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SPECIAL REPORT

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A68930 is a potent, full agonist at dopamine$_1$ (D_1) receptors in renal epithelial LLC-PK$_1$ cells

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The selective dopamine$_1$ (D_1) receptor agonists SK&F 82526 (fenoldopam) and A68930 and the mixed D_1/D_2 agonist SK&F 85174 were tested for their ability to stimulate adenosine 3':5'-cyclic monophosphate (cyclic AMP) accumulation in the porcine renal epithelial cell line, LLC-PK$_1$. SK&F 82526 and SK&F 85174 were potent stimulators of cyclic AMP accumulation (EC_{50}s 21.4 and 14.5 nM, respectively), but only partial agonists (intrinsic activities 31% and 46% of dopamine respectively). In contrast, A68930 was a potent, full agonist (EC_{50} 12.7 nM, intrinsic activity 102% of dopamine). The stimulatory effects of A68930 and dopamine on cyclic AMP accumulation were not additive, and the stimulation of cyclic AMP accumulation by A68930 was blocked by the D_1-selective antagonist, SCH 23390. These properties of A68930 suggest that it may be a useful D_1-selective agonist to study renal D_1 receptor mechanisms *in vitro* and *in vivo*.

Keywords: A68930 [(1**R**,3**S**)-1-aminomethyl-5,6-dihydroxy-3-phenylisochroman]; dopamine; adenylyl cyclase; dopamine$_1$ (D_1) receptors; LLC-PK$_1$ cells

Introduction Dopamine has robust natriuretic and diuretic properties when administered intravenously, effects which appear to be mediated primarily by dopamine$_1$ (D_1) receptors in the kidney (Felder *et al.*, 1989). The selective D_1 agonist SK&F 82526 (fenoldopam) is a potent natriuretic/diuretic agent and has been advanced as a possible pharmacological agent for treatment of hypertension and congestive heart failure (Holcslaw & Beck, 1990). Dopamine and SK&F 82526 have been shown to have both vascular and tubular actions in the kidney (Felder *et al.*, 1989), and low doses of dopamine or SK&F 82526 elicit a natriuresis/diuresis without altering renal haemodynamics (Hegde *et al.*, 1989a,b). Indeed, D_1 receptors in rat kidney are concentrated primarily on proximal tubules (Hegde *et al.*, 1989b; Huo & Healy, 1989), suggesting that tubular D_1 receptors may be important in mediating the dopamine-stimulated natriuretic/diuretic responses in the kidney.

D_1 receptors are generally classified biochemically as being linked to stimulation of adenylyl cyclase (Kebabian & Calne, 1979). We have shown recently that the proximal tubule-like porcine renal epithelial cell line, LLC-PK$_1$, expresses a D_1 receptor linked to stimulation of adenylyl cyclase and have suggested that these cells may be a useful model to study D_1 receptor mechanisms *in vitro* (Grenader & Healy, 1991b). We have further demonstrated that compared to dopamine, SK&F 82526 was only a partial agonist (intrinsic activity 37%) at the D_1 receptor in LLC-PK$_i$ cells (Grenader & Healy, 1991a). Thus, although SK&F 82526 is a potent D_1 agonist, the low efficacy of this drug would appear to limit its potential therapeutic utility in the kidney. Recently, the novel compound A68930 has been reported to be a partial agonist at D_1 receptors in the fish retina and a full agonist at D_1 receptors in the rat striatum (DeNinno *et al.*, 1991). The mixed D_1/D_2 agonist, SK&F 85174, an analogue of SK&F 82526, has also been shown to have D_1 receptor agonist activity in the kidney (Hieble *et al.*, 1990). Here we compared the ability of A68930, SK&F 82526, and SK&F 85174 to stimulate adenosine 3':5'-cyclic monophosphate (cyclic AMP) accumulation in LLC-PK$_1$ cells.

Methods LLC-PK$_1$ cells were grown in 24-well culture dishes and assayed for cyclic AMP accumulation as previously described (Grenader & Healy, 1991b). Cells were incubated for 10 min at 37°C with appropriate drugs in Hank's buffered salt solution containing 1 mM 3-isobutyl-1-methyl-xanthine (IBMX), 100 μM pargyline and 0.01% ascorbic acid. Cyclic AMP values were expressed as pmol cyclic AMP mg^{-1} of protein.

Materials A-68930 [1**R**,3**S**)-1-aminomethyl-5,6-dihydroxy-3-phenylisochroman] was kindly provided by Dr John Kebabian (Abbott Park, IL, U.S.A.). SK&F 82526 (fenoldopam; [6-chloro -7,8- dihydroxy -1- (4-hydroxyphenyl) -2,3,4,5-tetrahydro-1H-3-benzazepine]) and SK&F 85174-J [6-chloro-7,8-dihydroxy -1- (4-hydroxyphenyl) -3- (2-propen-1-yl) -2,3,4,5-tetrahydro-1H-3-benzazepine] were kindly provided by Dr J. Paul Hieble (King of Prussia, PA, U.S.A.). SCH 23390 (**R** (+) -8-chloro- 2,3,4,5- tetrahydro -3- methyl -5-phenyl-1H-3-benzazepine-7-ol HCl) a gift from Scherring (Bloomfield, NJ, U.S.A.). SCH 23388 (**S** (−) -8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-ol HCl) and pargyline were purchased from Research Biochemicals (Natick, MA, U.S.A.). All other chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.)

