be expected in fibres voltage-clamped to − 100 mV (following carbachol exposure) if staurosporine shifted the m.e.p.c. reversal potential to a value more negative than that of the control m.e.p.cs. Consequently, experiments were done to test whether staurosporine treatment altered the m.e.p.c. reversal potential either in the absence of carbachol exposure or during the recovery following a 5 min exposure to carbachol. Initially, the m.e.p.c. amplitude-voltage relationship was determined in control preparations and preparations pretreated with 0.5 μM staurosporine. Then the m.e.p.c. amplitude-voltage relationship was determined at control and staurosporine-treated endplates which had been exposed to 540 μM carbachol for 5 min and allowed to recover in carbachol-free solution for 5–15 min. Staurosporine pretreatment did not change the m.e.p.c. amplitude-voltage relationship in the absence of carbachol exposure (Figure 2a). However, following recovery from a 5 min exposure to carbachol, m.e.p.c. amplitudes at staurosporine-treated endplates were smaller at all voltages than at control (Figure 2b). Although the m.e.p.c. amplitudes were smaller at staurosporine-treated endplates after an exposure to carbachol, the decrease in amplitude occurred without a significant change in the value of the reversal potential (Figure 2b and Table 4).

Following carbachol exposure at staurosporine-treated endplates, mean channel conductance is decreased along with the m.e.p.c. amplitude

Previously, we concluded that in staurosporine-treated preparations, the decrease in m.e.p.c. amplitude during recovery from desensitization was not due to a presynaptic action of

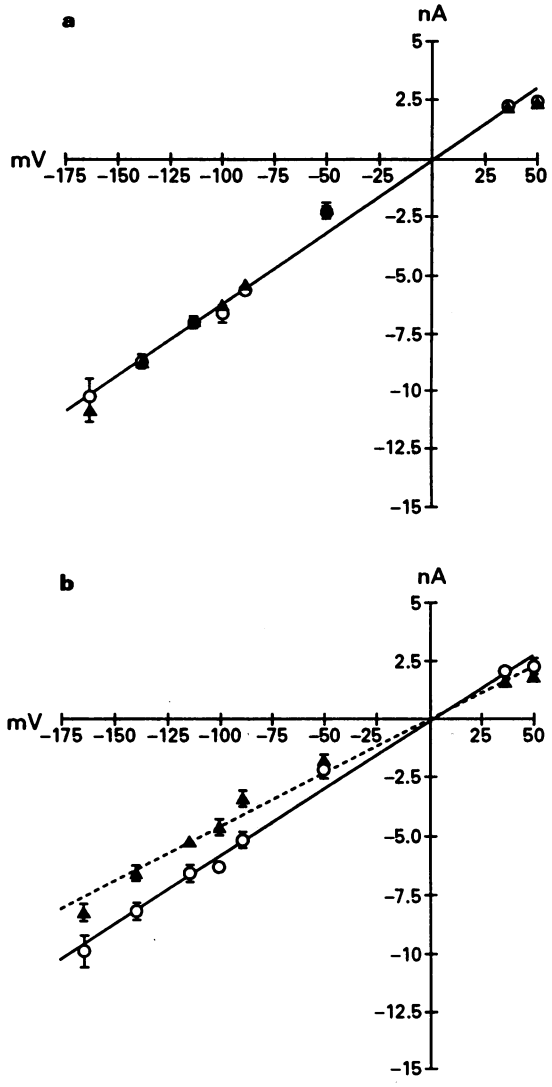


Figure 2 Current-voltage (*I-V*) relationships for miniature endplate currents before and after carbachol-induced desensitization. M.e.p.c. amplitude was determined at several different holding potentials in control (○) and staurosporine (0.5 μM)-treated preparations (▲) with and without incubation with 540 μM carbachol (5 min). (a) *I-V* curves from control and staurosporine-treated endplates prior to carbachol treatment show no difference in slope or reversal potential. (b) *I*-V curves from control and staurosporine-treated fibres following carbachol-induced desensitization show a decrease in the amplitude of m.e.p.cs with staurosporine treatment at all voltages with no change in the reversal potential. Points represent the mean ± s.e.mean (vertical bars) of 4 or more individual fibres and the lines are first order, least-squares regression lines.

Table 2 Comparison of mean channel conductance (γ) and m.e.p.c. amplitude at control and staurosporine-treated endplates

	γ (pS)	*m.e.p.c.* (nA)	n
Control	41 ± 8	− 7.81 ± 0.31	4
Control recovery	56 ± 5	− 6.48 ± 0.27	6
Staurosporine	52 ± 6	− 8.04 ± 0.22	4
Staurosporine recovery	23 ± 9*	− 5.22 ± 0.20*	5

Analysis of the endplate current noise produced during application of 20 μM carbachol at control and 0.5 μM staurosporine-treated preparations was performed before and after carbachol-induced desensitization and subsequent recovery. M.e.p.c. amplitudes were determined from separate fibres in the same preparations. Results shown are the means ± s.e.mean from *n* fibres for each condition. Staurosporine treatment resulted in a significant decrease in the estimated mean channel conductance and m.e.p.c. amplitude following recovery from desensitization.
*$P < 0.05$ by ANOVA.

staurosporine (Hardwick *et al.*, 1991). To provide further support for this conclusion we estimated the mean channel conductance using noise analysis. The results from noise analysis demonstrated that the mean channel conductance was not different at control and staurosporine-treated endplates in the absence of desensitization (Table 2). However, following recovery from a 5–10 min incubation with 540 μM carbachol, the mean channel conductance was decreased by 56% at staurosporine-treated endplates. In these preparations, the m.e.p.c. amplitude was decreased by 35%.

A small conductance channel is present during recovery from carbachol exposure at staurosporine-treated preparations

The results from the noise analysis suggested that the decrease in m.e.p.c. amplitude following recovery from carbachol exposure in staurosporine-treated preparations was due to a decrease in mean channel conductance. Therefore, single channel currents were measured to determine whether the conductance of all channels were decreased or whether a subpopulation of ACh receptor channel complexes exhibited a decrease in conductance. ACh-activated single channel currents were measured in cell-attached patches on the endplate region of fibres not exposed to carbachol and on fibres which were exposed for 5 min to 540 μM carbachol and then allowed to recover for 5–15 min in the isotonic potassium solution. Recordings were made from control preparations and preparations pretreated with 0.5 μM staurosporine for 15 min.

The initial single channel recordings were made with the patch held at − 100 mV. We found that the ACh-activated channels at both control and staurosporine-treated endplates in the absence of carbachol exposure exhibited one conductance level (Figure 3). At both control endplates and staurosporine-treated endplates kept in the isotonic potassium solution, the open time distribution of the ACh-activated channels did not always adequately fit as a single exponential function. Consequently, in roughly 70% of the patches made on both control and staurosporine-treated preparations, the open time distribution was fitted best as the sum of two exponential functions. There was no significant difference in the open time distributions at control and staurosporine-treated fibres (Table 3).

We found that single channel activity could not be recorded for prolonged periods at both control and staurosporine-treated endplates which had been exposed to 540 μM carbachol and then allowed to recover for 5–15 min prior to patch formation. Channel activity was evident immediately after patch formation, but then rapidly decreased so that the recording period was consistently shorter at these endplates than in preparations which had not been exposed to carbachol. However, only the frequency of channel activity and not the amplitude changed over the recording period. We attribute the rapid loss of channel activity to an acceleration of the rate of desensitization during recovery from a prior exposure to agonist (Scubon-Mulieri & Parsons, 1977; Wagoner & Pallotta, 1988). Even though the recording period was abbreviated and limited the time over which channel activity could be recorded in a given patch, we were able to compare the single channel properties of ACh-activated currents at control and staurosporine-treated preparations after recovery from exposure to carbachol. At control fibres, following recovery from a 5 min carbachol exposure, both the mean amplitude and the open time distribution of the ACh-activated currents recorded at − 100 mV were not significantly different from those estimated for endplates not exposed to carbachol (Table 3). Further, in 6 of 7 control endplates tested, only one population of ACh-activated currents was observed and the current amplitude remained essentially constant throughout the entire recording period (2

Table 3 Summary of single channel properties of acetylcholine (ACh)-activated channels from control and staurosporine-treated fibres before and after carbachol-induced desensitization

	Before		*Recovery*		
				Staurosporine	
	Control	*Staurosporine*	*Control*	*Small channel*	*Large channel*
Amplitude (pA)	− 4.40 ± 0.26 (7)	− 5.26 ± 0.26 (5)	− 4.76 ± 0.45 (6)	− 2.36 ± 0.29 (6)	− 5.23 ± 0.11 (7)
Open Times					
Single exponential τ (ms)	1.19 ± 0.13 (3)	1.47 ± 0.30 (2)	1.50 ± 0.33 (3)	0.26 (1)	1.57 ± 0.58 (2)
Double exponential τ_1 (ms)	0.53 ± 0.18 (4)	0.66 ± 0.12 (3)	0.44 ± 0.05 (3)	0.04 ± 0.01 (2)	0.98 ± 0.24 (2)
τ_2 (ms)	6.04 ± 1.39 (4)	4.76 ± 0.80 (3)	7.32 ± 1.32 (3)	3.88 ± 2.49 (2)	3.92 ± 1.63 (2)
Relative contributions ($weight_1/weight_2$)	0.62 ± 0.25	0.64 ± 0.28	0.36 ± 0.06	1.75 ± 0.07	0.85 ± 0.58

ACh (1 μM)-activated currents were recorded from cell-attached patches at control and staurosporine (0.5 μM)-treated preparations with and without desensitization by a 5 min incubation with 540 μM carbachol. Mean channel amplitude and open times at − 100 mV are shown. Channel open time distributions were fit with both one or two exponentials and the parameters of the best fit with the number of patches for each condition shown. Where two exponentials were found, the relative contributions of the two components is given as a ratio of weighting factor for τ_1 and the weighting factor for τ^2. For recovery patches, the number of events was often too small to estimate accurately the open time. Values for amplitude and open times represent the mean ± s.e.mean of *n* patches or mean ± s.d. when $n = 2$.

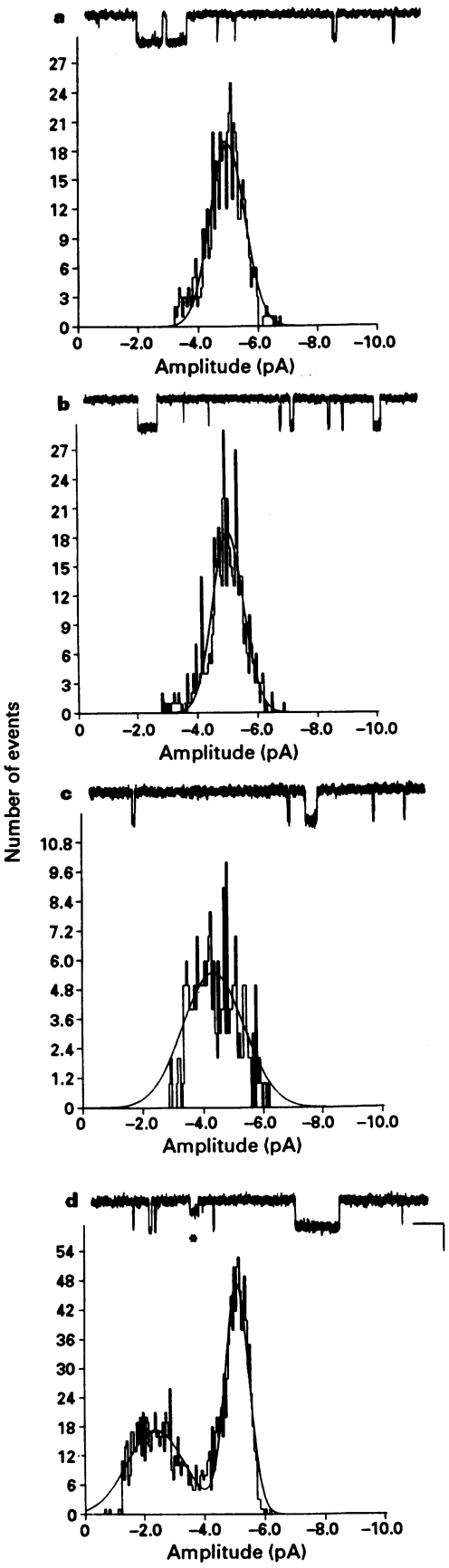


Figure 3 Acetylcholine (ACh)-induced single channel currents from cell-attached patches on control and staurosporine-treated endplates. ACh (1 μM)-induced single channel currents were recorded from cell-attached patches at fibres in (a) control isotonic potassium soluton, (b) isotonic potassium containing 0.5 μM staurosporine, (c) control preparations following 5 min incubation with 540 μM carbachol, or (d) staurosporine-treated preparations following 5 min incubation with 540 μM carbachol. Representative current traces and amplitude distributions are shown for each condition. A population of small amplitude channels was consistently seen in staurosporine-treated fibres following carbachol-induced desensitization (asterisk, d). The curves represent the best fit Gaussian distributions for each amplitude distribution with the following values (mean amplitude ± s.d.): (a) 4.99 pA ± 0.58, (b) 5.05 pA ± 0.52, (c) 4.35 pA ± 1.01 (d) 2.39 pA ± 0.96 (relative area = 41.5) and 5.10 pA ± 0.40 (relative area = 46.8). Calibration bars are X-axis 20 ms, Y-axis 5 pA.

to 3 min). However, in one patch from a control fibre following recovery from carbachol exposure, a second, smaller amplitude channel was recorded transiently. In this case, immediately after seal formation with the patch held at − 100 mV, the current amplitude of the ACh-activated currents was similar to that seen in the other 6 fibres. Approximately 30 s after seal formation, a second channel which had a much smaller current amplitude (approximately 3 pA) was activated. This channel exhibited activity for approximately 700 ms, then ceased and was not observed again during the remainder of the recording period (approximately 2 min).

At staurosporine-treated fibres exposed to 540 μM carbachol, activity of a small conductance ACh-activated channel was observed during recovery in 6 out of 7 successful patches held at − 100 mV. In these cases, the small conductance channel was observed throughout the recording period (approximately 2 min). This channel did not appear to be a sub-conductance state as no transitions from large to small conductance were seen. Neither the large amplitude nor small amplitude channel was observed in patches made off the endplate region. In 1 patch, only the small conductance channels were observed and in 5 other patches both large amplitude and small amplitude ACh-activated currents were recorded. The larger amplitude channel alone was seen in only 1 patch on an endplate from a staurosporine-treated preparation exposed to carbachol. The duration of single channel activity in many of the patches recorded from staurosporine-treated fibres which had been exposed to carbachol was not long enough to quantitate the open time distribution for the two channel types. However, the results do suggest that the open time distribution of the larger ACh-activated channel is similar to that of the large conductance channels recorded in the other preparations.

The current-voltage relationship was estimated for ACh-activated channels from control and staurosporine-treated preparations without carbachol exposure and following recovery from agonist application. Cell-attached patches were made initially at a holding potential of 0 mV at which no channel activity was observed. When voltage was applied to the patch to depolarize the intracellular membrane, the ACh-activated currents were outward and when the intracellular patch potential was made progressively negative, the ACh-activated currents were inward. We could not consistently voltage clamp patches to positive membrane potentials large enough to estimate accurately the ACh-activated current amplitudes and the frequency of channel activity was considerably less in the same patch at positive potentials than at negative potentials (Fiekers *et al.*, 1987). Consequently, we determined the mean current amplitudes only at negative membrane potentials. Unfortunately, it was often not possible to obtain recordings from the same patch at multiple voltages. This was especially true for those preparations which had been exposed to carbachol and then allowed to recover. Consequently, recordings were made at different voltages in different patches on fibres which had been maintained under similar conditions. The current-voltage relationship generated for the ACh-activated channels recorded from the different preparations is presented in Figure 4. The single channel conductance and reversal potentials determined by regression analysis of the current-voltage relationship for the large and small ACh-activated channels are summarized in Table 4.

Table 4 Reversal potential (V_{rev}) and slope conductance in control and staurosporine-treated fibres determined from m.e.p.c. and single channel *I-V* curves

	V_{REV} (mV ± s.e.mean)		*Slope conductance* (pS ± s.e.mean)	
	Before	*Recovery*	*Before*	*Recovery*
M.e.p.cs				
Control	1.64 ± 3.88	1.76 ± 3.90	62 ± 2	58 ± 2
Staurosporine	0.67 ± 4.72	− 0.54 ± 5.27	62 ± 3	46 ± 2
Single channels				
Control	− 8.74 ± 7.33	− 2.08 ± 10.96	40 ± 4	43 ± 6
Staurosporine	− 7.06 ± 12.75	− 2.06 ± 5.24 (L)	47 ± 7	53 ± 2 (L)
	ND	0.32 ± 2.50 (S)	ND	23 ± 1 (S)

ND = this class of channels was not detected in these fibres.
The reversal potential and slope conductance were determined by first order regression analysis of the *I-V* curve for each condition. The parameters are given along with their standard error as determined by the regression analysis. For the single channel data, two classes of channels were seen following recovery from desensitization, large (L) and small (S), in staurosporine-treated fibres.

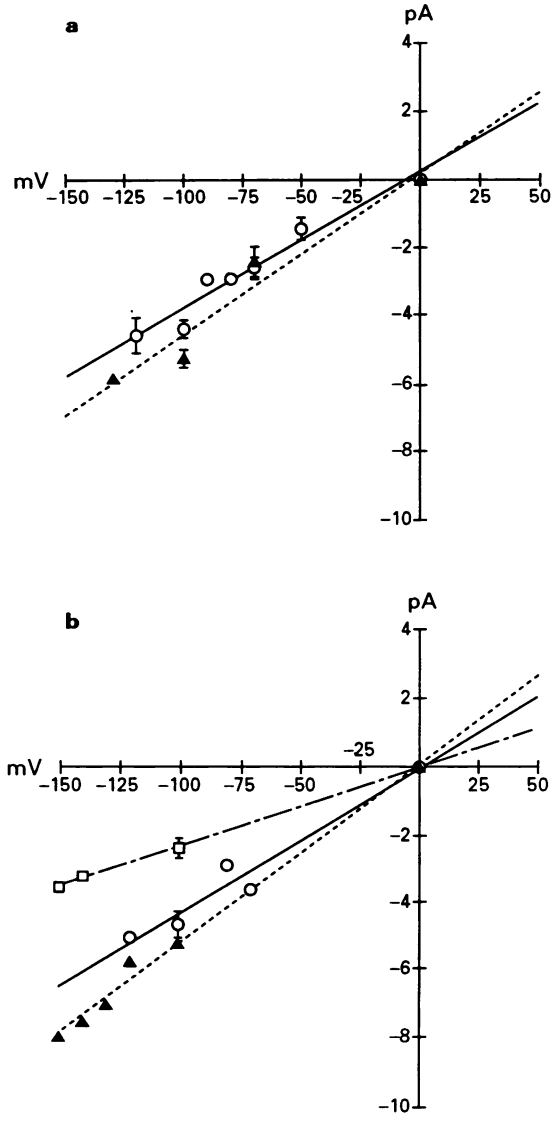


Figure 4 Current-voltage (*I-V*) relationship for acetylcholine (ACh)-induced single channels in control and staurosporine-treated preparations. ACh (1 μM)-induced single channel currents were measured at several holding potentials in control and staurosporine-treated preparations (a) without treatment with agonist and (b) following 5 min incubation with 540 μM carbachol. In the absence of agonist-treatment, there was no significant difference in the *I-V* curves for the control (○) and staurosporine-treated fibres (▲). Following carbachol-induced desensitization, a second population of channels was seen in the staurosporine-treated fibres. However, these small conductance channels (□) showed no difference in reversal potential from the larger conductance channels (▲). The points represent the mean ± s.e.mean (vertical bars) of 3 or more fibres for (a). Because of the rapid rate of desensitization, the number of patches for each voltage was less with recovery. In this case, points represent the mean of 2 fibres when no standard error is indicated. The lines represent first order, least-squares regression lines.

Discussion

In the first series of experiments, we found that staurosporine did not significantly decrease the m.e.p.c. amplitude following a 1 min exposure to carbachol. In previous studies, we showed that when 540 μM carbachol was microperfused locally onto the endplate region of fibres voltage clamped to + 30 mV, the carbachol (CCh)-induced current ($e.p.c._{CCh}$) peaked within 1–2 s and then gradually declined. By the end of a 1 min exposure, the $e.p.c._{CCh}$ had decayed by approximately 75% from the peak (Hardwick *et al.*, 1991). This observation demonstrated that even though many of the ACh receptors were desensitized at this time, other ACh receptors were still activated. Desensitization of essentially all activatable ACh receptors occurred when the duration of carbachol exposure was at least 2–3 min. Thus, in the present study, many ACh receptors should still have been activatable at the end of a 1 min bath application of carbachol. However, very few activatable ACh receptors would be expected to be present by the end of a 5–10 min carbachol exposure. Therefore, because the reduction in m.e.p.c. amplitude occurred only when carbachol was present sufficiently long to desensitize virtually all of the endplate ACh receptors, we assume that under the conditions of the present experiments, the primary action of staurosporine pretreatment is a decrease in the extent of recovery from full desensitization.

Desensitization is thought to be a biphasic process with fast and slow components (Feltz & Trautman, 1982; Connor *et al.*, 1984). Because the decrease in m.e.p.c. amplitude observed at stauropsorine-treated endplates following desensitization was evident only with the longer durations of carbachol applications, we suggest that complete inactivation of ACh receptors may be required for this effect and that sufficient inactivation may only be reached during the slow component of desensitization. Consequently, it is possible that it is the recovery from this kinetic state that is sensitive to staurosporine. This possibility needs to be explored in subsequent studies.

Pretreatment with staurosporine did not alter m.e.p.c. amplitude or the decay time constant at endplates which had not previously been exposed to carbachol. Our previous studies also showed that staurosporine had no affect on the rate of carbachol-induced desensitization (Hardwick *et al.*, 1991). The m.e.p.c. decay time constant was also not different in control and staurosporine-treated preparations following exposure to carbachol even though the m.e.p.c. amplitude

was reduced. The decrease in m.e.p.c. amplitude in staurosporine-treated preparations following recovery from desensitization occurred without any significant shift in the m.e.p.c. reversal potential. These observations indicate that the decrease in m.e.p.c. amplitude after desensitization at staurosporine-treated endplates was not due to an alteration in the basic kinetics of ACh receptor channel gating or due to a change in the ionic selectivity of the endplate channels.

Insight into the mechanism responsible for the decreased m.e.p.c. amplitude at staurosporine-treated fibres following recovery from desensitization was first obtained from noise analysis. Mean channel conductance was not significantly different at control and staurosporine-treated endplates in the absence of carbachol exposure. However, following carbachol-induced desensitization and recovery, the staurosporine-treated endplates showed a 56% decrease in mean channel conductance. This decrease in conductance was accompanied by a 35% decrease in m.e.p.c. amplitude. These results provide further support for the conclusion that the staurosporine-induced inhibition of recovery is due to a postsynaptic effect. These results also suggest that the decrease in m.e.p.c. amplitude is due, at least in part, to a decrease in the conductance of ACh receptor channel complexes. The percentage decrease in mean channel conductance was greater than the percentage decrease in m.e.p.c. amplitude. We suggest that this difference occurs because the m.e.p.c. amplitude at staurosporine-treated endplates following carbachol exposure is probably overestimated. At these endplates, very small amplitude m.e.p.cs could not be readily distinguished from the noise and therefore may not have been included in the averaged values. This problem is even more apparent in the slope conductance determined from the m.e.p.c. *I-V* curve. As m.e.p.c. amplitudes decrease at more positive amplitudes, the ability to distinguish small amplitude m.e.p.cs from the noise becomes more difficult. Thus, both the change in mean m.e.p.c. amplitude and the estimated slope conductance show smaller changes than the mean channel conductance due to experimental limitations.

Direct measurement of single channels confirmed the results of the noise analysis. Without carbachol exposure, the properties of the ACh-activated currents were similar in control and staurosporine-treated preparations. The slope conductance was approximately 45 pS for channels activated at control endplates and 49 pS for channels activated at staurosporine-treated endplates. In addition, we did not find any marked difference in the mean channel open times for control and staurosporine-treated preparations. We did find, however, that for both the control and staurosporine-treated preparations maintained in the isotonic potassium solution, the channel open times were not always described adequately by a single exponential function. This result differs from that reported previously for preparations kept in the normal sodium solution where the open time distribution has generally been fitted by a single exponential function (Dionne, 1989; Ruff & Spiegel, 1990). In the present study, we also observed a population of small amplitude channels at most staurosporine-treated preparations which had been exposed to carbachol and then allowed to recover in agonist-free isotonic potassium solution. These small conductance channels were not found in most control preparations following recovery from carbachol-induced desensitization. In the one control patch in which activity of a small conductance ACh channel was observed, the activity was very transient (<1 s), whereas the small conductance channels in patches from staurosporine-treated preparations remained active over the 2–3 min recording period.

The slope conductance of the small conductance channels was 23 pS. The conductance of the large ACh-activated channels recorded at either control or staurosporine-treated preparations following recovery from desensitization was similar to that found for channels recorded in patches on preparations not exposed to carbachol. The reversal potential for the small conductance channels was not different from that estimated for the large conductance channels which suggests that both channel types have similar permeability properties. This observation is consistent with the finding that the m.e.p.c. reversal potential also was not changed following recovery from desensitization at staurosporine-treated preparations.

The time course of recovery occurring under the conditions of the present experiments is too fast to be explained by either the synthesis of new ACh receptors or subunit substitution within existing receptors. Rather, we suggest that post-translational modification is a more likely mechanism for the recovery from desensitization in these experiments. The results of our previous studies led us to propose that the effect of staurosporine resulted from an inhibition of phosphorylation of ACh receptors or related proteins which was required for full recovery from desensitization (Hardwick *et al.*, 1991). The current studies suggest that, following carbachol-induced desensitization, either new receptors or receptors dephosphorylated in the activation-desensitization process, must be phosphorylated by a staurosporine-sensitive kinase. This phosphorylation step changes the conductance of the receptor-channel complex from the smaller value to the larger value typical of junctional ACh receptors. Pretreatment with staurosporine prevents this conversion so that the conductance remains smaller. Thus, we suggest that the m.e.p.c. amplitude at staurosporine-treated fibres following desensitization is reduced, at least in part, because ACh activates a mixture of small and large conductance ACh channels. However, a decrease in m.e.p.c. amplitude could also be due in part to a decrease in receptor density. Unfortunately, this cannot be tested directly in this preparation because garter snake ACh receptors are insensitive to the commonly used irreversible receptor ligands, such as α-bungarotoxin, normally used for the determination of receptor density (Burden *et al.*, 1975). The mean channel conductance estimated by noise analysis in staurosporine-treated fibres following recovery from desensitization was similar to the conductance of the small channels alone. However, noise analysis is not an accurate method for determining conductance values, but it is more useful in demonstrating relative changes in mean channel conductance. Therefore, we suggest that the actual mean channel conductance in these staurosporine-treated fibres lies somewhere between 23pS and 53pS, with the actual value dependent on the density of the two channel types.

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References

BETZ, W. & SAKMANN, B. (1973). Effects of proteolytic enzymes on function and structure of frog neuromuscular junctions. *J. Physiol,*. **230**, 673–688.

BURDEN, S.J., HARTZELL, H.C. & YOSHIKAMI, D. (1975). Acetylcholine receptors at neuromuscular synapses: phylogenetic differences detected by snake α-neurotoxins. *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 3245–3249.

CONNOR, E.A., FIEKERS, J.F., NEEL, D.S., PARSONS, R.L. & SCHNITZLER, R.M. (1984). Comparison of cholinergic activation and desensitization at snake twitch and slow muscle fibre endplates. *J. Physiol.*, **351**, 657–674.

DIONNE, V.E. (1989). Two types of nicotinic acetylcholine receptors channels at slow fibre end-plates of the garter snake. *J. Physiol.*, **409**, 313–331.

DIONNE, V.E. & PARSONS, R.L. (1981). Characteristics of the acetylcholine-operated channel at twitch and slow fibre neuromuscular junctions of the garter snake. *J. Physiol,*. **310,** 145–158.

FELTZ, A. & TRAUTMANN, A. (1982). Desensitization at the frog neuromuscular junction: a biphasic process. *J. Physiol,*. **322,** 257–272.

FIEKERS, J.F., NEEL, D.S. & PARSONS, R.L. (1987). Acceleration of desensitization by agonist pretreatment in the snake. *J. Physiol,*. **391,** 109–124.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. (1981). Improved patch-clamp techniques for high resolution current recording from cells and cell free membrane patches. *Pflügers Arch.*, **391,** 85–100.

HARDWICK, J.C., CONIGLIO, L.M. & PARSONS, R.L. (1991). Staurosporine inhibits the extent of acetylcholine receptor recovery from carbachol-induced desensitization in snake twitch fibres. *Br. J. Pharmacol.*, **104,** 879–886.

HUGANIR, R.L. & GREENGARD, P. (1990). Regulation of neurotransmitter receptor desensitization by protein phosphorylation. *Neuron*, **5,** 555–567.

KATZ, B. & THESLEFF, S. (1957). A study of the "desensitization" produced by acetylcholine at the motor end-plate. *J. Physiol.*, **138,** 63–80.

RUFF, R.L. & SPIEGEL, P. (1990). Ca sensitivity and acetylcholine receptor currents of twitch and tonic snake muscle fibres. *Am. J. Physiol.*, **259,** C911–C919.

SCUBON-MULIERI, B. & PARSONS, R.L. (1977). Desensitization and recovery at the frog neuromuscular junction. *J. Gen. Physiol.*, **69,** 431–447.

TAMAOKI, T., NOMOTO, H., TAKAHASHI, I., KATO, Y., MORIMOTO, M. & TOMITA, F. (1986). Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. *Biochem. Biophys. Res. Commun.*, **135,** 397–402.

WAGONER, P.K. & PALLOTTA, B.S. (1988). Modulation of acetylcholine receptor desensitization by forskolin is independent of cAMP. *Nature*, **240,** 1655–1657.

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ATP induced-relaxation in the mouse bladder smooth muscle

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1 The effect of adenosine 5'-triphosphate (ATP) on the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) as measured with the fluorescent Ca^{2+}-indicator fura-2, and on force was investigated in the intact smooth muscle strips of the mouse urinary bladder.

2 ATP elicited, when exogenously applied, a large increase of $[Ca^{2+}]_i$ with limited force development resulting in a marked Ca^{2+}-force dissociation.

3 Release of endogenous neurotransmitters by transmural electrical stimulation (TES) for 30 s induced a steady increase of $[Ca^{2+}]_i$ and a peak contraction, followed within 15 s by a relaxation.

4 In carbachol-prestimulated preparations, ATP elicited an initial rise of $[Ca^{2+}]_i$ followed by a return to the initial precontraction Ca^{2+}-level. Force in contrast presented a biphasic pattern, i.e. an initial contraction was followed by a sustained relaxation.

5 In the K^+-depolarized precontracted preparation, ATP elicited a slight initial rise of $[Ca^{2+}]_i$. The partial relaxation of the force during depolarization was not preceded by a transient contraction.

6 The ATP-induced relaxation of the K^+-prestimulated preparations was not inhibited by 8-phenyltheophylline, a potent P_1-purinoceptor antagonist.

7 The order of potency for relaxation of the ATP analogues was 2-MeSATP > ATP > βγMe-ATP, which is characteristic for P_{2y}-purinoceptors.

8 These results indicate that, besides its activating effect, ATP also relaxes the mouse urinary bladder. It is suggested that the relaxant effect, mediated through P_{2y}-purinoceptors, is mainly responsible for the low contractile potency of ATP in the bladder.

Keywords: ATP; P_2-purinoceptors; cytosolic Ca^{2+}; urinary bladder

Introduction

Receptors for extracellular purines have been described in numerous tissues (Gordon, 1986). The purinoceptors for adenosine 5'-triphosphate (ATP) in the smooth muscle tissues were subclassified into contracting P_{2x}- and relaxing P_{2y}-purinoceptors (Burnstock & Kennedy, 1985). The neurotransmitter function of ATP was initially reported in the smooth muscle of the rodent vas deferens in which co-transmission of ATP and noradrenaline occurs (Sneddon & Westfall, 1984). In this tissue, ATP elicits contraction through a Ca^{2+} influx induced by P_{2x}-purinoceptor activation (Friel, 1988). Co-transmission of ATP and acetylcholine was described in the smooth muscle of the rodent urinary bladder (Burnstock *et al.*, 1978), in which ATP also induces contraction through the P_{2x}-purinoceptors by Ca^{2+} influx (Acevedo & Contreras, 1989; Katsuragi *et al.*, 1990).

However, the contractile potency of ATP is very low both in the vas deferens (Fedan *et al.*, 1982) and in the bladder (Acevedo & Contreras, 1989; Hoyle & Burnstock, 1989; Katsuragi *et al.*, 1990). We observed recently that the low contractile effect of ATP in the mouse vas deferens occurs in spite of a marked increase of the free cytosolic calcium concentration ($[Ca^{2+}]_i$) and proposed that it mainly resulted from the binding of ATP to both P_{2x}- and P_{2y}-purinoceptors (Boland *et al.*, 1992). The present experiments were carried out to test the hypothesis that the low contractile potency of ATP observed in the bladder smooth muscle is due to the activation of both contracting and relaxing purinoceptors. We therefore measured the effect on $[Ca^{2+}]_i$ and force induced by ATP in resting and in precontracted bladder preparations.

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Methods

Muscle preparation

Adult male albino mice (NMRI, 3–4 month old, 30–40 g) were killed by cervical dislocation after anaesthesia with ether. The urinary bladder was isolated and transferred to the oxygenated HEPES-buffered Krebs solution at room temperature. The bladder was dissected free from its surrounding tissues and opened along its medial axis to allow removal of the thick mucosa by gentle rubbing. Intact smooth muscle strips (10 × 4 mm) were dissected.

Measurements of cytosolic Ca^{2+} and of force

The bladder strips were loaded for 3 h with 5 μM fura-2AM, as previously described (Katsuragi *et al.*, 1990). This loading procedure did not affect either the amplitude or the time-course of the force response to 10 μM carbachol ($n = 4$). After the loading, the strips were rinsed in the HEPES-Krebs solution for 30 min. The mounting of intact smooth muscle preparations under isometric conditions and the experimental set-up used for the simultaneous measurements of fura-2 fluorescence and force response have been described (Himpens & Somlyo, 1988). The bladder strips were stretched to a passive tension of 5 mN. The 340/380 nm fura-2 ratio was continuously recorded during the experiments. An internal calibration of the fura-2 fluorescence signals into free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was performed at the end of each experiment by use of the procedure designed by Himpens *et al.* (1988). The increases of $[Ca^{2+}]_i$ and of force induced by the stimulation with 10 μM carbachol were used as references. The changes of $[Ca^{2+}]_i$ are expressed in absolute values (nM) or as a percentage of the increase of $[Ca^{2+}]_i$ in 10 μM carbachol (100%). The force response is expressed in absolute values (mN) or as a percentage of the maximal force increase obtained with 10 μM carbachol (100%).

Stimulating procedure

The bladder strips were continuously superfused at 4 ml min^{-1}. Contraction was obtained by superfusing the preparation with 10 μM carbachol or by 70 mM K^+. The effect of ATP was examined both in the resting bladder and in the bladder prestimulated by carbachol or by 70 mM K^+. Transmural electrical stimulation (TES) specific for the nerve endings was applied as 30 s train of 1 ms square pulses of supramaximal voltage at 25 Hz through platinum electrodes lying in parallel to the smooth muscle preparation. The specificity of this TES for the nerve endings was indicated by the total abolition of the contractile response by 1 μM tetrodotoxin, as previously reported for similar pulse parameters (Parija et al., 1991).

Solutions

The normal HEPES-Krebs solution contained in mM: NaCl 135.5, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1.5, HEPES 11.6 and glucose 11.5. The isotonic 70 mM K^+ solution was obtained by replacing external Na^+ by an equivalent amount of K^+. Sodium salt of adenosine triphosphate (ATP), sodium salt of βγ-methylene ATP (βγme-ATP), carbachol and tetrodotoxin were from Sigma. Tetrasodium salt of 2-methylthioadenosine triphosphate (2-MeSATP) was obtained from ICN Biochemicals (Cleaveland, Ohio, U.S.A.). 8-Phenyltheophylline and ionomycin were from Calbiochem. Fura-2AM was from Molecular Probes (Eugene, OR, USA). All ATP analogues were the D-isomers. Drugs were dissolved in the HEPES-Krebs solution, except for 8PT which was dissolved in 80% methanol containing 0.2 M NaOH (Griffith et al., 1981). All other reagents were of analytical grade.

Statistics

The results are presented as means ± standard error of mean (s.e.mean), and n is the number of experiments. The data were evaluated for differences by Student's t test (paired two-tailed t test). A probability of less than 0.05 was considered significant.

Results

The mouse bladder smooth muscle did not show spontaneous contractile activity in the normal Krebs solution containing 1.5 mM Ca^{2+} at room temperature ($n = 10$), as previously reported (Acevedo & Contreras, 1989). During these resting conditions, the values of the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and of the force were 109 ± 4 nM and 5 ± 0.8 mN respectively. These levels were used as the basal references (0%) as mentioned in the methods. Our value of resting $[Ca^{2+}]_i$ in the mouse bladder muscle tissue (109 nM) is similar to that reported in the isolated smooth muscle cells of guinea-pig bladder (Ganitkevich & Isenberg, 1991). The effect of superfusion for 2 min with 10 μM carbachol, 70 mM K^+ and 100 μM ATP are compared in Figure 1. Carbachol (10 μM) increased the values of $[Ca^{2+}]_i$ to 242 ± 27 nM and of force to 25 ± 2 mN ($n = 9$). To these values, all the measurements described below were normalized (100%). During depolarization with 70 mM K^+, $[Ca^{2+}]_i$ and force attained a level of 351 ± 60 nM (181%) and of 23 ± 3 mN (89%) respectively ($n = 7$). In contrast, the rise of $[Ca^{2+}]_i$ elicited by 100 μM ATP was 265 ± 30 nM (117%) while force increased to only 6 ± 1 mN (2%) ($n = 6$). Two other preparations even failed to contract in response to ATP application, as reported in the human bladder (Hoyle et al., 1989), in spite of a marked elevation of $[Ca^{2+}]_i$. Thus, in spite of a large increase of $[Ca^{2+}]_i$, superfusion with exogenous ATP elicited only a very weak contraction.

In order to analyse the effects of endogenous ATP, we induced the release of neurotransmitters from the bladder by

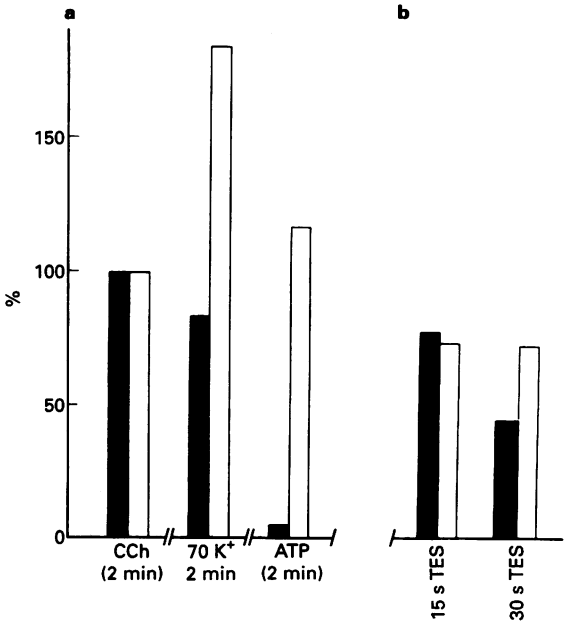


Figure 1 (a) Peak increases of $[Ca^{2+}]_i$ (open columns) and of force (solid columns) above the resting level induced by 70 mM K^+ and by 100 μM ATP, as normalized to the values elicited by 10 μM carbachol (CCh). (b) Comparison of the increases of $[Ca^{2+}]_i$ (open column) and of force (solid column) after 15 and 30 s of transmural electrical stimulation (TES).

transmural electrical stimulation (TES). TES with pulses of short duration is specific for the nerve endings, and does not activate the smooth muscle cells. The TES-induced increases of $[Ca^{2+}]_i$ and force for 15 and 30 s stimulation are displayed in Figure 1b while an example is presented in Figure 2. TES for 30 s with 1 ms square pulses of 40 V at 25 Hz induced within 15 s the peak of $[Ca^{2+}]_i$ to 207 ± 20 nM (73%) and of force to 20 ± 0.8 mN (76%) ($n = 8$) (Figure 2). Thereafter, during continued stimulation, $[Ca^{2+}]_i$ remained at about 200 nM while force declined by 40 ± 6%. At the end of the TES, $[Ca^{2+}]_i$ returned within 1 min to its basal level while the resting force level was reached in about 20 s. Incubation of the bladder preparation for 10 min with 1 μM tetrodotoxin completely abolished the response to TES indicating that this procedure elicited no direct stimulation of the smooth muscle cells ($n = 4$). The TES-induced contraction was reduced by 65 ± 7% by preincubation for 5 min with 1 μM atropine. In this condition, the force also started to decline after 15 s stimulation in spite of a maintained Ca^{2+} signal ($n = 3$).

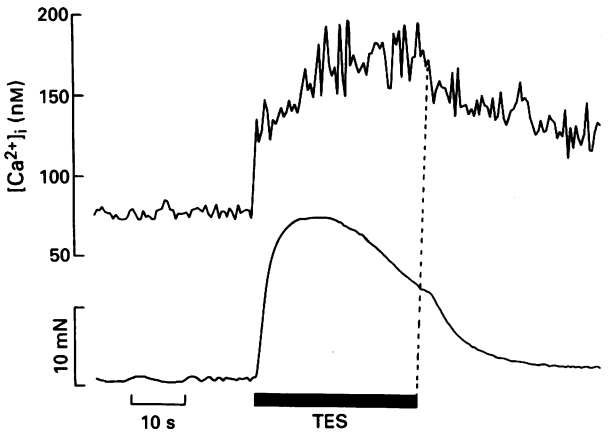


Figure 2 Record showing the changes in $[Ca^{2+}]_i$ (upper trace) and of force (lower trace) elicited during transmural electrical stimulation (TES) with 1 ms square pulses of 40 V at 25 Hz for 30 s. The dashed line represents the end of the electrical stimulation.

The force-Ca^{2+} dissociation observed during stimulation with both exogenous and endogenous ATP suggested the possibility of a relaxing effect by ATP. In order to investigate this possibility, we performed stimulations by ATP in bladder preparations precontracted by carbachol or by K^+-depolarization. A steady raised-tone was first obtained by stimulation with 10 μM carbachol. After the initial peak contraction (100%, see above) force stabilized within 20 min to a level of 20 ± 2% ($n = 8$). Preliminary data showed that application of 100 μM ATP for 5 min to this prestimulated preparation induced a contraction to 32% ($n = 6$), followed after 5 min by a return of the force to the previous raised-tone level (20%). However, during prolonged stimulation with 100 μM ATP for more than 10 min we found a force relaxation below the raised-tone level, which stabilized at 14 ± 2% ($n = 4$). We further examined the effect on $[Ca^{2+}]_i$ and force of 1000 μM ATP in the carbachol-precontracted bladder. First, $[Ca^{2+}]_i$ increased from 157 ± 20 to 275 ± 50 nM and force rose from 20 ± 2 to 29 ± 3% ($n = 5$) (Figure 3). Then, within 5 min, both $[Ca^{2+}]_i$ and force returned to their previous levels. Finally, after 10 min superfusion with 1000 μM ATP, $[Ca^{2+}]_i$ remained at 164 ± 28 nM while force relaxed to 7 ± 1%. On washing out ATP, both $[Ca^{2+}]_i$ and force returned to their previous carbachol-induced level. A second stimulation with 1000 μM ATP at 10 min interval elicited within 2 min slight rises of $[Ca^{2+}]_i$ to 202 ± 34 nM and of force to 26 ± 4% ($n = 5$) (Figure 3). Thereafter, during the prolonged ATP stimulation, $[Ca^{2+}]_i$ returned to 165 ± 21 nM while force relaxed close to the basal resting level (3 ± 1%). Again, on washing out ATP, $[Ca^{2+}]_i$ and force returned to 168 ± 25 nM and 18 ± 2% i.e. back to its control levels.

We also analysed the effects of ATP in the bladder smooth muscle prestimulated with a depolarizing solution containing 1.5 mM Ca^{2+} and 70 mM K^+. After 20 min stimulation with 70 mM K^+, $[Ca^{2+}]_i$ stabilized at 247 ± 35 nM and force at 10 ± 2% ($n = 7$). In this condition, application of 1000 μM ATP slightly elevated $[Ca^{2+}]_i$ within 2 min to 319 ± 69 nM while force hardly increased to 11 ± 3% ($n = 5$) (Figure 4). The ATP-induced increase of $[Ca^{2+}]_i$ was significantly lower in 70 mM K^+ (72 nM) than in 10 μM carbachol (118 nM) ($P < 0.05$). Thereafter, during prolonged stimulation with ATP $[Ca^{2+}]_i$ returned to 250 ± 38 nM and force declined to 5 ± 2%. In the K^+-precontracted bladder, incubation for 30 min with 10 μM 8-phenyltheophylline, a potent antagonist of P_1-purinoceptors, did not modify the relaxation induced by 100 μM ($n = 8$) or 1000 μM ATP ($n = 6$). The rank order of potency for relaxation of ATP analogues (100 μM) was examined in K^+ prestimulated preparations. Under these conditions, steady state force declined by 25 ± 3% after 5 min stimulation with ATP ($n = 3$), by 43 ± 3% after 2-MeSATP incubation and by 9 ± 2% in the presence of βγMe-ATP. The observed rank order of potency for relaxation was thus 2-MeSATP > ATP > βγMe-ATP. Finally, also in guinea-pig ($n = 6$) and rat ($n = 5$) bladder smooth muscles precontracted by 70 mM K^+, stimulation with ATP induced a reproducible, sustained and reversible relaxation (data not shown).

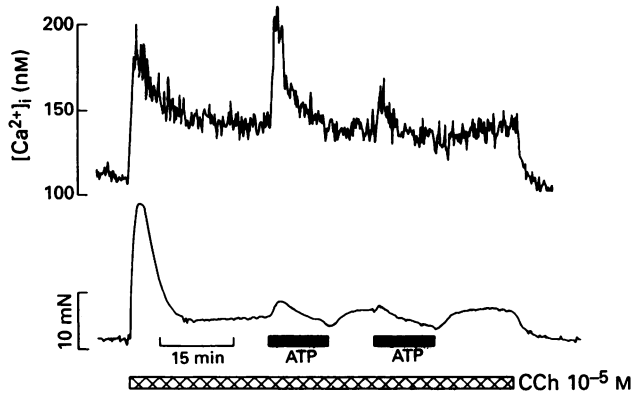


Figure 3 Effects on $[Ca^{2+}]_i$ (upper trace) and on force (lower trace) of two sequential applications of ATP 1000 μM for 10 min in the bladder preparation prestimulated by 10 μM carbachol (CCh).

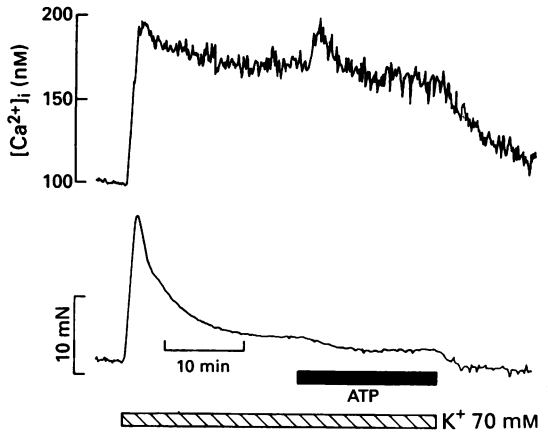


Figure 4 Effects on $[Ca^{2+}]_i$ (upper trace) and force (lower trace) of ATP (1000 μM) in the bladder preparation depolarized by 70 mM K^+. Note that the Ca^{2+} transient evoked by ATP in the K^+-precontracted bladder is smaller than the value in the carbachol-prestimulated bladder.

Discussion

Co-transmission of acetylcholine and ATP was described in the mammalian urinary bladder smooth muscle (Ambache & Aboo Zar, 1970; Burnstock *et al.*, 1972), in which transmural electrical stimulation of the nerve endings induces the activation of postsynaptic muscarinic receptors and purinoceptors (Fujii, 1988; Brading & Mostwin, 1989). In the bladder, the muscarinic receptors induce a release of Ca^{2+} from the internal stores and Ca^{2+} influx through receptor-operated ion channels (Maggi *et al.*, 1989; Iacovou *et al.*, 1990), while the P_{2x}-purinoceptors activate membranous ion permeability resulting in depolarization (Inoue & Brading, 1990) and Ca^{2+} influx (Katsuragi *et al.*, 1990). ATP elicits a large inward current in dispersed bladder smooth muscle cells (Inoue & Brading, 1991).

However, the force response to ATP was reported to be rather limited in the intact urinary bladder smooth muscle from the guinea-pig (Ambache & Aboo Zar, 1970; Burnstock *et al.*, 1978), the rat (Brown *et al.*, 1979; Bhat *et al.*, 1989), the mouse (Acevedo & Contreras, 1989) as well as from human bladder (Hoyle *et al.*, 1989). In these tissues, prolonged application of ATP up to 1000 μM induced only a weak and short contraction. These latter features were usually attributed either to extracellular breakdown of ATP by ectonucleotidases (Brown *et al.*, 1979; Inoue & Brading, 1991), or to the tachyphylaxis of the P_{2x}-purinoceptors (Kennedy, 1990). To investigate further the low potency of ATP, we studied simultaneously for the first time the ATP-induced changes of $[Ca^{2+}]_i$ and of force in intact bladder smooth muscle. The finding that ATP elicited a large increase of $[Ca^{2+}]_i$, which was abolished in the Ca^{2+}-free solution (Acevedo & Contreras, 1989; Bhat *et al.*, 1989; Katsuragi *et al.*, 1990), shows that ATP induces a marked Ca^{2+} influx through an effective activation of the P_{2x}-purinoceptors. This amplitude of the ATP-induced rise of $[Ca^{2+}]_i$ suggests thus that neither the breakdown of ATP nor the tachyphylaxis of the P_{2x}-purinoceptors account for the low contractive effect of ATP.

TES induced strong contractions in the mouse bladder. A force decline was already observed within 15 s while $[Ca^{2+}]_i$

in contrast remained elevated. Furthermore, in preparations preincubated with atropine, force induced by TES declined after 15 s, suggesting that a non-cholinergic mediator could be responsible for this Ca^{2+}-independent relaxation. Thus ATP, either exogenously or endogenously applied to the bladder smooth muscle, was associated with an early Ca^{2+}-force uncoupling. This observation suggested that ATP could bind to relaxing postsynaptic purinoceptors besides its activation of contracting P_{2x}-dependent purinoceptors. In order to reveal the possible relaxing effect of ATP, we analysed its effects in precontracted preparations.

In the carbachol-prestimulated bladder, ATP induced a biphasic force response, i.e. a marked contraction followed by a sustained relaxation (Figure 3). A biphasic effect of ATP has been reported in respiratory (Brown & Burnstock, 1981), vascular (Ralevic & Burnstock, 1991), digestive (Manzini *et al.*, 1985; Lefebvre & Burnstock, 1990) and genital (Boland *et al.*, 1992) smooth muscles, but to our knowledge not yet in the urinary bladder. The contractile effects of ATP were more pronounced in the carbachol-prestimulated bladder than under resting conditions. The mechanism underlying this increased potency of ATP for contraction is not known but could involve either a postsynaptic synergism between carbachol and ATP at the muscarinic receptor and the P_{2x}-purinoceptors as observed between noradrenaline and ATP for the vas deferens (Witt *et al.*, 1991) or an intracellular agonist-induced increase by carbachol of the sensitivy of the contractile filaments to Ca^{2+} (Himpens *et al.*, 1990).

In smooth muscle preparations containing both contracting and relaxing purinoceptors, the force response to ATP is the balance between two opposite effects. Here, during superfusion with 100 or 1000 μM ATP, the P_{2x}-dependent contractile effect predominated. In order to unmask the relaxation to ATP, we examined its effects in two conditions which are supposed to inhibit the P_{2x}-contraction. The first condition was to apply ATP twice successively in order to induce the tachyphylaxis which is specific for the P_{2x}-purinoceptors (Kennedy, 1990). In this condition, the ATP-induced rises of $[Ca^{2+}]_i$ and of force were decreased and the relaxation was more pronounced, i.e. the force level then declined close to the resting value. The decrease of the Ca^{2+}- and the contractile responses to the second ATP application can be explained by tachyphylaxis of the P_{2x} purinoceptors. The finding that relaxation induced by ATP in this condition did not decrease suggests that the two opposing effects are mediated through two different purinoceptors. The second experimental condition was to apply ATP in the K^+-depolarized bladder smooth muscle, in which the depolarizing effect of the P_{2x}-purinoceptors is expected to be markedly reduced, as well as their contractile effect. In fact, in the K^+-depolarized preparation, ATP induced a rise of $[Ca^{2+}]_i$ which was 56% of that observed in the carbachol-precontracted preparation. The contraction elicited by ATP was hardly observed, and was followed by a marked relaxation. In the two above mentioned experimental conditions, the relaxation induced by ATP occurred within about 30 s at an elevated $[Ca^{2+}]_i$, was sustained during the ATP application, and was reversible on washing out of ATP.

The relaxing purinoceptors in the smooth muscle are subclassified the P_{2y} and the A_1 adenosine receptor of the P_1-purinoceptor (Burnstock & Kennedy, 1985; Kennedy, 1990). Relaxing P_1-purinoceptors were reported in neonatal rat urinary bladder (Nicholls *et al.*, 1990) and are potently inhibited by 10 μM 8-phenyltheophylline (Griffith *et al.*, 1981). Our finding that the ATP-induced relaxation was not inhibited by 8-phenyltheophylline suggests that ATP acts on P_2-purinoceptors by itself, and not by its breakdown products on P_1-purinoceptors (Moody *et al.*, 1984). The P_2-type of the bladder relaxing purinoceptor activated by ATP is also supported by the observed rank order of potency for relaxation of the ATP analogues, which was characteristic for the P_{2y}-purinoceptors (Kennedy, 1990). Similar findings were recently reported in the mouse vas deferens smooth muscle (Boland *et al.*, 1992). To our knowledge, the relaxing effect of ATP has never been reported in the rodent bladder.

The physiological function of these relaxing purinoceptors is unknown. These inhibiting receptors could be activated *in vivo* during micturition, as suggested by the early relaxation observed during *in vitro* TES-induced stimulation. On the other hand, the continuous basal release of ATP (Burnstock *et al.*, 1978) could preferentially activate the relaxing purinoceptors during the bladder filling, thereby preventing the muscle contraction. The presence of two subtypes of purinoceptors in the bladder, mediating opposite mechanical effects, can explain both the striking Ca^{2+}-force dissociation induced by ATP and its low contractile potency.

In conclusion, besides its contractile effect through P_{2x}-purinoceptors, ATP also activates relaxing P_{2y}-purinoceptors in the mouse and other rodent bladder smooth muscles. It could be hypothesized that they counteract the bladder contraction during micturition or that they induce smooth muscle inhibition during the bladder filling. This latter hypothetical effect could explain in part the high compliance of the bladder smooth muscle.

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References

ACEVEDO, C.G. & CONTRERAS, E. (1989). Effect of extracellular calcium and calcium antagonists on ATP and field stimulation induced contractions of the mouse urinary bladder. *Gen. Pharmacol.*, **20**, 811–815.

AMBACHE, N. & ABOO ZAR, M. (1970). Non-cholinergic transmission by post-ganglionic motor neurones in the mammalian bladder. *J. Physiol.*, **210**, 761–783.

BHAT, M.B., MISHRA, S.K. & RAVIPRAKASH, V. (1989). Sources of calcium for ATP-induced contractions in rat urinary bladder smooth muscle. *Eur. J. Pharmacol.*, **164**, 163–166.

BOLAND, B., HIMPENS, B., GILLIS, J.M. & CASTEELS, R. (1992). ATP activates both contracting P_{2x}- and relaxing P_{2y}-purinoceptors in the smooth muscle of the mouse vas deferens. *Br. J. Pharmacol.*, **107**, 1152–1158.

BRADING, A.F. & MOSTWIN, J.L. (1989). Electrical and mechanical responses of guinea-pig bladder muscle to nerve stimulation. *Br. J. Pharmacol.*, **98**, 1083–1090.

BROWN, C.M. & BURNSTOCK, G. (1981). The structural conformation of the polyphosphate chain of the ATP molecule is critical for its promotion of prostaglandin biosynthesis. *Eur. J. Pharmacol.*, **69**, 81–86.

BROWN, C., BURNSTOCK, G. & COCKS, T. (1979). Effects of adenosine 5'-triphosphate (ATP) and β-γ-methylene ATP on the rat urinary bladder. *Br. J. Pharmacol.*, **65**, 97–102.

BURNSTOCK, G., COCKS, T., CROWE, R. & KASAKOV, L. (1978). Purinergic innervation of the guinea-pig urinary bladder. *Br. J. Pharmacol.*, **63**, 125–138.

BURNSTOCK, G., DUMSDAY, B.H. & SMYTHE, A. (1972). Atropine-resistant excitation of the urinary bladder: the possibility of the transmission via nerves releasing a purine nucleotide. *Br. J. Pharmacol.*, **44**, 451–461.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen. Pharmacol.*, **5**, 433–440.

FEDAN, J.S., HOGABOOM, G.K., WESTFALL, D.P. & O'DONNELL, J.P. (1982). Comparison of contractions of the smooth muscle of the guinea-pig vas deferens induced by ATP and related nucleotides. *Eur. J. Pharmacol.*, **81**, 193–204.

FRIEL, D.D. (1988). An ATP-sensitive conductance in single smooth muscle cells from the rat vas deferens. *J. Physiol.*, **401**, 361–380.

FUJII, K.G. (1988). Evidence for adenosine triphosphate as an excitatory transmitter to guinea-pig, rabbit and pig urinary bladder. *J. Physiol.*, **404**, 39–52.

GANITKEVICH, V.Y. & ISENBERG, G. (1991). Depolarization-mediated intracellular calcium transients in isolated smooth muscle cells of guinea-pig urinary bladder. *J. Physiol.*, **435**, 187–205.

GORDON, J.L. (1986). Extracellular ATP: effects, sources and fate. *Biochem. J.*, **233**, 309–319.

GRIFFITH, S.G., MEGHJI, P., MOODY, C.J. & BURNSTOCK, G. (1981). 8-Phenyltheophylline: a potent P_1-purinoceptor antagonist. *Eur. J. Pharmacol.*, **75**, 61–64.

HIMPENS, B., KITAZAWA, T. & SOMLYO, A.P. (1990). Agonist dependent modulation of the Ca^{2+}-sensitivity in rabbit pulmonary artery smooth muscle. *Eur. J. Physiol. (Pflüg. Arch.)*, **417**, 21–28.

HIMPENS, B., MATTHIJS, G., SOMLYO, A.V., BUTLER, T.M. & SOMLYO, A.P. (1988). Cytoplasmic free calcium, myosin light chain phosphorylation and force in phasic and tonic smooth muscle. *J. Gen. Physiol.*, **92**, 489–503.

HIMPENS, B. & SOMLYO, A.P. (1988). Free calcium and force transients during depolarization and pharmacomechanical coupling in guinea-pig smooth muscle. *J. Physiol.*, **395**, 507–530.

HOYLE, C.H., CHAPPLE, C. & BURNSTOCK, G. (1989). Isolated human bladder: evidence for an adenine dinucleotide acting on P_{2x}-purinoceptors and for purinergic transmission. *Eur. J. Pharmacol.*, **174**, 115–118.

IACOVOU, J.W., HILL, S.J. & BIRMINGHAM, A.T. (1990). Agonist-induced contraction and accumulation of inositol phosphates in the guinea-pig detrusor: evidence that muscarinic and purinergic receptors raise intracellular calcium by different mechanisms. *J. Urol.*, **144**, 775–779.

INOUE, R. & BRADING, A.F. (1990). The properties of the ATP-induced depolarization and current in single cells isolated from the guinea-pig urinary bladder. *Br. J. Pharmacol.*, **100**, 619–625.

INOUE, R. & BRADING, A.F. (1991). Human, pig and guinea-pig bladder smooth muscle cells generate similar inward currents in response to purinoceptor activation. *Br. J. Pharmacol.*, **103**, 1840–1841.

KATSURAGI, T., USUNE, S. & FURUKAWA, F. (1990). Antagonism by nifedipine of contraction and Ca^{2+}-influx evoked by ATP in guinea-pig urinary bladder. *Br. J. Pharmacol.*, **100**, 370–374.

KENNEDY, C. (1990). P_1- and P_2-purinoceptor subtypes – an update. *Arch. Int. Pharmacodyn.*, **303**, 30–50.

LEFEBVRE, R.A. & BURNSTOCK, G. (1990). Effect of adenosine triphosphate and related purines in the rat gastric fundus. *Arch. Int. Pharmacodyn.*, **303**, 199–215.

MAGGI, C.A., GIULIANI, S., PAPACCHINI, R., TURINI, D., BARBANTI, G., GIACHETTI, A. & MELI, A. (1989). Multiple sources of calcium for contraction of the human bladder muscle. *Br. J. Pharmacol.*, **98**, 1021–1031.

MANZINI, S., MAGGI, C.A. & MELI, A. (1985). Further evidence for involvement of adenosine-5'-triphosphate in non-adrenergic non-cholinergic relaxation of the isolated rat duodenum. *Eur. J. Pharmacol.*, **113**, 339–408.

MOODY, C.J., MEGHJI, P. & BURNSTOCK. G. (1984). Stimulation of P_1-purinoceptors by ATP depends partly on its conversion to AMP and adenosine and partly on direct activation. *Eur. J. Pharmacol.*, **97**, 47–54.

NICHOLLS, J., HOURANI, S.M. & KITCHEN, I. (1990). The ontogeny of purinoceptors in rat urinary bladder and duodenum. *Br. J. Pharmacol.*, **100**, 874–878.

PARIJA, S.C., RAVIPRAKASH, V. & MISHRA, S.K. (1991). Adenosine and α-β-methylene ATP-induced differential inhibition of cholinergic and non-cholinergic neurogenic responses in rat urinary bladder. *Br. J. Pharmacol.*, **102**, 396–400.

RALEVIC, V. & BURNSTOCK, G. (1991). Roles of P_2-purinoceptors in the cardiovascular system. *Circ.*, **84**, 1–14.

SNEDDON, P. & WESTFALL, D.P. (1984). Pharmacological evidence that adenosine triphosphate and noradrenaline are co-transmitters in the guinea-pig vas deferens. *J. Physiol.*, **347**, 561–580.

WITT, P.A., KRAMER, T.H. & BURKS, T.F. (1991). Norepinephrine and ATP are synergistic in the mouse vas deferens preparation. *Eur. J. Pharmacol.*, **204**, 149–155.

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Characterization and ontogeny of P_1-purinoceptors on rat vas deferens

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1 The P_1-purinoceptors which mediate the inhibition by adenosine of nerve-mediated contraction of the rat vas deferens have been investigated by use of the agonists N^6-cyclopentyladenosine (CPA) and 5'-N-ethylcarboxamidoadenosine (NECA) and the A_1-selective antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX). The ontogeny of the responses to adenosine and to the two co-transmitters which induce the contractions in this tissue, adenosine 5'-triphosphate (ATP) and noradrenaline (NA), have also been studied.

2 The order of potency for the adenosine agonists in inhibiting the nerve-mediated contractions was CPA = NECA > adenosine. Micromolar concentrations of DPCPX were required to antagonize the inhibition by adenosine and NECA of nerve-mediated responses, whereas the inhibitory effect of CPA was antagonized by nanomolar concentrations of the antagonist.

3 NECA and adenosine inhibited contractions induced by ATP (10 μM) or by NA (10 μM), NECA being at least ten fold more potent than adenosine, whereas CPA was inactive. Micromolar concentrations of DPCPX were required to antagonize the effect of adenosine on the contractions induced by ATP (10 μM).

4 Nerve-stimulated contractions could be observed in neonatal tissues from day 15 and increased with age, and could be inhibited by adenosine from this time, the potency of adenosine decreasing with age. Responses to ATP also appeared at day 15 and increased with age up to day 25, while responses to NA were present from day 10 (the earliest day tested) and decreased with age.

5 These results show that the rat vas deferens contains both prejunctional A_1-receptors and postjunctional A_2-receptors, and that adenosine acts on the latter populations to inhibit nerve-mediated contractions. The high potency of adenosine in the neonate and the parallel development of responses to ATP and to nerve-mediated contractions support suggestions that purinergic responses may be particularly important in neonatal tissues.

Keywords: P_1-purinoceptors; vas deferens; ontogeny; prejunctional; adenosine; ATP

Introduction

The purine nucleoside adenosine has a well-characterized role as a neuromodulator in the peripheral and central nervous systems, and has been shown to inhibit the release of many neurotransmitters. It also has direct post-junctional effects in many tissues, and can cause either contraction or, more commonly, relaxation of smooth muscle (White, 1988; Williams, 1989; Burnstock, 1990a). The effects of adenosine are mediated by specific receptors called P_1-purinoceptors of which two major classes exist, A_1 and A_2, which can be clearly distinguished in pharmacological and binding studies by the use of agonist potency orders and selective antagonists. On A_1-receptors, N^6-substituted analogues of adenosine such as N^6-cyclopentyladenosine (CPA) are more potent than 5'-substituted analogues such as 5'-N-ethylcarboxamidoadenosine (NECA) and the antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) has nanomolar affinity, whereas on A_2-receptors, NECA is more potent than CPA and DPCPX has micromolar affinity (Jacobson, 1990; Collis, 1990). A_2-receptors have been subdivided into A_{2a} and A_{2b} on the basis of radioligand binding in the brain and functional studies in fibroblasts (Bruns *et al.*, 1986), although this has not been widely investigated in smooth muscle, and a third class, A_3, has been proposed to exist in the heart and on nerve endings (Ribeiro & Sebastiao, 1986), but has not gained wide acceptance and the receptors here are normally considered to be of the A_1-type. As well as mediating prejunctional inhibition, A_1-receptors have also been shown to mediate the contractile effects of adenosine in smooth muscle, whereas the A_2-receptors mediate the relaxant effects (White, 1988; Burnstock, 1990a; Kennedy, 1990; Olsson & Pearson, 1990).

The adenine nucleotide adenosine 5'-triphosphate (ATP) also has effects on smooth muscle which are mediated by receptors called P_2-purinoceptors, of which at least two subclasses have been proposed to exist, P_{2X} and P_{2Y}, causing in general contraction and relaxation respectively (Burnstock & Kennedy, 1985; Burnstock, 1990a; Kennedy, 1990). ATP is rapidly and sequentially dephosphorylated ultimately to adenosine by ectonucleotidases present on smooth muscle, and the adenosine generated by this process may have its own effects via P_1-purinoceptors. There is good evidence that ATP has a transmitter or co-transmitter role in many tissues with a substantial non-adrenergic, non-cholinergic autonomic innervation, and adenosine derived from released ATP may play a role as a feedback inhibitor of transmitter release in these tissues (White, 1988; Burnstock, 1990b).

In the rat vas deferens, ATP causes contraction via P_{2x}-purinoceptors and the co-transmitter role of ATP released with noradrenaline (NA) is well established (White, 1988; Burnstock, 1990b; Von Kugelgen & Starke, 1991). In this tissue adenosine has been clearly shown to inhibit both NA release and nerve-stimulated contractions and has been reported not to inhibit contractions induced by NA; it has generally been assumed that the inhibitory effects of adenosine on the nerve-mediated contractions are due to prejunctional inhibition (Clanachan *et al.*, 1977; White, 1988; Paton, 1981a; 1989). Such prejunctional effects are likely to be mediated by A_1-receptors, and indeed N^6-substituted analogues are more potent than adenosine at inhibiting

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nerve-mediated contractions (Paton, 1981b) and the A_2-selective 2-substituted analogue 2-(phenylamino)adenosine (CV-1808) has been reported to be inactive (Major *et al.*, 1989), suggesting that an A_1-receptor is involved. However, in a detailed analysis of the structure-activity relationships of adenosine analogues in various tissues, the rat vas deferens emerged as a tissue which did not clearly fit into either of the two patterns (Paton, 1989), and in two recent brief reports it has been suggested that as well as prejunctional A_1-receptors there may also be postjunctional A_2-receptors which also inhibit the nerve-mediated contractions (Huidobro-Toro & Parada, 1989; Gustafsson *et al.*, 1990).

As part of a continuing study of the ontogeny of purinoceptors in smooth muscle we wished to use the rat vas deferens as a model tissue in which to study the development of prejunctional A_1-receptors to complement our previous work on rat urinary bladder, which contains A_2-receptors, and on the rat duodenum, which contains a mixture of A_1 and A_2 (Nicholls *et al.*, 1990; 1992). We therefore investigated the development with neonatal age of the inhibitory effects of adenosine on nerve-mediated contractions and of the development of responses to the two co-transmitters ATP and NA, and have also used CPA, NECA and DPCPX to characterize the adenosine receptors present. Some of these results have been briefly presented elsewhere (Lee *et al.*, 1991).

Methods

Adult male Wistar rats, >60 days old (200–250 g), and male neonatal rats up to 35 days old were killed by cervical dislocation. The day of birth was designated as day 1. The vasa deferentia were dissected out, freed of connective tissue and mounted in 10 ml organ baths containing magnesium-free Krebs solution of the following composition (mM): NaCl 118, $NaHCO_3$ 25, KH_2PO_4 1.2, KCl 4.8, $CaCl_2$ 2.5 and glucose 11, gassed with 95% O_2:5% CO_2 and maintained at 36°C. Magnesium-free Krebs was used because this enhances transmitter release and in the presence of magnesium, nerve-mediated responses cannot be detected in neonatal vas deferens. A resting tension of 0.5 g was applied to the tissues and isometric responses were recorded after equilibration for 45 min. Contractions were induced by the addition of ATP (0.1–100 μM), NA (0.01–100 μM), or by field stimulation (Grass S48 stimulator, twin pulses of 1 ms duration, 75 ms delay, 70 V) via parallel platinum electrodes (0.3 mm diameter, 0.5 cm apart), and expressed as a percentage of the maximal response induced by KCl (120–180 mM). Tetrodotoxin (TTX) (1 μM) was used to verify that the contractions induced by field stimulation were neurogenic. To measure the inhibitory effects of adenosine and its analogues on nerve-mediated contractions, the tissues were field-stimulated at a frequency of 0.1 Hz, the purines were added to the bath when the response to nerve stimulation had stabilized and left in contact with the tissue for 1 min or until maximum inhibition of the contractions were observed. The inhibition was expressed as % reduction of the nerve-mediated response. To measure the inhibitory effects of adenosine and its analogues on contractions induced by ATP and by NA, the tissues were preincubated with the purines for 1 min before addition of the contractile agonists (10 μM). This concentration was chosen as it was a submaximal concentration which reliably gave consistent contractile responses. The contractions induced by this concentration of NA were approximately equal to those induced by nerve stimulation, whereas those induced by this concentration of ATP were roughly one third as forceful. All concentration-response curves were constructed non-cumulatively, with 10 min allowed between doses, and pD_2 values (the negative logarithm of the EC_{50}) were calculated from linear regression analysis of the linear portion of the individual log concentration-response curves. In experiments to investigate the effect of DPCPX on the inhibition by adenosine or its analogues of the nerve-mediated responses, control concentration-response curves to the purines were constructed, followed by incubation with the antagonist for 30 min before repeating the concentration-response curves in the presence of DPCPX. Concentration-response curves repeated after 30 min incubation without DPCPX were not different from control curves. In experiments to investigate the effect of DPCPX on the inhibition by adenosine of ATP-induced responses, the control concentration-response curves and the concentration-response curves in the presence of DPCPX were carried out in parallel on separate tissues. This was necessary as the response to ATP tended to increase with time, making the determination of a second concentration-response curve to adenosine in the same tissue unreliable. Dose-ratios were calculated as the ratio of EC_{50} values in the presence and absence of DPCPX, and the dissociation constant (K_B) of the antagonist was calculated as the antagonist concentration divided by the dose-ratio − 1. pD_2 values for agonists in the presence and absence of DPCPX were compared by paired Student's *t* test in the case of experiments on nerve-mediated responses and unpaired Student's *t* test in the case of experiments on ATP-induced responses.

Drugs

DPCPX and CPA were obtained from Research Biochemicals Inc., and all other drugs were obtained from Sigma Chemical Co. CPA (10 mM) was dissolved in 20% ethanol and DPCPX (1 mM) was dissolved in 2% aqueous dimethylsulphoxide (DMSO) containing 6 mM NaOH, and after dilution corresponding to the final bath concentration of the substances used these solvents had no effect on the responses of the tissue. All other drugs were dissolved in distilled water.

Results

Field stimulation of the adult rat vas deferens induced contractions which were abolished by TTX (1 μM) (results not shown), and adenosine, NECA and CPA each inhibited these nerve mediated contractions with an order of potency of NECA = CPA > adenosine (Figure 1). The concentration-response curve for CPA was shifted to the right in a parallel fashion by nanomolar concentrations of DPCPX, with the dose-ratio for 3 nM DPCPX corresponding to a K_B value of 2.7 nM. DPCPX 1 μM resulted in a much larger dose-ratio which corresponded to a K_B value of 12.2 nM, although it should be noted that maximal responses to CPA could not be

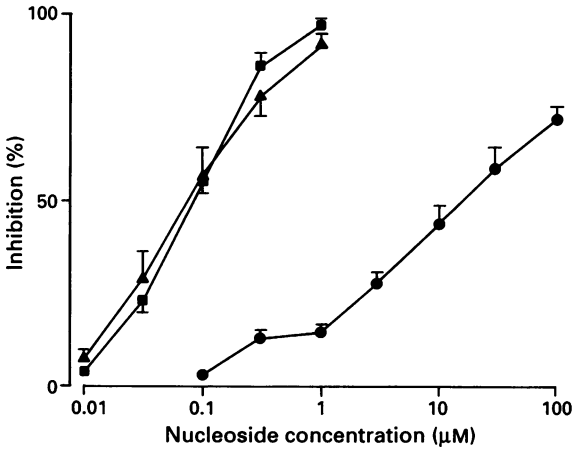


Figure 1 Effects of adenosine (●), 5'-N-ethylcarboxamidoadenosine (NECA, ■) and N^6-cyclopentyladenosine (CPA, ▲) on nerve-mediated contractions of the rat vas deferens. Each point is the mean of at least 6 determinations and the vertical bars show s.e.mean.

Table 1 Effect of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) on the inhibition by adenosine, 5'-N-ethylcarboxamidoadenosine (NECA) and N^6-cyclopentyladenosine (CPA) of nerve-mediated contractions of the rat vas deferens

	Agonist pD_2	*Dose-ratio (K_B)*
Adenosine alone	4.35 ± 0.16	3.87 (0.35 μM)
Adenosine + DPCPX (1 μM)	3.76 ± 0.20*	
NECA alone	7.03 ± 0.08	2.85 (0.54 μM)
NECA + DPCPX (1 μM)	6.58 ± 0.15**	
CPA alone	6.96 ± 0.23	2.10 (2.7 nM)
CPA + DPCPX (3 nM)	6.64 ± 0.16**	
CPA alone	6.91 ± 0.19	83.2 (12.2 nM)
CPA + DPCPX (1 μM)	4.99 ± 0.12***	

Agonist pD_2 values are the mean ± s.e.mean of 4 determinations, and were significantly lower in the presence of antagonist (Student's paired t test: $*P<0.05$; $**P<0.01$; $***P<0.001$).

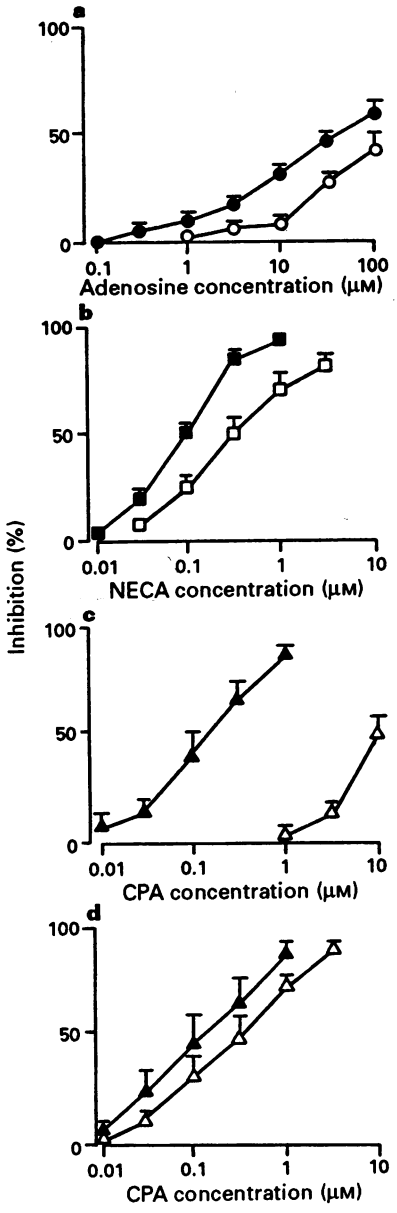


Figure 2 Antagonism by 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) of the effects of (a) adenosine (●,○); (b) 5'-N-ethylcarboxamidoadenosine (NECA, ■,□) or (c and d) N^6-cyclopentyladenosine (CPA, ▲,△) on nerve-mediated contractions of the rat vas deferens. The closed symbols show the effect of agonist alone, and the open symbols the effect of agonist in the presence of DPCPX at a concentration of 1 μM in (a), (b) and (c), and 3 nM in (d). Each point is the mean of at least 4 determination and the vertical bars show s.e.mean.

achieved due to its limited solubility (Figure 2). DPCPX 1 nM, gave a smaller non-significant shift of the concentration-response curve to CPA and did not inhibit the responses to NECA and adenosine (results not shown), which were inhibited by 1 μM DPCPX with dose-ratios corresponding to K_B values of 0.54 μM and 0.35 μM respectively (Figure 2). The pD_2 values for the agonists and the dose-ratios are detailed in Table 1. Adenosine and NECA also dose-dependently inhibited the responses to ATP (10 μM) and to NA (10 μM), but CPA was inactive at concentrations up to 1 μM. NECA was at least ten fold more potent than adenosine in each case, with pD_2 values of 6.50 ± 0.21 and 6.40 ± 0.22 for inhibition of ATP and NA respectively, while the corresponding pD_2 values for adenosine were 5.37 ± 0.10 and 4.85 ± 0.16 (Figure 3). The effect of adenosine on contractions induced by ATP (10 μM) was not reduced by

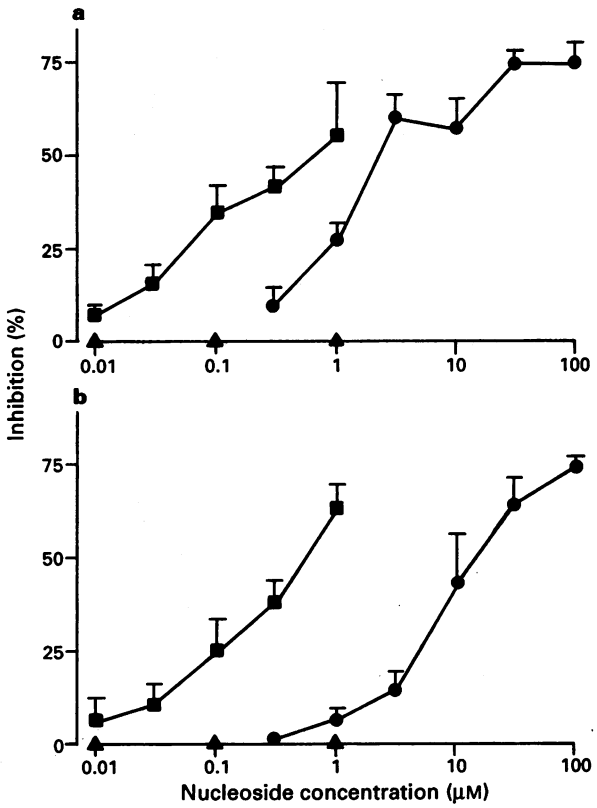


Figure 3 Effects of adenosine (●), 5'-N-ethylcarboxamidoadenosine (NECA, ■) or N^6-cyclopentyladenosine (CPA, ▲) on contractions of rat vas deferens induced by (a) ATP (10 μM) or (b) noradrenaline (NA, 10 μM). Each point is the mean of at least 4 determinations, and the vertical bars show s.e.mean.

DPCPX (1 nM) (results not shown), but was antagonized by DPCPX (1 μM), the pD_2 value being significantly ($P < 0.05$) reduced from 6.11 ± 0.16 to 5.47 ± 0.21 with a dose-ratio of 4.38 corresponding to an antagonist K_B of 0.30 μM (Figure 4).

The inhibitory effect of adenosine on nerve-mediated contractions was greatest at day 15, the earliest day at which field stimulation induced contractions, and the potency of adenosine declined with age until day 35 when it was equipotent with the adult (Figure 5). At each of these ages adenosine achieved the same maximal inhibition of the nerve-mediated contractions (approximately 90%) (results not shown). No contractions to KCl, ATP or NA were observed in 5 day-old vas deferens but in tissues from rats 10 days or older, contractile responses were obtained. Contractions to KCl increased with age and the effects of ATP and NA expressed relative to the contraction induced by KCl varied with age, but rather than a change in the EC_{50} value a change in the maximal response was observed. The maximal response to NA (100 μM) decreased with age from day 10 (the earliest day tested), while ATP (100 μM) had no effect at day 10 and its effect increased up to day 25, when it achieved a greater response than in the adult (Figure 5). No response to nerve stimulation was observed at day 10, and the response increased up to day 25, when it was equivalent to that in the adult (Figure 5).

Discussion

The order of potency of the adenosine analogues in inhibiting the nerve-mediated contractions of the adult rat vas deferens confirmed the suggestion made by Paton (1989) that the receptors mediating this effect do not fall clearly into the A_1 or A_2 classes, as NECA and CPA were equipotent and both much more potent than adenosine itself. This order of potency could be taken as support for the existence of an A_3 receptor subtype, at which NECA and CPA have been proposed to be equipotent (Ribeiro & Sebastiao, 1986), but an alternative explanation is that the rat vas deferens contains instead a mixture of prejunctional A_1-receptors and postjunctional A_2-receptors, as suggested by Huidobro-Toro & Parada (1989) and Gustafsson *et al.* (1990). The inhibition by adenosine of contractions induced by exogenous ATP and NA is in contrast to the results reported by Clanachan *et al.* (1977) and shows that there must be a postjunctional adenosine receptor. The finding that NECA also inhibits these contractions and is more potent than adenosine but that CPA does not, suggests strongly that this postjunctional adenosine receptor is of the A_2-class, and this was confirmed by the high (micromolar) concentration of DPCPX required to antagonize the inhibition by adenosine of contractions induced by ATP. That micromolar concentrations of DPCPX are also required to antagonize the inhibitory effects of adenosine and NECA on nerve stimulation shows that these agonists have their effect on the nerve-mediated contractions via this postjunctional A_2 receptor. The lack of effect reported by Major *et al.* (1989) of the A_2-selective agonist, CV-1808, could indicate that these A_2-receptors are of the A_{2b} subclass, as CV-1808 has selectivity for A_{2a}-receptors (Bruns *et al.*, 1986). That CPA inhibited the nerve-mediated contractions shows that there must also be a prejunctional adenosine receptor, and the low (nanomolar) concentrations of DPCPX which are effective in antagonizing this effect of CPA shows that this prejunctional receptor, as expected, is of the A_1-class. Overall therefore, these results provide evidence

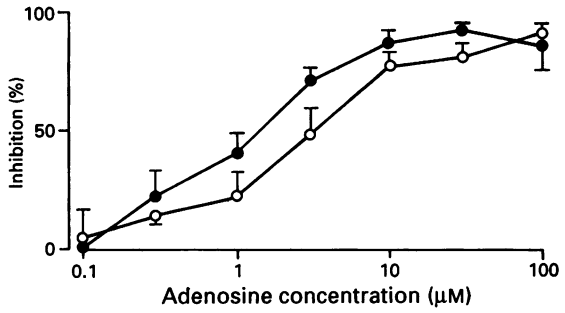


Figure 4 Effect of adenosine alone (●) or in the presence of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 1 μM) (○) on contractions of the rat vas deferens induced by ATP (10 μM). Each point is the mean of at least 4 determinations, and the vertical bars show s.e.mean.

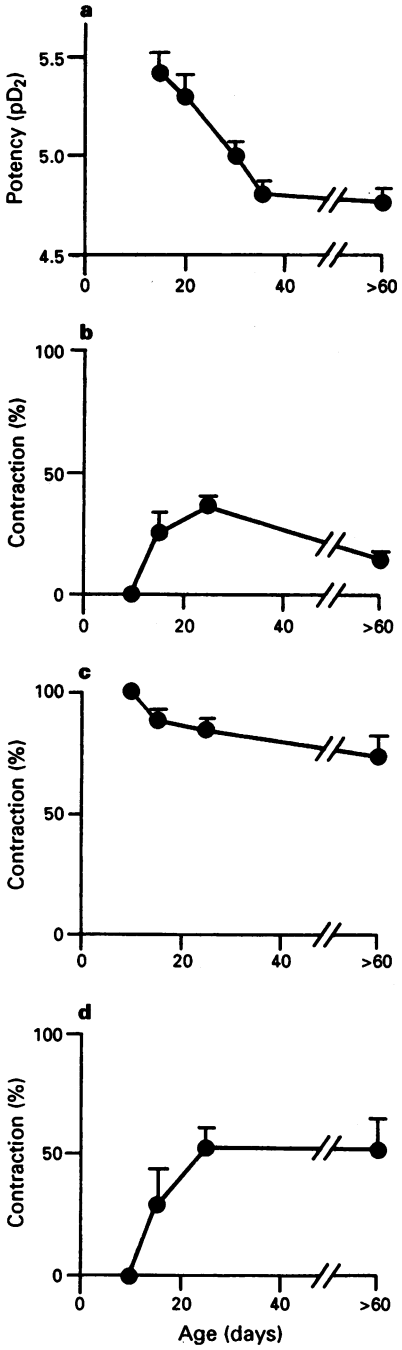


Figure 5 Variations in the responses of the rat vas deferens with age. (a) Variation in the potency of adenosine, expressed as pD_2 (the negative log of concentration producing 50% inhibition of the nerve-mediated contraction). (b, c and d) Variation in the contractile response induced by (b) ATP (100 μM), (c) noradrenaline (NA, 100 μM), or (d) nerve stimulation, expressed as a percentage of a maximal response to KCl. Each point is the mean of at least 4 determinations, and the vertical bars show s.e.mean.

that NECA and adenosine inhibit nerve-mediated contractions via postjunctional A_2-receptors while CPA acts via prejunctional A_1-receptors. As adenosine and NECA in general have a rather higher potency or affinity at A_1-receptors than they do at A_2 (White, 1988; Jacobson, 1990), it is perhaps surprising that their A_2 effects seem to dominate in this tissue, although we have also found this to be the case in the rat duodenum in which a mixed population of A_1- and A_2-adenosine receptors exists (Nicholls *et al.*, 1992).