Results The ability of A68930, SK&F 85174, SK&F 82526 to stimulate cyclic AMP accumulation in LLC-PK$_1$ cells was compared to dopamine (Figure 1). Dopamine stimulated cyclic AMP accumulation approximately eight fold over basal levels (9.1 ± 1.9 pmol mg^{-1} of protein) with an EC_{50} of 1.7 ± 0.8 μM and an E_{max} of 70.5 ± 12.9 pmol mg^{-1} of protein ($n = 3$). No increase in cyclic AMP accumulation in LLC-PK$_1$ cells was observed if the phosphodiesterase inhibitor IBMX was omitted (data not shown), indicating that cyclic AMP accumulation was due to adenylyl cyclase stimulation and not inhibition of phosphodiesterase. SK&F 85174 and SK&F 82526 were both potent stimulators of cyclic AMP accumulation (EC_{50} 14.5 ± 1.0 nM and 21.4 ± 3.1 nM, respectively, $n = 3$) but were only partial agonists, with E_{max}s 32.6 ± 3.5 pmol mg^{-1} protein (intrinsic activity 46% of dopamine) and 21.9 ± 1.0 pmol mg^{-1} protein (intrinsic activity 31% of dopamine), respectively. In contrast, A68930 was a potent full agonist (EC_{50} 12.7 ± 2.8 nM, E_{max} 72.0 ± 8.93

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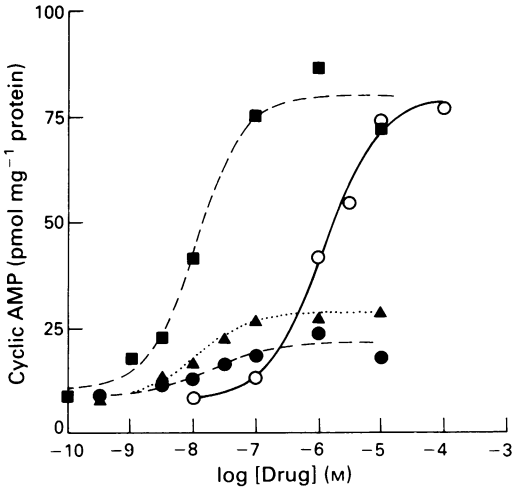


Figure 1 Representative dose-response curves for stimulation of cyclic AMP accumulation in LLC-PK$_1$ cells by dopaminergic receptor agonists. A68930 (■) and dopamine (○) were full agonists (EC_{50}s 12.7 ± 2.8 nM and 1.7 ± 0.8 μM, respectively, $n = 3$) and SK&F 85174 (◆) and SK&F 82526 (●) were partial agonists (EC_{50}s 14.5 ± 1.0 nM and 21.4 ± 3.1 nM, respectively, $n = 3$). Each point was done in duplicate.

pmol mg^{-1} of protein, intrinsic activity 102% of dopamine $n = 3$). In addition, concentrations of A68930 (0.1 μM) and dopamine (100 μM) that yielded maximum stimulation of cyclic AMP levels in LLC-PK$_1$ cells when incubated separately, were not additive when incubated together (Figure 2). Stimulation of cyclic AMP accumulation by 0.1 μM A68930 (77.5 ± 3.6 pmol mg^{-1} of protein, $n = 6$) was blocked by the D$_1$-selective antagonist SCH 23390 (0.1 μM) (Figure 2) but not by an equal dose of SCH 23388, the inactive stereoisomer of SCH 23390. Propranolol (0.1 μM), also failed to block the A68930-mediated increase in cyclic AMP (79.5 ± 4.5 pmol mg^{-1}of protein, $n = 3$).

Discussion A68930 stimulated cyclic AMP accumulation in LLC-PK$_1$ cells with an EC_{50} value similar to that reported for A68930-stimulated adenylyl cyclase activity in rat striatum (DeNinno *et al.*, 1991). Stimulation of cyclic AMP accumulation by A68930 was blocked by the selective D$_1$ receptor antagonist, SCH 23390, but not by its inactive stereoisomer SCH 23388 or the β-adrenoceptor antagonist, propranolol. Incubation of LLC-PK$_1$ cells with concentrations of A68930 and dopamine that maximally stimulated cyclic AMP accumulation when used alone, did not result in additive effects on cyclic AMP levels when incubated together.

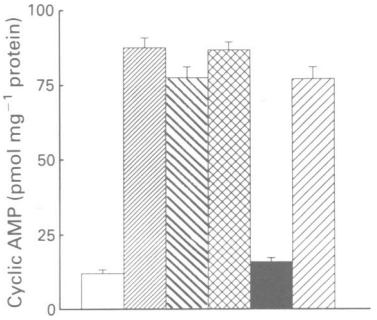


Figure 2 Characterization of the A68930-stimulated cyclic AMP accumulation in LLC-PK$_1$ cells. Cells were incubated with maximal concentrations of dopamine (100 μM, ▨) and A68930 (0.1 μM, ▧), alone and together (▩), or with A68930 plus the D$_1$ antagonist SCH 23390 (0.1 μM, ■) or its inactive stereoisomer SCH 23388 (0.1 μM, ▨). Basal cyclic AMP levels (□) were 11.9 ± 1.3 pmol mg^{-1} protein. Values represent the mean of six experiments, each point in triplicate; s.e.mean shown by vertical bars.

The lack of additivity suggests that A68930 and dopamine bind to the same D$_1$ receptor population of LLC-PK$_1$ cells. These results also provide further evidence that A68930 is a full agonist at D$_1$ receptors in LLC-PK$_1$ cells, since a potent partial agonist, such as SK&F 82526, will act as an antagonist in the presence of the full agonist dopamine resulting in cyclic AMP levels below that seen with dopamine alone (Grenader & Healy, 1991a). Together, these results indicate that A68930 is a highly potent, full agonist at D$_1$ receptors in LLC-PK$_1$ cells.

SK&F 82526 and SK&F 85174, on the other hand, were both potent stimulators of cyclic AMP accumulation in LLC-PK$_1$ cells but only partial agonists (intrinsic activity 31% and 46% of dopamine respectively). The results obtained here with SK&F 82526 were consistent with a previous report that SK&F 82526 was only a partial agonist at D$_1$ receptors in LLC-PK$_1$ cells (Grenader & Healy, 1991a).

The high potency and high intrinsic activity of A68930 at D$_1$ receptors in LLC-PK$_1$ cells suggests that A68930 may be a useful agent for study of D$_1$ receptor mechanisms in the kidney. The utility of A68930 as a natriuretic/diuretic agent in whole animal renal physiological studies has not yet been addressed but warrants further attention.

We would like to thank Dr John Kebabian (Abbott Laboratories, Abbot Park, IL, U.S.A.) for supplying A68930 and Dr J. Paul Hieble (SmithKline Beecham, Pharmaceuticals, King of Prussia, PA, U.S.A.) for supplying SK&F 85174 and SK&F 82526. This work was supported by the American Heart Association (Grant-in-Aid No. 91014490), the Irma T. Hirschl Foundation, and the National Institutes of Health (HL 42585). D.P.H. is an Established Investigator of the American Heart Association.

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Cannabinoid receptor agonists inhibit Ca current in NG108-15 neuroblastoma cells via a Pertussis toxin-sensitive mechanism

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Cannabinoid receptor ligands irreversibly inhibited peak voltage-activated Ca currents (44%) in NG108-15 cells; this inhibition was Pertussis toxin-sensitive. Inhibition was largely due to a reduction in the ω-conotoxin sensitive portion of high-voltage activated (HVA) current, although there was also a significant decrease in low-voltage activated current (56%) and in the nifedipine-sensitive portion of HVA current (41%).

Keywords: Cannabinoid; G-protein; Pertussis toxin; calcium current; neuroblastoma

Introduction Cannabinoids have a wide range of central actions (Howlett *et al.*, 1990), but their action at the cellular electrophysiological level is still unclear. A stereoselective receptor has been identified in brain, the activation of which causes inhibition of adenylyl cyclase (Howlett *et al.*, 1990). A cDNA for an appropriate G-protein coupled receptor has recently been cloned (Matsuda *et al.*, 1990). NG108-15 neuroblastoma x glioma hybrid cells also express this receptor (Matsuda *et al.*, 1990). In these cells, other receptors which couple negatively to adenylyl cyclase inhibit a voltage-gated calcium current (I_{Ca}) (e.g. α_2-adrenergic: Docherty & McFadzean, 1989; m2 and m4 muscarinic: Higashida *et al.*, 1990). We have therefore tested whether the cannabinoid receptor might also inhibit I_{Ca} in NG108-15 cells.

Methods I_{Ca} was recorded from chemically-differentiated NG108-15 cells as described by Caulfield *et al.* (1992). Whole-cell voltage-clamp recordings were made by use of a switching amplifier (Axon Instruments) operating at a sampling frequency of 4–10 kHz. Patch electrodes (3–5 MΩ) were filled with a solution containing (mM): CsCl 110, HEPES 40, EGTA 3, $MgCl_2$ 3, pH 7.4 with CsOH. Cells were superfused (5–10 ml min^{-1}) at 18°C–24°C with a modified Krebs solution containing (mM): tetraethylammonium chloride 120, KCl 3, $MgCl_2$ 1.2, HEPES 5, $NaHCO_3$ 23, $CaCl_2$ 2.5, D-glucose 11 and tetrodotoxin 0.5 μM (pH 7.4 when bubbled with 95% O_2: 5% CO_2). Cells were voltage-clamped at −90 mV and I_{Ca} evoked every 30 s by stepping for 500 ms to command potentials (V_c) in the range −100 mV to +60 mV. Current amplitudes were measured at peak or at the end of the voltage pulse, after leak subtraction (Caulfield *et al.*, 1992).

Acetylcholine chloride (ACh), nifedipine and Δ^9-tetrahydrocannabinol (THC) were purchased from Sigma Chemicals. *Bordatella pertussis* toxin was purchased from Porton International. CP55940 ([1α,2β(R)5α)-(-)-5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl)-cyclohexyl] phenol) was a gift from Pfizer Central Research. Synthetic ω-conotoxin GVIA (CgTx) was a generous gift of Dr A.J. Cross (Astra Neuroscience Research Unit, London).

Results The cannabinoid ligands THC (1–30 μM) and CP55940 (10 nM–1 μM) produced slowly-developing inhibition of I_{Ca}, which maximized after 2–15 min (Figure 1a). The maximum inhibition achieved was independent of drug concentration and inhibition did not recover detectably after up to 45 min washing ($n = 3$). This lack of reversibility precluded determination of meaningful concentration – response relationships. Subsequent studies were carried out with fixed concentrations of THC (30 μM) and CP55940 (1 μM). Cannabinoid ligands inhibited both peak (40% ± 6%; Figure 2a) and end-of-pulse current at $V_c = 0$ mV (47% ± 12%). Canna-

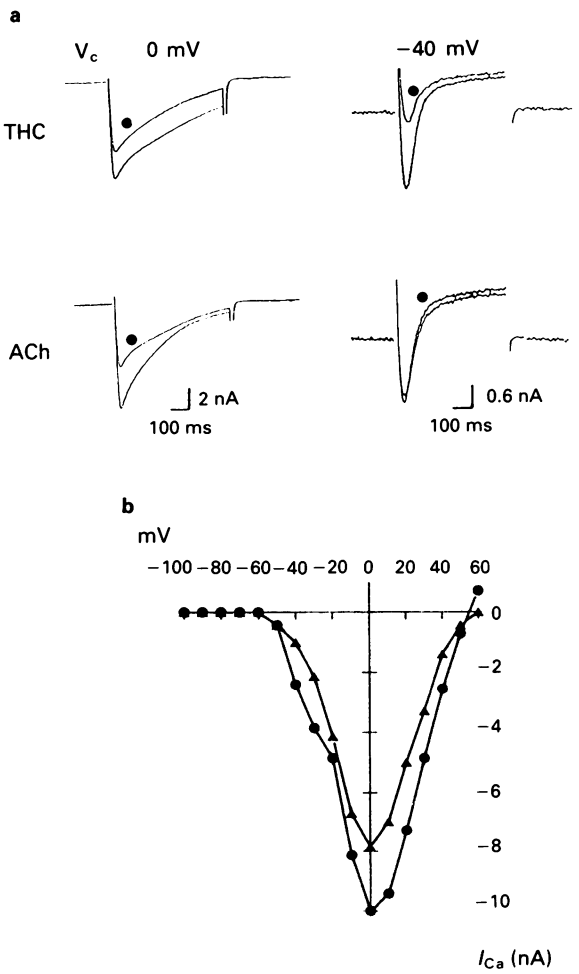


Figure 1 (a) Superimposed calcium currents are shown before and during (●) application of Δ^9-tetrahydrocannabinol (30 μM, THC) or acetylcholine (ACh, 30 μM). High voltage activated currents (V_c = 0 mV) are shown in the left panel and low voltage activated currents ($V_c = -40$ mV) are in the right panel. Holding potential −90 mV. (b) Leak-subtracted current-voltage relationships from the same experiment as (a) for control peak calcium currents (●) and currents in the presence of 30 μM THC (▲).