The potency of adenosine in inhibiting the nerve-mediated contractions decreased with age from day 15, the earliest age at which nerve-mediated contractions could be observed, and the greater potency of adenosine in the neonate in this tissue is in agreement with our previous findings in the rat bladder in which adenosine also inhibits contractions via A_2 receptors (Nicholls *et al.*, 1990; 1992). In the rat bladder however, ATP induced contractions via P_{2X} receptors from day 2 and was considerably more potent than in the adult between days 5 and 25 (Nicholls *et al.*, 1990), whereas in the vas deferens ATP had no effect at 10 days but again was more potent than in the adult at day 25. The ontogeny of the responses we observed to nerve stimulation mirrored those to ATP in that no contraction was observed at day 10 and that the effect increased up to day 25. The responses to NA showed a different pattern of development and decreased with age, and this suggests that in the neonatal tissue, ATP is more important as a transmitter than is NA. These results are in agreement with those of MacDonald & McGrath (1984), who showed that the vasa deferentia from sexually immature rats (3–4 weeks old) were more sensitive to NA than were the vasa from adult rats, but appeared to lack functional adrenergic transmission. Some workers have been able to distinguish a first non-adrenergic (purinergic) phase of the contractile response from a second adrenergic phase (McGrath, 1978; Amobi & Smith, 1987), although others (Major *et al.*, 1989) have concluded that both co-transmitters contribute to each phase. As we did not distinguish two phases to the nerve-stimulated contraction we were not able to follow each phase separately to see if they developed differentially in the neonatal tissues.

In conclusion, we have shown that the adult rat vas deferens contains both prejunctional A_1-receptors and postjunctional A_2-receptors and that adenosine acts via this latter population, and that there is no evidence for a third receptor class (A_3) in this tissue. Responses to ATP and to nerve stimulation are present from day 15 and increase with age up to day 25, whereas responses to NA are present from day 10 and decrease with age. Adenosine inhibits nerve-mediated responses as soon as these are detectable and its potency also decreases with age. These findings, together with our previous results with the rat bladder and duodenum (Nicholls *et al.*, 1990), suggest that purinergic responses may be particularly important in neonates.

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References

AMOBI, N. & SMITH, I.C.H. (1987). Effects of α,β-methylene ATP on biphasic responses of rat vas deferens. *Eur. J. Pharmacol.*, **133**, 75–82.

BRUNS, R.F., LU, G.H. & PUGSLEY, T.A. (1986). Characterization of the A_2 adenosine receptor labeled by [^{3}H]NECA in rat striatal membranes. *Mol. Pharmacol.*, **29**, 331–346.

BURNSTOCK, G. (1990a). Classification and characterization of purinoceptors. In *Purines in Cellular Signalling*. ed. Jacobson, K.A., Daly, J.W. & Manganiello, V. pp. 241–253. New York: Springer-Verlag.

BURNSTOCK, G. (1990b). Noradrenaline and ATP as cotransmitters in sympathetic nerves. *Neurochem. Int.*, **17**, 357–368.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen. Pharmacol.*, **16**, 433–440.

CLANACHAN, A.S., JOHNS, J.W. & PATON, D.M. (1977). Presynaptic inhibitory actions of adenine nucleotides and adenosine in the rat vas deferens. *Neuroscience*, **2**, 597–602.

COLLIS, M.G. (1990). Adenosine receptor sub-types in isolated tissues: antagonist studies. In *Purines in Cellular Signalling*. ed. Jacobson, K.A., Daly, J.W. & Manganiello, V. pp. 48–53. New York: Springer-Verlag.

DALY, J.W. (1990). Adenosine agonists and antagonists. In *Purines in Cellular Signalling*. ed. Jacobson, K.A., Daly, J.W. & Manganiello, V. pp. 3–12. New York: Springer-Verlag.

GUSTAFSSON, L.E., WIKLUND, C.U., WIKLUND, N.P. & STELIUS, L. (1990). Subclassification of neuronal adenosine receptors. In *Purines in Cellular Signalling*. ed. Jacobson, K.A., Daly, J.W. & Manganiello, V. pp. 200–205. New York: Springer-Verlag.

HUIDOBRO-TORO, J.P. & PARADA, S. (1989). Pharmacological characterization of A_1 and A_2-adenosine receptors in the rat vas deferens neuroeffector junction. In *Adenosine Receptors in the Nervous System*. ed. Ribeiro, J.A. p. 199. London: Taylor & Francis.

JACOBSON, K.A. (1990). Adenosine (P_1) and ATP (P_2) receptors. In *Comprehensive Medicinal Chemistry*, Vol. 3. *Membranes and Receptors*. ed. Emmett, J.C. pp. 601–642. Oxford: Pergamon Press.

KENNEDY, C. (1990). P_1- and P_2-purinoceptors subtypes – an update. *Arch. Int. Pharmacodyn.*, **303**, 30–50.

LEE, B.S.S., NICHOLLS, J., KITCHEN, I. & HOURANI, S.M.O. (1991). P_1-purinoceptors in adult and neonatal rat vas deferens. *Fund. Clin. Pharmacol.*, **5**, 461.

MACDONALD, A. & MCGRATH, J.C. (1984). Post-natal development of functional neurotransmission in rat vas deferens. *Br. J. Pharmacol.*, **82**, 25–34.

MCGRATH, J.C. (1978). Adrenergic and 'non-adrenergic' components in the contractile response of the vas deferens to a single indirect stimulus. *J. Physiol.*, **283**, 23–39.

MAJOR, T.C., WEISHAAR, R.E. & TAYLOR, D.G. (1989). Two phases of contractile response in rat isolated vas deferens and their regulation by adenosine and α-receptors. *Eur. J. Pharmacol.*, **167**, 323–331.

NICHOLLS, J., HOURANI, S.M.O. & KITCHEN, I. (1990). The ontogeny of purinoceptors in rat urinary bladder and duodenum. *Br. J. Pharmacol*, **100**, 874–878.

NICHOLLS, J., HOURANI, S.M.O. & KITCHEN, I. (1992). Characterisation of P_1-purinoceptors on rat duodenum and urinary bladder. *Br. J. Pharmacol.*, **105**, 639–642.

OLSSON, R.A. & PEARSON, J.D. (1990). Cardiovascular purinoceptors. *Pharmacol. Rev.*, **70**, 761–845.

PATON, D.M. (1981a). Presynaptic neuromodulation mediated by purinergic receptors. In *Purinergic Receptors. Receptors and Recognition Series B*, Volume 12. ed. Burnstock, G. pp. 199–219. London: Chapman & Hall.

PATON, D.M. (1981b). Structure-activity relations for presynaptic inhibition of noradrenergic and cholinergic transmission by adenosine: evidence for action on A_1 receptors. *J. Auton. Pharmacol.*, **1**, 287–290.

PATON, D.M. (1989). Function and structure of presynaptic adenosine receptors in peripheral adrenergic and cholinergic nerves. In *Adenosine Receptors in the Nervous System*. ed. Ribeiro, J.A. pp. 79–86. London: Taylor & Francis.

RIBEIRO, J.A. & SEBASTIAO, A.M. (1986). Adenosine receptors and calcium: basis for proposing a third (A_3) adenosine receptor. *Prog. Neurobiol.*, **26**, 179–209.

VON KUGELGEN, I. & STARKE, K. (1991). Noradrenaline-ATP cotransmission in the sympathetic nervous system. *Trends Pharmacol. Sci.*, **12**, 319–324.

WHITE, T.D. (1988). Role of adenine compounds in autonomic neurotransmission. *Pharmacol. Ther.*, **38**, 129–168.

WILLIAMS, M. (1989). Adenosine: the prototypic neuromodulator. *Neurochem. Int.*, **14**, 249–264.

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Tachykinin receptors of the NK_2 type involved in the acetylcholine release by nicotine in guinea-pig bladder

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1 The effects of guanethidine and tachykinins on nicotine- and electrical stimulation-induced cholinoceptor responses were studied in isolated urinary bladder from the guinea-pig.

2 Acetylcholine release and the contractile response stimulated by nicotine were partially reduced by a sympathetic nerve blocker, guanethidine. Neurokinin A (but not substance P methyl ester or senktide) enhanced both acetylcholine release and contraction by nicotine in the presence of guanethidine.

3 Frequency-contraction curves (1 to 50 Hz) for electrical field stimulation (EFS) were partially reduced by atropine (1 μM), and after desensitization to α,β-methylene adenosine 5'-triphosphate, the atropine-resistant contraction to EFS was completely abolished. Guanethidine, the tachykinin antagonist [D-Arg[1], D-Pro[2], Trp[7,9], Leu[11]]-substance P and application of neurokinin A or substance P did not change the contractile response to EFS. Preganglionic nerve stimulation (5 Hz and 20 Hz) also evoked a similar response to EFS and was not influenced at all by guanethidine or neurokinin A.

4 We conclude that the ability of nicotine to release acetylcholine is enhanced both by endogenous tachykinins (probably released from sympathetic nerves) and by exogenously applied tachykinins as a result of interaction with NK_2 receptors in the urinary bladder.

Keywords: NK_2 tachykinin receptor; nicotine; acetylcholine release; sympathetic nerve; guinea-pig bladder

Introduction

We have shown that nicotine produces a tetrodotoxin-resistant contraction of isolated detrusor strips of guinea-pig, and have suggested that the drug might interact with the presynaptic nicotinic receptors located on parasympathetic cholinergic nerves and non-sympathetic purinergic nerves to release acetylcholine and a purine nucleotide, since the contractile response is blocked by a combination of atropine and desensitization of P_2-purinoceptors by α,β-methylene adenosine 5'-triphosphate (Hisayama *et al.*, 1988). The nicotine-induced acetylcholine output was partially reduced by guanethidine and [D-Arg[1], D-Pro[2], Trp[7,9], Leu[11]]-substance P (rpwwL-SP) to much the same extent. Pretreatment with guanethidine abolished the inhibitory effects of rpwwL-SP and *vice versa*. We therefore proposed that nicotine was also stimulating release of a tachykinin from sympathetic nerves and that acetylcholine release evoked by nicotine was enhanced by their action (Hisayama *et al.*, 1989). However, the possibility that tachykinins from an unknown source stimulate release of a mediator from sympathetic nerve endings, remained, although a sympathetic component is not observed in the contraction to electrical field stimulation (Ambache & Zar, 1970; Burnstock *et al.*, 1978; Fujii, 1988).

We have now determined the effects of exogenously applied tachykinins on the cholinoceptor responses induced by nicotine or electrical stimulation.

Methods

Female Hartley strain guinea-pigs, weighing between 300 and 500 g, were reared on a standard diet and given tap water to drink. The guinea-pigs were stunned by a blow on the head and exsanguinated from the femoral artery. The urinary bladder was rapidly removed and a longitudinal strip (about 2 mm × 15 mm) of the detrusor muscle was prepared. Each strip was suspended vertically under a resting load of 1 g in a 5 ml organ bath which contained Krebs solution of the following composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.50, $MgSO_4$ 1.20, KH_2PO_4 1.20, $NaHCO_3$ 25.0 and glucose 10.0. The organ bath was maintained at 37°C and constantly gassed with carbogen (95% O_2 + 5% CO_2). The responses to drugs were recorded isotonically.

Electrical field stimulation was applied through a pair of platinum-wire electrodes by an electronic stimulator (Nihon Kohden, SEN-3301). Stimulus parameters were 0.5 ms duration, frequency of 1 to 50 Hz and supramaximal voltage for 2 s.

Preganglionic stimulation of the parasympathetic nerves innervating the bladder was performed as described by Hukovic *et al.* (1965). One ureter was dissected for about 20 mm from the bladder and thread was attached for passing the ureter through the electrodes; the detrusor strip was stimulated indirectly by rectangular pulses (50 V, duration 0.5 ms, 5 or 20 Hz for 2 s).

Desensitization to α,β-methylene adenosine 5'-triphosphate (α,β-MeATP) was produced by the method of Kasakov & Burnstock (1983).

When we determined the amount of acetylcholine released, each strip was incubated with 1 mM methanesulphonyl fluoride (MSF) for 30 min in order to inhibit acetylcholinesterase activity irreversibly. After priming 2 or 3 times with 300 nM carbachol, Krebs solutions containing various concentrations of drugs were superfused at a rate of 0.4 ml min^{-1} from the bottom and collected by aspiration at the top of an organ bath, and the effects of these drugs on acetylcholine output from the strips were evaluated. The superfusate was collected continuously on ice, divided into fractions for each 3 min period and assayed for acetylcholine as described by Shinkai *et al.* (1991).

Acetylcholine was determined by radioimmunoassay with rabbit antiserum raised against choline hemiglutarate-bovine serum albumin conjugates and tritiated acetylcholine with a specific activity of 86 Ci $mmol^{-1}$ according to the method of Kawashima *et al.* (1980, 1988). Assays were performed in duplicate at 4°C. To avoid overestimation by cross-reactivity with nicotine, the standard curve for acetylcholine was derived in the presence of nicotine as appropriate.

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A 200 μl portion of the superfusate was incubated overnight with 50 μl of the diluted antiserum (1:350) in Tris-HCl buffer (0.15 M, pH 7.4) containing 0.4% bovine gamma-globulin, 0.05% isofluorophosphate and 50 μl of tritiated acetylcholine (about 12.9 pg, 4200 c.p.m.). The same volume of superfusion fluid containing 0.1 mM nicotine served as a blank. Antibody-bound tritiated acetylcholine was separated from free by the ammonium sulphate method, and the radioactivity of the precipitates was quantified in a liquid scintillation counter.

Statistical analyses were performed by Student's t test. A P value of <0.05 was considered a significant difference.

Drugs used were nicotine bitartrate (Nakarai Chemicals, Ltd., Kyoto, Japan), atropine sulphate, α,β-MeATP, gamma-globulin (Sigma Chemical Co., MO, U.S.A.), hexamethonium dibromide (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan), tetrodotoxin (Sankyo Co., Ltd., Tokyo, Japan), neurokinin A (NKA), substance P (SP), SP methyl ester (SPOMe), senktide, rpwwL-SP (Peninsula Laboratories, Inc., Belmont, CA, U.S.A.), acetyl[methyl-^{3}H]-choline chloride (Amersham Japan Co., Tokyo, Japan) and methanesulphonyl fluoride (Aldrich Chemical Co., Inc., U.S.A.). Guanethidine sulphate was donated by Ciba-Geigy (Japan), Ltd. (Hyogo, Japan). All drugs used were of analytical grade.

Results

The effects of some drugs on acetylcholine release induced by nicotine (0.1 mM) are shown in Figure 1. The release evoked by nicotine was 74.2 ± 11.1 pg mg^{-1} tissue per 3 min ($n = 6$). The amount of acetylcholine released in the presence of test drugs is shown as % of that released to nicotine alone. The nicotine-induced acetylcholine output was greatly reduced by the sympathetic nerve blocker, guanethidine (3 μM) to 27.2 ± 6.5% ($n = 5$). The inhibitory effect of guanethidine was countered by the application of 1 nM neurokinin A (105.8 ± 12.5%, $n = 5$) but not the NK_1-selective agonist, SPOMe (22.3 ± 1.73%, $n = 5$) or the NK_3 selective agonist, senktide

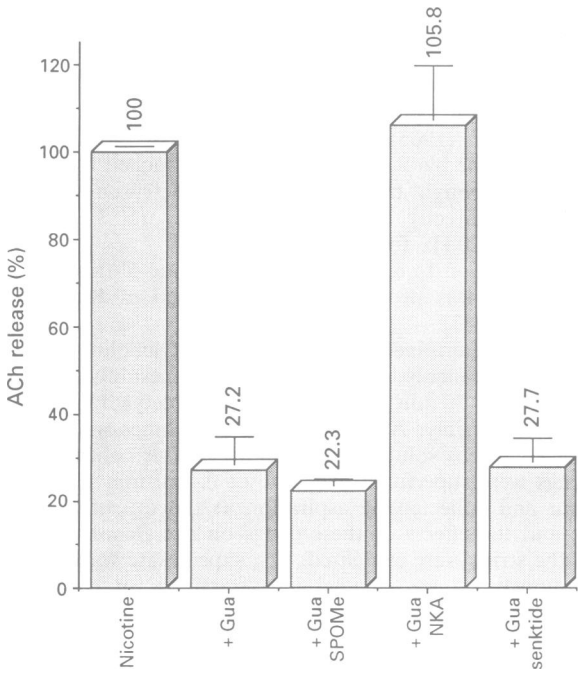


Figure 1 Effects of tachykinin receptor agonists on acetylcholine release evoked by nicotine from guinea-pig bladder. Gua: guanethidine 3 μM; NKA: neurokinin A 1 nM; SPOMe: substance P methyl ester 10 nM; senktide: senktide 10 nM. Nicotine-induced acetylcholine release was reduced by guanethidine to block modulator output from sympathetic nerves. Inhibitory effects of guanethidine were negated by neurokinin A but not by SPOMe or senktide.

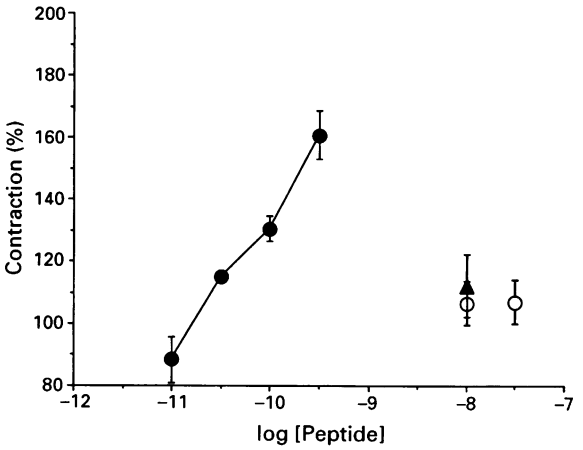


Figure 2 Effects of tachykinin receptor agonists on contractile responses induced by nicotine in the presence of guanethidine. Abscissa scale: log molar concentration of applied peptides. Ordinate scale: % of contraction induced by nicotine (0.1 mM) in the presence of guanethidine (3 μM): (●) neurokinin A; (○) senktide; (▲) substance P methyl ester (SPOMe). In the presence of guanethidine, nicotine-induced contraction was potentiated by neurokinin A but not by SPOMe or senktide.

(27.7 ± 5.5%, $n = 5$). The same was true of the contractile response to nicotine (0.1 mM): in the presence of guanethidine, the nicotine-induced contraction was also potentiated by neurokinin A (30 μM to 0.3 nM) but not by SPOMe (10 nM) or senktide (10 nM) (Figure 2). In the presence of guanethidine, none of the tachykinins tested in this study at the concentration of up to 10 nM produced a contraction.

Electrical field stimulation (EFS: frequency 1 to 50 Hz, duration 0.5 ms, trains 2 s, supramaximal voltage) produced tetrodotoxin-sensitive, transient contractions. The frequency-response curves were suppressed by atropine at all frequencies and the combined treatment of atropine and α,β-MeATP eliminated the nerve-mediated responses. The curves were not influenced by the treatment with guanethidine to block release of mediator from sympathetic nerve endings (Figure 3, Table 1).

Table 1 shows the effects of drugs on the contractions by EFS at 5 Hz and 20 Hz. Contractile responses to EFS were reduced by atropine and enhanced by physostigmine, indicating the existence of a cholinergic component. Hexamethonium, guanethidine, rpwwL-SP, neurokinin A, SPOMe and the metalloprotease inhibitor, phosphoramidon had no effect on the contraction caused by EFS.

Table 1 Effects of drugs on the contractile responses evoked by electrical field stimulation (EFS)

Treatment	*5 Hz*	*20 Hz*
Control	100	100
+ Hexamethonium, 100 μM, 5 min	102.3 ± 3.7	103.6 ± 3.9
+ Atropine, 1 μM, 5 min	51.9 ± 4.1*	61.0 ± 4.7*
+ Physostigmine, 0.1 μM, 30 min	136.0 ± 8.2*	146.7 ± 8.9*
+ Guanethidine, 3 μM, 30 min	106.7 ± 5.2	109.2 ± 5.1
+ rpwwL-SP, 10 μM, 5 min	113.2 ± 7.6	106.7 ± 5.3
+ Neurokinin A, 3 nM, 10 min	100.7 ± 6.1	104.5 ± 5.6
+ SPOMe, 10 μM, 10 min	95.6 ± 6.2	97.4 ± 2.8
+ Phosphoramidon, 10 μM, 20 min	105.6 ± 6.9	108.1 ± 6.2
+ Tetrodotoxin, 100 nM, 15 min	0.0 ± 0.0*	0.0 ± 0.0*

Each value shows mean ± s.e.mean of 6 experiments.
*Significant difference from control value (100%) at $P < 0.05$.
EFS (supramaximal voltage, duration 0.5 ms, 5 or 20 Hz for 2 s with 2 min intervals) was applied to the bladder. In contrast to results of nicotine-induced responses, contractions by EFS were not reduced by guanethidine and were not enhanced by neurokinin A.

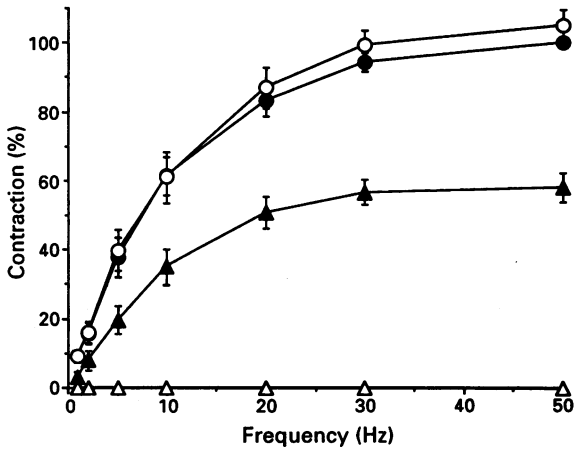


Figure 3 Effect of drugs on frequency-response curves in guinea-pig bladder. Abscissa scale: frequency. Ordinate scale: % of contraction induced by 50 Hz. (●) Control; (○) + guanethidine 3 μM; (▲) + atropine 1 μM; (△) + atropine after desensitization with α,β-methylene ATP 50 μM. Contractile responses to electrical field stimulation (EFS) were reduced by atropine and completely abolished by simultaneous treatment with atropine and α,β-methylene ATP. Though EFS induced a release of two excitatory transmitters, acetylcholine and a purine nucleotide as described in nicotine stimulation, guanethidine did not influence the curves.

When guinea-pig bladder preparations were stimulated indirectly through preganglionic nerves close to the ureter (5 Hz and 20 Hz), the evoked contractions were largely reduced by hexamethonium. Neurokinin A and guanethidine also had no effect on these contractile responses (Table 2).

Discussion

In this study, nicotine-induced acetylcholine output was reduced by guanethidine (Figure 1). Since chemical denervation with 6-hydroxydopamine abolished the inhibitory effect of guanethidine, and the drug did not inhibit the muscarinic receptor or purinoceptor mechanisms, it seems unlikely that the effect of guanethidine is non-specific (Hisayama *et al.*, 1988). We showed previously that nicotine contraction was not antagonized by bunazosin and yohimbine, and that exogenously applied noradrenaline did not cause a contraction even when the noradrenaline uptake mechanism and β-adrenoceptors were blocked (Hisayama *et al.*, 1988). The sympathomimetic effect of nicotine was non-adrenergic in nature, even if noradrenaline was released from the sympathetic nerve.

We previously determined that guanethidine and the tachykinin antagonist, rpwwL-SP, decreased the amount of acetylcholine released by nicotine to much the same degree. Pretreatment with guanethidine abolished the inhibitory effects of rpwwL-SP and *vice versa*. We proposed that acetylcholine release evoked by nicotine was increased by tachykinin released from sympathetic nerves (Hisayama *et al.*, 1989). But it is possible that tachykinins from an unknown site might have stimulated release of mediator from the sympathetic nerve endings. In this case, the excitatory effect of tachykinin (increasing acetylcholine output) would be abolished completely by a sympathetic nerve blocker.

Table 2 Effects of drugs on the contractile responses evoked by preganglionic stimulation

Treatment	*5 Hz*	*20 Hz*
Control	100	100
+ Hexamethonium, 100 μM, 5 min	53.9 ± 7.6*	62.9 ± 7.2*
+ Guanethidine, 3 μM, 30 min	106.6 ± 1.0	104.5 ± 8.9
+ Neurokinin A, 3 nM, 10 min	98.9 ± 6.6	101.5 ± 2.3
+ Tetrodotoxin, 100 nM, 15 min	0.0 ± 0.0*	0.0 ± 0.0*

*Significant difference from control value (100%) at $P<0.05$.
Each value shows mean ± s.e.mean of 5 experiments. Electrical stimulation (50 V, duration 0.5 ms, 5 or 20 Hz for 2 s with 2 min intervals) was applied to the supplying nerves (see Methods).

As shown in Figures 1 and 2, even in the presence of guanethidine which would block the release of any modulator from sympathetic nerves, neurokinin A enhanced acetylcholine output stimulated by nicotine. The possibility remains that nicotine could be releasing transmitters such as SP or neurokinin A from sensory nerve terminals. Densensitization to capsaicin, a drug that releases and depletes tachykinins from primary sensory neurones, had, however, no effect on the nicotine-induced contraction in guinea-pig bladder (Hisayama *et al.*, 1989). We may, therefore, reasonably conclude that sympathetic nerves are involved in the action of nicotine and are the site from which tachykinins are released.

As previously shown by Brading & Mostwin (1989) contractile responses to EFS were reduced by atropine and completely abolished by the stimultaneous treatment with α,β-MeATP and atropine (Figure 3). Since the response to nicotine is affected in the same way, it is likely that both methods of stimulation release the same excitatory transmitters. Two important differences were, however, noted between EFS and nicotine stimulation. Firstly, guanethidine had no effect on EFS-induced contraction at any frequency studied; this finding is in agreement with the accounts given by others (Ambache & Zar, 1970; Burnstock *et al.*, 1978; Fujii, 1988). One explanation for this lack of effect may be that modulator output from sympathetic nerves does not occur in response to EFS, but is initiated through presynaptic receptors and pharmacomechanical coupling. Secondly, neurokinin A (3 nM) did not enhance the contraction evoked by EFS (Table 1), although it clearly enhanced the ability of nicotine to release acetylcholine from nerve terminals. However, at this concentration, neurokinin A on its own has little contractile effect on the smooth muscle (Shinkai *et al.*, 1991). The metalloprotease inhibitor, phosphoramidon (10 μM) which potentiated pD_2 value and the maximal response of neurokinin A (unpublished observations), also had an influence on EFS-evoked responses.

Wessler *et al.* (1991) have suggested that transmural stimulation activates all excitable cell membranes, and because of this, presynaptic modulation cannot be observed using this technique. We therefore examined the response to presynaptic stimulation of vesical nerves as described by Hukovic *et al.* (1965); we found that hexamethonium largery reduced contractions evoked by presynaptic stimulation in contrast to its lack of effect on responses to EFS. Even with presynaptic stimulation, the responses are not influenced by guanethidine or neurokinin A (Table 2). The response to nerve stimulation is less susceptible to tachykinins than that to the nicotine stimulus.

Tachykinins facilitate the release of transmitter from the sympathetic nerves as a result of interaction with NK_2 receptors (Tousignant *et al.*, 1987; Regoli *et al.*, 1990). To determine the tachykinin receptor subtype involved in the acetylcholine output stimulated by nicotine, the effects of exogenous tachykinins in the presence of guanethidine to block the endogenous modulator release from sympathetic nerves were evaluated. Neither SPOMe nor senktide which were selective for NK_1 and NK_3 receptor subtypes, respectively, had any effect on the cholinoceptor responses in guinea-pig bladder. On the other hand, neurokinin A facilitated the acetylcholine output and cholinoceptor responses by nicotine at a concentration that had little effect on the smooth muscle.

In conclusion, the results presented confirm our previous suggestion that the nicotine-evoked release of acetylcholine from the bladder is enhanced by tachykinins simultaneously released from sympathetic nerves. The effect of the tachykinins is mediated by NK_2 receptors. These mechanisms do not appear to play a role in the response of normal bladder to excitatory nerve stimulation.

Our special thanks are due to Prof. Dr. Kawashima for providing us with the antiserum.

References

AMBACHE, N. & ZAR, M.A. (1970). Non-cholinergic transmission by postganglionic motor neurons in the mammalian bladder. *J. Physiol.*, **210**, 761–783.

BRADING, A.F. & MOSTWIN, J.L. (1989). Electrical and mechanical responses of guinea-pig bladder muscle to nerve stimulation. *Br. J. Pharmacol.*, **98**, 1083–1090.

BURNSTOCK, G., COCKS, T., CROWE, R. & KASAKOV, L. (1978). Purinergic innervation of the guinea-pig urinary bladder. *Br. J. Pharmacol.*, **63**, 125–138.

FUJII, K. (1988). Evidence for adenosine triphosphate as an excitatory transmitter in guinea-pig, rabbit and pig urinary bladder. *J. Physiol.*, **404**, 39–52.

HISAYAMA, T., SHINKAI, M., TAKAYANAGI, I. & TOYODA, T. (1988). Mechanism of action of nicotine in isolated urinary bladder of guinea-pig. *Br. J. Pharmacol.*, **95**, 465–472.

HISAYAMA, T., SHINKAI, M., TAKAYANAGI, I. & TOYODA, T. (1989). Mechanism of action of nicotine in isolated urinary bladder of guinea-pig: involvement of tachykinin(s) released by nicotine in the drug's sympathomimetic effect. *Arch. Int. Pharmacodyn. Ther.*, **301**, 277–284.

HUKOVIC, S., RAND, M.J. & VANOV, S. (1965). Observation on an isolated, innervated preparation of rat urinary bladder. *Br. J. Pharmacol.*, **24**, 178–188.

KASAKOV, L. & BURNSTOCK, G. (1983). The use of the slowly degradable analog, α,β-methylene ATP, to produce desensitization of the P_2-purinoceptor: effect on non-adrenergic, non-cholinergic responses of the guinea-pig urinary bladder. *Eur. J. Pharmacol.*, **86**, 291–294.

KAWASHIMA, K., FUJIMOTO, K., SUZUKI, T. & OOHATA, H. (1988). Direct determination of acetylcholine release by radioimmunoassay and presence of presynaptic M_1 muscarinic receptors in guinea pig ileum. *J. Pharmacol. Exp. Ther.*, **244**, 1036–1039.

KAWASHIMA, K., ISHIKAWA, H. & MOCHIZUKI, M. (1980). Radioimmunoassay for acetylcholine in the rat brain. *J. Pharmacol. Methods*, **3**, 115–123.

REGOLI, D., RHALEB, N.E., DION, S., TOUSIGNANT, C., ROUISSI, N., JUKIC, D. & DRAPEAU, G. (1990). Neurokinin A. A pharmacological study. *Pharmacol. Res.*, **22**, 1–14.

SHINKAI, M., TAKAYANAGI, I. & KATO, T. (1991). Contrasting effects of tachykinins and guanethidine on the acetylcholine output stimulated by nicotine from guinea-pig bladder. *Br. J. Pharmacol.*, **103**, 1191–1195.

TOUSIGNANT, C., DION, S., DRAPEAU, G. & REGOLI, D. (1987). Characterization of pre- and postjunctional receptors for neurokinins and kinins in rat vas deferens. *Neuropeptides*, **9**, 333–343.

WESSLER, I., KLEIN, A., POHAN, D., MACLAGAN, J. & RACKE, K. (1991). Release of [^{3}H]acetylcholine from the isolated rat or guinea-pig trachea evoked by preganglionic nerve stimulation; a comparison with transmural stimulation. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **344**, 403–411.

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The effect of N^G-nitro-L-arginine methyl ester upon basal blood flow and endothelium-dependent vasodilatation in the dog hindlimb

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1 The role played by the endothelium-derived relaxing factor (EDRF), nitric oxide (NO) in the regulation of blood flow to the skeletal muscle vasculature of the dog skinned hindlimb has been determined by examining the effects of the NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) upon (i) basal iliac artery blood flow, (ii) vasodilator responses to endothelium-dependent and -independent agonists and (iii) reactive hyperaemic responses to arterial occlusion.

2 L-NAME (0.1 – 3 mg min^{-1}) infused directly into the iliac artery dose-dependently reduced basal iliac artery blood flow by a maximum of 48.6 ± 6.9% ($n = 4$) and also increased mean systemic arterial blood pressure by 25.6 ± 5.0 mmHg ($n = 4$) (at 3 mg min^{-1} L-NAME).

3 Over the same dose range, L-NAME also inhibited the peak vasodilator responses to intra-arterially administered, submaximal bolus doses of the endothelium-dependent agonists, bradykinin (3–300 ng) and acetylcholine (30–300 ng) by ~40%. In contrast, peak vasodilator responses to the endothelium-independent agonists, sodium azide (3–30 μg) and adenosine (0.3–1 mg), and peak reactive hyperaemic responses to arterial occlusion (60 s) were largely unaffected by L-NAME.

4 The dose-related effects of L-NAME on basal iliac artery blood flow, mean systemic arterial blood pressure and endothelium-dependent vasodilator responses were significantly attenuated by pretreatment with L-arginine (100 mg min^{-1}) followed by co-infusion of L-arginine (100 mg min^{-1}) with L-NAME.

5 In conclusion, these data suggest that NO plays some role in regulating basal blood flow and in mediating the vasodilator responses to the endothelium-dependent agonists bradykinin and acetylcholine in the skeletal muscle vasculature of the dog hindlimb. The substantial component (~60%) of the peak vasodilator responses to bradykinin and acetylcholine, unaffected by L-NAME, may be independent of NO, or be mediated by an alternative EDRF-dependent but L-NAME-insensitive mechanism.

Keywords: Endothelium-derived relaxing factor (EDRF); nitric oxide (NO); N^G-nitro-L-arginine methyl ester (L-NAME); dog hindlimb circulation; endothelium-dependent vasodilatation; acetylcholine; bradykinin; sodium azide; adenosine; reactive hyperaemia

Introduction

The vascular endothelium produces potent vasoconstrictor factors including thromboxane A_2 (Engerman-Wojenski *et al.*, 1981), superoxide anions (Rosen & Freeman, 1984) and endothelin (Yanagisawa *et al.*, 1988). In addition, potent vasodilator substances are produced, such as prostacyclin (Moncada *et al.*, 1976) and endothelium-derived relaxing factor (EDRF, Furchgott & Zawadski, 1980). A balance between the production of these constrictor and dilator factors is believed to play a pivotal role in the regulation of vascular smooth muscle tone and therefore blood flow.

Nitric oxide (NO), or a closely related molecule, has been suggested to account for the biological activity of EDRF (Palmer *et al.*, 1987). NO is formed in vascular endothelial cells as a product of the enzymic conversion of L-arginine to L-citrulline via nitric oxide synthase (Palmer & Moncada, 1989). Recently, the N-substituted analogue of L-arginine, N^G-nitro-L-arginine has been reported to inhibit NO biosynthesis *in vitro* (Moore *et al.*, 1990). Similarly, *in vivo*, the methyl ester of N^G-nitro-L-arginine (L-NAME) produces inhibition of NO biosynthesis which is associated with marked increases in systemic arterial blood pressure reflecting vasoconstriction (Gardiner *et al.*, 1990a; Rees *et al.*, 1990). These findings are consistent with a pivotal role for L-arginine-derived NO formation in the regulation of basal blood flow under physiological conditions. In the present study, we have investigated the role of NO in the regulation of blood flow to the skeletal muscle vasculature of the dog hindlimb. The effects of L-NAME have been examined upon (i) basal iliac artery blood flow and systemic haemodynamics, (ii) vasodilator responses to endothelium-dependent and -independent vasodilator agents and (iii) reactive hyperaemia following release of an arterial occlusion.

A preliminary account of our findings has been presented to the British Pharmacological Society (White *et al.*, 1990).

Methods

Preparation of dogs and surgical procedures

Beagle dogs of either sex, weighing 10–12 kg after overnight fasting, were anaesthetized with pentobarbitone sodium (40 mg kg^{-1}, i.v.) and subsequently supplemented with the same anaesthetic at a maintenance dose of 6 mg kg^{-1} h^{-1}. Animals were respired with room air at a rate of 20 strokes min^{-1} and stroke volume of 13 ml kg^{-1} which was adjusted as necessary until blood gas tensions and blood pH fell within the range, P_{O_2} 70–110 mmHg; P_{CO_2} 35–45 mmHg and pH 7.35–7.45.

A laparotomy was performed and the lower intestines were deflected to expose the abdominal aorta and its bifurcation into the external iliac arteries. The left and right deep cir-

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cumflex iliac, the caudal mesenteric and sacral arteries were ligated to eliminate, as far as possible, any collateral blood flow, leaving the external iliac arteries as the only entry of blood into the hindlimbs. The right hindlimb was used as the test leg and iliac blood flow was monitored with a pulsed Doppler flow probe (TMI, IOWA), in both right and left external iliac arteries.

Distal to the flow probe on the right iliac artery, a small needle (26G) was inserted into the vessel lumen and connected to a 3-way tap for the administration of drugs. To maintain patency, saline (0.5 ml min^{-1}) was infused continuously through the needle.

In most experiments, in order largely to exclude the contribution of the sympathetic nervous system, the sciatic nerves were isolated and sectioned at the level of the popliteal space. In addition, ligatures were tied around the paw at the level of the ankle, to exclude arteriovenous shunts, and therefore non-nutritive blood flow. The skin from the test hindlimb was removed to eliminate the cutaneous circulation.

Systemic blood pressure was monitored from a catheter in the right carotid artery, via a Bell & Howell pressure transducer. Heart rate was derived electronically from the blood pressure signal via a Lectromed instantaneous ratemeter. All parameters were recorded on an 8 channel Device M19 chart recorder.

Experimental protocols

L-NAME and basal iliac artery blood flow and vasodilator responses Following surgery, dogs were pretreated with indomethacin (5 mg kg^{-1}) which was infused (0.5 ml min^{-1}) directly into the iliac artery over ~10 min period, to inhibit formation of endogenous prostanoids. Saline infusion (0.5 ml min^{-1}) was then recommenced. During saline infusion, a single, sub-maximal bolus dose of each vasodilator in the following dose ranges: bradykinin (3–300 ng), sodium azide (3–30 μg), acetylcholine (30–300 ng) and adenosine (0.3–1 mg) was given in the form of a bracket assay. This consisted of vasodilator responses to the endothelium-dependent agonists bradykinin (×2) and acetylcholine and the endothelium-independent agonists sodium azide and adenosine. In individual experiments, doses were chosen that elicited similar peak vasodilator responses. On completion of each bracket assay, the reactive hyperaemic response following 60 s total occlusion of the iliac artery was also measured. Bracket assays were repeated until reproducible. The saline infusion was then replaced with an infusion of L-NAME (0.5 ml min^{-1}) at 0.1, 0.3, 1 and 3 mg min^{-1}. All doses of L-NAME were infused for 10 min before starting a bracket assay and continued until measurement of the reactive hyperaemia response was completed (~30 min). In some experiments, the effects of L-NAME were examined in skin-intact hindlimbs in which the same protocol was adopted, except that L-NAME was infused at 0.1, 0.3 and 1 mg min^{-1} only.

L-Arginine/L-NAME and basal iliac artery blood flow and vasodilator responses After control (saline infusion) bracket assays had been established, L-arginine (100 mg min^{-1}) was infused into the iliac artery for 30 min prior to and then during a repeat bracket assay and reactive hyperaemia response. L-NAME (0.1–3 mg min^{-1}) was then infused in the continued presence of the L-arginine infusion and the bracket assay and reactive hyperaemia response again repeated as described above.

Expression of results and statistics

Basal iliac artery blood flow following L-NAME treatment is expressed as a percentage of the mean pretreatment value. The peak increase in blood flow to each vasodilator agonist, following L-NAME treatment, is expressed as a percentage of the mean peak increase in blood flow to the same agonist, obtained before administration of L-NAME. Reactive hyperaemia responses were calculated in the same way. Blood pressure (mmHg) and heart rate (beats min^{-1}) are expressed in absolute terms.

All data are expressed as the mean ± s.e.mean from (n) dogs. Comparisons within a group of animals were made by means of Students t test for paired data. Comparisons between groups of animals were made by an unpaired t test. A probability value of $P = 0.05$ or less was taken to indicate a significant difference.

Materials

Adenosine, L-arginine hydrochloride, bradykinin tri-acetate, indomethacin, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), and sodium azide, were obtained from Sigma. Acetylcholine hydrochloride was obtained from BDH Chemicals Ltd. Acetylcholine, bradykinin and sodium azide were dissolved in 0.9% w/v sodium chloride (saline) to form 5 mg ml^{-1} stock solutions, which were aliquoted and stored frozen at −20°C. L-Arginine (200 mg ml^{-1}) and L-NAME (60 mg ml^{-1}) were prepared fresh, daily, in saline and adjusted to pH ~6.0 (= pH saline) with 0.1 M sodium hydroxide solution. Adenosine (5 mg ml^{-1}) was dissolved in saline by the addition of a few drops of 2 N HCl. Indomethacin was dissolved in 3 ml of 10% w/v sodium bicarbonate solution and made up to a final volume of 5 ml with saline. All dilutions of compounds were made in saline and stored on ice during the experiments. Doses of drugs refer to those of the base.

Results

Effect of L-NAME upon basal iliac artery blood flow and systemic haemodynamics

In the dog skinned hindlimb the mean basal iliac artery blood flow was 49 ± 7 ml min^{-1} ($n = 4$). Infusions of L-NAME (0.1–3 mg min^{-1}) into the right iliac artery produced a dose-related reduction in mean iliac artery blood flow (Figure 1). A reduction in iliac blood flow of 41.1 ± 3.0% ($n = 4$) was achieved at 0.3 mg min^{-1} L-NAME, a dose which produced no significant effect on either mean systemic arterial blood pressure or heart rate (Figure 1). Although higher doses of L-NAME (1 and 3 mg min^{-1}) caused little further reduction in basal iliac artery blood flow, they produced an accompanying reduction in blood flow to the contralateral hindlimb (maximum of 23.4 ± 3.6%, $n = 4$). This effect of L-NAME (1 and 3 mg min^{-1}) was also associated with dose-related and significant increases in mean systemic arterial blood pressure from a pre-L-NAME value of 112 ± 6 mmHg to 128 ± 4 mmHg and 140 ± 5 mmHg at 1 and 3 mg min^{-1} L-NAME respectively (Figure 1). In experiments in which the test hindlimb was not skinned, L-NAME produced comparable effects on basal iliac artery blood flow, systemic blood pressure and heart rate (data not shown).

Effect of L-arginine on L-NAME-induced reduction in basal iliac artery blood flow and systemic haemodynamics

L-Arginine infusion (100 mg min^{-1} for 30 min), produced no effect on basal iliac artery blood flow, mean arterial blood pressure or heart rate. However, continuation of the infusion of L-arginine caused the dose-response curve for L-NAME-induced reduction in right iliac artery blood flow to be displaced approximately five fold to the right of that obtained with L-NAME alone (Figure 2). Co-administration of L-arginine with L-NAME also prevented the increase in mean systemic arterial blood pressure produced by the

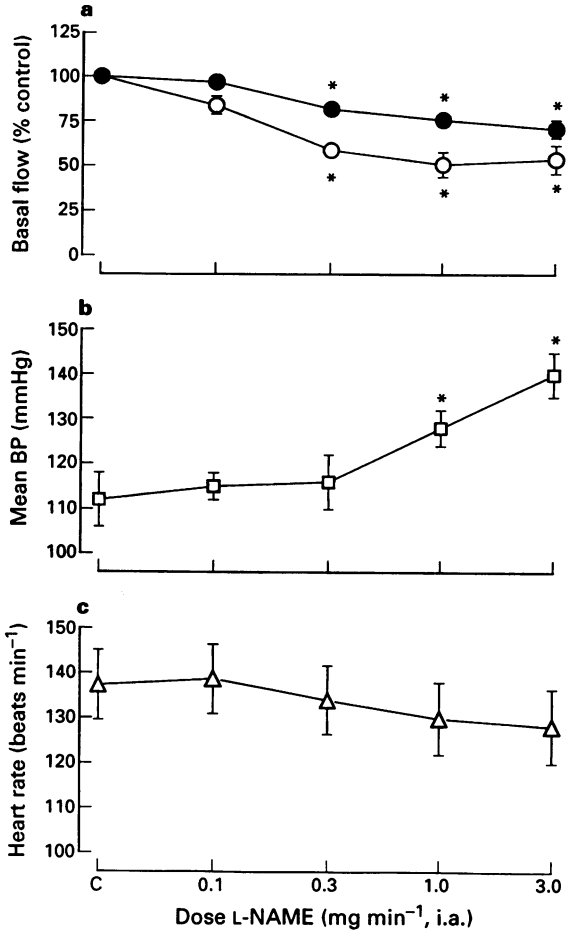


Figure 1 The effect of N^G-nitro-L-arginine methyl ester (L-NAME; 0.1–3.0 mg min^{-1}) on heart rate (c), mean systemic arterial blood pressure (b) and basal iliac artery blood flows (a) in the skinned (test ○) and intact (control ●) hindlimbs of the anaesthetized dog. Values are means ± s.e.mean ($n = 4$) where * represents a significant ($P < 0.05$) difference from control (C).

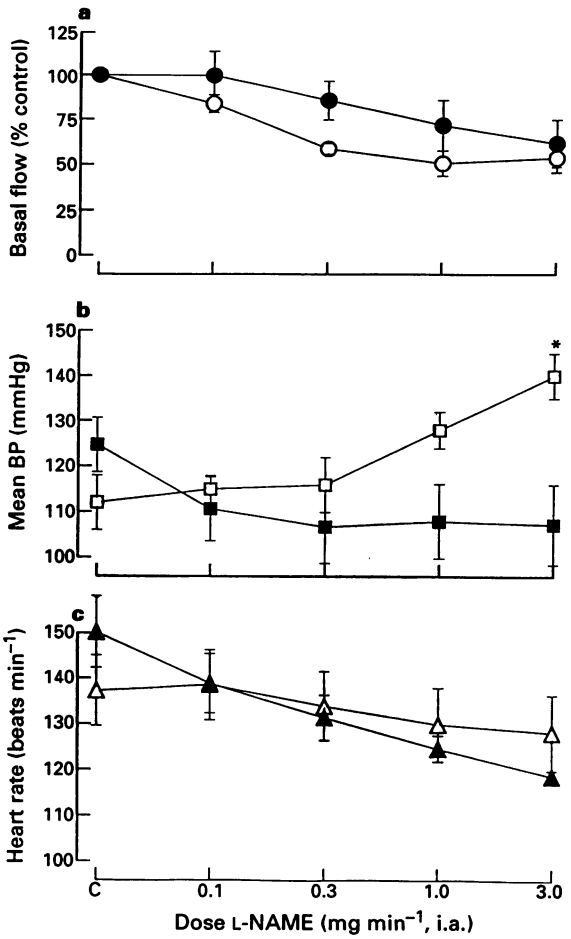


Figure 2 Comparison of the effect of N^G-nitro-L-arginine methyl ester (L-NAME; 0.1–3.0 mg min^{-1}) alone (open symbols) and in combination with L-arginine (100 mg min^{-1}; solid symbols) upon heart rate (c), mean systemic arterial blood pressure (b) and hindlimb basal iliac artery blood flow (a) in the anaesthetized dog. L-Arginine was administered 30 min prior to and during the infusions of L-NAME. The ED_{20} (dose of L-NAME required to produce a 20% reduction in basal iliac artery blood flow) for L-NAME alone was 1.5 ± 0.3 mg, a value significantly less ($P < 0.05$) than that obtained in the presence of L-arginine (7.9 ± 3.3 mg). Values are mean ± s.e.mean ($n = 4$) where * represents a significant ($P < 0.05$) difference from control (C).

highest dose of L-NAME alone (3 mg min^{-1}; Figure 2). No significant change in heart rate occurred with the combination of L-arginine and L-NAME treatments.

Effect of L-NAME on endothelium-dependent and -independent agonist-induced vasodilator responses and reactive hyperaemia

Peak vasodilator responses to intra-arterially administered single bolus doses of bradykinin, acetylcholine, sodium azide, adenosine and also following iliac artery occlusion (reactive hyperaemia) were reproducible. Over the dose-ranges tested, the agonist doses selected in individual experiments were approximately equieffective in increasing iliac artery blood flow (approximately 2–3 fold over the mean basal blood flow of 49 ± 7 ml min^{-1}; $n = 4$). The peak reactive hyperaemia response following total occlusion of the iliac artery (60 s) was similar in magnitude to the vasodilator responses produced by the agonists.

Infusion of L-NAME (0.1–3 mg min^{-1}) attenuated, by a maximum of 43.4 ± 11 and 38.6 ± 9.5% ($n = 4$), the peak vasodilator responses to the endothelium-dependent agonists bradykinin and acetylcholine respectively (Figure 3). In contrast, over the same dose-range, L-NAME produced little or no effect on the peak vasodilator responses to the endothelium-independent agents, adenosine and sodium azide (Figure 3). In the case of sodium azide, however, L-NAME (1 mg min^{-1}) produced a 17.8 ± 1.8% ($n = 4$) reduction of the peak vasodilator response, although no such effect was observed at the higher dose of L-NAME (3 mg min^{-1}; Figure 3). The peak reactive hyperaemic response following iliac artery occlusion was also unaffected by L-NAME treatment (0.1–3 mg min^{-1}). In hindlimb preparations from which the skin was not removed, L-NAME, in the same dose-range produced similar effects on the agonist-induced vasodilator responses (data not shown).

The effect of L-arginine alone and in combination with L-NAME on endothelium-dependent and -independent vasodilator responses

L-Arginine (100 mg min^{-1} infusion) produced no significant effect on the peak vasodilator responses to bradykinin, acetylcholine, sodium azide, adenosine and reactive hyperaemia. However, during the continued infusion of L-arginine, the inhibitory effects of L-NAME (0.1–3 mg min^{-1}) on the peak vasodilator responses to bradykinin and acetylcholine were significantly attenuated (Figure 4). In contrast, the peak vasodilator responses to the endothelium-

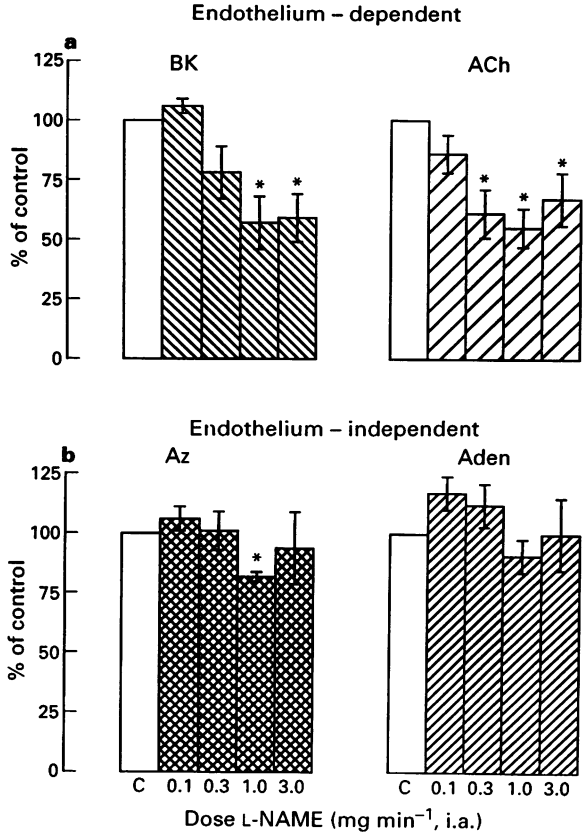


Figure 3 Effect of N^G-nitro-L-arginine methyl ester (L-NAME; 0.1–3.0 mg min^{-1}) on (a) bradykinin-(BK; 3–300 ng), acetylcholine-(ACh; 3–300 ng) and (b) sodium azide-(Az; 3–30 μg) and adenosine-(Aden; 0.3–1.0 mg)-induced peak vasodilator responses in the hindlimb (skinned) of the anaesthetized dog. The agonist dose-ranges used are shown in parentheses. Columns represent mean ± s.e.mean (vertical bars) ($n = 4$) values where * represents a significant ($P < 0.05$) difference from control value (C).

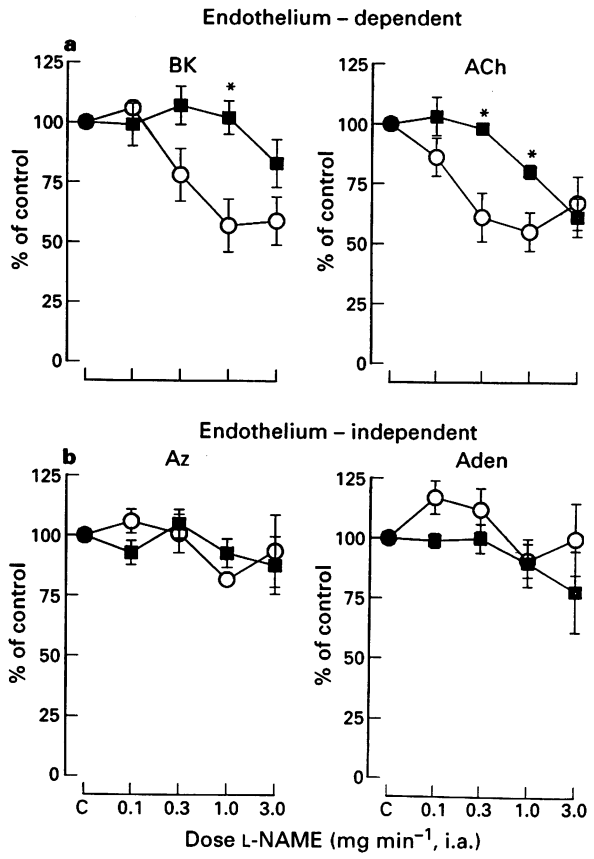


Figure 4 Effect of N^G-nitro-L-arginine methyl ester (L-NAME; 0.1–3.0 mg min^{-1}) on (a) bradykinin-(BK; 3–300 ng), acetylcholine-(ACh; 3–300 ng) and (b) sodium azide-(Az; 3–30 μg) and adenosine-(Aden; 0.3–1.0 mg)-induced peak vasodilator responses in the presence (■) and absence (○) of L-arginine (100 mg min^{-1}) in the hindlimb of the anaesthetized dog. The agonist dose-ranges used are shown in parentheses. Data are mean ± s.e.mean (vertical bars) ($n = 4$) values where * represents a significant ($P < 0.05$) difference from the corresponding dose of L-NAME in the absence of L-arginine.

independent agents, sodium azide and adenosine (Figure 4) and reactive hyperaemia responses (data not shown), were unaffected by the co-infusion of L-arginine with L-NAME.

Discussion

The role of NO in the regulation of blood flow to the skeletal muscle vasculature of the dog hindlimb has been determined by examining the effect of the NO synthesis inhibitor, L-NAME, on basal iliac artery blood flow and endothelium-dependent, agonist-induced vasodilator responses, as measures of basal and stimulated NO formation respectively.

In the dog hindlimb, with or without a cutaneous circulation, local administration of L-NAME caused a dose-dependent reduction (maximum ~50%) of basal iliac artery blood flow. A dose-related fall in basal iliac artery blood flow also occurred in the contralateral (control) hindlimb, but the maximum reduction achieved here was ~20%. An explanation for this differential effect is that, in the test hindlimb, the local concentration of L-NAME was higher than that present systemically following recirculation. An additional, or alternative, explanation for the vasoconstriction observed in the contralateral hindlimb following administration (into the test leg) of a low dose of L-NAME (0.3 mg min^{-1}, i.a.), that produced no change in systemic pressure or heart rate, may be deduced from the observations of Klabunde *et al.* (1991). They showed that, in beagle dogs, N^G-nitro-L-arginine reduced cardiac output and increased systemic vascular resistance at doses having no effect on systemic pressure. Thus, the vasoconstriction seen in the contralateral limb could, at least in part, be a passive effect in response to a reduced inflow of blood. L-NAME also caused a significant increase (~30–40 mmHg) in systemic arterial blood pressure. This was most evident at doses of L-NAME (1 and 3 mg min^{-1}) greater than those necessary to elicit a near maximum reduction in iliac artery blood flow in the test hindlimb. This increase in blood pressure almost certainly reflects systemic vasoconstriction, caused by L-NAME-induced inhibition of basal NO formation in other vascular beds (see below). Since perfusion pressure is an important determinant of blood flow, it is clear from the present study that the L-NAME-induced maximum reduction in basal iliac artery blood flow, under conditions when systemic driving pressure into the limb was increasing, underestimates the true degree of local vasoconstriction.

In an attempt to confirm that the effect of L-NAME on local and systemic haemodynamics was mediated via NO inhibition, experiments were conducted with its precursor L-arginine. Pretreatment followed by the continuous administration of L-arginine, significantly attenuated the haemodynamic effects of subsequently administered L-NAME. These findings suggest the effects of L-NAME are mediated via inhibition of endogenously produced NO. Furthermore, these data are consistent with an important role for an L-arginine-derived NO in the control of vascular tone and,

therefore, blood flow in the skeletal muscle vasculature of the dog hindlimb. Interestingly, comparable data in the human forearm have emerged from the work of Vallance and colleagues (1989) who have shown basal blood flow in the brachial artery to be reduced by ~50% following the local administration of the arginine analogue, N^G-monomethyl-L-arginine (L-NMMA) which, like L-NAME, stereospecifically inhibits the formation of NO in vascular tissue (Moncada *et al.*, 1989). Thus, NO appears to play an important role in the regulation of basal blood flow in both the human forearm and in the dog hindlimb.

Similar findings with L-NAME have been observed in other species. For example, studies in the rabbit (Persson *et al.*, 1991) and rat (Gardiner *et al.*, 1990a,b; Rees *et al.*, 1990) have shown that intravenous administration of L-NAME causes widespread vasoconstriction and thus increases in systemic blood pressure, both effects being reversible with L-arginine. Inhibition of endothelial cell basal NO production (Griffith *et al.*, 1984) is the most likely explanation of these observations, although additional consequences of L-NAME-induced inhibition of NO formation cannot be discounted. For example, Boulanger & Lücsher (1990) have reported that NO inhibits the release of the potent vasoconstrictor peptide, endothelin, from porcine endothelium. Thus, L-NAME-induced reductions in blood flow and the concomitant increases in arterial blood pressure may be a consequence of the vasoconstrictor actions of increased endothelium-derived endothelin release, as well as a loss of basal NO production.

In the dog hindlimb, L-arginine alone produced no significant effect on basal iliac artery blood flow or systemic arterial blood pressure, in agreement with other studies with L-arginine in the guinea-pig, rat and rabbit (Aisaka *et al.*, 1989; Rees *et al.*, 1990; Persson *et al.*, 1990). An interpretation of these observations is that the availability of L-arginine is not rate limiting for basal NO production and/or that endogenous L-arginine substrate is sufficiently available to saturate the NO synthase for basal NO production. However, L-arginine was effective in attenuating the L-NAME-induced reduction in basal iliac artery blood flow and increase in systemic pressure when administered prior to, and in conjunction with, L-NAME.

A second aim of the present study was to examine the effect of L-NAME on NO release stimulated by endothelium-dependent vasodilators and on reactive hyperaemia responses to artery occlusion. Our data show that L-NAME dose-dependently reduced the peak vasodilator responses to the endothelium-dependent agonists, bradykinin and acetylcholine by approximately 40%. Although not shown, a comprehensive analysis of the influence of L-NAME upon the duration of the bradykinin and acetylcholine vasodilator responses revealed no effect. L-NAME was also largely without effect on the peak or duration of the vasodilator responses to the endothelium-independent agonists sodium azide, (a nitrovasodilator which, following conversion to NO, can act directly on the guanylate cyclase of vascular smooth muscle) and adenosine (an adenylate cyclase-dependent vasodilator). These data suggest that L-NAME acts specifically on NO-mediated responses. L-NAME also produced no inhibitory effect on the peak reactive hyperaemia responses. L-Arginine treatment alone failed to affect vasodilator responses to any of the agonists tested, but attenuated the L-NAME-sensitive component of the endothelium-dependent responses, again consistent with NO mediating a substantial component (~40%) of the bradykinin and acetylcholine vasodilator responses. Similar conclusions have been drawn from the effects of L-NMMA on acetylcholine-induced vasodilator responses in the human forearm (Vallance *et al.*, 1989) and also in conscious rats, when L-NAME attenuated bradykinin and acetylcholine-induced vasodilator responses in the hindquarter, renal and mesenteric vascular beds, but where a substantial component of the vasodilator response to these agonists was insensitive to L-NAME (Gardiner *et al.*, 1990a). This phenomenon appears to be less evident in endothelium-intact, *isolated* vascular smooth muscle preparations such as the rat and rabbit aorta (Rees *et al.*, 1990; Moore *et al.*, 1990) and in canine coronary and femoral arteries (Richard *et al.*, 1990) in which L-NAME and N^G-nitro-L-arginine substantially inhibit endothelium-dependent relaxant responses to acetylcholine. These observations and those in the present study therefore raise some intriguing points. Firstly, do differences exist between endothelium-dependent components of vasodilator responses in the microcirculation and in conduit vessels? Secondly, in our studies *in vivo* a substantial component of the vasodilator responses to bradykinin and acetylcholine appear to be NO-independent and may therefore involve another mediator which we have not investigated. Since the dogs in the present experiments were indomethacin pretreated, we can however, exclude the involvement of vasodilator prostanoids such as prostacyclin. Non-prostanoid vasodilators such as endothelium-derived hyperpolarizing factor (EDHF; Komori & Suzuki; 1987) may, however, be involved. Another consideration, exemplified by a recent *in vitro* study (Martin *et al.*, 1992), is that L-arginine analogues demonstrate tissue- and agonist-dependence with respect to inhibition of receptor-mediated events involving the liberation of NO. Incomplete inhibition of bradykinin- and acetylcholine-induced vasodilatation might therefore be expected if these agonists are efficiently coupled to NO generation in the dog hindlimb vasculature. A further possibility is that a preformed storage pool of NO may exist which does not require *de novo* synthesis from L-arginine and is inaccessible and/or insensitive to the actions of L-NAME (Aisaka *et al.*, 1989), yet may be released by endothelium-dependent agonists.

The potent vasodilator activity of NO raises the possibility of its involvement in reactive hyperaemia, the physiological response observed in certain vascular beds following release of artery occlusion. At present, the precise mechanisms regulating this response are unclear, although an interaction between myogenic and metabolic regulatory mechanisms has been proposed (Johnson *et al.*, 1976; Tuma *et al.*, 1977). In the present study, the lack of effect of L-NAME upon peak reactive hyperaemia responses indicates NO is probably not involved. This finding supports those of Björnberg *et al.* (1990) who have reported the NO synthase inhibitor, L-NMMA to have no inhibitory effect on reactive hyperaemia in the cat hindlimb. Thus, a major role for NO in mediating reactive hyperaemia responses seems unlikely.

In conclusion, our observations indicate that NO plays a significant role in modulating basal vascular tone, and in mediating vasodilator responses to bradykinin and acetylcholine in the skeletal muscle vasculature of the dog hindlimb. The substantial component of the vasodilator response to bradykinin and acetylcholine unaffected by L-NAME, may be independent of NO, or mediated by an alternative NO-dependent, but L-NAME insensitive, mechanism. The present study has focused on the role of EDRF in basal blood flow to non-working skeletal muscle. L-NAME may be a useful agent for elucidating whether NO release contributes to the functional hyperaemia associated with skeletal muscle contraction. In this context, recent studies with L-NMMA in rabbit tenuissimus muscle microvasculature suggest that NO release does not contribute to the hyperaemic response induced by motor nerve stimulation (Persson *et al.*, 1991).

References

AISAKA, K., GROSS, S.S., GRIFFITH, O.W. & LEVI, R. (1989). L-arginine availability determines the duration of acetylcholine-induced systemic vasodilation in vitro. *Biochem. Biophys. Res. Commun.*, **163**, 710–712.

BJÖRNBERG, J., ALBERT, U. & MELLANDER, S. (1990). Resistance responses in proximal arterial vessels, arterioles and veins during reactive hyperaemia in skeletal muscle and their underlying regulatory mechanisms. *Acta Physiol. Scand.*, **139**, 535–550.

BOULANGER, G. & LÜSCHER, T.F. (1990). Release of endothelin from porcine aorta. Inhibition by endothelium-derived nitric oxide. *J. Clin. Invest.*, **85**, 587–590.

ENGERMAN-WOJENSKI, C., SILVER, M.J., SMITH, J.B. & MACARAK, E. (1981). Bovine endothelial cells in culture produce thromboxane as well as prostacyclin. *J. Clin. Invest.*, **67**, 1292–1296.

FURCHGOTT, R.F. & ZAWADSKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **228**, 373–376.

GARDINER, S.M., COMPTON, A.M., KEMP, P.A. & BENNETT, T. (1990a). Regional and cardiac haemodynamic responses to glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1 in conscious rats: effects of N^G-nitro-L-arginine methyl ester. *Br. J. Pharmacol.*, **101**, 632–639.

GARDINER, S.M., COMPTON, A.M., KEMP, P.A. & BENNETT, T. (1990b). Regional and cardiac haemodynamic effects of N^G-nitro-L-arginine methyl ester in conscious, Long Evans rats. *Br. J. Pharmacol.*, **101**, 625–631.

GRIFFITH, T.M., EDWARDS, D.H., LEWIS, M.J., NEWBY, A.C. & HENDERSON, A.H. (1984). The nature of endothelium-derived relaxing factor. *Nature*, **308**, 645–647.

JOHNSON, P.C., BURTON, K.S., HENRICH, H. & HENRICH, U. (1976). Effect of occlusion duration on reactive hyperaemia in sartorius muscle capillaries. *Am. J. Physiol.*, **230**, 715–719.

KLABUNDE, R.E., RITGER, R.C. & HELGREN, R.C. (1991). Cardiovascular actions of inhibitors of endothelium-derived relaxing factor (nitric oxide) formation/release in anaesthetized dogs. *Eur. J. Pharmacol.*, **199**, 51–59.

KOMORI, K. & SUZUKI, H. (1987). Heterogeneous distribution of muscarinic receptors in rabbit saphenous artery. *Br. J. Pharmacol.*, **92**, 657–664.

MARTIN, G.R., BOLOFO, M. & GILES, H. (1992). Inhibition of endothelium-dependent vasorelaxation by arginine analogues: a pharmacological analysis of agonist and tissue dependence. *Br. J. Pharmacol.*, **105**, 643–652.

MONCADA, S., GRYGLEWSKI, R.J., BUNTING, S. & VANE, J.R. (1976). An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature*, **263**, 663–665.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1989). Biosynthesis of nitric oxide from L-arginine: A pathway for the regulation of cell function and communication. *Biochem. Pharmacol.*, **38**, 1709–1715.

MOORE, P.K., AL-SWAYEH, O.A., CHONG, N.W.S., EVANS, R.A. & GIBSON, A. (1990). L-N^G-nitroarginine (L-NOARG), a novel L-arginine-reversible inhibitor of endothelium-dependent vasodilatation *in vitro*. *Br. J. Pharmacol.*, **99**, 408–412.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

PALMER, R.M.J. & MONCADA, S. (1989). A novel citrulline-forming enzyme implicated in the formation of nitric oxide by vascular endothelial cells. *Biochem. Biophys. Res. Commun.*, **158**, 348–352.

PERSSON, M.G., GUSTAFSSON, L.E., WIKLUND, N.P., HEDQVIST, P. & MONCADA, S. (1990). Endogenous nitric oxide as a modulator of rabbit skeletal muscle microcirculation *in vivo*. *Br. J. Pharmacol.*, **100**, 463–466.

PERSSON, M.G., WIKLUND, N.P. & GUSTAFSSON, L.E. (1991). Nitric oxide requirement for vasomotor nerve-induced vasodilatation and modulation of resting blood flow in muscle microcirculation. *Acta Physiol. Scand.*, **141**, 49–56.

REES, D.D., PALMER, R.M.J., SCHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterisation of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **101**, 746–752.

RICHARD, V., GOSNACH, M., BERDEAUX, A. & GIUDICELLI, J.F. (1990). *In vitro* and *in vivo* effect of N-nitro-L-arginine an inhibitor of nitric oxide formation. *Arch. Int. Pharmacodyn. Ther.*, **305**, 274.

ROSEN, G.M. & FREEMAN, B.A. (1984). Detection of superoxide generated by endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 7269–7273.

TUMA, R.F., LINDBOM, L. & ARFORS, K.E. (1977). Dependence of reactive hyperaemia in skeletal muscle on oxygen tension. *Am. J. Physiol.*, **233**, 289–294.

VALLANCE, P., COLLIER, J. & MONCADA, S. (1989). Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet*, **ii**, 997–999.

WHITE, D.G., DREW, G.M., GURDEN, J.M. PENNY, D.M. ROACH, A.G. & WATTS, I.S. (1990). Effect of L-N^G-nitro-arginine upon basal blood flow and endothelium-dependent and -independent vasodilator agents in the dog hindlimb. *Br. J. Pharmacol.*, **102**, 129P.

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

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Differential effects of captopril on regional haemodynamic responses to angiotensin I and bradykinin in conscious rats

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1 Conscious, Long Evans rats were chronically instrumented with pulsed Doppler flow probes and intravascular catheters to allow regional haemodynamic (coeliac, mesenteric and hindquarters vascular beds) responses to i.v. bradykinin to be assessed in the absence and presence of captopril and of ganglion blockade (with mecamylamine).

2 Bradykinin (3 nmol kg^{-1}, i.v. bolus) had no effect on mean arterial blood pressure, although it caused hyperaemic vasodilatation in the coeliac, mesenteric and hindquarters vascular beds. Following administration of captopril at a dose (28 nmol kg^{-1}) which had no effect on responses to angiotensin I, the hypotensive and coeliac and mesenteric vasodilator responses to bradykinin were enhanced. However, there was a temporal dissociation between these events indicating that changes in cardiac output must have been contributing to the changes in mean arterial blood pressure.

3 Captopril at a higher dose (280 nmol kg^{-1}) caused reversible inhibition of the pressor and coeliac and mesenteric vasoconstrictor effects of angiotensin I, but the inhibition of the mesenteric vascular responses was significantly less than that of the coeliac vascular responses. Under the same conditions, the mesenteric vasodilator effects of bradykinin were less enhanced than the coeliac vasodilator effects, consistent with greater inhibition of angiotensin-converting enzyme (i.e., kininase II) in the coeliac than in the mesenteric vascular bed. But, since the hypotensive action of bradykinin was markedly enhanced in these circumstances, the possibility existed that baroreflex responses influenced the haemodynamic effects of bradykinin. However, assessment of the haemodynamic changes following bradykinin administration (bolus or infusion) in the presence of ganglion blockade showed that only the hindquarters vasodilator response to bradykinin was enhanced, while the coeliac and mesenteric vasodilator responses were diminished. Thus, additional factors must have been influencing the latter responses.

4 The results show that inhibition of angiotensin-converting enzyme (kininase II) can have differential effects on the regional haemodynamic responses to angiotensin I and bradykinin. The results provide a striking illustration of our previous assertion that the measurement of arterial blood pressure alone cannot provide sufficient information to allow interpretation either of the effects of vasoactive substances, or of the influence of drugs thereupon.

Keywords: Bradykinin; angiotensin I; captopril; regional haemodynamics

Introduction

The traditional picture of the renin-angiotensin system (RAS) is one in which release of renin from the granular cells of the juxtaglomerular apparatus stimulates the production of angiotensin I (AI) from circulating angiotensinogen, and the AI is then converted into angiotensin II (AII) by the action of angiotensin-converting enzyme (ACE) in the pulmonary vascular bed. However, it is now known that all the components of the RAS may be located within individual tissues (see Campbell, 1987; Dzau, 1988, for reviews), and hence the regional haemodynamic responses to systemic, i.v. administration of exogenous AI could be due to its local conversion to AII, in addition to its pulmonary conversion. If this were the case, then pretreatment with an ACE inhibitor, particularly at submaximal doses, might not influence the regional haemodynamic effects of exogenous AI uniformly, especially if the extent of the local production of AII, from exogenous AI, differed in different vascular beds, and/or the degree of ACE inhibition was not the same in all vascular beds. However, if the exogenous AI were converted into AII in the pulmonary vascular bed, and the AII then exerted systemic effects, treatment with an ACE inhibitor should reduce all the regional haemodynamic actions of AI proportionately. Thus, our first objective was to determine which of these two possibilities pertains *in vivo*. Since the renal vascular bed is so markedly influenced by the renal RAS, it is feasible that the functional consequences of the activity of any local vascular RAS in the kidney would be difficult to detect, and hence measurement of renal blood flow might not help to answer the question we were asking. To avoid this potential problem, and in order to obtain maximum information from each experiment, we compared the effects of exogenous AI, given i.v., on the coeliac, mesenteric and hindquarters vascular beds, in the absence and in the presence of the ACE inhibitor, captopril, at doses submaximal for ACE inhibition (Muller *et al.*, 1990). We judged the use of such doses of captopril would optimise our chances of detecting regional differences in the effects of the drug.

ACE is kininase II, and bradykinin is a better substrate for this enzyme than is AI, since its K_m is about 10^{-7} M compared to AI, which has a K_m about 10^{-5} M (see Erdös, 1975). Thus, in the presence of an ACE inhibitor, the regional haemodynamic effects of AI and bradykinin might be influenced to different extents, even though additional enzymes might be involved in the degradation of bradykinin (see Erdös, 1975, Regoli & Barabé, 1980). Furthermore, for the reasons given above, treatment with an ACE inhibitor could have a regionally differentiated influence on the haemodynamic responses to exogenous bradykinin. So, our second objective was to delineate the regional haemodynamic responses to exogenous bradykinin, given i.v., in the absence and presence of the submaximal doses of captopril, in the same animals that received AI.

The results of this experiment indicated that the haemo-

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dynamic effects of bradykinin in the presence of captopril might have been influenced by reflex mechanisms. Therefore, in a separate experiment we assessed regional haemodynamic responses to i.v. bolus injection and infusion of bradykinin in the absence and presence of the ganglion blocker, mecamylamine, and also in the presence of mecamylamine and captopril.

Methods

All experiments were carried out on male, Long Evans rats (350–450 g) bred in Nottingham. Under anaesthesia (sodium methohexitone, 60 mg kg^{-1} i.p., supplemented as required) pulsed Doppler probes (Haywood *et al.*, 1981) were sutured around the coeliac and superior mesenteric arteries and the distal abdominal aorta (to monitor hindquarters flow) (Gardiner *et al.*, 1990a). Probe wires were fed subcutaneously to emerge at the back of the neck, where they were anchored by a suture. An i.m. injection of ampicillin (7 mg kg^{-1}; Penbritin, Beecham) was given before animals were returned to individual home cages with free access to food and water. At least 7 days after probe implantation, rats were anaesthetized (sodium methohexitone, 40 mg kg^{-1}, i.p.) and had an intra-arterial (distal abdominal aorta via ventral caudal artery) and 3 intravenous (right jugular vein) catheters implanted. The catheters ran subcutaneously to emerge at the same site as the wires from the pulsed Doppler probes. The latter were soldered into a microconnector (Microtech Inc., Boothwyn, USA) that was held in a clamp attached to a harness worn by the rat. A flexible spring, through which the catheters ran, was connected to the harness, and the lead connection to the probes was taped to the spring. The spring assembly was supported by a counter-balanced lever system that allowed the rat unrestrained movement. Only animals with acceptable signals (signal:noise > 20:1) from all 3 probes were entered into experimental protocols, and only after they had had their intravascular catheters implanted for at least 24 h. During the experiments individual rats were kept in their home cages with free access to food and water.

Experiment I: Effects of captopril on responses to angiotensin I and bradykinin

In this experiment we assessed the haemodynamic effects of bolus i.v. injections of AI (250 pmol kg^{-1}) and bradykinin (3 nmol kg^{-1}) before and about 10 and 60 min after bolus i.v. injection of captopril (28 or 280 nmol kg^{-1}); these doses were chosen as being submaximal for ACE inhibition (Muller *et al.*, 1990). Animals ($n = 9$) received AI and bradykinin in that order (because of the prolonged effects of the latter in the presence of captopril). The challenges before and after captopril were carried out on separate days for each dose of captopril, but the lower dose of captopril was always given first, in order to avoid any possible carry-over effects.

Experiment 2: Effects of mecamylamine and mecamylamine plus captopril on responses to bradykinin

In this experiment, a separate group of rats ($n = 7$) received i.v. bolus injections (3 nmol kg^{-1}) and 3 min infusions (36 nmol kg^{-1} min^{-1}) of bradykinin before and 20 min after the onset of mecamylamine administration (50 μmol kg^{-1} h^{-1}; Gardiner *et al.*, 1992) and again about 10 min after the i.v. bolus injection of captopril (280 nmol kg^{-1}) in the presence of mecamylamine.

Drugs and peptides

AI and bradykinin (Bachem, UK) were dissolved in isotonic (0.9%) NaCl containing 1% bovine serum albumin (Sigma, UK). Captopril (Squibb, UK) was dissolved in isotonic saline and buffered to pH 7.0 with NaOH. Mecamylamine hydrochloride (Sigma, UK) was dissolved in isotonic saline. All bolus injections were given in 0.1 ml flushed in with 0.1 ml saline; mecamylamine infusions were given at 0.3 ml h^{-1}, and bradykinin infusions were given at 0.15 ml min^{-1}. Administration of vehicle at these volumes and rates had no significant cardiovascular effects.

Data analysis

Continuous recordings (on a Gould ES 1000 system) were made of phasic and mean arterial blood pressure (MAP), instantaneous heart rate and phasic and mean Doppler shift signals with a modified (Gardiner *et al.*, 1990b) VF-1 mainframe (Crystal Biotech, Holliston, USA). The mean Doppler shift signals were taken as indices of flow (Gardiner *et al.*, 1990a), and vascular conductances were calculated by dividing the mean Doppler shift signal by the mean arterial blood pressure (Gardiner *et al.*, 1992). Within an experimental run, responses to any intervention were assessed by Friedman's test (Theodorsson-Norheim, 1987) applied to changes relative to baseline. Comparison of responses, in the same group under different experimental conditions, was made using Friedman's test applied to areas under or over curves (AUC and AOC, respectively); resting values were compared with the Kruskal-Wallis test. A P value < 0.05 was taken as significant.

Results

Experiment 1: Effects of captopril on responses to angiotensin I and bradykinin

Responses to angiotensin I Since the responses to AI were so rapid in onset and offset, only maximum changes were measured. AI caused a marked rise in MAP (56 ± 2 mmHg) (Figure 1) and a substantial bradycardia (− 104 ± 7 beats min^{-1}). There were reductions in coeliac (− 2.2 ± 0.4 kHz) and mesenteric (− 3.2 ± 0.4 kHz) flows, in association with marked, and similar falls in vascular conductances (coeliac = − 43 ± 3; mesenteric = − 40 ± 4 [kHz $mmHg^{-1}$]10^3) (Figure 1); hindquarters flow and vascular conductance showed significantly smaller reductions (flow = − 0.6 ± 2 kHz; conductance = − 17 ± 3 [kHz $mmHg^{-1}$]10^3) (Figure 1).

The lower dose of captopril (28 nmol kg^{-1}) had no effects on the regional haemodynamic responses to AI (Figure 1).

Prior to administration of the higher dose of captopril (280 nmol kg^{-1}), the regional haemodynamic responses to AI were not different from those before or after administration of the lower dose of captopril (Figure 1). However, about 10 min after the administration of the higher dose of captopril, AI evoked a significantly reduced pressor effect (16 ± 1 mmHg) (Figure 1), and bradycardia (− 37 ± 7 beats min^{-1}). There were no significant reductions in coeliac or hindquarters flows, although mesenteric flow still showed a marked reduction. Thus, although the falls in coeliac and mesenteric vascular conductances (coeliac = − 7 ± 2; mesenteric = − 27 ± 5 [kHz $mmHg^{-1}$]10^3) were significantly attenuated, the response in the latter bed was significantly greater than in the former. There was no hindquarters vasoconstriction (Figure 1).

About 1 h after the higher dose of captopril, the rise in MAP (50 ± 1 mmHg) (Figure 1), and the bradycardia (− 90 ± 6 beats min^{-1}) and the reductions in coeliac, mesenteric and hindquarters flows (coeliac = − 1.6 ± 0.3; mesenteric = − 3.2 ± 0.4; hindquarters = − 0.3 ± 0.2 kHz) and vascular conductances (coeliac = − 38 ± 4; mesenteric = − 39 ± 4; hindquarters = − 13 ± 2 [kHz $mmHg^{-1}$]10^3) in response to AI were not different from those before captopril (Figure 1).

Responses to bradykinin Bradykinin caused a slight rise in MAP (maximum = 8 ± 4 mmHg; AUC, 12 ± 3 mmHg min),

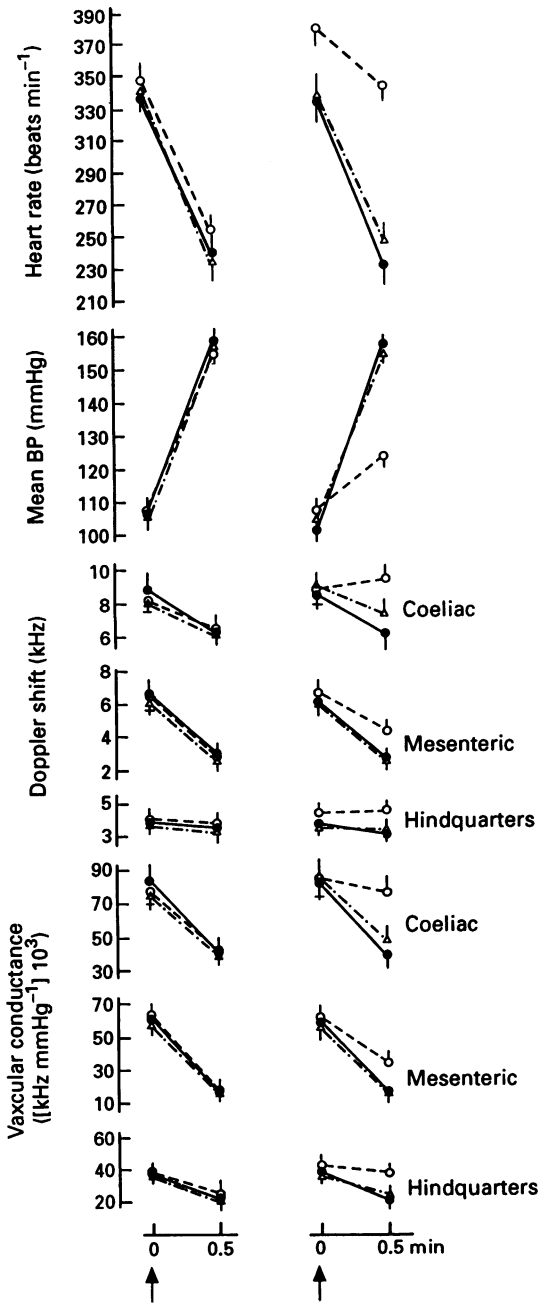


Figure 1 Cardiovascular responses (maxima) to bolus i.v. injection of angiotensin I (250 pmol kg^{-1} at arrow) in the same conscious Long Evans rats ($n = 9$) in the absence (●) and about 10 min (○) and 1 h (△) after administration of captopril at a dose of 28 nmol kg^{-1} (left-hand panels) or 280 nmol kg^{-1} (right-hand panels). Values are mean and bars show s.e.mean; for clarity statistics are given in the text.

but a marked tachycardia (maximum = 116 ± 8 beats min^{-1} at 0.25 min after injection, falling to 46 ± 14 beats min^{-1} after 2 min; AUC, 130 ± 22 beats) (Figure 2). There were early increases in coeliac and mesenteric flows (AUC, coeliac = 1.6 ± 0.5; mesenteric = 2.6 ± 0.7 kHz min) and a later increase in hindquarters flow (AUC = 2.6 ± 0.5 kHz min) (Figure 2). The changes in vascular conductances showed a similar pattern (AUC, coeliac = 13 ± 4; mesenteric = 21 ± 6; hindquarters = 20 ± 3 [kHz $mmHg^{-1}$]10^3 min) (Figure 2).

About 10 min after the lower dose (28 mmol kg^{-1}) of captopril, bradykinin caused a fall in MAP (nadir = − 16 ± 4 mmHg; AOC, 12 ± 3 mmHg min) and marked tachycardia (maximum 120 ± 8 beats min^{-1}; AUC, 148 ± 18 beats). The change in MAP was significantly different from that elicited by bradykinin before captopril (Figure 2), but the tachycardia was not; there were increases in coeliac, mesenteric and hindquarters flows as before, although the patterns of change differed (Figure 2); we did not compare these formally because of the differences in MAP. However, the coeliac and mesenteric vasodilator responses to bradykinin were significantly enhanced (AUC, coeliac = 42 ± 8; mesenteric = 49 ± 12 [kHz $mmHg^{-1}$]10^3 min) whereas the hindquarters vasodilatation was not changed significantly (AUC = 25 ± 6 [kHz $mmHg^{-1}$]10^3 min) (Figure 2).

About 1 h after the lower dose of captopril, the changes in MAP (AUC, 9 ± 3 mmHg min) (Figure 2) and heart rate (maximum = 104 ± 12 beats min^{-1}, AUC = 115 ± 11 beats) in response to bradykinin were not different from those before captopril, but the change in MAP was significantly different from that seen 10 min after captopril (Figure 2). The coeliac vasodilatation (AUC = 16 ± 4 [kHz $mmHg^{-1}$] 10^3 min) and the mesenteric vasodilatation (AUC = 31 ± 9 [kHz $mmHg^{-1}$]10^3 min) were less than those seen following bradykinin administration 10 min after captopril, and not different from the responses prior to captopril. As before, the hindquarters vasodilator response to bradykinin (AUC, 15 ± 3 [kHz $mmHg^{-1}$]10^3 min) was unchanged (Figure 2).

Before administration of the higher dose (280 mmol kg^{-1}) of captopril the responses to bradykinin (MAP, AOC 7 ± 2 mmHg min; heart rate, AUC 103 ± 25 beats; coeliac flow, AUC, 1.6 ± 0.5; mesenteric flow, AUC 2.6 ± 0.8; hindquarters flow, AUC 2.2 ± 0.2 kHz min; coeliac conductance, AUC 18 ± 5; mesenteric conductance, AUC 27 ± 8; hindquarters conductance, AUC 21 ± 3 [kHz $mmHg^{-1}$]10^3 min) were not different from those seen before administration of the lower dose of captopril (Figure 2). However, about 10 min after the higher dose of captopril the bradykinin-induced fall in MAP was markedly increased (nadir = − 48 ± 2 mmHg; AOC = 46 ± 4 mmHg min), but the tachycardia was unchanged (maximum = 97 ± 15 beats min^{-1}, AUC = 84 ± 16 beats). As after the lower dose of captopril, the patterns of change in regional blood flows in response to bradykinin were altered, there being significant reductions in coeliac and mesenteric blood flows (Figure 2), and no change in hindquarters. Although the flows were not formally compared, the calculated vascular conductances showed significant enhancement of coeliac (AUC = 35 ± 8 [kHz $mmHg^{-1}$] 10^3 min), but not of the mesenteric (AUC = 23 ± 6 [kHz $mmHg^{-1}$]10^3 min), or hindquarters (AUC = 29 ± 5 kHz $mmHg^{-1}$]10^3 min) vasodilatations (Figure 2).

About 1 h after the higher dose of captopril the hypotensive response to bradykinin was still enhanced (nadir = − 28 ± 5 mmHg; AOC = 20 ± 5 mmHg min), but significantly less so than the response 10 min after captopril (Figure 2). However, the tachycardia was still not changed significantly (maximum = 111 ± 11 beats min^{-1}, AUC = 177 ± 20 beats). Coeliac, mesenteric and hindquarters flows increased in response to bradykinin (Figure 2) and the vasodilatations in the coeliac (AUC = 45 ± 8 [kHz $mmHg^{-1}$)10^3 min) and mesenteric (54 ± 9 [kHz $mmHg^{-1}$]10^3 min) vascular beds were significantly greater than the control responses. The increase in mesenteric vascular conductance was also significantly greater than that seen following bradykinin administration about 10 min after the higher dose of captopril. Although there was a tendency towards enchancement of the hindquarters vasodilator response to bradykinin (AUC = 34 ± 9 [kHz mmHg]10^3 min), this did not reach significance.

Experiment 2: Effects of mecamylamine and mecamylamine plus captopril on responses to bradykinin

Cardiovascular status prior to administration of bradykinin in each phase of this experiment is shown in Table 1. The hypotensive effect of mecamylamine was accompanied by coeliac and hindquarters vasodilatation, but a mesenteric

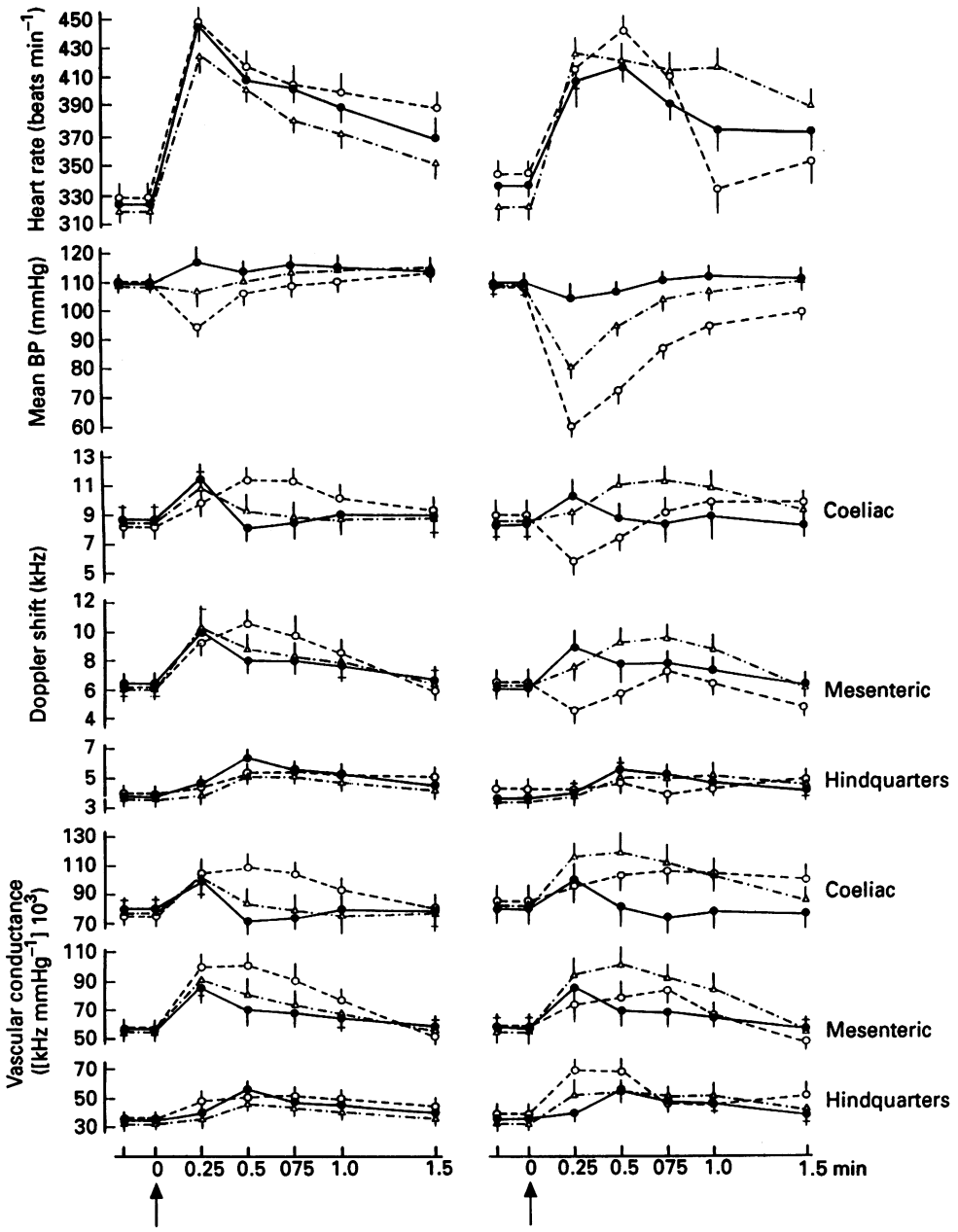


Figure 2 Cardiovascular responses to bolus i.v. injection of bradykinin (3 nmol kg^{-1} at arrow) in the same conscious Long Evans rats ($n = 9$) in the absence (●) and about 10 min (○) and 1 h (△) after administration of captopril at a dose of 28 nmol kg^{-1} (left-hand panels) or 280 nmol kg^{-1} (right-hand panels). Values are mean and bars show s.e.mean; for clarity statistics are given in the text.

vasoconstriction. The values given in the presence of mecamylamine and captopril were obtained at least 10 min after the onset of captopril infusion in the presence of mecamylamine and so do not show the acute haemodynamic responses to ACE inhibition.

Responses to bolus injections of bradykinin In this group of animals there was a small, but significant, hypotensive response to bolus injection of bradykinin in the untreated state (Table 2), and this effect was accompanied by tachycardia and coeliac, mesenteric and hindquarters vasodilatations (Table 2).

In the presence of mecamylamine, the hypotensive, and hindquarters vasodilator effects of bradykinin were enhanced, but the tachycardia was reduced and the coeliac and mesenteric vasodilator responses were not changed significantly (Table 2). In the presence of mecamylamine and captopril there was an augmentation of the tachycardic effect of bradykinin, but all other changes were not different from those seen in the presence of mecamylamine alone (Table 2).

Responses to infusion of bradykinin Infusion of bradykinin elicited substantial hypotensive, tachycardic and coeliac, mesenteric and hindquarters vasodilator responses (Table 3). In the presence of mecamylamine, the hypotensive effect of bradykinin was significantly increased, and the tachycardia was significantly reduced; the coeliac, mesenteric and hindquarters vasodilator responses were not significantly different from those in the absence of mecamylamine (Table 3). During co-administration of mecamylamine and captopril there was further enhancement of the hypotensive effect of bradykinin; the tachycardia was also increased significantly relative to that seen in the presence of mecamylamine alone, but it was still less than the control response (Table 3). The hind-

Table 1 Resting cardiovascular variables in the same, conscious, Long Evans rats ($n = 7$) under control conditions, in the presence of mecamylamine and in the presence of mecamylamine and captopril

		Control	*Mecamylamine*	*Mecamylamine + captopril*
Heart rate (beats min^{-1})		320 ± 7	282 ± 11*	287 ± 11*
Mean arterial blood pressure (mmHg)		107 ± 1	84 ± 3*	90 ± 5*
Flow (kHz)	Coeliac	7.4 ± 0.7	6.6 ± 0.7	7.0 ± 0.8
	Mesenteric	5.8 ± 0.3	3.3 ± 0.1	3.2 ± 0.2
	Hindquarters	3.2 ± 0.3	3.1 ± 0.3	3.3 ± 0.5
Vascular	Coeliac	69 ± 6	79 ± 9*	77 ± 8*
conductance	Mesenteric	54 ± 3	39 ± 2*	36 ± 3*
([kHz $mmHg^{-1}$]10^3)	Hindquarters	29 ± 3	38 ± 6*	38 ± 9*

Values are mean ± s.e.mean.
*$P < 0.05$ versus control values.
Flows were not compared under the different experimental conditions because of the marked differences in mean arterial blood pressure.

Table 2 Cardiovascular changes (areas under or over curves to 5 min) after bolus injection of bradykinin (3 nmol kg^{-1}) in the same, conscious, Long Evans rats ($n = 7$) under control conditions, in the presence of mecamylamine and in the presence of mecamylamine and captopril

		Control	*Mecamylamine*	*Mecamylamine + captopril*
Heart rate (AUC; beats)		180 ± 32*	138 ± 32*[a]	324 ± 59*[ab]
Mean arterial blood pressure (AOC; mmHg min)		−19 ± 4*	−65 ± 18*[a]	−63 ± 13*[a]
Flow (AUC; kHz min)	Coeliac	8.7 ± 1.5	5.4 ± 1.3	3.1 ± 1.0
	Mesenteric	4.2 ± 1.0	2.9 ± 0.7	2.5 ± 0.9
	Hindquarters	5.0 ± 1.2	6.7 ± 2.3	7.5 ± 1.5
Conductance	Coeliac	88 ± 18*	105 ± 27*	45 ± 12*
(AUC:	Mesenteric	48 ± 10*	52 ± 12*	44 ± 14*
([kHz $mmHg^{-1}$]10^3)min)	Hindquarters	52 ± 12*	116 ± 28*[a]	119 ± 17*[a]

Values are mean ± s.e.mean.
*$P < 0.05$ versus baseline; [a]$P < 0.05$ versus control; [b]$P < 0.05$ versus mecamylamine.
Flow changes were not compared under the different experimental conditions because of the marked differences in the mean arterial blood pressure changes.

Table 3 Cardiovascular changes (areas under or over curves) during 3 min infusions of bradykinin (36 nmol kg^{-1} min^{-1}) in the same, conscious Long Evans rats ($n = 7$) under control conditions, in the presence of mecamylamine and in the presence of mecamylamine and captopril

		Control	*Mecamylamine*	*Mecamylamine + captopril*
Heart rate (AUC; beats)		303 ± 25*	165 ± 12*[a]	214 ± 20*[ab]
Mean arterial blood pressure (AOC; mmHg min)		−33 ± 12*	−60 ± 9*[a]	−117 ± 19*[ab]
Flow (AUC; kHz min)	Coeliac	8.5 ± 1.0	3.0 ± 0.8	0.2 ± 0.1
	Mesenteric	8.8 ± 0.9	6.6 ± 1.2	1.9 ± 1.4
	Hindquarters	5.7 ± 1.3	4.2 ± 0.8	2.0 ± 0.7
Conductance	Coeliac	106 ± 9*	101 ± 13*	54 ± 11*[ab]
(AUC:	Mesenteric	103 ± 9*	132 ± 17*	75 ± 26*[ab]
([kHz $mmHg^{-1}$]10^3)min)	Hindquarters	71 ± 20*	96 ± 18*	95 ± 20*

Values are mean ± s.e.mean.
*$P < 0.05$ versus baseline; [a]$P < 0.05$ versus control; [b]$P < 0.05$ versus mecamylamine.
Flow changes were not compared under the different experimental conditions because of the marked differences in the mean arterial blood pressure changes.

quarters vasodilator effects of bradykinin in the presence of mecamylamine and captopril were not significantly different from those in the presence of mecamylamine alone, or under control conditions, but the increases in coeliac and mesenteric vascular conductance were significantly less than under both those conditions (Table 3).

Discussion

In the present work we used lower doses of captopril than are frequently employed (see Muller *et al.*, 1990) and assessed responses to AI and bradykinin soon after (about 10 min), and around 1 h subsequent to administering the ACE

inhibitor. We considered this protocol would optimise our ability to detect subtle changes in the regional haemodynamic effects of AI and bradykinin, under the influence of captopril. We also attempted to assess any possible involvement of reflex changes in the responses to bradykinin by measuring its effects in ganglion-blocked animals in the absence and presence of captopril.

The lower dose of captopril (28 nmol kg^{-1}), while having no influence on responses to bolus injection of AI, caused significant, but reversible, enhancement of the hypotensive and coeliac and mesenteric vasodilator effects of a bolus injection of bradykinin. This observation is entirely consistent with the greater affinity of bradykinin than AI for ACE (Erdös, 1975). However, the enhancement of the hypotensive response to bradykinin did not coincide with the peak of its coeliac and mesenteric hyperaemic vasodilator effects (which occurred later). One possible explanation of the enhanced hypotensive effect of bradykinin after captopril is that there was a reduction in cardiac output under these conditions, probably because inhibition of pulmonary ACE allowed a larger proportion of the administered bradykinin to gain access to the heart. Certainly, the proportionately similiar increase in the effects of bradykinin on coeliac and mesenteric vascular beds after administration of the lower dose of captopril argues in favour of the effect of captopril being due to its action in the pulmonary vascular bed rather than on ACE in the coeliac and mesenteric vascular beds. The failure of captopril to enhance the hindquarters vasodilator effect of bradykinin could have been attributable to interference with adrenaline release (MacLean & Ungar, 1986) which underlies this event (Gardiner *et al.*, 1992). However, additional factors were probably involved (see below).

Soon (about 10 min) after the higher dose (280 nmol kg^{-1}) of captopril there was substantial inhibition of the AI-induced pressor and coeliac vasoconstrictor effects, but less marked reduction of the mesenteric vasoconstriction. Since there was no apparent difference in the sensitivity of the coeliac and mesenteric vascular beds to AI under control conditions, the most likely explanation of the differential antagonism by captopril of the response to AI is that this was due to greater inhibition of ACE in the coeliac than mesenteric vascular bed; clearly, an action confined to inhibition of ACE in the pulmonary vascular bed could not have produced the picture seen. It is notable that the coeliac vasodilator effect of bradykinin was also enhanced soon after the higher dose of captopril, whereas the mesenteric vasodilator effect was not. Hence, these results are also consistent with greater inhibition of ACE in the coeliac than the mesenteric vascular bed. However, this interpretation is too simplistic, since the lower dose of captopril clearly enhanced the mesenteric vasodilator effects of bradykinin (see above), and 60 min after the higher dose of captopril the coeliac and mesenteric vasodilator effects of bradykinin were both enhanced. One explanation of this paradox is that the marked hypotensive effect of bradykinin soon after the higher dose of captopril triggered baroreflex responses that offset the mesenteric vasodilator action of bradykinin (see below).

By 60 min after captopril, all effects of AI were back to control levels, while those to bradykinin were not. Once again, this is consistent with the greater affinity of bradykinin than AI for ACE. Moreover, the dissociation between the augmentation of the hypotensive effect of bradykinin (which was greatest 10 min after the higher dose of captopril) and its coeliac and mesenteric vasodilator effects (which were greatest 60 min after captopril) is in line with the proposition that baroreflex responses to the hypotension acted to suppress the regional vasodilatations. However, this cannot be the full explanation; we suspect that changes in cardiac output must have been contributing differently to the fall in MAP in the various experimental circumstances, otherwise there should have been a direct relationship between the magnitudes and time courses of the hypotensive and regional vasodilator responses to bradykinin.

The use of ganglion blockade to assess reflex contributions to the overall response to bradykinin under normal conditions is not without problems. Administration of mecamylamine caused clear changes in baseline cardiovascular status (Table 1) which, themselves, could have influenced responses to bradykinin. Interestingly, the mecamylamine-induced hypotension was associated with coeliac vasodilatation and mesenteric vasoconstriction. While this disparate change is consistent with the differential effects of vasopressin in these two vascular beds (Gardiner *et al.*, 1990a), and it is known that ganglion-blockade triggers vasopressin-mediated haemodynamic events in conscious rats (Gardiner & Bennett, 1985), a contribution from the 'hepatic buffer' response (Richardson & Withrington, 1981) cannot be excluded. Mecamylamine-induced hindquarters vasodilatation is in line with an important vasomotor tone in that vascular bed and its relative insensitivity to vasopressin (Gardiner *et al.*, 1990a).

Notwithstanding these incidental complications, it was clear that, in the presence of mecamylamine, the hypotensive response to bradykinin was augmented, as was its hindquarters vasodilator effect. While these findings are consistent with our suspicion that reflex mechanisms might have been offsetting the haemodynamic actions of bradykinin, it appears that additional, presently unknown, factors must have been contributing to the changes seen in the coeliac and mesenteric vascular beds, since mecamylamine did not cause significant enhancement of bradykinin-induced vasodilatation at these sites. Furthermore, the finding that, in the presence of mecamylamine, captopril had no additional effect on the response to bolus injection of bradykinin could indicate that the influence of captopril under normal circumstances was due to interference with the vasoconstrictor and prejunctional actions of AII (Zimmerman, 1981; Reid, 1992), rather than to inhibition of bradykinin degradation. While we do not know for sure that mecamylamine was without effect on ACE, it is unlikely this fully explains the results since, in the presence of mecamylamine, captopril caused additional augmentation of the hypotensive response to infusion of bradykinin. However, even in this setting, as in the presence of mecamylamine alone, it is notable that the augmentation of the hypotensive effect of bradykinin infusion was not associated with enhanced coeliac, mesenteric or hindquarters vasodilatation. As before, this indicates differential contributions from changes in cardiac output to the hypotension following bradykinin administration in different experimental circumstances. Clear, albeit indirect, evidence of this came from the finding that, in the presence of mecamylamine and captopril the enhancement of the hypotensive response to bradykinin infusion was accompanied by diminished coeliac and mesenteric vasodilator responses compared to those seen in the control experiment. Our explanation of the latter observation is that there was enhancement of the vasoconstrictor effects of bradykinin (Fasciolo *et al.*, 1990).

While this study has raised more questions than it has answered, the questions have an important bearing on our understanding of the ways in which ACE inhibition might influence integrated cardiovascular control. Apart from specific conclusions relating to the regional haemodynamic changes under our different experimental conditions, a major outcome of the present investigation is the affirmation it gives to our previous proposal that measurement of blood pressure alone may provide insufficient data if one is seeking to understand the ways in which drugs and peptides impinge on cardiovascular function *in vivo*.

References

CAMPBELL, D.J. (1987). Circulating and tissue angiotensin systems. *J. Clin. Invest.*, **79**, 1–5.

DZAU, V.J. (1988). Circulating versus local renin-angiotensin system in cardiovascular homeostasis. *Circulation*, **77**, (suppl. I), I-4–I-13.

ERDÖS, E.G. (1975). Angiotensin I converting enzyme. *Circ. Res.*, **36**, 247–255.

FASCIOLO, J.C., VARGAS, L., LAMA, M.C. & NOLLY, H. (1990). Bradykinin-induced vasoconstriction in rat mesenteric arteries precontracted with noradrenaline. *Br. J. Pharmacol.*, **101**, 344–348.

GARDINER, S.M. & BENNETT, T. (1985). Interactions between neural mechanisms, the renin-angiotensin system and vasopressin in the maintenance of blood pressure during water deprivation: studies in Long Evans and Brattleboro rats. *Clin. Sci.*, **68**, 647–657.

GARDINER, S.M., COMPTON, A.M. & BENNETT, T. (1990a). Differential effects of neuropeptides on coeliac and superior mesenteric blood flows in conscious rats. *Regul. Pept.*, **29**, 215–227.

GARDINER, S.M., COMPTON, A.M., BENNETT, T. & HARTLEY, C.J. (1990b). Can pulsed Doppler technique measure changes in aortic blood flow in conscious rats? *Am. J. Physiol.*, **259**, H448–H456.

GARDINER, S.M., KEMP, P.A., BENNETT, T., BOSE, C., FOULKES, R. & HUGHES, B. (1992). Involvement of β_2-adrenoceptors in the regional haemodynamic responses to bradykinin in conscious rats. *Br. J. Pharmacol.*, **105**, 839–848.

HAYWOOD, J.R., SHAFFER, R., FASTENOW, C., FINK, G.D. & BRODY, M.J. (1981). Regional blood flow measurement with pulsed Doppler flowmeter in conscious rat. *Am. J. Physiol.*, **241**, H273–H278.

MACLEAN, M.R. & UNGAR, A. (1986). Effects of the renin-angiotensin system on the reflex response of the adrenal medulla to hypotension in the dog. *J. Physiol.*, **373**, 343–352.

MULLER, A.F., GARDINER, S.M., COMPTON, A.M. & BENNETT, T. (1990). Regional haemodynamic effects of captopril, enalaprilat and lisinopril in conscious water-replete and water-deprived Brattleboro rats. *Clin. Sci.*, **79**, 393–401.

REGOLI, D. & BARABÉ, J. (1980). Pharmacology of bradykinin and related kinins. *Pharmacol. Rev.*, **32**, 1–45.

REID, I.A. (1992). Interactions between ANG II, sympathetic nervous system, and baroreceptor reflexes in regulation of blood pressure. *Am. J. Physiol.*, **262**, E763–778.

RICHARDSON, P.D.I. & WITHRINGTON, P.G. (1981). Liver blood flow I. Intrinsic and nervous control of liver blood flow. *Gastroenterology*, **81**, 159–173.

THEODORSSON-NORHEIM, E. (1987). Friedman and Quade tests: BASIC computer program to perform non-parmetric two-way analysis of variance and multiple comparisons on ranks of several related samples. *Comput. Biol. Med.*, **17**, 85–99.

ZIMMERMAN, B.G. (1981). Adrenergic facilitation by angiotensin: does it serve a physiological function? *Clin. Sci.*, **60**, 343–348.

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Mediation via different receptors of the vasoconstrictor effects of endothelins and sarafotoxins in the systemic circulation and renal vasculature of the anaesthetized rat

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1 Using endothelin-1 (ET-1), endothelin-3 (ET-3), sarafotoxin 6b (SX6b) and sarafotoxin 6c (SX6c) as agonists and BQ-123 as a selective ET_A receptor antagonist, we have examined the endothelin receptor subtypes mediating the systemic pressor and renal vasoconstrictor effects of the ET/SX family of peptides.

2 In anaesthetized rats, bolus intravenous injections of ET-1, ET-3, SX6b or SX6c (0.1, 0.25 and 0.50 nmol kg^{-1}) produced initial transient depressor responses followed by sustained and dose-dependent increases in mean arterial pressure (MAP) with the following rank order of potency: SX6b > ET-1 >> SX6c > ET-3. In contrast, in the renal vasculature these peptides caused equipotent dose-dependent falls in renal blood flow (RBF) (ET-1 = ET-3 = SX6b = SX6c).

3 BQ-123 (1 mg kg^{-1}, i.v. bolus) significantly reduced the systemic pressor effects of all the peptides but was largely ineffective against the renal vasoconstrictions.

4 These results indicate that although the systemic pressor effects of the ET/SX peptides are mediated via ET_A receptors, the vasoconstriction in the kidney *in vivo* may be mediated predominantly via ET_B-like receptors. This may be of therapeutic relevance, for an ET_A-receptor-selective antagonist could offer only poor protection of the renal circulation from the deleterious effects of endogenously produced members of this peptide family.

Keywords: Endothelin; sarafotoxin; kidney; BQ-123

Introduction

In the kidney, locally administered endothelin-1 (ET-1) induces vasoconstriction resulting in an increase in renal vascular resistance (RVR) and a decrease in renal blood flow (Badr *et al.*, 1989; King *et al.*, 1989; Kon *et al.*, 1989; Miller *et al.*, 1989). ET-1 also contracts mesangial cells which results in a further decrease in the glomerular filtration rate (Badr *et al.*, 1989; King *et al.*, 1989). Circulating levels of ET-1 have been reported to be increased in both experimental models and clinical studies of acute and chronic renal failure (Firth *et al.*, 1988; Koyama *et al.*, 1989; Warrens *et al.*, 1990; Brooks *et al.*, 1991) and infusion of anti-ET-1 antibodies protects the renal vasculature against ischaemia/reperfusion injury or cyclosporin nephrotoxicity (Kon *et al.*, 1989; 1990). Thus, there is good evidence that endothelins may be involved in renal diseases and that selective ET-receptor antagonists might be useful in the therapy of such diseases.

Two ET-receptor subtypes have been characterized. The ET_A receptor, which has a higher affinity for ET-1, ET-2 and sarafotoxin (SX) 6b than for ET-3 or SX6c (Arai *et al.*, 1990), and the ET_B receptor which is non-isopeptide selective (Sakurai *et al.*, 1990). It has been generally assumed (Masaki *et al.*, 1991) that the ET_A receptor is located on vascular smooth muscle cells, where it mediates the constrictor responses, and the ET_B receptor on the endothelial cell, where it mediates prostacyclin and endothelium-derived relaxing factor (EDRF) release by endothelins (De Nucci *et al.*, 1988; Warner *et al.*, 1989). There may also be a third subtype of ET receptor selective for ET-3 and SX6c (Kloog *et al.*, 1989; Emori *et al.*, 1990; Samson *et al.*, 1990; Harrison *et al.*, 1992).

We have studied which endothelin receptor subtype(s) mediate(s) renal vasoconstriction. For these experiments we used the ET/SX peptides as selective agonists, and the cyclic pentapeptide BQ-123 (cyclo(D-Asp-L-Pro-D-Val-L-Leu-D-trp) (Ihara *et al.*, 1992), an ET_A-receptor-selective antagonist.

Methods

Surgical procedures

Wistar rats (male, 300–400 g) were anaesthetized with thiopentone sodium (120 mg kg^{-1}, i.p.). The trachea was cannulated to facilitate respiration and body temperature was maintained at 37°C by means of a rectal probe connected to a homeothermic blanket (Bioscience, Sheerness, Kent). The right carotid artery was cannulated and connected to a pressure transducer (Transamerica type 4-422-001) for the measurement of arterial blood pressure which was displayed on a polygraph (Grass 7D, Grass Instruments, Quincy, MA, U.S.A.). Mean arterial blood pressure (MAP) was calculated as diastolic pressure plus one third of the difference between the diastolic and systolic pressures. The left jugular vein was cannulated for the administration of drugs and the right femoral vein for the administration of saline (1.5 ml h^{-1}) to compensate for any fluid loss.

The left kidney was exposed via a mid-line laparotomy and the renal artery was carefully isolated. An ultrasonic flow probe (1RB, internal diameter = 1 mm), embedded in a silicone cuff to provide optimal alignment, was placed around the left renal artery for measurement of total renal blood flow (RBF) using a Transonic T206 Small Animal Flowmeter (Transonic Systems Inc., New York, U.S.A.). A small amount of acoustical couplant (100 mg Nalco 1181, mixed with 10 ml distilled water; Nalco Chemical Co., IL., U.S.A.) was deposited in the acoustic window of the probe adjacent to the artery, in order to displace all air. Renal vascular resistance

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(RVR) was calculated by dividing MAP by RBF. Under these conditions in preliminary control experiments MAP, RBF and autoregulation were stable up to 6 h ($n = 3$).

Experimental procedures

After surgery and a stabilization period of at least 30 min, animals ($n = 4$ for each group) were given bolus intravenous injections of 0.1, 0.25, 0.5 and 1 nmol kg^{-1} of ET-1, ET-3, SX6b or SX6c. The time between each injection was 1 h. Five minutes prior to each dose of peptide, animals received either BQ-123 (1 mg kg^{-1}) or vehicle (0.9% w/v sodium chloride containing 10 mM sodium bicarbonate), as i.v. bolus injections of 1 ml kg^{-1}. This dose and time course of BQ-123 administration is effective in antagonizing the pressor effects of the ET/SX peptides (McMurdo *et al.*, 1993).

Changes in MAP and RBF were calculated 3 min after the injection of the ET/SX peptides at which time the initial depressor response was ended, but the renal vasoconstriction was still maintained for all doses of the ET/SX peptides.

Materials

Trapanal (thiopentone sodium) was obtained from Byk Gulden (Konstanz, Germany). ET-1, ET-3 and SX6b were purchased from the Peptide Institute (Osaka, Japan), and SX6c from Peninsula Laboratories Inc. (Belmont, U.S.A.). The peptides were reconstituted in 0.1% v/v acetic acid and then dissolved in 0.9% w/v saline containing 1% w/v bovine serum albumin and 10 mM sodium bicarbonate. We thank Drs A. Doherty and W. Cody of the Chemistry Department of Parke-Davis (Ann Arbor, MI, USA) for providing the BQ-123 which was dissolved in 0.9% w/v saline containing 10 mM sodium bicarbonate.

Statistics

Statistical differences between points were determined by an unpaired two tail Student's t test and $P < 0.05$ was taken to reflect significant difference.

Results

Systemic and renal effects of ET/SX peptides

Basal values for the MAP, RBF and RVR were 120 ± 2 mmHg, 9.4 ± 0.3 ml min^{-1} and 13.2 ± 0.5 mmHg min^{-1} ml^{-1}, respectively ($n = 32$, for each). These values were unaffected by vehicle or BQ-123 at the (1 mg kg^{-1}) dose used in this study.

Injection of all peptides produced similar initial and transient falls in MAP (data not shown). This depressor effect was followed by a sustained, dose-dependent and isopeptide-selective increase in MAP (Figure 1a, Figure 3). The maximum increase in MAP was obtained within 10 min for all peptides except SX6c, in which case the peak occurred at 20 min.

In the kidney, all the peptides produced similar falls in RBF (Figure 2a, Figure 3) associated with a dramatic increase in RVR. The constrictor effect in the renal vasculature reached a maximum within 1 min (Figure 3). At doses greater than 0.25 nmol kg^{-1} the RBF was still affected 1 h after injection although MAP had returned to control levels.

Inhibition by BQ-123 of ET/SX pressor responses

BQ-123 (1 mg kg^{-1}) significantly reduced the systemic pressor effects of SX6b at all doses and of the other ET/SX peptides at doses of 0.25 mmol kg^{-1} and higher (Figure 1b, Figure 3). For instance, 3 min after injection, the increases in MAP induced by ET-1, SX6b, SX6c and ET-3 at 0.5 nmol kg^{-1} were 10 ± 1 mmHg, 4 ± 1 mmHg, 0 ± 4 mmHg and 3 ± 1 mmHg,

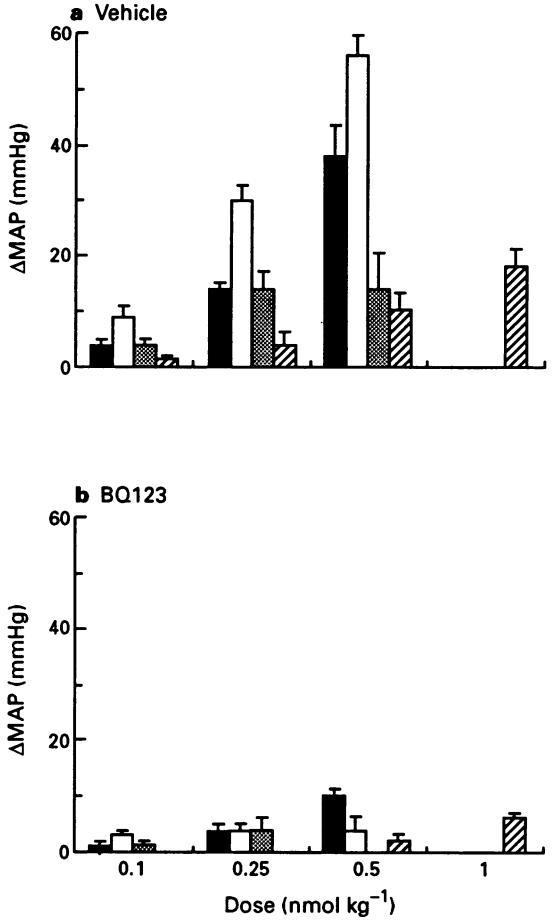


Figure 1 The effect of BQ-123 on the pressor effects of endothelin-1 (ET-1), sarafotoxin 6b (SX6b), SX6c or ET-3 in the anaesthetized rat. (a) The ET/SX peptides (0.1–1 nmol kg^{-1}) produced dose-dependent elevations in blood pressure, 3 min after i.v. administration. Data for 1 nmol kg^{-1} is only shown for ET-3, due to the high mortality in the other groups. (b) Responses to the same doses of the ET/SX peptides in animals pretreated with BQ-123 at 1 mg kg^{-1}, 5 min prior to the peptide. Closed columns, ET-1; open columns, SX6b; stippled columns, SX6c; hatched columns, ET-3. Each column with a vertical bar represents the mean ± s.e.mean from 4 observations.

respectively. In the control experiments the values were 38 ± 6 mmHg, 56 ± 3 mmHg, 14 ± 6 mmHg and 10 ± 4 mmHg ($n = 4$ for each, $P < 0.05$). BQ-123 did not affect the magnitude of the falls in MAP induced by any of the peptides but prolonged their durations (data not shown).

Lack of effect of BQ-123 on renal vasoconstrictions induced by ET/SX peptides

BQ-123 failed to prevent the fall in RBF induced by the ET/SX peptides (Figure 2b, Figure 3). For instance, 3 min after injection, the falls in RBF induced by ET-1, SX6b, SX6c or ET-3 at 0.5 nmol kg^{-1} were 84 ± 5%, 84 ± 9%, 70 ± 9%, 64 ± 5%, respectively. In the control experiments the values were 69 ± 6%, 90 ± 3%, 81 ± 7%, 74 ± 6% ($n = 4$ for each, $P > 0.05$). When RVR values were calculated, BQ-123 significantly decreased the renal vasoconstrictor effects of ET-1 (0.1 nmol kg^{-1}) and SX6b (0.1 and 0.25 nmol kg^{-1}) (Table 1). At higher doses of either ET-1 or SX6b, the very large increases in RVR, due to the great reductions in RBF, were not significantly attenuated by BQ-123. BQ123 did not affect the changes in RVR induced by either ET-3 or SX6c (Table 1). BQ-123 also did not prevent the prolonged effects of the ET/SX peptides at doses greater than 0.25 nmol kg^{-1}.

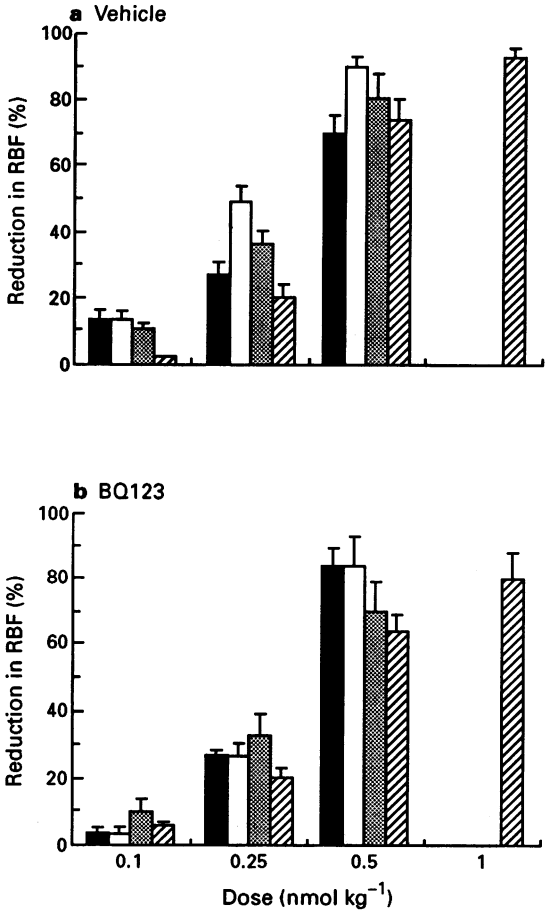


Figure 2 Lack of effect of BQ-123 on the reductions in renal blood flow induced by endothelin-1 (ET-1), sarafotoxin 6b (SX6b), SX6c or ET-3 in the anaesthetized rat. (a) The ET/SX peptides (0.1–1 nmol kg^{-1}) produced dose-dependent falls in renal blood flow 3 min after i.v. administration, expressed as the % reduction in flow from the level immediately prior to peptide injection. Data for 1 nmol kg^{-1} is only shown for ET-3, due to the high mortality in the other groups. (b) Effects of the same doses of the ET/SX peptides in animals pretreated with BQ-123 at 1 mg kg^{-1}, 5 min prior to the peptide. Closed columns, ET-1; open columns, SX6b; stippled columns, SX6c; hatched columns, ET-3. Each column with a vertical bar represents the mean ± s.e.mean from 4 observations.

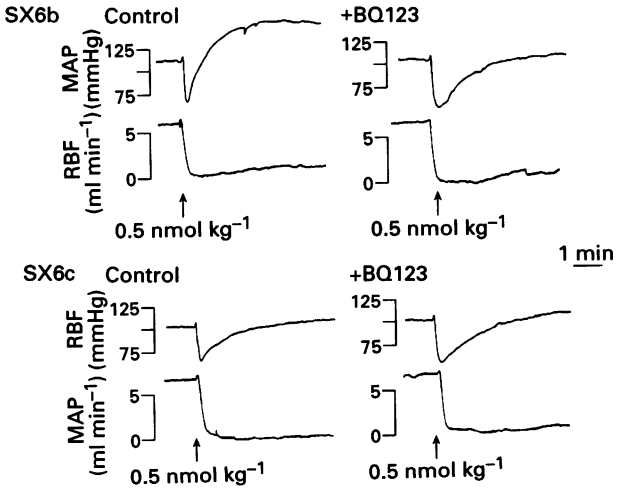


Figure 3 Representative traces of the effect of BQ-123 on the mean arterial blood pressure and renal vasoconstrictor responses to sarafotoxin 6b (SX6b) or SX6c. SX6b or SX6c were administered as i.v. bolus injections (0.5 nmol kg^{-1}). Vehicle or BQ-123 (1 mg kg^{-1}) was administered as an i.v. bolus 5 min prior to the peptide injection. MAP, mean arterial pressure; RBF, renal blood flow.

Table 1 The changes in renal vascular resistances calculated 3 min after bolus intravenous injections of endothelin-1 (ET-1), ET-3, sarafotoxin 6b (SX6b) or SX6c (0.1 or 0.2 nmol kg^{-1})

	0.1 nmol kg^{-1}		*0.25 nmol kg^{-1}*	
	Control	*+ BQ-123*	*Control*	*+ BQ-123*
ET-1	2.1 ± 0.4	0.6 ± 0.2*	7.7 ± 0.3	5.3 ± 1.3
SX6b	3.5 ± 1.0	−0.2 ± 0.1*	23.3 ± 2.3	6.3 ± 0.9*
SX6c	2 ± 0.5	1.9 ± 0.8	11.9 ± 2.2	8.9 ± 2.8
ET-3	0.4 ± 0.2	0.4 ± 0.2	3.6 ± 0.7	2.8 ± 0.5

*Indicates significant differences in the RVR calculated in control versus BQ-123 pretreated (1 mg kg^{-1}, 5 min prior to peptide) animals.

Discussion

This study demonstrates that the vasoconstrictor effects of the ET/SX peptides in the systemic circulation and renal vasculature are mediated by pharmacologically distinct receptor subtypes. The rises in MAP were isopeptide-selective, for ET-1 or SX6b were clearly more potent than ET-3 or SX6c and the elevations in MAP were largely, but not completely, blocked by BQ-123, a selective ET_A antagonist, as described previously (Ihara *et al.*, 1991a; Pollock *et al.*, 1992; McMurdo *et al.*, 1993). This is indicative of a dominant role for the ET_A receptor in these responses. At the same time it is noteworthy that BQ-123 also decreased the pressor effects of SX6c, which has been described as an agonist selective for ET_B receptors (Williams *et al.*, 1991). This may suggest either the presence of another receptor type or that there is an interaction between SX6c and ET_A receptors, or between BQ-123 and ET_B receptors. The vasoconstriction induced by the ET/SX peptides in the renal vascular bed was not isopeptide selective, as the peptides were equipotent, and BQ-123 failed to prevent the ET/SX-induced fall in RBF. At the same time SX6b at a dose of 0.5 nmol kg^{-1} caused a greater increase in RVR than the other ET/SX peptides at the same dose. Taken together with the finding that BQ-123 depressed the reductions in RVR caused by the lowest doses of ET-1 or SX6b, these data are consistent with the premise that the vasoconstriction in the renal vasculature may be mediated via a mixed receptor population. ET_A receptors appear to mediate a proportion of the response to the more selective ET_A agonists whereas the renal vasoconstrictions induced by other ET/SX peptides or to higher doses of ET-1 or SX6b are mediated by another, possibly ET_B, receptor the activation of which can compensate for the blockade of the ET_A-mediated pathway.

Until recently, it was generally thought that the ET_A receptor was the predominant subtype mediating the vasoconstrictor and pressor responses to ET/SX peptides (Ihara *et al.*, 1991a; Masaki *et al.*, 1991; Pollock *et al.*, 1992). The suggestion of non-ET_A-receptors mediating vasoconstrictions first came from *in vitro* studies with selective ET_A receptor antagonists, for in some tissues these antagonists did not fully antagonise endothelin-induced contractions (Ihara *et al.*, 1991b; Fukuroda *et al.*, 1992; Warner *et al.*, 1992). In addition, we have also shown that BQ-123 cannot completely block the pressor effects of ET/SX peptides in the anaesthetized ganglion-blocked rat (McMurdo *et al.*, 1993). The pharmacological characteristics of the responses in most of these reports suggest that this non-ET_A-mediated vasoconstriction is due to ET_B receptor activation (Ihara *et al.*, 1991b; Williams *et al.*, 1991; Fukuroda *et al.*, 1992).

It has been shown previously that ET-1 elicits a strong renal arteriolar vasoconstriction (Badr *et al.*, 1989; King *et al.*, 1989; Miller *et al.*, 1989; Kon *et al.*, 1989). Although the receptor subtype responsible for this effect has not been characterized, [^{125}I]ET-1 binding studies have suggested that binding sites in kidney homogenates are principally of the

ET_B-receptor subtype (Ihara *et al.*, 1991a). These receptors may be located on the vascular smooth muscle of the kidney, or they may be present on another cell type, not necessarily within the kidney, and cause renal vasoconstriction via an indirect mechanism such as activation of the renin-angiotensin system (Miller *et al.*, 1989) or release of a vasoconstrictor autacoid like thromboxane A_2 (Rae *et al.*, 1989). These systems may even be activated by the substantial initial depressor response which would affect autoregulation of kidney blood flow and GFR leading to elevation of angiotensin II, for instance, and so renal vasoconstriction. On the other hand the rapidity of the renal vasoconstriction does argue for a more direct mechanism, at least for the initial phase of the response.

In conclusion, this study demonstrates that in the anaesthetized rat the pressor effects of the ET/SX peptides and the vasoconstrictions they induce in the renal vasculature are predominantly mediated via two different receptor subtypes, respectively ET_A and probably ET_B. This finding attests to the fact that there is a heterogeneity in the distribution of ET/SX receptor subtypes in the circulation. This observation may have therapeutic relevance for an ET_A-receptor selective antagonist might not protect the renal circulation from the deleterious effects of endogenously produced members of this peptide family.

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References

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

BADR, K.F., MURRAY, J.J., BREYER, M.D., TAKAHASHI, K., INAGAMI, T. & HARRIS, R.C. (1989). Mesangial cell, glomerular and renal vascular responses to endothelin in the rat kidney. *J. Clin. Invest.*, **83**, 336–342.

BROOKS, D.P., CONTINO, L.C., STORER, B. & OHLSTEIN, E.H. (1991). Increased endothelin excretion in rats with renal failure induced by partial nephrectomy. *Br. J. Pharmacol.*, **104**, 987–989.

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

EMORI, T., HIRATA, Y. & MARUMO, F. (1990). Specific receptors for endothelin-3 in cultured bovine endothelial cells and its cellular mechanism of action. *FEBS Lett.*, **263**, 261–264.

FIRTH, J.D., RATCLIFFE, P.J., RAINE, A.E.G. & LEDINGHAM, J.G.G. (1988). Endothelin: an important factor in acute renal failure? *Lancet*, **ii**, 1179–1181.

FUKURODA, T., NISHIKIBE, M., OHTA, Y., IHARA, M., YANO, M., ISHIKAWA, K., FUKAMI, T. & IKEMOTO, F. (1992). Analysis of responses to endothelins in isolated porcine blood vessels by using a novel endothelin antagonist, BQ-153. *Life Sci.*, **50**, Pl-107-Pl-112.

HARRISON, V.J., RANDRIANTSOA, A. & SCHOEFFTER, P. (1992). Heterogeneity of endothelin-sarafotoxin receptors mediating contraction of pig coronary artery. *Br. J. Pharmacol.*, **105**, 511–513.

IHARA, M., FUKURODA, T., SAEKI, T., NISHIKIBE, M., KOJIRI, K., SUDA, H. & YANO, M. (1991a). An endothelin receptor (ET_A) antagonist isolated from *Streptomyces misakiensis*. *Biochem. Biophys. Res. Commun.*, **178**, 132–137.

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

IHARA, M., SAEKI, T., FUNABASHI, K., NAKAMICHI, K., YANO, M., FUKURODA, T., MIYAJI, M., NISHIKIBE, M. & IKEMOTO, F. (1991b). Two endothelin receptor subtypes in porcine arteries. *J. Cardiovasc. Pharmacol.*, **17**, Suppl 7, S119–S121.

KING, A.J., BRENNER, B.M. & ANDERSON, S. (1989). Endothelin: a potent renal and systemic vasoconstrictor peptide. *Am. J. Physiol.*, **256**, F1051–F1058.

KLOOG, Y., BOUSSO-MITLER, D., BDOLAH, A. & SOKOLOVSKY, M. (1989). Three apparent receptor subtypes for the endothelin/sarafotoxin family. *FEBS Lett.*, **253**, 199–202.

KON, V., SUGIARA, M., INAGAMI, T., HARVIE, B.R., ICHIKAWA, I. & HOOVER, R.L. (1990). Role of endothelin in cyclosporine-induced glomerular dysfunction. *Kidney Int.*, **37**, 1487–1491.

KON, V., YOSHIOKA, T., FOGO, A. & ICHIKAWA, I. (1989). Glomerular actions of endothelin in vivo. *J. Clin. Invest,*. **83**, 1762–1767.

KOYAMA, H., TABATA, T., NISHZAWA, Y., INOUE, T., MORII, H. & YAMAJI, T. (1989). Plasma endothelin levels in patients with uraemia. *Lancet*, **i**, 991–992.

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

MCMURDO, L., CORDER, R., THIEMERMANN, C. & VANE, J.R. (1993). Incomplete inhibition of the pressor effects of ET-1 and related peptides in the anaesthetised rat with BQ-123 provides evidence for a further vasoconstrictor receptor. *Br. J. Pharmacol.*, (in press).

MILLER, W.L., REDFIELD, M.M. & BURNETT, J.C. (1989). Integrated cardiac, renal and endocrine actions of endothelin. *J. Clin. Invest,*. **83**, 317–320.

POLLOCK, D.M., DIVISH, B.J., SULLIVAN, G.M., SHIOSAKI, K. & OPGENORTH, T.J. (1992). Effect of an endothelin type A receptor antagonist, BQ-123, on pressor responses to endothelin family peptides. *J. Vasc. Res.*, **29**, 183, Abstract.

RAE, G.A., TRYBULEC, M., DE NUCCI, G. & VANE, J.R. (1989). Endothelin-1 releases eicosanoides from rabbit isolated perfused kidney and spleen. *J. Cardiovasc. Pharmacol,*. **13** (suppl. 5), S89–S92.

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

SAMSON, W.K., SKALA, K.D., ALEXANDER, R.B. & HUANG, F.L.S. (1990). Pituitary site of action of endothelin: selective inhibition of prolactin release in vitro. *Biochem. Biophys. Res. Commun.*, **169**, 737–743.

WARNER, T.D., MITCHELL, J.A., DE NUCCI, G. & VANE, J.R. (1989). Endothelin-1 and endothelin-3 release EDRF from isolated perfused arterial vessels of the rat and the rabbit. *J. Cardiovasc. Pharmacol,*. **13** (suppl. 5), S85–S88.

WARNER, T.D., ALLCOCK, G.H., CORDER, R. & VANE, J.R. (1992). BQ-123 and different isolated tissue preparations reveal heterogeneity in the receptors mediating contractions to endothelin-1. *Br. J. Pharmacol.*, **107**, 103P.

WARRENS, A.N., CASSIDY, M.J.D., TAKAHASHI, K., GHATEI, M.A. & BLOOM, S.R. (1990). Endothelium in renal failure. *Nephrol. Dial. Transplant.*, **5**, 418–422.

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

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Amelioration by the Ca^{2+} antagonist, nimodipine of an existing neuropathy in the streptozotocin-induced, diabetic rat

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1 Neuropathy is a frequently diagnosed complication in diabetic patients but an effective treatment does not exist.

2 The development of neuropathy in streptozotocin-induced diabetic rats was monitored by measuring the motor and sensory nerve conduction velocity in the sciatic nerve.

3 A significant decrease in sensory and motor nerve conduction velocity was apparent in young, 14-week-old diabetic rats as compared to non-diabetic, age-matched controls 4 weeks after the induction of diabetes with streptozotocin.

4 Intraperitoneal treatment with the Ca^{2+} channel blocker, nimodipine, from week 4 onwards, in a dosage of 10 mg kg^{-1} or 20 mg kg^{-1} intraperitoneally per 48 h, resulted in a significant increase in sensory and motor nerve conduction velocity whereas treatment with 5 mg kg^{-1} intraperitoneally per 48 h was not effective.

5 One-year-old, adult, diabetic rats treated with nimodipine 20 mg kg^{-1} (treatment started again 4 weeks after induction of diabetes mellitus) also showed an increase of both sensory and motor nerve conduction velocity as compared to diabetic rats treated with placebo.

6 It is concluded that nimodipine ameliorates existing experimental diabetic neuropathy in streptozotocin-induced diabetic rats in both young and adult animals.

Keywords: Diabetes mellitus; neuropathy; nerve conduction velocity; peripheral nerve regeneration; Ca^{2+} antagonist; nimodipine

Introduction

Although insulin treatment has achieved enormous improvement in the quality of life in diabetic patients, it is unclear whether absolute normoglycaemia will prevent the development of complications such as nephropathy, retinopathy and neuropathy. A major focus of research is the prevention and treatment of these complications from both type 1 and type 2 diabetes mellitus (Pirart, 1978). To date, irrespective of the pathogenesis, there is no treatment of neuropathy other than attempting to decrease the symptoms, such as paraesthesia, pain, muscle weakness and postural hypotension. Over the years numerous studies in experimental diabetic neuropathy (EDN) have been carried out to assess the efficacy of putative pharmacotherapy. Streptozotocin (STZ) is frequently used to destroy the β cells in the islets of Langerhans of rats and so to induce diabetes mellitus (Rerup, 1970). Although neither this nor any other model can mimic the full detail of human diabetes mellitus, the pathology of diabetic neuropathy is close to that observed in man. In EDN, both in the STZ-diabetic rat and in the spontaneously diabetic rat (BB/Wor), several abnormalities such as biochemical, histomorphological and nerve conduction velocity level disturbances have been described (Crisa *et al.*, 1992). Commonly, sensory and motor nerve conduction velocities are greatly decreased in diabetic patients (Thomas & Eliasson, 1975). For this reason, reductions in peripheral sensory and motor nerve conduction velocity (SNCV and MNCV) are frequently taken as indices of EDN and are used to determine the possible effects of pharmacotherapy in rats (Greene *et al.*, 1987).

There are several hypotheses about the pathogenesis of diabetic neuropathy. One hypothesis points to the disregulation of neural glucose metabolism that leads to an increased activity of the polyol pathway with accumulation of sorbitol and fructose and a subsequent depletion of myo-inositol. Presumably one of the consequences of a myo-inositol depletion is a reduction of the Na,K ATPase activity and thus an impairment of nerve function (Finegold *et al.*, 1983; Dyck *et al.*, 1987). The other major hypothesis points to the reduction of endoneural bloodflow and oxygen tension in nerves which have been extensively described in diabetic rats (Tuck *et al.*, 1984; Yasuda & Dyck, 1987). Rats subjected to chronic hypoxia (10%) developed electrophysiological abnormalities that were similar to those of EDN but in the absence of a disregulation of the glucose level (Low *et al.*, 1984; 1986; 1987; Hendriksen *et al.*, 1992).

Different putative pharmacotherapies have been tested over the years that were aimed to prevent or improve diabetic neuropathy. One line of research focused on the efficacy of compounds that would interfere with the presumed aetiology of diabetic neuropathy e.g. regulating blood glucose levels by treatment with insulin (Greene *et al.*, 1975; Finegold *et al.*, 1983) or preventing flux through the polyol pathway by treatment with aldose reductase inhibitors (Mayer & Tomlinson, 1983; Tomlinson *et al.*, 1984; 1985). Another experimental approach tries to capitalize on the possibility that putative neurotrophic drugs may possess neuroprotective properties as well. In this respect it was demonstrated that the neurotrophic peptide, Org 2766 (a synthetic analogue of $ACTH_{4\text{-}9}$) known to facilitate recovery of function following a mechanical lesion of the sciatic nerve in the rat (Koning de & Gispen, 1987) also prevents the development of peripheral neuropathy induced by cisplatin (Hamers *et al.*, 1990) or by streptozotocin (EDN) (Van der Zee *et al.*, 1989).

The use of the Ca^{2+}antagonist, nimodipine, in the treatment of peripheral neuropathies is based on a similar rationale. In general, Ca^{2+}antagonists potentially modulate numerous aspects of nervous system function (Bär *et al.*, 1990).

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Following the discovery of specific binding sites for Ca^{2+} antagonists of the 1,4-dihydropyridine type in neural tissue (Belleman *et al.*, 1983) it became clear that these dihydropyridines exert profound neuropharmacological effects (Traber & Gispen, 1989). Special attention was paid to nimodipine (isopropyl(2-methoxy-ethyl) 1,4-dihydro 2,6 dimethyl-4(3-nitrophenyl)-3,5-pyridine dicarboxylate) as this compound readily penetrates the blood brain barrier (Hoffmeister *et al.*, 1985). The neurotrophic and neuroprotective action of nimodipine was investigated, under a variety of experimental conditions: neurones in culture (Azmitia, 1989), post-lesion recovery of function in PNS and CNS (Van der Zee *et al.*, 1987; 1991; Nyakas *et al.*, 1989; Gispen *et al.*, 1991) age-related brain and behavioural deficits in rodents (Schuurman *et al.*, 1987; Disterhoft *et al.*, 1989; Landfeld, 1989) and cisplatin-induced, peripheral neuropathy in rats (Hamers *et al.*, 1990). Furthermore as a member of the dihydropyridine class of Ca^{2+}channels blockers, nimodipine may exhibit vasodilating effects that may have a beneficial effect on endoneural bloodflow (Brandt *et al.*, 1981). In view of the interesting combination of pharmacological effects as discussed above, it was decided to test the putative efficacy of nimodipine in EDN. Recently we demonstrated that treatment of STZ-induced diabetic rats with nimodipine 20 mg kg^{-1} i.p. from the start of the diabetes counteracts the development of EDN (Kappelle *et al.*, 1992). In view of these preventive properties of nimodipine in EDN, it was of interest to investigate the possibility that nimodipine might also ameliorate an existing EDN. We therefore studied the effect of nimodipine on existing neuropathy in young and adult STZ-induced, diabetic rats.

Methods

Animals

Male Wistar rats of an inbred strain (TNO, Zeist, NL) weighing approximately 300 g at the start of the first two experiments (age 13–14 weeks) were used. In a third experiment, rats weighing approximately 570 g (age 12 months) were used. The rats were housed on sawdust in macrolon cages, 2 rats per cage and maintained on a 12:12 h light-dark cycle with food and water *ad libitum*. The rats were made diabetic with a single intravenous injection of streptozotocin (Zanosar, Upjohn Company, Kalamozoo, Michigan, U.S.A.) at a dose of 40 mg kg^{-1} body weight in experiments 1 and 2. In experiment 3 the rats received a single intravenous injection of STZ at a dosage of 20 mg kg^{-1}. Samples for blood glucose monitoring were obtained from the tail vein. After 48 h blood glucose levels were determined with a Minilab (Bayer GmbH, Munich, Germany). Rats with glucose levels $>$ 15 mmol l^{-1} 48 h following STZ injection were considered diabetic. Age-matched, non-diabetic rats served as controls.

Drugs

Nimodipine (Bayer AG, Elberfeld, Germany) as a micronized powder was dissolved in polyethylene glycol 1 ml kg^{-1} under dim light and injected intraperitoneally as a freshly prepared solution. Control rats received 1 ml kg^{-1} polyethylene glycol 400 i.p. In all 3 experiments nimodipine or placebo (polyethylene glycol) treatment began once a peripheral neuropathy had developed i.e. 4 weeks following STZ treatment.

Electrophysiology

Measurements of nerve conduction velocities were carried out under general anaesthesia (Hypnorm, Duphar, Weesp, NL, containing fluanisone 10 mg ml^{-1} and fentanyl citrate 0.2 mg ml^{-1}, dose 0.8 ml kg^{-1} body weight, administered subcutaneously). To minimize effects of differences in body temperature on conduction velocity, the animals were placed under a 40-W light bulb (at a distance of 25 cm) 5 min prior to testing. During this procedure, the rectal temperature varied from 37–38°C. The method is described in detail by De Koning & Gispen (1987). In short, the sciatic and tibial nerves were stimulated at the sciatic notch and ankle, respectively, by means of monopolar needle electrodes. The anode was placed 5 mm proximal to the cathode. Upon stimulation of these mixed peripheral nerves, two responses were recorded from the small muscles of the foot by means of surface electrodes: the short-latency M-response, due to stimulation of α-motor fibres, and the long-latency H-response, due to stimulation of afferent Ia-fibres, which excite α-motor neurones in the spinal cord monosynaptically (Waxman, 1980; Stanley, 1981). The motor nerve conduction velocity (MNCV) and H-reflex related sensory nerve conduction velocity (SNCV) can be calculated from the latencies of these responses and the distance between the two stimulation points. The measurements were performed in a 'blind' fashion to ensure that the experimenter, who measured the NCVs, was unaware of the treatment a rat had received.

Outline of the study

In all experiments one group served as an age-matched, control group (Con) and one group served as a diabetic control group (Plac), both treated with 1 ml kg^{-1} polyethylene glycol i.p. every 48 h. In the first and the third experiment a third, diabetic, group (Nimo 20) was treated with nimodipine 20 mg kg^{-1} i.p. every 48 h. In the second experiment one diabetic group (Nimo 5) was treated with nimodipine 5 mg kg^{-1} i.p. every 48 h and another diabetic group (Nimo 10) was treated with nimodipine 10 mg kg^{-1} i.p. every 48 h. In all experiments the nimodipine or placebo (polyethylene glycol) treatment was initiated 4 weeks after the STZ injection when EDN was manifest and continued for 6 weeks. SNCV and MNCV were measured at week 0, 4, 6, 8 and 10.

Data analysis

The treatment code was broken at the end of the experiment to allow analysis of the data obtained for the various treatment groups. All data are presented as means ± s.e.mean. The results of SNCV and MNCV and body weights were analysed by a multivariate analysis of variance with repeated measurements (MANOVA) followed by supplemental *t* tests.

Results

Body weight and blood glucose levels

The STZ-induced diabetic rats developed high blood glucose levels which increased within 48 h. The blood glucose levels remained $>$15 mmol l^{-1} throughout the experimental period of 10 weeks. Additional treatment with nimodipine did not influence the blood glucose levels in any experiment (Table 1).

There was a marked reduction in body weight after the induction of diabetes (ten weeks after STZ administration a mean loss of 36.7 ± 7.7 g in experiment 1, of 41.7 ± 6.1 g in experiment 2 and of 176 ± 10.1 g in experiment 3). The mean weight of the nimodipine and the polyethylene glycol-treated diabetic rats in all experiments were not significantly different during the studies (data not shown).

Effect of streptozotocin-induced diabetes on nerve conduction velocity

Age-matched controls showed an increase of MNCV and SNCV in experiments 1 and 2 while they showed hardly any change in the fully grown rats in experiment 3. In all 3 experiments the differences in both MNCV and SNCV

Table 1 Blood glucose level (mmol l^{-1} ± s.e.mean) in diabetic rats

		Week 0	Week 4	Week 10
Exp 1:	Con	5.8 ± 0.5	6.4 ± 0.8	7.4 ± 0.7
	Plac	28.4 ± 1.4	28.5 ± 1.3	31.6 ± 1.8
	Nimo 20	27.0 ± 1.5	26.7 ± 0.8	29.4 ± 1.7
Exp 2:	Con	6.2 ± 0.7	6.7 ± 0.6	7.0 ± 0.5
	Plac	22.0 ± 0.4	25.2 ± 1.0	28.6 ± 1.1
	Nimo 5	25.1 ± 1.5	26.8 ± 1.4	29.2 ± 1.5
	Nimo 10	23.4 ± 0.8	27.1 ± 1.1	29.2 ± 1.5
Exp 3:	Con	7.8 ± 0.5	5.9 ± 0.9	7.2 ± 0.9
	Plac	28.1 ± 2.4	27.5 ± 2.0	29.6 ± 1.7
	Nimo 20	27.6 ± 1.3	27.7 ± 1.8	29.0 ± 2.1

The mean glucose levels ± s.e.mean 48 h after streptozotocin injection (Week 0), at the start of the treatment (Week 4) and at the end of the 3 experiments (Week 10) of the age-matched control groups (Con), the placebo-treated groups (Plac) and the nimodipin 5 mg kg^{-1}, 10 mg kg^{-1} and 20 mg kg^{-1}-treated groups (Nimo 5, Nimo 10, Nimo 20).

between age-matched controls and diabetic rats reached significance within 4 weeks (ANOVA followed by supplemental t test; experiment 1 and 2: SNCV and MNCV $P<0.001$; experiment 3: SNCV, $P<0.005$ and MNCV, $P<0.001$). Diabetic rats treated with vehicle displayed hardly any change in MNCV and SNCV after STZ administration in experiment 1 and 2 but a marked decrease in experiment 3 (Figures 1–3).

Effect of nimodipine treatment on nerve conduction velocity

In all experiments, nimodipine treatment was started 4 weeks after STZ administration. In experiment 1, treatment with nimodipine, 20 mg kg^{-1} ($n = 12$) resulted in an increase in SNCV and MNCV as compared to the placebo treatment ($n = 12$) (SNCV: $F(1,22) = 14.37$, $P<0.001$; MNCV: $F(1,22) = 13.79$, $P<0.005$) (Figure 1).

In order to obtain more information on the dose-response relationship, two lower doses were tested. Nimodipine 10 mg kg^{-1} treatment ($n = 12$) also resulted in an increase of SNCV and MNCV as compared to the placebo treatment (SNCV: $F(1,20) = 8.24$, $P<0.01$; MNCV: $F(1,20) = 7.76$, $P<0.05$) but the gain in velocity was smaller than that observed following treatment with nimodipine 20 mg kg^{-1}. Nimodipine 5 mg kg^{-1} had no beneficial effect on SNCV or MNCV (SNCV: $F(1,21) = 0.00$, $P = 0.985$; MNCV: $F(1,21) = 0.00$, $P = 0.953$) (Figure 2).

In the last experiment we tested whether the beneficial effect of nimodipine was also present in fully mature, adult rats. Placebo-treated, one-year-old rats showed a severe decrease of SNCV and MNCV whereas treatment with nimodipine 20 mg kg^{-1} resulted in a slight increase of SNCV and MNCV (SNCV: $F(1,17) = 11.86$, $P<0.005$; MNCV: $F(1,17) = 8.74$, $P<0.01$) (Figure 3).

Discussion

The STZ-induced diabetic rat is used in several laboratories as a possible model for diabetic neuropathy. STZ causes chronic hyperglycaemia and a severe reduction in peripheral NCVs of the diabetic rats (Mordes & Rossini, 1981). The model allows the study of the efficacy of agents to counteract some of the effect of chronic hyperglycaemia on peripheral nerves. Based on the neuroprotective effects of nimodipine in the development of EDN in the STZ rat model (Kappelle *et al.*, 1992) it was decided to test the effect of nimodipine on an existing diabetic neuropathy. Such an effect could lead to a better understanding of the defects in diabetic rats and would

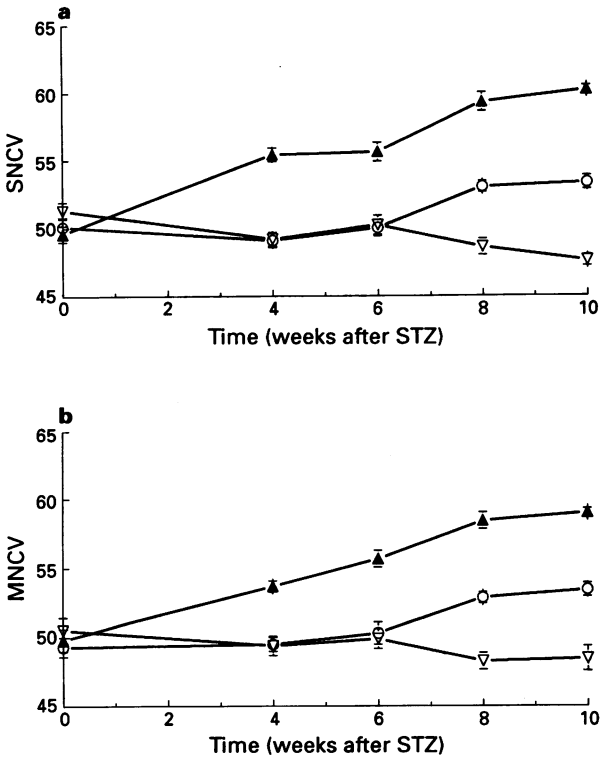


Figure 1 Sensory nerve conduction velocity (SNCV) (a) and motor nerve conduction velocity (MNCV) (b) ± s.e.mean (vertical bars) in diabetic rats. Treatment started at 4 weeks after streptozotocin (STZ) administration. Group 1 (▲, $n = 12$), a non-diabetic, age-matched control group, in which rats were treated with placebo (polyethylene glycol). Group 2 (▽, $n = 12$), in which diabetic rats were also treated with placebo i.p. every 48 h. Group 3 (○, $n = 12$) in which diabetic rats were treated with nimodipine 20 mg kg^{-1} i.p. every 48 h. Treatment with nimodipine (20 mg kg^{-1}), significantly enhanced the SNCV compared to that of the treatment of placebo ($F(1,22) = 14.37$, $P<0.001$). Treatment with nimodipine (20 mg kg^{-1}) also significantly enhanced the MNCV as compared to that of the placebo-treated group ($F(1,22) = 13.79$, $P<0.005$).

be helpful in the development of putative treatment strategies in patients with type 1 or type 2 diabetes mellitus with neuropathy.

Nimodipine treatment affected neither body weight nor blood glucose levels and therefore did not interfere with the diabetic state. It also has no effects on the NCV of young, non-diabetic, control rats (Hamers *et al.*, 1991). As both MNCV and SNCV of the non-diabetic, mature, control rats in experiment 3 did not significantly change throughout the treatment period it seems that polyethylene glycol did not influence NCVs. It is clear from the electrophysiological data that nimodipine ameliorates an existing diabetic neuropathy in a dose-dependent manner. The drug is effective in a dosage of 10 mg kg^{-1} and 20 mg kg^{-1} per 48 h whereas 5 mg kg^{-1} is not. It is of interest to note that the observed dose-response relationship is identical to that observed for the neurotrophic action of nimodipine on the recovery of function following a crush lesion of the sciatic nerve in rats (Gispen *et al.*, 1991). The increase of SNCV from week 4 (start of the treatment) until week 10 (end of the treatment) in the nimodipine 20 mg kg^{-1}-treated group was 4.2 m s^{-1} and 4.0 m s^{-1} for the MNCV, whereas the nimodipine 10 mg kg^{-1}-treated group showed an increase of 2.1 m s^{-1} for SNCV and 3.4 m s^{-1} for MNCV. The use of young adult rats has the disadvantage that these rats are still maturing and hence the NCVs are increasing the time to approximately 60 m s^{-1} (Kappelle *et al.*, 1992). Therefore drug effects under these experimental conditions might be related not only to EDN but also to

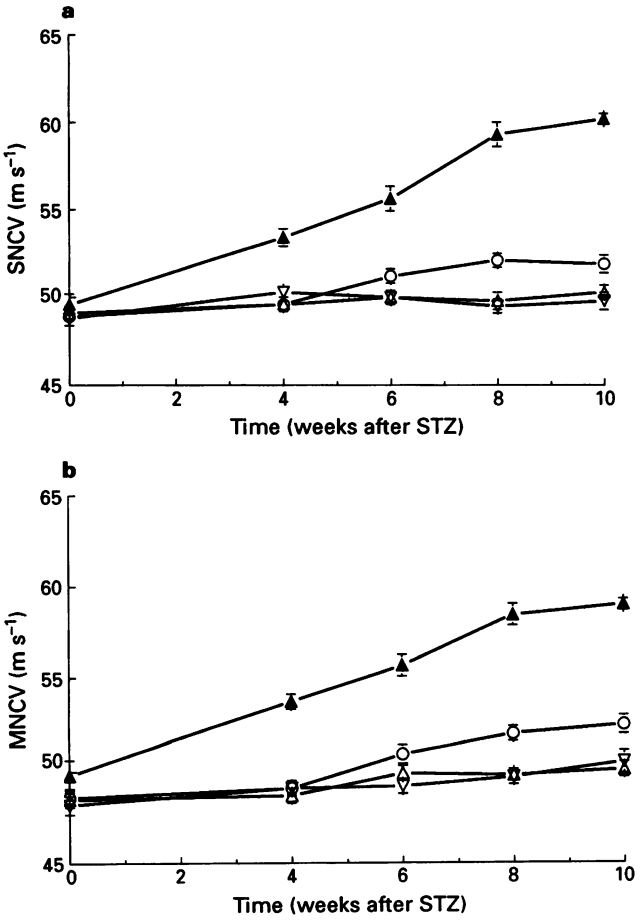


Figure 2 Sensory nerve conduction velocity (SNCV) (a) and motor nerve conduction velocity (MNCV) (b) ± s.e.mean in diabetic rats. Treatment started at 4 weeks after streptozotocin (STZ) administration. Group 1 (▲, $n = 10$), a non-diabetic, age-matched control group, was treated with placebo (polyethylene glycol) i.p. every 48 h. Group 2 (▽, $n = 12$), diabetic rats were also treated with placebo i.p. every 48 h. Group 3 (△, $n = 12$) diabetic rats which were treated with nimodipine 5 mg kg^{-1} i.p. every 48 h. Group 4 (○, $n = 12$), diabetic rats which were treated with nimodipine 10 mg kg^{-1} i.p. every 48 h. Treatment with nimodipine 10 mg kg^{-1} significantly enhanced the SNCV as compared to that of the placebo-treated group ($F(1,20) = 8.24$, $P < 0.01$). Treatment with nimodipine 5 mg kg^{-1} body weight did not significantly enhance the SNCV as compared to that of the placebo-treated group ($F(1,21) = 0.00$, $P = 0.985$). Treatment with nimodipine 10 mg kg^{-1} also significantly enhanced the MNCV as compared to that of the placebo-treated group ($F(1,20) = 7.76$, $P < 0.05$). Treatment with nimodipine 5 mg kg^{-1} did not significantly enhance the MNCV as compared to that of the placebo-treated group ($F(1,21) = 0.00$, $P = 0.953$).

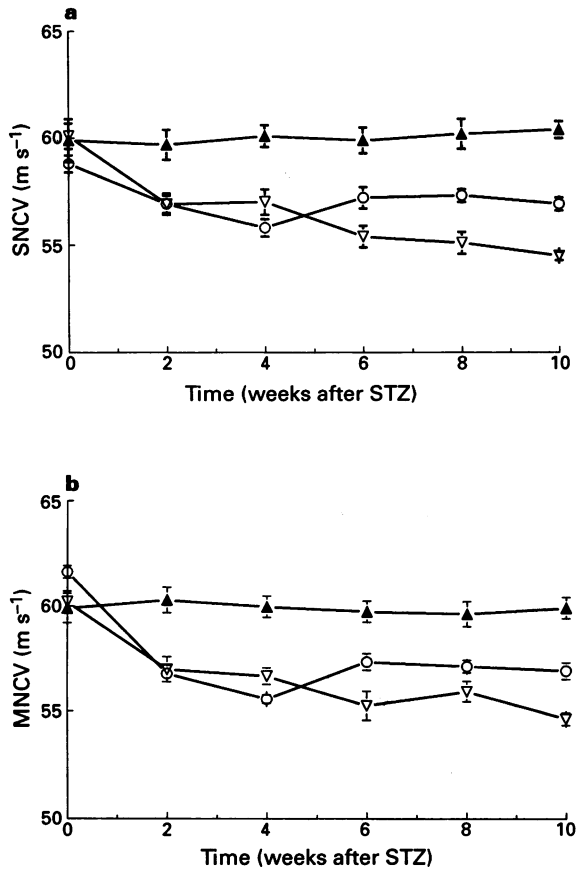


Figure 3 Sensory nerve conduction velocity (SNCV) (a) and motor nerve conduction velocity (MNCV) (b) ± s.e.mean in diabetic rats. Treatment started at 4 weeks after streptozotocin (STZ) administration. Group 1 (▲, $n = 10$), a non-diabetic, age-matched control group, in which rats were treated with placebo (polyethylene glycol) i.p. every 48 h. Group 2 (▽, $n = 10$), in which diabetic rats were also treated with placebo i.p. every 48 h. Group 3 (○, $n = 10$) diabetic rats which were treated with nimodipine 20 mg kg^{-1} i.p. every 48 h. Treatment with nimodipine 20 mg kg^{-1} significantly enhanced the SNCV as compared to that of the placebo-treated group ($F(1,17) = 11.86$, $P < 0.005$). Treatment with nimodipine 20 mg kg^{-1} also significantly enhanced the MNCV as compared to that of the placebo-treated group ($F(1,17) = 8.74$, $P < 0.01$).

maturation processes. Hence we investigated whether nimodipine could ameliorate EDN in STZ-induced, one-year-old diabetic rats that were fully matured. Indeed we demonstrated that nimodipine 20 mg kg^{-1} not only ameliorates EDN in young adult rats but also in fully grown, one-year-old, adult rats.

Within the experimental period there was no complete restoration of NCVs by nimodipine as compared to the level of the non-diabetic, age-matched controls. This could be due to the fact that diabetic rats have high blood glucose levels, which results in a worsening of their physical condition. Since diabetic neuropathy is a multifactoral disease, nimodipine could ameliorate just one aspect of the disease, whereas other factors such as depletion of trophic factors also need to be corrected to find complete restoration.

The mechanism of the neurotrophic and neuroprotective action of nimodipine is still unclear. There is increasing evidence suggesting a vascular implication in the pathogenesis of diabetic neuropathy. Reduced nerve blood flow and endoneurial hypoxia have been demonstrated (Low *et al.*, 1987). It is possible that nimodipine has a vasodilator effect on the microneurial vessels (Brandt *et al.*, 1981), improving endoneurial bloodflow and hence oxygen delivery to the peripheral nerves. A similar mechanism has been demonstrated using angiotensin converting enzyme (ACE) inhibition (Cameron *et al.*, 1991). Increased oxygen delivery could improve oxygen-dependent metabolic processes, leading to better nerve function. In this respect it is of interest to note that nimodipine treatment may affect the endoneurial blood flow of the sciatic nerve in the STZ-induced diabetic rat. Treatment started 4 weeks after the induction of diabetes and lasted for a period of 6 weeks. Nimodipine restored the deficit in endoneurial bloodflow by about 50% (Kappelle *et al.*, unpublished observations). Alternatively enhanced blood supply may also originate from the reported influence of nimodipine on microvessel growth (Yuan *et al.*, 1990).

Accumulating evidence suggests that Ca^{2+} plays an important role in cell injury. Changes in [Ca^{2+}] might play a role in the pathogenesis of diabetic neuropathy since [Ca^{2+}] is in-

creased in the axoplasm from myelinated fibres of diabetic rats (Lowery *et al.*, 1990). Changes in [Ca^{2+}] could lead to a diminished action of the Na,K ATP-ase (Pan & Janus, 1984; Glossmann *et al.*, 1989). Na,K ATP-ase is an important enzyme for maintaining the exact balance between the intracellular potassium and extracellular sodium concentration, which is crucial in the propagation of action potentials in the peripheral nerves.

It should be noted that we and others have previously described neuroprotective effects in EDN of compounds that clearly have quite different mechanisms of action. The compounds studied include aldose reductase inhibitors (Mayer *et al.*, 1983), myo-inositol (Finegold *et al.*, 1983) and neurotrophic peptides (Van der Zee *et al.*, 1989; Bravenboer *et al.*, 1991). Apparently the complex nature of the pathogenesis of EDN allows improvement of nerve function through interference with different components of the disease process or with interference of the etiological mechanisms via different routes.

We conclude that nimodipine is not only effective in preventing the development of EDN (Kappelle *et al.*, 1992), but also effective in ameliorating existing EDN. Further studies are necessary to unravel the mechanisms by which nimodipine is capable of ameliorating an existing EDN.

References

AZMITIA, E.C. (1989). Nimodipine attenuates NMDA- and MDMA-induced toxicity of cultured fetal serotonergic neurons: evidence for a generic model of calcium toxicity. In *Nimodipine and Central Nervous System Function: New Vistas.* ed. Traber, J. & Gispen, W.H. pp. 141–159. Stuttgart-New York: Schattauer.

BÄR, P.R., TRABER, J., SCHUURMAN, T. & GISPEN, W.H. (1990). CNS and PNS effects of nimodipine. *J. Neural Transm.*, **31**, 55–71.

BELLEMAN, R., SCHADE, A. & TOWART, R. (1983). Dihydropyridine receptor in rat brain labelled with (3H)Nimodipine. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 2356–2360.

BRANDT, L., ANDERSSON, K.E., EDVINSSON, L. & LIUNGGREN, B. (1981). Effect of extracellular calcium antagonists on the contractile responses of isolated human pial en mesenteric arteries. *J. Cerebr. Blood Flow Metabol.*, **3**, 339–347.

BRAVENBOER, B., KAPPELLE, A., BUREN VAN, T., ERKELENS, D.W. & GISPEN, W.H. (1991). ACTH-analogue Org 2766 can ameliorate existing neuropathy in diabetic rats. *Diabetologia*, **34**, 619.

CAMERON, N.E., COTTER, M.A. & ROBERTSON, S. (1991). Angiotensin converting enzyme inhibition prevents development of muscle and nerve dysfunction and stimulates angiogenesis in streptozotocin-diabetic rats. *Diabetologia*, **35**, 12–18.

CRISA, L., MORDES, J.P. & ROSSINI, A.A. (1992). Autoimmune diabetes mellitus in the BB rat. *Diabetes Met. Rev.*, **8**, 9–37.

DISTERHOFT, J.F., DEYO, R.A., BLACK, J., JONGE DE, M., STRAUBE, K.T. & THOMPSON, L.T. (1989). Associative learning in aging rabbits is facilitated by nimodipine. In *Nimodipine and Central Nervous System Function: New Vistas.* ed. Traber, J. & Gispen, W.H. pp. 209–226. Stuttgart-New York: Schattauer.

DYCK, P.J., JAMES, J. & O'BRIEN, P.C. (1987). Diagnosis staging and classification of diabetic neuropathy and association with other complications. In *Diabetic Neuropathy*, ed. Dyck, P.J., Thomas, P.K., Asbury, A.K., Winegrad, A.I. & Porte, D. pp. 36–44. Philadelphia: WB Saunders Co.

FINEGOLD, D., LATTIMER, S.A., NOLLE, S., BERNSTEIN, M. & GREENE, D.A. (1983). Polyol pathway activity and myo-inositol metabolism: a suggested relationship in pathogenesis of diabetic neuropathy. *Diabetes*, **32**, 988–992.

GISPEN, W.H., SPOREL-ÖZAKAT, E., DUCKERS, E. & EDWARDS, P.M. (1991). The Ca^{2+} antagonist nimodipine and the recovery of function following peripheral nerve damage in the rat. *Neurosci. Res. Commun.*, **8**, 175–184.

GLOSSMANN, H., ZERNIG, G., GRAZIADEI, I. & MOSHAMMER, T. (1989). Non L-type Ca^{2+} channel linked receptors for 1,4-dihydropyridines and phenylalkylamines. In *Nimodipine and Central Nervous System Function: New Vistas.* ed. Traber, J. & Gispen, W.H. pp. 51–67. Stuttgart-New York: Schattauer.

GREENE, D.A., LATTIMER, S.A. & SIMA, A.A.F. (1987). Sorbitol, phosphoinositides and sodium-potassium-ATP-ase in the pathogenesis of diabetic complications. *N. Engl. J. Med.*, **316**, 599–606.

GREENE, D.A., DEJESUS, P.V. JR & WINEGRAD, A.I. (1975). Effects of insulin and dietary myoinositol on impaired peripheral motor nerve conduction velocity in acute streptozotocin diabetes. *J. Clin. Invest.*, **55**, 1326–1336.

HAMERS, F.P.T., GERRITSEN VAN DER HOOP, R., TRABER, J., NEIJT, J.P., JENNEKENS, F.G.I. & GISPEN, W.H. (1990). The calcium antagonist nimodipine in cisplatin-induced neuropathy in the rat. *Eur. J. Pharmacol.*, **183**, 1710–1711.

HAMMERS, F.P.T., GERRITSEN VAN DER HOOP, R., STEERENBURG, P.A., NEIJT, J.P. & GISPEN, W.H. (1991). Putative neurotrophic factors in the protection of cisplatin-induced peripheral neuropathy in rats. *Toxicol. Appl. Pharmacol.*, **111**, 514–522.

HENDRIKSEN, P.H., OEY, P.L., WIENEKE, G.H., HUFFELEN VAN, A.C. & GISPEN, W.H. (1992). Hypoxic neuropathy versus diabetic neuropathy – an electrophysiological study in rats. *J. Neurol. Sci.*, **110**, 99–106.

HOFFMEISTER, F., BELLEMANN, H.P., BENZ, U. & VAN DEN KERCKHOFF, W. (1985). Psychotrophic actions of nimodipine. In *Nimodipine: Pharmacological and Clinical Properties.* ed. Betz, E., Deck, K. & Hoffmeister, F. pp. 77–97. Stuttgart-New York: Schattauer.

KAPPELLE, A.C., BRAVENBOER, B., TRABER, J., ERKELENS, D.W. & GISPEN, W.H. (1992). The Ca^{2+} antagonist nimodipine counteracts the onset of an experimental diabetic neuropathy in streptozotocin-induced diabetic rats. *Neurosci Res. Commun.*, **10**, 95–104.

KONING DE, P. & GISPEN, W.H. (1987). Org 2766 improves functional and electrophysiological aspects of regenerating sciatic nerve in the rat. *Peptides*, **8**, 415–422.

LANDFIELD, P.W. (1989). Nimodipine modulation of aging-related increases in hippocampal calcium currents. In *Nimodipine and Central Nervous System Function: New Vistas.* ed. Traber, J. & Gispen, W.H. pp. 227–238. Stuttgart-New York: Schattauer.

LOW, P.A., SCHMELZER, J.D., WARD, K.K. & YAO, J.K. (1986). Experimental chronic hypoxic neuropathy; relevance to diabetic neuropathy. *Am. J. Physiol.*, **250**, E94–99.

LOW, P.A., TUCK, R.R., DYCK, P.J., SCHMELZER, J.D. & YAO, J.K. (1984). Prevention of some electrophysiologic and biochemical abnormalities with oxygen supplementation in experimental diabetic neuropathy. *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 6894–6898.

LOW, P.A., TUCK, R.R. & TAKEUCHI, M. (1987). Nerve microenvironment in diabetic neuropathy. In *Diabetic Neuropathy.* ed. Dyck, P.J., Thomas, P.K., Asbury, A.K., Winegrad, A.I. & Porte, D. pp. 266–278. Philadelphia: Saunders.

LOWERY, J.M., EICHBERG, J., SAUBERMANN, A.J. & LOPACHIN JR, M. (1990). Distribution of elements and water in peripheral nerve of streptozotocin-induced diabetic rats. *Diabetes*, **39**, 1498–1503.

MAYER, J.H. & TOMLINSON, D.R. (1983). Prevention of defects of axonal transport and nerve conduction velocity by oral administration of myo-inositol or an aldose-reductase inhibitor in streptozotocin-diabetic rats. *Diabetologia*, **25**, 433–438.

MORDES, J.P. & ROSSINI, A.A. (1981). Animal models of diabetes. *Am. Med.*, **70**, 353–360.

NYAKAS, C., MARKEL, E., KRAMERS, R.J.K., GASPAR, E., BOHUS, B. & LUITEN, P.G.M. (1989). Effects of nimodipine on hypoxia-induced learning and memory deficits. In *Nimodipine and Central Nervous System Function: New Vistas.* ed Traber, J. & Gispen, W.H. pp. 175–194. Stuttgart-New York: Schattauer.

PAN, M. & JANUS, R.A. (1984). Stimulation of Na,K-ATPase of isolated smooth muscle membranes by the Ca^{2+} channel inhibitors, nimodipine and nitrendipine. *Biochem. Pharmacol.*, **33**, 787–793.

PIRART, J. (1978). Diabetes mellitus and its degenerative complications: a prospective study of 4400 patients observed between 1947 and 1973. *Diabetes Care*, **1**, 168–252.

RERUP, C.C. (1970). Drugs producing diabetes through damage of the insulin secreting cells. *Pharmacol. Rev.*, **22**, 485–518.