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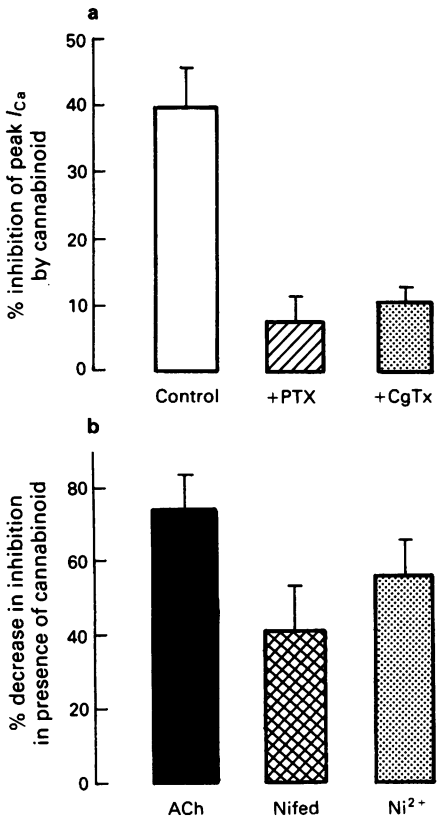


Figure 2 Mean inhibition of peak Ca current ($V_c = 0$ mV) by either 30 μM Δ^9-tetrahydrocannabinol (THC) or 1 μM CP55940 ($n = 10$; control) and after pretreatment of cells with 500 ng ml^{-1} Pertussis toxin (PTX; $n = 5$) or in the presence of 100 nM ω-conotoxin GVIA (CgTx; control inhibition 36% ± 10%, $n = 5$). Bars show s.e.mean. (b) Control inhibitions of peak I_{Ca} at $V_c = 0$ mV were: ACh (30 μM) 44% ± 14%; nifedipine (Nifed; 1–2 μM) 28% ± 6%; at $V_c = -20$ mV, $NiCl_2$ (Ni^{2+}; 20 μM) was 50% ± 7%. Histograms show the percentage reduction of the inhibitory effect of these agents in the presence of either THC (30 μM) or CP55940 (1 μM). Bars show s.e.mean.

binoid-sensitive currents showed a peak at −3 mV ± 2 mV ($n = 10$; Figure 1b), but there was also significant inhibition of I_{Ca} at negative potentials (e.g. at $V_c = -40$ mV there was 24% ± 11% inhibition; $n = 8$).

The inhibitory effect of cannabinoids was reduced to 8% ± 6% at $V_c = 0$ mV (Figure 2a) after pretreatment of cells with Pertussis toxin ($n = 5$; 500 ng ml^{-1}; 8–48 h); in these cells, cannabinoid inhibition of peak I_{Ca} at $V_c = -40$ mV was 7% ± 6%, and inhibition of end-of-pulse current at $V_c = 0$ mV was 8% ± 6%. I_{Ca} in NG108-15 cells can be subdivided pharmacologically into a low-voltage activated (LVA) component, selectively blocked by Ni^{2+}, and two high-voltage activated (HVA) components inhibited by CgTx and nifedipine respectively (Caulfield *et al.*, 1992). In the presence of CgTx, the cannabinoid-sensitive current was reduced to 7% ± 3% (Figure 2a). The inhibitory action of ACh is restricted to the CgTx-sensitive component (Caulfield *et al.*, 1992) and as expected from this, pre-application of cannabinoids largely occluded the inhibitory effect of ACh (Figure 2b). However, unlike ACh (see Caulfield *et al.*, 1992), cannabinoids also reduced the inhibitory effects of nifedipine and Ni^{2+} (Figure 2b).

Discussion In common with other neurotransmitter agonists which inhibit adenylyl cyclase (see MacFadzean & Docherty, 1989; Higashida *et al.*, 1990), the cannabinoid receptor ligands THC and CP55940 inhibit I_{Ca} in NG108-15 cells in a Pertussis toxin-sensitive manner. However, the precedents from studies with other neurotransmitters suggest that G_o, rather than G_i, might be the G-protein responsible for I_{Ca} inhibition (McFadzean *et al.*, 1989). Further, the low potency of THC and CP55940 as inhibitors of I_{Ca} relative to their potency in inhibiting cyclase (see Howlett *et al.*, 1990) implies less efficient coupling of cannabinoid receptors to the G-protein mediating I_{Ca} inhibition than to G_i.

Although ACh inhibition was greatly reduced by cannabinoids, indicating considerable overlap of action, there were nevertheless significant differences between ACh inhibition and cannabinoid inhibition of I_{Ca}. Thus, not only did the cannabinoid inhibition not reverse, but cannabinoids also significantly inhibited both end-of-pulse HVA current and LVA current. This latter effect is relatively unusual for G-protein coupled receptors (see review by Scott *et al.*, 1991). Also, some residual inhibitory effect of cannabinoids persisted in the presence of CgTx, and (unlike ACh) the cannabinoids produced significant inhibition (about 40%) of the nifedipine-sensitive portion of peak HVA current and Ni^{2+}-sensitive LVA current. The inhibition of multiple components of Ca current by cannabinoids may imply the existence of multiple receptor subtypes, each inhibiting a different current component, or possibly coupling of one receptor type to more than one type of G-protein (there are two subtypes of G_o in NG108-15 cells; Mullaney & Milligan, 1990).