SCHUURMAN, T., KLEIN, H., BENEKE, M. & TRABER, J. (1987). Nimodipine and motor deficits in the aged rat. *Neurosci. Res. Commun.*, **1**, 9–15.

STANLEY, E.F. (1981). Sensory and motor nerve conduction velocities and the latency of the H-reflex during growth of the rat. *Exp. Neurol.*, **71**, 497–506.

THOMAS, P.K. & ELIASSON, S.G. (1975). Diabetic neuropathy. In *Peripheral Neuropathy*. ed. Dyck, P.J., Thomas, P.K. & Lambert, E.M. pp. 956–981. Philadelphia: P.A. Saunders.

TOMLINSON, D.R., MORIARTY, R.J. & MAYER, J.H. (1984). Prevention and reversal of defective axonal transport and motor nerve conduction velocity in rats with experimental diabetes by treatment with the aldose reductase inhibitor sorbinil. *Diabetes*, **33**, 470–476.

TOMLINSON, D.R., TOWNSEND, J. & FRETTEN, P. (1985). Prevention of defective axonal transport in STZ diabetic rats by treatment of Statil (ICI 128,436), an aldose reductase inhibitor. *Diabetes*, **34**, 907–912.

TRABER, J. & GISPEN, W.H. (1989). *Nimodipine and the Central Nervous System*: New Vistas. Stuttgart-New York: Schattauer.

TUCK, R.R., SCHMELZER, J.D. & LOW, P.A. (1984). Endoneurial blood flow and oxygen tension in sciatic nerves of rats with experimental diabetic neuropathy. *Brain*, **107**, 935–950.

WAXMAN, S.G. (1980). Determination of conduction velocity in myelinated nerve fibres. *Muscle Nerve*, **3**, 141–150.

YASUDA, H. & DYCK, P.J. (1987). Abnormalities of endoneurial microvessels and sural nerve pathology in diabetic neuropathy. *Neurology*, **37**, 20–28.

YUAN, X.Q., SMITH, T.L., PROUGH, D.S., WITT DE, D.S., DUSSEAU, J.W., LYNCH, C.D., FULTON, J.M. & HUTCHINS, P.M. (1990). Long-term effects of nimodipine on pial microvasculature and systemic circulation in conscious rats. *Am. J. Physiol.*, **258**, H1395–H1401.

ZEE VAN DER, C.E.E.M., BRAKKEE, J.H. & GISPEN, W.H. (1991). Putative neurotrophic factors and functional recovery from peripheral nerve damage in the rat. *Br. J. Pharmacol.*, **103**, 1041–1046.

ZEE VAN DER, C.E.E.M., GERRITSEN VAN DER HOOP, R. & GISPEN, W.H. (1989). Beneficial effect of peptide ORG 2766 ($ACTH_{4-9}$ analog) in the treatment of peripheral neuropathy in streptozocin-diabetic rats. *Diabetes*, **38**, 225–230.

ZEE VAN DER, C.E.E.M., SCHUURMAN, T., TRABER, J. & GISPEN, W.H. (1987). Oral administration of nimodipine accelerates functional recovery following peripheral nerve damage in the rat. *Neurosci. Lett.*, **83**, 143–148.

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Nitric oxide-mediated hyporeactivity to noradrenaline precedes the induction of nitric oxide synthase in endotoxin shock

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1 The role of an enhanced formation of nitric oxide (NO) and the relative importance of the constitutive and inducible NO synthase (NOS) for the development of immediate (within 60 min) and delayed (at 180 min) vascular hyporeactivity to noradrenaline was investigated in a model of circulatory shock induced by endotoxin (lipopolysaccharide; LPS) in the rat.

2 Male Wistar rats were anaesthetized and instrumented for the measurement of mean arterial blood pressure (MAP) and heart rate. In addition, the calcium-dependent and calcium-independent NOS activity was measured *ex vivo* by the conversion of [^{3}H]-arginine to [^{3}H]-citrulline in homogenates from several organs obtained from vehicle- and LPS-treated rats.

3 *E. coli* LPS (10 mg kg^{-1}, i.v. bolus) caused a rapid (within 5 min) and sustained fall in MAP. At 30 and 60 min after LPS, pressor responses to noradrenaline (0.3, 1 or 3 μg kg^{-1}, i.v.) were significantly reduced. The pressor responses were restored by N^G-nitro-L-arginine methyl ester (L-NAME, 1 mg kg^{-1}, i.v. at 60 min), a potent inhibitor of NO synthesis. In contrast, L-NAME did not potentiate the noradrenaline-induced pressor responses in control animals.

4 Dexamethasone (3 mg kg^{-1}, i.v., 60 min prior to LPS), a potent inhibitor of the induction of NOS, did not alter initial MAP or pressor responses to noradrenaline in control rats, but significantly attenuated the LPS-induced fall in MAP at 15 to 60 min after LPS. Dexamethasone did not influence the development of the LPS-induced immediate (within 60 min) hyporeactivity to noradrenaline. However, dexamethasone pretreatment prevented the hypotension and vascular hyporeactivity at 180 min.

5 At 60 min after LPS a moderate increase in the activity of a calcium-independent (inducible) NOS activity was detected in the aorta, but not in any of the other tissues studied. However, at 180 min after LPS, a significant NOS induction was observed in the lung, liver, spleen, mesentery, heart and aorta. This NOS induction was substantially prevented by pretreatment with dexamethasone.

6 These results suggest that the immediate hypotension and vascular hyporeactivity to noradrenaline in endotoxin shock is caused by an enhanced formation of NO due to activation of the constitutive enzyme. The delayed hypotension and vascular hyporeactivity, however, is due to enhanced NO formation by the LPS-induced enzyme.

Keywords: Lipopolysaccharide; hypotension; circulatory shock; catecholamines; rat, *in vivo*; nitric oxide synthase

Introduction

The formation of nitric oxide (NO) from the guanidino nitrogen group of L-arginine is catalyzed by an enzyme termed NO synthase (NOS) (Moncada *et al.*, 1991). Several isoforms of NOS are known (Förstermann *et al.*, 1991; Moncada *et al.*, 1991). A constitutive isoform, which is present in vascular endothelial cells is calcium-dependent. A continuous release of NO produced by the constitutive enzyme keeps the vasculature in an active state of vasodilatation and is, hence, of paramount importance for the maintenance of a normal blood pressure (Rees *et al.*, 1989) and the regulation of organ blood flow distribution (Gardiner *et al.*, 1990; Walder *et al.*, 1991; Benyó *et al.*, 1991; Humphries *et al.*, 1991).

In addition, bacterial lipopolysaccharide (LPS, endotoxin) and a number of cytokines including tumour necrosis factor (TNF) and interleukin-1 induce an isoform of NOS in macrophages, resulting in NO formation which destroys bacterial pathogens and tumour cells (Marletta *et al.*, 1990). However, LPS and various cytokines also induce NOS activity in endothelial cells (Radomski *et al.*, 1990; Gross *et al.*, 1991) and vascular smooth muscle (Busse & Mülsch, 1990) *in vitro*. Induction of NOS has only been demonstrated after 3 h of endotoxaemia *in vivo* (Salter *et al.*, 1991), and hence may well contribute to the delayed hyporeactivity to adrenoceptor agonists (Julou-Schaeffer *et al.*, 1990) as well as the delayed peripheral vascular failure (Wright *et al.*, 1992) associated with septic shock.

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However, the hyporeactivity to adrenoceptor agonists occurs within 60 min of endotoxaemia in the anaesthetized rat, while an induction of NOS in the rat aorta *ex vivo* was only demonstrated at 4 h after injection of LPS (Julou-Schaeffer *et al.*, 1990). Moreover, the rapid (within 5–20 min) development of hypotension in response to LPS and TNF in the anaesthetized dog and rat are also mediated by an enhanced formation of NO (Thiemermann & Vane, 1990; Kilbourn *et al.*, 1990a,b).

Here we have studied the time-course of NOS induction and the cardiovascular changes in response to endotoxaemia in the anaesthetized rat. In addition, we investigated the relative importance of the activation of the constitutive and/or inducible NOS for the immediate and delayed hypotension and vascular hyporeactivity in endotoxin shock by (i) inhibition of both NOS isoforms with N^G-nitro-L-arginine methyl ester (L-NAME) (Moore *et al.*, 1990; Hecker *et al.*, 1990; Thiemermann *et al.*, 1990); (ii) inhibition of NOS induction by dexamethasone (Radomski *et al.*, 1990; Knowles *et al.*, 1990b) and (iii) measurement of calcium-dependent and calcium-independent (i.e. constitutive and inducible) NOS activity *ex vivo* in tissue homogenates (Bredt & Snyder, 1990; Mitchell *et al.*, 1991) from control animals as well as 30, 60 and 180 min after LPS administration. In addition, we investigated the potential contribution of cyclo-oxygenase products for the immediate (within 60 min) cardiovascular changes (hypotension and hyporeactivity) associated with endotoxaemia.

An account of some of these results was presented to the British Pharmacological Society in Dublin (Szabó *et al.*, 1992).

Methods

Haemodynamic measurements

Male Wistar rats (260–320 g; Glaxo Laboratories Ltd., Greenford, Middx.) were anaesthetized with thiopentone sodium (Trapanal; 120 mg kg^{-1}, i.p.). The trachea was cannulated to facilitate respiration and the rectal temperature was maintained at 37°C by means of a rectal probe connected to a homeothermic blanket (BioSciences, Sheerness, Kent, U.K.). The right carotid artery was cannulated and connected to a Transamerica type 4-422-0001 pressure transducer for the measurement of phasic and mean blood pressure (MAP) and heart rate (HR) on a Grass model 7D polygraph recorder (Grass Instruments, Quincy, Mass, U.S.A.). The left and right femoral veins were cannulated for the administration of drugs.

Endotoxin-induced immediate (within 60 min) cardiovascular alterations

Ten min after the completion of the surgical procedure, a dose-response curve to noradrenaline (NA) (0.3, 1 or 3 μg kg^{-1}, i.v. bolus) was constructed. One group of animals then received *E. coli* LPS (10 mg kg^{-1}, i.v., $n = 7$) as a slow bolus injection (0.3 ml volume) over 2 min. The dose-response curve to NA was repeated at 30 and 60 min after LPS administration. After constructing a second dose-response curve to NA (at 60 min), the animals received L-NAME (1 mg kg^{-1}, i.v.). At 10 min after L-NAME injection, another dose-response curve to NA was constructed. In a separate series of experiments, using the same protocol, L-arginine (100 mg kg^{-1}, i.v., $n = 4$) was administered 2 min prior to L-NAME.

In separate experiments, rats were pretreated with dexamethasone (3 mg kg^{-1}, i.v.; 60 min prior to LPS administration, $n = 5$). To investigate the role of cyclo-oxygenase products in the early cardiovascular events following LPS injection, rats were pretreated with indomethacin (5 mg kg^{-1}, i.v.; 20 min prior to LPS administration, $n = 6$). In both, dexamethasone- and indomethacin-pretreated animals the above experimental protocol was repeated.

The effects of L-NAME (1 mg kg^{-1}, i.v.) in the absence ($n = 6$) and in the presence ($n = 5$) of L-arginine (100 mg kg^{-1}, i.v.) was also investigated in sham-operated control animals without LPS treatment.

To test whether dexamethasone interfered with the pressor response caused by L-NAME, animals not subjected to LPS were given dexamethasone (3 mg kg^{-1}, i.v.) and the pressor effect of L-NAME (1 mg kg^{-1}, i.v.) was investigated 5 min ($n = 4$) or 60 min ($n = 3$) later.

Endotoxin-induced delayed cardiovascular events (180 min after LPS)

After the completion of the surgical procedure, the pressor effect of 1 μg kg^{-1} NA was investigated. The animals then received *E. coli* LPS (10 mg kg^{-1}, i.v., $n = 5$) as a slow bolus injection (0.3 ml volume) over 2 min. Injections of NA (1 μg kg^{-1}, i.v.) were repeated at 60, 120 and 180 min after LPS administration. In separate experiments, rats were pretreated with dexamethasone (3 mg kg^{-1}, i.v.; 60 min prior LPS administration, $n = 5$) and the same experimental protocol was performed.

Nitric oxide synthase assay

Lung, liver, spleen, mesentery, kidney, heart and thoracic aorta were quickly removed and immediately frozen in liquid nitrogen. NOS activity was measured in tissue samples from vehicle-treated rats and from rats killed at 30, 60 and 180 min after LPS (10 mg kg^{-1}, $n = 3$–4 in each group). At 180 min, NOS activity was also measured in the lung and aorta of dexamethasone (3 mg kg^{-1}, i.v., 60 min prior to LPS) pretreated animals ($n = 4$). Frozen organ samples were homogenized on ice in an Ultra-Turrax T 25 homogenizer (Janke & Kunkel, IKA Labortechnik, Staufen i Br, Germany) in the following buffer: Tris/HCl 50 mM, EDTA 0.1 mM, EGTA 0.1 mM, 2-mercaptoethanol 12 mM and phenylmethylsulphonyl fluoride 1 mM (pH 7.4). Conversion of [^{3}H]-arginine to [^{3}H]-citrulline was measured in the homogenates according to the method described by Bredt & Snyder (1990). Briefly, 50 μl of tissue homogenates (approx. 100 μg protein) were incubated in the presence of L-arginine/[^{3}H]-L-arginine (10 μM, 5000 Bq/tube), and NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 μM) and calcium (2 mM) for 20 min in HEPES (pH 7.5). The reaction was stopped by the addition of HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). The reaction mixture was applied to Dowex 50W (Na form) columns and the eluted [^{3}H]-citrulline activity was measured by scintillation counting (Beckman, LS 3801; Fullerton, CA, U.S.A). Parallel experiments without NADPH or without calcium determined the extent of [^{3}H]-citrulline formation independent of NOS activity and the NO production of the inducible NOS, respectively (Mitchell *et al.*, 1991). Protein concentration was measured spectrophotometrically in 96-well plates using Bradford reagent (Bradford, 1976) with bovine serum albumin as standard.

Materials

L-Arginine hydrochloride, calmodulin, dexamethasone 21-phosphate disodium salt, indomethacin, lipopolysaccharide (from *E. coli*, serotype No. 0127-B8), NADPH, N^G-nitro-L-arginine methyl ester, noradrenaline and Dowex anion exchange resin were obtained from Sigma Chemical Co. (Poole, Dorset). L-[2,3,4,5-^{3}H]-arginine hydrochloride was obtained from Amersham (Buckinghamshire). Tetrahydrobiopterin was obtained from Dr B. Schirks Laboratories (Jona, Switzerland).

Statistical comparison

All values in the figures and text are expressed as mean ± s.e.mean of n observations. Student's paired or unpaired t tests were used to compare means within and between groups, respectively. A P-value less than 0.05 was considered statistically significant.

Results

LPS-induced immediate (within 60 min) cardiovascular changes in the anaesthetized rat

Baseline values for MAP and HR of the different animal groups studied ranged from 110 ± 6 to 122 ± 5 mmHg and from 408 ± 10 to 444 ± 16 beats per min (beats min^{-1}), and between groups were not significantly different (Figure 1). Before injection of LPS, NA caused dose-dependent increases in MAP (Figure 2) and HR (0.3 μg kg^{-1}: 6 ± 1 beats min^{-1}; 1 μg kg^{-1}: 18 ± 2 beats min^{-1}; 3 μg kg^{-1}: 46 ± 4 beats min^{-1}).

Intravenous administration of *E. coli* LPS induced a rapid (within 5 min) fall in MAP from 120 ± 6 to 65 ± 4 mmHg ($n = 7$). The MAP remained below the baseline value throughout the observation period (Figure 1a). Heart rate was unchanged within the first hour (Figure 1b). Pressor responses to NA (0.3, 1 or 3 μg kg^{-1}) were significantly attenuated at both 30 and 60 min after LPS administration (Figure 2b). Endotoxaemia for 60 min did not influence the increase in HR in response to lower concentrations of NA

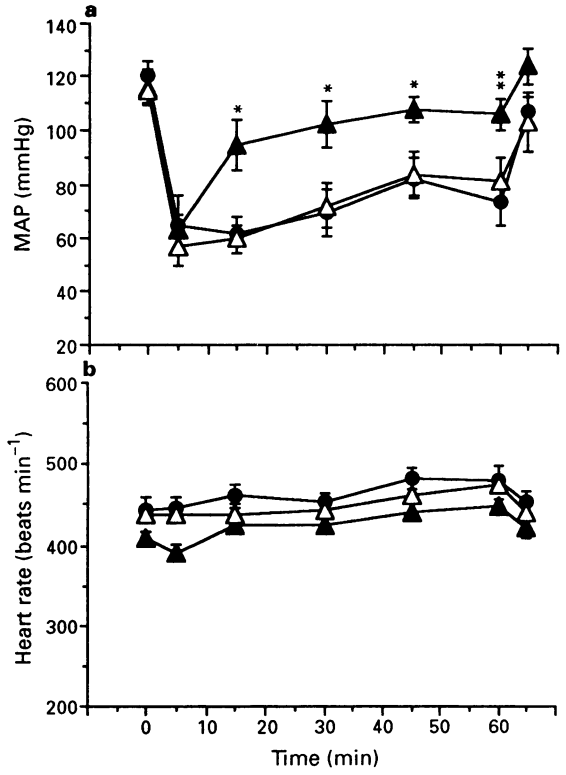


Figure 1 Effect of *E. coli* lipopolysaccharide (LPS, 10 mg kg^{-1}, i.v. at time 0) and N^G-nitro-L-arginine methyl ester (L-NAME, 1 mg kg^{-1}, i.v. at 60 min) on mean arterial blood pressure (a) and heart rate (b) in the anaesthetized rat. Different groups of animals were treated with vehicle (●, $n = 7$), dexamethasone (▲, 3 mg kg^{-1}, i.v. 60 min prior to LPS, $n = 5$) or inomethacin (△, 5 mg kg^{-1}, i.v. 20 min prior to LPS, $n = 6$). Data are expressed as means ± s.e.mean (vertical bars) of n observations. Significant differences when compared to control at the same time point: $^*P<0.05$ and $^{**}P<0.01$.

(0.3 μg kg^{-1} and 1 μg kg^{-1}). However, the increase in HR seen with 3 μg kg^{-1} of NA was significantly reduced to 24 ± 2 beats min^{-1} at 60 min after LPS injection ($P<0.05$).

At 60 min after LPS injection, L-NAME (1 mg kg^{-1}, i.v.; $n = 7$) caused a 30 ± 3 mmHg rise in MAP (Figure 1a), a moderate bradycardia (34 ± 8 beats min^{-1}, $P<0.05$) and a significant enhancement of the pressor responses to NA (Figure 2b; $P<0.01$). These effects of L-NAME were prevented by L-arginine (100 mg kg^{-1}) ($n = 4$, Figure 2b). In contrast, L-NAME did not affect the pressor responses to NA in control animals ($n = 6$, Figure 2a). Moreover, the pressor effect of L-NAME (1 mg kg^{-1}) 60 min after LPS (30 ± 3 mmHg, $n = 7$) was significantly larger ($P<0.05$) than its pressor effect in control rats (22 ± 3 mmHg, $n = 6$). L-NAME did not affect the increases in HR caused by NA either before (control) or after injection of LPS (data not shown).

Dexamethasone (3 mg kg^{-1}; 60 min prior to LPS) had no effect on baseline MAP or HR values nor on the control pressor responses to NA (Figures 1a, 3). Moreover, the rapid fall in the MAP after LPS (occurring within 5 min) in the dexamethasone-treated animals was similar to the one observed in LPS-treated controls (54 ± 8 mmHg, $n = 5$). Dexamethasone-pretreated animals, however, maintained significantly higher MAP values at 15–60 min after LPS when compared to LPS-treated controls (Figure 1a).

Dexamethasone did not alter the development of the LPS-induced hyporeactivity to NA occurring during the first 60 min of endotoxaemia (Figure 3). In addition, dexamethasone had no effect on the increase in HR seen with NA either before or after injection of LPS (not shown). Normal pressor responses to NA were restored by L-NAME in dexamethasone treated animals (Figure 3). The L-NAME-induced bradycardia (27 ± 10 beats min^{-1}) was similar, whereas the increase in MAP was significantly smaller ($P<0.05$) in the dexamethasone and LPS-treated group of animals (14 ± 2 mmHg, $n = 5$) than in the LPS-treated control group.

In sham-operated controls, the pressor response to L-NAME (22 ± 3 mmHg, $n = 6$) was unaltered at 5 or 60 min after dexamethasone (3 mg kg^{-1}, i.v.) (20 ± 2 mmHg, $n = 4$ and 23 ± 8 mmHg, $n = 3$, respectively).

Indomethacin pretreatment had no effect on baseline haemodynamic parameters, pressor responses to NA before LPS injection, or on the development of hypotension and vascular hyporeactivity to NA occurring within 60 min after LPS (Figures 1 and 3). In addition, indomethacin had no effect on the increase in HR seen with NA either before or after injection of LPS (not shown). At 60 min after LPS, both the pressor effect and bradycardia in response to L-NAME in indomethacin-treated rats (23 ± 4 mmHg, and 28 ± 7 beats min^{-1}, respectively) were similar to the responses obtained with the NOS inhibitor in LPS-treated rats in the absence of indomethacin-pretreatment.

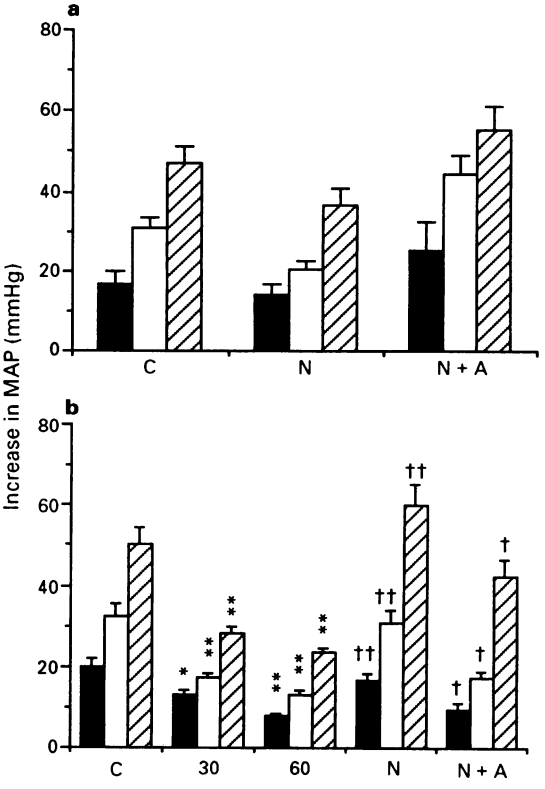


Figure 2 (a) Effect of N^G-nitro-L-arginine methyl ester (L-NAME, 1 mg kg^{-1}, i.v., $n = 6$) or L-NAME (1 mg kg^{-1}, i.v.) and L-arginine (L-Arg, 100 mg kg^{-1}, 2 min prior to L-NAME; i.v., $n = 4$) on pressor responses induced by intravenous noradrenaline (NA, 0.3 μg kg^{-1}: solid columns; 1 μg kg^{-1}: open columns; or 3 μg kg^{-1}: hatched columns) in anaesthetized control rats. (b) Effect of *E. coli* lipopolysaccharide (10 mg kg^{-1}, i.v.) on pressor responses induced by NA (0.3 μg kg: solid columns; 1 μg kg^{-1}: open columns; or 3 μg kg^{-1}: hatched columns) at 30 and 60 min after LPS ($n = 7$), and the effects of L-NAME ($n = 7$) or L-NAME and L-Arg ($n = 4$) at 60 min. C: Control response; N: pressor response in the presence of L-NAME; N + A: pressor response in the presence of L-NAME and L-Arg. Data are expressed as means ± s.e.mean (vertical bars) of n observations. *, ** represent significant differences compared to control, †, †† represent significant differences of the N group compared to the response at 60 min or significant differences of the N + A group compared to the N group ($P<0.05$ and 0.01, respectively).

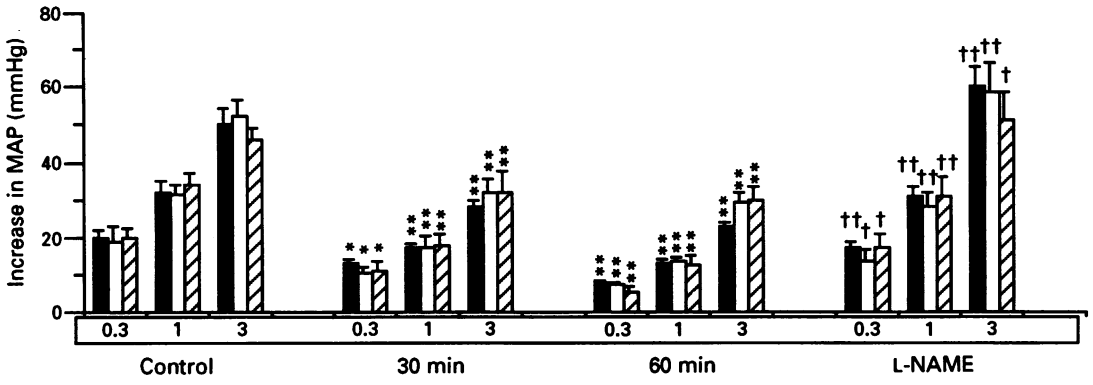


Figure 3 Effect of *E. coli* lipopolysaccharide (10 mg kg^{-1}, i.v.) on pressor responses induced by noradrenaline (NA, 0.3, 1 or 3 μg kg^{-1}, i.v.) before (control) or 30 and 60 min after LPS. Different groups of animals received vehicle (solid columns, $n = 7$), dexamethasone (3 mg kg^{-1}, i.v., open columns, $n = 5$) and indomethacin (5 mg kg^{-1}, i.v., hatched columns, $n = 6$) prior to LPS. All animals received N^G-nitro-L-arginine methyl ester (L-NAME) at 60 min. Data are expressed as means ± s.e.mean of n observations. *, ** represent significant differences compared to control; †, †† represent significant differences after L-NAME administration compared to the response at 60 min ($P<0.05$ and 0.01, respectively).

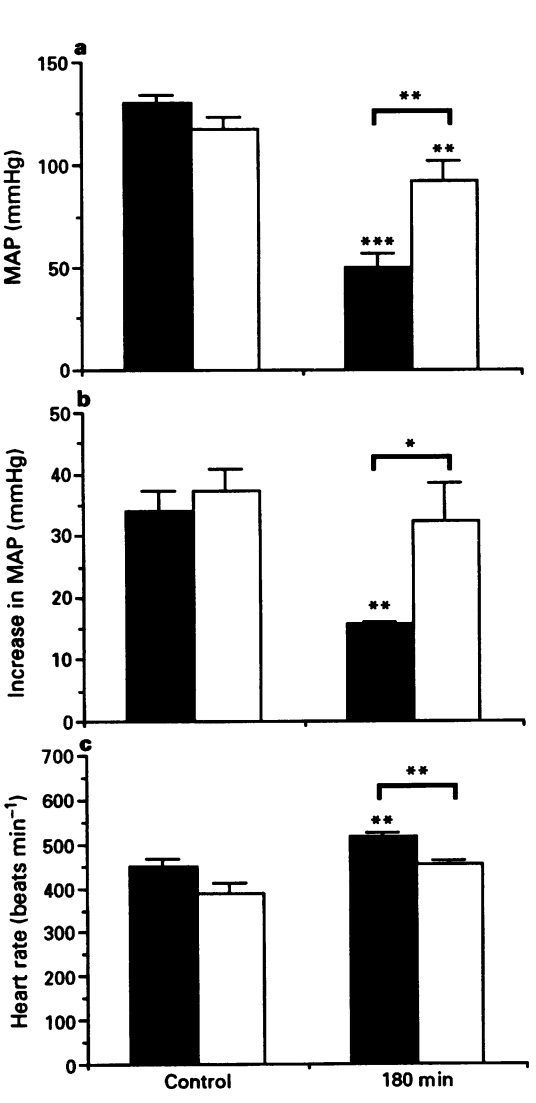


Figure 4 Mean arterial blood pressure (a), pressor responses to noradrenaline (NA, 1 μg kg^{-1}, i.v.) (b) and heart rate (c) in anaesthetized rats before (Control) and 180 min after administration of LPS (10 mg kg^{-1}, i.v.). Different groups of animals received vehicle (solid columns, $n = 5$), or dexamethasone (3 mg kg^{-1}; open columns, $n = 5$) prior to LPS. Data are expressed as means ± s.e.mean of n observations. Significant differences when compared to control: *$P<0.05$, **$P<0.01$; and ***$P<0.001$.

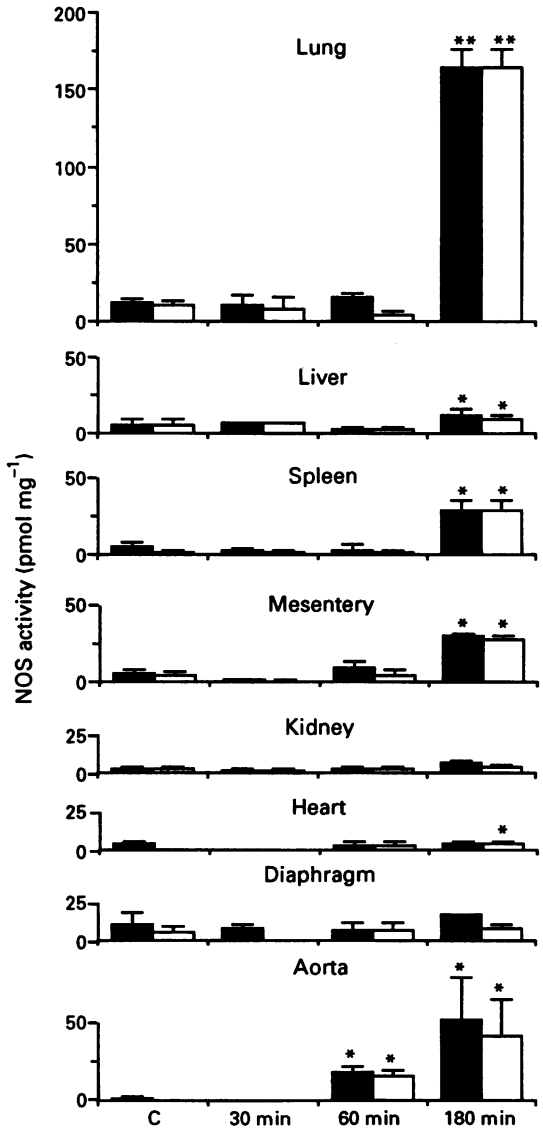


Figure 5 Total (solid columns) and calcium-independent (inducible, open columns) nitric oxide synthase (NOS) activity in the lung, liver, spleen, mesentery, kidney, heart and aorta of vehicle-treated control rats and 30, 60 and 180 min after *E. coli* lipopolysaccharide (10 mg kg^{-1}, i.v.) ($n = 3$–4). Data are expressed as means ± s.e.mean of n observations. Significant difference when compared to control: $P<0.05$ and **$P<0.001$.

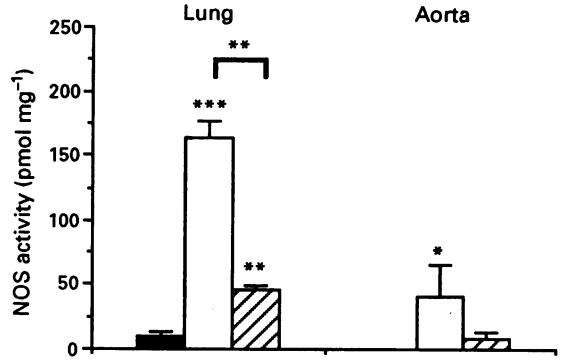


Figure 6 Calcium-independent (inducible) nitric oxide synthase (NOS) activity in the lung and aorta obtained from vehicle-treated control rats (solid columns) and from vehicle-pretreated (hatched columns) or dexamethasone-pretreated (3 mg kg^{-1}, i.v.; open columns) rats at 180 min after *E. coli* lipopolysaccharide (10 mg kg^{-1}, i.v.) in ($n = 3$–4). Data are expressed as means ± s.e.mean of n observations. Significant difference when compared to control: *$P<0.05$, **$P<0.01$ and ***$P<0.001$.

LPS-induced delayed cardiovascular changes (at 180 min)

After 180 min of endotoxaemia, the MAP had declined from 118 ± 6 mmHg to 50 ± 7 mmHg ($P<0.001$) (Figure 4a). Pressor responses to NA (1 μg kg^{-1}) were reduced from 34 ± 3 mmHg to 16 ± 1 mmHg ($P<0.01$) (Figure 4b). In addition, 180 min of endotoxaemia was associated with a significant tachycardia ($n = 5$, Figure 4c). Dexamethasone pretreatment significantly ($P<0.01$) prevented the LPS-induced fall in MAP at 180 min (Figure 4a). Although the pressor responses to NA were significantly reduced at 30 and 60 min after LPS injection in dexamethasone-treated rats (Figure 3), pressor responses to NA after dexamethasone-pretreatment recovered to normal values at 180 min of endotoxaemia (Figure 4b). Thus, dexamethasone prevented the delayed hyporeactivity to NA in endotoxaemia. In the dexamethasone-pretreated rats, no significant increase in the heart rate was observed at 180 min (Figure 4c).

Nitric oxide synthase activity after LPS administration

There was no increase in the calcium-dependent (constitutive) or -independent (inducible) NOS activity in any of the organs studied at 30 min after LPS administration. At 60 min, an increase in the calcium-independent NOS activity was demonstrated in the aorta, but not in any of the other tissues (Figure 5). At 180 min a significant NOS induction was seen in the lung, liver, spleen, mesentery, heart and aorta. The most pronounced NOS induction was observed in the lung. No significant NOS induction was observed in the aorta of dexamethasone-pretreated animals after 180 min (Figure 6). Similarly, dexamethasone pretreatment strongly reduced (by 73%) NOS induction in the lung (Figure 6). In the liver and kidney, NADPH-independent citrulline formation was also detected (57.8 ± 7.5 and 21.2 ± 8.7 pmol mg^{-1}, respectively), which was unaffected by LPS (not shown).

Discussion

Here we demonstrate that the vascular hyporeactivity to NA and the fall in blood pressure occurring within 60 min of endotoxaemia in the anaesthetized rat is mediated by an enhanced formation of NO by the constitutive NOS. In addition, endotoxaemia for 180 min, but not for 30 or 60 min, results in a significant induction of a calcium-independent NOS activity which contributes to the vascular hyporeactivity to NA and the circulatory failure seen after prolonged periods of endotoxic shock.

The vascular hyporeactivity to NA occurring within 60 min of endotoxaemia is caused by an enhanced release of NO for (i) it was reversed by L-NAME, an inhibitor of both constitutive and inducible NOS; (ii) L-arginine prevented the reversal of the LPS-induced hyporeactivity by L-NAME and (iii) L-NAME did not affect the pressor responses to NA in control animals. In addition, the pressor effect of L-NAME after 60 min of endotoxaemia was greater than in control animals supporting the hypothesis (Thiemermann & Vane, 1990; Kilbourn *et al.*, 1990b) that an enhanced formation of NO contributes to the early hypotension in endotoxaemia.

This enhanced release of NO (within 60 min) is due to an activation of the constitutive NOS in the vascular endothelium, for dexamethasone did not prevent the vascular hyporeactivity to NA at 30 and 60 min after LPS injection. In addition, no evidence for the induction of a calcium-independent NOS was found in any of the organs studied at 30 min after LPS administration; a time at which a substantial reduction of the pressor responses to NA was already established. Moreover, L-NAME restored the reduced pressor response to NA (at 60 min after LPS) even in rats pretreated with dexamethasone.

The mechanism(s) responsible for an activation of the constitutive NOS in response to LPS are unclear. It is, however, conceivable that LPS results in the release of a variety of mediators which trigger the release of NO from the endothelium. For instance, LPS causes the release of bradykinin (Fleming *et al.*, 1992) and NO (Salvemini *et al.*, 1990; Fleming *et al.*, 1992) from cultured endothelial cells *in vitro* and increases plasma levels of bradykinin (within 5 min) in the anaesthetized rat (Katori *et al.*, 1989). In addition, injection of LPS results in a rapid increase in plasma levels of catecholamines, 5-hydroxytryptamine, angiotensin II and histamine (see: Parratt, 1983) which also stimulate the release of NO from the endothelium (see: Gryglewski *et al.*, 1988).

Interestingly, dexamethasone reduced the degree of hypotension occurring within 30 min after LPS. Inhibition of NOS induction by dexamethasone cannot account for this effect of the steroid, as no induction of NOS activity had occurred at this time. Clearly, steroids exert many pharmacological effects apart from the inhibition of NOS induction (Radomski *et al.*, 1990), including inhibition of both the production of cyclo-oxygenase metabolites (Flower & Blackwell, 1979) and of the induction of cyclo-oxygenase (Fu *et al.*, 1990; Masferrer *et al.*, 1992). In contrast to previous studies in anaesthetized cats or pigs (Parratt & Surgess, 1974; Feuerstein *et al.*, 1981; Mózes *et al.*, 1991), this study demonstrates that indomethacin does not affect the LPS-induced hypotension in the anaesthetized rat. Thus, the prevention by dexamethasone of the fall in blood pressure in response to LPS is not related to inhibition of the formation cyclo-oxygenase metabolites. Whether other well documented actions of glucocorticosteroids, such as inhibition of the release of endorphins (Simatov, 1979) or platelet-activating factor (Braquet *et al.*, 1987), and/or inhibition of neutrophil activation (Goldstein *et al.*, 1976; Hammerschmidt *et al.*, 1979; Schubitz *et al.*, 1981) contribute to the observed haemodynamic effects of dexamethasone in endotoxic shock warrants further investigation.

This study also confirms (Knowles *et al.*, 1990a; Salter *et al.*, 1991) that prolonged periods of endotoxaemia (180 min) led to the induction of a calcium-independent (inducible) NOS activity, a process which was inhibited by dexamethasone. In addition, we demonstrate that dexamethasone pretreatment prevented the vascular hyporeactivity to NA as well as the secondary fall in blood pressure observed after 180 min of endotoxaemia suggesting that an enhanced formation of NO by the inducible NOS contributes to the peripheral vascular failure associated with prolonged periods of endotoxaemia. It has been previously reported that the vascular hyporeactivity to adrenoceptor agonists in anaesthetized rats occurs within 60 min, while an induction of NOS in rat aortic rings *ex vivo* was only demonstrated at 4 h

of endotoxaemia (Julou-Schaeffer *et al.*, 1990). The present study demonstrates that both the early and the delayed vascular hyporeactivity in endotoxaemia are mediated by an enhanced formation of NO. However, the release of NO in the early (within 60 min) and late phase of endotoxaemia (e.g. at 180 min) is triggered by two distinct mechanisms, namely by the activation of the constitutive and the inducible NOS, respectively. In addition, the enhanced formation of NO by the constitutive NOS accounts for the immediate hypotension in response to LPS, while the induction of NOS contributes to the delayed hypotension observed after endotoxaemia. Similarly, haemorrhagic shock in the anaesthetized rat also results (within 90 min) in an enhanced formation of NO due to activation of the constitutive NOS which accounts for the early vascular hyporeactivity to adrenoceptor agonists. Prolonged periods of haemorrhagic hypotension, however, are associated with an induction of NOS, which contributes to the delayed vascular decompensation (Thiemermann *et al.*, 1992). Thus, endotoxic and haemorrhagic shock result in an enhanced NO formation due to activation of both constitutive and induced NOS.

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References

BENYÓ, Z., KISS, G., SZABÓ, C., CSÁKI, C. & KOVÁCH, A.G.B. (1991). Importance of basal nitric oxide synthesis in the regulation of myocardial blood flow. *Cardiovasc. Res.*, **25**, 700–703.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantification of protein dye binding. *Anal. Biochem.*, **72**, 248–254.

BRAQUET, P., TOUQUI, L., SHEN, T.Y. & VARGAFTIG, B.B. (1987). Perspectives in platelet-activating factor research. *Pharmacol. Rev.*, **39**, 97–145.

BREDT, D.S & SNYDER, S.H. (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 682–685.

BUSSE, R. & MÜLSCH, A. (1990). Induction of nitric oxide by cytokines in vascular smooth muscle cells. *FEBS Lett.*, **275**, 87–90.

FEUERSTEIN, G., DIMICCO, J.A., RAMU, A. & KOPIN, I.J. (1981). Effect of idomethacin on the blood pressure and plasma catecholamine response to acute endotoxaemia. *J. Pharm. Pharmacol.*, **33**, 576–579.

FLEMING, I., DAMBACHER, T. & BUSSE, R. (1992). Endothelium-derived kinins account for the immediate response of endothelial cells to bacterial lipopolysaccharide. *J. Vasc. Res.*, **29**, 114 (abstr.).

FLOWER, R.J. & BLACKWELL, G.J. (1979). Anti-inflammatory steroids induce biosynthesis of a phospholipase A2 inhibitor which prevents prostaglandin generation. *Nature*, **278**, 456–459.

FÖRSTERMANN, U., SCHMIDT, H.H.H.W., POLLOCK, J., SHENG, H., MITCHELL, J.A., WARNER, T.D., NAKANE, M. & MURAD, F. (1991). Isoforms of nitric oxide synthase. Characterization and purification from different cell types. *Biochem. Pharmacol.*, **42**, 1849–1857.

FU, J.Y., MASFERRER, J.L., SEIBERT, K., RAZ, A. & NEEDLEMAN, P. (1990). The induction and suppression of prostaglandin H_2 synthase (cyclooxygenase) in human monocytes. *J. Biol. Chem.*, **265**, 16737–16740.

GARDINER, S.M., COMPTON, A.M., BENNETT, T., PALMER, R.M.J. & MONCADA, S. (1990). Control of regional blood flow by endothelium-derived nitric oxide. *Hypertension*, **15**, 486–492.

GOLDSTEIN, J.M., ROOS, D., WEISMAN, G. & KAPLAN, H.B. (1976). Influence of corticosteroids on human polymorphonuclear leukocyte function in vitro: reduction of lysosomal enzyme release and superoxide production. *Inflammation*, **1**, 305–315.

GROSS, S.S., JAFFE, E.A., LEVI, R. & KILBOURN, R.G. (1991). Cytokine-activated endothelial cells express an isotype of nitric oxide synthase which is tetrahydrobiopterin-dependent, calmodulin-independent and inhibited by arginine analogs with a rank-order of potency characteristic of activated macrophages. *Biochem. Biophys. Res. Commun.*, **187**, 823–829.

GRYGLEWSKI, R.J., BOTTING, R. & VANE, J.R. (1988). Mediators produced by the endothelial cell. *Hypertension*, **12**, 530–548.

HAMMERSCHMIDT, D.E., WHITE, J.G., CRADDOCK, P.R. & JACOB, H.S. (1979). Corticosteroids inhibit complement-induced granulocyte aggregation. *J. Clin. Invest.*, **63**, 798–803.

HECKER, M., MITCHELL, J.A., HARRIS, H.J., KATSURA, M., THIEMERMANN, C. & VANE, J.R. (1990). Endothelial cells metabolize N^G-monomethyl-L-arginine to L-citrulline and subsequently to L-arginine. *Biochem. Biophys. Res. Commun.*, **167**, 1037–1043.

HUMPHRIES, R.G., CARR, R.D., NICHOL, A.K., TOMLINSON, W. & O'CONNOR, S.E. (1991). Coronary vasoconstriction in the conscious rabbit following intravenous infusion of L-N^G-nitroarginine. *Br. J. Pharmacol.*, **102**, 565–566.

JULOU-SCHAEFFER, G., GRAY, G.A., FLEMING, I., SCHOTT, C., PARRATT, J.R. & STOCLET, J.C. (1990). Loss of vascular responsiveness induced by endotoxin involves L-arginine pathway. *Am. J. Physiol.*, **259**, H1038–H1043.

KATORI, M., MAJIMA, M., ODOI-ADOME, R., SUNAHARA, N. & UCHIDA, Y. (1989). Evidence for the involvement of a plasma kallikrein-kinin system in the immediate hypotension produced by endotoxin in anaesthetized rats. *Br. J. Pharmacol.*, **98**, 1383–1391.

KILBOURN, R.G., GROSS, S.S., JUBRAN, A., ADAMS, J., GRIFFITH, O.W., LEVI, R. & LODATO, R.F. (1990a). N^G-methyl-L-arginine inhibits tumor necrosis factor-induced hypotension: implications for the involvement of nitric oxide. *Proc. Natl. Acad. Sci. U.S.A*, **87**, 3629–3632.

KILBOURN, R.G., JUBRAN, A., GROSS, S.S., GRIFFITH, O.W., LEVI, R., ADAMS, J. & LODATO, R.F. (1990b). Reversal of endotoxin-mediated shock by N^G-methyl-L-arginine, an inhibitor of nitric oxide synthesis. *Biochem. Biophys. Res. Commun.*, **172**, 1132–1138.

KNOWLES, R.G., MERRETT, M., SALTER, M. & MONCADA, S. (1990a). Differential induction of brain, lung and liver nitric oxide synthase by endotoxin in the rat. *Biochem. J.*, **270**, 833–836.

KNOWLES, R.G., SALTER, M., BROOKS, S.L. & MONCADA, S. (1990b). Anti-inflammatory glucocorticoids inhibit the induction by endotoxin of nitric oxide synthase in the lung, liver and aorta of the rat. *Biochem. Biophys. Res. Commun.*, **172**, 1042–1048.

MARLETTA, M.A., TAYEH, M.A. & HEVEL, J.M. (1990). Unrevealing the biological significance of nitric oxide. *Biofactors*, **2**, 219–225.

MASFERRER, J.L., SEIBERT, K., ZWEIFEL, B.S. & NEEDLEMAN, P. (1992). Endogenous glucocorticoids regulate an inducible cyclooxygenase enzyme. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 3917–3921.

MITCHELL, J.A., SHENG, H., FORSTERMANN, U. & MURAD, F. (1991). Characterization of nitric oxide synthase in non-adrenergic-non-cholinergic nerve containing rat anococcygeus. *Br. J. Pharmacol.*, **104**, 289–291.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.*, **43**, 109–141.

MOORE, P.K., AL-SWAYEH, O.A., CHONG, N.W.S., EVANS, R.A. & GIBSON, A. (1990). L-N^G-nitroarginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilatation *in vitro*. *Br. J. Pharmacol.*, **99**, 408–412.

MÓZES, T., ZIJLSTRA, F.J., HEILIGERS, J.P.C., TAK, C.J.A.M., BENEFRAIN, S., BONTA, I.L. & SAXENA, P.R. (1991). Sequential release of tumour necrosis factor, platelet activating factor and eicosanoids during endotoxin shock in anaesthetized pigs: protective effects of indomethacin. *Br. J. Pharmacol.*, **104**, 691–699.

PARRATT, J.R. (1983). Neurohumoral agents and their release in shock. In *Handbook of Shock and Trauma*. ed. Altura, B.M. pp. 311–336. New York: Raven Press.

PARRATT, J.R. & SURGESS, R.M. (1974). The effect of indomethacin on the cardiovascular and metabolic responses to E. coli endotoxin in cat. *Br. J. Pharmacol.*, **50**, 177–183.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1990). Glucocorticoids inhibit the expression of an inducible, but not the constitutive nitric oxide synthase in vascular endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 10043–10047.

REES, D., PALMER, R.M.J. & MONCADA, S. (1989). Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 3375–3378.

SALTER, M., KNOWLES, R.G. & MONCADA, S. (1991). Widespread tissue distribution, species distribution and changes in activity of calcium-dependent and calcium-independent nitric oxide synthases. *F.E.B.S.*, 291, 145–149.

SALVEMINI, D., KORBUT, R., ANGGARD, E. & VANE, J.R. (1990). Immediate release of a nitric oxide-like factor from bovine aortic endothelial cells by Eschericia coli lipopolysaccharide. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 2593–2597.

SCHUBITZ, K.M., CRADDOCK, P.R., HAMMERSCHMIDT, D.E. & AUGUST, T.J. (1981). Corticosteroids block binding of chemotactic peptide to its receptor on granulocytes and cause disaggregation of granulocyte aggregates in vitro. *J. Clin. Invest.*, **68**, 13–20.

SIMATOV, R. (1979). Glucocorticoids inhibit endorphin synthesis by pituitary cells. *Nature*, **280**, 684.

SZABÓ, C., THIEMERMANN, C., MITCHELL, J.A. & VANE, J.R. (1992). Activation of constitutive and cytokine-inducible NO synthase accounts for the reduced pressor response in endotoxin shock. *Br. J. Pharmacol.*, **107**, 81P.

THIEMERMANN, C. & VANE, J.R. (1990). Inhibition of nitric oxide synthesis reduces the hypotension induced by bacterial lipopolysaccharides in the rat in vivo. *Eur. J. Pharmacol.*, **182**, 591–595.

THIEMERMANN, C., MUSTAFA, M., MESTER, P.A., MITCHELL, J.A., HECKER, M. & VANE, J.R. (1990). Inhibition of the release of endothelium-derived relaxing factor *in vitro* and *in vivo* by dipeptides containing N^G-nitro-L-arginine. *Br. J. Pharmacol.*, **104**, 31–38.

THIEMERMANN, C., SZABÓ, C., MITCHELL, J.A. & VANE, J.R. (1993). Vascular hyporeactivity to vasoconstrictor agents and haemodynamic decompensation in hemorrhagic shock is mediated by nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.*, (in press).

WALDER, C., THIEMERMANN, C. & VANE, J.R. (1991). Endothelium-derived relaxing factor plays an important role in the regulation of renal cortical blood flow in the rat. *Br. J. Pharmacol.*, **102**, 967–973.

WRIGHT, C.E., REES, D.D. & MONCADA, S. (1992). Protective and pathological roles of nitric oxide in endotoxin shock. *Cardiovasc. Res.*, **26**, 48–57.

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Higher potency of RP 67580, in the mouse and the rat compared with other nonpeptide and peptide tachykinin NK_1 antagonists

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1 This study was undertaken to compare the potency and selectivity of the nonpeptide (RP 67580, (±)-CP-96,345 and its chloro-derivative [(±)-*cis*-3-(2-chlorobenzylamino)-2-benzhydrylquinuclidine] (CP-Cl)) and peptide (GR 71,251 and spantide) neurokinin$_1$ (NK_1) antagonists in mouse and rat preparations.

2 Among the NK_1 antagonists tested, RP 67580 was the most potent in inhibiting the specific binding of [^{125}I]-Bolton Hunter substance P ([^{125}I]-BHSP) to crude synaptosomes from the rat brain (K_i: 2.9 nM). (±)-CP-96,345 was about ten fold less potent (K_i: 31 nM) than RP 67580 while other compounds exhibited even less affinity.

3 All NK_1 antagonists inhibit competitively the activation of phospholipase C by [Pro^9]substance P ([Pro^9]SP) in cultured cortical astrocytes from the newborn mouse, a preparation rich in NK_1 receptors but devoid of NK_2 and NK_3 receptors. pA_2 values for the most potent compounds, RP 67580 and (±)-CP-96,345, were 8.28 and 7.08 respectively. When used alone, all antagonists showed some agonist activity at 10^{-5} M, except spantide which was already effective at 10^{-6} M.

4 An excellent correlation was found between the potency of the NK_1 antagonists in blocking the stimulation by [Pro^9]SP of phosphoinositide breakdown in cortical astrocytes and in inhibiting [^{125}I]-BHSP specific binding to rat brain synaptosomes.

5 As shown on single cells by use of the Indo-1 microfluorometric method, RP 67580 (10^{-7} M) prevented reversibly the elevation of cytosolic calcium concentration induced by [Pro^9]SP (10^{-8} M) in cultured cortical astrocytes.

6 Several experiments indicated that the antagonists were highly selective for NK_1 receptors. RP 67580 did not modify the noradrenaline-evoked activation of phospholipase C in cortical astrocytes; when used at 10^{-5} M all antagonists had no or only little affinity for NK_2 or NK_3 binding sites and did not block the NKA (10^{-8} M)-induced activation of phospholipase C in the hamster urinary bladder (a selective NK_2 test).

7 In conclusion, RP 67580 appears to be a potent NK_1 antagonist in the mouse and the rat. Results obtained with (±)-CP-96,345 confirm the lower potency of this compound in these two species when compared with reported data obtained in the guinea-pig or man.

Keywords: NK_1 tachykinin receptors; NK_1 selective nonpeptide and peptide antagonists; mouse astrocytes; phospholipase C; calcium; binding studies

Introduction

Binding studies made on peripheral and/or brain tissues and experiments performed on peripheral isolated organs have distinguished three main types of tachykinin receptor: the neurokinin$_1$ (NK_1), NK_2 and NK_3 receptors (Buck & Burcher, 1986; Maggio, 1988; Quirion & Dam, 1988; Regoli *et al.*, 1989; Guard & Watson, 1991; Torrens *et al.*, 1991). These receptors, which have recently been cloned, were shown to belong to the large family of receptors coupled to G proteins characterized by their seven transmembrane domains (Gerard *et al.*, 1990, 1991; Nakanishi, 1991; Takeda *et al.*, 1991). In mammals, the endogenous ligands of NK_1, NK_2 and NK_3 receptors are substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) respectively. Two other longer peptides, neuropeptide K (Tatemoto *et al.*, 1985) and neuropeptide gamma (Kage *et al.*, 1988) present in two precursor molecules of SP and NKA have also been shown to be potent agonists of NK_2 receptors (Beaujouan *et al.*, 1988; Krause *et al.*, 1990).

For several years, the characterization of tachykinin receptors in tissues containing more than one type of tachykinin receptor was particularly difficult since SP, NKA and NKB show only a moderate selectivity for their respective receptors (Lavielle *et al.*, 1988; Regoli *et al.*, 1989; Guard & Watson, 1991). In addition, only a few peptide antagonists with limited potency and selectivity were available. Progressively, potent and selective peptide agonists were synthesized. One of them, [Pro^9]SP, the most selective NK_1 agonist, will be used in the present study (Lavielle *et al.*, 1986; Petitet *et al.*, 1991). Among the first tachykinin peptide antagonists available, spantide ([D-Arg^1,D-Trp^{7,9},Leu^{11}]SP) has been particularly used (Folkers *et al.*, 1984). However, peptide antagonists have several disadvantages: their affinity is significantly lower than that of the natural agonist, they show in most cases a rather poor selectivity and their sensitivity to peptidase inactivation limits their use in *in vivo* studies. In addition, some of them have been shown to be neurotoxic (Hökfelt *et al.*, 1981; Freedman *et al.*, 1989). Slightly more potent and selective antagonists were then obtained when constrained tachykinin analogues of the (γ)-spirolactam type substituted in positions 7, 8, 9 and 10 such as GR 71,251 and GR 82,334

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were synthesized (Ward et al., 1990). More definitive progress has recently been made for NK_1 antagonists with the discovery of two potent and selective nonpeptide compounds (Snider et al., 1991a; Garret et al., 1991). Thus, Snider et al. (1991a) first reported that CP-96,345, a quinuclidine derivative was highly potent in blocking the SP-induced relaxation of the dog carotid artery previously contracted with noradrenaline (NA). In addition, they demonstrated not only that the biological activity of CP-96,345 was confined to the (2S,3S)-enantiomer, but also binding studies showed NK_1-receptor selectivity and enantiomeric selectivity. This compound, which has also been shown to antagonize SP-induced activation of locus coeruleus neurones (McLean et al., 1991), as well as SP-induced salivation (Snider et al., 1991b) or hypotension (Constantine et al., 1991) appears however to have a greater affinity for bovine and human NK_1 binding sites than for those in the mouse (Snider et al., 1991a). Since, Garret et al. (1991) found that RP 67580, a perhydroisoindolone derivative, not only inhibited competitively the binding of [^{3}H]-SP to NK_1 receptors in rat brain membranes but also, when injected intravenously in low doses, had the capacity to block totally plasma extravasation induced by either SP in the urinary bladder or antidromic electrical stimulation of the saphenous nerve in the hind paw skin. In addition, these authors indicated that this compound was as active as morphine in two analgesic tests in mice: phenylbenzoquinone-induced writhing and the formalin test.

The present study was undertaken to examine further the pharmacological properties of RP 67580 and to compare its antagonist potency with those of some other NK_1 antagonists such as (±)-CP-96,345, its chloro-derivative (CP-Cl), GR 71,251 and spantide. Experiments were performed mainly on cortical astrocytes of newborn mice in primary culture which possess NK_1 but not NK_2 or NK_3 receptors (Beaujouan et al., 1991) and in which SP or NK_1 agonists have been shown to stimulate significantly phospholipase C activity (Torrens et al., 1989) and to increase the cytosolic concentration of calcium (Delumeau et al., 1991).

Methods

Primary cultures of cortical astrocytes

Cells (2×10^5) from the cerebral cortex of 1-day-old Swiss mice (IFFA Credo, France) were dissociated and plated onto culture dishes (16 mm diameter wells) precoated for 30 min with poly-L-ornithine (1.5 μg ml^{-1}; Sigma). For calcium experiments, 4×10^6 cells were plated onto glass slides (45 mm × 70 mm) previously coated for 30 min with poly-L-ornithine (1.5 μg ml^{-1}; Sigma) and for 4 h with laminin (2 μg ml^{-1}; Gibco); slides were then placed in 100 mm culture dishes. In all cases the culture medium, which consisted of a mixture of minimal essential medium (MEM) and F-12 nutrient (GIBCO Europe, France) (1:1 v/v) supplemented with 33 mM glucose, 2 mM glutamine, 3 mM $NaHCO_3$, 5 mM HEPES (pH 7.2) and 10% (v/v) foetal bovine serum (Imperial Laboratories, West Portway, U.K.), was changed every 3 days. Glial cells formed a confluent monolayer devoid of neurones in the 3- to 4-week-old cultures used in most experiments. Immunohistochemistry using a rabbit antibody against glial fibrillary acidic protein (GFAP) (Dakopatts, Denmark) revealed that at least 95% of the cells were GFAP immunoreactive.

Binding assays

NK_1 binding sites: cortical astrocytes in primary culture and rat brain synaptosomes Routinely, 3- to 4-week-old cortical astrocyte cultures were incubated for 45 min at 20°C with 60 pM [^{125}I]-BHSP (25,000 c.p.m. per dish) in either the presence or absence of various concentrations of each tachykinin or antagonist tested. The incubation medium (0.2 ml per dish) consisted of a Krebs-Ringer phosphate buffer (in mM: NaCl 120, KCl 4.8, $CaCl_2$ 1.2, $MgSO_4$ 1.2 and NaH_2PO_4 15.6, pH 7.0) containing bovine serum albumin (BSA) (0.4 mg ml^{-1}; Calbiochem), bacitracin (0.3 mg ml^{-1}; Sigma); thiorphan (1 μM; Sigma), and glucose (6 mg ml^{-1}). At the end of the incubation, the supernatant was discarded, and the cells washed three times with 0.5 ml of cold (4°C) Krebs-Ringer phosphate buffer. Cells were then detached with 0.2% Triton X-100 (0.25 ml) containing BSA (1 mg ml^{-1}), and radioactivity bound to tissues was estimated. Assays were performed in quadruplicate.

Crude synaptosomes from the brain (minus the cerebral cortex) of Sprague Dawley male rats were used and prepared as previously described (Viger et al., 1983), the final pellet being resuspended in Krebs-Ringer phosphate buffer containing BSA (0.4 mg ml^{-1}), bacitracin (0.03 mg ml^{-1}) and glucose (1 mg ml^{-1}). Synaptosomes were incubated for 10 min in Eppendorf tubes at 20°C in 200 μl of buffer containing 60 pM of [^{125}I-BHSP and increasing concentrations of each tachykinin agonist or antagonist tested. The samples were then centrifuged, washed and the radioactivity bound to the pellets was estimated.

NK_2 binding sites: rat duodenal membranes Membranes from rat duodenal smooth muscle were prepared according to a procedure previously described (Bergström et al., 1987). The membrane suspension was incubated for 25 min at 20°C with [^{3}H]-NKA (0.9 nM) and increasing concentrations of each antagonist in 200 μl of 50 mM Tris HCl buffer, pH 7.4, containing BSA (0.4 mg ml^{-1}), bacitracin (0.03 mg ml^{-1}), leupeptin (4 μg ml^{-1}), chymostatin (4 μg ml^{-1}), kelatorphan (1 μM; provided by B.P Roques, Paris, France) and $MnCl_2$ (3 mM). The samples were then centrifuged, washed and the radioactivity contained in the pellets was estimated.

NK_3 binding sites: rat cerebral cortex synaptosomes Crude synaptosomes from the cerebral cortex of Sprague Dawley male rats were used and prepared as described previously (Beaujouan et al., 1984), the final pellet being resuspended in Krebs-Ringer phosphate buffer containing BSA (0.4 mg ml^{-1}), bacitracin (0.03 mg ml^{-1}) and glucose (1 mg ml^{-1}). Synaptosomes were incubated for 15 min in Eppendorf tubes at 20°C in 200 μl of buffer containing 60 pM of [^{125}I]-BHE and increasing concentrations of each agonist and/or antagonist. The samples were then centrifuged, washed and the radioactivity contained in the pellets was estimated.

Breakdown of inositol phospholipids

Cortical astrocytes in primary culture Three-week-old astrocyte cultures were incubated for 24 h in a culture medium containing 0.5 μCi/0.5 ml per dish of *myo*-2-[^{3}H]-inositol (18 Ci mmol^{-1}; 6×10^{-8} M; Amersham). Cultures were washed four times with Krebs-phosphate buffer (in mM: NaCl 120, KCl 4.8, $CaCl_2$ 1.2, $MgSO_4$ 1.2 and NaH_2PO_4 15.6; pH 7.0) containing BSA (0.4 mg ml^{-1}), bacitracin (0.3 mg ml^{-1}) and glucose (6 mg ml^{-1}). Each antagonist was added 15 min before and during the 60 min incubation at 37°C in the presence of various concentrations of [Pro^9]SP. The 60 min incubation was carried out in the presence of 500 μl of Krebs phosphate buffer containing LiCl (10 mM). Incubations were terminated by adding 500 μl of Triton X-100 (0.1%) to each well. Cells were harvested with the supernatant and transferred into glass tubes. The accumulation of [^{3}H]-inositol phosphates was estimated according to the method of Berridge et al. (1982) with slight modifications. To each tube, 1 ml of chloroform/methanol (1:2 v/v) and 0.5 ml of chloroform were successively added. After centrifugation, 1 ml of the upper phase diluted with water (5 ml) was passed through an AG 1-X8 (200–400 mesh, formate form) column (Bio-Rad Laboratories, Richmond, CA). The initial eluate containing free *myo*-[^{3}H]-inositol was collected into vials (95% of total radioactivity contained in the cells). Columns were then

washed with 0.1 M formic acid (10 ml), and [^{3}H]-inositol phosphates retained on the columns were eluted with 5 ml of 1 M ammonium formate/0.1 M formic acid, and eluates were used for the estimation of radioactivity.

Hamster urinary bladder slices

Urinary bladders from three male Golden Syrian hamsters (sufficient for 42 determinations) were chopped in three directions (350 × 350 × 350 μm) with a McIlwain apparatus. Tissues were then dispersed, and washed for 30 min at 37°C in gassed (95% O_2/5% CO_2) Krebs-Ringer bicarbonate buffer (in mM: NaCl 118, KCl 4.7, $CaCl_2$ 1.25, $MgSO_4$ 1.2, KH_2PO_4 1.2 and $NaHCO_3$ 25; pH 7.4) containing BSA (1 mg ml^{-1}), bacitracin (0.03 mg ml^{-1}) and glucose (2 mg ml^{-1}). After elimination of the supernatant, urinary bladder slices (150 μg protein per assay) were incubated for 90 min at 37°C in 6 ml of Krebs buffer containing 48 μCi of *myo*-[^{3}H]-inositol (Amersham, S.A: 18 Ci mmol^{-1}). After 6 washes with Krebs-Ringer bicarbonate in order to eliminate non-incorporated *myo*-[^{3}H]-inositol, aliquots of urinary bladder slices were incubated for 20 min at 37°C in Krebs buffer (final volume 250 μl) containing LiCl (10 mM) and NKA. The antagonists were incubated 15 min before the addition of NKA. The incubation was stopped by adding 1 ml of chloroform/methanol solution (1:2 v/v), followed by 500 μl of chloroform and 500 μl of water. After centrifugation (3000 r.p.m., 5 min) the aqueous phase (containing *myo*-[^{3}H]-inositol monophosphates) diluted with water (5 ml) was passed through anion-exchange chromatography columns (700 mg of Dowex AG 1-X8, 200–400 mesh, formate form). The initial eluate containing free *myo*-[^{3}H]-inositol was collected into vials. Columns were then washed with 10 ml of ammonium formate solution (60 mM) containing 5 mM sodium tetraborate and finally *myo*-[^{3}H]-inositol monophosphates were eluted with 10 ml of ammonium formate solution (200 mM) containing formic acid (100 mM), the eluates being directly collected into vials. Radioactivity was estimated in a β-liquid scintillation spectrometer after addition of Aquasol-2 (10 ml).

Cytosolic calcium measurement

Measurement of intracellular calcium concentration was carried out under dual emission microfluorimetry using Indo-1 (Calbiochem) as described by Delumeau *et al.* (1991). Cells were loaded for 90 min with 12 μM of the cell-permeant indo-1 acetoxymethylester in the perfusion saline-HEPES buffer (in mM: HEPES 20, glucose 5.5, NaCl 145, KCl 5.5, $MgCl_2$ 0.8 and $CaCl_2$ 1.2). After loading, the glass slide was placed in a perfusion chamber kept at room temperature and cells were excited with a 75 W Xenon light, filtered at 360 nm with a 10 nm wide interference filter. Excitation and emission spectra were separated by a 380 nm dichroic long-pass filter and the emission spectra were then divided in two halves by a dichroic long-pass filter. Two discriminant bands were selected from the two halves at 400–410 nm and 470–480 nm, and both fluorescence signals were estimated by photometers (opticals and photometers were obtained from Nikon). Cells were exposed to various substances dissolved in the perfusion saline buffer using a multichannel cell superfusion device. Calcium concentrations were calculated from the fluorescence ratio (R) measured respectively at 400–410 nm and 470–480 nm and a K_d of Indo-1 for ionised calcium of 250 mM, according to the equation described by Grynkiewicz *et al.* (1985):

$$[Ca^{2+}]_i = K_d \times (F480f/F480b) \times (R-R_{min})/(R_{max}-R)$$

in which F480f is the fluorescence of free Indo-1 and F480b the fluorescence of Indo-1 bound to calcium, R is the ratio between fluorescences measured at 405 and 480 nm; R_{min} and R_{max} were determined in the presence of ionomycin (10 μM, Calbiochem) and either EGTA (2 mM) or $CaCl_2$ (2 mM) respectively.

Ligands and drugs

[^{125}I]-Bolton Hunter substance P ([^{125}I]-BHSP) and [^{125}I]-Bolton Hunter eledoisin ([^{125}I]-BHE) were obtained by coupling the [^{125}I]-Bolton Hunter reagent (Amersham: monoiododerivative, S.A: 2000 Ci mmol^{-1}) to SP or eledoisin as previously described (Beaujouan *et al.*, 1984; Torrens *et al.*, 1991), [^{3}H]-NKA was synthesized by reacting [^{3}H]-methyl-4 tolyl sulphonate (Sp. Act. 75 Ci mmol^{-1}) with [HCys^{10}]NKA thiolactone according to a procedure previously described (Chassaing *et al.*, 1985). After removal of acetonitrile and lyophilisation, [^{3}H]-NKA was aliquoted into water in the presence of 0.2% mercaptoethanol and then kept under liquid nitrogen. High performance liquid chromatography (h.p.l.c.) analysis indicated that the retention time of the tritiated peptide was identical to that of the corresponding unlabelled compound. SP, [L-Pro^9]SP and NKA were synthesized and purified by S. Lavielle (University, Paris 6). The nonpeptide antagonists synthesized and purified by Rhône-Poulenc Rorer were CP-96,345 (racemic form) [(±)-*cis*-3-(2-methoxybenzylamino)-2-benzhydrylquinuclidine], its (2-chlorobenzylamino) analogue (CP-Cl) (racemic form) and RP 67580 [(3 a**R**, 7 a**R**)-7,7-diphenyl-2[1-imino-2-(2-methoxy-phenyl)-ethyl] perhydroisoindol-4-one]. The peptide antagonists were GR 71,251 (a gift from Dr R.M. Hagan, Glaxo Lab.) and spantide ([D-Arg^1,D-Trp^{7,9},Leu^{11}] SP) purchased from Peninsula Laboratories, Inc. (Belmont, CA, U.S.A.), noradrenaline (NA) was purchased from Calbiochem (San Diego, CA, U.S.A.).

Results

Binding affinities of tachykinin antagonists for NK_1 binding sites

Like [Pro^9]SP, a selective NK_1 agonist, all tachykinin antagonists tested inhibited competitively and in a concentration-dependent manner the high-affinity specific binding of [^{125}I]-BHSP to rat brain synaptosomes (Figure 1). Hill coefficients for these compounds were in all cases close to unity. The nonpeptide antagonists, RP 67580, (±)-CP-96,345 and CP-Cl were more potent competitors of [^{125}I]-BHSP binding than the two peptide antagonists spantide and GR 71,251 (Figure 1, Table 1). Among the nonpeptide antagonists, RP 67580

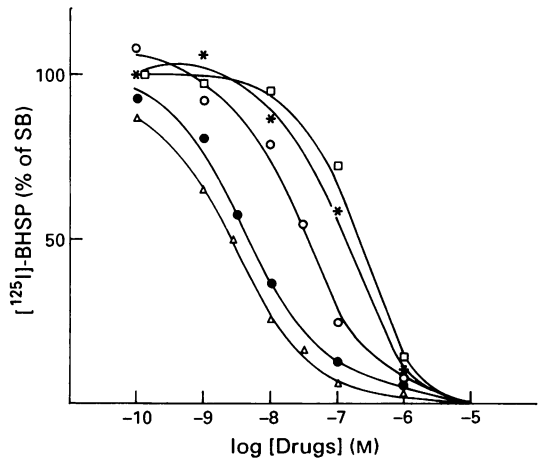


Figure 1 Competitive inhibition by tachykinin antagonists of [^{125}I]-Bolton Hunter substance P ([^{125}I]-BHSP) binding to rat brain synaptosomes (NK_1 sites). Crude synaptosomes were incubated with [^{125}I]-BHSP (40 pM) and increasing concentrations of [Pro^9]SP (Δ) or tachykinin antagonists, RP 67580 (●), (±)-CP-96,345 (○), CP-Cl (*) and spantide (□) as described in Methods. Each point is the mean of data obtained in at least three experiments, each value being determined in triplicate. Results are expressed as percentages of [^{125}I]-BHSP specific binding.

Table 1 Binding affinities of peptide and nonpeptide NK_1 antagonists for NK_1, NK_2 and NK_3 selective binding sites

Compounds	K_i (nM) NK_1[a]	NK_2[b]	NK_3[c]
Agonists			
Substance P	0.6	206	130
[Pro^9]SP	2.6	$>10^{-5}$	$>10^{-5}$
Nonpeptide antagonists			
RP 67580	2.9	$>10^{-5}$	$>10^{-5}$
(±)-CP-96,345	31	$>10^{-5}$	$>10^{-5}$
CP-Cl	125	16900	$>10^{-5}$
Peptide antagonists			
Spantide	230	8150	$>10^{-5}$
GR 71,251	243	2750	$>10^{-5}$

Binding studies were performed as described in methods using [^{125}I]-Bolton Hunter substance P ([^{125}I]-BHSP) and rat brain synaptosomes (a); [^{3}H]-neurokinin A ([^{3}H]-NKA) and rat duodenal membranes (b) and [^{125}I]-Bolton Hunter eledoisin ([^{125}I]-BHE) and rat cortical synaptosomes (c) for NK_1, NK_2 and NK_3 binding sites respectively. Apparent inhibition constants (K_i values) were obtained according to the Cheng-Prussoff formula, $K_i = IC_{50}/[1 + (L_o/K_d)]$. IC_{50} values were determined from Hill plots of data obtained in competition studies, results being the mean values determined from at least three experiments each determined in triplicate; s.d. for each K_i value was $<50\%$ of the mean; n_H values were close to unity.

was particularly potent since its affinity for NK_1 binding sites was similar to that of [Pro^9]SP ($K_i = 2.9$ nM). (±)-CP-96,345 and CP-Cl inhibited [^{125}I]-BHSP binding with one-tenth and one-fortieth the potency of RP 67580, respectively. The two peptide molecules spantide and GR 71,251 inhibited [^{125}I]-BHSP binding with a very low potency compared to RP 67580 (one-eightieth) (Table 1).

Efficacy of tachykinin antagonists in inhibiting the [Pro^9]SP-evoked accumulation of [^{3}H]-inositol phosphates in cortical astrocytes from the newborn mouse

As previously shown, cortical astrocytes from the newborn mouse in primary culture possess a high density of NK_1 binding sites and are devoid of NK_2 and NK_3 binding sites (Beaujouan *et al.*, 1991). Moreover, NK_1 agonists including [Pro^9]SP, the most potent and selective agonist of NK_1 receptors, have been shown to stimulate markedly the formation of [^{3}H]-inositol phosphates in these cells (Torrens *et al.*, 1989). Therefore, this preparation represents a very suitable and valid test for the potency of tachykinin antagonists on central NK_1 receptors (Beaujouan *et al.*, 1991). Furthermore, the K_i values observed for inhibition of [^{125}I]-BHSP binding by RP 67580 on intact cortical astrocytes (2.7 ± 0.9, $n = 3$) and on rat brain synaptosomes (2.9 ± 0.3 nM, $n = 3$) were identical.

Except spantide, which had a weak agonist action (EC_{50}: $6.9 \pm 2.5 \times 10^{-6}$ M; $n = 4$), all other antagonists, when tested alone at 10^{-6} M, were without effect on the activity of phospholipase C. These antagonists show very weak non significant stimulatory effects at 10^{-5} M.

All tachykinin antagonists inhibited in a concentration-dependent manner the [Pro^9]SP-induced production of [^{3}H]-inositol phosphates in cultured astrocytes. As illustrated in Figure 2 for RP 67580, in all cases, parallel shifts to the right of the [Pro^9]SP-dose-response curves were seen with no significant reduction of the maximal NK_1 agonist-evoked response. This indicates that inhibitory effects of the antagonists are of the competitive type. The pA_2 value for RP 67580 was 8.28 ± 0.04 ($n = 3$) with a slope not significantly different from 1 (1.03 ± 0.13) which is again consistent with a mechanism of competitive inhibition. Providing evidence for its selectivity of action, RP 67580 (10^{-6} M) was without effect on the noradrenaline-induced accumulation of [^{3}H]-inositol phosphates (Figure 2).

Estimates of pA_2 or pK_B values for all antagonists indicated that RP 67580 is much more potent than other nonpeptide and peptide compounds, the potency of (±)-CP-96,345 being, however, higher than that of other molecules (Table 2). Finally, as shown in Figure 3, a good correlation was found between the pK_B values of all antagonists tested for inhibition of the [Pro^9]SP-induced accumulation of [^{3}H]-inositol phosphates in cortical astrocytes and their K_i values

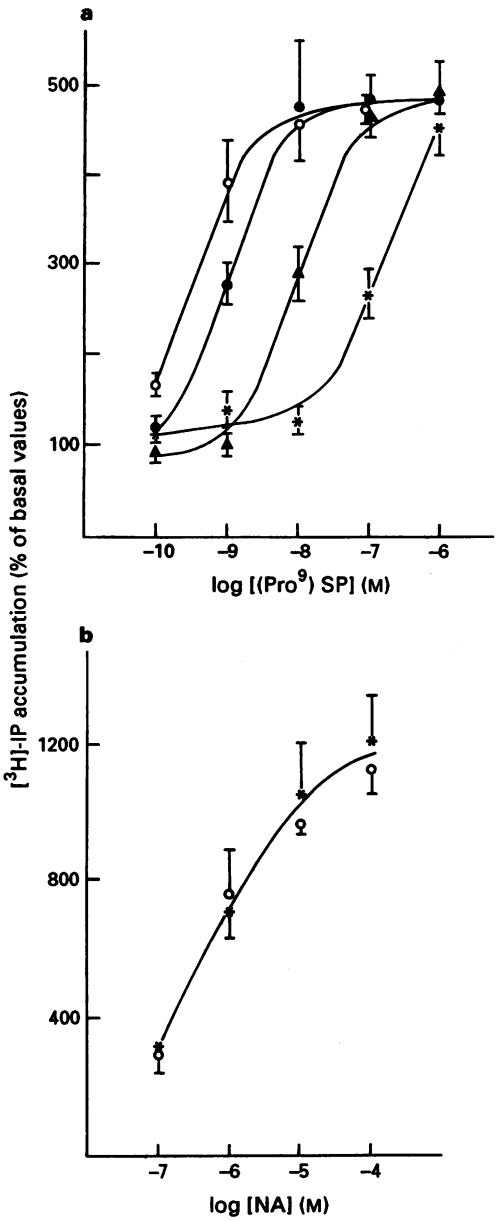


Figure 2 Competitive and selective antagonism by RP 67580 of [Pro^9] substance P ([Pro^9]SP)-induced activation of phospholipase C in cultured cortical astrocytes from the newborn mouse. Concentration-response curves to [Pro^9]SP or noradrenaline (NA) in control (○) or in the presence of various concentrations of RP 67580: (●, 10^{-8} M; ▲, 10^{-7} M; *, 10^{-6} M). (a) RP 67580 was added to the incubation medium 15 min before the selective NK_1 agonist ([Pro^9]SP) used at various concentrations, the accumulation of [^{3}H]-inositol phosphates ([^{3}H]-IP) being determined at the end of the 60 min incubation as described in Methods. Results corresponding to the accumulation of [^{3}H]-inositol phosphates are expressed as percentage of control values. Each point corresponds to the mean ± s.e.mean (vertical bars) of four determinations obtained in a typical experiment. (b) Lack of antagonistic effect of RP 67580 on the NA-induced accumulation of [^{3}H]-inositol phosphates in cortical astrocytes.

Table 2 Blockade by NK_1 antagonist of the [Pro^9]substance P ([Pro^9]SP)-induced accumulation of [^{3}H]-inositol phosphates in cultured cortical astrocytes from the newborn mouse: pK_B and or pA_2 values

Antagonists	pK_B	pA_2
RP 67580	8.04 ± 0.21	8.28 ± 0.04
(±)-CP-96,345	7.19 ± 0.24	7.08 ± 0.59
CP-Cl	6.84 ± 0.20	ND
Spantide	6.42 ± 0.21	ND
GR 71,251	6.90 ± 0.10	ND

Experiments were done as described in Figure 2. Each value is the mean ± s.e.mean of 3–5 independent determinations, each being performed in quadruplicate. ND: not determined.

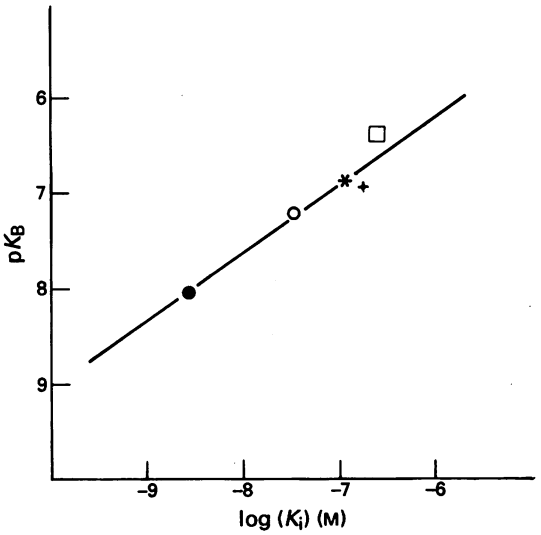


Figure 3 Comparative potency of NK_1 antagonists: Correlation between the potency of NK_1 antagonists (RP 67580, ●; (±)-CP-96,345, ○; CP-Cl, *; GR 71,251, +; spantide, □), in blocking the [Pro^9] substance P ([Pro^9]SP)-induced activation of phospholipase C in cortical astrocytes and in inhibiting [^{125}I]-BHSP specific binding to rat brain synaptosomes (R = 0.95). pK_B and K_i values for each compound are those shown in Tables 2 and 1.

for inhibition of [^{125}I]-BHSP binding on rat brain synaptosomes (R = 0.95). Moreover, pK_B and K_i values were most identical, the slope of the line being 0.71.

Blockade by RP 67580 of the [Pro^9]SP-induced elevation of cytosolic calcium in cultured cortical astrocytes from the newborn mouse

As shown in Figure 4, in most single astrocytes tested ($n = 50$), the prolonged application of [Pro^9]SP (10^{-8} M) evoked a biphasic elevation of the cytosolic calcium concentration. Indeed, a rapid and transient increase in cytosolic calcium immediately followed by a prolonged plateau (up to 6 min) was observed. A rapid return of the cytosolic concentration of calcium to basal levels occurred at the end of the [Pro^9]SP application. Finally, repeated applications of [Pro^9] SP at 10^{-8} M did not result in a significant tachyphylaxis of the responses.

When used at 10^{-7} M, RP 67580 antagonized completely the [Pro^9]SP-induced elevation of the cytosolic calcium concentration in astrocytes. This was observed when either RP-67580 was applied with [Pro^9]SP or added after [Pro^9] SP during the plateau period. Indeed, in the latter case, cytosolic calcium decreased rapidly to basal levels immediately after the beginning of the antagonist application and this effect was rapidly reversible as soon as RP 67580 was removed (Figure 4).

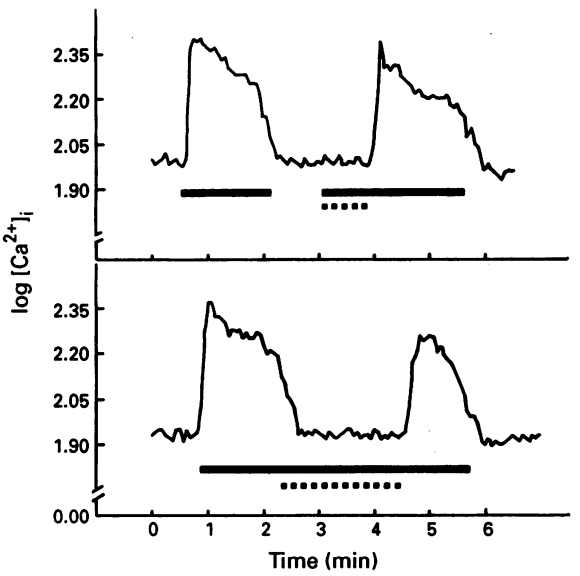


Figure 4 Blockade by RP 67580 of the [Pro^9] substance P ([Pro^9] SP)-induced elevation of cytosolic calcium in cortical astrocytes of the newborn mouse. Changes in cytosolic calcium concentrations were estimated on single cells by the Indo-1 microfluorometric method. This figure illustrates two typical experiments in which RP 67580 (---10^{-7} M) was applied simultaneously with the selective NK_1 agonist [Pro^9]SP (——10^{-8} M) (upper part) or after the start of the NK_1 agonist application (lower part). In both cases it can be seen that the inhibitory effect of RP 67580 is reversible. Similar results were obtained in 10 other cells.

Lack of effect of RP 67580 and other NK_1 tachykinin antagonists on NK_2 and NK_3 receptors

In a first series of experiments, the effects of RP 67580 and other NK_1 tachykinin antagonists were examined on the binding of either [^{3}H]-NKA to rat duodenal membranes or [^{125}I]-BHE to rat cerebral cortex synaptosomes, these two preparations being respectively rich in NK_2 and NK_3 binding sites. As shown in Table 1, the most potent NK_1 antagonists, RP 67580 and (±)CP-96,345 were without affinity for NK_2 and NK_3 binding sites. This was also the case for CP-Cl, spantide and GR 71,251 on NK_3 binding sites but these three compounds had a low affinity for NK_2 binding sites.

The lack of effect of RP 67580 and other NK_1 tachykinin antagonists on NK_2 receptors was further confirmed by studying their ability to antagonize the NKA-induced accumulation of [^{3}H]-inositol monophosphate in the hamster urinary bladder, a preparation particularly rich in NK_2 receptors. NKA was used at a concentration of 10^{-8} M, corresponding to its EC_{50} value. Even when used at a high concentration (10^{-5} M), none of the antagonists tested blocked the NKA-evoked response (Table 3). In addition, these antagonists did not show agonist properties, since they did not stimulate the formation of [^{3}H]-inositol monophosphate when applied alone at 10^{-5} M.

Discussion

The present study indicates that, in the rat or the mouse, the recently discovered nonpeptide compound RP 67580 is a highly selective and much more potent NK_1 antagonist than the other molecules tested including (±)-CP-96,345.

As first shown by binding studies, RP 67580 was found to be the most potent in inhibiting competitively the binding of [^{125}I]-BHSP to rat brain synaptosomes or to intact cortical astrocytes from the newborn mouse, its inhibition constant (K_i) in these two preparations being 2.9 nM and 2.7 nM respectively. These values are closely similar to those found

Table 3 Lack of effect of nonpeptide and peptide NK_1 antagonists on the neurokinin A (NKA)-induced activation of phospholipase C in the hamster urinary bladder

Compounds	*IP1 accumulation*
NKA (10^{-8} M)	100
NKA (10^{-8} M) + RP 67580 (10^{-5} M)	121 ± 23
NKA (10^{-8} M) + (±)-CP-96,345 (10^{-5} M)	93 ± 12
NKA (10^{-8} M) + CP-Cl (10^{-5} M)	91 ± 22
NKA (10^{-8} M) + GR 71,251 (10^{-5} M)	108 ± 15
NKA (10^{-8} M) + spantide (10^{-5} M)	110 ± 11

Slices from the hamster urinary bladder which possess NK_2 receptors coupled to phospholipase C were incubated for 20 min with NKA (10^{-8} M) in order to estimate the formation of [^{3}H]-inositol monophosphates (IP 1) as indicated in Methods. The stimulatory effect of NKA (10^{-8} M) represented 522% the control value. All antagonists were added at a concentration of 10^{-5} M, 15 min before NKA. Data are expressed as percentage ± s.e.mean of the mean value obtained for the NKA-evoked response. Similar results were obtained with RP 67580, (±)-CP-96,345 or CP-Cl when NKA was used at various concentrations from 10^{-9} M up to 10^{-6} M (data not shown). Results are the mean ± s.e.mean of 3 determinations.

with the selective NK_1 agonist ([Pro^9]SP (2.6 nM and 0.42 nM) (Lavielle *et al.*, 1986; Beaujouan *et al.*, 1991). These results agree with those of Garret *et al.* (1991), since these authors reported that RP 67580 inhibits competitively the binding of [^{3}H]-SP to rat brain membranes with a K_i of 4.2 nM.

(±)-CP-96,345 was about 10 times less potent than RP 67580 in inhibiting [^{125}I]-BHSP binding to rat brain synaptosomes (K_i: 31 nM), while other compounds, including the chloro-derivative of (±)-CP-96,345, had a much lower potency (1 to 2% that of RP 67580). Results concerning (±)-CP-96,345 are comparable to those of Snider *et al.* (1991a) since these authors have observed that this compound has a relatively low affinity for NK_1 binding sites in the rat or the mouse (IC_{50}: 240 nM). However, a much higher affinity of (±)-CP-96,345 for NK_1 binding sites has been found in the bovine brain (IC_{50}: 0.5 nM) (Snider *et al.*, 1991a), the IM 9 human B-lymphoblastoid cell line (IC_{50}: 0.35 nM) and the U 373 human intact astrocytoma cell line (IC_{50}: 0.4 nM) (Gitter *et al.*, 1991). Extending these observations, a pronounced species difference in the affinities of nonpeptide NK_1 antagonists of the quinuclidine family was reported since both the methoxy ((±)-CP-96,345) and chloro (CP-Cl) compounds were found to be more potent inhibitors at NK_1 binding sites in human, bovine, guinea-pig and rabbit brain tissues when compared to those of the rat, mouse and chicken (Gitter *et al.*, 1991). This can now be easily explained since cloning and sequencing studies recently made on human tissues have indicated that NK_1 receptors from the human and rat differ by 22 amino acids randomly distributed in the receptor molecule (Takeda *et al.*, 1991). Interestingly enough, a recent report demonstrated that a species difference can also be observed with RP 67580 (Fardin *et al.*, 1992).

To our present knowledge, cortical astrocytes from the newborn mouse in primary culture represent one of the most suitable preparations to look for the potency of agonists and antagonists acting on central NK_1 receptors (Beaujouan *et al.*, 1991). Indeed, in this preparation which is devoid of NK_2 and NK_3 receptors, SP and other NK_1 agonists induce a marked activation of phospholipase C (Torrens *et al.*, 1989; Beaujouan *et al.*, 1991) and increase the cytosolic concentration of calcium (Delumeau *et al.*, 1991). In addition, IC_{50} values for NK_1 agonists in inhibiting [^{125}I]-BHSP binding to intact astrocytes are identical to their EC_{50} values for increasing [^{3}H]-inositol phosphates production (Torrens *et al.*, 1989; Beaujouan *et al.*, 1991). In the present study, a good correlation was found between the efficacy of the different antagonists tested in inhibiting the binding of [^{125}I]-BHSP to rat brain synaptosomes and the [Pro^9]SP-induced accumulation of [^{3}H]-inositol phosphates in cortical astrocytes, all compounds acting in a competitive manner. Therefore, as in binding studies, RP 67580 was the most potent in antagonizing the [Pro^9]SP-evoked response in cortical astrocytes, its pA_2 value being 8.3 with a slope not significantly different from 1, which is again consistent with a mechanism of competitive inhibition. As expected, when used at a concentration of 10^{-7} M, RP 67580 totally, but reversibly, blocked the [Pro^9]SP (10^{-8} M)-induced elevation of cytosolic calcium in cultured cortical astrocytes.

As already indicated, RP 67580 is also particularly potent in inhibiting plasma extravasation in the rat urinary bladder or hind paw skin induced respectively by SP and antidromic electrical stimulation of the saphenous nerve (Garret *et al.*, 1991). In addition, RP 67580 has been shown to inhibit competitively the SP-evoked contraction in the guinea-pig ileum with a pA_2 value of 7.16 (Garret *et al.*, 1991) which is significantly lower than that found for its antagonistic effect toward the [Pro^9]SP-induced activation of phospholipase C in cortical astrocytes of the mouse (pA_2: 8.3). Species differences in the properties of NK_1 receptors could again explain the latter observation. Confirming this statement and in agreement with our results of binding studies, the pA_2 value of (±)-CP-96,345 for inhibiting the [Pro^9]SP-induced accumulation of [^{3}H]-inositol phosphates in cortical astrocytes was 7.1, a value much lower than that determined with this compound in other biological tests of NK_1 receptors performed on peripheral isolated organs of the guinea-pig, rabbit or dog (pA_2 between 8.7–9.6) (Snider *et al.*, 1991a; Rouissi *et al.*, 1991). This provides further indication that care must be taken in comparing the potency of potential NK_1 antagonists.

The possible existence of NK_1 subtypes should be also taken into account in comparing the efficacy of NK_1 antagonists. Indeed, we have recently suggested that septide, substance P methyl ester, [Apa$^{9-10}$]SP and [Pro^{9,10}]SP which are as potent as [Pro^9]SP in contracting the guinea-pig ileum exert their contractile effect by acting on a new tachykinin receptor, possibly a NK_1 receptor subtype (Petitet *et al.*, 1992). Thus, as determined by their potency to inhibit the highly specific high affinity binding of [^{3}H]-[Pro^9]SP, septide and the three other mentioned peptides have a relatively low affinity for NK_1 binding sites in the guinea-pig ileum when compared to [Pro^9]SP (Petitet *et al.*, 1992). In addition, GR 71,251 which appears to be much less potent than RP 67580 and (±)-CP-96,345 in antagonizing the [Pro^9]SP-evoked response in cortical astrocytes had a much higher potency in inhibiting the septide- than the [Pro^9]SP-evoked contraction in the guinea-pig ileum (Petitet *et al.*, 1992). RP 67580 has been shown to be as potent in inhibiting contractions evoked by septide (pA_2: 7.59) as those evoked by SP (pA_2: 7.16) in the guinea-pig ileum (Garret *et al.*, 1991), suggesting that this powerful NK_1 antagonist could also be an antagonist of tachykinin receptors highly sensitive to septide in the guinea-pig ileum.

In their studies, Garret *et al.* (1991) and Snider *et al.* (1991a) indicated that RP 67580 and CP-96,345 respectively were highly selective antagonists of NK_1 receptors, since these compounds showed insignificant binding to NK_2 and NK_3 receptors. In addition, these compounds were shown to be without effect on the binding of ligands for several other peptide or nonpeptide receptors. However, CP-96,345 and (±)-CP-96,345 were found to displace binding to Ca^{2+} channel binding sites in rat cardiac and brain tissues (Guard & Watling, 1992; Schmidt *et al.*, 1992). In our study the activity of (±)-CP-96,345 seemed to be exerted solely through NK_1 receptors in mouse cortical astrocytes since a good correlation was observed for this compound between its antagonistic activity on phospholipase C activator and its affinity for NK_1 binding sites. Furthermore, in addition to the other antagonists, (±)-CP-96,345 acts in a competitive manner at the levels of the NK_1 receptors. Neither RP 67580 nor CP-96,345

modified the contractile responses evoked by either NKA or senktide in different peripheral organs used as biological assays for NK_2 or NK_3 receptors (Garret *et al.*, 1991; Snider *et al.*, 1991a). Further indicating the selectivity of RP 67580, this compound did not modify the contractile response evoked by either acetylcholine, histamine or bradykinin in the guinea-pig ileum. Similarly (±)-CP-96,345 did not antagonize acetylcholine-induced salivation in the rat.

In our study, experiments were also designed to look for the selectivity of the action of RP 67580 and other antagonists tested on NK_1 receptors. Firstly, when used at a concentration up to 10^{-5} M, neither RP 67580 nor (±)-CP-96,345 inhibited the binding of either [^{3}H]-NKA or [^{125}I]-BHE to rat duodenal membranes or rat cortical synaptosomes, two preparations used to estimate the affinity of tachykinin agonists or antagonists on NK_2 and NK_3 binding sites respectively (Beaujouan *et al.*, 1984; Bergström *et al.*, 1987). The other antagonists (CP-Cl, spantide, GR 71,251) were also without effect on [^{125}I]-BHE binding to rat cortical synaptosomes, but a slight inhibitory effect was seen, particularly with GR 71,251, on the binding of [^{3}H]-NKA to rat duodenal membranes. Secondly, RP 67580, the most potent antagonist in blocking the [Pro^9]SP-induced activation of phospholipase C in cortical astrocytes of the newborn mouse did not antagonize the noradrenaline-evoked response. Thirdly, experiments were carried out on the hamster urinary bladder, which is particularly suitable to look for the activation of phospholipase C linked to the stimulation of NK_2 receptors (Bristow *et al.*, 1987). Even when used in high concentration (10^{-5} M), neither RP 67580, nor (±)-CP-96,345 antagonized the NKA-induced activation of phospholipase C and similar observations were made with the other antagonists tested. Unfortunately, similar experiments could not be carried out for NK_3 receptors coupled to phospholipase C, since, although some tissues such as the guinea-pig ileum or the spinal cord of the newborn rat possess NK_3 receptors coupled to phospholipase C, they also contain NK_1 receptors coupled to this enzyme (Guard & Watson, 1991).

Altogether, our comparative study provides further evidence indicating that the two nonpeptide NK_1 antagonists at present available, RP 67580 and (±)-CP-96,345, are much more potent and selective than peptide NK_1 antagonists. In addition, RP 67580 appears to have a higher potency in the rat and the mouse than (±)-CP-96,345, which could be relevant for pharmacological investigations on peripheral and central functions of SP in these species.

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References

BEAUJOUAN, J.-C., SAFFROY, M., PETITET, F., TORRENS, Y. & GLOWINSKI, J. (1988). Neuropeptide K, scyliorhinin I and II: new tools in the tachykinin receptor field. *Eur. J. Pharmacol.*, **151**, 353–354.

BEAUJOUAN, J.-C., TEUTSCH, B., SAFFROY, M., PETITET, F., TORRENS, Y. & GLOWINSKI, J. (1991). NK-1 receptors are the only class of tachykinin receptors found on mouse cortical astrocytes. *Peptides*, **12**, 813–820.

BEAUJOUAN, J.C., TORRENS, Y., VIGER, A. & GLOWINSKI, J. (1984). A new type of tachykinin binding site in the rat brain characterized by specific binding of a labeled eledoisin derivative. *Mol. Pharmacol.*, **26**, 248–254.

BERGSTRÖM, L., BEAUJOUAN, J.C., TORRENS, Y., SAFFROY, M., GLOWINSKI, J., LAVIELLE, S., CHASSAING, G., MARQUET, A., D'ORLÉANS-JUSTE, P., DION, S. & REGOLI, D. (1987). ^{3}H-neurokinin A labels a specific tachykinin-binding site in the rat duodenal smooth muscle. *Mol. Pharmacol.*, **32**, 764–771.

BERRIDGE, M.J., DOWNES, C.P. & HANLEY, M.R. (1982). Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. *Biochem. J.*, **206**, 587–595.

BRISTOW, D.R., CURTIS, N.R., SUMAN-CHAUHAN, N., WATLING, K.J. & WILLIAMS, B.J. (1987). Effects of tachykinins on inositol phospholipid hydrolysis in slices of hamster urinary bladder. *Br. J. Pharmacol.*, **90**, 211–217.

BUCK, S.H. & BURCHER, E. (1986). The tachykinins: a family of peptides with a brood of 'receptors'. *Trends Pharmacol. Sci.*, **7**, 65–68.

CHASSAING, G., LAVIELLE, S., JULIEN, S. & MARQUET, A. (1985). Homocysteine thiolactone as precursor of methionine amide: application to the modification of peptides of the tachykinin family. *Tetrahedron Lett.*, **26**, 623–626.

CONSTANTINE, J.W., LEBEL, W.S. & WOODY, H.A. (1991). Inhibition of tachykinin-induced hypotension in dogs by CP-96,345, a selective blocker of NK-1 receptors. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **344**, 471–477.

DELUMEAU, C., PETITET, F., CORDIER, J., GLOWINSKI, J. & PRÉMONT, J. (1991). Synergistic regulation of cytosolic Ca^{2+} concentration in mouse astrocytes by NK1 tachykinin and adenosine agonists. *J. Neurochem.*, **57**, 2026–2035.

FARDIN, V., FOUCAULT, F., BOCK, M.D. & GARRET, C. (1992). Binding profile of RP 67580, a new non-peptide substance P antagonist: comparison with CP-96,345. *Br. J. Pharmacol.*, **105**, 80P.

FOLKERS, K., HAKANSON, R., HÖRIG, J., XU, J.-C. & LEANDER, S. (1984). Biological evaluation of substance P antagonists. *Br. J. Pharmacol.*, **83**, 449–456.

FREEDMAN, J., HÖKFELT, T., POST, C., BRODIN, E., SUNDSTRÖM, E., JONSSON, G., TERENIUS, L., LEANDER, S., FISCHER, J.A. & VERHOFSTAD, A. (1989). Immunohistochemical and behavioral analysis of spinal lesions induced by a substance P antagonist and protection by thyrotropin releasing hormone. *Exp. Brain Res.*, **74**, 279–292.

GARRET, C., CARRUETTE, A., FARDIN, V., MOUSSAOUI, S., PEYRONEL, J.-F., BLANCHARD, J.-C. & LADURON, P. (1991). Pharmacological properties of a potent and selective nonpeptide substance P antagonist. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 10208–10212.

GERARD, N.P., EDDY, R.L.Jr., SHOWS, T.B. & GERARD, C. (1990). The human neurokinin A (substance K) receptor. Molecular cloning of the gene, chromosome localization, and isolation of cDNA from tracheal and gastric tissues. *J. Biol. Chem.*, **265**, 20455–20462 and corrections GERARD, N.P. *et al.*, (1991). *J. Biol. Chem*,. **266**, 1354.

GITTER, B.D., WATERS, D.C., BRUNS, R.F., MASON, N.R., NIXON, J.A. & HOWBERT, J.J. (1991). Species differences in affinities of nonpeptide antagonists for substance P receptors. *Eur. J. Pharmacol.*, **197**, 237–238.

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

GUARD, S. & WATLING, K.J. (1992). Interaction of the non-peptide NK_1 tachykinin receptor antagonist (±)-CP-96,345 with L-type calcium channels in rat cerebral cortex. *Br. J. Pharmacol*,. **106**, 37P.

GUARD, S. & WATSON, S.P. (1991). Tachykinin receptor subtypes: classification and membrane signalling mechanisms. *Neurochem. Int.*, **18**, 149–165.

HÖKFELT, T., VINCENT, S., HELLSTEN, L., ROSELL, S., FOLKERS, K., MARKEY, K., GOLDSTEIN, M. & CUELLO, C. (1981). Immunohistochemical evidence for a 'neurotoxic' action of (D-Pro^2,D-Trp^{7,9})-substance P, an analogue with substance P antagonistic activity. *Acta Physiol. Scand.*, **113**, 571–573.

KAGE, R., MCGREGOR, G.P., THIM, L. & CONLON, J.M. (1988). Neuropeptide-γ: a peptide isolated from rabbit intestine that is derived from γ-preprotachykinin. *J. Neurochem.*, **50**, 1412–1417.

KRAUSE, J.E., HERSHEY, A.D., DYKEMA, P.E. & TAKEDA, Y. (1990). Molecular biological studies on the diversity of chemical signalling in tachykinin peptidergic neurons. *Ann. N.Y. Acad. Sci.*, **579**, 254–272.

LAVIELLE, S., CHASSAING, G., JULIEN, S., MARQUET, A., BERGSTRÖM, L., BEAUJOUAN, J.-C., TORRENS, Y. & GLOWINSKI, J. (1986). Specific recognition of SP or NKB receptors by analogues of SP substituted at positions 8 and 9. *Eur. J. Pharmacol.*, **125**, 461–462.

LAVIELLE, S., CHASSAING, G., PLOUX, O., LOEUILLET, D., BESSEYRE, J., JULIEN, S., MARQUET, A., CONVERT, O., BEAUJOUAN, J.-C., TORRENS, Y., BERGSTRÖM, L., SAFFROY, M. & GLOWINSKI, J. (1988). Analysis of tachykinin binding site interactions using constrained analogues of tachykinins. *Biochem. Pharmacol.*, **37**, 41–49.

MAGGIO, J.E. (1988). Tachykinins. *Annu. Rev. Neurosci.*, **11**, 13–28.

MCLEAN, S., GANONG, A.H., SEEGER, T.F., BRYCE, D.K., PRATT, K.G., REYNOLDS, L.S., SIOK, C.J., LOWE III, J.A. & HEYM, J. (1991). Activity and distribution of binding sites in brain of a nonpeptide substance P (NK_1) receptor antagonist. *Science*, **251**, 437–439.

NAKANISHI, S. (1991). Mammalian tachykinin receptors. *Annu. Rev. Neurosci.*, **14**, 123–136.

PETITET, F., BEAUJOUAN, J.-C., SAFFROY, M., TORRENS, Y., CHASSAING, G., LAVIELLE, S., BESSEYRE, J., GARRET, C., CARRUETTE, A. & GLOWINSKI, J. (1991). Further demonstration that [Pro^9]-substance P is a potent and selective ligand of NK-1 tachykinin receptor. *J. Neurochem.*, **56**, 879–889.

PETITET, F., SAFFROY, M., TORRENS, Y., LAVIELLE, S., CHASSAING, G., LOEUILLET, D., GLOWINSKI, J. & BEAUJOUAN, J.C. (1992). Possible existence of a new tachykinin receptor subtype in the guinea-pig ileum. *Peptides*, **13**, 383–388.

QUIRION, R. & DAM, T.V. (1988). Multiple neurokinin receptors: recent developments. *Reg. Pept.*, **22**, 18–25.

REGOLI, D., DRAPEAU, G., DION, S. & D'ORLÉANS-JUSTE, P. (1989). Receptors for substance P and related neurokinins. *Pharmacol.*, **38**, 1–15.

ROUISSI, N., GITTER, B.D., WATERS, D.C., HOWBERT, J.J., NIXON, J.A. & REGOLI, D. (1991). Selectivity and specificity of new, non-peptide, quinuclidine antagonists of substance P. *Biochem. Biophys. Res. Commun,*. **176**, 894–901.

SCHMIDT, A.W., MCLEAN, S. & HEYM, J. (1992). The substance P receptor antagonist CP-96,345 interacts with Ca^{2+} channels. *Eur. J. Pharmacol,*. **215**, 351–352.

SNIDER, R.M., CONSTANTINE, J.W., LOWE, J.A. III, LONGO, K.P., LEBEL, W.S., WOODY, H.A., DROZDA, S.E., DESAI, M.C., VINICK, F.J., SPENCER, R.W. & HESS, H.-J. (1991a). A potent nonpeptide antagonist of the substance P (NK_1) receptor. *Science*, **251**, 435–437.

SNIDER, R.M., LONGO, K.P., DROZDA, S.E., LOWE III, J.A. & LEEMAN, S.E. (1991b). Effect of CP-96,345, a nonpeptide substance P receptor antagonist, on salivation in rats. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 10042–10044.

TAKEDA, Y., CHOU, K.B., TAKEDA, J., SACHAIS, B.S. & KRAUSE, J.E. (1991). Molecular cloning, structural characterization and functional expression of the human substance P receptor. *Biochem. Biophys. Res. Commun.*, **179**, 1232–1240.

TATEMOTO, K., LUNDBERG, J.M., JÖRNVALL, H. & MUTT, V. (1985). Neuropeptide K; Isolation, structure and biological activities of a novel brain tachykinin. *Biochem. Biophys. Res. Commun.*, **128**, 947–953.

TORRENS, Y., BEAUJOUAN, J.-C., DIETL, M., SAFFROY, M., PETITET, F. & GLOWINSKI, J. (1991). Tachykinins receptors: binding and cellular activity assays. In *Methods in Neurosciences*, Vol. 5, *Neuropeptide Technology: Gene Expression* and *Neuropeptide Receptors*. pp. 243–267. ed. Conn, M. New York: Academic Press Inc.

TORRENS, Y., DAGUET DE MONTETY, M.C., EL ETR, M., BEAUJOUAN, J.C. & GLOWINSKI, J. (1989). Tachykinin receptors of the NK1 type (substance P) coupled positively to phospholipase C on cortical astrocytes from the newborn mouse in primary culture. *J. Neurochem.*, **52**, 1913–1918.

VIGER, A., BEAUJOUAN, J.C., TORRENS, Y. & GLOWINSKI, J. (1983). Specific binding of a ^{125}I-substance P derivative to rat brain synaptosomes. *J. Neurochem.*, **40**, 1030–1039.

WARD, P., EWAN, G.B., JORDAN, C.C., IRELAND, S.J., HAGAN, R.M. & BROWN, J.R. (1990). Potent and highly selective neurokinin antagonists. *J. Med. Chem.*, **33**, 1848–1851.

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A comparison of capsazepine and ruthenium red as capsaicin antagonists in the rat isolated urinary bladder and vas deferens

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1 The ability of capsazepine, a recently developed capsaicin receptor antagonist, to prevent the effects of capsaicin on the rat isolated urinary bladder (contraction) and vas deferens (inhibition of electrically-evoked twitches) was compared to that of ruthenium red, a dye which behaves as a functional antagonist of capsaicin.

2 In the rat bladder, capsazepine (3–30 μM) produced a concentration-dependent rightward shift of the curve to capsaicin without any significant depression of the maximal response to the agonist. By contrast, ruthenium red (10–30 μM) produced a non-competitive type of antagonism, characterized by marked depression of the maximal response attainable. Similar findings were obtained in the rat isolated vas deferens in which capsazepine (10 μM) produced a rightward shift of the curve to capsaicin while ruthenium red (3 μM) depressed the maximal response to the agonist.

3 At the concentrations used to block the effect of capsaicin, neither capsazepine nor ruthenium red affected the contractile response of the rat urinary bladder produced by either neurokinin A or electrical field stimulation or the twitch inhibition produced by rat α-calcitonin gene-related peptide (αCGRP) in the vas deferens.

4 These findings provide additional evidence that both capsazepine and ruthenium red are valuable tools for exploration of the function of capsaicin-sensitive primary afferent neurones. The antagonism of the action of capsaicin by capsazepine is entirely consistent with the proposed interaction of this substance with a vanilloid receptor located on primary afferents, while the action of ruthenium red apparently involves a more complex, non-competitive antagonism.

Keywords: Capsaicin, primary afferent neurones; efferent function of sensory nerves; capsazepine; ruthenium red

Introduction

In recent years, much interest has developed in the pharmacological modulation of sensory neurone function (Maggi & Meli, 1988; Holzer, 1988; 1991; Maggi, 1991). A specific receptor on the cell membrane of primary afferent neurones has been recently discovered, which recognizes capsaicin and other natural pungent principles, including the ultrapotent capsaicin analogue, resiniferatoxin (Szallasi & Blumberg, 1990). This capsaicin or 'vanilloid' receptor fulfils several criteria to be considered the molecular target through which capsaicin and its congeners produce their specific actions on primary afferent neurones, which are characterized by long-lasting desensitization. Capsaicin also possesses non-specific actions (i.e. not restricted to primary afferent neurones) on nerves and smooth muscle which do not exhibit desensitization (Maggi & Meli, 1988; Holzer, 1991 for reviews). Studies from various laboratories have indicated that stimulation of the 'vanilloid' receptor on the membrane of primary afferent neurones is followed by the opening of a novel type of receptor-operated ion channel, which admits both sodium and calcium ions (Bevan & Szolcsanyi, 1991). It has recently been proposed that two different substances act as capsaicin antagonists, the inorganic dye ruthenium red (see Amann & Maggi, 1991 for review) which does not interact with the vanilloid receptor (Szallasi & Blumberg, 1990) and the benzazepine derivative, capsazepine (Bevan *et al.*, 1991; 1992; Dray *et al.*, 1991; Dickenson *et al.*, 1991; Urban & Dray, 1991), which was proposed as the first competitive antagonist of the capsaicin or vanilloid receptor.

Owing to the different mechanisms by which capsazepine and ruthenium red are thought to act as capsaicin antagonists, a direct comparison of these two drugs appeared of interest. With this aim we have chosen two preparations, the rat isolated urinary bladder and vas deferens, in which the selective action of capsaicin has been well characterized. In both preparations ruthenium red has been shown to antagonize the action of capsaicin by acting at a prejunctional site to inhibit the release of sensory neuropeptides which mediate the response to capsaicin (Maggi *et al.*, 1988a,b). The resultant contraction of the rat isolated bladder is mediated through the release of endogenous tachykinins (Maggi *et al.*, 1991); inhibition of electrically-evoked (field stimulation) twitches of the rat isolated vas deferens is mediated by the release of calcitonin gene-related peptide (CGRP) (Maggi *et al.*, 1987; Santicioli *et al.*, 1988). In both preparations a cumulative concentration-response curve to capsaicin has been used in order to determine the nature of the antagonism produced by capsazepine and ruthenium red.

Methods

Male albino rats of Wistar strain (body wt. 340–360 g) were stunned and bled. The whole urinary bladder and vasa deferentia were excised and placed in oxygenated (96% O_2 and 4% CO_2, pH 7.4) Krebs solution of the following composition (mM): NaCl 119, KCl 4.7, $MgSO_4$ 1.5, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11.

Contractile responses of the urinary bladder and vas deferens (pars prostatica) were studied as described previously (Maggi *et al.*, 1987; 1991). Briefly, iosmetric tension

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recording was made from 1 cm long longitudinal strips of rat detrusor muscle and segments of the vas deferens 1.5 cm in length under a load of 10 mN (bladder) and 5 mN (vas deferens). In both preparations, electrical field stimulation was carried out by means of two wire platinum electrodes placed at the top and the bottom of the organ bath which were connected to a GRASS S88 stimulator. Square wave pulses (pulse width 0.5 ms, 60 V) were automatically delivered at a frequency of 0.01 Hz (bladder) and 0.2 Hz (vas deferens).

Experiments started after a 60 min equilibration period. In the rat bladder the contractile response to KCl (80 mM) was determined at 15 min intervals until reproducible responses were obtained. This response to KCl was used as an internal standard to evaluate the maximal response to capsaicin.

The effects of capsazepine and ruthenium red were investigated on a concentration-response curve produced by addition of capsaicin (10 nM–10 μM). In order to avoid or minimize capsaicin desensitization (see Results), the capsaicin concentration was increased by a factor of 10 for each successive dose of the cumulative nerve. Each dose of the cumulative curve was added when the effect of the preceding one had reached its maximum. Only one concentration-response curve to capsaicin was obtained in each preparation. Several preparations were made from the same animal, one of which served as control while the others received capsazepine or ruthenium red before capsaicin application.

Contact time for capsazepine and ruthenium red was 30 min. To check whether capsazepine and ruthenium red may have affected the capsaicin response at the postjunctional level, their effect was studied on the contraction of the rat urinary bladder produced by neurokinin A (1–30 nM) and inhibition of twitch contractions of the vas deferens produced by rat αCGRP (1–300 nM).

Statistical analysis of the data was performed by means of the Student's *t* test for paired or unpaired data or by analysis of variance, when applicable. All values in the text, figures and table are means ± s.e.mean.

Stock solutions were prepared as follows: capsaicin to given a final concentration of 10–100 mM, was dissolved in absolute ethanol and then diluted in water. Ruthenium red (1–10 mM, Aldrich) was dissolved in water. Capsazepine (10–100 mM) was dissolved in dimethylsulphoxide. Rat αCGRP and neurokinin A (Peninsula) were dissolved in water. The vehicles used to dissolve capsaicin (0.01% ethanol) or capsazepine (0.03% dimethylsulphoxide) had no effect on the preparations.

Results

General

Cumulative addition of capsaicin (10 nM–10 μM) with a ten fold increase in concentration between doses produced a concentration-dependent contraction of the rat isolated bladder. The maximal effect was observed at 1 μM, after which tension declined and 10 μM capsaicin did not evoke a larger response. The maximal response to capsaicin obtained with this protocol of cumulative addition (48 ± 4% of the response to 80 mM KCl, mean ± s.e.mean, $n = 10$) was slightly less than the response (55 ± 3% of KCl response, $n = 8$) observed when 1 μM capsaicin was added to the bath as a single concentration.

In the rat isolated vas deferens, cumulative addition of capsaicin (10 nM–10 μM) produced concentration-dependent inhibition of electrically-evoked twitches. The maximal inhibitory effect was observed with 1 μM capsaicin and was followed by a gradual recovery of the twitches amplitude. Addition of 1 μM capsaicin to the bath as a single dose produced a transient inhibitory effect (58 ± 4%, $n = 6$, cf. Maggi *et al.*, 1987) which was not different from that obtained in response to 1 μM capsaicin using a cumulative protocol (54 ± 3% inhibition, $n = 8$).

In the vas deferens, the twitch inhibition is ascribable to a specific action of capsaicin on sensory nerves and release of CGRP (Maggi *et al.*, 1987). In this preparation, at a threshold concentration of 10 μM, capsaicin produces an opposite effect, twitch enhancement (not shown), as originally described by Moritoki *et al.* (1987). This effect, which is repeatedly observed upon consecutive application of capsaicin in the same preparation, is ascribable to a nonspecific effect of the drug (i.e. not involving activation of sensory nerves, Maggi & Meli, 1988) and limits the possibility of exploring the effects of capsazepine over a large range of concentrations.

Effect of capsazepine and ruthenium red in the rat urinary bladder

Neither capsazepine nor ruthenium red affected spontaneous motility of the rat urinary bladder.

Capsazepine (3–30 μM) produced a concentration-dependent rightward shift of the concentration-response curve to capsaicin in the rat bladder (Figure 1). In the presence of 3 μM capsazepine, the response to a threshold concentration of capsaicin (10 nM) was blocked but the maximal response to capsaicin was obtained at the same concentration (1 μM) as in controls. Ten and 30 μM capsazepine produced a further inhibition of the response to capsaicin and in this case the maximal response to the agonist was obtained at 10 μM capsaicin (Figure 1).

The maximal response to capsaicin in the presence of 3–30 μM capsazepine was not significantly different from that found in control experiments (Table 1).

Ruthenium red (3–10 μM) produced a concentration-dependent inhibition of the response to capsaicin in the rat bladder. As can be noted from curves presented in Figure 1b, the maximal response to capsaicin in the presence of ruthenium red was depressed as compared to controls.

Neither capsazepine (10–30 μM) nor ruthenium red (30 μM) significantly affected contractions produced by neurokinin A (Figure 1c and d) nor twitch contractions of the rat bladder produced by electrical field stimualtion at 0.01 Hz (Table 1).

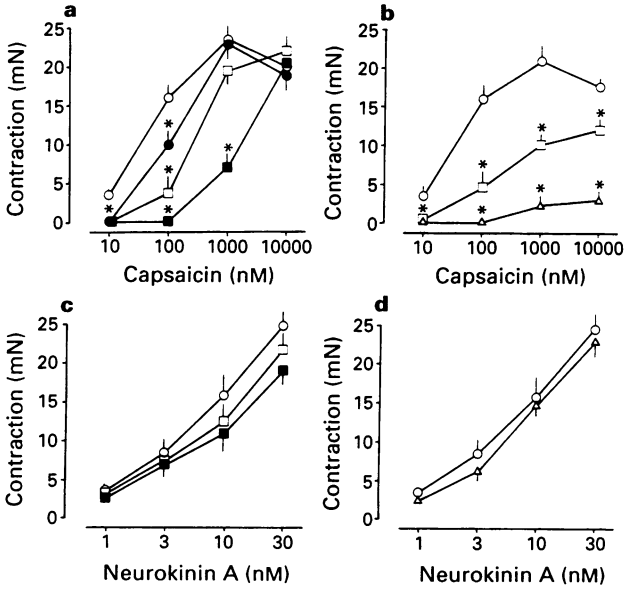


Figure 1 Effect of capsazepine (CPZ, a,c) or ruthenium red (RR, b,d) on concentration-response curve for capsaicin-induced (a,b) or neurokinin A-induced (c,d) contraction of the rat isolated urinary bladder. In (a) and (c): control (○); CPZ 3 μM (●); 10 μM (□); 30 μM (■). In (b) and (d): control (○); RR 10 μM (□); 30 μM (△). Each value is mean ± s.e.mean (vertical lines) of 4–12 experiments.

Table 1 Effect of capsazepine and ruthenium red on contractile responses of the rat isolated urinary bladder to various agents

	Response to EFS (mN	*Response to NKA* (30 nM) (mN)	*Maximal response to capsaicin* (mN)
Controls	13.8 ± 1.5	25 ± 2	23 ± 2
Capsazepine 3 μM	14.0 ± 2.5	NT	23 ± 6
Capsazepine 10 μM	13.2 ± 4.0	21 ± 2	22 ± 4
Capsazepine 30 μM	11.0 ± 1.2	19 ± 3	20 ± 4
Ruthenium red 10 μM	11.7 ± 3.0	NT	12 ± 2*
Ruthenium red 30 μM	12.5 ± 2.0	23 ± 2	3 ± 1*

Contractile response to electrical field stimulation (EFS, 0.01 Hz, 0.5 ms pulse width, maximal voltage), neurokinin A (NKA) or capsaicin are mean ± s.e.mean.
Number of experiments is 9–12 in the control group and 4–6 for each group with capsazepine or ruthenium red.
Significantly different from control: $*P<0.05$.

Effect of capsazepine and ruthenium red in the rat vas deferens

Capsazepine (10 μM) produced a slight (10–20%) enhancement of twitch contractions of the rat isolated vas deferens to electrical stimulation (0.2 Hz, 0.5 ms pulse width, maximal voltage). Capsazepine (10 μM) produced a parallel rightward shift of the concentration-response curve to capsaicin, with no apparent depression of maximal inhibitory response (Figure 2). In the presence of 10 μM capsazepine, the response to 10–100 nM capsaicin was virtually abolished and a concentration of 10 μM capsaicin was required to produce a transient maximal twitch inhibition. This effect was followed by a prompt recovery of twitch amplitude, possibly related to the nonspecific potentiating effect produced by capsaicin at 10 μM, as described above.

Ruthenium red (3 μM) inhibited twitch amplitude by 38 ± 5% ($n = 4$). In the presence of ruthenium red, the concentration-response curve to capsaicin was shifted to the right and maximum effect was depressed compared to controls (Figure 2). In Figure 2, the inhibitory effect of capsaicin in the presence of ruthenium red (and rat αCGRP, see below) is expressed as % reduction of the new baseline. The maximal twitch inhibition produced by 10 μM capsaicin in the presence of 3 μM ruthenium red (21 ± 3% inhibition) was significantly less than the maximal response obtained in either control preparations (55 ± 4% inhibition at 1 μM capsaicin) or in the presence of capsazepine (54 ± 3% inhibition at 10 μM capsaicin).

Rat αCGRP (10 nM–0.3 μM) produced a concentration-dependent inhibition of twitch amplitude in the rat isolated vas deferens, as described previouisly (Maggi *et al.*, 1987). The inhibitory effect of CGRP was unaffected by capsazepine (10 μM) or ruthenium red (3 μM) (Figure 2b).

Discussion

In this study we have used a cumulative protocol of capsaicin administration to investigate capsaicin antagonism by capsazepine and ruthenium red. It is well known that capsaicin produces a functional desensitization of primary afferent neurones. In descriptive terms, this means that, after application of the drug, the primary afferents become insensitive to further applications of capsaicin itself as well as to other agents which stimulate the primary afferent neurones. The capsaicin desensitization phenomenon is concentration-, time- and temperature-dependent (e.g. Amann, 1990) and probably involves different mechanisms such as a true desensitization (tachyphylaxis) of the vanilloid receptor (Dray *et al.*, 1989; Maggi *et al.*, 1990), blockade of transmitter release through the inactivation of voltage-sensitive calcium channels (Bleakman *et al.*, 1990; Docherty *et al.*, 1991) and degenerative changes in primary afferents owing to the marked influx of cations produced by capsaicin (Bevan & Szolcsanyi, 1990; Maggi, 1991; Holzer, 1991).

To overcome this problem, increasing concentrations of capsaicin were added to the bath with a ten fold increase in concentration at each step, while the effect of antagonists was compared to control curves obtained in preparations from

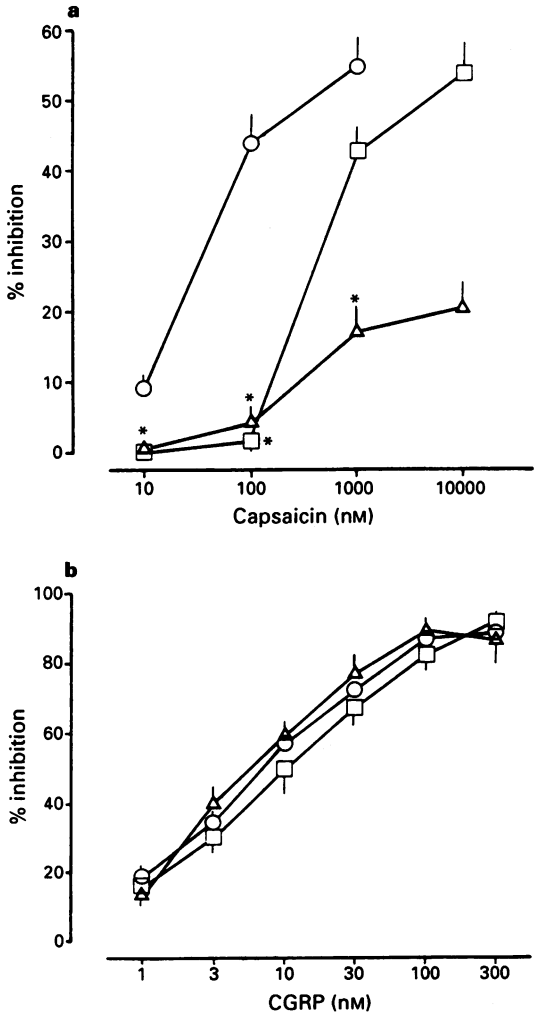


Figure 2 Effect of capsazepine (CPZ) or ruthenium red (RR) on concentration-response curve for capsaicin-induced (a) or rat α-calcitonin gene-related peptide (αCGRP)-induced (b) inhibition of twitch contractions of the rat isolated vas deferens: control (○); CPZ 10 μM (□); RR 3 μM (△). Each value is mean ± s.e.mean (vertical lines) of 4–12 experiments.

the same animal. The maximal response produced by cumulative application of capsaicin was only slightly less (bladder) or not significantly different (vas deferens) from that produced by the administration of a 1 μM single dose of capsaicin.

A drawback of the technique we have used is that the whole curve obtained with the agonist, either in the absence or presence of antagonists, is very steep and the few experimental points obtained in this way do not lend themselves to a conventional analysis for establishing whether competitive antagonism occurred (i.e. measurement of ED_{50}s, dose-ratios and Schild plots). In spite of this limitation, the different nature of antagonism by capsazepine and ruthenium red was very evident in both preparations investigated. Capsazepine essentially produced a rightward shift of the concentration-response curve to capsaicin without any significant depression of the maximum response. The failure of capsazepine to modify significantly the contractile response to neurokinin A in the bladder or to CGRP in the vas deferens ruled out a postjunctional action to inhibit the effect of endogenous neuropeptides released by capsaicin. Likewise, the failure of capsazepine to affect the amplitude of twitch contractions in both the bladder and the vas deferens excludes a nonspecific neuronal depressant action.

Overall, the present results are in good agreement with the competitive antagonist character of capsazepine action at the vanilloid receptor, as demonstrated in other test systems (Bevan *et al.*, 1991; 1992; Dray *et al.*, 1991).

In sharp contrast, ruthenium red displaced the curve to capsaicin in a manner that is indicative of non-competitive antagonism. The ability of ruthenium red to antagonize the action of capsaicin in the rat bladder and vas deferens at a prejunctional site of action was demonstrated in previous studies (Maggi *et al.*, 1988a,b) but the nature of antagonism (competitive vs. noncompetitive) had not been investigated. In contrast to capsazepine, ruthenium red at the concentration required to antagonize capsaicin, may have a neuronal depressant action not restricted to primary afferent neurones. This aspect of ruthenium red pharmacology, already noted in previous investigations (e.g. Chahl, 1989), is further confirmed here by its depressant action on twitch contractions of the isolated vas deferens. This effect of ruthenium red is however very variable from one preparation to another and cannot account for the ability of the dye to act as functional capsaicin antagonist (Amann & Maggi, 1991). Evidence has been presented (Dray *et al.*, 1990; Bleakman *et al.*, 1990) indicating that ruthenium red acts at the level of the cell membrane of primary afferent neurones to prevent the opening of the vanilloid receptor-operated cation channel. Although the molecular mechanism by which ruthenium red acts as a functional capsaicin antagonist has not yet been established, the non-competitive antagonism seen in this study is in keeping with its proposed site of action at a step beyond the occupation of the vanilloid receptor by the agonist.

The results of the present study, while confirming that both capsazepine and ruthenium red are useful tools to explore sensory neurone function, indicate that these two agents act at distinct steps in the sequence of events which is activated by capsaicin application. The different nature of the antagonism by capsazepine and ruthenium red is strongly reminiscent of the manner in which other drugs (e.g. bicuculline and picrotoxin at the $GABA_A$ receptor) act to inhibit receptor-operated ion channels and this parallel may be kept in mind in planning further experiments analysing the pharmacology of the vanilloid receptor and functional responses coupled to its activation.

References

AMANN, R. (1990). Desensitization of capsaicin-evoked neuropeptide release – influence of Ca^{2+} and temperature. *Naunyn Schmiedebergs Arch. Pharmacol.*, **342**, 671–676.

AMANN, R. & MAGGI, C.A. (1991). Ruthenium red as a capsaicin antagonist. *Life Sci.*, **49**, 849–856.

BEVAN, S.J., HOTHI, S., HUGHES, G., JAMES, I.F., RANG, H.P., SHAH, K., WALPOLE, C.S.J. & YEATS, J.C. (1992). Capsazepine: a competitive antagonist of the sensory neurone excitant capsaicin. *Br. J. Pharmacol.*, **107**, 544–552.

BEVAN, S.J., JAMES, I.F., RANG, H.P., SHAH, K. & YEATS, J.C. (1991). The development of a capsaicin antagonist for the sensory neurone excitant, capsaicin. *Br. J. Pharmacol.*, **102**, 77P.

BEVAN, S. & SZOLCSANYI, J. (1991). Sensory neuron-specific actions of capsaicin: mechanisms and applications. *Trends Pharmacol. Sci.*, **11**, 330–333.

BLEAKMAN, D., BRORSON, J.R. & MILLER, R.J. (1990). The effect of capsaicin on voltage-gated calcium currents and calcium signals in cultured dorsal root ganglion cells. *Br. J. Pharmacol.*, **101**, 423–431.

CHAHL, L.A. (1989). The effects of ruthenium red on the response of guinea-pig ileum to capsaicin. *Eur. J. Pharmacol.*, **169**, 241–247.

DICKENSON, A.H., DRAY, A., HUGHES, G.A. & WALPOLE, C.S.J. (1991). The selective antagonist capsazepine inhibits capsaicin induced antinociception: electrophysiological studies in rodents. *Br. J. Pharmacol.*, **102**, 79P.

DOCHERTY, R.J., ROBERTSON, B. & BEVAN, S. (1991). Capsaicin causes prolonged inhibition of voltage-activated calcium currents in adult rat dorsal root ganglion neurons in culture. *Neuroscience*, **40**, 513–521.

DRAY, A., BETTANEY, J. & FORSTER, P. (1989). Capsaicin desensitization of peripheral nociceptive fibers does not impair sensitivity to other noxious stimuli. *Neurosci. Lett.*, **99**, 50–54.

DRAY, A., FORBES, C.A. & BURGESS, G.M. (1990). Ruthenium red blocks the capsaicin-induced increase in intracellular calcium and activation of membrane currents in sensory nurones, as well as the activation of peripheral nociceptors in vitro. *Neurosci. Lett.*, **110**, 52–59.

DRAY, A., CAMPBELL, E.A., HUGHES, G.A., PATEL, I.A., PERKINS, M.N., RANG, H.P., RUEFF, A., SENO, N., URBAN, L. & WALPOLE, C.S.J. (1991). Antagonism of capsaicin-induced activation of C fibres by a selective capsaicin antagonist capsazepine. *Br. J. Pharmacol.*, **102**, 78P.

HOLZER, P. (1988). Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. *Neuroscience*, **24**, 739–768.

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

MAGGI, C.A. (1991). Capsaicin and primary afferent neurons: from basic science to human therapy? *J. Autonom. Nerv. System*, **33**, 1–14.

MAGGI, C.A., ASTOLFI, M., DONNERER, J. & AMANN, R. (1990). Which mechanisms account for the sensory neuron blocking action of capsaicin on primary afferents in the rat urinary bladder? *Neurosci. Lett.*, **110**, 267–272.

MAGGI, C.A., GIULIANI, S., SANTICIOLI, P. & MELI, A. (1987). Capsaicin-induced inhibition of motility of the rat isolated vas deferens: do multiple neuropeptides mediate the visceromotor effects of capsaicin? *J. Autonom. Pharmacol.*, **7**, 243–255.

MAGGI, C.A. & MELI, A. (1988). The sensory-efferent function of capsaicin-sensitive sensory neurons. *Gen. Pharmacol.*, **19**, 1–43.

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

MAGGI, C.A., PATACCHINI, R., SANTICIOLI, P., GIULIANI, S., GEPPETTI, P. & MELI, A. (1988a). Protective action of ruthenium red toward capsaicin desensitization of sensory fibers. *Neurosci. Lett.*, **88**, 201–205.

MAGGI, C.A., SANTICIOLI, P., GEPPETTI, P., PARLANI, M., ASTOLFI, M., PRADELLES, P., PATACCHINI, R. & MELI, A. (1988b). The antagonism induced by ruthenium red of the actions of capsaicin on the peripheral terminals of sensory neurons: further studies. *Eur. J. Pharmacol.*, **154**, 7–10.

MORITOKI, H., IWAMOTO, T., KANAYA, J., ISHIDA, Y., ANDO, K. & KITAGAWA, K. (1987). Capsaicin enhances the nonadrenergic twitch response of rat vas deferens. *Br. J. Pharmacol.*, **92**, 469–475.

SANTICIOLI, P., MAGGI, C.A., GEPPETTI, P., DEL BIANCO, E., THEODORSSON, E. & MELI, A. (1988). Release of calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) from organs of the genitourinary tract in rats. *Neurosci. Lett.*, **92**, 197–201.

SZALLASI, A. & BLUMBERG, P.M. (1990). Specific binding of resiniferatoxin: an ultrapotent capsaicin analog by dorsal root ganglion membranes. *Brain Res.*, **524**, 106–111.

URBAN, L. & DRAY, A. (1991). Capsazepine, a novel capsaicin antagonist, selectively antagonises the effects of capsaicin in the mouse spinal cord in vitro. *Neurosci. Lett.*, **134**, 9–11.

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Effects of the central analgesic tramadol on the uptake and release of noradrenaline and dopamine *in vitro*

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1 The centrally acting analgesic, tramadol, has low affinity for opioid receptors and therefore presumably other mechanisms of analgesic action. Neurotransmitter release and uptake experiments were used to characterize the effects of tramadol on the central noradrenergic and dopaminergic systems.

2 Tramadol inhibited the uptake of [^{3}H]-noradrenaline into purified rat hypothalamic synaptosomes with an IC_{50} of 2.8 μM; the (−)-enantiomer was about ten times more potent than the (+)-enantiomer. Results with the principal metabolite O-desmethyltramadol were very similar. Inhibition of dopamine uptake into purified rabbit caudate nucleus synaptosomes was very weak with 62% inhibition at 100 μM.

3 Rat occipital cortex slices were preincubated with [^{3}H]-noradrenaline and rabbit caudate nucleus slices with [^{3}H]-dopamine, then superfused and stimulated electrically. Tramadol, 1 and 10 μM, enhanced the stimulation-evoked [^{3}H]-noradrenaline overflow by 25 and 69%, respectively; the (−)-enantiomer was more potent than the racemate or the (+)-enantiomer. Tramadol, 10 μM, had no effect on dopamine release.

4 The effects of tramadol on the stimulation-evoked [^{3}H]-noradrenaline release were abolished when uptake sites were already blocked by a high concentration of cocaine.

5 The metabolite O-desmethyltramadol showed a slight facilitation of the stimulation-evoked noradrenaline release; the effect was more pronounced in the presence of a high concentration of naloxone. In the presence of cocaine, inhibition of the release was observed similar to the effect of morphine but less potent.

6 The results show that tramadol blocks noradrenaline uptake with selectivity as compared to dopamine uptake. The interaction with the noradrenaline transporter is stereoselective. The principal metabolite O-desmethyltramadol shows in addition to noradrenaline uptake inhibition, opioid inhibition of noradrenaline release.

Keywords: Tramadol; noradrenaline; dopamine; uptake; release

Introduction

Tramadol, (1**RS**; 2**RS**)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)-cyclohexanol hydrochloride, is a centrally acting analgesic with affinity for opioid receptors in the micromolar range (Hennies *et al.*, 1988; Raffa *et al.*, 1992), although its analgesic and antinociceptive potency is only five to ten fold lower than that of morphine. Actions other than opioid mechanisms may therefore contribute to its analgesic efficacy. This assumption is supported by the observation that in some experiments effects of tramadol are not fully antagonized by naloxone (Carlsson & Jurna, 1987; Kayser *et al.*, 1991; Raffa *et al.*, 1992). The noradrenergic system is involved in exogenously induced opioid and non-opioid antinociception (Sawynok, 1989), and a slight to moderate inhibition of noradrenaline uptake has been reported previously (Hennies *et al.*, 1982; Raffa *et al.*, 1992). Hence we investigated the interference of tramadol with the noradrenergic system more closely as regards specificity and stereoselectivity and examined in release studies, whether the effects were pure uptake inhibition since effects of tramadol in the 5-hydroxytryptaminergic system were more consistent with an action like an indirect mimetic (Driessen & Reimann, 1992).

Methods

General

Female Chinchilla cross rabbits weighing 2.5–4.2 kg and male Sprague Dawley rats weighing 180–380 g were killed by decapitation. The brain was quickly removed and chilled. Rabbit caudate nuclei were bluntly detached from the capsula interna, and rat hypothalami were dissected according to Glowinski & Iversen (1966). Slices from rabbit caudate nucleus (0.4 mm thick) and rat occipital cortex (0.4 mm thick, 5 mm diameter) were prepared as described and characterized previously (Starke *et al.*, 1978; Reimann *et al.*, 1981).

Outflow of tritium from slices after preincubation with ^{3}H-labelled catacholamines

Slices from rat occipital cortex were preincubated with 0.1 μM (−)-[^{3}H]-noradrenaline, and slices from rabbit caudate nucleus with 0.1 μM [^{3}H]-dopamine at 37°C for 30 min. After incubation, the slices were rinsed 3 times with medium and transferred to glass superfusion chambers equipped with platinum electrodes. They were superfused at 37°C with ^{3}H-catecholamine-free medium at a rate of 1 ml min^{-1} for 115–165 min. Five minute samples of the superfusate were collected 50 min after starting the superfusion. The slices were stimulated electrically for two periods of 2 min each, after 60 and 95 min ((−)-[^{3}H]-noradrenaline) or 60 and 120 min ([^{3}H]-dopamine) of superfusion (S_1, S_2). Rectangular pulses of 2 ms duration, 24 mA current strength and a frequency of 3 Hz were delivered from a stimulator constructed by the Biomedical Technics Department of Grünenthal. The slices were solubilized in 0.5 ml Soluene 350 (Packard) at the end of the experiment. The radioactivity in superfusates and slices was measured after addition of Ready Gel (Beckman) or Ready Safe (Beckman), respectively.

The incubation and superfusion medium contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 1.3, $MgSO_4$ 1.2, $NaHCO_3$ 25,

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KH_2PO_4 1.2, glucose 11, ascorbic acid 0.57, disodium EDTA 0.03; the medium was saturated with 5% CO_2 in O_2. The pH was adjusted to 7.4 by the addition of NaOH. Drugs were added 15 min before S_2 or were present throughout superfusion as indicated.

Accumulation of ^{3}H-labelled catecholamines in synaptosomes

Rat hypothalami or rabbit caudate nuclei were homogenized in 0.32 M ice-cold sucrose (3 ml 100 mg^{-1} tissue) in a glass homogenizer with a loosely fitting teflon pestle using ten full up and down strokes at 800 revolutions per min. The homogenate was filtered through Thomapor gauze 100 μm mesh and centrifuged at 1000g for 15 min at 4°C. Further subfractionation was performed according to Dodd *et al.* (1981). The supernatant was layered on 4.5 ml of 1.2 M sucrose and centrifuged at 190 000 g for 25 min. Particles at the gradient interface were collected in a volume of about 2.5 ml and diluted with ice-cold 0.32 M sucrose to a final volume of 8.0 ml. This suspension was layered on 4.5 ml 0.8 M sucrose and centrifuged again for 25 min at 190,000 g. The resulting synaptosomal pellet was gently resuspended in 4 ml ice-cold incubation medium/100 mg original tissue weight by use of a teflon/glass homogenizer.

The composition of the incubation medium was as follows (mmol l^{-1}): NaCl 119, KCl 3.9, $CaCl_2$ 0.51, $MgSO_4$ 0.65, Na_2HPO_4 15.6, NaH_2PO_4 3.4, glucose 10, ascorbic acid 0.57. Nialamide, 0.0125 mmol l^{-1}, was added for inhibition of monoamine oxidase. The pH was adjusted to 7.4 by addition of NaOH.

The synaptosomes were preincubated for 5 min at 37°C in the absence or presence of the drugs to be tested. The uptake was started by addition of 10 μl (−)-[^{3}H]-noradrenaline or [^{3}H]-dopamine yielding a concentration of 0.1 μM. The final incubation volume was 1 ml, and the protein concentration, determined according to Lowry *et al.* (1951), was 99–135 μg ml^{-1} in noradrenaline experiments and 21–74 μg ml^{-1} in dopamine experiments. The incubation lasted for 2 min in noradrenaline and 30 s in dopamine experiments if not stated otherwise. It was stopped by addition of 6 ml ice-cold medium, immediately followed by vacuum filtration through nitrocellulose membranes (pore size 0.65 μm; Sartorius) presoaked in incubation medium. Filters were washed 3 times with 4 ml cold medium. For determination of radioactivity, the wet filters were solubilized in 2 ml ethylene glycol monoethyl ether. Radioactivity was counted for after addition of 10 ml Ready Safe (Beckman). Values were corrected for the respective accumulation of radioactivity in synaptosomes incubated at 0°C. All assays were performed in triplicate.

Calculations and statistics

The fractional rate of tritium outflow in release experiments was calculated by dividing the tritium content in the superfusate by the tritium content in the slice at the start of the respective collection period. The stimulation-evoked overflow of tritium was calculated by subtraction of the estimated basal outflow and was expressed as a percentage of the tissue tritium content at the start of stimulation.

K_m and V_{max} were estimated by adaptation of the data to the Michaelis-Menten hyperbola and IC_{50}s by adaptation to the IB_1 or IB_2 model of the TOPFIT programme package (Thomae) on a Wang 2236-D type computer.

Means ± s.e.mean are given throughout. Differences between means were tested for significance by the Mann-Whitney test.

Compounds

(−)-[ring-2,5,6-^{3}H]-noradrenaline, specific activity 40.8 and 42.1 Ci $mmol^{-1}$, 3,4-[ring-2,5,6-^{3}H]-dopamine hydrochloride, specific activity 41 and 45.7 Ci $mmol^{-1}$ (New England Nuclear); cocaine HCl, morphine HCl (E. Merck); desipramine HCl (Sigma); naloxone HCl (DuPont); yohimbine HCl (Roth); tramadol HCl, (−)- and (+)-enantiomer (E 381, E382), O-desmethyltramadol HCl, (−)- and (+)-enantiomer (EM 724, EM 723; Grünenthal) were used. All drugs were dissolved in distilled water.

Results

Effects on tritium overflow from slices preincubated with [^{3}H]-noradrenaline

Slices from rat brain occipital cortex were preincubated with (−)-[^{3}H]-noradrenaline, then superfused with either drug-free medium or medium containing the drugs indicated and stimulated electrically for two periods (S_1, S_2). The test drugs were added to drug-free or drug-containing medium 15 min before S_2. The basal outflow of tritium was monitored in the collection periods preceding the stimulation. Drugs added before S_2 had only minor effects on the basal outflow of tritium not exceeding 12% deviation. Electrical stimulation at S_1 enhanced the overflow of tritium, about 5% of the tritium in the tissue appeared in the medium when no drug was present, and stimulation at S_2 induced on overflow of about the same amount as shown by the ratio S_2/S_1 near unity when no further drug was added. Tramadol, 1 and 10 μM, enhanced the stimulation-evoked overflow by 25 and 69%, respectively (Table 1; Figure 1). When the enantiomers were tested, the (−)-enantiomer was more active then the (+)-enantiomer, which showed significant effects only at 10 μM. Effects by tramadol were not changed, when α_2-adrenoceptors were blocked by a high concentration of yohimbine (Table 1). However, blockade of the noradrenaline uptake mechanism by cocaine abolished the facilitatory effects on the stimulation-evoked overflow almost completely (Table 1; Figure 2).

The O-demethylated metabolite of tramadol had only a weak facilitatory effect on the stimulation-evoked overflow, 17% at 10 μM, when no further drug was present (Table 1). The facilitatory effect became more prominent when naloxone was added to the medium as shown in Figure 3. An inhibition by O-desmethyltramadol of the stimulation-evoked overflow, 12% at 10 μM, became apparent in the presence of cocaine (Figure 3) and a similar effect was also observed, when desipramine, 1 μM, was added to the medium (not shown). In the presence of both drugs, cocaine and naloxone, no further effect by O-desmethyltramadol could be observed (Figure 3). In contrast, combination of the α_2-blocker yohimbine with naloxone did not attenuate the facilitation induced by O-desmethyltramadol (Table 1).

Morphine when added at S_2 depressed the stimulation-evoked overflow in a concentration-related manner; this effect was completely antagonized by the presence of naloxone (Table 1; Figure 3). The presence of cocaine had no distinct effect on the action of morphine. Naloxone, added at S_2, had no influence on the stimulation-evoked overflow, and cocaine itself effected a pronounced facilitation which was already observed at 0.1 μM and reached 58% at 1 μM (Table 1).

Effects on tritium overflow from slices preincubated with [^{3}H]-dopamine

Slices from rabbit caudate nucleus were preincubated with [^{3}H]-dopamine, then superfused with drug-free medium and stimulated electrically for two periods (S_1, S_2). Stimulation at S_1 evoked an overflow of 7.8 ± 0.3% ($n = 9$) of the tritium in the tissue. When no drug was added at S_2, the ratio S_2/S_1 was 0.98 ± 0.02 ($n = 3$). After addition of 10 μM of either tramadol or O-desmethyltramadol 15 min before S_2 the S_2/S_1 ratios were 1.04 ± 0.03 ($n = 3$) and 0.98 ± 0.00 ($n = 3$), respectively. Thus no changes of the stimulation-evoked overflow were

Table 1 Effects of tramadol, O-desmethyltramadol and reference compounds on the stimulation-evoked overflow of tritium from rat cerebral cortex slices preincubated with (−)-[^{3}H]-noradrenaline

Drug added at S_2	*Drug added throughout superfusion*	*S_2/S_1 at a drug concentration of* 0	0.1 μM	1 μM	10 μM
Tramadol	–	0.96 ± 0.01 (7)	0.96 ± 0.01 (7)	1.20 ± 0.06 (8)*	1.62 ± 0.03 (8)*
Tramadol (−)-enantiomer	–	0.94 ± 0.03 (6)	0.99 ± 0.04 (6)	1.18 ± 0.05 (6)*	1.82 ± 0.02 (6)*
Tramadol (+)-enantiomer	–	0.99 ± 0.02 (6)	0.96 ± 0.04 (6)	0.99 ± 0.03 (6)	1.17 ± 0.03 (6)*
Tramadol	Yohimbine 1 μM	0.96 ± 0.01 (5)		1.23 ± 0.02 (5)*	1.94 ± 0.06 (5)*
Tramadol	Cocaine 30 μM	1.03 ± 0.01 (6)	1.01 ± 0.02 (6)	1.03 ± 0.01 (6)	1.07 ± 0.01 (6)*
O-Desmethyltramadol	–	0.94 ± 0.01 (12)	0.91 ± 0.02 (5)	0.96 ± 0.03 (10)	1.10 ± 0.04 (11)*
O-Desmethyltramadol	Cocaine 30 μM	1.01 ± 0.01 (6)		0.96 ± 0.02 (6)*	0.89 ± 0.01 (6)*
O-Desmethyltramadol	Naloxone 1 μM	0.94 ± 0.02 (6)	0.95 ± 0.02 (5)	1.07 ± 0.02 (5)*	1.36 ± 0.06 (5)*
O-Desmethyltramadol	Cocaine 30 μM + Naloxone 1 μM	1.04 ± 0.03 (6)		0.99 ± 0.02 (6)	1.03 ± 0.03 (6)
O-Desmethyltramadol	Yohimbine 1 μM + Naloxone 1 μM	0.91 ± 0.01 (4)		1.06 ± 0.01 (5)*	1.51 ± 0.03 (6)*
Morphine	–	0.91 ± 0.02 (6)	0.83 ± 0.03 (6)	0.72 ± 0.03 (6)*	0.67 ± 0.03 (6)*
Morphine	Naloxone 1 μM	0.98 ± 0.02 (8)	0.95 ± 0.03 (6)	0.95 ± 0.02 (7)	0.94 ± 0.01 (8)
Morphine	Cocaine 30 μM	1.00 ± 0.04 (6)	0.97 ± 0.01 (6)	0.86 ± 0.01 (6)	0.83 ± 0.04 (6)*
Naloxone	–	1.01 ± 0.03 (4)	0.97 ± 0.01 (4)	1.06 ± 0.07 (4)	0.99 ± 0.04 (4)
Cocaine	–	0.96 ± 0.02 (6)	1.09 ± 0.01 (6)*	1.52 ± 0.07 (6)*	

After preincubation, the slices were superfused with medium containing no drug or the drugs indicated, and were stimulated electrically for two periods (S_1, S_2). Drugs given at S_2 were added to the medium 15 min prior to the onset of stimulation. Stimulation at S_1 evoked an overflow of 4.8 ± 0.1% (no drug added, $n = 174$), 9.3 ± 0.3% (yohimbine, $n = 15$), 9.2 ± 0.1% (cocaine, $n = 66$), 5.2 ± 0.1% (naloxone, $n = 50$), 8.2 ± 0.2% (cocaine and naloxone, $n = 18$) and 9.1 ± 0.4% (yohimbine and naloxone, $n = 18$) of the tritium in the tissue at the start of stimulation. Significant differences from corresponding controls (concentration of drug added at $S_2 = 0$): $^*P < 0.05$. Means ± s.e.mean of numbers of experiments indicated in parentheses.

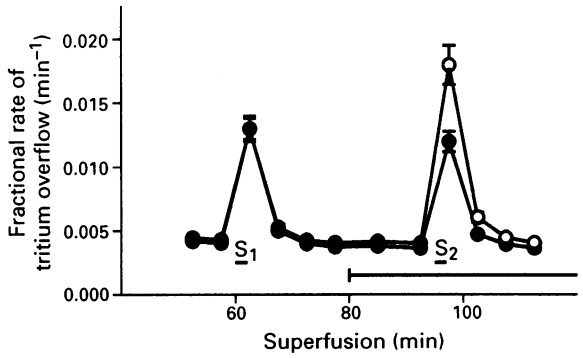


Figure 1 Effect of tramadol on the outflow of tritium from brain cortical slices preincubated with (−)-[^{3}H]-noradrenaline. After preincubation, slices were superfused with drug-free medium and were stimulated electrically for two periods (S_1, S_2). (●) Controls ($n = 7$); (○) tramadol, 10 μM, was added 15 min before S_2 as indicated by the bar ($n = 8$). s.e.mean shown by vertical bars.

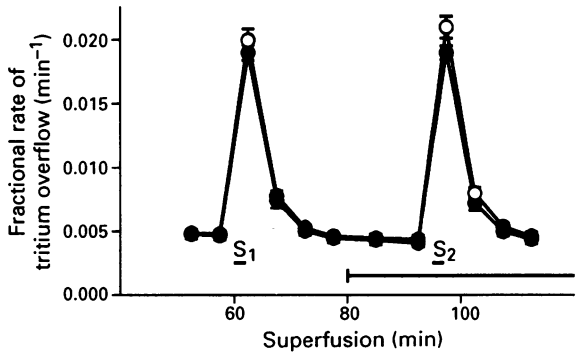


Figure 2 Effect of tramadol on the outflow of tritium from brain cortical slices preincubated with (−)-[^{3}H]-noradrenaline. After preincubation, slices were superfused with medium containing 30 μM cocaine and were stimulated electrically for two periods (S_1, S_2). (●) Controls ($n = 6$); (○) tramadol, 10 μM, was added 15 min before S_2 as indicated by the bar ($n = 6$). s.e.mean shown by vertical bars.

obvious, and nor were significant changes of the basal outflow observed.

Effects on the accumulation of [^{3}H]-noradrenaline in synaptosomes

Experiments evaluating the time course of (−)-[^{3}H]-noradrenaline accumulation in the purified hypothalamic synaptosomes showed linear uptake of tritium for 2 min (not shown). So this time period seemed adequate to approach the initial rate of uptake. Kinetic analysis showed an apparent K_m of 0.11 ± 0.02 μM and V_{max} was 16.2 ± 3.1 pmol 2 min^{-1} mg^{-1} protein ($n = 3$ with 7 concentrations per experiment).

Synaptosomes were incubated with (−)-[^{3}H]-noradrenaline either in the presence or absence of drugs. Accumulation of tritium under control conditions (no drug present) was 4.5 ± 0.2 pmol [^{3}H]-noradrenaline equivalent mg^{-1} protein ($n = 58$). The accumulation of radioactivity was potently inhibited by desipramine, the concentration-response relationship showed an obviously biphasic course (Figure 4). When the inhibition curve was analyzed with the IB2 model, the high potenty part of the inhibition with an IC_{50} value in the nanomolar range (Table 1) comprised 72 ± 2% of the total uptake; the other part of the uptake was inhibited with an IC_{50} in the micromolar range and constituted 28 ± 2% of the total uptake ($n = 6$). Cocaine, tramadol, O-desmethyltramadol (Figure 4) and both enantiomers of either tramadol or O-desmethyltramadol (not shown) displayed a steady inhibition of uptake in the concentration-range tested. IC_{50}s are summarized in Table 2. The uptake inhibition by the (−)-enantiomer of tramadol is about twice that of the racemate, and is about one order of magnitude more potent than that of the (+)-enantiomer. Results with O-desmethyltramadol and its enantiomers were very similar to those of

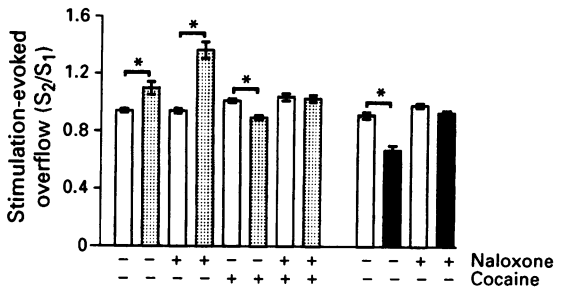


Figure 3 Effects of O-desmethyltramadol and morphine on the stimulation-evoked overflow of tritium from brain cortical slices preincubated with (−)-[^{3}H]-noradrenaline. After preincubation, slices were superfused with medium containing no drug or the drugs indicated below the abscissa scale. They were then stimulated electrically for two periods (S_1, S_2). Open columns represent control experiments with the indicated drugs contained in the medium. O-desmethyltramadol, 10 μM (stippled column) or morphine, 10 μM (solid column) were added 15 min before S_2. Columns represent mean ratios S_2/S_1 of 5–8 experiments; s.e.mean shown by vertical bars.

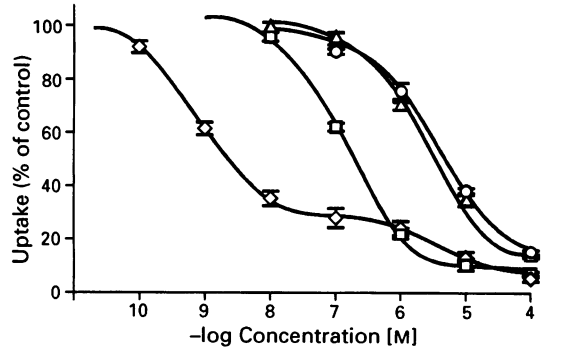


Figure 4 Effect of various drugs on the accumulation of tritium in hypothalamic synaptosomes incubated with 0.1 μM (−)-[^{3}H]-noradrenaline. The accumulation in the presence of desipramine (◇), cocaine (□), tramadol (△) and O-desmethyltramadol (○) is expressed as a percentage of control. Each data point represents the mean ± s.e.mean (vertical bars) of 4–10 experiments.

tramadol and its enantiomers (Table 2). Maximum inhibition at 100 μM amounted to 92.7 ± 0.7% with cocaine, 86.5 ± 0.5% with tramadol, 87.9 ± 0.8% with the (−)-enantiomer of tramadol, 75.1 ± 0.9% with the (+)-enantiomer, 82.7 ± 1.0% with O-desmethyltramadol, 86.5 ± 1.0% with its (−)-enantiomer and 60.5 ± 1.8% with its (+)-enantiomer. No inhibition of tritium accumulation by morphine could be observed (4.7 ± 4.6% inhibition at 100 μM; $n = 3$).

Effects on the accumulation of [^{3}H]-dopamine in synaptosomes

Preliminary experiments confirmed that the rate of accumulation of tritium in rabbit caudate nucleus synaptosomes was declining already at 30 s. This time period was therefore chosen for subsequent experiments. Kinetic analysis showed an apparent K_m of 0.11 ± 0.01 μM and V_{max} was 53.2 ± 15.9 pmol 30 s^{-1} mg^{-1} protein ($n = 4$ with 7 concentrations per experiment).

Synaptosomes were incubated with [^{3}H]-dopamine either in the presence or absence of drugs. Accumulation of tritium under control conditions was 21.7 ± 7.2 pmol [^{3}H]-dopamine equivalent mg^{-1} protein ($n = 9$). The accumulation was inhibited by nomifensine with an IC_{50} of 0.15 ± 0.02 μM ($n = 6$). Tramadol inhibited the uptake very weakly; only

Table 2 Effect of drugs on the accumulation of tritium in hypothalamic synaptosomes incubated with 0.1 μM (−)-[^{3}H]-noradrenaline

Drug	IC_{50} (μM)	n
Desipramine		
high potency range	0.00079 ± 0.00007	6
low potency range	7.2 ± 1.7	
Cocaine	0.16 ± 0.01	6
Tramadol	2.8 ± 0.3	6
Tramadol (−)-enantiomer	1.6 ± 0.3	6
Tramadol (+)-enantiomer	14.7 ± 0.7	6
O-Desmethyltramadol	4.8 ± 0.6	10
O-Desmethyltramadol (−)-enantiomer	1.4 ± 0.2	6
O-Desmethyltramadol (+)-enantiomer	41.4 ± 4.4	9
Morphine	>100	3

In each experiment, at least four drug concentrations were used. Means ± s.e.mean of numbers of individual experiments indicated.

small effects were observed at 1 μM and inhibition at 100 μM was 61.6 ± 5.9% ($n = 3$).

Discussion

Release of radiolabelled neurotransmitters from isolated tissues maintained *in vitro* is a frequently used model for the evaluation of presynaptic auto- and heteroreceptors (Langer, 1981; Starke, 1981; Chesselet, 1984; Starke *et al.*, 1989), and also of the noradrenaline uptake mechanisms as increased overflow can be observed with uptake inhibitors which reduce the fraction of the previously released transmitter which is normally subject to re-uptake (Starke *et al.*, 1971). The facilitating effect of the uptake inhibitor, cocaine, on the electrically-evoked noradrenaline overflow has been described (Starke & Montel, 1973) and was observed in the present experiments as well. An enhancement of the evoked overflow was also seen with tramadol. Differentiation between the possible mechanisms, α_2-autoreceptor antagonism or uptake blockade, was carried out either by means of receptor blockade by yohimbine or uptake site blockade by cocaine. Since effects were no longer observed when the function of the uptake sites was eliminated, in contrast to receptor blockade, evidence for an uptake blocking action of tramadol was provided. Efficacy seemed about one order of magnitude less than that of cocaine. The uptake inhibiting properties reside obviously in the (−)-enantiomer of tramadol. Direct α_2-adrenoceptor activation by tramadol which could have been masked by the effects of uptake inhibition was not observed in the presence of cocaine and can therefore be ruled out.

The naloxone-sensitive inhibition by morphine of noradrenaline release has long been known (Montel *et al.*, 1974; Henderson *et al.*, 1979) and was also observed in the present investigation. The lack of effect of naloxone alone (Montel *et al.*, 1974) indicates that noradrenaline release from cortical neurones is not tonically inhibited by endogenous opioids.

The principal metabolite O-desmethyltramadol has in [^{3}H]-naloxone binding experiments an approx. ten fold higher affinity for opioid receptors as compared to tramadol (IC_{50} 0.88 μM vs. 6.1 μM; Hennies *et al.*, 1988); it occurs in many species although in lower amounts in man as compared e.g. to rats and mice (Lintz *et al.*, 1981). In contrast to tramadol, its action on the stimulation-evoked noradrenaline overflow is apparently a combination of both noradrenaline uptake inhibition and opioid receptor activation. This becomes obvious when uptake sites are blocked by cocaine or opioid receptors are blocked by naloxone resulting in either inhibition or further enhancement of the evoked noradrenaline overflow. The assumuption of a dual mode of action is confirmed by the observation that in the presence of both

cocaine and naloxone, but not of yohimbine and naloxone, O-desmethyltramadol has no further effects on the release.

Actions by tramadol and its metabolite O-desmethyltramadol seem to be rather selective for uptake inhibition of the catecholamine noradrenaline since no effects were observed in dopamine release experiments. Previous experience, however, has shown that dopamine agonists and antagonists as well as uptake inhibitors have effects in these experiments (Starke et al., 1978; Reimann et al., 1979).

Although uptake inhibition can be specifically observed in functional studies such as neurotransmitter release experiments, quantification of the effects is usually performed in uptake experiments using synaptosomes. In order to approach true uptake, i.e. unidirectional transmitter flow, only the initial linear rate of uptake should be measured (Graefe, 1976). This precondition was met in the present investigation. Since transmitters may accumulate in other than their own terminals, e.g. noradrenaline is readily taken up in dopaminergic terminals (Iversen, 1974), regions for the preparation of synaptosomes were carefully chosen. The hypothalamus, which has a low amount of dopaminergic terminals, was used for noradrenaline uptake. The specific noradrenaline inhibitor desipramine inhibits the accumulation of [^{3}H]-noradrenaline in the nanomolar range and micromolar concentrations are necessary to inhibit the uptake in dopaminergic terminals. It has been shown that hypothalamic synaptosomes accumulate 74% of total [^{3}H]-noradrenaline in noradrenergic and 26% in presumably dopaminergic terminals (Michel et al., 1984). Our results concur with these findings and provide evidence that our synaptosomal preparation represented mainly noradrenergic terminals. Purification of the synaptosomes by gradient centrifugation was performed in order to minimize uptake in or attachment to other cell organelles.

Inhibition by desipramine and cocaine of noradrenaline uptake in our study was comparable to previously published data (Koe, 1976; Richelson & Pfenning, 1984). Inhibition by cocaine of total uptake was monophasic and almost complete; this is probably due to the potent and well-known interaction of cocaine with the dopamine transporter (Galloway, 1988). Tramadol inhibited more than 72% of the total uptake; the weak interference with the dopamine carrier observed in the dopamine uptake experiments is therefore presumed to contribute to the overall effect. If effects were only related to the noradrenergic proportion of the synaptosomes (= 72% of total uptake), a higher efficacy with an IC_{50} of about 1.5 μM became apparent. The efficacy in the uptake experiments is in good agreement with the effects observed in the release experiments. It can also be observed, that effects of the metabolite on uptake inhibition are very similar to those of the parent substance.

When the effects of tramadol on noradrenaline and dopamine uptake are compared, it is obvious that there is a selectivity of about two orders of magnitude towards the noradrenergic system. The noradrenergic carrier shows, in contrast to the dopaminergic carrier, stereoselectivity for noradrenaline uptake (Iversen, 1974); the stereoselective inhibition by tramadol and O-desmethyltramadol, the (−)-enantiomer being one order of magnitude more potent, provides further evidence for a specific interaction with the noradrenaline uptake system.

Apparent neurotransmitter uptake inhibition in experiments measuring accumulation of transmitter may be the result of the induction of neurotransmitter release (Baumann & Maitre, 1976; Maxwell et al., 1976). This misinterpretation of uptake inhibition can be avoided by the concomitant investigation of the release. These effects would impose as acceleration of the basal outflow. This effect of tramadol was observed in 5-hydroxytryptamine release experiments (Driessen & Reimann, 1992). However, concentrations of tramadol which inhibited the noradrenaline uptake in mainly noradrenergic synptosomes had no distinct effect on the basal outflow of noradrenaline in a tissue slice release model, providing evidence for a true interaction with the uptake mechanism. This assumption is further supported by the functional interaction of tramadol and O-desmethyltramadol with the uptake inhibitor cocaine.

Morphine exerts its antinociceptive effects in part by supraspinal activation of spinal descending noradrenergic pathways (Yaksh, 1988). Locally, at the nerve terminal, morphine interferes with the release of noradrenaline and may thus attenuate its own efficacy. Reinforcement of the noradrenergic neurotransmission might therefore add to opioid efficacy, and, in fact, it has been shown in rats, that the noradrenaline uptake inhibitor desipramine enhanced the antinociceptive actions of systemically or centrally administered morphine (Ossipov et al., 1982). Relevant tramadol concentrations in the CNS can be supposed, since experiments on antinociception in the mouse tail-flick test have shown, that plasma concentrations of tramadol range from 0.8 μM at the threshold to 10.8 μM at the maximum effective dose (Friderichs & Becker, 1991). Investigations of positron emission following [^{11}C]-tramadol injection showed, within minutes, at least four fold higher concentrations in the CNS as compared to plasma levels in the mouse and in man while O-desmethyltramadol levels only slowly approach those in plasma (G. Stoecklin, KFA Jülich, personal communication). Further support for the assumption of a participation of the noradrenaline uptake inhibition in the analgesic effect of tramadol is derived from the observation that spinal antinociceptive effects of tramadol are antagonized by the α_2-adrenoceptor blocker, yohimbine (Driessen et al., 1990; Raffa et al., 1992).

In conclusion, tramadol and its principal metabolite O-desmethyltramadol inhibited noradrenaline uptake in a synaptosomal preparation in a specific and stereoselective fashion. The functional consequences of this mode of action were confirmed in the release experiments. In these experiments the mode of action was further confirmed by the functional interaction of tramadol and O-desmethyltramadol with the uptake inhibitor, cocaine. Antinociception by opioids is in part mediated by supraspinal activation of spinal descending noradrenergic pathways (Yaksh, 1988; Sawynok, 1989). While morphine interferes locally with the release of noradrenaline and may thus attenuate its own effects, reinforcement of noradrenergic neurotransmission by tramadol may add to its analgesic efficacy.

Results are part of the doctoral thesis of B.D.

References

BAUMANN, P.A. & MAITRE, L. (1976). Is drug inhibition of dopamine uptake a misinterpretation of in vitro experiments? *Nature*, **264**, 789–790.

CARLSSON, K.H. & JURNA, I. (1987). Effects of tramadol on motor and sensory responses of the spinal nociceptive system in the rat. *Eur. J. Pharmacol.*, **139**, 1–10.

CHESSELET, M.-F. (1984). Presynaptic regulation of neurotransmitter release in the brain: facts and hypothesis. *Neuroscience*, **12**, 347–375.

DODD, P.R., HARDY, J.A., OAKLEY, A.E., EDWARDSON, J.A., PERRY, E.K. & DELAUNOY, J.-P. (1981). A rapid method for preparing synaptosomes: comparison with alternative procedures. *Brain Res.*, **226**, 107–118.

DRIESSEN, B. & REIMANN, W. (1992). Interaction of the central analgesic, tramadol, with the uptake and release of 5-hydroxytryptamine in the rat brain *in vitro*. *Br. J. Pharmacol.*, **105**, 147–151.

DRIESSEN, B., SCHLÜTZ, H. & REIMANN, W. (1990). Evidence for a non-opioid component in the analgesic action of tramadol. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **341**, R104.

FRIDERICHS, E. & BECKER, R. (1991). Correlation of tramadol and M_1 serum levels with antinociceptive activity in mice. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **343**, R9.

GALLOWAY, M.P. (1988). Neurochemical interactions of cocaine with dopaminergic systems. *Trends Pharmacol. Sci.*, **9**, 451–454.

GLOWINSKI, J. & IVERSEN, L.L. (1966). Regional studies of catecholamines in the rat brain. I. The disposition of [^{3}H]norepinephrine, [^{3}H]dopamine and [^{3}H]DOPA in various regions of the brain. *J. Neurochem.*, **13**, 655–669.

GRAEFE, K.-H. (1976). Methodology of catecholamine transport studies: definition of terms. In *The Mechanism of Neuronal and Extraneuronal Transport of Catecholamines*. ed. Paton, D.M. pp. 7–35. New York: Raven Press.

HENDERSON, G., HUGHES, J. & KOSTERLITZ, H.W. (1979). Modification of catecholamine release by narcotic analgesics and opioid peptides. In *The Release of Catecholamines from Adrenergic Neurons*. ed. Paton, D.M. pp. 217–228. Oxford: Pergamon Press.

HENNIES, H.-H.,FRIDERICHS, E. & SCHNEIDER, J. (1988). Receptor binding, analgesic and antitussive potency of tramadol and other selected opioids. *Arzneim-Forsch./Drug Res.*, **38**, 877–880.

HENNIES, H.-H., FRIDERICHS, E., WILSMANN, K. & FLOHÉ, L. (1982). Effect of the opioid analgesic tramadol on inactivation of norepinephrine and serotonin. *Biochem. Pharmacol.*, **31**, 1654–1655.

IVERSEN, L.L. (1974). Uptake mechanisms for neurotransmitter amines. *Biochem. Pharmacol.*, **23**, 1927–1935.

KAYSER, V., BESSON, J.-M. & GUILBAUD, G. (1991). Effects of the analgesic agent tramadol in normal and arthritic rats: comparison with the effects of different opioids, including tolerance and cross-tolerance to morphine. *Eur. J. Pharmacol.*, **195**, 37–45.

KOE, B.K. (1976). Molecular geometry of inhibitors of the uptake of catecholamines and serotonin in synaptosomal preparations of rat brain. *J. Pharmacol. Exp. Ther.*, **199**, 649–661.

LANGER, S.Z. (1981). Presynaptic regulation of the release of catecholamines. *Pharmacol. Rev.*, **32**, 337–362.

LINTZ, W., ERLAÇIN, S., FRANKUS, E. & URAGG, H. (1981). Metabolismus von Tramadol bei Mensch und Tier. *Arzneim. Forsch./Drug Res.*, **31**, 1932–1943.

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

MAXWELL, R.A., FERRIS, R.M. & BURCSU, J.E. (1976). Structural requirements for inhibition of noradrenaline uptake by phenethylamine derivatives, desipramine, cocaine, and other compounds. In *The Mechanisms of Neuronal and Extraneuronal Transport of Catecholamines*. ed. Paton, D.M. pp. 95–153. New York: Raven Press.

MICHEL, M., HIEMKE, C. & GHRAF, R. (1984). Preferential uptake of norepinephrine into dopaminergic terminals of a synaptosomal preparation from rat cerebral cortex. *Brain Res.*, **301**, 149–152.

MONTEL, H., STARKE, K. & WEBER, F. (1974). Influence of morphine and naloxone on the release of noradrenaline from rat brain cortex slices. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **283**, 357–369.

OSSIPOV, M.H., MALSEED, R.T. & GOLDSTEIN, F.J. (1982). Augmentation of central and peripheral morphine analgesia by desipramine. *Arch. Int. Pharmacodyn.*, **259**, 222–229.

RAFFA, R.B., FRIDERICHS, E., REIMANN, W., SHANK, R.P., CODD, E.E. & VAUGHT, J.L. (1992). Opioid and nonopioid components independently contribute to the mechanism of action of tramadol, an 'atypical' opioid. *J. Pharmacol. Exp. Ther.*, **260**, 275–285.

REIMANN, W., STEINHAUER, H.B., HEDLER, L., STARKE, K. & HERTTING, G. (1981). Effect of prostaglandins D_2, E_2 and $F_{2\alpha}$ on catecholamine release from slices of rat and rabbit brain. *Eur. J. Pharmacol.*, **69**, 421–427.

REIMANN, W., ZUMSTEIN, A., JACKISCH, R., STARKE, K. & HERTTING, G. (1979). Effect of extracellular dopamine on the release of dopamine in the rabbit caudate nucleus: evidence for a dopaminergic feedback inhibition. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **306**, 53–66.

RICHELSON, E. & PFENNING, M. (1984). Blockade by antidepressants and releated compounds of biogenic amine uptake into rat brain synaptosomes: most antidepressants selectively block norepinephrine uptake. *Eur. J. Pharmacol.*, **104**, 277–286.

SAWYNOK, J. (1989). The 1988 Merck Frosst Award. The role of ascending and descending noradrenergic and serotonergic pathways in opioid and non-opioid antinociception as revealed by lesion studies. *Can. J. Physiol. Pharmacol.*, **67**, 975–988.

STARKE, K. (1981). Presynaptic Receptors. *Annu. Rev. Pharmacol. Toxicol.*, **21**, 7–30.

STARKE, K., GÖTHERT, M. & KILBINGER, H. (1989). Modulation of neurotransmitter release by presynaptic autoreceptors. *Physiol. Rev.*, **69**, 964–989.

STARKE, K. & MONTEL, H. (1973). Alpha-receptor-mediated modulation of transmitter release from central noradrenergic neurones. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **279**, 53–60.

STARKE, K., MONTEL, H. & WAGNER, J. (1971). Effect of phentolamine on noradrenaline uptake and release. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **271**, 181–192.

STARKE, K., REIMANN, W., ZUMSTEIN, A. & HERTTING, G. (1978). Effect of dopamine receptor agonists and antagonists on release of dopamine in the rabbit caudate nucleus in vitro. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **305**, 27–36.

YAKSH, T.L. (1988). CNS mechanisms of pain and analgesia. *Cancer Surv.*, **7**, 5–28.

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Use-dependent block of Ca^{2+} current by moricizine in guinea-pig ventricular myocytes: a possible ionic mechanism of action potential shortening

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1 Whole cell patch clamp techniques were used to study the effects of moricizine on membrane currents in guinea-pig ventricular myocytes.