Notwithstanding this apparent complexity, inhibition of I_{Ca} might provide a cellular electrophysiological basis for some of the central actions of cannabinoids. It would therefore be interesting to know whether similar effects occur in central neurones and if so, whether they result in significant changes in neuronal excitability or transmitter release.

Similar findings were reported in preliminary form by Mackie & Hille (1991, *Soc. Neurosci. Abstr.*, **21**, 435.6) while this work was in progress (Mackie & Hill, 1992; *Proc. Natl. Acad. Sci. U.S.A.*, in press).

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Evidence that the apparent complexity of receptor antagonism by angiotensin II analogues is due to a reversible and syntopic action

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1 The interactions between angiotensin II (AII), two non-peptide antagonists DuP 753 and IMI, and eight peptide analogues of AII were investigated on the rabbit isolated aorta assay. DuP 753 and IMI behaved as simple competitive antagonists (pK_B values 8.4 and 6.8, respectively). To different degrees, all the AII-peptide analogue interactions failed to meet the basic criteria for simple competition. In addition to rightward shift, the most significant feature was a concentration-dependent saturable depression of the upper asymptote of the AII concentration-effect curves.

2 'Washout' and combined dose-ratio analysis experiments, in which DuP 753 was used as a reference antagonist, indicated that the profile of peptide antagonism was solely due to a reversible and syntopic action at the AII receptor.

3 By use of an operational model of agonism (Black & Leff, 1983) as a starting point, it was possible to account for the data with a new model which describes reversible receptor occupancy and occupied receptor-determined, saturable reduction in the efficacy of AII. Model-fitting gave estimates of pK_B values for the peptide analogues and agonist affinity and efficacy parameters for AII.

4 The model was successfully tested by applying it to qualitatively similar results obtained in a cross-tissue analysis on guinea-pig aorta, ileum and stomach.

5 A 'molecular' interpretation of the efficacy changes, based on the concepts of receptor internalisation and expression, is offered.

Keywords: Angiotensin II; receptor; antagonist; agonist; aorta, ileum, stomach; DuP 753

Introduction

Angiotensin II (AII), peptide-analogue, antagonists were first described by Khairallah *et al.* (1970) and Marshall *et al.* (1970). Since then, although a large number of similar AII analogues have been described, all of them have shown more or less complex pharmacological behaviour. For example, it has been reported that some peptide analogues did not behave in a simple competitive manner with AII, but produced 'non-competitive' or 'insurmountable' antagonism (Regoli *et al.*, 1974; Freer *et al.*, 1980a,b; Koziarz & Moore, 1989; Bovy & Blaine, 1989). The antagonism of [Sar[1],Ile[8]]AII, however, has been described as both 'essentially competitive' (Turker *et al.*, 1972) and 'pseudo-irreversible' (Scanlon & Moore, 1988). Most peptide analogues have been found to produce partial agonist effects *in vivo* (Streeten & Anderson, 1984; Holck *et al.*, 1989; Bovy *et al.*, 1989) and some *in vitro* (Regoli *et al.*, 1974; Scanlon *et al.*, 1984; Criscione *et al.*, 1990).

In contrast, recently described, non-peptide, AII-receptor ligands, exemplified by DuP 753 (Chiu *et al.*, 1990; Timmermans *et al.*, 1991) and IMI (Koekpe *et al.*, 1990), have been described as simple competitive antagonists free from partial agonist activity.

In this paper we have used DuP 753 as a reference competitive antagonist to analyse the complex behaviour of eight peptide analogues of AII in isolated preparations of rabbit aorta and guinea-pig aorta, ileum and stomach.

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Methods

Isolated smooth muscle preparations

Rabbit and guinea-pig aorta ring preparations Aortic ring segments (approximately 3 mm long) from rabbit (male, New Zealand White, approximately 2.5 kg) and guinea-pig (male, Dunkin Hartley, 350 to 450 g) aortae were prepared following Stollak & Furchgott (1983). Briefly, descending thoracic aortae were excised, cleaned of extraneous tissues, and the endothelium was removed. Rings were suspended between 2 stainless steel wires in 20 ml organ baths containing Krebs-Henseleit (K-H) solution of the following composition (in mM): Na^+ 143, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128, HPO_4^{2-} 1.0, SO_4^{2-} 1.2, HCO_3^- 25, D-glucose 10, constantly gassed with 95% O_2 and 5% CO_2, and maintained at 37°C. Tension was continuously recorded with isometric transducers, following the application of 1 g and 4 g pre-loads in the guinea-pig and rabbit preparations, respectively.

Guinea-pig ileum longitudinal muscle preparation Sections of the distal ileum were removed from guinea-pigs and prepared essentially as described by Rang (1964). Longitudinal muscle strips of approximately 2 cm were suspended between 2 stainless steel hooks in K-H solution at 30°C, and gassed as above. A 1 g pre-load was applied and isometric tension was continuously recorded.

Guinea-pig stomach smooth muscle preparation Whole stomachs were removed from guinea-pigs and the smooth muscle from the main body region was separated from the mucosa by injecting K-H solution to form a blister. Thin strips (approximately 0.5 × 1.5 cm) were cut from the blistered region and mounted on isotonic transducers, in

gassed K-H solution at 30°C, for continuous displacement recording.

Experimental protocols

Six preparations were used simultaneously and allocated to control and treatment groups by a randomised block design. Preparations were allowed to stabilize for 60 min during which the organ bath fluid was replaced with pre-warmed K-H solution at 15 min intervals.

Analysis

Logistic curve-fitting Agonist concentration-effect (E/[A]) data from individual preparations were fitted to a logistic function of the form,

$$E = \frac{\alpha\ [A]^p}{[A_{50}]^p + [A]^p} \quad (1)$$

to provide estimates of α (upper asymptote), $\log[A_{50}]$ (midpoint location) and p (midpoint slope index). These parameters, expressed as mean ± s.e., were used for subsequent analysis and display of data (see Black & Shankley, 1985 for details).

Competitive analysis Competitive analysis was performed according to the procedure described previously (Black *et al.*, 1985b). If the Schild plot slope parameter, b, was found to be different from unity, or if differences were found in the midpoint slope indexes or upper asymptotes of the AII E/log[A] curves in the absence and presence of an antagonist, a pA_2 value was estimated without prejudice to mechanism of action.

Combined dose-ratio (r) analysis This analysis was performed as described previously (Shankley *et al.*, 1988) with DuP 753 used as reference antagonist to test for syntopic, competitive, antagonism. In brief, the theoretical relations which describe the syntopic (additive, $r_{B+C} = r_B + r_C - 1$) and allotopic (multiplicative, $r_{BC} = r_B.r_C$) interaction between two antagonists were expressed as test statistics (S) in terms of the experimentally-estimable $\log[A_{50}]$ values,

$$S_A = \log[A_{50}]_{B+C} - \log([A_{50}]_B + [A_{50}]_C - [A_{50}]) \quad (2)$$

$$S_M = \log[A_{50}]_{BC} - \log[A_{50}]_B - \log[A_{50}]_C + \log[A_{50}] \quad (3)$$

Test statistic values of zero are expected for model compliance.

Experimental data were directly fitted to the mathematical models described below with the 'AR' programme (derivative-free, non-linear, regressional analysis) within the BMDP statistical software package (Dixon, 1990).

Drugs

Drug stock solutions were prepared in distilled water, stored frozen below −20°C and used within 4 weeks, except noradrenaline which was prepared freshly, stoichiometrically with ascorbic acid. The latter had no significant effect on tissue responsiveness in control experiments. The total volume added to the 20 ml organ bath did not exceed 400 µl. Drugs and their sources were as follows: AII (Asp[1]-Arg[2]-Val[3]-Tyr[4]-Ile[5]-His[6]-Pro[7]-Phe[8]), Cambridge Research Biochemicals, U.K.; [Sar[1],Ala[8]]AII, [Sar[1],Val[5],Ala[8]]AII, [Sar[1],Ile[8]]AII, [Sar[1],Leu[8]]AII and noradrenaline hydrochloride (NA), Sigma Chemical Co. Ltd., U.K.; [Sar[1], Tyr(Me)[4]]AII, a gift from Professor G.J. Moore, University of Calgary, Canada and Peninsula Laboratories Inc., U.K.; [Sar[1]]AII(1-7)NH_2 and IMI (4'((2-butyl-4-chloro-5-(hydroxymethyl)-1H-imidazol-1-yl)methyl)-(1,1'-biphenyl)-2-carboxylic acid), gifts from Dr P.R. Bovy, Searle Research and Development, U.S.A.; [Sar[1],Thr[8]]AII, a gift from Dr M.C. Khosla, The Cleveland Clinic Foundation, U.S.A.; [Sar[1],Phe(2,3,4,5,6-Br_5)[8]]AII, a gift from Dr E. Escher, University of Sherbrooke, Canada; DuP 753, also designated as losartan and MK 954, (1-(2'-(5-tetrazoyl)phenyl)benzyl-2-butyl-4-chloro-5-methanolimidazole, potassium salt), a gift from E.I. Du Pont De Nemours & Co., U.S.A.

Results

Angiotensin concentration-effect relations in rabbit aorta

AII concentration-effect (E/[A]) data obtained by cumulative dosing in half-log dose increments at peak responses, could be fitted to the general logistic function (equation (1)). As fitted, the AII E/log[A] curves were symmetrical sigmoids with mean (± s.e.) parameter values as follows: $\log[A_{50}] = -8.86 \pm 0.05$, $\alpha = 6.28 \pm 0.11$ g, $p = 1.55 \pm 0.07$ (d.f. = 59). The phasic relaxation seen with high concentrations did not appear to accumulate suggesting that rapid desensitization was not occurring. Thus, when AII was given as single additions, one per preparation, the average E/[A] curve was not significantly different from that obtained by cumulative dosing. AII behaved as a partial agonist in the contractile system having an α value of about 60% of the NA maximum response (Figure 1).

Simple competitive antagonism of AII-receptors in rabbit aorta

DuP 753 and IMI, non-peptides, classified as competitive AII-receptor antagonists with pA_2 values of 8.5 (Chiu *et al.*, 1990) and 7.1 (Koepke *et al.*, 1990), have been used to validate the assay and characterize the AII-receptor population. When AII E/log[A] curves were obtained after incubation for 30 min with these ligands they were, with respect to control curves, displaced in parallel with no depression of upper asymptotes as expected for simple competitive antagonism. These concentration-dependent displacements were fitted closely by the Gaddum-Schild equation and pK_B values of 8.44 ± 0.05 (d.f. = 34) and 6.81 ± 0.03 (d.f. = 28) were estimated for DuP 753 and IMI, respectively. Judged by goodness-of-fit and the value of the pK_B's the system was apparently in equilibrium at all antagonist concentrations (Figure 2).

Therefore, these non-peptide ligands are behaving as simple competitive antagonists of AII at a homogeneous population of receptors in rabbit aorta.

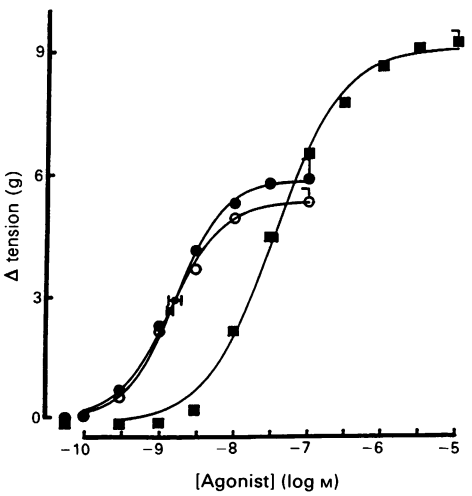


Figure 1 Angiotensin II (AII) E/[A] curves obtained by cumulative (●) and single (○) dosing. The noradrenaline (NA) E/[A] curve obtained by cumulative dosing (■) is shown for comparison (see text for details).