2 Application of moricizine caused reversible depression of the time-dependent outward K^+ current.

3 The Na^+/Ca^{2+} exchange current was not directly affected by moricizine.

4 Although moricizine hardly affected the L-type Ca^{2+} current when cells were stimulated at a frequency of 0.1 Hz, it suppressed the current at depolarized holding potentials in a use-dependent manner at 1 Hz.

5 Developments of use-dependent block of the Ca^{2+} current in the presence of moricizine were best expressed by two exponentials. Binding to both activated and inactivated states of the Ca^{2+} channel were supported from the binding kinetics study.

6 We concluded that moricizine suppressed the L-type Ca^{2+} current in a use-dependent manner and this might explain, at least in part, action potential shortening by the drug.

Keywords: Moricizine; action potential duration; use-dependent block of Ca^{2+} current; guinea-pig ventricular myocytes

Introduction

Moricizine hydrochloride (ethmozine) has as its main action the depression of cardiac Na^+ channels, displaying potent local anaesthetic and membrane-stabilizing properties (Fitton & Buckley, 1990). Since cellular electrophysiological studies provided slow onset and offset kinetics on Na^+ current depression (Makielski *et al.*, 1988; Rosenshtraukh *et al.*, 1987), moricizine is classified as a class Ic antiarrhythmic agent (Vaughan Williams, 1991), although different views as to its subclassification have been presented (Mann, 1990; Fitton & Buckley, 1990; Lee & Rosen, 1991). Although the clinical efficacy of moricizine – a decreased mortality of patients after myocardial infarction with asymptomatic ventricular depolarizations – has not been proved (The Cardiac Arrhythmia Suppression Trial (CAST) Investigators, 1989), it has beneficial effects in the treatment of symptomatic life-threatening arrhythmias.

In addition to depressing the Na^+ current, moricizine has been shown to exert additional effects on electrophysiological properties. Moricizine speeds up repolarization to shorten action potential duration (APD), and to abolish early afterdepolarization (Danilo *et al.*, 1977; Ruffy *et al.*, 1979; Tsuji *et al.*, 1983; January *et al.*, 1988). The drug suppresses normal automaticity in Purkinje fibres, not mediated through effects on slow diastolic depolarization (Dangman & Hoffman, 1983), and depresses delayed afterdepolarization in the subacute phase of myocardial infarction and in digitalis toxicity (Danilo *et al.*, 1977; Hewett *et al.*, 1983).

Drug effects on action potential repolarization are regarded as an important factor for determining antiarrhythmic as well as proarrhythmic actions. Since the ionic mechanism by which moricizine shortens APD has not been elucidated, we studied its effects on membrane currents forming the repolarization phase of ventricular action potentials in guinea-pig ventricular myocytes.

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Methods

Myocyte preparations

Single ventricular myocytes from guinea-pig hearts were prepared by an enzymatic dissociation procedure as described previously (Hirano & Hiraoka, 1988). Isolated single cells were transferred to a recording chamber placed on the stage of an inverted phase-contrast microscope (Diaphot TMD, Nikon Co., Tokyo). The temperature of the bath chamber was maintained at 34–35°C.

Solutions

The composition of the Tyrode solution was (mM): NaCl 144.0, KCl 4.0, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5, NaH_2PO_4 0.33, and HEPES 5.0; pH was adjusted to 7.3–7.4 by addition of NaOH. The standard pipette solution was composed of (mM): KCl 130.0, K_2ATP 5.0, K_2creatine phosphate 5.0, EGTA 0.2 and HEPES 5.0. For measuring the Na^+/Ca^{2+} exchange current, 20 μM ouabain and 2 μM verapamil were added to the external solution to block Na-K ATPase and the Ca^{2+} current, respectively. The external solution contained (mM): $CaCl_2$ 0.1 or 1, NaCl 140, $MgCl_2$ 2, HEPES 5, CsCl 2, $BaCl_2$ 2, and the pipette solution contained (mM): NaCl 20, CsCl 130, $CaCl_2$ 21, EGTA 42 (pCa 7.2), tetraethylammonium (TEA)-Cl 20, aspartic acid 42, MgATP 10, K_2 creatine phosphate 5, $MgCl_2$ 3 and HEPES 5; pH was adjusted to 7.4 with CsOH. For isolation of the L-type Ca^{2+} current, a TEA-rich solution was used as the external perfusate which had the following composition (mM): TEA-Cl 154, $CaCl_2$, 1.8, $MgCl_2$ 2, glucose 5.5 and HEPES 5. The pipette solution contained (mM): CsCl 140, $CaCl_2$ 1, MgATP 5, K_4BAPTA 5 and HEPES 10.

Recording techniques

Membrane currents were recorded by a whole-cell patch-clamp technique using an amplifier (EPC-7, List Co., Darm-

stadt, Germany). Details of the recording technique have been described in our previous paper (Hirano & Hiraoka, 1988). At the start of each experiment, the junction potential was adjusted to zero immediately before the cell was attached and it was checked again after the end of experiments. If the difference in the two values exceeded ± 2 mV, membrane potentials were corrected accordingly. The series resistance compensation was done to minimize the capacitive surge in the current record just after the cell membrane was ruptured, and then voltage steps of various protocols were applied as indicated in the text. The resistance of the pipette filled with standard pipette solution was 2–4 MΩ. Current and voltage signals were stored on a magnetic tape of a data-recorder (A-45, SONY Co., Tokyo, Japan) using a PCM converter system (RP-880, NF Instruments, Yokohama, Japan) for later analysis.

Data analysis and curve fitting

To obtain the kinetics of the Ca^{2+} current (I_{Ca}) block by the drug, the time course of development was assessed by a curve-fitting procedure using a simple nonlinear least-squares algorithm on a personal computer (IBM-AT). The formula for the curve fitting used in this study is:

$$I_{Ca} = A_1\exp(-bC1) + A_2\exp(-bC_2) + A_3\exp(-bC_3) + A$$

where b is the pulse number and C_1, C_2, and C_3 are onset rates. Applicability of either one, two or three exponential functions was always examined using the F test, and $P<0.05$ was considered significant. The F test was assessed if adding an additional exponential to the fit resulted in a better fit. All the values were expressed as mean ± s.e.mean. Statistical analysis except curve fitting was carried out by the paired or unpaired t test, and $P<0.05$ was considered significant.

Results

Effects of moricizine on membrane currents forming the repolarization of action potentials

With the voltage clamp experiments, we studied the effects of moricizine on transmembrane currents forming the plateau and repolarization phase of action potentials. First we tested the effect on time-dependent outward K^+ current in the Tyrode solution. Outward tail currents upon repolarization following 500 ms voltage steps from a holding potential of −40 mV to a test potential of +40 mV were elicited at a pulse frequency of 0.1 Hz. Application of 10 μM moricizine was started at 7 min and continued up to 13 min after the formation of whole cell configuration. Figure 1 shows the relations between the time course and the relative value of the peak tail currents normalized by the value at 3 min after the rupture of the membrane. In the absence of drug, the current decayed with time and the average value of relative currents at 20 min was 0.48 ± 0.11 ($n = 4$). In the presence of 10 μM moricizine, the tail currents decreased progressively with time and the minimum value of 0.29 ± 0.07 ($n = 4$) was reached at 13 min after rupture of the membrane (or 6 min after the application of moricizine). The tail currents showed significant recovery after the wash-out of the drug in all four cases.

It is suggested that the Na^+/Ca^{2+} exchange current plays an important role in maintaining action potential duration (Carmeliet, 1990) and the action of moricizine on this current is implicated as a possible factor for APD shortening (Vaughan Williams, 1991). Therefore, we tested the effect of moricizine on the Na^+/Ca^{2+} exchange current. Figure 2a shows a typical example of control I-V curve obtained by applying a ramp pulse of ± 100 mV s^{-1} from a holding potential of −30 mV. Changing external Ca^{2+} from 0.1 to 1 mM induced an outward current. Since 5 mM Ni^+ almost completely blocked the increased outward current by raising

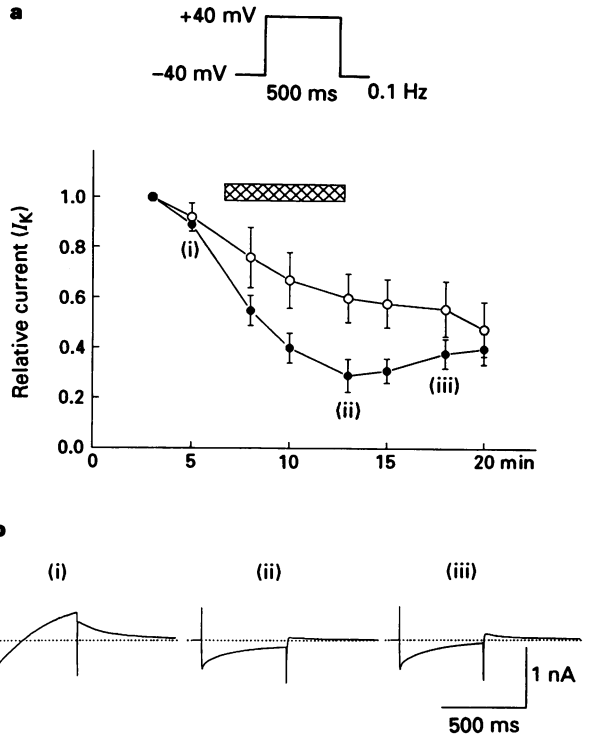


Figure 1 Effect of moricizine on the time-dependent outward K^+ current: (a) shows the relations between the time course and the relative value of the peak tail currents normalized by the value at 3 min. Outward tail currents were induced by a voltage protocol indicated at the top at a pulse frequency of 1 Hz. (○) Control; (●) moricizine. The crosshatched bar indicates the period during which moricizine 10 μM was applied. The relative currents in the presence of moricizine were significantly depressed compared to the control and the current recovered after wash-out of the drug. Values and bars indicate the mean ± s.e.mean of 4 experiments. $^*P<0.05$. (b) shows examples of the current record from a typical experiment at the time indicated in (a). Dashed line represents the holding current level. Notice that the tail current was decreased in (ii) and recovered in (iii).

external Ca^{2+} (not shown), the outward current was considered to be generated by the electrogenic Na^+/Ca^{2+} exchange (Kimura *et al.*, 1987). While 10 μM moricizine did not affect the current in 1 mM Ca^{2+} solution, reduction of external Ca^{2+} to 0.1 mM caused a decrease in the outward current (Figure 2b). Essentially similar results were confirmed in three other myocytes.

Since the L-type Ca^{2+} current is an important determinant of the plateau and repolarization phase of cardiac action potentials (Carmeliet, 1990), we examined the effects of moricizine on this current. It is well known that class I antiarrhythmic agents produce tonic and use-dependent block of Na^+ current (Hondeghem & Katzung, 1977). This raises a question whether moricizine exhibits tonic and/or use-dependent block of Ca^{2+} channels similar to its actions on the fast Na^+ channel (Rosenshtraukh *et al.*, 1987; Makielski *et al.*, 1988). We first studied the tonic block of isolated L-type Ca^{2+} current (I_{Ca}) by moricizine. Tonic block was defined as a diminution in the peak I_{Ca} of the first depolarizing pulse after exposure to moricizine compared to that in the control. In the tonic block study, a holding potential was set at −80 mV to obtain the rested state of the L-type Ca^{2+} channel and a test potential of + 20 mV was chosen, where the T-type Ca^{2+} current was small at this voltage (Bean, 1985). As shown in Figure 3, 10 μM moricizine did not suppress the L-type Ca^{2+} current. On average, tonic block at 10 μM moricizine was 4.7 ± 2.8% ($n = 4$), which was not

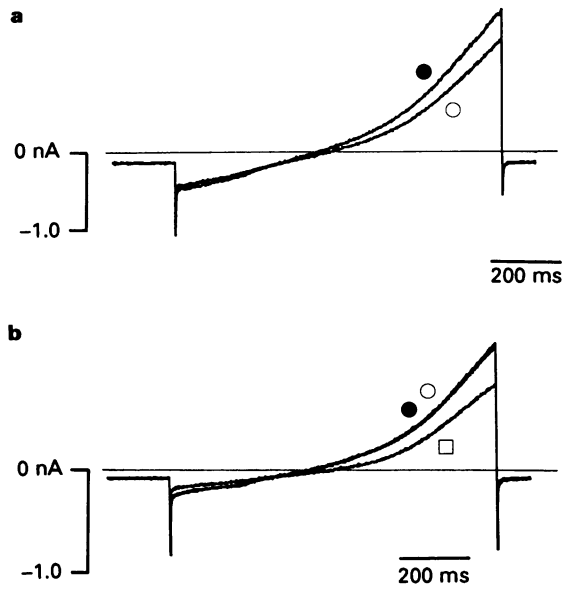


Figure 2 Effect of moricizine on Na^+/Ca^{2+} exchange current: (a) demonstrates the outward current elicited by increasing external Ca^{2+} concentration from 0.1 mM (○) to 1 mM (●). The increased outward current under these conditions was thought to be outward Na^+/Ca^{2+} exchange current. (b) Shows the records from another cell with external Ca^{2+} concentration of 1 mM before (○) and after (●) application of 10 μM moricizine. After application of moricizine for 5 min, without effects on the current, the external Ca^{2+} concentration was decreased to 0.1 mM (□). The current was elicited by ramp pulses of ± 100 mV s^{-1} from a holding potential of −30 mV at a frequency of 0.1 Hz. Horizontal line represents the zero-current level.

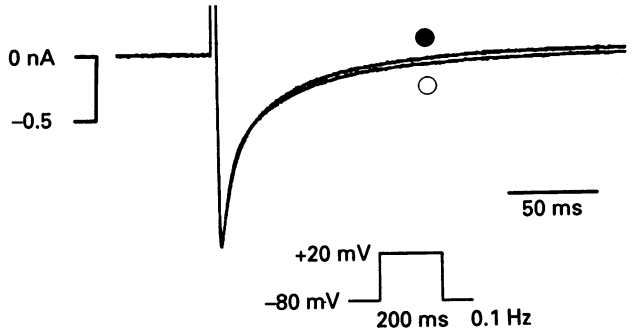


Figure 3 Effects of moricizine on the isolated L-type Ca^{2+} current at the rested state. The records were obtained from a cell treated with the external tetraethylammonium (TEA) and internal Cs^+ to block the K^+ currents. Voltage protocol is indicated below the figure. A holding potential was set at −80 mV to obtain the rested state of the Ca^{2+} channel. The traces are Ca^{2+} current records before (○) and 5 min after (●) application of 10 μM moricizine.

significantly different from the control, and, therefore, the drug seemed to have no effects on the rested state of the Ca^{2+} channel.

We next studied the possibility of use-dependent suppression of the Ca^{2+} current by moricizine, an effect that had been proposed by Scamps *et al.* (1989) on frog ventricular cells. The use-dependent block of the Ca^{2+} current was examined by changing the holding potential of −80 mV to −40 mV or −60 mV and by applying 30 depolarizing pulses of 200 ms duration at 1 Hz. Figure 4 shows an example of use-dependent block of I_{Ca} by moricizine. When a train of 30 depolarizing pulses were applied to + 20 mV from a holding potential of −40 mV, the peak Ca^{2+} currents were decreased by 15% during the pulses in the absence (Figure 4a) and by 40% in the presence of the drug (Figure 4b). A reduction in I_{Ca} in control conditions was due to the incomplete recovery from the inactivated states of the Ca^{2+} channel. Here, the decrease in I_{Ca} at the 30th pulse relative to the 1st one was tentatively regarded as degree of 'use-dependent block'. On average, the degree of use-dependent block in the control condition was 30.5 ± 7.3% ($n = 4$) and 9.6 ± 1.7% ($n = 6$) with the holding potential at −40 mV and −60 mV, respectively. After exposure to 10 μM moricizine, the degrees were significantly increased to 47.4 ± 8.1% ($P<0.05$) with the holding potential at −40 mV and 22.9 ± 1.9% ($P<0.01$) at −60 mV.

Blocking kinetics of the Ca^{2+} channel by moricizine

As shown in Figure 5, while the fall in peak I_{Ca} was best fitted by a single exponential in the control condition, double exponential function of the block development was observed after exposure to 10 μM moricizine in 3 preparations examined. On average, in the control, peak I_{Ca} fell with the very slow time constant of 0.0027 ± 0.0008 $pulse^{-1}$ ($n = 3$), while during the perfusion with 10 μM moricizine it fell with fast and slow time constants of 0.4681 ± 0.0115 $pulse^{-1}$ and 0.0018 ± 0.0002 $pulse^{-1}$, respectively.

As the results in Figures 4 and 5 may suggest that moricizine has affinity for the inactivated state of the channel, we examined the steady-state inactivation curve in the absence and presence of the drug. Inactivation induced by a 1000 ms prepulse to various membrane potentials was measured during a 200 ms test pulse to + 20 mV separated from the prepulse by a 4 ms return to the holding potential at −40 mV. Figure 6a shows the relationship between the steady-state inactivation (f_∞) and the membrane potential (Vm) obtained by normalizing the current evoked from a holding potential without inactivation as 1.0. Lines are drawn by the fits to the Boltzmann equation:

$$f_\infty = 1/\{1 + \exp[(Vm-Vh)/s]\}$$

where Vh is the membrane voltage at which I_{Ca} is half maximal, and s is a slope factor. In control condition, the averaged value of Vh was −30.4 ± 1.27 mV and s was 5.11 ± 0.16 ($n = 10$). The curve shifted to hyperpolarizing direction in Vh by 5.6 ± 1.8 mV with 10 μM moricizine ($n = 5$) and by 11.0 ± 1.2 mV with 30 μM ($n = 5$). Such a shift in the availability curve indicates that the drug has an affinity for the inactivated state of the channel. To estimate the binding kinetics of moricizine to the Ca^{2+} channel, we next performed a double pulse experiment in which prepulses of varying duration (10∼2000 ms) from −80 mV to a conditioning potential of + 20 mV were followed by a 200 ms test pulse after a quiescent interval of 800 ms. As shown in Figure 6b, decline of the Ca^{2+} current in the absence of the drug was less than 10% (90.7 ± 0.3% of the control peak I_{Ca}, $n = 5$). In the presence of 30 μM moricizine, an initial rapid and later slow decline of I_{Ca} were noted with further increase ($P<0.05$) in the degree of the block (84.6 ± 1.6% of the control, $n = 5$). Although the average decline of I_{Ca} in the absence of the drug was fitted to a single exponential with an uptake time constant of 2.06 s, it was fitted to two exponential functions in the presence of the drug with fact and slow uptake time constants of 0.029 s and 1.21 s, respectively. These results suggest that moricizine may undergo additional binding to the activated state of the Ca^{2+} channel.

Discussion

This study demonstrated that moricizine, at a near therapeutic concentration, caused blockade of the L-type Ca^{2+} current in a use-dependent manner, while the drug depressed the delayed outward K^+ current without any direct

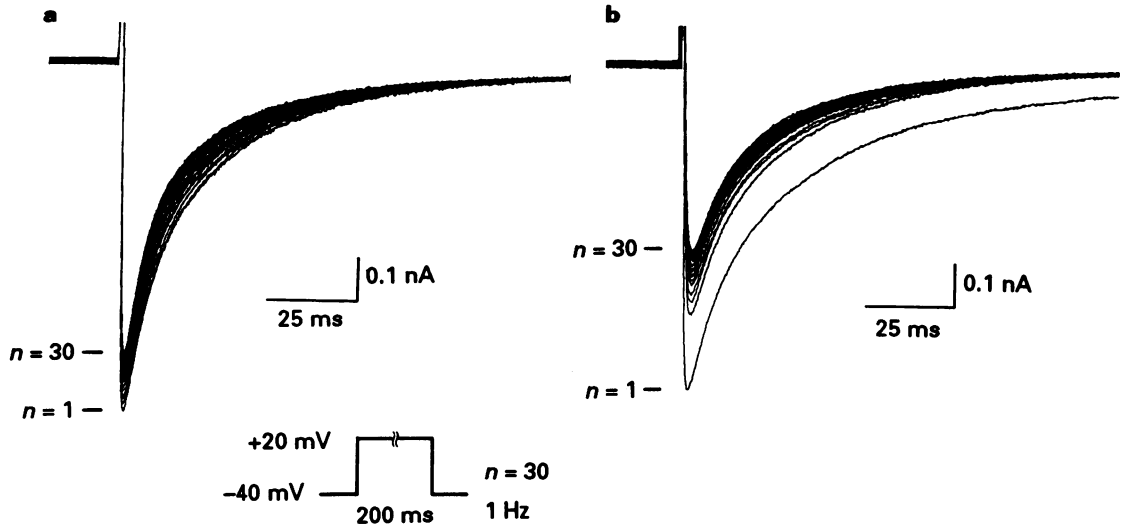


Figure 4 Use-dependent block of I_{Ca} by moricizine. (a) Superimposed I_{Ca} records elicited by pulse train in the control. (b) Superimposed I_{Ca} recording during exposure to 10 μM moricizine. A train of 30 depolarizing pulses of 200 ms duration was applied from a holding potential of −40 mV to +20 mV at 1 Hz with and without moricizine. A 2-min interval in control and a 3-min interval in moricizine were allowed between pulse trains to permit full recovery from inactivated states. (a) and (b) are from the same cell.

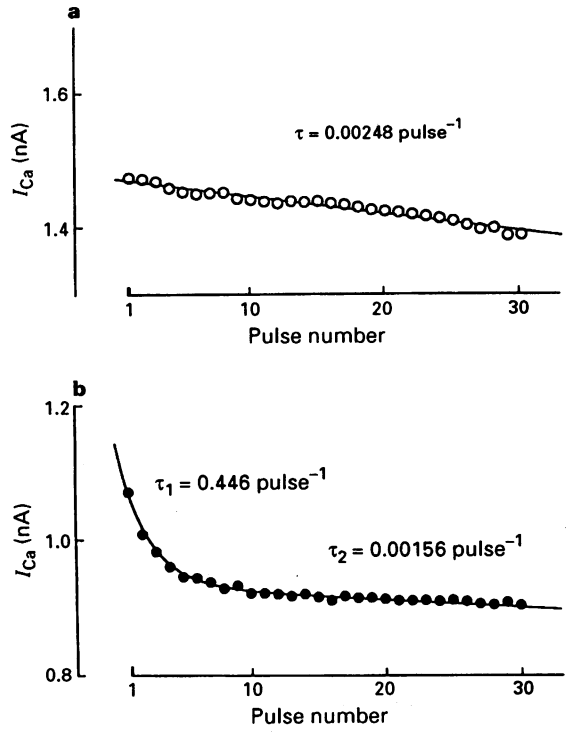


Figure 5 Development of I_{Ca} block by moricizine. Peak I_{Ca} was plotted against pulse numbers in a train of depolarizations under the control (a) and presence of 10 μM moricizine (b). In the control, the fall in peak I_{Ca} was best fitted to a single exponential. After exposure to 10 μM moricizine, the block development was best fitted to two exponential functions. The onset rates are indicated by a unit of per pulse (pulse^{-1}). Stimulus protocol was the same as the one shown in Figure 4 except that the holding potential was −60 mV. (a) and (b) were obtained from the same cell.

effects on Na^+/Ca^{2+} exchange current. As the basis of the Ca^{2+} channel block, moricizine was shown to have affinity for the inactivated as well as the activated states of the channel.

Although moricizine is classified as a class I antiarrhythmic agent due to its main action of the Na^+ channel, universal agreement as to its subclassification into Ia, Ib or Ic has not been achieved. The reason for this confusion is due to conflicting results as to the blocking kinetics of the Na^+ channel by moricizine as well as its effects on action potential configuration (Mann, 1990; Fitton & Buckley, 1990; Vaughan Williams, 1991). While moricizine shortens action potential duration like class Ib agents, the blocking kinetics on the Na^+ channels have been characterized either as intermediate (Lee & Rosen, 1991) or slow (Rosenshtraukh *et al.*, 1987; Makielski *et al.*, 1988). Furthermore, the ionic nature of action potential shortening has not been clarified. Action potential duration (APD) is regulated by several membrane currents such as delayed outward K^+ current, L-type Ca^{2+} current, late (window) Na^+ current, and Na^+/Ca^{2+} exchange current (Carmeliet, 1990). We investigated the action of moricizine on each of these currents.

In sheep Purkinje fibres, Carmeliet & Saikawa (1982) observed a shortening of action potential duration by quinidine and they attributed this action to the suppression of the Na^+-window current. Salata & Wasserstrom (1988) also reported that the effect of quinidine on action potential duration of canine ventricular myocytes was partly derived from suppression of the tetrodotoxin (TTX)-sensitive, steady-state Na^+ window current. The Na^+ window current seemed not to be prominent in forming action potential repolarization in guinea-pig ventricular myocytes (Hiraoka *et al.*, 1986). We could not consistently detect a steady inward current sensitive to TTX (not shown), with similar voltage protocol as those demonstrated by Kiyosue & Arita (1989). With the current clamp experiments, we observed that moricizine further shortened APD on TTX (50 μM)-treated cells (not shown). All of these results suggest that block of the window Na^+ current was not a main factor for shortening of APD by moricizine but an additional factor might be present.

A time-dependent outward K^+ current plays a significant role for the repolarization of ventricular myocytes (Carmeliet, 1990) and it was affected by class Ia agents in ventricular preparations (Hiraoka *et al.*, 1986; Salata & Wasserstrom, 1988). Moricizine (10 μM) suppressed this current (Figure 1) confirming the findings of Rosenshtraukh *et*

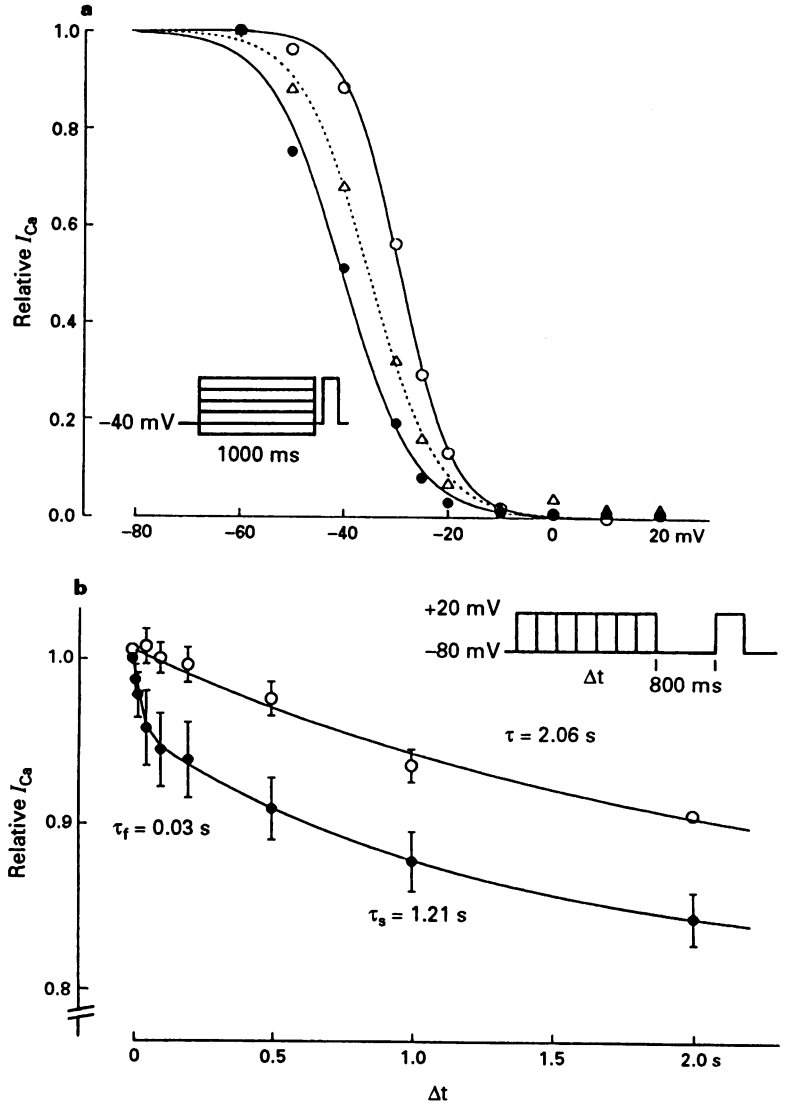


Figure 6 (a) Effects of moricizine on the voltage-dependent inactivation of the L-type Ca^{2+} current. Inactivation of L-type I_{Ca} was induced by a 1000 ms prepulse to various membrane potentials and was measured by during 200 ms test pulse to +20 mV preceded by a 4 ms repolarization to −40 mV. Relative amplitude of L-type I_{Ca} was plotted against prepulse voltages. The amplitudes of L-type I_{Ca} evoked by the test pulse were normalized to that without a conditioning prepulse. Lines are the fits of the Boltzmann equation to the data with Vh values of −29.2, −40.4, −35.3 mV and s values of 5.0, 6.9, 6.5 before (○), during (●), and washing out (△) of 30 μM moricizine, respectively. (b) Development of I_{Ca} block by moricizine. Uptake kinetics of moricizine were evaluated by measuring I_{Ca} as a function of prepulse duration. Conditioning pulses to +20 mV were applied for durations ranging from 10 ms to 2000 ms followed by a 200 ms test pulse to +20 mV interposed by a repolarization to −80 mV for 800 ms. The amplitude of I_{Ca} was normalized with respect to the amplitude obtained without a conditioning prepulse. Values and bars indicate the mean ± s.e.mean of 4 experiments. In the presence of 30 μM moricizine, relative I_{Ca} decreased with a fast and slow time course.

al. (1987). This suppressive effect on the delayed outward K^+ current, however, must be responsible for the prolongation of the final repolarization phase of the action potential, and cannot explain the shortening of APD.

Another possible explanation for the shortening of APD by moricizine, which was proposed by Vaughan Williams (1991), might be an inhibition of the Na^+/Ca^{2+} exchange mechanism. Intracellular Ca^{2+} rises as the action potential is initiated and then it falls to the original low level after the plateau phase. This fall in internal Ca^{2+} may be partly due to Ca^{2+} uptake by the sarcoplasmic reticulum and partly due to Ca^{2+} extrusion by Na^+/Ca^{2+} exchange. We induced an outward current by increasing the external Ca^{2+} concentration with the same procedure reported by Kimura *et al.* (1987). While 5 μM Ni^+ almost completely blocked the outward current induced by raising external Ca^{2+} which was attributed to the Na^+/Ca^{2+} exchange current, 10 μM moricizine did not cause any change in this current component. Therefore, moricizine appears not to influence directly the Na^+/Ca^{2+} exchange current, but it may modulate the exchange mechanism indirectly through its effects on Na^+ influx resulting in a decrease in internal Na^+.

The L-type Ca^{2+} current has been thought to maintain the plateau phase of action potential (Carmeliet, 1990), and moricizine might have a depressive action on this current to shorten the plateau. As shown in Figure 3, moricizine (10 μM) did not produce tonic block of the Ca^{2+} current. This result suggests that the affinity of moricizine for the rested state of the L-type Ca^{2+} channel is negligible. Scamps *et al.* (1989) reported that 10 μM moricizine caused use-dependent block of I_{Ca} by about 10% at stimulus frequency of 1 Hz without a tonic block. They further concluded that moricizine did not have an affinity for the inactivated state of the Ca^{2+} channel, since the availability curve was barely

shifted by 10 μM moricizine. Our results, however, showed the increases in drug-induced use-dependent block by about 17% and 13% were seen with lowering the holding potential from −80 mV to −40 mV and −60 mV, respectively. When the steady-state availability curve was analyzed, it was shifted toward the hyperpolarizing direction in a concentration-dependent manner by moricizine. This indicates that moricizine binds to the inactivated state of the Ca^{2+} channel. Activated state block was also suggested with the result of the block onset study (Figure 6b), in which an apparent fast component was detected in the uptake time constants ($\tau = 0.029$ s). These results are different from those of Scamps *et al.* (1989) possibly because of differences in species and experimental conditions (temperature, etc). This use-dependent reduction of the L-type Ca^{2+} current might well be able to explain the shortening of APD by moricizine.

Furthermore, we observed two exponential developments of the use-dependent block of I_{Ca} by moricizine (Figure 5b). Since the time constant of the slower component was similar to that in the control condition, they might reflect the same process, that is the slow recovery from the inactivated state of the Ca^{2+} channel (Yatani *et al.*, 1983). On the other hand, the fast component was not seen in the control condition and clearly depended on the presence of the drug. As to the use-dependent block of the Na^+ current by class I antiarrhythmic drugs, our previous report demonstrated that lignocaine produced two components of the block development dependent on both the channel states and the drug forms, whereas disopyramide showed two components of the block, dependent on the drug form itself (Sunami *et al.*, 1991). From the above results, moricizine was assumed to bind to both activated and inactivated states of the Ca^{2+} channel and two components of block development in addition to a component due to normal slow recovery would be expected after moricizine treatment. There was, however, only a single (fast) component to be detected without a new development of an additional slow component. This discrepancy might reflect a shortcoming in our experimental protocol. Trains of 30 pulses were too brief to estimate precisely the slower component newly developed after the drug. Although we carried out the protocol with trains of 100 pulses, it damaged the cells so severely that we could not get reproducible results with accurate analysis. We speculate that one more (intermediate) component might exist in the presence of moricizine and be concealed within the slow component of the block development of I_{Ca} in the present study. Further study is necessary to characterize the nature and kinetics of the I_{Ca} block.

We conclude that moricizine suppresses the L-type Ca^{2+} current use-dependently and it may explain, at least in part, its shortening effect on the action potential duration. To our knowledge, there have been few reports to describe the use-dependent block of the Ca^{2+} current in detail with class I antiarrhythmic drugs on single cardiac myocytes.

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References

BEAN, B.P. (1985). Two kinds of calcium channels in canine atrial cells. Differences in kinetics, selectivity, and pharmacology. *J. Gen. Physiol.*, **86**, 1–30.

CARMELIET, E. (1990). The cardiac action potential. In *Cardiac Electrophysiology: A Textbook*. ed. Rosen, M.R., Janse, M.J. & Wit, W.L. pp. 55–62. Mount Kisco, NY: Futura Publishing Company.

CARMELIET, E. & SAIKAWA, T. (1982). Shortening of the action potential and reduction of pacemaker activity by lidocaine, quinidine, and procainamide in sheep cardiac Purkinje fibres: an effect on Na or K currents? *Cir. Res.*, **50**, 257–272.

DANGMAN, K.H. & HOFFMAN, B.F. (1983). Antiarrhythmic effects on ethmozin in cardiac Purkinje fibers: suppression of automaticity and abolition of triggering. *J. Pharmacol. Exp. Ther.*, **227**, 578–586.

DANILO, P. JR., LANGAN, W.B., ROSEN, M.R. & HOFFMAN, B.F. (1977). Effects of the phenothiazine derivative analog EN 313 on ventricular arrhythmias in dogs. *Eur. J. Pharmacol.*, **45**, I27–I39.

FITTON, A. & BUCKLEY, M.M.T. (1990). Moricizine: a review of its pharmacological properties, and therapeutic efficacy in cardiac arrhythmias. *Drugs*, **40**, 138–167.

HEWETT, K., GESSMAN, L. & ROSEN, M.R. (1983). Effects of procainamide, quinidine and ethmozin on delayed afterdepolarizations. *Eur. J. Pharmacol.*, **96**, 21–28.

HIRANO, Y. & HIRAOKA, M. (1988). Barium-induced automatic activity in isolated ventricular myocytes from guinea-pig hearts. *J. Physiol.*, **395**, 455–472.

HIRAOKA, M., SAWADA, K. & KAWANO, S. (1986). Effects of quinidine on plateau currents of guinea-pig ventricular myocytes. *J. Mol. Cell. Cardiol.*, **18**, 1097–1106.

HONDEGHEM, L.M. & KATZUNG, B.G. (1977). Time- and voltage-dependent interactions of antiarrhythmic drugs with cardiac sodium channels. *Biochem. Biophys. Acta*, **472**, 373–398.

JANUARY, C.T., RIDDLE, J.M. & SALATA, J.J. (1988). A model for early afterdepolarizations: induction with the Ca^{2+} channel agonist Bay K 8644. *Circ. Res.*, **62**, 563–571.

KIMURA, J., MIYAMAE, S. & NOMA, A. (1987). Identification of sodium-calcium exchange current in single ventricular cells of guinea-pig. *J. Physiol.*, **384**, 199–222.

KIYOSUE, T. & ARITA, M. (1989). Late sodium current and its contribution to action potential configuration in guinea-pig ventricular myocytes. *Circ. Res.*, **64**, 389–397.

LEE, J.H. & ROSEN, M.R. (1991). Use-dependent actions and effects on transmembrane action potentials of flecainide, encainide, and ethmozine in canine Purkinje fibers. *J. Cardiovasc. Pharmacol.*, **18**, 285–292.

MAKIELSKI, J.C., UNDROVINAS, A.I., HANCK, D.A., SHEETS, M.F., NESTERENKO, V.V., ALPERT, L.A., ROSENSHTRAUKH, L.V. & FOZZARD, H.A. (1988). Use-dependent block of sodium current by ethmozin in voltage-clamped internally perfused canine cardiac Purkinje cells. *J. Mol. Cell. Cardiol.*, **20**, 255–265.

MANN, H.J. (1990). Moricizine: a new class I antiarrhythmic. *Clin. Pharmacol.*, **9**, 842–852.

ROSENSHTRAUKH, L.V., ANYUKHOVSKY, E.P., NESTERENKO, V.V., UNDROVINAS, A.I., SHUGUSHEV, K.KH., PORTNOY, V.F. & BURNASHEV, N.A. (1987). Electrophysiological aspects of moricizine HCl. *Am. J. Cardiol.*, **60**, 27F–34F.

RUFFY, R.R., ROSENSHTRAUKH, L.V., ELHARRAR, V. & ZIPES, D.P. (1979). Electrophysiological effects of ethmozin on canine myocardium. *Cardiovas. Res.*, **13**, 354–363.

SALATA, J.J. & WASSERSTROM, J.A. (1988). Effects of quinidine on action potentials and ionic currents in isolated canine ventricular myocytes. *Circ. Res.*, **62**, 324–337.

SCAMPS, F., UNDROVINAS, A. & VASSORT, G. (1989). Inhibition of I_{Ca} in single frog cardiac cells by quinidine, flecainide, ethmozin, and ethacizin. *Am. J. Physiol.*, **256** (Cell Physiol. **25**), C549–559.

SUNAMI, A., FAN, Z., NITTA, J. & HIRAOKA, M. (1991). Two components of use-dependent block of Na^+ current by disopyramide and lidocaine in guinea pig ventricular myocyte. *Cir. Res.*, **68**, 653–661.

THE CARDIAC ARRHYTHMIA SUPPRESSION TRIAL (CAST) INVESTIGATORS. (1989). Preliminary report: effect of encainide and flecainide on mortality in a randomized trial of arrhythmia suppression after myocardial infarction. *N. Engl. J. Med.*, **321**, 406–412.

TSUJI, Y., NISHIMURA, M., OSADA, M. & WATANABE, Y. (1983). Membrane action of ethmozin on normoxic and hypoxic canine Purkinje fibers. *J. Cardiovasc. Pharmacol.*, **5**, 961–967.

VAUGHAN WILLIAMS, E.M. (1991). Classification of the antiarrhythmic action of moricizine. *J. Clin. Pharmacol.*, **31**, 216–221.

YATANI, A., WILSON, D.L. & BROWN, A.M. (1983). Recovery of Ca current from inactivation: the roles of Ca influx, membrane potential, and cellular metabolism. *Cell. Mol. Neurobiol.*, **3**, 381–395.

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Effects of carbamazepine and baclofen on 4-aminopyridine-induced epileptic activity in rat hippocampal slices

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1 Rat transverse hippocampal slices exposed to 100 μM 4-aminopyridine (4-AP) generate spontaneous epileptic discharges ranging in duration from short 50 ms 'interictal' bursts to long 0.5–2 s 'polyspike' activity.

2 Here we compared the effects of the commonly used anticonvulsant, carbamazepine (40 μM) and the antispastic drug, baclofen (2 μM) on the various types of burst.

3 Carbamazepine completely abolished long bursts whilst leaving shorter bursts intact. This is consistent with its known anticonvulsant properties.

4 Baclofen greatly reduced the frequency of short bursts but did not block the long bursts. Rather, they became significantly more prolonged, indicating that baclofen does not have an anticonvulsant action, and may be proconvulsant.

5 These results conflict with conclusions based on studies using models that exhibited only interictal bursts, and emphasize the need to use experimental epilepsies which generate several types of epileptic discharge to evaluate the effects of putative anticonvulsant drugs.

6 The present findings suggest that $GABA_B$ receptors play a role in the transition of benign interictal bursts to longer polyspike activity which could develop into seizures in the whole animal.

Keywords: 4-Aminopyridine; epilepsy; anticonvulsant drugs; $GABA_B$ receptors; carbamazepine; baclofen; hippocampal slice; CA3 pyramidal cell

Introduction

4-Aminopyridine (4-AP) induces epileptiform activity *in vivo* (Szente & Pongracz, 1979), *in vitro* (Rutecki *et al.*, 1987), and clinically (Spyker *et al.*, 1980). It differs from the most frequently used convulsant drugs, such as bicuculline, picrotoxin and penicillin, which induce epileptic activity by blocking fast synaptic inhibition (Wong *et al.*, 1986; Jefferys, 1992). Instead, 4-AP blocks the intrinsic neuronal potassium currents – I_A and I_D, (Storm, 1988) and may augment calcium currents (Rogawski & Barker, 1983). This enhances both excitatory and inhibitory synaptic transmission (Rutecki *et al.*, 1987). The presence of inhibition confers some specific characteristics on the synchronization of the neuronal population (Ives & Jefferys, 1990). Hippocampal slices exposed to 4-AP also generate several types of spontaneous discharges, including two types of short burst (Voskuyl & Albus, 1985) and long polyspike bursts. The short bursts resemble interictal spikes *in vivo*, whilst longer bursts more closely resemble the onset of epileptic seizures. The relative sensitivity of these burst types to pharmacological manipulation may prove valuable in assessing the anticonvulsant properties of carbamazepine and baclofen.

Carbamazepine is a commonly used and effective anticonvulsant in man (Rogawski & Porter, 1990), particularly against complex partial (usually temporal lobe) seizures and generalized tonic-clonic seizures. It is effective at suppressing seizures in several models of epilepsy *in vivo* (David & Grewal, 1976), and inhibits epileptic activity induced *in vitro* by various convulsants (Smith & Swann, 1987; Psarropoulou & Haas, 1989). Its mechanisms of action have yet to be fully elucidated, but may involve blockade of sodium channels (McLean & MacDonald, 1987). Baclofen is an antispastic agent which has been shown to be an agonist at both pre- and post-synaptic $GABA_B$ receptors (Bowery *et al.*, 1981; Newbury & Nicholl, 1985) and to inhibit synaptic transmission (Ault & Nadler, 1982). From these findings, it might be expected that baclofen would exhibit anticonvulsant properties by inhibiting neuronal firing. Several studies have shown that baclofen has antiepileptic properties (Brady & Swann, 1984; Ault *et al.*, 1986). However, short interictal bursts were converted to long ictal type bursts in the low magnesium model of epilepsy (Swartzwelder *et al.*, 1987) and abnormal neuronal synchronization could be induced (Lewis *et al.*, 1989). Clinically, baclofen has also been reported to have pro-convulsant effects at both overdose and therapeutic levels in patients being treated for spasticity (Rush & Gibberd, 1990). In these cases a disinhibitory action, possibly via pre-synaptic $GABA_B$-mediated inhibition of γ-aminobutyric acid (GABA) release, appears to predominate. Given the conflicting results outlined above, the present study was designed to investigate the effects of baclofen on both the short and long epileptic discharges generated by the 4-AP model of epilepsy.

Methods

Adult male Wistar rats (Harlan OLAC, Bicester, UK) weighing between 250 and 500 g were stunned and killed by cervical dislocation. The brain was quickly removed, bisected and immersed in ice-cold artificial cerebrospinal fluid (a.CSF). Transverse hippocampal slices, 400 μm thick, were cut with a Vibroslice (Campden Instruments, Loughborough, UK). Slices were maintained in a recording chamber at 34°C at the interface between a warmed, water-saturated oxygen/

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carbon dioxide mixture and warmed, oxygenated aCSF. This consisted of (in mM): NaCl 135, $NaHCO_3$ 16, KCl 3, $CaCl_2$ 2, $MgCl_2$ 1, NaH_2PO_4 1.25, glucose 10, and was perfused at a rate of 0.5 ml min^{-1}. Extracellular recordings were made from the CA3 pyramidal cell layer by use of glass electrodes filled with 3 M NaCl and with tip resistances of 2–20 MΩ. Recordings were made through an Axoprobe (Axon Instruments, Burlingame, USA) and Neurolog amplifiers and filters (Digitimer, Welwyn, UK) and data analysed by a CED 1401 laboratory computer system running SIGAVG and SPIKE2 software (Cambridge Electronic Design, UK).

4-Aminopyridine (4-AP) was purchased from Sigma Chemicals, UK. Carbamazepine (5H-dibenzo[bf]azepine-5-carboxamide, Tegretol A.S.) and baclofen (β-(4-chlorophenyl)GABA, Lioresal) were both from Geigy Pharmaceuticals (Horsham, UK). All were dissolved in a small volume of warmed distilled H_2O before dilution to the required concentration in aCSF.

After a minimum 1 h incubation, the convulsant drug 4-AP was added to the bathing medium at a concentration of 100 μM. Once the rate of both short and long duration spontaneous activity had stabilized, a 5 min control recording was made 10 min before adding either 40 μM carbamazepine or 2 μM baclofen to the perfusate; only one drug was tested on each slice. Drugs were applied for 15 min, but changes in burst frequency usually plateaued within 5 min. Effects were measured 5 min into the 15 min drug application and 5 and 30 min after the start of washout. Burst frequency was counted over 5 min intervals, and results are expressed as the frequency per minute (mean ± s.e.mean), and as a percentage of the control frequency.

Data were analysed statistically by the relevant two-tailed parametric or non-parametric test for paired data. Tests were run on the RS/1 statistics package (BBN Inc, Cambridge, USA) with a significance level set at 0.01.

Results

All slices exposed to 100 μM 4-AP exhibited spontaneous population bursts. Bursts occurred regularly at a rate which varied between slices in the range 10 to 30 per minute; longer bursts were followed by brief periods of inactivity before re-emergence of short bursts (Figure 1a). Simultaneous extracellular recordings from a fixed electrode and one positioned various distances along the pyramidal layer showed that all the spontaneous bursts originated in the hilar/$CA3_{b/c}$ region and propagated towards CA1 at 0.15 ± 0.03 m s^{-1} (5 slices). Bursts were categorized by their duration and general form (Figure 1). The short 50 ms bursts, analogous to interictal activity were termed 'type 1' (Figure 1b), whilst the double bursts lasting 150–400 ms were called 'type 2' (Figure 1c). The second part of the type 2 bursts is likely to be equivalent to the non-synaptic type II bursts described by Voskuyl & Albus (1985) and originated at the CA3/CA1 border. Long 'type 3' bursts were also seen here. These were relatively infrequent and consisted usually of a type 2 burst followed by a train of repetitive short bursts lasting up to several seconds (Figure 1d).

The duration of each burst was measured and plotted on a log scale against its latency from the start of the recording period (Figure 2a). Type 1 bursts were always found to be between 45 and 70 ms duration, whilst type 2 bursts were of 150–400 ms duration. Any bursts longer than 400 ms were taken to be type 3 bursts. Classification of the bursts was by superimposing these limits on graphs of burst duration (Figures 2 and 3). The frequency of each type of burst was also measured and expressed in relation to the frequency of that burst type prior to drug application (Figure 2b). Overall frequency of bursts in control conditions, incorporating types 1, 2 and 3 for all experiments, was 22.6 ± 2.7 min^{-1} (mean ± s.e.mean, 11 slices).

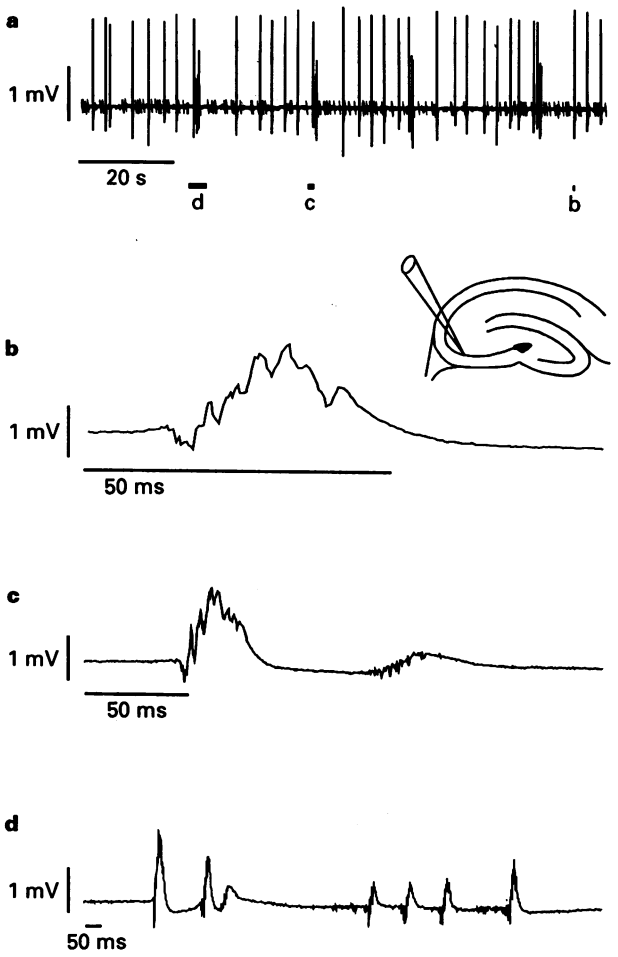


Figure 1 Spontaneous field bursts by 4-aminopyridine (4-AP). (a) Chart record showing 60 s of spontaneous activity recorded in the CA3b region of a slice in the presence of 4-AP (100 μM). (b) Short 'type 1' burst expanded from chart record in (a), (c) double 'type 2' burst; (d) long 'type 3' burst.

Effects of carbamazepine

Carbamazepine completely abolished the type 3 long bursts in all experiments from a control frequency of 0.9 ± 0.1 min^{-1} (6 slices, $P = 0.0001$), with incomplete recovery during the time course of the experiment (0.5 ± 0.2 min^{-1}, 30 min after washout, $P = 0.08$). The frequency of shorter type 1 bursts also fell during drug application, from 19.7 ± 4.7 to 11.9 ± 4.5 min^{-1} ($P = 0.0003$), but recovered quickly following washout (17.8 ± 3.5 min^{-1}, 5 min after washout, $P = 0.24$). Frequency of type 2 bursts did not change from control levels (1.4 ± 0.2 min^{-1}) during carbamazepine application ($P = 0.68$), but became significantly more frequent immediately afterwards (2.2 ± 0.1 min^{-1}, $P = 0.004$) before recovering to previous levels ($P = 0.23$) with longer washout. The start of type 3 bursts often resembled the type 2 bursts seen separately. It was therefore possible that carbamazepine was converting type 3 bursts to type 2's. If type 2 and type 3 bursts are taken as one category and their frequency expressed as a proportion of the total burst frequency (i.e. type 1, 2 and 3 bursts together), we can see what proportion of bursts are not short type 1's. In fact this analysis reveals very little change in the proportion of combined type 2 and 3 bursts during the time course of the experiment, changing from 0.15 ± 0.04 before carbamazepine to 0.19 ± 0.08 during application, and 0.17 ± 0.06 upon recovery ($P > 0.28$ for all time points, Figure 2c). Thus the abolition of type 3 bursts was balanced by an increase in type 2, suggesting that carbamazepine converted the type 3 bursts to type 2's.

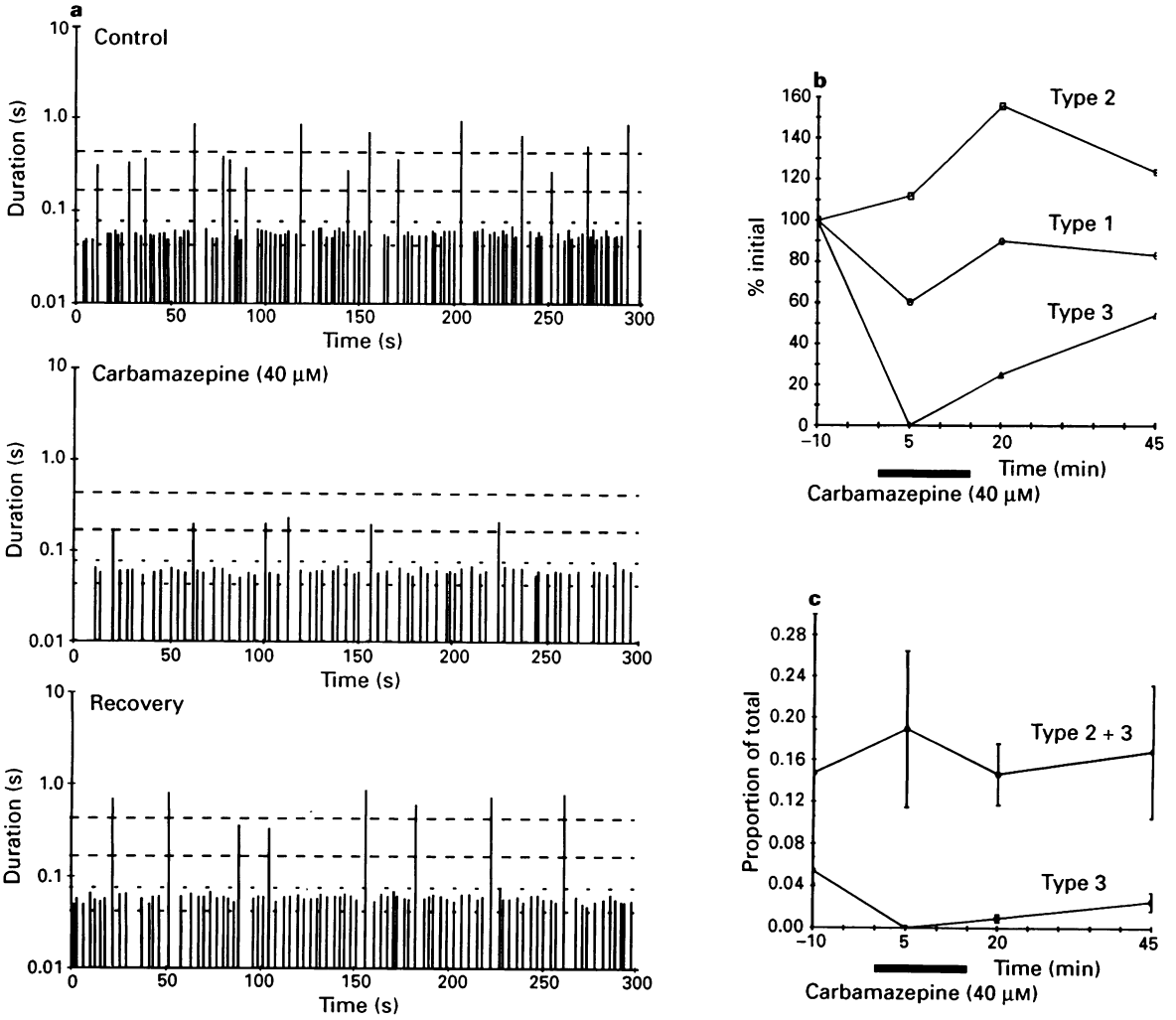


Figure 2 Burst duration with application of carbamazepine (40 μM). Duration of each burst occurring during a recording period 10 min prior to, 5 min into the 30 min after carbamazepine application is shown on a log scale. Horizontal bars show limits for accepting a burst as 'type 1' (lower set) or 'type 2' (upper set). Any bursts of longer duration are classified as 'type 3'. (b) Burst frequency for each burst type with carbamazepine (40 μM) expressed as a percentage of the control value. (c) Burst frequency expressed as a proportion of total burst frequency (types 1, 2 and 3 combined) for type 3 bursts (lower trace), and for type 2 and 3 burst frequency combined (upper line). The proportion of type 2 + 3 bursts remains fairly constant throughout the experiment, but during carbamazepine perfusion this is made up totally of type 2 bursts (mean ± s.e.mean).

Effects of baclofen

Application of 2 μM baclofen produced very different effects from carbamazepine. During the 15 min application of 2 μM baclofen, burst frequency was radically reduced (Figure 3a). At first sight this would appear to support the proposed antiepileptic action of this drug. However, closer inspection of the burst types and burst duration reveals that although type 1 bursts were almost completely abolished by baclofen, type 2 and type 3 bursts were still present. The most obvious effect of the baclofen was a considerable reduction in the frequency of short type 1 bursts from 21.5 ± 2.4 to 1.0 ± 0.3 min^{-1} during drug application (5 slices, $P = 0.0001$), which took a long time to recover following removal of baclofen from the bathing medium (17.6 ± 1.7 min^{-1}, 30 min after washout, $P = 0.02$). Thus if only type 1 bursts had been present, it would have appeared that baclofen was having an antiepileptic action. The frequency of the type 3 bursts was also reduced under baclofen, from control levels of 1.0 ± 0.1 to 0.4 ± 0.1 min^{-1} ($P = 0.003$). However they were not abolished, in contrast with their total abolition by carbamazepine. These type 3 bursts also recovered quickly following washout of the baclofen (1.1 ± 0.2 min^{-1}, 30 min after washout, $P = 0.37$), indicating that the effect of baclofen on longer type 3 bursts wears off quickly than for type 1 bursts. The frequency of type 2 bursts increased from 0.8 ± 0.2 min^{-1} to 2.4 ± 0.4 min^{-1} during application of baclofen ($P = 0.01$) and also recovered quickly following removal of the drug (1.5 ± 0.3 min^{-1} just after washout, $P = 0.05$).

Although the frequency of type 3 bursts fell during baclofen application, the remaining type 3 bursts appeared longer. Measurement of burst duration before, during and after baclofen application was plotted (Figure 3c) and showed that bursts increased in duration from 1.5 ± 0.3 s before baclofen to 2.9 ± 0.6 s during application ($P = 0.001$, 5 slices). Following a 30 min washout these bursts recovered to their control durations (1.3 ± 0.5 s, $P = 0.45$).

Discussion

The epileptic activity induced in hippocampal slices by 4-AP is complex. It can be categorized into three burst types by duration. Type 1 bursts are analogous to the benign interictal spikes seen in human epileptics. Type 3 bursts are probably analogous to polyspike activity in human subjects and may represent the initiation of bursts which, in the intact brain, would develop into seizure activity. The presence and

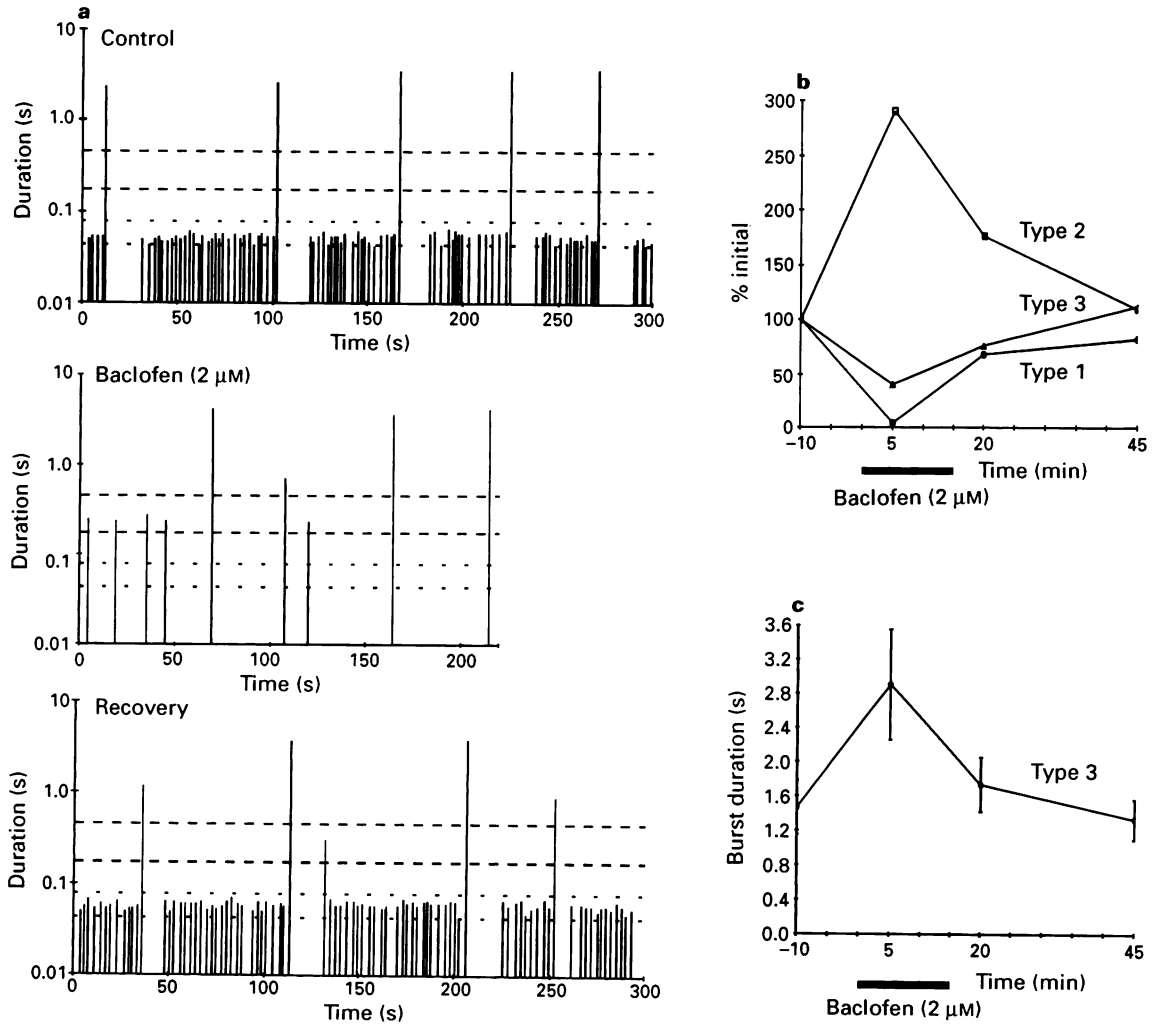


Figure 3 Burst duration with application of baclofen (2 μM). Duration of each burst occurring during a recording period 10 min prior to, 5 min into and 30 min after baclofen application is shown on a log scale. As for Figure 2, horizontal bars show limits for accepting a burst as 'type 1' (lower set) or 'type 2' (upper set); any bursts of longer duration are classified as 'type 3'. (b) Burst frequency for each burst type with application of baclofen (2 μM) expressed as a percentage of the control value. (c) Duration of type 3 bursts with baclofen perfusion (mean ± s.e.mean).

relatively high frequency of type 3 polyspike activity generated by 4-AP is unusual in that most epilepsy models *in vitro* exhibit only short interictal type bursts.

Carbamazepine completely inhibits the long type 3 bursts without preventing the shorter type 1 or type 2 bursts. This supports the hypothesis that carbamazepine blocks the development of longer activity which could develop into seizure episodes. A similar effect of carbamazepine has also been described in other *in vitro* models of epilepsy induced by low magnesium (Psarropoulou & Haas, 1989) and by penicillin in tissue from immature rats (Smith & Swan, 1987), and is consistent with the *in vivo* action of carbamazepine in the alumina cream model of epilepsy in the monkey (David & Grewal, 1976), where it blocks the spread of epileptic activity from the focus but does not prevent the initiation of short discharges within the focus. The lack of a major effect of carbamazepine on shorter burst types at the therapeutic concentrations used here indicates that they are relatively unimportant in the generation of seizures. Indeed, the literature suggests that whilst interictal discharges are a feature of epileptic EEGs, there is no correlation within individual patients between their incidence and the probability of a seizure (Katz *et al.*, 1991). The most likely mechanism of action of carbamazepine is a frequency-dependent block of sodium channels (McLean & Macdonald, 1987), so that longer bursts, but not initial population spikes, are blocked at therapeutic concentrations of carbamazepine. Alternative actions at adenosine receptors (Skerritt *et al.*, 1982) or N-methyl-D-aspartate (NMDA) receptors (Lampe & Bigalke, 1990) have also been proposed. In fact, NMDA receptor antagonists and adenosine have been shown to inhibit epileptiform bursting (Löscher *et al.*, 1988; Ault & Wang, 1986). Whatever its mechanism of action, the selective blockade of polyspike, but not interictal, activity by carbamazepine shown in the present study supports its role as a proven and widely used anticonvulsant.

In contrast, the effects of baclofen indicate that it would not be useful as an anticonvulsant agent because, although it greatly reduces the frequency of type 1 interictal bursts, it does not abolish long type 3 bursts and the duration of these type 3 bursts also increases. This result agrees with other reports of a pro-epileptic effect of baclofen in the low magnesium model, where it converts interictal bursts to long ictal-type events (Swartzwelder *et al.*, 1987). Indeed, apart from one study on entorhinal cortex slices (Jones, 1989), the 'anticonvulsant' actions of baclofen have solely been described for models of focal epilepsies exhibiting short interictal bursts only (Brady & Swann, 1984; Ault *et al.*, 1986) where the profound inhibition of these short bursts could easily be mistaken for an antiepileptic action. If related to

human epilepsies, it can be seen that although the benign bursts would be abolished, the incidence of seizure episodes would not be reduced by baclofen, and indeed they may be exacerbated. In fact, several reports have shown baclofen to have a proconvulsant effect clinically (Rush & Gibberd, 1990) and to increase mortality in NMDA-induced seizures in mice (Czuczwar *et al.*, 1985). Baclofen has also been shown to be proconvulsant in models of absence seizures (e.g. Snead, 1992), even though these have fundamentally different cellular mechanisms from the focal experimental epileptic activity induced by agents such as 4-AP (reviewed in Jefferys, 1992).

The 2 μM dose of baclofen used here was at the low end of the range used in the literature (of the order of 1–100 μM). Presynaptic receptors appear to be more sensitive to baclofen than postsynaptic (respectively 0.5–1 μM vs 5–50 μM for detectable effects; Davies *et al.*, 1990; Yoon & Rothman, 1991). Therefore the pro-epileptic effect of baclofen in this model is most probably due to an action at pre-synaptic $GABA_B$ receptors on inhibitory interneurones, inhibiting GABA release and producing a functional disinhibition. Whatever the mechanism of action, these studies using baclofen have highlighted the possible role of $GABA_B$ receptors in the conversion of benign interictal bursts to longer ictal-type discharges.

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References

AULT, B. & NADLER, J.V. (1982). Baclofen selectivity inhibits transmission at synapses made by axons of CA3 pyramidal cells in the hippocampal slice. *J. Pharmacol. Exp. Ther.*, **223**, 291–297.

AULT, B. & WANG, C.M. (1986). Adenosine inhibits epileptiform activity arising in the hippocampal area CA3. *Br. J. Pharmacol.*, **87**, 695–703.

AULT, B., GRUENTHAL, M., ARMSTRONG, D., NADLER, J.V. & WANG, M.Y. (1986). Baclofen suppresses bursting activity induced in hippocampal slices by different convulsants. *Eur. J. Pharmacol.*, **126**, 289–292.

BOWERY, N.G., HILL, D.R., HUDSON, A.L., DOBLE, A., MIDDLEMISS, D.N., SHAW, J.S. & TURNBULL, M.J. (1981). Baclofen decreases neurotransmitter release in the mammalian CNS by an action on a novel GABA receptor. *Nature*, **283**, 92–94.

BRADY, R.J. & SWANN, J. (1984). Postsynaptic actions of baclofen associated with its antagonism of bicuculline-induced epileptogenesis in hippocampus. *Cell. Mol. Neurobiol.*, **4**, 403–408.

CZUCZWAR, S.J., FREY, H.-H. & LÖSCHER, W. (1985). Antagonism of NMDA induced convulsions by antiepileptic drugs and other agents. *Eur. J. Pharmacol.*, **108**, 273.

DAVID, J. & GREWAL, R.S. (1976). Effect of carbamazepine (Tegretol) on seizure and EEG patterns in monkeys with alumina-induced focal motor and hippocampal foci. *Epilepsia*, **17**, 415–422.

DAVIES, C.H., DAVIES, S.N. & COLLINGRIDGE, G.L. (1990). Paired-pulse depression of monosynaptic GABA-mediated inhibitory postsynaptic responses in rat hippocampus. *J. Physiol.*, **424**, 513–531.

IVES, A.E. & JEFFERYS, J.G.R. (1990). Synchronization of epileptiform bursts induced by 4-aminopyridine in the in vitro hippocampal slice preparation. *Neurosci. Lett.*, **112**, 239–245.

JEFFERYS, J.G.R. (1992). The pathophysiology of epilepsies. In *The Epilepsies*. ed. J. Laidlaw, J., Richens, A. & Chadwick, D.W. pp. 241–276. Edinburgh: Churchill Livingstone.

JONES, R.S.G. (1989). Ictal epileptiform events induced by removal of extracellular magnesium in slices of entorhinal cortex are blocked by baclofen. *Exp. Neurol.*, **104**, 155–161.

KATZ, A., MARKS, D.A., MCCARTHY, G. & SPENCER, S.S. (1991). Does interictal spiking change prior to seizures? *EEG Clin. Neurophysiol.*, **79**, 153–156.

LAMPE, H. & BIGALKE, H. (1990). Carbamazepine blocks NMDA-activated currents in cultured spinal cord neurons. *Neuropharmacol. Neurotox.*, **1**, 8–10.

LEWIS, D.V., JONES, L.S. & MOTT, D.D. (1989). Baclofen induces spontaneous, rhythmic sharp waves in the rat hippocampal slice. *Exp. Neurol.*, **106**, 181–186.

LÖSCHER, W., NOLTING, B. & HÖNACK, D. (1988). Evaluation of CPP, a selective NMDA antagonist, in various rodent models of epilepsy. Comparison with other NMDA antagonists & with diazepam and phenobarbital. *Eur. J. Pharmacol.*, **152**, 9–17.

MCLEAN, M.J. & MACDONALD, R.L. (1987). Carbamazepine and 10,11-epoxycarbamazepine produce use- and voltage-dependent limitation of rapidly firing action potentials of mouse central neurons in cell culture. *J. Pharmacol. Exp. Ther.*, **238**, 727–738.

NEWBURY, N.R. & NICOLL, R.A. (1985). Comparison of the action of baclofen with GABA on rat hippocampal pyramidal cells in vitro. *J. Physiol.*, **360**, 161–185.

PSARROPOULOU, C. & HAAS, H.L. (1989). Action of anticonvulsants on hippocampal slices in Mg-free medium. *Naunyn Schmiedebergs Arch. Pharmacol.*, **339**, 613–616.

ROGAWSKI, M.A. & BARKER, J.L. (1983). Effects of 4-aminopyridine on calcium action potentials and calcium current under voltage clamp in spinal neurons. *Brain Res.*, **280**, 180–185.

ROGAWSKI, M.A. & PORTER, R.J. (1990). Antiepileptic drugs: pharmacological mechanisms and clinical efficacy with consideration of promising developmental stage compounds. *Pharmacol. Rev.*, **42**, 223–286.

RUSH, J.M. & GIBBERD, F.B. (1990). Baclofen-induced epilepsy. *J.R. Soc. Med.*, **83**, 115–116.

RUTECKI, P.A., LEBEDA, F.J. & JOHNSTON, D. (1987). 4-Aminopyridine produces epileptiform activity in hippocampus and enhances synaptic excitation and inhibition in the rat. *J. Neurophysiol.*, **57**, 1911–1924.

SKERRITT, J.H., DAVIES, L.P. & JOHNSTON, G.A.R. (1982). A purinergic component in the anticonvulsant action of carbamazepine? *Eur. J. Pharmacol.*, **82**, 195–197.

SMITH, K.L. & SWANN, J.W. (1987). Carbamazepine suppresses synchronous after discharging in disinhibited immature rat hippocampus in vitro. *Brain Res.*, **400**, 371–376.

SNEAD, O.C., III (1992). Evidence for $GABA_B$-mediated mechanisms in experimental generalized absence seizures. *Eur. J. Pharmacol.*, **213**, 343–349.

SPYKER, D.A., LYNCH, C., SHABANOWITZ, J. & SINN, J.A. (1980). Poisoning with 4-aminopyridine: report of three cases. *Clin. Toxicol.*, **16**, 487–497.

STORM, J.F. (1988). Temporal integration by a slowly inactivating potassium current in hippocampal neurons. *Nature*, **336**, 379–381.

SWARTZWELDER, H.S., LEWIS, D.V., ANDERSON, W.W. & WILSON, W.A. (1987). Seizure-like events in brain slices: suppression by interictal activity. *Brain Res.*, **410**, 362–366.

SZENTE, M.B. & PONGRACZ, F. (1979). Aminopyridine induced seizure activity. *EEG Clin. Neurophysiol.*, **46**, 605–608.

VOSKUYL, R.A. & ALBUS, H. (1985). Spontaneous epileptiform discharges in hippocampal slices induced by 4-aminopyridine. *Brain Res.*, **342**, 54–66.

WONG, R.K.S., TRAUB, R.D. & MILES, R. (1986). Cellular basis of neuronal synchrony in epilepsy. In *Advances in Neurology* Vol. 44. ed. Delgado-Escueta, A.V., Ward, A.A., Woodbury, D.M. & Porter, R.J. pp. 583–592, New York: Raven Press.

YOON, K.-W. & ROTHMAN, S.M. (1991). The modulation of rat hippocampal synaptic conductances by baclofen and gamma-aminobutyric acid. *J. Physiol.*, **442**, 377–390.

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Leukotriene D_4 receptors are not negatively coupled to adenylyl cyclase in guinea-pig lung parenchyma

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1 The possibility that receptors for the peptide-containing leukotrienes may be negatively coupled to adenylyl cyclase in guinea-pig lung parenchyma was investigated by comparing the effect of leukotriene D_4 (LTD_4) on the intracellular cyclic nucleotide (cyclic AMP and cyclic GMP) content and on the activity of cyclic AMP-dependent protein kinase (PKA). In addition, the potential association between changes in the cyclic nucleotide content and the ability of LTD_4 to increase lung parenchymal tone was also evaluated.

2 Non-cumulative challenge of parenchymal lung strips with LTD_4 elicited concentration-dependent contractions ($pD_2 = 8.23$) that were paralleled by concentration-related increases in the intracellular level of cyclic AMP and cyclic GMP, and in the activation state of PKA ($K_{act} = 33$ nM). Temporally, these biochemical effects of LTD_4 were transient, peaking after ~5 min drug contact thereafter decaying, despite the continued generation of tone. Both the biochemical and mechanical effects of LTD_4 were antagonized by the LTD_4-receptor blocking drug, ICI 198,615 (1 μM for 60 min), indicating that they were receptor-mediated events.

3 Challenge of guinea-pig lung with LTD_4 (200 nM; EC_{100} for tension generation) stimulated a 150 and 70 fold increase in the elaboration of thromboxane B_2 (TXB_2) and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) respectively, relative to that generated spontaneously.

4 Pretreatment of lung strips for 60 min with an irreversible inhibitor of cyclo-oxygenase, flurbiprofen, at a concentration (8 μM) that abolished both basal and LTD_4 (200 nM)-induced TXB_2 and 6-keto-$PGF_{1\alpha}$ release, relaxed rapidly the spontaneous tone of the tissues, reduced the cyclic AMP content by ~50% and lowered the PKA activity ratio from 29% to 17%. In addition, flurbiprofen abolished the ability of LTD_4 (200 nM) to increase the cyclic AMP content and to activate PKA. Functionally, the magnitude of LTD_4 (200 nM)-induced tone and the increase in cyclic GMP content were attenuated by approximately 20% and 50% respectively in flurbiprofen-treated tissues.

5 In flurbiprofen-treated tissues, isoprenaline (10 μM for 10 min) increased the cyclic AMP content (from 4 to 27 pmol mg^{-1} protein) and activated PKA (from 15% to 26%). Preincubation (30 s or 5 min) of lung with LTD_4 (200 nM) did not inhibit (or enhance) these isoprenaline-induced effects.

6 Pretreatment of lung strips for 60 min with the thromboxane synthetase inhibitor, dazmegrel (10 μM), relaxed the spontaneous tone of the tissues, abolished the LTD_4 (200 nM)-stimulated release of TXB_2 and significantly enhanced (~two fold) the elaboration of 6-keto-$PGF_{1\alpha}$. In addition, dazmegrel attenuated (by ~50%) LTD_4 (200 nM)-induced cyclic GMP accumulation but approximately doubled both the cyclic AMP content and PKA activity ratio. LTD_4-induced contractions, in contrast, were not affected by dazmegrel.

7 EP 092 (1 μM for 60 min), a selective TP-receptor blocking drug, had no effect on spontaneous tone, eicosanoid formation or on the cyclic GMP content of guinea-pig lung parenchymal strips. Likewise, EP 092 exerted no significant mechancial effect in lung challenged with LTD_4 (200 nM) although it did potentiate, to a small extent, the ability of LTD_4 (200 nM) to increase the cyclic AMP content.

8 It is concluded that LTD_4 can increase the intracellular level of cyclic AMP in guinea-pig parenchyma and activate PKA by a leukotriene-receptor-mediated mechanism sensitive to ICI 198,615. However, these biochemical actions of LTD_4 are induced indirectly by an arachidonic acid-derived cyclo-oxygenase product(s) other than TXA_2. Thus, contrary to reports of other investigators, no evidence was found to corroborate the finding that stimulation of leukotriene receptors on guinea-pig lung parenchyma results in a rapid lowering of the cyclic AMP content even in cyclo-oxygenase-blocked tissues. These data, therefore, do not support the hypothesis that leukotriene-induced tension generation is dependent upon a prior reduction in the cyclic AMP content.

Keywords: Guinea-pig lung, effect of leukotriene D_4; excitation-contraction coupling; cyclic AMP; cyclic AMP-dependent protein kinase; cyclic GMP; signal transduction; leukotriene receptors, blockade by ICI 198,615; thromboxane receptors, blockade by EP 092; cyclo-oxygenase, inhibition by flurbiprofen; thromboxane synthetase, inhibition by dazmegrel

Introduction

Since the discovery that the peptide-containing leukotrienes account for the activity of slow reacting substance of anaphylaxis (SRS-A; Lewis *et al.*, 1980), many studies have been performed with the objective of understanding further the biochemical mechanism(s) by which leukotrienes elicit contraction of airways smooth muscle.

Evidence accrued from radioligand binding experiments indicates that leukotriene D_4 (LTD_4), the most potent of the peptido-leukotrienes, interacts with distinct receptors (Hoga-

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boom *et al.*, 1983; Mong *et al.*, 1984) that are located exclusively on the extracellular facing surface of the plasmalemma (Mong *et al.*, 1988). The binding of [^{3}H]-LTD$_4$ to membranes prepared from guinea-pig lung is stereoselective and is displaced by LT-receptor agonists with a rank order of potency that correlates well with their ability to elicit smooth muscle contraction (Pong & DeHaven, 1982; Cheng & Townley, 1984; Cheng *et al.*, 1985; Mong *et al.*, 1985a). The interaction of LTC$_4$ and LTD$_4$ with their receptors is modulated by monovalent and divalent cations and by guanine nucleotides (Pong & DeHaven, 1982; Bruns *et al.*, 1983; Mong *et al.*, 1985b) in much the same way as agonists which have efficacy at a_2-adrenoceptors, dopamine D_2-receptors, adenosine A_1-receptors, muscarinic cholinoceptors, and receptors for angiotensin II, δ opioids and somatostatin (see Jakobs *et al.*, 1984). These latter observations prompted several investigators to postulate that the LT-receptor may be negatively coupled, through an inhibitory guanine nucleotide-binding protein (G_i, or variant thereof), to adenylyl cyclase (Hedman & Andersson, 1982a; Bruns *et al.*, 1983; Mong *et al.*, 1985b). Indeed, Andersson and his colleagues (Andersson *et al.*, 1982; 1983; Hedman & Andersson, 1982a,b) have found that LTC$_4$, LTD$_4$ and immunologically generated SRS-A produce a rapid reduction in the basal level of adenosine 3':5'-cyclic monophosphate (cyclic AMP) in guinea-pig tracheae, that precedes the development of tension. Similar results have been reported by Barnes & Grandordy (1986) in guinea-pig lung. Further support for the involvement of a G_i-like GTP-binding protein in LT-receptor-effector coupling is the observation that pretreatment of guinea-pig minced lung with pertussis toxin attenuates the ability of LTD$_4$ to promote thromboxane (TX) and prostacyclin (PGI_2) biosynthesis (Mong *et al.*, 1986). Based upon these findings it has been proposed that a rapid reduction in the cyclic AMP content may be linked intimately to LTD$_4$-induced tension development (Andersson *et al.*, 1982).

In spite of these data, however, the effect of the peptido-leukotrienes on the cellular cyclic AMP content and on the activity of PKA has not been systematically studied in any tissue which express receptors for these lipid mediators. This may be a consequence, in part, of several more recent reports which provide compelling evidence that LTC$_4$ and LTD$_4$ can stimulate the hydrolysis of phosphatidylinositol (4,5)-bisphosphate by a receptor-mediated mechanism (e.g. Mong *et al.*, 1987; 1988; Sarau *et al.*, 1987) that, in some tissues, is sensitive to pertussis toxin (e.g. Howard *et al.*, 1992).

In view of the observations of Andersson *et al.* (1982, 1983) together with the other circumstantial evidence cited above, consideration should be given to the possibility that the peptido-leukotrienes can activate more than one signal transduction mechanism. Demonstration of such a phenomenon would be analogous to the effects of cholinomimetics on airways smooth muscle cells where they elicit an atropine-sensitive inhibition of adenylyl cyclase (Jones *et al.*, 1987), reduce the basal and isoprenaline-stimulated cyclic AMP content (Langlands *et al.*, 1987) and promote the reassociation (inactivation) of the regulatory and catalytic subunits of PKA (Langlands & Rodger, 1992), events associated temporally with tension generation. Over the same concentration-range these cholinomimetics also promote inositol polyphosphate accumulation, an intimate step in Ca^{2+}-dependent force generation in smooth muscle (see Hall & Chilvers, 1989, for review).

In an attempt to reconcile the reported action of the peptido-leukotrienes on the cyclic AMP content in guinea-pig trachea and lung tissue (Andersson *et al.*, 1982a,b; Hedman & Andersson, 1982,a,b; Barnes & Grandordy, 1986) with the currently accepted theory regarding LT-receptor-effector coupling (Mong *et al.*, 1987; 1988; Sarau *et al.*, 1987), the effect of LTD$_4$ on the cyclic nucleotide content and on the activity of PKA was examined in guinea-pig lung parenchyma and compared temporally to its established spasmogenic activity in this tissue.

Methods

Isolation, preparation and treatment of lung tissue

Male Camm-Hartley guinea-pigs (250–300 g) were used. Each animal was heparinised (140 units kg^{-1}), left for 10 min and then killed by cervical dislocation and exsanguinated. The thorax was opened and the lungs were perfused *in situ* with heparinised Krebs-Henseleit solution (KHS) through the right ventricle and pulmonary artery; the left atrium was removed from the heart to permit the outflow of the perfusion fluid. When the perfusate was free of blood the lungs were excised and placed in a petri dish containing KHS. The lower lung lobes were isolated from the major airways and blood vessels, and tissue showing significant haemorrhage was discarded. Sub-pleural parenchymal lung strips (~3 mm × 3 mm × 15 mm) were then cut and mounted vertically, under an initial stretching tension of 20 mN, in 10 ml tissue baths containing KHS maintained at 37°C. The KHS was bubbled gently with a gaseous mixture containing 95% O_2 and 5% CO_2 so as to produce a Po_2 = 70–73 kPa, Pco_2 = 5.1–5.3 kPa and a pH of 7.4. Each strip was left to equilibrate for 60 min before the addition of drugs. Cyclic nucleotide content and PKA activity was measured in tissues challenged with various concentrations of LTD$_4$ pretreated with either flurbiprofen, ICI 198,615, dazmegrel, EP 092 or their respective vehicles (as indicated in the text). In these studies LTD$_4$ was given as a bolus and at the peak of the induced mechanical response, or at pre-determined times (see text), tissues were freeze-clamped using tongs pre-cooled in liquid nitrogen and processed for cyclic nucleotide estimation and PKA activity as described below. Changes in tension resulting from drug treatment were measured isometrically by Grass FT03.c force-displacement transducers and recorded on a Grass 79D curvilinear, ink-writing polygraph. In some experiments the KHS bathing the lung strips was sampled for the estimation of TXB_2- and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$)-like immunoreactivity (see below). The composition of the KHS was as follows (mM): NaCl 118, KCl 4.7, $MgSO_4.7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.7, $CaCl_2.6H_2O$ 2.5.

Assay of cyclic AMP, cyclic GMP, 6-keto-PGF$_{1\alpha}$ and TXB$_2$

For cyclic nucleotide measurements each individual lung strip was weighed before being placed in a 2 ml capacity Teflon vial (pre-cooled in liquid nitrogen) containing 1 ml trichloroacetic acid (TCA, 6% w/v) and a 0.9 mm stainless steel grinding ball. Tissues were then disrupted (Braun Micro-Dismembrator II) for 30 s. The pulverised material was transferred to a 5 ml polypropylene centrifugation tube, residual material being removed from the teflon vial with a further 1 ml TCA. Each tube was then centrifuged at 11300 g_{max} for 15 min at 4°C. After centrifugation the supernatant was decanted into a large Pyrex tube and extracted six-times with 10 ml ice-cold water-saturated diethylether to ensure removal of the TCA and lipids in the extract. After the final extraction residual traces of ether were evaporated by heat in a water bath and the samples stored at −20°C. Before assaying for cyclic nucleotide content, samples were lyophilised and reconstituted in ice-cold Tris 20 mM/EDTA 2 mM buffer (pH 7.4). Tissue levels of cyclic AMP and guanosine 3':5'-cyclic monophosphate (cyclic GMP) were estimated with a commercially available competitive protein binding and radioimmunoassay kit respectively.

The concentrations of TXA_2 and PGI_2 released from guinea-pig lung into the bathing buffer was measured, as their stable non-enzymatic degradation products, TXB_2 and 6-keto-$PGF_{1\alpha}$ respectively, by radioimmunoassay as described in detail previously (Giembycz & Rodger, 1987). It was assumed that the conversion of TXA_2 to TXB_2 and PGI_2 to 6-keto-$PGF_{1\alpha}$ followed a 1:1 stoichiometry.