AII-receptor antagonism by peptide analogues of angiotensin II

Incubation times for each ligand were chosen by determining the time (in 30 min increments) required for the rightward shifting effect on the AII curve to saturate with the lowest concentrations (data not shown). All of the eight peptide analogues required longer periods of incubation than the non-peptides at the lowest concentrations tested. For most of them 60 min was sufficient but three needed 120 min (Table 1).

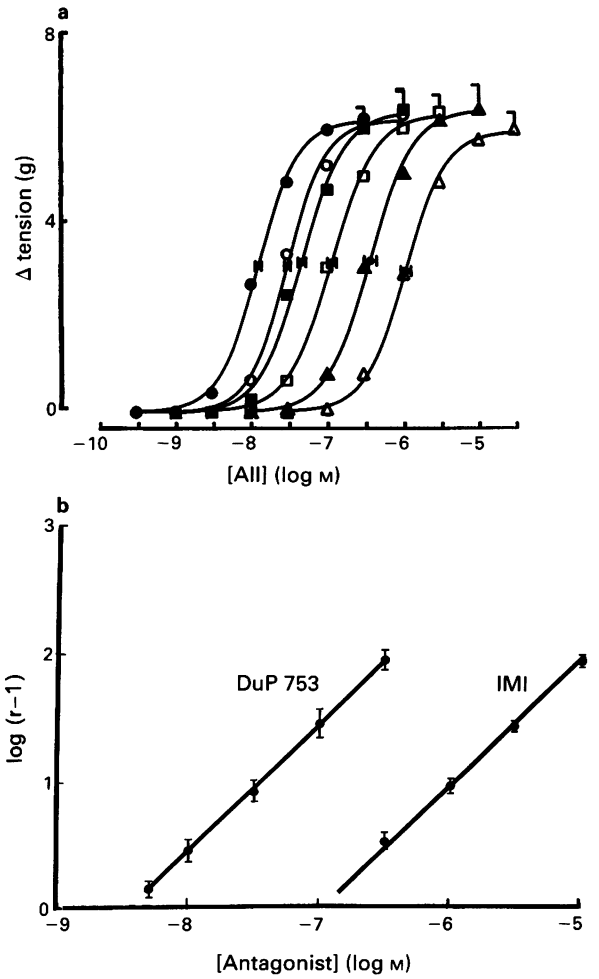


Figure 2 (a) Angiotensin II (AII) E/[A] curves obtained in the absence (●) and presence of 5 (○), 10 (■), 30 (□), 100 (▲), 300 (△) nM DuP 753. (b) Schild plots obtained from the interaction of AII with Dup 753 and IMI on rabbit aorta (see text for details).

Only two of the peptide analogues, [Sar^1,Tyr(Me)^4]AII and [Sar^1,Leu^8]AII, produced significant agonist responses (12 ± 4% and 3 ± 1%, respectively) which were totally blocked by DuP 753 at 0.3 μM. The partial agonist effect by [Sar^1,Leu^8]AII faded away during incubation (Figure 3d).

Simple competitive antagonism is manifested by parallel displacement of E/log[A] curves with no change in upper asymptote, so that only the log[A_{50}] parameter is changed. However, for this set of peptide analogues of AII although all of the data could be fitted by the logistic function, changes in all three of the parameters were found (Figure 3). In addition, in four cases the Schild plot slope parameters were significantly greater than unity (Table 1), another indication of the failure to adhere to the conditions of simple competitive antagonism. In spite of this and without prejudice to mechanism, we used the relationship between [B] and [A_{50}] in Schild plot space to estimate pA_2 values as a measure of the relative potencies of the ligands (Table 1).

For most of the compounds no significant change in the value of the slope index (p) of the AII E/log[A] curves could be detected by analysis of variance. However, with two compounds, [Sar^1,Ile^8]AII and [Sar^1,Leu^8]AII, a significant and apparently saturable, reduction in p occurred. These were also the compounds which produced the most depression of the AII E/log[A] curve maxima. Although, obviously, the relation between log [B] and log [A_{50}] did not saturate, it looked (Figure 3) as though the relationship between log [B] and the change in α, the depressant effect, did. The appearance of saturation was not due to inadequate incubation (i.e. equilibration) times because increased incubation did not result in either increased rightward shift or further depression of α (data not shown).

In order to define this observation more precisely, the interaction between one of the ligands which produced the most depression, [Sar^1,Leu^8]AII, and AII was re-examined using extra [Sar^1,Leu^8]AII concentrations (Figure 4). The data were fitted to the following logistic function.

$$\frac{\alpha - \alpha_B}{\alpha} = \frac{D_m \cdot [B]^{p'}}{[B_{50}]^{p'} + [B]^{p'}} \quad , \qquad (4)$$

where α and α_B are the upper asymptotes from the logistic curve-fitting (equation (1)) in the absence and presence of

Table 1 Analysis of angiotensin II (AII)-receptor ligands: rabbit aorta

Ligand	D_m[a]	pA_2[b]	$p[B_{50}]$[c]	*Model-fitting*[d] β	*Model-fitting*[d] pK_B
(incubation time)					
[Sar^1,Ala^8]AII (60 min)	37 ± 4	9.01 ± .06[e]	8.47 ± .02	0.43 ± .05	8.74 ± .06
[Sar^1,Val^5,Ala^8]AII (60 min)	34 ± 4	9.03 ± .06	8.34 ± .07	0.34 ± .05	8.69 ± .05
[Sar^1,Ile^8]AII (120 min)	82 ± 3	9.47 ± .10[e]	8.91 ± .14	0.68 ± .06	8.97 ± .05
[Sar^1,Leu^8]AII (120 min)	77 ± 2	9.04 ± .06[e]	8.86 ± .07	0.69 ± .05	8.93 ± .05
[Sar^1,Tyr(Me)^4]AII[f] (60 min)	21 ± 5	6.95 ± .09	6.94 ± .17	0.22 ± .05	6.75 ± .08
[Sar^1]AII(1–7)NH_2 (60 min)	42 ± 4	7.37 ± .08[e]	6.84 ± .12	0.49 ± .06	7.33 ± .06
[Sar^1,Thr^8]AII (60 min)	38 ± 4	8.80 ± .07	8.40 ± .08	0.35 ± .05	8.49 ± .05
[Sar^1,Phe(2,3,4,5,6-Br_5)^8]AII (120 min)	47 ± 2	8.41 ± .06	8.48 ± .02	0.41 ± .06	8.28 ± .06
DuP 753 (30 min)		8.44 ± .05			
IMI (30 min)		6.81 ± .03			

[a]The maximum percentage change from the mean control AII E/log[A] curve maximum (see text and equation (4) for details).
[b]Estimated by Schild analysis.
[c]-log concentration of ligand required to produce half maximum depression of α.
[d]Obtained by fitting of the mean experimental data to the model equations (10), (11), (15) and (16) (see text for details).
[e]Schild plot slopes greater than unity.
[f]Expressed significant partial agonism. Therefore, an estimate of τ was made ($\tau_B = 0.35 \pm .08$) (see text for details).