Preparation and assay of soluble PKA

Previous studies have demonstrated that guinea-pig lung contains primarily the Type II isoenzyme of PKA (Giembycz & Diamond, 1990a). To prevent the inactivation of this enzyme following drug treatment each frozen tissue was homogenized (Polytron PT 10) for 1 × 15 s burst at setting 8 in 20 vol (w/v) of ice-cold phosphate buffer (5 mM KH_2PO_4, 5 mM K_2HPO_4, pH 6.8) containing 10 mM EDTA, 10 mM DTT and 500 μM IBMX and supplemented with 20 mM NaCl and 140 mM KCl (Cherrington *et al.*, 1976). The resulting homogenate was centrifuged (Beckman J2 21) at 31000 g_{max} for 15 min at 4°C in a fixed arm JA 17 rotor (r_{av} = 9.6 cm) to form soluble (supernatant) and particulate (pellet) fractions. The soluble enzyme was diluted 16 fold in phosphate buffer immediately before use.

Soluble PKA activity was measured as described previously (Giembycz & Diamond, 1990b). One unit of enzyme activity is defined as that amount of PKA which catalysed the incorporation of 1 pmol of phosphate from ATP into the peptide substrate, Kemptide, in 1 min at 30°C. In some experiments PKA activity is expressed as an activity ratio which represents the specific activity of PKA in the absence of exogenous cyclic AMP divided by the specific activity of PKA in the presence of a concentration of cyclic AMP required maximally to activate the enzyme.

Measurement of haemoglobin content

The protein concentration attributable to haemoglobin in guinea-pig lung varied widely (6 to 24%) from tissue to tissue even after perfusion of >100 ml of heparinised KHS through the pulmonary circulation. The contribution made by this haemoglobin was thus determined for each tissue and subtracted from the total measured protein. Haemoglobin content was determined spectrophotometrically by measuring the absorbance, at 411 nm, of a suitably diluted aliquot of the soluble fraction of the original homogenate. The amount of protein corresponding to this absorbance was quantified by reference to a standard curve constructed using human haemoglobin.

Protein determination

The protein content of soluble fractions was measured according to the method of Lowry *et al.* (1951) with bovine serum albumin (BSA) used as standard. For quantifying the cyclic nucleotide data the protein in the pellet was first solubilised with 2% (w/v) sodium deoxycholate and then precipitated with TCA (24% w/v).

Chemical, drugs and analytical reagents

The following were purchased from the Sigma Chemical Company: ATP (free acid), bovine serum albumin (BSA; grade III) cyclic AMP, cyclic GMP, (±)-1,4-dithiothreitol (DTT), EDTA (free acid), human haemoglobin (fraction III), heparin, 3-isobutyl-1-methyl-xanthine (IBMX) and Kemptide. All other chemicals, drugs and analytical reagents were obtained from the following sources: [γ^{32}P]-ATP (20–40 Ci mmol^{-1}), [^{3}H]-cyclic AMP competitive protein binding assay kit, TRK 432 and [^{3}H]-cyclic GMP radioimmunoassay kit, TRK 500 (Amersham), dazmegrel (UK-38,485; 3-(1H-imidazol-1-yl-methyl)-2-methyl-1H-indole propanoic acid, Pfizer), EP 092 ((±)-5-endo-(6'carboxyhex-2'Z-enyl)-6-exo-{[1''-(phenylthiocarbamoyl)-hydrozono]-ethyl}-bicyclo [2,2,1] heptane; University of Edinburgh), flurbiprofen Na$^+$ (Boots), LTD_4 (Merck Frosst & Bayer), ICI 198,615 ([1-[[2-methoxy-4-[[phenylsulphonyl) amino]carbonyl] phenyl] methyl]-1H-indazol-6-yl] carbamic acid cyclopentyl ester; ICI) and salts for buffers and constituents of the assay cocktail (BDH).

Stock solutions of all drugs and reagents were made up in distilled water with the following exceptions: ICI 198,615 and dazmegrel were dissolved in 0.1 M NaOH to form their respective soluble sodium salts. IBMX was dissolved in 20% (v/v) ethanol.

LTD_4 was shipped to the laboratory in dry ice and stored at −80°C. Under these storage conditions the non-enzymatic conversion of LTD_4 to the inactive 11-*trans* isomer was minimized to a rate not exceeding 1.5% per month as assessed by reverse phase high performance liquid chromatography (h.p.l.c.). The molar concentration of stock solutions of LTD_4 was determined from its u.v. absorbance at 280 nm using its molar extinction coefficient of 4.03 × 10 cm^2 mmol^{-1}.

Statistical analysis

Values in the text represent the mean ± s.e.mean of n independent determinations. All data were analysed parametrically with the statistical package 'Number Cruncher Statistical System'. Student's t test (two-tailed) was used to test the significance between paired and unpaired variates. When more than two groups of unpaired data were compared significance was tested by one-way analysis of variance (ANOVA) and assessed with the Newman-Keuls modified t test. The null hypothesis was rejected when $P<0.05$.

Results

Effect of LTD_4 on tension, intracellular cyclic nucleotide content and on the elaboration of TXB_2 and 6-keto-$PGF_{1\alpha}$

Figure 1 illustrates the non-cumulative effect of LTD_4 on tension and on the intracellular cyclic nucleotide (cyclic AMP and cyclic GMP) content of guinea-pig isolated lung parenchyma. In agreement with previous studies (e.g. Dahlen *et al.*, 1983), LTD_4 (200 pM to 200 μM) evoked concentration-

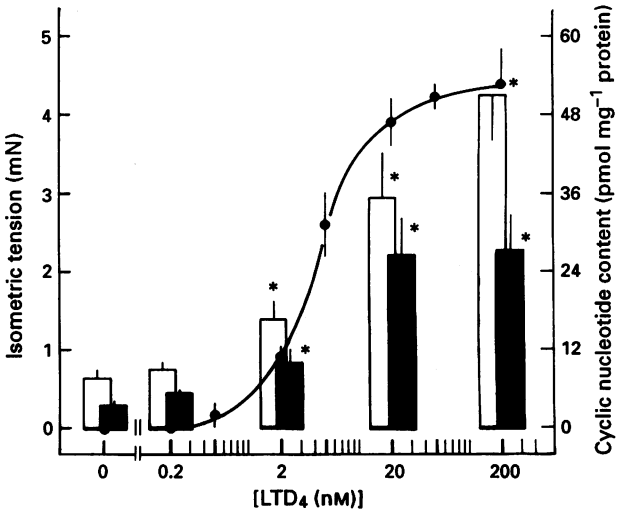


Figure 1 Concentration-response relationship for leukotriene D_4 (LTD_4)-induced tension generation (●), and cyclic AMP (solid columns) and cyclic GMP (open columns) accumulation in guinea-pig isolated lung parenchyma. Tissues were set up for isometric tension recording in KHS and after the equilibration period had elapsed challenged non-cumulatively with LTD_4 (0.2–200 nM). Changes in tension were subsequently measured at the peak of the resulting contracture. For cyclic nucleotide measurements, lung strips were incubated with LTD_4 (0.2–200 nM) for 5 min and then frozen rapidly by submersion in liquid nitrogen. Cyclic AMP and cyclic GMP were then extracted from tissues and their respective masses estimated as described in Methods. Data represent the mean ± s.e.mean (vertical lines) of six independent determinations. *$P<0.05$, significantly different from resting cyclic nucleotide levels as assessed by one-way ANOVA/Newman Keuls test.

dependent contractions of lung strips with a pD_2 and maximum force of 8.23 ± 0.09 ($n = 6$) and 4.3 ± 0.5 mN ($n = 6$) respectively. Pretreatment (60 min) of tissues with ICI 198,615 (1 μM), a potent and highly selective LTD_4-receptor antagonist (Snyder *et al.*, 1987), had no effect on the spontaneous tone generated by the lung but markedly antagonized LTD_4 (200 nM)-induced tension generation (Figure 2a) indicating that this response was mediated through stimulation of LTD_4-receptors.

Associated with the development of force, LTD_4 (200 pM to 200 μM) also promoted a concentration-dependent accumulation of both cyclic AMP and cyclic GMP (Figure 1), and enhanced over basal levels the elaboration of TXB_2 and 6-keto-$PGF_{1\alpha}$ into the bathing buffer (Figure 3). At the concentration of LTD_4 (200 nM) that produced the maximum contracture, the intracellular content of both cyclic nucleotides was increased four to seven fold (Figures 1, 2; Table 1) while the concentration of immunoreactive TXB_2 and 6-keto-$PGF_{1\alpha}$ present in the bathing buffer was increased ~150- and 70 fold respectively relative to time-matched controls (Figure 3).

Consistent with the effect of ICI 198,615 on LTD_4-induced tension generation, the increase in cyclic nucleotide content and the elaboration of TXB_2 and 6-keto-$PGF_{1\alpha}$ evoked by LTD_4 was attenuated markedly following pretreatment (60 min) of lung with ICI 198,615 (1 μM; Figure 2b,c; Figure 3a,b). ICI 198,615 did not, however, inhibit the spontaneous release of eicosanoids from the lung or alter the the resting cyclic nucleotide content (data not shown).

The kinetics of the mechanical and cyclic nucleotide-elevating effects of LTD_4 on guinea-pig lung are shown in Figure 4 and in Table 1. LTD_4 (200 nM) produced slow contractures of lung strips that developed over the 15 min period of study and which persisted even after the tissue was washed free of drug. Temporally, the accumulation of both cyclic AMP and cyclic GMP occurred either in parallel to or, more often, lagged behind tension development (Figure 4; Table 1). Indeed, the mean data of six independent experiments show that LTD_4 promoted a significant increase in tone 30 s post-drug challenge without affecting the cyclic AMP content (Figure 4). The cyclic nucleotide-elevating effect of LTD_4 occurred only transiently peaking after 5 min drug contact thereafter decaying despite the continued generation of tone (Figure 4).

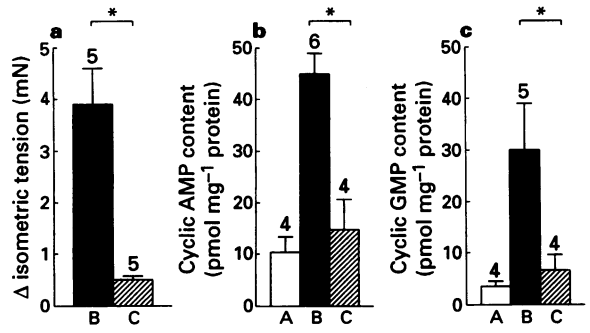


Figure 2 Effect of the leukotriene D_4 (LTD_4)-receptor antagonist ICI 198,615 on LTD_4-induced tension generation (a), cyclic AMP accumulation (b) and cyclic GMP accumulation (c) in guinea-pig isolated lung parenchyma. Lung strips were incubated with ICI 198,615 (1 μM for 60 min) or its vehicle and then challenged with LTD_4 (200 nM). At the peak of the resulting contracture tissues were frozen rapidly by submersion in liquid nitrogen and cyclic nucleotides subsequently extracted and their concentration measured as described in Methods. (A) resting cyclic nucleotide levels; (B) responses induced by LTD_4; (C) responses induced by LTD_4 in ICI 198,615-pretreated tissues. Data represent the mean ± s.e.mean (vertical lines) of n independent observations shown at the top of each column. $^*P < 0.05$, significant inhibition of LTD_4-induced responses as assessed by Student's unpaired t test.

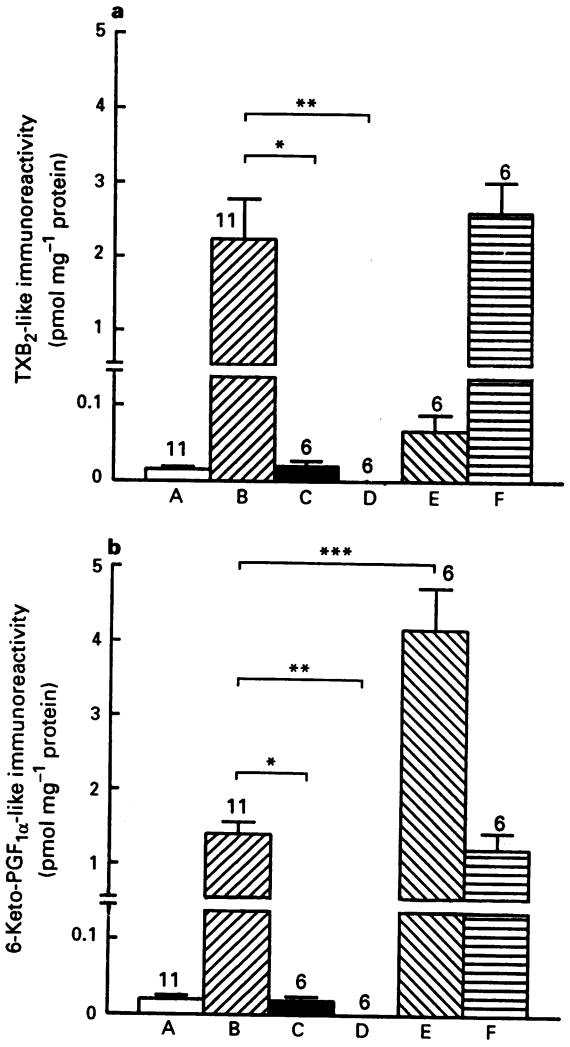


Figure 3 Pharmacological modulation of the release of thromboxane B_2 (TXB_2) (a) and 6-keto-prostaglandin$_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) (b) by leukotriene D_4 (LTD_4) from guinea-pig isolated lung parenchyma. Lung strips were incubated for 60 min with either ICI 198,615 (1 μM), flurbiprofen (8 μM), dazmegrel, (10 μM), EP 092 (1 μM) or vehicle. Tissues were then challenged with LTD_4 (200 nM). Immediately before, and 15 min after LTD_4 the KHS was sampled and assayed for immunoreactive TXB_2 and 6-keto-$PGF_{1\alpha}$ as described in Methods. The columns depict the total release (i.e. LTD_4 + spontaneous) of eicosanoids from the lung with respect to time-matched controls. (A) spontaneous; (B) LTD_4; (C) LTD_4 + ICI 198,615; (D) LTD_4 + flurbiprofen; (E) LTD_4 + dazmegrel; (F) LTD_4 + EP 092. Data represent the mean ± s.e.mean (vertical bars) of n independent observations shown at the top of each column. $^*P < 0.001$ and $^{**}P < 0.0001$, significant inhibition of LTD_4-evoked eicosanoid release. $^{***}P < 0.05$, significant augmentation of LTD_4-induced 6-keto-$PGF_{1\alpha}$ release. Data analysed by one-way ANOVA/Newman-Keuls test.

Effect of LTD_4 on PKA activity

Since receptor-mediated increases in the tissue cyclic AMP content are associated generally with smooth muscle relaxation (Diamond, 1978; Giembycz & Raeburn, 1991) studies were performed to determine if LTD_4-induced cyclic AMP accumulation was coupled to the activation of PKA, the enzyme through which all the actions of cyclic AMP are believed to be mediated (see Beebe & Corbin, 1986). The data in Figures 5 and 6 show the results of these experiments. In the absence of LTD_4, the specific activity of PKA extracted from the soluble fraction of lung homogenates was 1.93 ± 0.25 nmol min^{-1} mg^{-1} protein ($n = 6$) which was

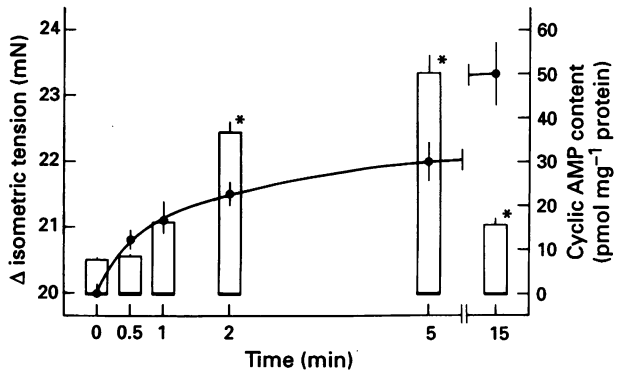


Figure 4 Kinetics of leukotriene D_4 (LTD_4)-induced tension generation (●) and cyclic AMP accumulation (columns) in guinea-pig isolated lung parenchyma. Lung strips were challenged with LTD_4 (200 nM) and after 0, 0.5, 1, 2, 5 and 15 min frozen rapidly by submersion in liquid nitrogen. Cyclic nucleotides were then extracted from each tissue and the cyclic AMP content subsequently measured as described in Methods. Data represent the mean ± s.e.mean (vertical bars) of eight independent observations. $*P < 0.05$, significantly different from resting cyclic AMP levels as assessed by one-way ANOVA/Newman Keuls test.

Table 1 Effect of flurbiprofen, dazmegrel and EP 092 on leukotriene D_4 (LTD_4)-induced tension development and on cyclic GMP accumulation in guinea-pig lung parenchyma

Treatment	*Contact Time* (min)	*Δ Tension* (mN)	*Cyclic GMP content* (pmol mg^{-1} protein)
Control	− 60	−	3.9 ± 0.4
($n = 8$)	0	0.3 ± 0.1	3.7 ± 0.7
	0.5	0.8 ± 0.2	6.3 ± 1.2
	1	1.1 ± 0.1	7.8 ± 2.5*
	2	1.5 ± 0.3	21.7 ± 5.3*
	5	2.0 ± 0.3	26.5 ± 2.8*
	15	3.3 ± 0.5	14.0 ± 2.8*
+ Flurbiprofen	− 60	−	6.1 ± 1.4
(8 μM; $n = 7$)	0	1.4 ± 0.3	3.0 ± 0.3
	0.5	0.2 ± 0.1	7.3 ± 1.6
	1	0.6 ± 0.1	10.6 ± 0.9*
	2	0.8 ± 0.2	8.9 ± 1.6
	5	1.5 ± 0.4	10.2 ± 3.9*
	15	2.1 ± 0.4	11.0 ± 3.8*
+ Dazmegrel	− 60	−	2.3 ± 0.1
(10 μM; $n = 5$)	0	1.2 ± 0.2	2.6 ± 0.3
	0.5	0.8 ± 0.1	4.9 ± 2.1
	1	0.9 ± 0.2	6.3 ± 1.4*
	2	1.7 ± 0.5	10.9 ± 0.7*
	5	2.3 ± 0.1	13.6 ± 0.7*
	15	2.8 ± 0.5	10.6 ± 2.4*
+ EP 092	− 60	−	4.1 ± 0.7
(10 μM; $n = 5$)	0	0.2 ± 0.1	3.5 ± 0.6
	0.5	0.7 ± 0.1	6.8 ± 1.1
	1	1.2 ± 0.4	11.7 ± 1.0*
	2	1.9 ± 0.2	14.7 ± 3.4*
	5	2.4 ± 0.4	19.1 ± 5.7*
	15	2.8 ± 0.5	17.4 ± 4.4*

Strips of guinea-pig lung were set up for isometric tension recording as described in Methods. After the initial equilibration period tissues were incubated for 60 min with either vehicle, flurbiprofen (8 μM), dazmegrel (10 μM) or EP 092 (10 μM) before being challenged with LTD_4 (200 nM). At the time points indicated changes in tension were measured and tissues were snap frozen in liquid nitrogen for subsequent cyclic GMP determinations. Data represent the mean ± s.e.mean of n independent determinations indicated in parentheses.
$*P < 0.05$ with respect to '0 min' control values (one-way ANOVA/Newman-Keuls test).

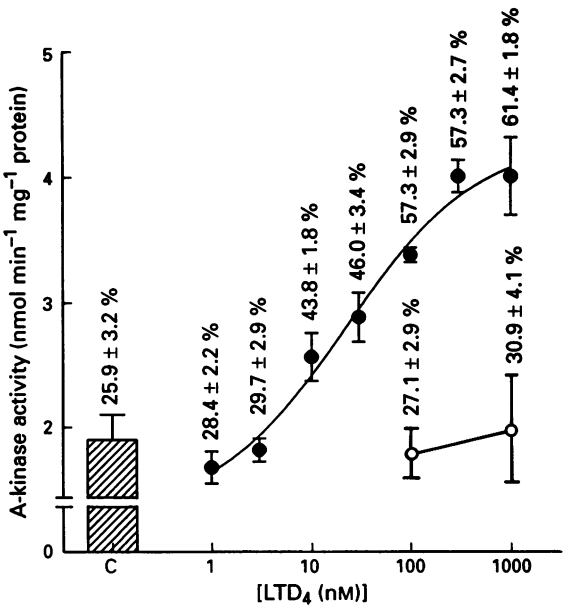


Figure 5 Concentration-dependent activation of PKA in guinea-pig isolated lung parenchyma by leukotriene D_4 (LTD_4). Lung strips were pretreated for 60 min with 1 μM ICI 198,615 (○) or its vehicle (●) and challenged non-cumulatively with LTD_4 (1–1000 nM). After 5 min drug contact, tissues were frozen rapidly by submersion in liquid nitrogen and PKA activity in the soluble fraction of lung homogenates measured as described previously (Giembycz & Diamond, 1990b). The bar indicates A-kinase activity in the absence of LTD_4 while the figures at each point refer to the percentage A-kinase present in an active form. Data represent the mean ± s.e.mean of six independent determinations measured in duplicate.

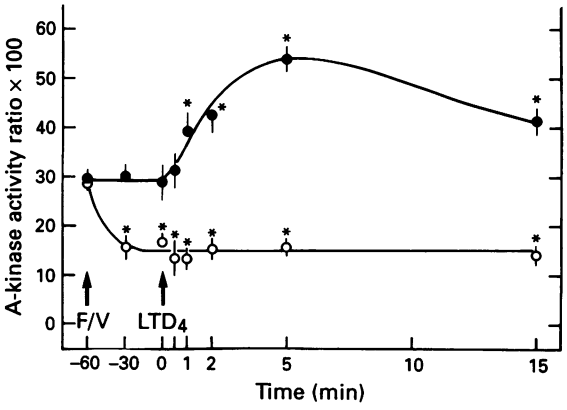


Figure 6 Kinetics of leukotriene D_4 (LTD_4)-induced PKA activation in guinea-pig isolated lung parenchyma and the effect of flurbiprofen. Lung strips were pretreated for 60 min with flurbiprofen (○) or its vehicle (●) and then challenged with LTD_4 (200 nM). At predetermined times, indicated in the figure, tissues were frozen rapidly by submersion in liquid nitrogen and A-kinase activity in the soluble fraction of lung homogenates measured as described previously (Giembycz & Diamond, 1990b). Data represent the mean ± s.e.mean of six determinations at each time-point. $*P < 0.05$, significantly different from resting PKA activity ratio as assessed by one-way ANOVA/Newman-Keuls test.

equivalent to PKA being ~26% active (i.e. present as free catalytic subunits) under resting conditions. Incubation of guinea-pig lung for 5 min with LTD_4 (1 nM to 1 μM) produced a concentration-dependent stimulation of PKA with a K_{act} of 32.6 ± 6.4 nM ($n = 6$) and maximum specific activity of 4.02 ± 0.31 nmol min^{-1} mg^{-1} protein ($n = 6$). Under these

conditions LTD_4 (1 μM for 5 min) more than doubled (to 61 ± 1.8%, $n = 6$) the proportion of PKA present in an active state (Figure 5). Kinetically, the profile of LTD_4 (200 nM)-induced PKA activation paralleled the changes in the cyclic AMP content described above peaking after 5 min contact then returning towards control activity (Figure 6). Moreover, no significant activation of PKA was detected at 30 s post-LTD_4 challenge despite a detectable increase in lung parenchymal tone (cf. Figure 4 with Figure 6).

Pre-incubation of guinea-pig lung with ICI 198,615 (1 μM for 60 min) abolished the stimulation of PKA induced by LTD_4 (100 nM and 1 μM) confirming that this effect of LTD_4 was receptor-mediated (Figure 5).

It is important to note that the observed activation of PKA was the result of an increase in the concentration of free (active) catalytic subunits since total cyclic AMP-dependent phosphotransferase activity was unchanged (data not shown).

Effect of inhibition of cyclo-oxygenase on the mechanical and biochemical actions of LTD_4

It is well documented that LTD_4 promotes the *de novo* biosynthesis and release of arachidonic acid-derived cyclo-oxyenase products in guinea-lung (e.g. Weichman *et al.*, 1982; Robinson *et al.*, 1984; Mong *et al.*, 1985a,b; Giembycz & Rodger, 1987). Since these eicosanoids exert marked biochemical and mechanical effects, themselves, their evoked release could mask effectively the direct actions of LTD_4. To examine the cyclo-oxygenase-independent actions of LTD_4, therefore, guinea-pig lung was pre-incubated (60 min) with the cyclo-oxygenase inhibitor, flurbiprofen (Rome & Lands, 1975), at a concentration (8 μM), which abolished both the spontaneous and LTD_4 (200 nM)-stimulated release of TXB_2 and 6-keto-$PGF_{1\alpha}$ (Figure 3).

Addition of flurbiprofen to guinea-pig lung strips resulted in a prompt reduction of spontaneous tone and an associated 47% decrease in the tissue cyclic AMP content (Figure 7a). Under these conditions, LTD_4 (200 nM) elicited contractures that were slower in onset and slightly less in magnitude than those seen in control tissues (cf. Figure 4 with Figure 7a) an observation consistent with the fact that LTD_4-induced tone is dependent, in part, upon the release of cylco-oxygenase products (e.g. Weichman *et al.*, 1982; Giembycz & Rodger, 1987). Moreover, flurbiprofen abolished the ability of LTD_4 to elevate the cyclic AMP content at any time-point, and also suppressed (by ~68%) the increase in cyclic GMP (Table 1).

The effect of flurbiprofen on basal and LTD_4-stimulated PKA activity is shown in Figure 6. In agreement with the cyclic AMP results described above, flurbiprofen reduced the PKA activity ratio from ~29% at rest to 17% after 60 min pre-incubation with the cyclo-oxygenase inhibitor. Subsequent challenge of guinea-pig lung with LTD_4 (200 nM) neither increased nor decreased further the PKA activity ratio over the 15 min post-challenge period studied (Figure 6).

It is clear from the results described above that the effect of LTD_4 on cyclic AMP levels and PKA activity do not corroborate the observations of either Andersson and co-workers (Andersson *et al.*, 1982; 1983; Hedman & Andersson, 1982a,b) and Barnes & Grandordy (1986). In an attempt to explain this discrepancy it was reasoned that LTD_4 may only be able to reduce the cyclic AMP content and lower the PKA activity ratio in flurbiprofen-treated tissue in the presence of an activator of adenylyl cyclase. To examine this possibility, the ability of LTD_4 to inhibit isoprenaline-stimulated cyclic AMP accumulation and PKA activity was evaluated. Consistent with our previous report (Giembycz & Diamond, 1990b), isoprenaline (10 μM for 10 min) elevated significantly the cyclic AMP content (from 3.9 ± 0.9 to 26.7 ± 5.1 pmol mg^{-1} protein, $n = 4$) and almost doubled the PKA activity ratio (from 14.6 ± 2.9% to 26.3 ± 5.5%, $n = 5$). The addition of LTD_4 (1 μM) to lung strips either 30 s or

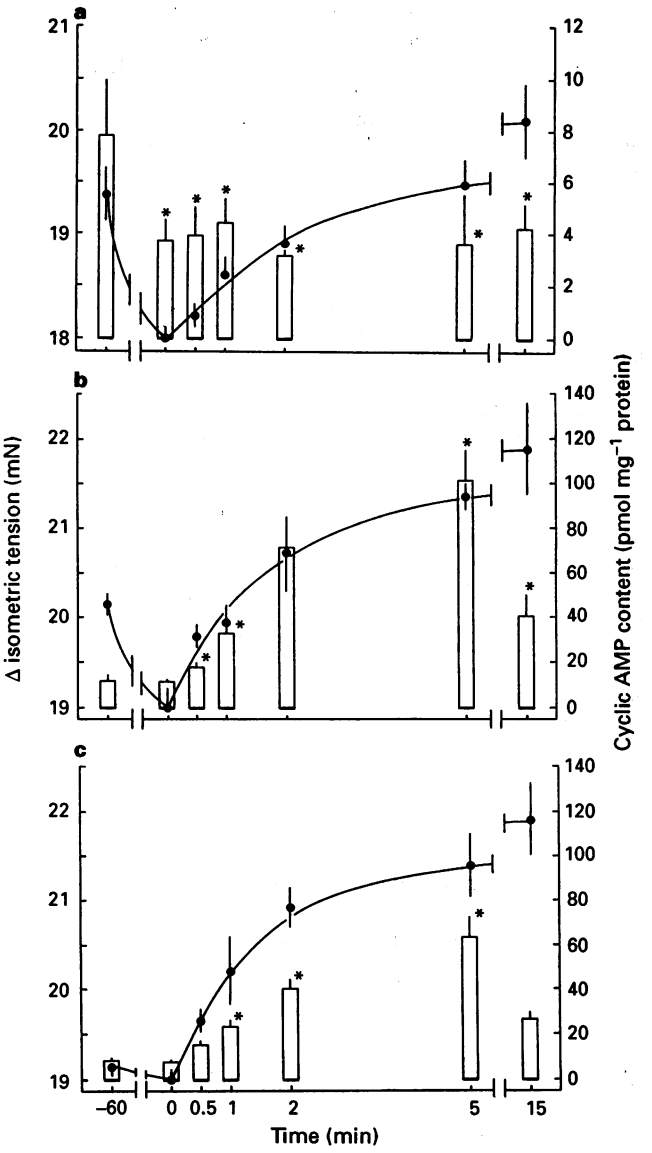


Figure 7 Kinetics of leukotriene D_4 (LTD_4)-induced tension generation and cyclic AMP accumulation in guinea-pig isolated lung parenchyma pretreated for 60 min with flurbiprofen (8 μM; a), dazmegrel (10 μM; b) and EP 092 (1 μM; c). See legend to Figure 4 and text for further details. Data represent the mean ± s.e.mean of seven, five and five independent observations at each time-point for flurbiprofen-, dazmegrel- and EP 092-pretreated tissues respectively. $*P < 0.05$, significantly different from resting cyclic AMP levels as assessed by one-way ANOVA/Newman-Keuls test.

5 min prior to isoprenaline, however, failed to inhibit cyclic AMP accumulation or PKA activation (data not shown).

Effect of inhibition of thromboxane synthetase and of TP-receptor blockade on the mechanical and biochemical actions of LTD_4

The results obtained from the experiments with flurbiprofen, described above, suggested that LTD_4-induced cyclic nucleotide accumulation and PKA activation were dependent upon the generation of a prostaglandin(s) and/or TXA_2. A number of studies were, therefore, performed to gain further information on the identity of the cyclo-oxygenase product(s) responsible for these effects.

Pretreatment of guinea-pig lung with dazmegrel (10 μM for 60 min), a selective inhibitor of thromboxane synthetase (Parry *et al.*, 1982) relaxed spontaneous tone of the tissue but, unlike flurbiprofen, did not affect either the basal cyclic

nucleotide content (Table 1; Figure 7b) or the PKA activity ratio (data not shown). LTD_4-induced tension development was similarly unaffected in dazmegrel-pretreated lung strips. In contrast, dazmegrel attenuated (by ~50%) the ability of LTD_4 to promote the accumulation of cyclic GMP, whereas the increase in the cyclic AMP content 5 min after challenge with LTD_4 was potentiated approximately two fold (Table 1; Figure 7b). Similarly, dazmegrel potentiated LTD_4-induced PKA activation (from $31.3 \pm 3.4\%$ to $74.1 \pm 7.9\%$ 5 min post LTD_4 challenge, $n = 5$).

To ensure that dazmegrel had, indeed, inhibited thromboxane synthetase in guinea-pig lung, the concentration of immunoreactive TXB_2 and 6-keto-$PGF_{1\alpha}$ was measured in the bathing buffer immediately before and after challenge of tissues with LTD_4 (200 nM). As shown in Figure 3, dazmegrel (10 μM for 60 min) reduced, to levels achieved spontaneously, the elaboration of TXB_2 from the lung but augmented (~2.5-fold) the LTD_4-stimulated release of 6-keto-$PGF_{1\alpha}$ (Figure 3b).

Pretreatment of guinea-pig lung with EP 092 (1 μM for 60 min), a potent and selective TP-receptor antagonist (Jones *et al.*, 1984), had no effect on spontaneous tone, the elaboration of TXB_2 and 6-keto-$PGF_{1\alpha}$ or on the basal level of cyclic GMP (Table 1; Figures 3 and 7c). Similarly, LTD_4-induced tension generation was unaffected by EP 092 although there was a small potentiation of LTD_4-induced cyclic AMP accumulation at all time-points where measurements were made except at 2 min (Figure 7c).

Discussion

The results of these studies demonstrate that under equilibrium conditions LTD_4-induced tension development of guinea-pig isolated lung parenchyma is associated with, but divorced temporally from, a cyclo-oxygenase-dependent increase in the cyclic AMP content and in the activation state of PKA. Moreover, under conditions where the elaboration of cyclo-oxygenase products is prevented, LTD_4 affects neither of these biochemical parameters irrespective of whether the experiment is performed in the absence or presence of the β-adrenoceptor agonist, isoprenaline. It is clear, therefore, that the results obtained herein with LTD_4 in flurbiprofen-pretreated tissues contradict data published previously where LTC_4, LTD_4 and immunologically-generated SRS-A are reported to reduce rapidly the intracellular cyclic AMP content in guinea-pig tracheae and lung parenchyma (Andersson *et al.*, 1982; 1983; Hedman & Andersson, 1982a,b; Barnes & Grandordy, 1986) and, in the case of SRS-A, lower the PKA activity ratio (Hedman & Andersson, 1982b). These data, therefore, do not support the suggestion that receptors for the peptido-leukotrienes can couple directly to adenylyl cyclase in guinea-pig lung and thus argue against the assertion that a reduction in intracellular cyclic AMP is related to LTD_4-induced tension generation in these tissues (Andersson *et al.*, 1982; 1983; Hedman & Andersson, 1982a,b; Barnes & Grandordy, 1986). This conclusion is supported by preliminary data from Langlands *et al.* (1987), who also failed to detect any rapid decrease in the cyclic AMP content and PKA activity ratio in guinea-pig tracheae challenged with LTD_4. Moreover, since submission of this manuscript, Howard *et al.* (1992) reported that LTD_4 produced no demonstrable effect on the cyclic AMP content of guinea-pig cultured tracheal smooth muscle cells either in the absence or presence of the adenylyl cyclase activator forskolin.

It was reasoned that one explanation for the inability of LTD_4 to lower the cyclic AMP content in flurbiprofen-treated lung may relate to a low basal adenylyl cyclase activity such that inhibition of this enzyme would not result in a detectable decrement in the cyclic AMP content when whole cell cyclic nucleotide measurements were made. This reasoning was seemingly incorrect since LTD_4 failed also to attenuate isoprenaline-induced cyclic AMP accumulation and PKA activation in this tissue.

Another explanation given consideration was derived from the knowledge that distinct receptors for LTC_4 and LTD_4 are expressed by guinea-pig lung (e.g. Hogaboom *et al.*, 1983; Mong *et al.*, 1985a) and trachea (e.g. Krell *et al.*, 1983; Weichman & Tucker, 1985). Conceivably a reduction in cyclic AMP levels may be affected only following activation of the LTC_4 receptor. Although a tenable hypothesis, this is unlikely (unless the LTC_4 and LTD_4 receptors in the lung are different from those expressed by guinea-pig trachea) since both LTC_4 and LTD_4 reduce rapidly the cyclic AMP content in guinea-pig tracheae (Andersson *et al.*, 1982, 1983). At the present time, therefore, we can provide no satisfactory explanation for the discrepancy between the data presented in this study and results reported previously (see above).

The results obtained with flurbiprofen indicate that the LTD_4-stimulated increase in cyclic AMP mass and in the activity of PKA is mediated indirectly via the *de novo* biosynthesis and subsequent release of one or more prostanoids catalysed by the cyclo-oxygenation of arachidonic acid. The observation that pretreatment of lung tissue with the TP-receptor blocking drug, EP 092, did not inhibit the increase in cyclic AMP induced by LTD_4 suggests that TXA_2 does not mediate these biochemical effects. This conclusion is corroborated by the results obtained with the thromboxane synthetase inhibitor, dazmegrel, which enhanced (rather than inhibited) LTD_4-induced cyclic AMP accumulation and activation of PKA. These data thus suggest that pretreatment of lung with dazmegrel leads to the accumulation and subsequent diversion of the prostaglandin endoperoxide intermediates to the synthesis of cyclic AMP-generating cyclo-oxygenase products in response to LTD_4. Further support for this proposal was the finding that dazmegrel augmented more than two fold the ability of LTD_4 to release the prostacyclin metabolite, 6-keto-$PGF_{1\alpha}$, from the lung.

Despite the fact that LTD_4 enhanced the generation of prostacyclin (this study), and prostaglandins E_2 and $F_{2\alpha}$ from guinea-pig lung (Robinson *et al.*, 1984), the identity of the cyclic AMP-generating prostanoid(s) released is not clear and was outside the objectives of this study. However, the finding of Gardiner (1986) that butaprost, a highly selective EP_2-receptor agonist (which, unlike the naturally occurring prostaglandins, does not evoke TP-receptor-mediated contraction of guinea-pig lung) inhibits LTD_4-induced tension generation in this tissue indicates that this response may be mediated, at least in part, by prostaglandins which stimulate inhibitory EP_2-receptors which are reported to be positively coupled to adenylyl cyclase (Jumblatt & Paterson, 1991; Nakahata, 1992). It is noteworthy, however, that since lung parenchyma is a mixture of more than 40 different cell types (Stahlman & Gray, 1978) other adenylyl cyclase-coupled prostanoid receptors on non-contractile tissues may contribute significantly to the cyclic AMP-elevating action of LTD_4.

In addition to increasing the concentration of cyclic AMP in guinea-pig lung, LTD_4 also elevated the cyclic GMP content in a concentration-dependent manner which is consistent with the fact that many smooth muscle spasmogens promote cyclic GMP accumulation (Kuo & Kuo, 1973; Diamond, 1978). The precise biochemical events which culminate in an increase in cyclic GMP mass are uncertain though in many tissues an elevation in the cytosolic free Ca^{2+} concentration will stimulate soluble guanylyl cyclase (Schultz *et al.*, 1973; Spies *et al.*, 1980). Furthermore, a variety of long chain poly-unsaturated fatty acids, including arachidonate, can stimulate cyclic GMP biosynthesis (Gruetter & Ignarro, 1979; Lewicki *et al.*, 1983; Gerzer *et al.*, 1983) which has led to the proposal that arachidonic acid or one of its metabolites can function as an endogenous regulator of soluble guanylyl cyclase (e.g. Gerzer *et al.*, 1983). The results reported herein are consistent with this latter hypothesis given the observation that the elevation in cyclic GMP seen in response to LTD_4 was attenuated significantly in tissues pretreated with

the cyclo-oxygenase inhibitor, flurbiprofen. This result is comparable to the study of Gruetter *et al.* (1983) who reported that the activation of soluble guanylyl cyclase isolated from guinea-pig lung by arachidonate was dependent upon active cyclo-oxygenase.

Although the identity of the lipid species responsible for stimulating cyclic GMP biosynthesis is unknown, the observation that dazmegrel attenuated the LTD_4-induced increase in this cyclic nucleotide to a similar degree as flurbiprofen suggests that TXA_2 or one of its metabolites is a principal regulator of soluble guanylyl cyclase in guinea-pig lung. Further support for this idea derives from the finding that the TP-receptor antagonist, EP 092, which did not affect the cyclo-oxygenation of arachidonate as assessed by the elaboration of TXB_2, exerted little effect on the time course or magnitude of LTD_4-induced cyclic GMP accumulation.

In conclusion, the results presented herein do not confirm previous studies which document a cyclic AMP lowering action of peptido-LT's on guinea-pig tracheae and lung parenchyma. As such, these data do not support the hypothesis that LTD_4-induced tension generation of these tissues requires a decrement in the cyclic AMP content.

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References

ANDERSSON, R.G.G., GUSTAFSSON, L.E., HEDMAN, S.E., HEDQVIST, P. & SAMUELSSON, B. (1982). Leukotriene C_4-induced cyclic nucleotide changes and contractile responses in guinea-pig trachea. *Acta Physiol Scand.*, **116**, 97–99.

ANDERSSON, R.G.G., HEDMAN, S.E. & HULTGREN, A. (1983). Leukotrienes, leukotriene-receptive substances and the cyclic AMP system of guinea-pig airways. *Adv. Cyclic Nucl. Protein. Phosphorylation. Res.*, **17A**, 117.

BARNES, P.J. & GRANDORDY, B. (1986). Leukotriene C_4 elicits inositol phosphate formation and inhibits adenylate cyclase in guinea-pig lung. *Br. J. Pharmacol.*, **89**, 783P.

BEEBE, S.J. & CORBIN, J.D. (1986). Cyclic nucleotide-dependent protein kinases. *The Enzymes*, **17**, 43–111.

BRUNS, R., THOMSEN, W.J. & PUGSLEY, T.A. (1983). Binding of leukotriene C_4 and D_4 to membranes from guinea-pig lung: regulation by ions and nucleotides. *Life Sci.*, **33**, 645–653.

CHENG, J.B., LANG, D., BENTRA, A. & TOWNLEY, R.G. (1985). Tissue distribution and functional correlation of [^{3}H] leukotriene C_4 and [^{3}H] leukotriene D_4 binding sites in guinea-pig uterus and lung preparations. *J. Pharmacol. Exp. Ther.*, **232**, 80–87.

CHENG, J.B. & TOWNLEY, R.G. (1984). Identification of leukotriene D_4 receptor binding sites in guinea-pig lung homogenates using [^{3}H] leukotriene D_4. *Biochem. Biophys. Res. Commun.*, **118**, 20–26.

CHERRINGTON, A.D., ASSIMACOPOULOS, F.D., HARPER, S.C., CORBIN, J.D., PARK, C.R. & EXTON, J.H. (1976). Studies on the *a*-adrenergic activation of hepatic glucose output. II. Investigation of the roles of adenosine cyclic 3',5'-monophosphate and guanosine cyclic 3',5'-monophosphate-dependent protein kinase in the actions of phenylephrine in isolated hepatocytes. *J. Biol. Chem.*, **251**, 5209–5218.

DAHLEN, S.-E., HEDQVIST, P., WESTLUND, P., GRANDSTROM, E., HAMMARSTROM, S., LINDGREN, J.A. & RADMARK, O. (1983). Mechanisms of leukotriene-induced contractions of guinea-pig airways: leukotriene C_4 has a potent direct action whereas leukotiene B_4 acts indirectly. *Acta Physiol. Scand.*, **118**, 393–403.

DIAMOND, J. (1978). Role of cyclic nucleotides in control of smooth muscle contraction. *Adv. Cyclic Nucl. Res.*, **9**, 327–340.

GARDINER, P.J. (1986). Characterization of prostanoid relaxant/inhibitory receptors (ψ) using a highly selective agonist, TR4979. *Br. J. Pharmacol.*, **87**, 45–56.

GERZER, R., HAMET, P., ROSS, A.H., LAWSON, J.A. & HARDMAN, J.G. (1983). Calcium-induced release from platelets of fatty acids that modulate soluble guanylate cyclase. *J. Pharmacol. Exp. Ther.*, **226**, 180–186.

GIEMBYCZ, M.A. & DIAMOND, J. (1990a). Characterisation of cyclic AMP-dependent protein kinases in guinea-pig lung employing the novel synthetic substrate, Kemptide: In vitro sensitivity of the soluble enzyme to isoprenaline, forskolin, methacholine and leukotriene D_4. *Biochem. Pharmacol.*, **39**, 1297–1312.

GIEMBYCZ, M.A. & DIAMOND, J. (1990b). Evaluation of Kemptide, a serine-containing heptapeptide, for the estimation of cyclic AMP-dependent protein kinase activity in respiratory tissues. *Biochem. Pharmacol.*, **39**, 271–283.

GIEMBYCZ, M.A. & RAEBURN, D. (1991). Putative substrates for cyclic nucleotide-dependent protein kinases and the control of airway smooth muscle tone. *J. Autonomic Pharmacol.*, **11**, 365–398.

GIEMBYCZ, M.A. & RODGER, I.W. (1987). Does thromboxane mediate the indirect contractile action of leukotriene D_4 in guinea-pig isolated lung parenchyma under equilibrium conditions? *Can. J. Physiol. Pharmacol.*, **65**, 1467–1477.

GRUETTER, D.Y. & IGNARRO, L.J. (1983). Arachidonic acid activation of guinea-pig lung guanylate cyclase by two independent mechanisms. *Prostaglandins*, **18**, 541–556.

HALL, I.P. & CHILVERS, E.R. (1989). Inositol phosphates and airway smooth muscle. *Pulm. Pharmacol.*, **2**, 113–120.

HEDMAN, S.E. & ANDERSSON, R.G.G. (1982a). Effect of SRS (slow reacting substance) on cyclic nucleotides in guinea-pig tracheal smooth muscle. *Acta Pharmacol. Toxicol.*, **50**, 30–34.

HEDMAN, S.E. & ANDERSSON, R.G.G. (1982b). The cyclic AMP system in sensitized and desensitized guinea-pig tracheal smooth muscle. *Eur. J. Pharmacol.*, **83**, 107–112.

HOGABOOM, G.K., MONG, S., WU, H.-L. & CROOKE, S.T. (1983). Peptidoleukotrienes: distinct receptors of leukotriene C_4 and D_4 in the guinea-pig lung. *Biochem. Biophys. Res. Commun.*, **116**, 1136–1143.

HOWARD, S., CHAN-YEUNG, M., MARTIN, L., PHANEUF, S. & SALARI, H. (1992). Polyphosphoinositide hydrolysis and protein kinase C activation in guinea-pig tracheal smooth muscle cells in culture by leukotriene D_4 involve a pertussis toxin sensitive G-protein. *Eur. J. Pharmacol - Mol. Pharmacol. Section.*, **227**, 123–129.

JAKOBS, K.H., AKTORIES, K. & SCHULTZ, G. (1984). Mechanisms and components involved in adenylate cyclase inhibition by hormones. *Adv. Cyclic Nucl. Protein. Phosphorylation. Res.*, **17**, 135–143.

JONES, C.A., MADISON, J.M., TOM-MOY, M. & BROWN, J.K. (1987). Muscarinic cholinergic inhibition of adenylate cyclase in airway smooth muscle. *Am. J. Physiol.*, **253**, C97–C104.

JONES, R.L., SMITH, G.M. & WILSON, N.H. (1984). The effect of synthetic prostanoid EP 092 on intravascular platelet aggregation and bronchoconstriction. *Br. J. Pharmacol.*, **81**, 100P.

JUMBLATT, M.M. & PATERSON, C.A. (1991). Prostaglandin E_2 effects on corneal cyclic adenosine monophosphate synthesis and cell shape change are mediated by a receptor of the EP_2 subtype. *Invest. Ophthalmol. Visual. Sci.*, **32**, 360–365.

KRELL, R.D., TSAI, B.S., BERDOULAY, A., BARONE, M. & GILES, R.E. (1983). Heterogeneity of leukotriene receptors in guinea-pig trachea. *Prostaglandins*, **25**, 171–178.

KUO, J.F. & KUO, W.-N. (1973). Regulation by β-adrenergic receptor and muscarinic cholinergic receptor activation of intracellular cyclic AMP and cyclic GMP levels in rat lung slices. *Biochem. Biophys. Res. Commun.*, **55**, 660–665.

LANGLANDS, J.M. & RODGER, I.W. (1992). The effect of methacholine and histamine on cyclic AMP-dependent protein kinase activity in the guinea-pig isolated trachea. *Can. J. Physiol. Pharmacol.*, **70**, 344–348.

LANGLANDS, J.M., RODGER, I.W. & WATSON, J. (1987). Effect of methacholine and leukotriene D_4 on cyclic AMP content and cyclic AMP-dependent protein kinase activity in airway smooth muscle. *Br. J. Pharmacol.*, **92**, 633P.

LEWICKI, J.A., WALDMAN, S.A., CHANG, B. & MURAD, F. (1983). Membrane-dependent regulation of soluble guanylate cyclase by snake venom phospholipase A_2. *Fed. Proc.*, **42**, 902.

LEWIS, R.A., AUSTEN, K.F., DRAZEN, J.M., CLARCK, D.M., MARFAT, A. & COREY, E.J. (1980). Slow reacting substance of anaphylaxis: identification of leukotriene C-1 and D from human rat sources. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 3710–3714.

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

MONG, S., CHI-ROSSO, G., HAY, D.W.P. & CROOKE, S.T. (1988). Subcellular localization of leukotriene D_4 receptors in sheep tracheal smooth muscle. *Mol. Pharmacol,*. **34**, 590–596.

MONG, S., MILLER, J., WU, H.-L. & CROOKE, S.T. (1987). Leukotriene D_4 receptor-mediated hydrolysis of phosphoinositide and mobilization of calcium in sheep tracheal smooth muscle cells. *J. Pharmacol. Exp. Ther.*, **244**, 508–515.

MONG, S., WU, H.-L., CLARK, M.A., GLEASON, J.G. & CROOKE, S.T. (1986). Leukotriene D_4 receptor-mediated synthesis and release of arachidonic acid metabolites in guinea-pig lung: induction of thromboxane and prostacyclin biosynthesis by leukotriene D_4. *J. Pharmacol. Exp. Ther.*, **239**, 63–70.

MONG, S., WU, H.-L., HOGABOOM, K.H., CLARK, M.A. & CROOKE, S.T. (1984). Characterization of the leukotriene D_4 receptor in guinea-pig lung. *Eur. J. Pharmacol.*, **102**, 1–11.

MONG, S., WU, H.-L., HOGABOOM, K.G., CLARK, M.A., STADEL, J.M. & CROOKE, S.T. (1985b). Regulation of ligand binding to leukotriene D_4 receptors: effects of cations and guanine nucleotides. *Eur. J. Pharmacol,*. **106**, 241–253.

MONG, S., WU, H.-L., MILLER, J., HALL, R.F., GLEASON, J.G. & CROOKE, S.T. (1988). SKF 104353, a high affinity antagonist for human and guinea-pig lung leukotriene D_4 receptor, blocked phosphatidylinositol metabolism and thromboxane synthesis induced by leukotriene D_4. *Mol. Pharmacol.*, **32**, 223–229.

MONG, S., WU, H.-L., SCOTT, M.O., LEWIS, M.A., CLARK, M.A., WEICHMAN, B.M., KINZIG, C.M., GLEASON, J.G. & CROOKE, S.T. (1985a). Molecular heterogeneity of leukotriene receptors: correlation of smooth muscle contraction and radioligand binding in guinea-pig lung. *J. Pharmacol. Exp. Ther.*, **234**, 330–335.

NAKAHATA, N. (1992). Signal transduction of prostaglandin E_2 and thromboxane A_2. *Folia Pharmacol. Jap.*, **99**, 1–11.

PARRY, M.J., RANDALL, M.J., HAWKSWOOD, E., CROSS, P.E. & DICKINSON, R.P. (1982). Enhanced production of prostacyclin in blood after treatment with selective thromboxane synthetase inhibitor, UK-38,485. *Br. J. Pharmacol.*, **77**, 547P.

PONG, S.S. & DE HAVEN, R.N. (1983). Characterization of a leukotriene D_4 receptor in guinea-pig lung. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 7415–7420.

ROBINSON, C., HOULT, J.R.S., WADDELL, K.A., BLAIR, J.A. & DOLLERY, C.T. (1984). Total profiling by GC/NICIMS of the major cyclo-oxygenase products from antigen and leukotriene-challenged guinea-pig lung. *Biochem. Pharmacol.*, **33**, 395–400.

ROME, L.H. & LANDS, W.E.M. (1975) Structural requirements for time-dependent inhibition of prostaglandin biosynthesis by anti-inflammatory agents. *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 4863–4865.

SARAU, H.M., MONG, S., FOLEY, J.J., WU, H.-L. & CROOKE, S.T. (1987). Identification and characterization of leukotriene D_4 receptors and signal transduction processes in rat basophilic leukemia cells. *J. Biol. Chem.*, **262**, 4034–4041.

SCHULTZ, G., HARDMAN, J.G., SCHULTZ, K., BAIRD, C.E. & SUTHERLAND, E.W. (1973). The importance of calcium ions for the regulation of guanosine 3',5'-monophosphate levels. *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 3889–3893.

SNYDER, D.W., GILES, R.E., KEITH, R.A., YEE, Y.K. & KRELL, R.D. (1987). *In vitro* pharmacology of ICI 198,615: a novel, potent and selective leukotriene antagonist. *J. Pharmacol. Exp. Ther.*, **243**, 548–556.

SPIES, C., SCHULTZ, K.-D. & SCHULTZ, G. (1980). Inhibitory effects of mepacrine and eicosatetraynoic acid on cyclic GMP elevations caused by calcium and hormonal factors in rat ductus deferens. *Naunyn-Schmiedebergs. Arch. Pharmacol.*, **311**, 71–77.

STAHLMAN, M.T. & GRAY, M.E. (1978). Anatomical development and maturation of the lung. *Clin. Perinatol,*. **5**, 225–236.

WEICHMAN, B.M., MUCCITELLI, R.M., OSBORNE, R.R., HOLDEN, D.A., GLEASON, J.G. & WASSERMAN, M.A. (1982). In vitro and in vivo mechanisms of leukotriene-mediated bronchoconstriction in the guinea-pig. *J. Pharmacol. Exp. Ther.*, **222**, 202–208.

WEICHMAN, B.M. & TUCKER, S.S. (1985). Differentiation of the mechanisms by which leukotrienes C_4 and D_4 elicit contraction of guinea-pig trachea. *Prostaglandins*, **29**, 694–699.

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Feedback inhibition of nitric oxide synthase activity by nitric oxide

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1 A murine macrophage cell line, J774, expressed nitric oxide (NO) synthase activity in response to interferon-gamma (IFN-γ, 10 u ml^{-1}) plus lipopolysaccharide (LPS, 10 ng ml^{-1}). The enzyme activity was first detectable 6 h after incubation, peaked at 12 h and became undetectable after 48 h.

2 The decline in the NO synthase activity was not due to inhibition by stable substances secreted by the cells into the culture supernatant.

3 The decline in the NO synthase activity was significantly slowed down in cells cultured in a low L-arginine medium or with added haemoglobin, suggesting that NO may be involved in a feedback inhibitory mechanism.

4 The addition of NO generators, S-nitroso-acetyl-penicillamine (SNAP) or S-nitroso-glutathione (GSNO) markedly inhibited the NO synthase activity in a dose-dependent manner. The effect of NO on the enzyme was not due to the inhibition of *de novo* protein synthesis.

5 SNAP directly inhibited the inducible NO synthase extracted from activated J774 cells, as well as the constitutive NO synthase extracted from the rat brain.

6 The enzyme activity of J774 cells was not restored after the removal of SNAP by gel filtration, suggesting that NO inhibits NO synthase irreversibly.

Keywords: Nitric oxide; nitric oxide synthase inhibition; macrophage; J774 cells

Introduction

Nitric oxide (NO), derived from molecular oxygen and the guanidino nitrogen of L-arginine, is involved in a variety of biological functions (reviewed in Moncada *et al.*, 1991). Nitric oxide is the transduction mechanism for the soluble guanylate cyclase responsible for endothelium-dependent vascular relaxation, modulation of platelet aggregation and some forms of central and peripheral neurotransmission (reviewed in Moncada *et al.*, 1991). Nitric oxide also contributes to the cytotoxic and cytostatic actions of macrophages activated by various immunological stimuli (Hibbs *et al.*, 1988). The production of NO is catalysed by the enzyme NO synthase, of which there are at least two different types. One is constitutive and is Ca^{2+}/calmodulin-dependent. The other is inducible by cytokines and bacterial lipopolysaccharide (LPS) and is Ca^{2+}-independent. The activity of these enzymes can be specifically and stoichiometrically inhibited by structural analogues of L-arginine such as L-N^G-monomethyl arginine (L-NMMA).

The generation of NO by the inducible NO synthase is tightly regulated. Indeed, a number of cytokines such as transforming growth factor-beta (TGF-β) (Ding *et al.*, 1990), interleukin-4 (IL-4) (Liew *et al.*, 1991) and IL-10 (Cunha *et al.*, 1992) are effective in inhibiting the expression of the NO synthase. Furthermore, the induction of NO synthase in macrophages *in vitro* is accompanied by a steep increase in nitrite (NO_2^-) accumulation in the culture supernatant, which reaches a plateau 40–60 h after the initial stimulus (Stuehr & Marletta, 1987; Takema *et al.*, 1991). This suggests that there may also be a self-regulatory mechanism modulating the activity of NO synthase. We show here that NO can act as a negative feedback mechanism for its own synthesis.

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Methods

Materials

The murine macrophage cell line J774 was obtained from American Tissue Culture Collection (ATCC). Cells were maintained in culture medium (DMEM [Gibco] supplemented with 10% foetal calf serum [FCS], 2 mM L-glutamine, 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 20 mM glucose). For this standard DMEM, the initial L-arginine concentration was 351 ± 10.2 μM ($n = 9$). In some experiments, DMEM with a low L-arginine concentration (13.5 ± 0.5 μM; $n = 3$) was used. The residual L-arginine was due to the amino acid present in FCS which was not dialysed. Murine recombinant IFN-γ was kindly provided by Dr G. Adolf, Ernst Boehringer-Institut fur Arzneimettel-Forschung, Vienna, Austria. LPS from *E. coli* (026:B6) was obtained from Difco. S-nitroso-acetyl-penicillamine (SNAP) was kindly provided by Dr H. Hodson (Department of Medicinal Chemistry, The Wellcome Research Laboratories, Beckenham). S-nitroso-glutathione (GSNO), synthesized according to the method of Hart (1985), was kindly provided by Dr A. Noronha-Dutra (Middlesex Hospital, University of London). 8-Bromo-guanosine 3':5'-cyclic monophosphate (8-Br-cyclic GMP) was purchased from Sigma. L-[^{3}H]-leucine (specific activity 120 Ci mmol^{-1}; 4.44 TBq mmol^{-1}) and L-[2,3,4,5-^{3}H]-arginine (specific activity 62 Ci mmol^{-1}; 2.3 TBq mmol^{-1}) were obtained from the Radiochemical Centre, Amersham (UK). All other reagents were purchased from Sigma (Poole, England).

Induction of NO synthase activity and NO_2^- synthesis

J774 cells were cultured for 2 h at 37°C in an atmosphere of 5% CO_2 in 12-well Costar plates at 2 × 10^6 cells per 2 ml of DMEM. To each well, 2 ml of medium alone (control) or medium containing the stimulus (10 u ml^{-1} IFN-γ plus

10 ng ml^{-1} LPS) was added. The activity of NO synthase and the NO_2^- concentration were determined 6, 12, 24 and 48 h after activation.

Effect of cell supernatant on NO synthase levels

Cells were left in contact with or without the stimulus for 4 h, washed and cultured in fresh medium for another 12 h. Preliminary experiments demonstrated that the levels of NO synthase attained with this short period of exposure were the same as when the stimulus was left for longer periods. The supernatant was collected, filtered through a 0.2 μm filter and diluted 1:1 with fresh DMEM. Fresh J774 monolayers were pre-incubated with this culture supernatant (2 ml per well) for 12 h followed by stimulation with IFN-γ plus LPS. Nitric oxide synthase activity was determined 12 h after stimulation.

Effects of NO donors, 8-Br-cyclic GMP and haemoglobin on NO synthase levels

All experiments with SNAP, GSNO or 8-Br-cyclic GMP were carried out in low L-arginine medium to avoid interference by endogenously-produced NO. The NO donors and the cyclic nucleotide analogue were added together with the stimulus and the enzyme activity was assayed 12 h later. To test the effects of these compounds on the total protein synthesis, cells were pulsed with L-[^{3}H]-leucine (1 μCi per well; 2 h, 37°C) 4 h or 6 h after the stimulation. The medium was removed and the cells were washed with PBS containing 2 mM cold L-leucine and finally with 10% and 5% trichloracetic acid. The radioactivity in the monolayers was counted in a liquid scintillation counter (LKB, Sweden). The experiments with haemoglobin were carried out in standard DMEM.

Effect of SNAP on the inducible and constitutive NO synthases

Cytosol (20,000 *g*) from J774 cells was prepared by sonicating cells activated by 12 h incubation with the stimulus and used as the source of inducible enzyme. Rat brain cytosol was prepared by homogenizing brains in 0.1 M HEPES/1 mM dithiothreitol (DTT), pH 7.4, in a ratio of 1:5 (w/v) followed by centrifugation (20,000 *g*). The supernatant was used as the source of constitutive enzyme. Aliquots of both cytosols containing 5 μM tetrahydrobiopterin were incubated for 30 min at room temperature in the reaction mixture for the citrulline assay (see below) containing 0, 30, 100, 300 or 1000 μM of SNAP. The addition of SNAP or GSNO did not alter the pH of the medium. The citrulline assay was used because NO released by SNAP interferes with the spectrophotometric assay (see below).

To assess whether removal of SNAP would restore the enzyme activity, stimulated J774 cytosol was incubated with 1 mM of SNAP or buffer for 1 h at room temperature. These mixtures were then passed through a Sephadex G-50 (2.5 ml volume) column which was equilibrated and eluted in 0.1 M HEPES/1 mM DTT/1 mM EDTA/100 μM NADPH/5 μM tetrahydrobiopterin to avoid loss of enzyme activity by removal of cofactors. Fractions of 0.25 ml were collected. Peak enzyme activity appeared in fraction 6 while the SNAP appeared from fraction 10 onwards. The enzyme activity in the various fractions and in the controls was determined by the citrulline assay (see below).

Assays of NO synthase activities

Spectrophotometric assay The cell monolayers were washed with PBS and sonicated after addition of 0.25 ml of buffer (0.1 M HEPES/1 mM DTT/1 mM EDTA, pH 7.4). The cytosol (20,000 *g*) was assayed for NO synthase activity by the spectrophotometric assay based on the conversion of oxy- to methaemoglobin as described previously (Feelisch & Noack, 1987). The protein content of the cytosol was determined by the Coomassie Blue binding method (Pierce Chemical). Activity was expressed as pmol of NO mg^{-1} protein min^{-1}. The NO synthase activity in the positive controls varies between 600–1000 pmol NO mg^{-1} protein min^{-1} in different experiments. This probably reflects stages of differentiation of the cell line in different passages.

Citrulline assay This was performed by the conversion of radioactive L-arginine to L-citrulline as previously described (Salter *et al.*, 1991), except that it was carried out at room temperature for 30 min and [^{3}H]-L-arginine was used. Activity was expressed as pmol of citrulline mg^{-1} protein min^{-1}.

Measurement of NO_2^-

The concentration of NO_2^- in the supernatants was determined by chemiluminescence as previously described (Palmer *et al.*, 1987).

Statistical analysis

Results are expressed as mean ± s.e.mean and statistical significance ($P<0.05$) was analysed by Student's *t* test. In the figures, vertical bars = 1 s.e.mean; where no bar is seen, it is covered by the symbol.

Results

Time course of NO synthase activation and NO_2^- production

After 6 h of exposure to IFN-γ plus LPS a small amount of NO_2^- was detectable in the culture supernatant, increasing until 24 h and remaining essentially the same at 72 h (Figure 1). The NO synthase activity (measured by the spectrophotometric method) in the cytosolic fraction showed a high activity by 6 h, which peaked at 12 h, decreased thereafter to a low level by 48 h and was not detectable by 72 h (Figure 1). At this time 70–80% of the cells remained viable, as judged by trypan blue exclusion (data not shown).

There was no difference in the levels of enzyme activity in

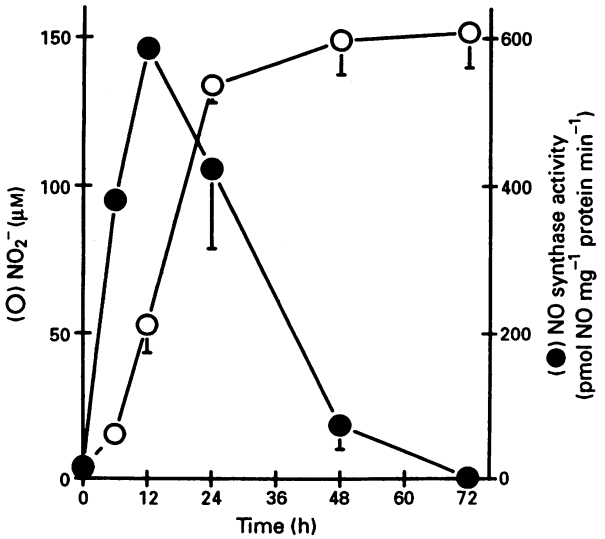


Figure 1 Time-course of NO_2^- accumulation (○) and NO synthase activity (●) in J774 cells stimulated with interferon gamma (IFN-γ, 10 u ml^{-1}) plus lipopolysaccharide (LPS, 10 ng ml^{-1}). Medium was standard DMEM. NO synthase activity was assayed by the spectrophotometric method. Vertical bars = 1 s.e.mean of triplicate wells. Results are representative of 3 experiments.

the cells which were pre-incubated with the supernatant derived from stimulated and non-stimulated cells before stimulation with IFN-γ plus LPS for 12 h (611 ± 73 vs 519 ± 19 pmol NO mg^{-1} protein min^{-1}, respectively, $n = 3$; $P > 0.05$).

Effects of haemoglobin or low L-arginine on NO synthase levels

Low concentrations of NO_2^- (10 ± 1.03 μM; $n = 3$) were found in the supernatant of cells stimulated in low L-arginine DMEM when compared to the NO_2^- concentrations in the supernatant from cells stimulated for 12 h in standard DMEM (137.1 ± 3.4 μM, $n = 3$). The presence of haemoglobin also reduced the NO_2^- concentrations in the supernatant (71.7 ± 1.6 mM; Figure 2a). In contrast, when the enzyme activity from those cells was studied by the spectrophotometric method, the peak enzyme activity in low L-arginine DMEM was markedly increased and the decay of this activity was slowed down when compared to that observed after activation in standard DMEM. The presence of haemoglobin also increased the peak activity of the enzyme by 50% and reduced the rate of its decay (Figure 2b).

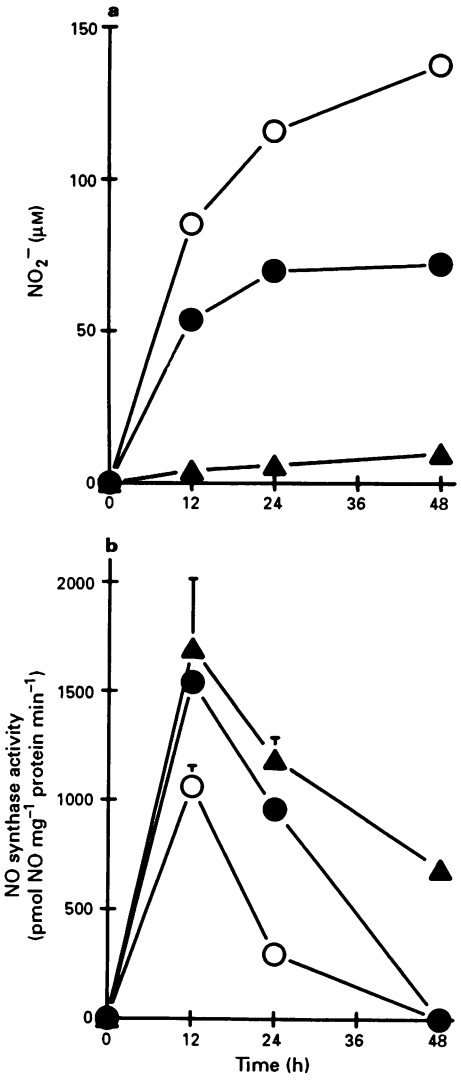


Figure 2 Time-course of NO_2^- accumulation (a) and NO synthase activity (b) of J774 cells activated with interferon gamma (IFN-γ, 10 u ml^{-1}) plus lipopolysaccharide (LPS, 10 ng ml^{-1}), in standard DMEM (○), in the presence of 200 μM of haemoglobin (●), or in low L-arginine DMEM (▲). NO synthase activity was assayed by the spectrophotometric method. Each point is the mean ± s.e.mean of triplicate wells. Results are representative of 2 experiments.

Effects of SNAP, GSNO and 8-Br-cyclic GMP

When added to the cells at the same time as the stimulus, the NO donors SNAP and GSNO inhibited the enzyme activity in a dose-dependent manner, whereas 8-Br-cyclic GMP was without effect (Figure 3). The inhibitory effect of SNAP and GSNO was due to NO, since neither acetyl-penicillamine nor glutathione (which do not release NO) had any effect on NO synthase activity (779 ± 144 and 734 ± 90 pmol NO mg^{-1} protein min^{-1}, respectively, $n = 3$). Neither SNAP nor 8-Br-cyclic GMP (10–100 μM) affected the macrophage total protein synthesis assayed 4, 8 or 12 h after stimulation ($n = 3$ for each concentration and each time).

Effect of SNAP on NO synthase activity in a cell-free system

When SNAP was incubated with the cytosolic fraction of stimulated J774 cells, it inhibited the NO synthase activity (measured by the citrulline assay) in a dose-dependent manner (Figure 4). In parallel experiments, the enzyme activity of the constitutive NO synthase extracted from rat brain was similarly inhibited by SNAP (Figure 4). The removal of SNAP by gel filtration did not restore the NO synthase activity (Figure 5).

Discussion

The NO synthase activity induced in macrophages following activation by immunological stimuli decays rapidly. Since supernatants of stimulated cells failed to affect the production of NO by other stimulated J774 cells, such decay is not likely to be due to inhibitory stable substances produced by the cells and present in the culture supernatant. We have now provided evidence that the decay in NO synthase activity is due, at least in part, to feedback inhibition by NO. Thus, low concentrations of L-arginine (which limit NO synthesis), or the presence of haemoglobin (which scavenges NO), increased the activity and slowed the decay of NO synthase activity. In contrast, the addition of SNAP and GSNO,

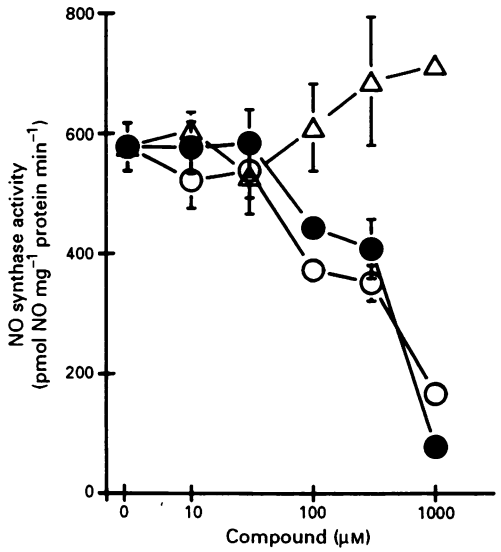


Figure 3 Effect of NO donors and 8-Br-cyclic GMP on the NO synthase activity in J774 cells. S-nitroso-acetyl-penicillamine (SNAP, ○), S-nitroso-glutathione (GSNO, ●) or 8-Br-cyclic GMP (△) were added together with the stimuli (10 u ml^{-1} interferon gamma plus 10 ng ml^{-1} lipopolysaccharide) and the enzyme activity assayed 18 h later by the spectrophotometric assay. Each point is the mean ± s.e.mean of triplicate wells. Results are representative of 2 experiments.

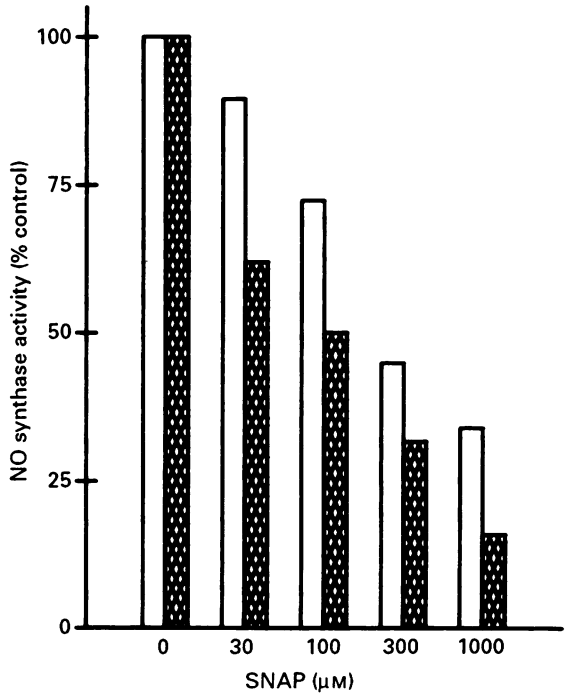


Figure 4 Inhibitory effect of S-nitroso-acetyl-penicillamine (SNAP) on NO synthase extracted from the cytosol of stimulated J774 cells (open columns) and from rat brain (cross-hatched columns). Aliquots of both cytosols were incubated with SNAP in the presence of 5 μM tetrahydrobiopterin. After 30 min at room temperature, the NO synthase activity was assayed by the citrulline assay. Original activities were 1000 and 300 pmol citrulline mg^{-1} protein min^{-1} for J774 and rat brain cytosol, respectively. Similar results were obtained with a 60 min incubation time. Data are representative of 2 experiments each with triplicate samples.

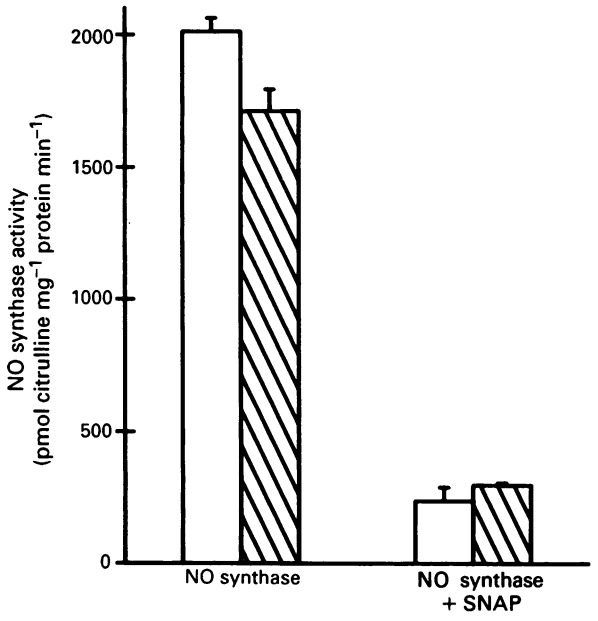


Figure 5 Gel-filtration analysis of S-nitroso-acetyl-penicillamine (SNAP)-inactivated NO synthase. Aliquots of cytosol extracted from activated J774 cells and containing 5 μM tetrahydrobiopterin were incubated with buffer or with 1 mM of SNAP for 60 min at room temperature. Part of the mixtures was then gel filtered through a Sephadex G-50 column. NO synthase activity was assayed by the citrulline assay. The data are total activity of non-fractionated enzyme (open columns) or of fractionated (pooled fractions 5–7) enzyme (hatched columns). Results represent mean ± s.e.mean of 3 experiments.

which produce NO, strongly inhibited the enzyme activity either in whole cells or in a cell-free system. The latter finding clearly indicates that NO inhibits the enzyme directly. The requirement of relatively high concentrations of NO donors in this system is probably due to the slow release of NO by these compounds. NO is released by GSNO at a rate of 0.02 nmol min^{-1} (Radomski *et al.*, 1992). Since the dose-response curves of SNAP and GSNO on the inhibition of NO synthase are very similar, it is likely that SNAP releases NO at a similar rate.

SNAP also inhibited the activity of the constitutive brain NO synthase. An inhibition of the particulate but not the soluble constitutive NO synthase in endothelial cells caused by superoxide has been suggested (Mitchell *et al.*, 1991). The mechanism by which NO inhibits NO synthase activity is at present unknown. The concentrations of NO released by SNAP, although sufficient to inhibit the enzyme activity, had no effect on the total protein synthesis of the macrophages. The inhibition did not appear to be mediated by cyclic GMP, since 8-Br-cyclic GMP had no effect. It is also unlikely that NO inhibits the NO synthase activity indirectly by affecting the levels of NADPH or tetrahydrobiopterin, co-factors essential for NO synthase activity, since excess concentrations of these compounds were added in the NO synthase assays. It may be that NO has a direct effect on the activity of NO synthase. This is consistent with our finding that SNAP could inhibit the enzyme activity in a cell-free system. Furthermore, the removal of SNAP from the incubate did not restore the enzyme activity. This suggests that the inhibition of NO synthase by NO may be irreversible. Whether other free radicals can also inhibit NO synthase is at present unknown.

Nitric oxide has been shown to induce S-nitrosylation of four cysteine residues of glyceraldehyde-3-phosphate dehydrogenase which are essential for the catalytic function, thereby inhibiting the enzyme activity (Molina y Vedio *et al.*, 1992). Mouse macrophage inducible enzyme has been shown to have 24 cysteine residues (Lyons *et al.*, 1992; Xie *et al.*, 1992), the potential sites of S-nitrosylation. Nitric oxide has also been shown to react with amino groups of proteins (Moriguchi *et al.*, 1992). It was recently shown that NO synthase is a haem protein (White & Marletta, 1992). It is possible that NO binds directly to the haem moiety of the enzyme. Whether NO inhibits the synthase activity by these or other mechanisms remains to be established. It is also unclear whether, in addition to its direct inhibitory effect on the enzyme, NO also inhibits the induction process leading to the expression of the NO synthase.

Earlier studies have demonstrated that a number of cytokines (TGF-β, IL-4 and IL-10) can inhibit the induction of NO synthase in macrophages. Our present data suggest an additional regulatory process of NO synthesis via a feedback mechanism by NO.

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References

CUNHA, F.Q., MONCADA, S. & LIEW, F.Y. (1992). Interleukin-10 (IL-10) inhibits the induction of nitric oxide synthase by interferon-γ in murine macrophages. *Biochem. Biophys. Res. Commun.*, **186**, 1155–1159.

DING, A., NATHAN, C.F., GRAYCAR, J., DERYNCK, R., STUEHR, D.J. & SRIMAL, S. (1990). Macrophage deactivating factor and transforming growth factors-β1, β2 and β3 inhibit induction of macrophage nitrogen oxide synthesis by IFN-γ. *J. Immunol.*, **145**, 940–944.

FEELISCH, M. & NOACK, E.A. (1987). Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur. J. Pharmacol.*, **139**, 19–30.

HART, T.W. (1985). Some observations concerning the S-nitroso and S-phenylsulphonyl derivatives of L-cysteine and glutathione. *Tetrahedron Lett.*, **26**, 2013–2016.

HIBBS, J.B.Jr., TAINTOR, R.R., VAVRIN, Z. & RACHLIN, E.M. (1988). Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.*, **157**, 87–94.

LIEW, F.Y., LI, Y., SEVERN, A., MILLOTT, S., SCHMIDT, J., SALTER, M. & MONCADA, S. (1991). A possible novel pathway of regulation by murine T helper type-2 (Th_2) cells of a T_h1 cell activity via the modulation of the induction of nitric oxide synthase on macrophages. *Eur. J. Immunol.*, **21**, 2489–2494.

LYONS, C.R., ORLOFF, G.J. & CUNNINGHAM, J.M. (1992). Molecular cloning and functional expression of an inducible nitric oxide from a murine macrophage cell line. *J. Biol. Chem.*, **287**, 6370–6374.

MITCHELL, J.A., SHENG, H., WARNER, T.D., POLLOCK, J.S., FORSTERMANN, U. & MURAD, F. (1991). Relationship between endothelial cell activation and subsequent NO synthase activity: inhibition by oxygen derived free radicals. *Br. J. Pharmacol.*, **104**, 117P.

MOLINA Y VEDIO, L., McDONALD, B., BRUNE, B. & LAPETINA, E.G. (1992). Nitric oxide-induced S-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase inhibits enzymatic activity and increases endogenous ADP-ribosylation. *J. Biol. Chem.*, (in press).

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.

MORIGUCHI, M., MANNING, L.R. & MANNING, J.M. (1992). Nitric oxide can modify amino acid residues in proteins. *Biochem. Biophys. Res. Commun.*, **183**, 598–604.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

RADOMSKI, M.W., REES, D.D., DUTRA, A. & MONCADA, S. (1992). S-nitroso-glutathione inhibits platelet activation *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **107**, 745–749.

SALTER, M., KNOWLES, R.G. & MONCADA, S. (1991). Widespread tissue distribution, species distribution and changes in activity of Ca^{2+}-dependent and Ca^{2+}-independent nitric oxide synthases. *FEBS Lett.*, **291**, 145–149.

STUEHR, D.J. & MARLETTA, M.A. (1987). Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines or interferon-γ. *J. Immunol.*, **139**, 518–525.

TAKEMA, M., INABA, K., UNO, K., KAKIHARA, K.-I., TAWARA, K. & MURAMATSU, S. (1991). Effect of L-arginine on the retention of macrophage tumoricidal activity. *J. Immunol.*, **146**, 1928–1933.

WHITE, K.A. & MARLETTA, M.A. (1992). Nitric oxide synthase is a cytochrome P-450 type hemoprotein. *Biochemistry*, **31**, 6627–6631.

XIE, Q., CHO, H.J., CALAYCAY, J., MUMFORD, R.A., SWIDEREK, K.M., LEE, T.D., DING, A., TROSO, T. & NATHAN, C. (1992). Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science*, **256**, 225–228.

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Endothelin-1 in the rabbit: interactions with cyclo-oxygenase and NO-synthase products

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1 Endothelin-1 infusion (5–40 pmol kg^{-1} min^{-1}) in the normal anaesthetized rabbit, produced a dose-dependent increase in mean arterial blood pressure (MAP) and reduced renal blood flow (RBF) and glomerular filtration rate (GFR), when compared with an equivalent infusion of physiological saline.

2 Endothelin, 20 pmol kg^{-1} min^{-1}, was also assessed in animals pretreated with either indomethacin (2 mg kg^{-1}), methylene blue (1.6 mg kg^{-1} h^{-1}) or N^G-monomethyl L-arginine (L-NMMA, 10 mg kg^{-1} h^{-1}).

3 The effect of endothelin on MAP and RBF was enhanced ($P = 0.05$ and < 0.01 respectively) by the cyclo-oxygenase inhibitor, indomethacin, without any significant change in the effect on GFR.

4 Methylene blue and L-NMMA, inhibitors of endothelium-derived relaxant factor (EDRF), enhanced the effect of endothelin on each of the parameters measured ($P < 0.01$).

5 Our results are consistent with endothelin having a predominant effect on pre-glomerular vascular resistance to reduce GFR. Endothelin appears to stimulate the release of vasodilator prostanoids and EDRF which oppose its effects. Thus endothelin may have an important role in the complex control of GFR in the rabbit.

Keywords: Endothelin; endothelium-derived relaxing factor; glomerular filtration rate; NO synthase; cyclo-oxygenase

Introduction

Endothelin-1 is a 21 amino acid peptide with potent vasoconstrictor properties (Yanagisawa *et al.*, 1988). It is produced by endothelial cells in culture and is coded in the genome of several species. Endothelium-dependent constricting factors are released in response to stimuli such as hypoxia and increased transmural pressure (Rubanyi & Vanhoutte, 1985) and endothelin is thought to be one such factor. Endothelin has a prolonged effect *in vivo* but a short half-life in the circulation (Spokes *et al.*, 1989). This is consistent with avid uptake at specific binding sites, which have been demonstrated widely in the vasculature and in the parenchyma of several organs including kidney, brain and heart (Koseki *et al.*, 1989).

Endothelin increases renal vascular resistance and decreases renal function in both rats (Badr *et al.*, 1989; López-Farré *et al.*, 1989) and dogs (Goetz *et al.*, 1988; Miller *et al.*, 1989). Kon and her colleagues (1989) infused endothelin-1 into the first order branch of the left main renal artery in Munich-Wistar rats and found a 35% reduction in single nephron glomerular filtration rate (GFR) compared with unexposed glomeruli in the same kidney. Furthermore, in models of ischaemic acute renal failure (Kon *et al.*, 1989) and acute cyclosporin nephrotoxicity (Kon *et al.*, 1990) in the same model, the infusion of a polyclonal anti-endothelin antibody protected against the characteristic renal vasoconstriction in each case. This supports the idea that endothelin might have an important physiological and pathophysiological role in the kidney (Firth *et al.*, 1988; Cairns *et al.*, 1988).

The control of glomerular blood flow is critical in the maintenance of homeostasis and the rabbit kidney exhibits autoregulation of its flow (Forster & Maes, 1947). There is a complex interaction between neural and humoral, constrictor and dilator and local and systemic mechanisms contributing to this control. The two best documented regulatory mechanisms involved are the renin-angiotensin system and the renal eicosanoids; the principal renal prostaglandins are prostaglandin E_2 (PGE_2) and PGI_2 (Terragno *et al.*, 1978), which are both vasodilator in the kidney. Since the isolated perfused kidney can still autoregulate (Kastner *et al.*, 1984), local factors are likely to be important, in particular, the importance of the endothelium as a source of vasoactive agents is increasingly recognized.

We have therefore studied the systemic and renal effects of endothelin in the rabbit and assessed its role in the regulation of renal blood flow by inhibiting some of these other systems: cyclo-oxygenase with indomethacin and the action and generation of endothelium-derived relaxing factor (EDRF: nitric oxide, Ignarro *et al.*, 1987), with methylene blue (MB) an inhibitor of soluble guanylate cyclase (Ignarro *et al.*, 1986) and N^G monomethyl-L-arginine (L-NMMA) a competitive inhibitor of the enzyme NO synthase (Palmer & Moncada, 1989).

Methods

Surgical preparation

Normal adult New Zealand White rabbits (6–10 in each group) of either sex and weighing 2.5–3.5 kg (Rosemead, U.K.) were fluid loaded with physiological saline (50 ml) and given 2 mg frusemide via the ear vein, to induce a temporary diuresis prior to cannulation of the ureter. Anaesthesia was induced with pentobarbitone 15 mg kg^{-1} (Sagatal, May & Baker, Dagenham, U.K.) and althesin 0.2 ml kg^{-1} (containing 9 mg alfaxalone and 3 mg alphadolone ml^{-1}) (Saffan, Glaxovet, U.K.) as intravenous boluses, and maintained with a continuous infusion of althesin 1.2–1.5 ml kg^{-1} h^{-1} into

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the ear vein.

After formation of a tracheostomy, the animals were ventilated with room air supplemented with 100% oxygen. The left carotid artery was cannulated for blood sampling and for continuous measurement of blood pressure via a Statham blood pressure transducer on a Watanabe (Type WA281) pen chart recorder. A second catheter was introduced by the Seldinger technique, over a guide wire, into the right femoral artery and thence into the aorta. The catheter tip was positioned approximately one centimeter above the level of the renal arteries by palpation and was flushed with heparinised saline for the subsequent infusion of endothelin.

At laparotomy both ureters were cannulated so that timed urine collections could be made and an electromagnetic flow probe (1.5 mm, Gould) was placed around the left renal artery and connected to a flowmeter (Gould Inc., Oxnard, Ca. model SP2202) for measurement of renal blood flow. The midline wound was then closed with clips. Animals were hydrated throughout with 0.9% saline, 20 ml kg^{-1} h^{-1}, and 0.5 MBq of [^{51}Cr]-EDTA was administered by the carotid cannula as a bolus, followed by a further 0.5 MBq in isotonic saline as a continuous infusion (0.3 ml min^{-1}), via the ear vein.

In addition in six animals, indomethacin was given as an intravenous bolus (2 mg kg^{-1}) followed by an infusion at 1 mg kg^{-1} h^{-1} in normal saline, to block the cyclo-oxygenase enzyme (Flower, 1974).

Methylene blue (MB), 1.6 mg kg^{-1} h^{-1} and L-NMMA, 10 mg kg^{-1} h^{-1}, were infused for 140 and 60 min respectively from the end of the recovery period in a further 5 animals each. These doses had been shown in our preliminary experiments to inhibit the effect of acetylcholine 10 nmol kg^{-1} min^{-1} in the normal rabbit.

Experimental procedure

After completion of the surgical preparation and infusions of [^{51}Cr]-EDTA and drugs, the animals were allowed to recover from the operation for a period of 30–40 min. Two consecutive baseline 10 min collections of urine were then made with recordings of arterial pressure (MAP) renal blood flow (RBF) and plasma sampling at the mid-point of the collection. Glomerular filtration rate (GFR) was subsequently calculated as the clearance of [^{51}Cr]-EDTA during each collection period. In the animals treated with methylene blue or L-NMMA, repeateed baseline measurements were made after 10 min of administration. Endothelin-1 (Peptide Institute, Osaka, Japan), 250 nM in normal saline, was then infused into the aortic cannula to allow adequate mixing of the drug in the blood before reaching the renal circulation whilst minimizing the systemic effect. The infusion continued for a total of 40 min during which three sets of measurements were made at 5, 10 and 30 min respectively.

After completing the infusion a 70 min recovery period was allowed with three similar observation periods at 55, 80 and 110 min from the start of the infusion. Endothelin was infused at 5, 20 and 40 pmol kg^{-1} min^{-1} in preliminary studies to assess the dose response ($n = 5–6$) and thereafter, 20 pmol kg^{-1} min^{-1} was infused in each experiment.

Results are expressed, at each time point, as the percentage change from the mean of the two baseline measurements (absolute baseline values are shown in Table 1). The GFR results reported are from the right kidney. The rabbit renal artery is sensitive to manipulation and placement of the flow probe on the left resulted in a reduction in renal function on that side which recovered to a variable degree in each case. In 27 of 122 different experiments employing this preparation renal function was preserved on the left (mean GFR 69.2% of that on the right, range 10.7–268%). In these cases both kidneys responded similarly to pharmacological manipulation, and we have therefore considered it valid to compare measurements of function on the right with recordings of blood flow from the left kidney.

Statistical analyses were by multifactor analysis of variance (ANOVA).

Results

Endothelin alone

Endothelin infusion (20 pmol kg^{-1} min^{-1}) produced an increase in MAP from baseline 49.3 ± 5 mmHg to 56.8 ± 5 mmHg (mean ± s.e.mean) at the end of the infusion period. This was accompanied by a reduction in GFR (4.2 ± 0.5 to 2.1 ± 0.5 ml min^{-1}) and RBF (12.9 ± 2.3 to 8 ± 2 ml min^{-1}) (Figure 1). The changes were dose related, in the range 5–40 pmol kg^{-1} min^{-1}, developed immediately and progressed throughout the course of the infusion period to a maximum at 30 min in most cases. There was no initial fall in MAP. After the infusion, mean arterial pressure returned to baseline values by 55 min at the lower dose levels of endothelin but did not recover after 40 pmol kg^{-1} min^{-1}. RBF was not affected by the lowest dose of endothelin

Table 1 Absolute data at baseline before infusion of control saline or endothelin at various doses with or without other named agents

	MAP (mmHg)	*RBF* (ml min^{-1})	*GFR* (ml min^{-1})	*PP* (mmHg)
Control	53.1 (3.1)	17.7 (2.6)	3.3 (0.6)	33.7 (3.0)
Endothelin 20 pmol kg^{-1} min^{-1}	49.3 (5.1)	12.8 (2.3)	4.2 (0.5)	29.9 (2.4)
Endothelin 40 pmol kg^{-1} min^{-1}	44.0 (5.0)	20.0 (5.3)	3.1 (1.0)	26.3 (5.9)
Endothelin 5 pmol kg^{-1} min^{-1}	70.2 (4.8)	16.6 (2.4)	5.1 (0.5)	33.6 (2.1)
Endothelin 20 + indomethacin	69.8 (2.7)	25.9 (3.7)	5.8 (1.0)	26.6 (2.1)
Endothelin 20 + Meth. blue	69.9 (1.7)	15.1 (2.4)	4.5 (1.0)	29.7 (1.8)
Endothelin 5 + Meth. blue	74.8 (5.8)	14.4 (4.0)	1.7 (0.3)	29.9 (2.3)
Endothelin 1 + Meth. blue	66.5 (11.2)	16.1 (3.3)	3.0 (0.8)	28.3 (2.9)
Endothelin 20 + L-NMMA	74.5 (6.9)	24.7 (3.0)	4.8 (0.9)	25.6 (3.0)

Values are mean ± s.e.mean. MAP = mean arterial pressure; RBF = renal blood flow; GFR = glomerular filtration rate; PP = pulse pressure. L-NMMA = N^G-monomethyl L-arginine; Meth blue = methylene blue.

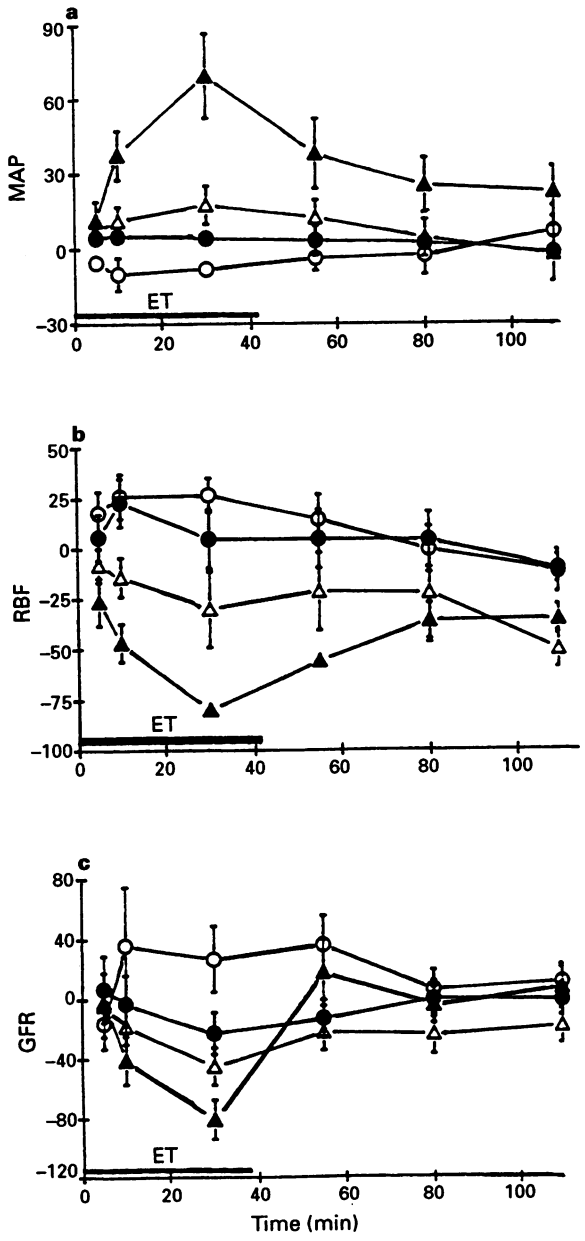


Figure 1 Dose-response curve to a 40 min endothelin-1 infusion. (a) Percentage change in mean arterial pressure (MAP); (b) percentage change in renal blood flow (RBF); (c) percentage change in glomerular filtration rate (GFR): (○) saline control; (●) endothelin 5 pmol kg^{-1} min^{-1}; (△) endothelin 20 pmol kg^{-1} min^{-1}; (▲) endothelin 40 pmol kg^{-1} min^{-1}. Horizontal line indicates endothelin infusion period.

(5 pmol kg^{-1}) but at 40 pmol kg^{-1} min^{-1} the marked reduction (80%) had not returned to the baseline value by the end of the observation period. In contrast, there was a fall in GFR after only 5 pmol kg^{-1} endothelin ($P = 0.06$ v control) and the profound reduction in GFR (81 ± 13.2%) induced by endothelin 40 pmol kg^{-1} min^{-1} returned to the pre-infusion level 15 min after the infusion had been discontinued. A progressive fall in pulse pressure also occurred throughout the infusion and did not recover (data not shown).

Endothelin with indomethacin

Administration of indomethacin alone increases basal blood pressure and GFR (24.8 and 15.4% respectively, $n = 3$); results following infusion of endothelin were therefore expressed as percentages from this new baseline. Indomethacin enhanced the effect of endothelin infusion (20 pmol kg^{-1}) on RBF (25.9 ± 3.7 ml min^{-1} falling to 8 ± 1.4 ml min^{-1}) (that is − 66.5% of baseline compared with − 30.3%, $P = 0.02$) and MAP (69.8 ± 2.7 to 88.9 ± 3.5 mmHg or 27.4% against 17.8% for endothelin alone, $P = 0.35$), neither MAP nor RBF had returned to pre-infusion values by the end of the experiment. Despite these changes, the fall in GFR after administration of indomethacin (5.6 ± 0.9 to 2.6 ± 0.6 ml min^{-1}) was not different from that produced by endothelin alone (Figure 2).

Endothelin with methylene blue and N^G-monomethyl-L-arginine

MB potentiated the effect of endothelin on MAP (+ 57% v 17.8%, $P<0.01$), RBF (− 75% v − 30.3%, $P<0.001$) and GFR (− 91.4% v − 45%, $P<0.001$) (Figure 3). A dose-response curve to endothelin (1–20 pmol kg^{-1} min^{-1}) in the presence of MB showed a shift to the left (Figure 4) indicating that endothelin is more potent in the absence of soluble guanylate cyclase activity. Similarly, in the presence of L-NMMA 10 mg kg^{-1} h^{-1}, all three parameters showed an exaggerated response to endothelin 20 pmol kg^{-1} min^{-1} (MAP + 39%, $P<0.01$; RBF − 74%, $P<0.02$; GFR − 87%, $P<0.02$, Figure 3). L-NMMA also caused a basal increase in systemic blood pressure of 10%, but without a statistically significant change in RBF (data not shown). There was no difference between the effects of MB and L-NMMA on the response to endothelin 20 pmol kg^{-1} min^{-1} ($P<0.6$), thus this enhancement of the effect of endothelin is likely to be due to inhibition of EDRF.

Control

Infusion of physiological saline at the same rate as endothelin served as the control experiment (Figure 1), and produced a 10% fall in MAP and a rise in RBF (20%) and GFR (35%).

These changes may represent the effect of a local infusion of fluid into the aorta, but more likely represent the instability of the baseline values, since they did not progress during the infusion. The changes seen in response to endothelin are opposite to those in the control animals, hence any error introduced by the early response to saline will tend to minimize the observed effect of endothelin, enhancing the validity of our conclusions.

Discussion

Our results demonstrate that endothelin is a very potent constrictor of the systemic and renal vasculature. The constrictor effect is markedly potentiated by inhibition of either the cyclo-oxygenase enzyme system or the action or generation of EDRF, indicating that vasodilator prostaglandins and EDRF are released *in vivo* to offset the effect of endothelin alone. The reduction of GFR induced by endothelin is also potentiated by inhibitors of EDRF but not by indomethacin suggesting that the interaction of endothelin and cyclo-oxygenase products in the control of GFR is more complex.

The reduction in renal perfusion and glomerular filtration rate observed *in vivo*, confirms our earlier finding in the isolated perfused kidney of the rabbit (Cairns *et al.*, 1989) and is consistent with other studies of endothelin infusion *in vivo*, in both rat (Badr *et al.*, 1989) and dog (Goetz *et al.*, 1988; Miller *et al.*, 1989) and with the effect of endothelin infused at low dose (0.16 pmol min^{-1}) directly into a segmental renal artery in rats (Kon *et al.*, 1989). The dose-related reduction in renal blood flow and GFR, despite a significant increase in mean arterial blood pressure, suggests that

endothelin is acting predominantly on pre-glomerular resistance vessels to reduce the net intraglomerular hydraulic pressure difference (Δ-P), and glomerular plasma flow. Preferential afferent arteriolar constriction by endothelin has been shown by direct observation in the split hydronephrotic rat kidney model (Loutzenhiser *et al.*, 1990). Micropuncture studies in Munich-Wistar rats (Badr *et al.*, 1989) have confirmed that endothelin infusion (10 pmol min^{-1}) increases the resistance of both afferent and efferent arterioles, lowering both the single nephron GFR and the calculated ultrafiltration co-efficient (K_f). In support of the latter, endothelin has been shown to induce contraction of mesan-

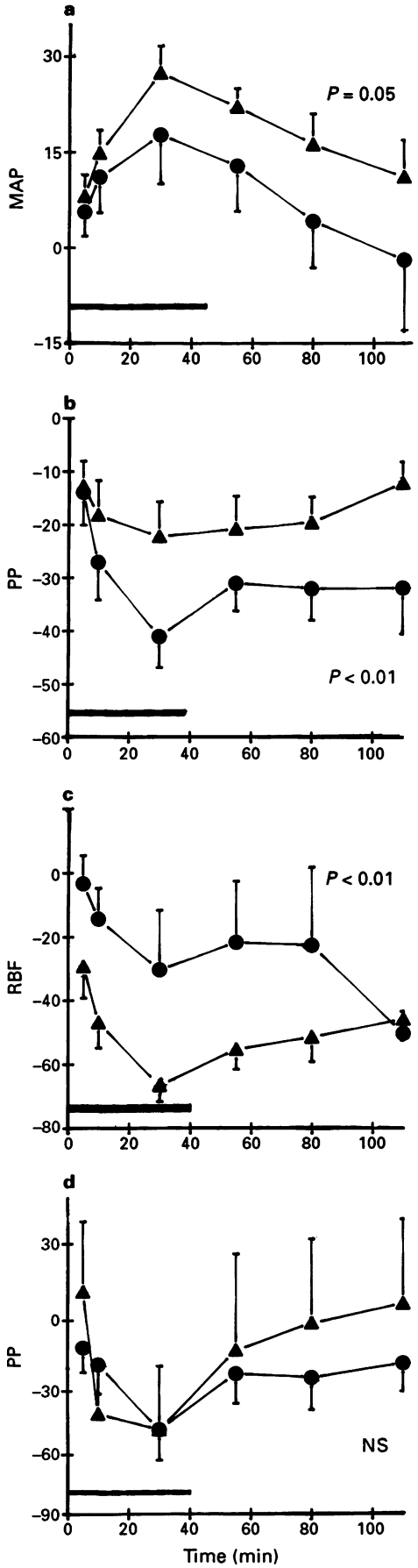


Figure 2 Percentage change in (a) mean arterial blood pressure (MAP), (b) pulse pressure (PP), (c) renal blood flow (RBF) (d) glomerular filtration rate (GFR) following infusion of endothelin 20 pmol kg^{-1} min^{-1} alone (●); endothelin 20 pmol kg^{-1} min^{-1} with indomethacin 2 mg kg^{-1} (▲). Horizontal line indicates endothelin infusion period.

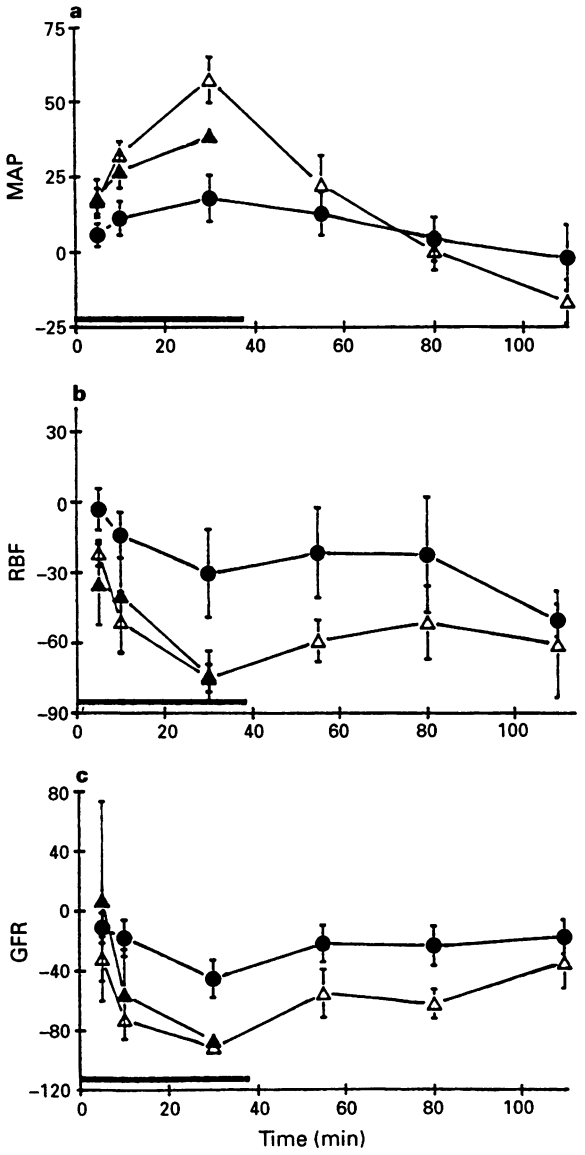


Figure 3 Percentage change in (a) mean arterial blood pressure (MAP); (b) renal blood flow (RBF) and (c) glomerular filtration rate (GFR) following infusion of endothelin 20 pmol kg^{-1} min^{-1} alone (●); endothelin 20 pmol kg^{-1} min^{-1} with methylene blue 1.6 mg kg^{-1} h^{-1} (Δ); endothelin 20 pmol kg^{-1} min^{-1} with N^G-monomethyl-L-arginine 10 mg kg^{-1} (▲). Horizontal line indicates endothelin infusion.

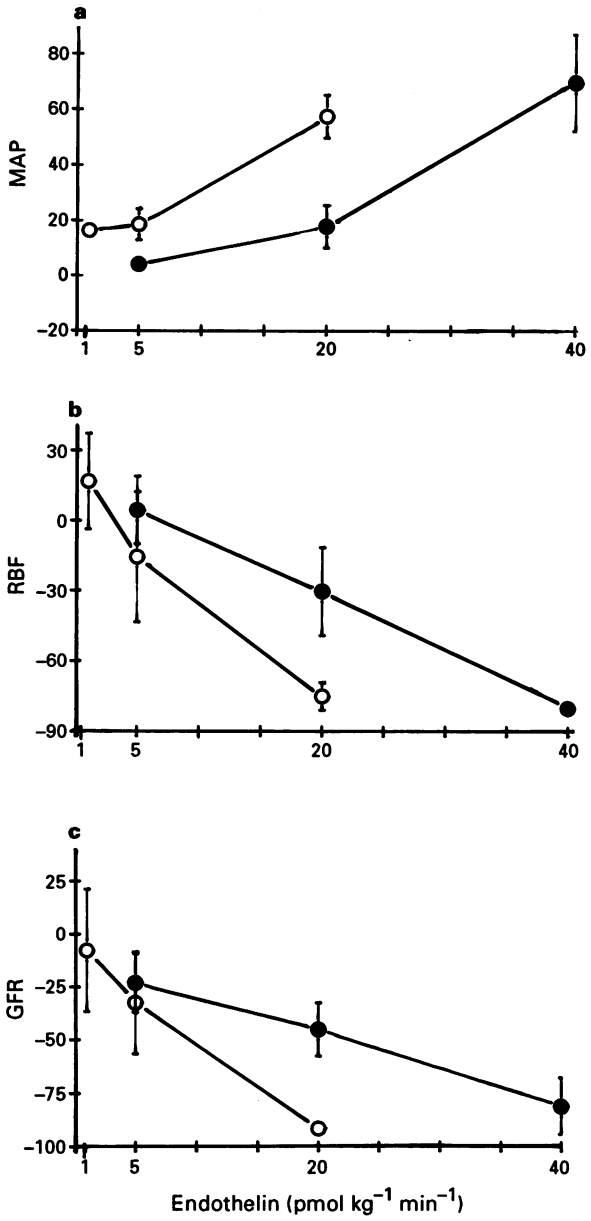


Figure 4 Maximal change in (a) mean arterial pressure (MAP), (b) renal blood flow (RBF) and (c) glomerular filtration rate (GFR), at variable doses of endothelin, with or without methylene blue (1.6 mg kg^{-1} h^{-1}): (●) endothelin alone; (○) endothelin with methylene blue 1.6 mg kg^{-1} h^{-1}.

gial cells in culture (Badr *et al.*, 1989; Simonson *et al.*, 1989), Kon however, found no change in K_f (Kon *et al.*, 1989). Other studies in anaesthetized rabbits also showed an increase in both pre and post glomerular vascular resistances, with a delayed fall in GFR (Denton & Anderson, 1990). The overall effect of endothelin on GFR may therefore be complex, involving changes in both vascular resistance and mesangial cell tone.

The striking reduction in pulse pressure seen during endothelin infusion implies that its hypertensive effect may be mediated by an effect on the peripheral vasculature rather than on cardiac function. However an additional inotropic effect, either positive or negative, cannot be excluded.

Endothelin stimulates the release of prostaglandins from guinea-pig and rat isolated lungs (de Nucci *et al.*, 1988), rat mesenteric arteries (Warner *et al.*, 1989) and from rabbit isolated perfused kidney and spleen (Rae *et al.*, 1989), and it is known to activate phospholipase A_2 in isolated vascular smooth muscle cells in culture (Resink *et al.*, 1989). Our results show that indomethacin enhances the constrictor effects of endothelin infusion (20 pmol kg^{-1} min^{-1}) both systemically and in the kidney, suggesting that indomethacin has inhibited the production of predominantly vasodilator eicosanoids. Some of this effect might also be explained by a switch of arachidonic acid from the cyclo-oxygenase to the 5-lipoxygenase pathway and the production of constrictor mediators. In contrast to RBF, GFR is preserved in the presence of indomethacin indicating that the control of GFR is more complex than just the change in renal vascular resistance and intraglomerular hydrostatic pressure, and may involve other parameters such as mesangial tone where the balance between eicosanoids and other mediators including endothelin is different. Whether phopholipase A_2 is directly activated by endothelin or whether this is a secondary response to the haemodynamic changes induced by the infusion *in vivo* is unknown.

Methylene blue and L-NMMA are inhibitors of the action and formation, respectively, of EDRF. EDRF, which is now characterized as nitric oxide, is a short lived vasodilator released from the vascular endothelium in response to shear stress as well as pharmacological stimuli such as acetylcholine and substance P (Vanhoutte, 1988). The role of EDRF in the control of vascular tone *in vivo* is not yet established although a dose-related increase in basal blood pressure has been reported in rabbits following infusion of L-NMMA (Rees *et al.*, 1989), suggesting a basal dilator tone induced by EDRF. Our results confirm the haemodynamic effect on basal systemic tone (MAP increased by 10% following L-NMMA) but without change in renal blood flow. This suggests that EDRF may have a role in the control of resting systemic blood pressure, but in this model its role in the renal circulation is more impressive in pathological states e.g. in reversing the effect of a potent vasoconstrictor substance such as endothelin.

We did not observe a preliminary vasodilator effect following endothelin infusion in the rabbit. In rats and dogs, such an effect has been attributed to release of dilator prostaglandins and EDRF. Our results suggest that both of these systems are activated throughout the course of the endothelin effect, but that the constrictor effect is dominant.

In summary, we have shown that endothelin is a potent vasoconstrictor in the rabbit, and that it reduces glomerular filtration rate, probably by preferential constriction of the afferent renal arteriole. The response to endothelin is modulated *in vivo* by the production of vasodilator prostaglandins and EDRF and to a lesser extent by activation of the renin-angiotensin system (Rogerson *et al.*, 1989). The cellular or sub-cellular level of this interaction remains to be determined. The control of renal vascular resistance and glomerular filtration rate involves a complex network of many factors. Our results suggest that endothelin-1 may be one of these.

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References

BADR, K.F., MURRAY, J.J., BREYER, M.D., TAKAHASHI, K., INAGAMI, T. & HARRIS, R.C. (1989). Mesangial cells, glomerular and renal vascular responses to endothelin in the rat kidney. *J. Clin. Invest.*, **83**, 336–342.

BANKS, R.O. (1989). Cardiovascular and renal effects of endothelin in the dog and in the rat. *Kidney Int.*, **35**, 309.

CAIRNS, H.S., ROGERSON, M.E., FAIRBANKS, L.D., NEILD, G.H. & WESTWICK, J. (1989). Endothelin induces an increase in renal vascular resistance and a fall in glomerular filtration rate in the rabbit isolated perfused kidney. *Br. J. Pharmacol.*, **98**, 155–160.

CAIRNS, H.S., ROGERSON, M., FAIRBANKS, L.D., WESTWICK, J. & NEILD, G.H. (1988). Endothelin and cyclosporin nephrotoxicity. *Lancet*, **ii**, 1496–1497.

DENTON, K.M. & ANDERSON, W.P. (1990). Vascular actions of endothelin in the rabbit kidney. *Clin. Exp. Pharmacol. Physiol.*, **17**, 861–872.

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

FIRTH, J.D., RATCLIFFE, P.J., RAINE, A.E.G. & LEDINGHAM, J.G.G. (1988). Endothelin: an important factor in acute renal failure? *Lancet*, **ii**, 1179–1181.

FLOWER, R.J. (1974). Drugs which inhibit prostaglandin biosynthesis. *Pharmacol. Rev.*, **26**, 33–67.

FORSTER, R.P. & MAES, J.P. (1947). Effect of experimental neurogenic hypertension on renal blood flow and glomerular filtration rates in intact denervated kidneys of unanaesthetized rabbits with adrenal glands demedullated. *Am. J Physiol.*, **150**, 534–540.

GOETZ, K.L., WANG, B.C., MADWED, J.B., ZHU, J.L. & LEADLEY, R.J. Jr. (1988). Cardiovascular, renal and endocrine responses to intravenous endothelin in conscious dogs. *Am. J. Physiol.*, **255**, R1064–R1068.

IGNARRO, L.J., BUGA, G.M., WOOD, K.S., BYRNS, R.E. & CHAUDHURI, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 9265–9269.

IGNARRO, L.J., HARBISON, R.G., WOOD, K.S. & KADOWITZ, P.J. (1986). Activation of purified soluble guanylate cyclase by endothelium-derived relaxing factor from intrapulmonary artery and vein: stimulation by acetylcholine, bradykinin and arachidonic acid. *J. Pharmacol. Exp. Ther.*, **237**, 893–900.

KASTNER, P.R., HALL, J.E. & GUYTON, A.C. (1984). Control of glomerular filtration rate: role of intrarenally formed angiotensin II. *Am. J. Physiol.*, **246**, F897–F906.

KON, V., TOSHIOKA, T., FOGO, A. & ICHIKAWA, I. (1989). Glomerular actions of endothelin in vivo. *J. Clin. Invest.*, **83**, 1762–1767.

KON, V., SUGIURA, M., INAGAMI, T., HARVIE, B.R., ICHIKAWA, I. & HOOVER, R.L. (1990). Role of endothelin in cyclosporine-induced glomerular dysfunction. *Kidney Int.*, **37**, 1487–1491.

KOSEKI, C., IMAI, M., HIRATA, Y., YANAGISAWA, M. & MASAKI, T. (1989). Autoradiographic distribution in rat tissues of binding sites for endothelin: a neuropeptide? *Am. J. Physiol.*, **256**, R858–R866.

LÓPEZ-FARRÉ, A., MONTAÑÉS, I., MILLÁS, I. & LÓPEZ-NOVOA, J.M. (1989). Effect of endothelin on renal function in rats. *Eur. J. Pharmacol.*, **163**, 187–189.

LOUTZENHISER, R., EPSTEIN, M., HAYASHI, K. & HORTON, C. (1990). Direct visualisation of effects of endothelin on the renal microvasculature. *Am. J. Physiol.*, **258**, F61–68.

MATSUMURA, Y., HISAKI, K., OHYAMA, T., HAYASHI, K. & MORIMOTOT, S. (1989). Effects of endothelin on renal function and renin secretion in anaesthetised rats. *Eur. J. Pharmacol.*, **166**, 577–580.

MILLER, W.L., REDFIELD, M.M. & BURNETT, J.C. Jr. (1989). Integrated cardiac, renal, and endocrine actions of endothelin. *J. Clin. Invest.*, **83**, 317–320.

PALMER, R.M.J. & MONCADA, S. (1989). A novel citrulline-forming enzyme implicated in the formation of nitric oxide by vascular endothelial cells. *Biochem. Biophys. Res. Commun.*, **158**, 348–352.

RAE, G.A., TRYBULEC, M., DE NUCCI, G. & VANE, J.R. (1989). ET-1 releases eicosanoids from rabbit isolated perfused kidney and spleen. *J. Cardiovasc. Pharmacol.*, **13**, suppl. 5, S89–92.

REES, D.D., PALMER, R.M.J. & MONCADA S. (1989). Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 3375–3378.

RESINK, T.J., SCOTT-BURDEN, T. & BUHLER, F.R. (1989). Activation of phospholipase A2 by endothelin in cultured vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.*, **158**, 279–286.

ROGERSON, M.E., CAIRNS, H.S., FAIRBANKS, L.D., NEILD, G.H. & WESTWICK, J. (1989). Endothelin in the anaesthetized rabbit: actions and interactions in the renal microcirculation. *Br. J. Pharmacol.*, **98**, 841P.

RUBANYI, G.M. & VANHOUTTE, P.M. (1985). Hypoxia releases a vasoconstrictor substance from the canine vascular endothelium. *J. Physiol.*, **364**, 45–56.

SIMONSON, M.S., WANN, S., MENE, P., DUBYAK, G.R., KESTER, M., NAKAZATO, Y., SEDOR, J.R. & DUNN, M.J. (1989). Endothelin stimulates phospholipase C, Na^+/H^+ exchange, c-fos expression, and mitogenesis in rat mesangial cells. *J. Clin. Invest.*, **83**, 708–712.

SPOKES, R.A., BRETHERTON-WATT, D., GHATEI, M.A. & BLOOM, S.R. (1989). Endothelin infusion in the anaesthetised rat: plasma endothelin concentrations and blood pressure changes. *Br. J. Pharmacol.*, **96**, 107P.

TAKAGI, M., MATSUOKA, H., ATARASHI, K. & YAGI, S. (1988). Endothelin: a new inhibitor of renin release. *Biochem. Biophys. Res. Commun.*, **157**, 1164–1168.

TERRAGNO, N.A., TERRAGNO, A., EARLY, J.A., ROBERTS, M.A. & MCGIFF, J.C. (1978). Endogenous prostaglandin synthesis inhibitor in the renal cortex. Effects on production of prostacyclin by renal blood vessels. *Clin. Sci. Mol. Med.*, **55**, 199s–202s.

TOLINS, J.P., PALMER, R.M., MONCADA, S. & RAIJ, L. (1990). Role of endothelium-derived relaxing factor in regulation of renal hemodynamic responses. *Am. J. Physiol.*, **258**, H655–662.

VANHOUTTE, P.M. (1988). The endothelium-modulator of vascular smooth-muscle tone. *New Engl. J. Med.*, **319**, 512–513.

WARNER, T.D., MITCHELL, J.A., DE NUCCI, G. & VANE, J.R. (1989). Endothelin-1 and Endothelin-3 release EDRF from isolated perfused arterial vessels of the rat and rabbit. *J. Cardiovasc. Pharmacol*, **13**, suppl 5, S85–88.

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

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Effects of two novel tachykinin antagonists, FK224 and FK888, on neurogenic airway plasma exudation, bronchoconstriction and systemic hypotension in guinea-pigs *in vivo*

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1 We compared the effects of two novel tachykinin receptor antagonists, FK888 (selective at the tachykinin NK_1 receptor) and FK224 (dual antagonist at NK_1 and NK_2 tachykinin receptors) on stimulus-evoked airway plasma exudation, bronchoconstriction and systemic hypotension in guinea-pigs *in vivo*. Plasma exudation was induced by substance P (SP), synthetic tachykinin receptor agonists, platelet activating factor (PAF), electrical stimulation of the cervical vagus nerves or by inhalation of cigarette smoke. Changes in airway tone and in carotid artery blood pressure (BP) were induced by synthetic tachykinin agonists, PAF and vagal stimulation.

2 Both FK224 and FK888 dose-dependently inhibited SP-induced plasma exudation in the lower trachea and main bronchi (ID_{50} values respectively of 1.1 and 0.1 μmol kg^{-1} in lower trachea, and of 0.5 and 0.1 μmol kg^{-1} in main bronchi) with complete inhibition at both airway levels at 10 μmol kg^{-1} for FK224 and at 2 μmol kg^{-1} for FK888.

3 The NK_1-selective tachykinin receptor agonist, [Sar^9,$Met(O_2)^{11}$]substance P ([Sar]SP), induced plasma exudation, a response which was blocked by both FK888 and FK224. The NK_2-selective agonist, [β-Ala^8]neurokinin A-(4-10) ([β-Ala]NKA), did not induce plasma exudation: neither FK888 nor FK224 affected this lack of response to [β-Ala]NKA.

4 [β-Ala]NKA induced bronchoconstriction, a response which was blocked by FK224 but which was completely unaffected by FK888. [Sar]SP induced a small but significant bronchoconstriction which was completely inhibited by both tachykinin antagonists.

5 In animals pretreated with capsaicin to deplete sensory neuropeptides, PAF induced both plasma exudation and bronchoconstriction. Neither response to PAF was inhibited by either FK888 or FK224.

6 Both FK888 and FK224 inhibited plasma exudation induced by vagus nerve stimulation or by cigarette smoke, with FK888 more potent than FK224.

7 FK224 inhibited non-cholinergic bronchoconstriction induced by vagal stimulation, whereas FK888, at doses inhibiting vagally-induced plasma exudation, did not.

8 Decreases in BP induced by SP or [Sar]SP were blocked by both FK888 and FK224. In contrast, neither antagonist had any significant inhibitory effect on the decrease in BP induced by vagal stimulation (in the presence of atropine) or PAF. [β-Ala]NKA did not decrease BP and neither tachykinin antagonist had any significant effect on this lack of response.

9 We conclude that in guinea-pig airways, plasma leakage induced by endogenous tachykinins is mediated predominantly via NK_1-receptors, whereas bronchoconstriction is mediated predominantly via NK_2-receptors. In addition, SP-evoked decreases in BP are also mediated via NK_1 receptors, whereas the contribution of endogenous tachykinins to vagally-induced decreases in BP appears to be minimal. Development of selective tachykinin receptor antagonists will be important in understanding the involvement of tachykinins in airway physiology and pathophysiology, whereas potent dual tachykinin receptor antagonists such as FK224 may have greater therapeutic potential in certain airway diseases in which tachykinins have been implicated in pathogenesis, including asthma and chronic bronchitis associated with cigarette smoking.

Keywords: FK224; FK888; tachykinin antagonist; NK_1-receptor; NK_2-receptor; plasma exudation; bronchoconstriction; vagus nerve; cigarette smoke; asthma

Introduction

Three types of tachykinin receptor (i.e. NK_1, NK_2, NK_3) are currently recognized, at which substance P (SP), neurokinin A (NKA) or NKB respectively are thought to be the most relevant natural agonists (Guard & Watson, 1991). Among these, SP and NKA may be more important in certain lung diseases, including asthma, in terms of their possible role in neurogenic inflammation and smooth muscle contraction (Barnes *et al.*, 1991).

Electrical stimulation of the cervical vagus nerves, in the presence of atropine, in experimental animals leads to airway plasma exudation and non-cholinergic bronchoconstriction, responses which are medited via neuropeptides released from capsaicin-sensitive sensory nerves (Lundberg *et al.*, 1983b). Cigarette smoke also induces plasma exudation, again induced by endogenously released neuropeptides from sensory nerves (Lundberg *et al.*, 1983a). In guinea-pig airways *in vivo* the order of potency of SP > NKA > NKB in causing plasma leakeage (Rogers *et al.*, 1988) indicates that NK_1-receptors mediate the response, a suggestion consistent with inhibition of neurogenic plasma exudation by a selective

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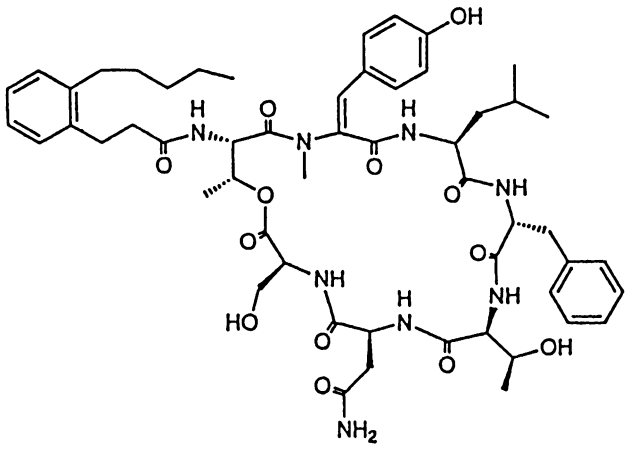
non-peptide NK_1-antagonist, CP-96,345 (Delay-Goyet & Lundberg, 1991; Eglezos *et al.*, 1991; Lei *et al.*, 1992). In contrast, in guinea-pig airways *in vitro* agonist potencies (Advenier *et al.*, 1987) and the activity of synthetic antagonists in inhibiting non-cholinergic neurogenic airway contraction (Maggi *et al.*, 1991b) suggest a predominant role for NK_2 receptors mediating smooth muscle constriction with a smaller contribution from NK_1 receptors. In human bronchus *in vitro*, agonist potencies indicate that only NK_2 receptors mediate bronchoconstriction (Advenier *et al.*, 1987).

In the present study, we compared the effects of two new tachykinin receptor antagonists, FK224 and FK888 (Figure 1), on plasma exudation induced by exogenous SP, the synthetic tachykinin agonists [Sar^9,Met $(O_2)^{11}$]substance P ([Sar]SP) and [β-Ala^8]neurokinin A-(4-10) ([β-Ala]NKA) which are selective for the NK_1 and NK_2 receptor respectively (Maggi *et al.*, 1990a,b), platelet activating factor (PAF), electrical stimulation of the vagus nerves or by inhalation of cigarette smoke. FK224 is a dual antagonist at both NK_1 and NK_2 receptors (Morimoto *et al.*, 1992; Murai *et al.*, 1992), whereas FK888 is a highly selective antagonist at NK_1 receptors (Fujii *et al.*, 1992). We also compared the effects of both compounds on bronchoconstriction and hypotension induced by [β-Ala]NKA, [Sar]SP, PAF and vagal stimulation. The airway effects of PAF may be mediated via sensory nerve activation and release of sensory neuropeptides including SP and NKA (Martins *et al.*, 1991). Consequently, we used animals pretreated with capsaicin (Holzer, 1991) to eliminate sensory neuropeptide involvement in the studies with PAF.

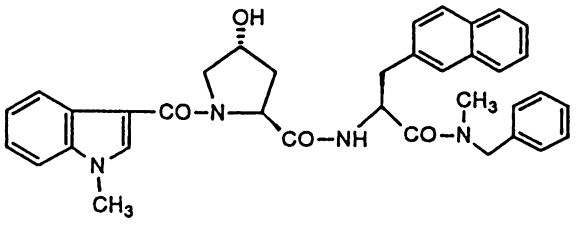
Methods

Animal preparation

Male Dunkin-Hartley outbred guinea-pigs (Charles River U.K. Ltd., Kent) weighing 260–440 g were anaesthetized with urethane (2 g kg^{-1}, i.p.). A tracheal cannula was inserted via a tracheostomy into the lumen of the trachea and connected to a constant-volume mechanical ventilator (Harvard Apparatus Limited, Edenbridge, Kent). A tidal volume of 10 ml kg^{-1} and a frequency of 60 breaths min^{-1} were used to ventilate the animals. Pulmonary insufflation pressure (PIP) was measured by means of a pressure transducer (Sensortechnics, Rugby, Warwks.) connected to a side arm in the expiratory limb of the ventilation tubing. Blood pressure was monitored throughout the experiments via a polyethylene catheter filled with heparin-saline (10 u ml^{-1}) inserted into the left carotid artery and linked to a pressure transducer (Bell and Howell, Basingstoke) and two channel recorder (Ormed Ltd., Welwyn Garden City). All drugs were administered via the jugular veins.



FK224



FK888

Figure 1 The structures of FK224 and FK888.

Measurement of plasma exudation

To assess plasma exudation, Evans blue dye (25 mg kg^{-1}) was used as a plasma marker (Rogers *et al.*, 1989b) and was injected 1 min before each experimental procedure. Five min after vagus nerve stimulation or 15 min after SP or cigarette smoke, the systemic circulation of the animals was perfused with saline via the aorta to expel intravascular dye. The lower half of the trachea and the main bronchi were removed and the Evans blue dye in the tissue was extracted in formamide (37°C for 16 h) and quantified spectrophotometrically by measuring dye absorbance at 620 nm wavelength. Tissue dye content was expressed as ng dye mg^{-1} wet weight tissue.

Protocol

In the experiments involving vagus nerve stimulation, animals were first given atropine (1 mg kg^{-1}). After 8 min, both cervical vagus nerves were cut at the level of the fifth tracheal cartilage ring. Two min later, the caudal ends of the nerves were stimulated with bipolar platinum electrodes at 5 V, 5 ms, 10 Hz for 3 min (Belvisi *et al.*, 1990). Control animals were prepared similarly but without electrical stimulation (sham stimulation).

The cigarettes used were commercially available unfiltered U.K. government category 'Middle Tar' (nicotine content 1.2 mg per cigarette, carbon monoxide content 11 mg per cigarette) and were lit in a fume cupboard where a laminar flow prevented smoke accumulation. Smoke was collected into a 50 ml-syringe and blown into the trachea via 3-way tap immediately rostral to the endotracheal tube. One tidal volume of cigarette smoke was given every five to six ventilated breaths of room air. Control animals inhaled air drawn through an unlit cigarette.

In the experiments involving PAF, one group of animals were pretreated with capsaicin (or its vehicle) to deplete sensory neuropeptides. To reduce capsaicin-induced bronchoconstriction, animals were given aminophylline (25 mg kg^{-1}, i.p.) and terbutaline (0.1 mg kg^{-1}, s.c.). These administrations were repeated 20 min later. Five min after the second administration of bronchodilators, the animals were anaesthetized with xylazine (0.1 mg kg^{-1}, i.m.) and ketamine hydrochloride (50 mg kg^{-1}, i.m.) and injected with capsaicin (50 mg kg^{-1}, s.c.) or equivalent volume of vehicle (10% ethanol and 10% Tween 80, v v^{-1} in saline).

SP (1 nmol kg^{-1}), [Sar]SP (1 nmol kg^{-1}) or [β-Ala]NKA (10 nmol kg^{-1}) (Maggi *et al.*, 1991a) or their vehicle (saline), or C_{16}-PAF (5 μg kg^{-1}) or its vehicle (0.5% ethanol in saline) were injected intravenously 1 min after Evans blue dye. Tachykinin antagonists or their vehicle (dimethylsulphoxide, DMSO; see Drugs below) were injected 5 min before SP, [Sar]SP, [β-Ala]NKA, PAF, vagus nerve stimulation or administration of cigarette smoke.

Drugs

The following drugs were used: SP, DMSO, formamide, C_{16}-PAF, capsaicin and urethane (Sigma Chemical Co. Ltd., Poole, Dorset), [Sar]P (Peninsula Labs., Merseyside), atropine sulphate (Phoenix Pharmaceuticals Ltd., Gloucester), Evans blue dye (Aldrich Chemical Co. Ltd., Gillingham, Kent), saline (0.9% sodium chloride BP for intravenous infusion, FL Manufacturing Ltd., Basingstoke). [β-Ala]NKA was a gift from A. Menarini Pharmaceuticals, Florence, Italy. Stock solutions of 0.1 μmol ml^{-1} SP, of 3 μmol ml^{-1} [Sar]SP and [β-Ala]NKA in saline, and of 1 mg ml^{-1} PAF in ethanol were each stored at − 20°C and diluted in saline on each day of experimentation.

FK224 and FK888 were provided by the Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan). FK224 is a modified cyclic peptide with the structure N-(N^2-(*N*-(*N*-(*N*-(2,3-didehydro-*N*-methyl-*N*-(*N*-(3-(,2-penthylphenyl)-propionyl)-L-threonyl]tyrosyl-L-leucynyl]-D-phenylalanyl)-L-*allo*-threonyl)-L-asparaginyl-L-serine-υ-lactone. FK888 is a synthetic dipeptide with the structure N^2-[(4**R**)-4-hydroxy-1-(1-methyl-1H-indol-3-yl)carbonyl-L-prolyl]-*N*-methyl-*N*-phenylmethyl-3-(2-napthyl)-L-alaninamide). Both antagonists were dissolved in DMSO (20 μmol ml^{-1} for FK888, 100 μmol ml^{-1} for FK224), aliquoted and stored at 4°C.

In a number of instances the effect of DMSO on plasma exudation, bronchoconstriction and hypotension was compared with no administration. DMSO significantly ($P < 0.05$) affected the magnitude of plasma exudation compared with no treatment in air controls and sham-stimulated controls: the percentage difference between the mean values for DMSO and no DMSO respectively for each group was 42% and 8% for the lower trachea and 19% and 30% for the main bronchi. DMSO affected the magnitude of cigarette smoke or vagally stimulated plasma exudation to a lesser degree (maximum percentage difference between the means of 12%). Bronchoconstriction was 4% higher in sham-stimulated animals and 11% higher in stimulated animals with DMSO compared with no treatment. Blood pressure was minimally affected by DMSO (maximum percentage difference between mean values for 7 pairs of experimental groups was 5%). Thus, to account for the effects of DMSO in each study in Results involving the antagonists, DMSO, at the appropriate dilution in saline for the highest dose of antagonist used, was administered at appropriate time-points to both stimulated and unstimulated animals.

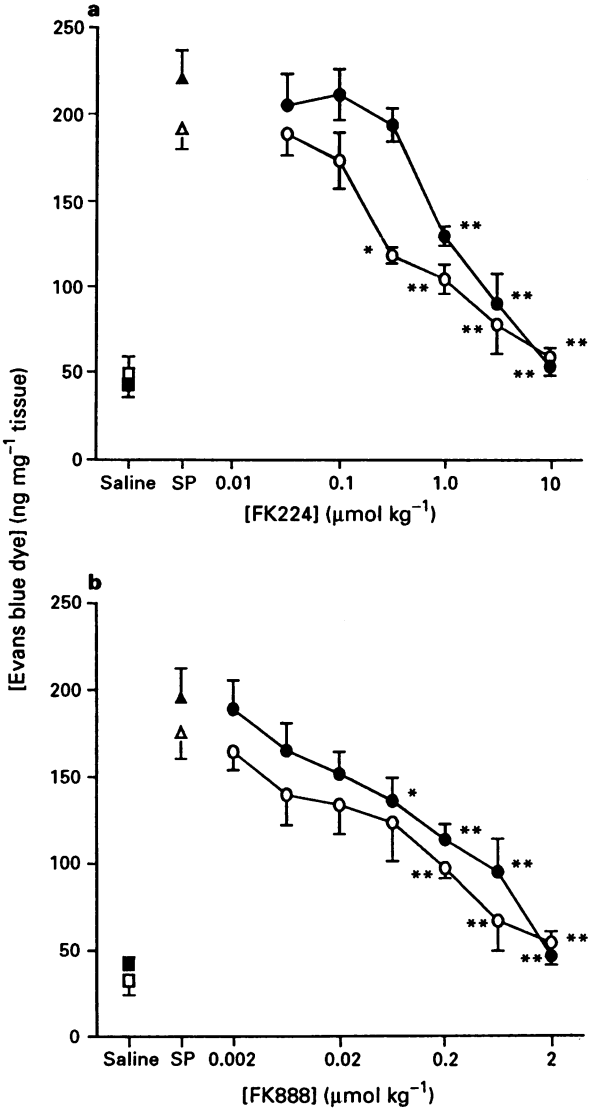


Figure 2 Effects of FK224 (a) and FK888 (b) on substance P (SP 1 nmol kg^{-1})-induced plasma exudation *in vivo* in guinea-pig lower trachea (solid symbols) or main bronchi (open symbols): (□,■) DMSO (vehicle for FK compounds) + saline; (△,▲) DMSO + SP (1 nmol kg^{-1}); (○,●) FK224 or FK888 + SP. All drugs given intravenously. Data are expressed as mean tissue content of the plasma marker Evans blue dye for 5–10 animals; s.e.mean shown by vertical bars. *$P < 0.05$; **$P < 0.01$ vs DMSO + SP.

Data analyses

Data in the Results section are mean and one s.e.mean. Increases in PIP induced by vagus nerve stimulation were expressed as percentage increases for the peak of the response above the pre-stimulation value. Mean blood pressure was calculated from the recorded traces as diastolic pressure + 0.33 (systolic pressure − diastolic pressure). Decreases in mean BP were expressed as a percentage of the mean BP just before nerve stimulation or drug administration. The dose of antagonist causing 50% inhibition (ID_{50}) was calculated by non-linear regression. The significance of differences between groups was assessed by the Mann-Whitney U-test. The null hypothesis was rejected at $P < 0.05$ (two-tail test). Inhibition was considered to be complete when the response to stimulation in the presence of antagonist was significantly different from the response to stimulation without the antagonist and not significantly different from unstimulated controls.

Results

Effects of FK224 and FK888 on plasma exudation

The inhibitory effects of FK224 and FK888 were initially determined on SP-induced plasma exudation in the lower trachea and main bronchi (Figure 2). Both compounds gave a dose-dependent inhibition and, at the highest doses used (10 μmol kg^{-1} FK224 or 2 μmol kg^{-1} FK888), totally abolished the response. FK888 was 5–10 fold more potent than FK224 in inhibiting the response; ID_{50} values for FK224 and FK888 were respectively 1.1 and 0.1 μmol kg^{-1} (1.10 and 0.06 mg kg^{-1}) in lower trachea, and 0.5 and 0.1 μmol kg^{-1} (0.51 and 0.06 mg kg^{-1}) in main bronchi.

[Sar]SP (1 nmol kg^{-1}) significantly increased plasma exudation by 185% above saline controls (Figure 3). Both FK888 (2 μmol kg^{-1}) and FK224 (10 μmol kg^{-1}) abolished [Sar]SP-induced plasma exudation (Figure 3). In contrast, [β-Ala]NKA did not induce significant plasma exudation (Figure 3): neither antagonist significantly affected this lack of response ($n = 5$ for each antagonist; data not shown).

In animals treated one week previously with the vehicle for capsaicin or with capsaicin itself, PAF increased plasma exudation in the main bronchi (Table 1). The magnitude of PAF-induced plasma exudation was 21% greater in vehicle-

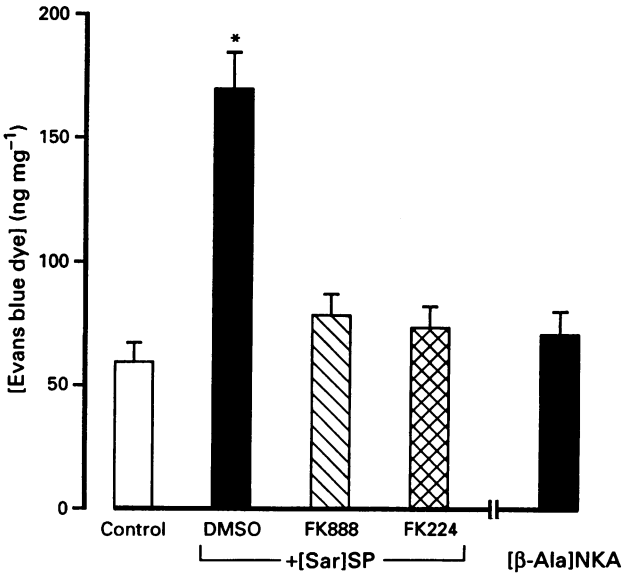


Figure 3 Effects of FK888 (2 μmol kg^{-1}) and FK224 (10 μmol kg^{-1}) on plasma exudation induced *in vivo* in guinea-pigs by [Sar]SP (NK_1 agonist, 1 nmol kg^{-1}). *$P < 0.05$ compared with control. The NK_2-selective agonist [β-Ala]NKA (10 nmol kg^{-1}) did not induce significant plasma exudation. All drugs given intravenously. Data are expressed as mean tissue content of the plasma marker Evans Blue dye for 5 animals; s.e.mean shown by vertical bars.

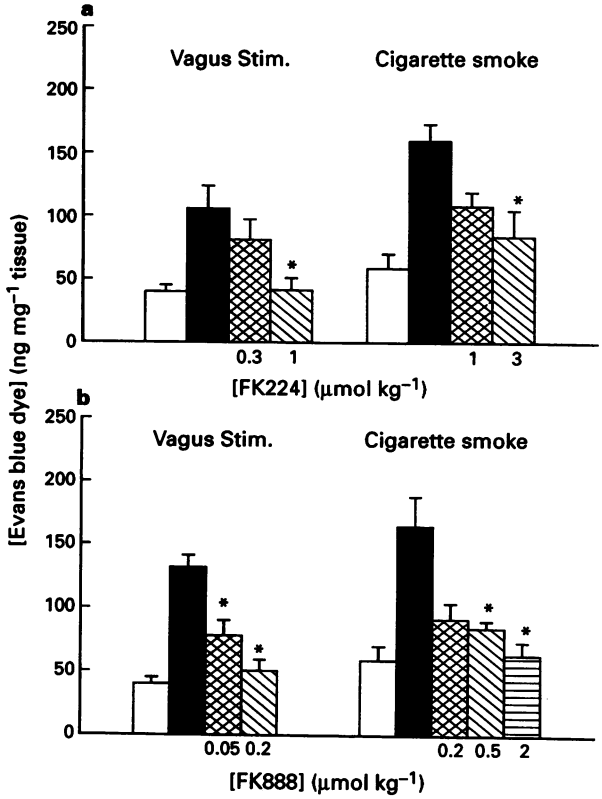


Figure 4 Effects of FK224 (a) and FK888 (b) on vagus nerve stimulation or cigarette smoke-induced plasma exudation *in vivo* in main bronchi of guinea-pigs. Open column, control; solid column, response to stimulation; hatched and cross-hatched columns, effects of FK224 or FK888. All drugs given intravenously. Data are expressed as mean tissue content of the plasma marker Evans blue dye for 5 or 6 animals; s.e.mean shown by vertical bars. *$P < 0.05$ vs stimulation.

pretreated compared with capsaicinized animals, although there was no significant difference between the two groups. Neither FK888 nor FK224 inhibited PAF-induced plasma exudation (Table 1).

Electrical stimulation of the vagus nerves or cigarette smoke inhalation induced significant increases in the tissue content of Evans blue dye in both the lower trachea (by 165–292% above controls) and main bronchi (the latter shown in Figure 4). Both FK224 and FK888 inhibited these responses. FK224 at 1 μmol kg^{-1} or FK888 at 0.2 μmol kg^{-1} completely abolished the increase in plasma exudation induced by vagus nerve stimulation in both the lower trachea and main bronchi (Figure 4). FK224 and FK888 partially but significantly ($P < 0.05$) inhibited the cigarette smoke-induced response in the lower trachea (by approximately 50% at the highest dose of each compound used) and induced complete inhibition in the main bronchi (Figure 4).

Effects of FK224 and FK888 on bronchoconstriction

[β-Ala]NKA increased pulmonary insufflation pressure by 259% above baseline (Figure 5). FK888 did not significantly affect this response (in fact induced a potentiation of 39%), whereas FK224 inhibited [β-Ala]NKA-induced bronchoconstriction by 87% (Figure 5). [Sar]SP induced a small (16% above baseline) but significant increase in PIP, a response that was inhibited by 95% by FK888 and by 100% by FK224 (Figure 5).

In capsaicin or vehicle pretreated animals, PAF-induced increases in PIP were greater (although not significantly so) by 24% in vehicle-pretreated compared with capsaicinized animals. PAF-induced bronchoconstriction was not significantly affected by either of the tachykinin receptor antagonists under study (Table 1).

Electrical stimulation of the vagus nerves caused non-cholinergic bronchoconstriction as well as plasma exudation (Figure 6). The increase in PIP reached a peak approximately 1 min after the start of stimulation. FK224 induced a significant inhibition of 89% at 1 μmol kg^{-1} (Figure 6)

Table 1 Effect of tachykinin receptor antagonists on plasma exudation and bronchoconstriction induced by platelet activating factor (PAF)

	[Evans blue dye] (ng mg^{-1} wet wt. tissue)	*PIP* (cmH_2O)
Vehicle for capsaicin + DMSO + PAF	212.0 ± 20.2	328.8 ± 32.4
Capsaicinization + DMSO + PAF	175.9 ± 30.2	264.7 ± 49.1
Capsaicinization + FK888 + PAF	164.0 ± 12.7	305.0 ± 39.4
Capsaicinization + FK224 + PAF	175.7 ± 34.0	299.2 ± 41.7

Guinea-pigs were pretreated with capsaicin, to deplete sensory neuropeptides, or its vehicle. One week later animals were anaesthetized and given PAF (5 μg kg^{-1}) to induce exudation of the plasma marker Evans blue dye into the main bronchi and to increase pulmonary insufflation pressure (PIP), a measure of airway patency, either with the tachykinin antagonists FK888 (2 μmol kg^{-1}) or FK224 (10 μmol kg^{-1}), or their vehicle (dimethylsulphoxide, DMSO). All drugs given intravenously. Data are mean ± s.e.mean for 5 animals per group. There were no significant differences between treatment groups for plasma exudation or PIP.

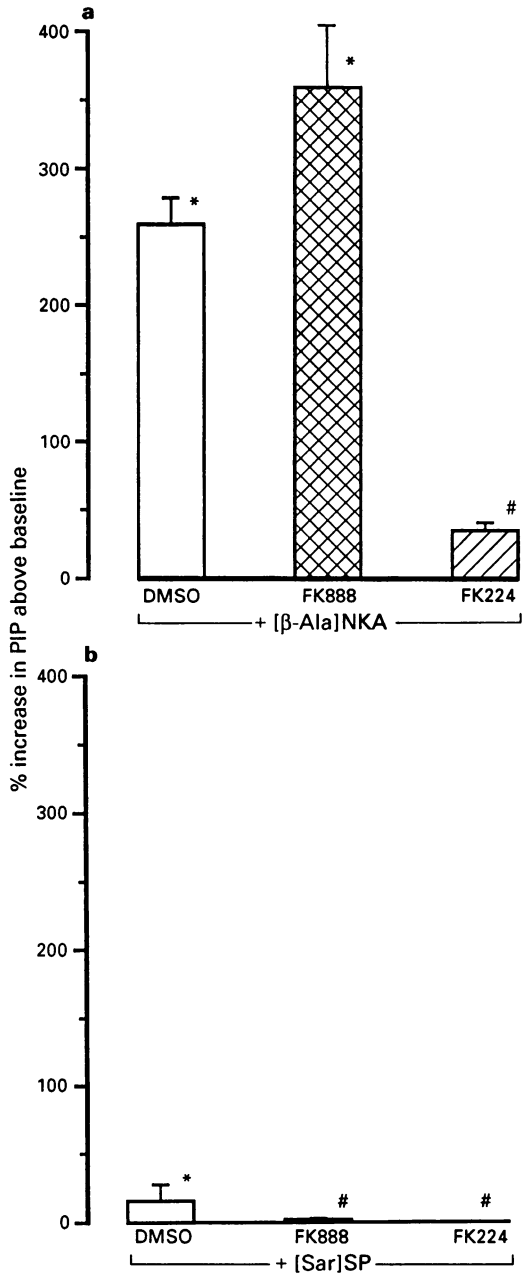


Figure 5 Effect of FK888 (2 μmol kg^{-1}) and FK224 (10 μmol kg^{-1}) on the increase in pulmonary insufflation pressure (PIP), a measure of airway patency, induced *in vivo* in guinea-pigs by the NK_2 agonist, [β-Ala]NKA (10 nmol kg^{-1}) (a) or the NK_1 agonist, [Sar]SP (1 nmol kg^{-1}) (b). All drugs given intravenously. Data are expressed as mean for 5 animals; s.e.mean shown by vertical bars. $*P < 0.05$ compared with baseline; $\dagger P < 0.05$ compared with value for agonist (with antagonist vehicle, dimethylsulphoxide (DMSO)).

whereas FK888 showed no significant effect on the response even at 0.2 μmol kg^{-1}, the dose giving complete inhibition of neurogenic plasma exudation induced by vagus nerve stimulation.

Cigarette smoke did not induce bronchoconstriction, and this lack of response was not affected by either FK888 or FK224 (data not shown).

Effects of FK224 and FK888 on decreases in blood pressure

Administration of 1 nmol kg^{-1} SP decreased BP in the carotid artery with a maximal fall approximately 1 min after

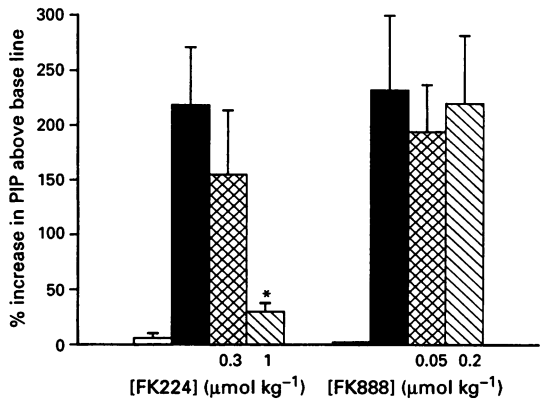


Figure 6 Effects of FK224 or FK888 on vagus nerve stimulation-induced increases in pulmonary insufflation pressure (PIP), a measure of airway patency, *in vivo* in guinea-pigs. Open columns, control; solid column, response to stimulation; hatched and cross-hatched columns, effects of FK224 or FK888. All drugs given intravenously. Data are expressed as mean for 5 animals; s.e.mean shown by vertical bars. $*P < 0.05$ vs stimulation.

SP injection. The SP-induced decrease in BP was significantly inhibited by both FK224 and FK888 (Figure 7). Similarly, [Sar]SP significantly ($P < 0.05$) reduced BP by 58.2 ± 6.9% ($n = 5$). Both FK888 and FK224 significantly ($P < 0.05$) inhibited [Sar]SP-induced decreases in BP (16.6 ± 13.7% increase with FK888 and 12.2 ± 4.7% decrease with FK224, $n = 5$ each). In capsaicinized animals, PAF significantly ($P < 0.05$) reduced BP by 48.6 ± 5.0%. Neither FK888 nor FK224 significantly affected this response (reduction of 26.2 ± 17.0% with FK888 and 39.0 ± 9.7% with FK224, $n = 5$ each).

During electrical stimulation of the vagus nerves, BP fell continuously (Figure 8). Although FK224 showed a tendency to inhibit the BP-decrease, the effect was not statistically significant ($P = 0.06$ at 1 μmol kg^{-1}, 3 and 4 min after the start of stimulation). FK888 showed no tendency to any significant inhibitory effect on the BP decrease at either of the doses used or at any of the time points examined.

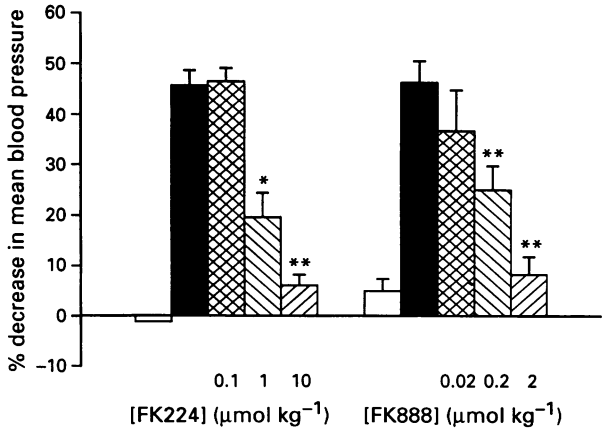


Figure 7 Effects of FK224 or FK888 on substance P (SP, 1 nmol kg^{-1})-induced decrease in mean carotid artery blood pressure *in vivo* in guinea-pigs. Open column, dimethylsulphoxide (DMSO) + saline; solid column, DMSO + SP (1 nmol kg^{-1}); hatched and cross-hatched columns, effects of FK224 or FK888. All drugs given intravenously. The datum of the control group for FK224 is the average for 2 animals. The other data are expressed as mean for 4–8 animals; s.e.mean shown by vertical bars. $*P < 0.05$; $**P < 0.01$ vs DMSO + saline.

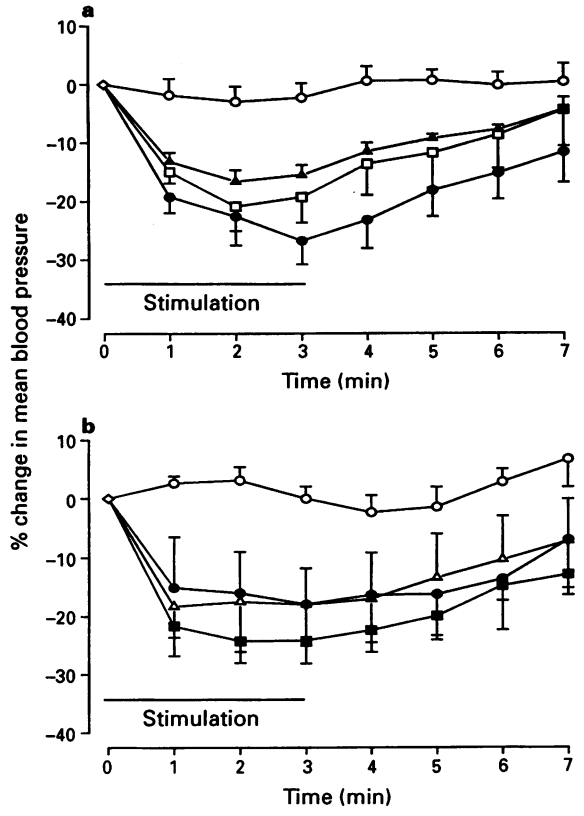


Figure 8 Effects of FK224 (a) and FK888 (b) on vagus nerve stimulation-induced changes in carotid artery blood pressure *in vivo* in guinea-pigs. Dimethylsulphoxide (DMSO) + sham stimulation (○); DMSO + vagus nerve stimulation (●); effects of FK224 at 0.3 μmol kg^{-1} (□) or 1 μmol kg^{-1} (▲) in (a), effects of FK888 at 0.05 μmol kg^{-1} (■) or 0.2 μmol kg^{-1} (△) in (b). All drugs given intravenously. Data are expressed as mean for 5 animals; s.e.mean shown by the vertical bars.

Discussion

In the present *in vivo* study, we found that both FK224 (NK_1 and NK_2 dual tachykinin antagonist) (Murai *et al.*, 1992; Morimoto *et al.*, 1992) and FK888 (NK_1-selective antagonist) (Fujii *et al.*, 1992) inhibited plasma exudation induced in guinea-pig airways by exogenous SP, the selective NK_1 agonist [Sar]SP (Maggi *et al.*, 1990), bilateral vagus nerve stimulation or by inhalation of cigarette smoke. These data extend those of others who found that these antagonists inhibited plasma exudation (as well as broncoconstriction for FK224) induced by SP, NKA or capsaicin *in vivo* in guinea-pigs (Fujii *et al.*, 1992; Murai *et al.*, 1992). Natural agonist studies (Rogers *et al.*, 1988) indicated that the tachykinin NK_1 receptor was the more important in mediating plasma exudation evoked by tachykinins. Recently, an NK_1-selective non-peptide antagonist, CP-96,345 (Snider *et al.*, 1991), has been used to demonstrate that neurogenic plasma exudation in rat or guinea-pig airways is mediated predominantly via NK_1-receptors (Delay-Goyet & Lundberg, 1991; Eglezos *et al.*, 1991; Lei *et al.*, 1992). Our present results provide further evidence for the involvement of NK_1-receptors in neurogenic plasma exudation, and demonstrate that both FK224 and FK888 are potent tachykinin antagonists *in vivo*. *In vitro* data using pA_2 values indicate that FK888 is approximately 300 times more potent than FK224 as an NK_1 receptor antagonist (Fujii *et al.*, 1992; Morimoto *et al.*, 1992). Our present *in vivo* data for the effect of these antagonists in inhibiting SP-induced plasma exudation demonstrate that FK888 is only 5–10 times more potent than FK224. Similar data have been reported recently (Fujii *et al.*, 1992; Murai *et al.*, 1992). The precise reasons for the discrepancy between the *in vitro* and *in vivo* data are unclear. One possibility is differences in pharmacokinetics (tissue distribution, bioavailability, metabolism) of the compounds given intravenously, since by intratracheal installation *in vivo* FK888 is 100 times more potent than FK224 (Y. Hirayama, unpublished observations). Alternatively, the discrepancy in potencies may indicate that SP-induced plasma exudation is not mediated exclusively via NK_1 receptors. The involvement of NK_2 receptors in the response is unlikely because the NK_2-selective agonist, [β-Ala]MKA (Maggi *et al.*, 1990b), did not induce plasma exudation in the present study. A non-specific action is also unlikely because neither tachykinin antagonist inhibited plasma exudation (or indeed bronchoconstriction or systemic hypotension) induced by PAF. PAF, histamine and sulphidopeptide leukotrienes induce bronchoconstriction and changes in systemic blood pressure (Barnes *et al.*, 1988). Of these, PAF is markedly the most potent in inducing plasma exudation in guinea-pig airways (Evans *et al.*, 1989). However these three mediators have also been found to release SP- and NKA-like immunoreactivity in guinea-pig lungs (Martins *et al.*, 1991), although in the absence of neutral endopeptidase inhibition, PAF releases the least amounts of tachykinin-like immunoreactivity. Thus, PAF was the most appropriate choice of available mediators to determine any non-selectivity of FK888 and FK224 for tachykinin receptor-mediated plasma exudation, bronchoconstriction or hypotension in the present study. In order to exclude the effects of PAF-induced release of tachykinins, we pretreated animals with capsaicin to deplete sensory neuropeptides, including tachykinins, from sensory nerves (Hozler, 1991). The reduced exudative and broncoconstrictor responses in capsaicinized compared with vehicle pretreated animals (albeit not significant) may indicate a small amount of PAF-induced tachykinin release. The complete lack of inhibitory effect of FK888 or FK224 on PAF-induced plasma exudation or bronchoconstriction in capsaicinized animals argues against a non-specific action for these antagonists.

The NK_1-selective antagonist, FK888, showed no significant inhibitory effect on vagus nerve stimulation-induced non-cholinergic bronchoconstriction, although it gave complete inhibition of plasma leakage induced by vagus nerve stimulation in the same animals. In contrast, the dual antagonist, FK224, inhibited the bronchoconstriction. These data indicate that bronchoconstriction *in vivo* induced by endogenously-released tachykinins is mediated predominantly via NK_2-receptors, which is consistent with *in vitro* data in human bronchus using natural tachykinin potencies (Advenier *et al.*, 1987) and NK_2 antagonist inhibition of contraction of electrically stimulated-guinea-pig bronchi *in vitro* (Maggi *et al.*, 1991b). In the present study the NK_1-selective agonist, [Sar]SP (Maggi *et al.*, 1990b), also induced significant, although small, bronchoconstriction, a response markedly inhibited by both FK888 and FK224. These data indicate that NK_1 receptors contribute a minor role to bronchoconstriction in the guinea-pig, a suggestion consistent with that of others (Ireland *et al.*, 1991; Maggi *et al.*, 1991b). Our present agonist data indicate the involvement of both NK_1 and NK_2 receptors in tachykinin-induced bronchoconstriction which is in contrast to our observed lack of effect of FK888 on neurogenic non-cholinergic bronchoconstriction. We interpret this latter result as reflecting the relatively minor role for NK_1 receptors in bronchoconstriction and the differences in resistance of SP and [Sar]SP to degradation by endogenous peptidases. [Sar]SP, despite its resistance to peptidase degradation, induced here only a very small increase in airway pressure. Thus, any small intrinsic effect of endogenous SP in inducing bronchoconstriction will be further reduced by enzyme action which makes the involvement of NK_1 receptors in neurogenic bronchoconstriction difficult to demonstrate. The latter suggestion is consistent with the finding *in vitro* that the minor relative contribution of NK_1 receptors to neurogenic bronchoconstriction is increased in the presence of peptidase inhibitors (Maggi *et al.*, 1991b).

Both FK224 and FK888 inhibited hypotension induced by SP, indicating that the vasodilator activity of SP is mediated via NK_1-receptors, a suggestion which is consistent with a recent report using exogenously-administered tachykinins in dogs (Constantine *et al.*, 1991). In contrast, neither compound, at doses that significantly inhibited SP-induced decreases in BP and induced complete inhibition of neurogenic plasma exudation, had any significant inhibitory effect on the decrease in BP induced by vagus nerve stimulation, although FK224 showed a tendency to partial inhibition of the response. This effect of FK224 may be due to an inhibitory effect on bronchoconstriction, which can induce hypoxia with subsequent bradycardia. The contribution of endogenous tachykinins, SP and/or NKA, to neurogenic hypotension would, therefore, appear to be minimal. Other neuropeptides released simultaneously together with SP, including calcitonin gene-related peptide (CGRP) (Saria *et al.*, 1988), may be involved in the hypotensive response. Antidromically released CGRP may affect arterial pressure via a direct action on heart function or via vasodilator activity, which could change blood flow resistance. We also cannot exclude the possibility that adrenergic pathways affected the response. Development of selective and potent CGRP receptor antagonists would serve to elucidate the role of this peptide in neurogenic hypotension.

An axon reflex has been proposed as a mechanism amplifying the chronic inflammation and hyperresponsiveness of asthma (Barnes, 1986), in which neuropeptides released antidromically from sensory C-fibres play a critical role. Increased microvascular permeability with consequent plasma exudation and oedema is a feature of a number of airway diseases including asthma (Rogers & Evans, 1992), although no evidence is available for induction of plasma exudation by tachykinins in human airways, or about the receptor type(s) involved. Bronchoconstriction may be induced in human airways via NKA-activation of NK_2 receptors (Advenier *et al.*, 1987). Similarly, activation of capsaicin-sensitive sensory nerves in human bronchi induces mucus secretion (Rogers & Barnes, 1989). Natural agonist potencies indicate that NK_1 receptors mediate tachykinin-induced mucus secretion in human airways (Rogers *et al.*, 1989a), although this has not been definitively proven by the use of receptor-selective agonists and antagonists. Nevertheless, the evidence for the involvement of tachykinins in asthma is circumstantial. Tachykinins appear to be important pathophysiological regulators of airway function in guinea-pigs, but their role in human airway disease is substantially unsettled. Similar considerations apply to the receptors involved in the actions of tachykinins in the airways. Data in the species which has been more investigated, the guinea-pig, are not yet confirmed for human airways. Development of selective and potent tachykinin receptor antagonists will be important in understanding the involvement of tachykinins in airway physiology and pathophysiology. If tachykinins are shown to be important in airway pathophysiology, development of potent but non-selective (i.e. dual) tachykinin antagonists may have greater therapeutic potential in asthma and in airway diseases associated with cigarette smoking, including chronic bronchitis.

Note added in proof

Ichinose, M. *et al.* (1992) report that FK224 inhibits bradykinin-induced bronchoconstriction in asthmatics (*Lancet*, **340**, 1248–1251).

References

ADVENIER, C., NALINE, E., DRAPEAU, G. & REGOLI, D. (1987). Relative potencies of neurokinins in guinea-pig trachea and human bronchus. *Eur. J. Pharmacol.*, **139**, 133–137.

BARNES, P.J. (1986). Asthma as an axon reflex. *Lancet*, **i**, 242–245.

BARNES, P.J., BARANIUK, J.N. & BELVISI, M.G. (1991). Neuropeptides in the respiratory tract. *Am. Rev. Respir. Dis.*, **144**, 1391–1399.

BARNES, P.J., CHUNG, K.F. & PAGE, C.P. (1988). Inflammatory mediatiors and asthma. *Pharmacol. Rev.*, **40**, 49–84.

BELVISI, M.G., BARNES, P.J. & ROGERS, D.F. (1990). Neurogenic inflammation in the airways: characterisation of electrical parameters for vagus nerve stimulation in the guinea pig. *J. Neurosci. Methods*, **32**, 159–167.

CONSTANTINE, J.W., LEBEL, W.S. & WOODY, H.A. (1991). Inhibition of tachykinin-induced hypotension in dogs by CP-96,345, a selective blocker of NK-1 receptors. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **344**, 471–477.

DELAY-GOYET, P. & LUNDBERG, J.M. (1991). Cigarette smoke-induced airway oedema is blocked by the NK_1 antagonist, CP-96,345. *Eur. J. Pharmacol.*, **203**, 157–158.

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

EVANS, T.W., ROGERS, D.F., AURSUDKIJ, B., CHUNG, K.F. & BARNES, P.J. (1989). Regunal and time-dependent effects of inflammatory mediators on airway microvascular permeability in the guinea-pig. *Clin. Sci.*, **76**, 479–485.

FUJII, T., MURAI, M., MORIMOTO, H., MAEDA, Y., YAMAOKA, M., HAGIWARA, D., MIYAKE, H., IKARI, N. & MATSUO, M. (1992). Pharmacological profile of a high affinity dipeptide NK_1 receptor antagonist, FK888. *Br. J. Pharmacol.*, **107**, 785–789.

GUARD, S. & WATSON, S. (1991). Tachykinin receptor subtypes: classification and membrane signalling mechanisms. *Neurochem. Int.*, **18**, 149.

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

IRELAND, S.J., BAILEY, F., COOK, A., HAGAN, R.M., JORDAN, C.C., STEPHENS-SMITH, M.L. (1990). Receptors mediating tachykinin-induced contractile responses in guinea-pig trachea. *Br. J. Pharmacol.*, **103**, 1463–1469.

LEI, Y.-H., BARNES, P.J. & ROGERS, D.F. (1992). Inhibition of neurogenic plasma exudation in guinea-pig airways by CP-96,345, a new non-peptide NK_1 receptor antagonist. *Br. J. Pharmacol.*, **105**, 261–262.

LUNDBERG, J.M., MARTLING, C.R., SARIA, A., FOLKERS, K. & ROSELL, S. (1983a). Cigarette smoke-induced airway oedema due to activation of capsaicin-sensitive vagal afferents and substance P release. *Neuroscience*, **10**, 1361–1368.

LUNDBERG, J.M., SARIA, A., BRODIN, E., ROSELL, S. & FOLKERS, K. (1983b). A substance P antagonist inhibits vagally induced increase in vascular permeability and bronchial smooth muscle contraction in the guinea-pig. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 1120–1124.

MAGGI, C.A., GIULIANI, S., BALLATI, L., ROVERO, P., ABELLI, L., MANZINI, S., GIACHETTI, A. & MELI, A. (1990a). *In vivo* pharmacology of [β-Ala^8]neurokinin A-(4-10), a selective NK_2 tachykinin receptor agonist. *Eur. J. Pharmacol.*, **177**, 81–87.

MAGGI, C.A., GIULIANI, S., BALLATI, L., LECCI, A., MANZINI, S., PATACCHINI, R., RENZETTI, A.R., ROVERO, P., QUARTARA, L. & GIACHETTI, A. (1991a). In vivo evidence for tachykininergic transmission using a new NK-2 receptor-selective antagonist, MEN-10,376. *J. Pharmacol. Exp. Ther.*, **257**, 1172–1178.

MAGGI, C.A., PATACCHINI, R., QUARTARA, L., ROVERO, P. & SANTICIOLI, P. (1990b). Tachykinin receptors in the guinea-pig isolated bronchi. *Eur. J. Pharmacol.*, **197**, 167–174.

MAGGI, C.A., PATACCHINI, R., ROVERO, P. & SANTICIOLI, P. (1991b). Tachykinin receptors and noncholinergic bronchoconstriction in guinea-pig isolated bronchi. *Am. Rev. Respir. Dis.*, **144**, 363–367.

MARTINS, M.A., SHORE, S.A. & DRAZEN, J.M. (1991). Release of tachykinins by histamine, methacholine, PAF, LTD_4, and substance P from guinea pig lungs. *Am. J. Physiol.*, **261**, L449–L455.

MORIMOTO, H., MURAI, M., MAEDA, Y., YAMAOKA, M., NISHIKAWA, M., KIYOTOH, S. & FUJII, T. (1992). FK224, a novel cyclopeptide substance P antagonist with NK_1 and NK_2 receptor selectivity. *J. Pharmacol. Exp. Ther.*, **262**, 398–402.

MURAI, M., MORIMOTO, H., MAEDA, Y., KIYOTOH, S., NISHIKAWA, S. & FUJII, T. (1992). Effects of FK224, a novel compound NK_1 and NK_2 receptor antagonist, on airway constriction and airway edema induced by neurokinins and sensory nerve stimulation in guinea pigs. *J. Pharmacol. Exp. Ther.*, **262**, 403–408.

ROGERS, D.F., AURSUDKIJ, B. & BARNES, P.J. (1989a). Effects of tachykinins on mucus secretion in human bronchi *in vitro*. *Eur. J. Pharmacol.*, **174**, 283–286.

ROGERS, D.F. & BARNES, P.J. (1989). Opioid inhibition of neurally mediated mucus secretion in human bronchi. *Lancet*, **i**, 930–932.

ROGERS, D.F., BELVISI, M.G., AURSUDKIJ, B., EVANS, T.W. & BARNES, P.J. (1988). Effects and interactions of sensory neuropeptides on airway microvascular leakage in guinea-pigs. *Br. J. Pharmacol.*, **95**, 1109–1116.

ROGERS, D.F., BOSCHETTO, P. & BARNES, P.J. (1989b). Correlation between Evans blue dye and radiolabeled albumin in guinea pig airway in vivo. *J. Pharmacol. Methods*, **21**, 309–315.

ROGERS, D.F. & EVANS, T.W. (1992). Plasma exudation and oedema in asthma. *Br. Med. Bull.*, **48**, 120–134.

SARIA, A., MARTLING, C.-R., YAN, Z., THEODORSSON-NOHEIM, E., GAMSE, R. & LUNDBERG, J.M. (1988). Release of multiple tachykinin from capsaicin-sensitive sensory nerve in the lung by bradykinin, histamine, dimethylphenyl piperazinium, and vagal nerve stimulation. *Am. Rev. Respir. Dis.*, **137**, 1330–1335.

SNIDER, R.M., CONSTANTINE, J.W., LOWE, J.A., LONGO, K.P., LEBEL, W.S., WOODY, H.A., DROZDA, S.E., DESAI, M.C., VINICK, F.J., SPENCER, R.W. & HESS, H.-J. (1991). A potent non-peptide antagonist of the substance P (NK_1) receptor. *Science*, **251**, 435–437.

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Erratum

Br. J. Pharmacol. (1992) **106**, 986–995

Staffan Uhlén, Yun Xia, Vijay Chhajlani, Christian C. Felder & Jarl E.S. Wikberg. [^{3}H]-MK 912 binding delineates two α_2-adrenoceptor subtypes in rat CNS one of which is identical with the cloned pA2d α_2-adrenoceptor.

In the above article, which appeared in the August 1992 issue of the journal, in Figure 1e and f the label of the y-axis should be [^{3}H]-MK 912 bound (pM); in Figure 1e the lowest value above the origin on the x-axis should be 0.5; and in Figure 4b the label of the y-axis should be [^{3}H]-MK 912 bound (nM).

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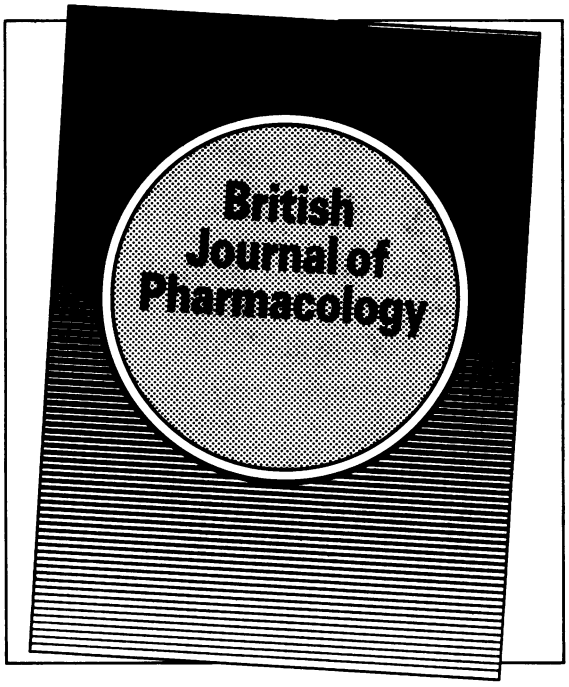
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10. **Figures**—Both labelled and non-labelled Figures to be prepared in accordance with current requirements of the Journal (see *Instructions to Authors*, 1993, **108,** 275–281) and provided with Figure Number and Authors' names on back (*in pencil*).
    —each legend to be typed on a separate page and carrying keys to symbols.
    —keys to symbols and histograms must not appear on the figures themselves, but in the respective legends.
    —'box style' figures are not in keeping with the Journal style; line drawings etc must have only left-hand and bottom axes.

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    (c) the same material is neither 'in press' (i.e. is in proof or has definitely been accepted for publication) nor under consideration elsewhere. Furthermore it will not be submitted or published elsewhere before a decision has been reached by the Editorial Board of the *British Journal of Pharmacology* and will not be submitted elsewhere if accepted by the *British Journal of Pharmacology*.
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13. **Reminder**—Packaging to be sufficiently robust to protect Figures and to withstand mailing.

Failure to comply with *Instructions to Authors* may lead to substantial delays in processing, review and publication and may even jeopardize acceptance of the manuscript.

NOMENCLATURE

Authors are reminded that accepted receptor and associated terminology is laid out in *Nomenclature Guidelines for Authors,* as published in the *British Journal of Pharmacology, Br. J. Pharmacol.*, 1993, **108,** 282–284.

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