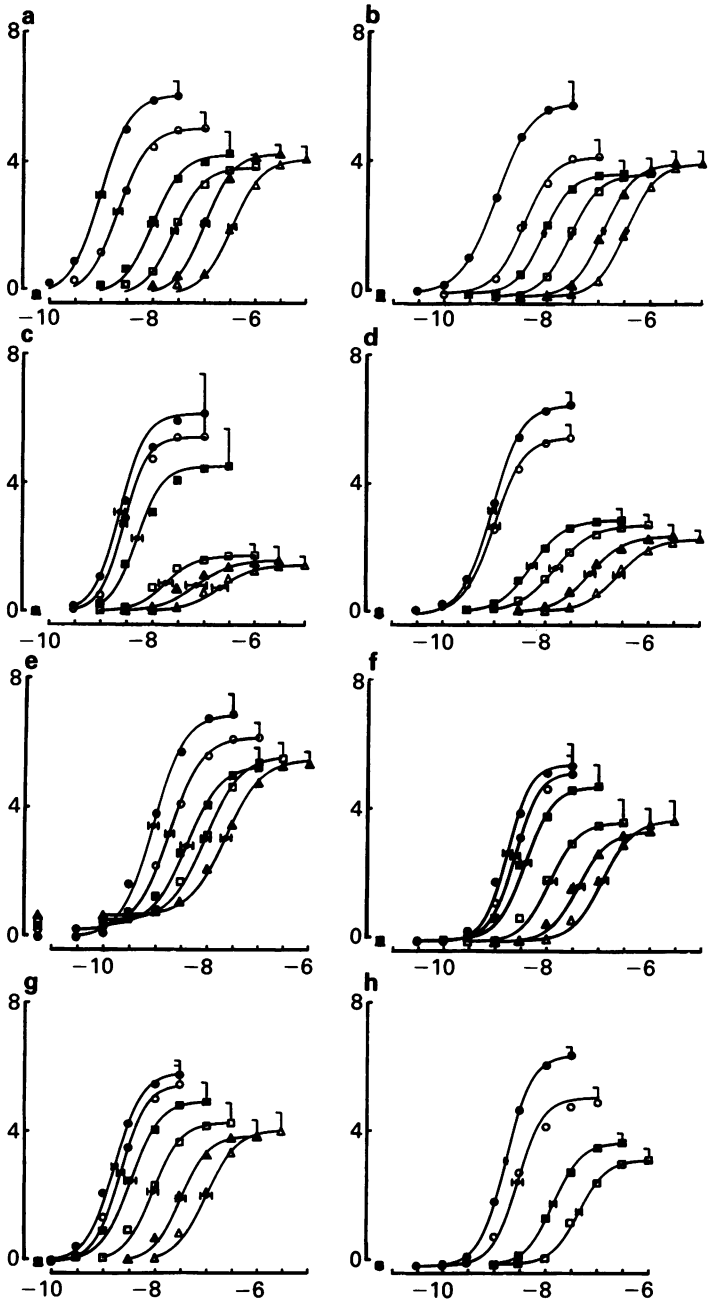


Figure 3 Angiotensin II (AII)-receptor antagonism by 8 peptide analogues of AII in rabbit aorta. AII E/[A] curves ($n = 6$ to 8) obtained in the absence (●) and presence (nM) of (a) [Sar^1,Ala^8]AII, 3 (○), 10 (■), 30 (□), 100 (▲), 300 (△); (b) [Sar^1,Val^5,Ala^8]AII, 3 (○), 10 (■), 30 (□), 100 (▲), 300 (△); (c) [Sar^1,Ile^8]AII, 0.3 (○), 1 (■), 3 (□), 10 (▲), 30 (△); (d) [Sar^1,Leu^8]AII, 0.3 (○), 1 (■), 3 (□), 10 (▲), 30 (△); (e) [Sar^1,Tyr(Me)^4]AII, 100 (○), 300 (■), 1000 (□), 3000 (▲); (f) [Sar^1]AII(1–7)NH$_2$, 30 (○), 100 (■), 300 (□), 1000 (▲), 3000 (△); (g) [Sar^1,Thr^8]AII, 1 (○), 3 (■), 10 (□), 30 (▲), 100 (△); (h) [Sar^1,Phe(2,3,4,5,6-Br$_5$)^8]AII, 3 (○), 30 (■), 100 (□). The curves drawn through the mean experimental data points were simulated using the parameter values obtained from the logistic curve-fitting procedure (see Methods). Error bars represent s.e.mean. For the incubation-times see Table 1. Abscissae: [AII]: log M. Ordinates: Δ tension (g).

[B]. D_m, $[B_{50}]$ and p′ are the fitting parameters for the maximum depression, midpoint location and midpoint slope index, respectively. From the logistic curve-fitting (Figure 4), estimates of D_m and $p[B_{50}]$ were obtained. Similarly, parameter estimates were obtained with the existing data (Figure 3) for the other peptide analogues (Table 1). The D_m values varied between 21 ± 5% and 82 ± 3%, but no correlation was found between this effect and the potency of the antagonists as defined by the pA_2 ($r = 0.62$, $t = 1.95$, d.f. = 6). However, a high correlation was found between the pA_2 and the potency of the depressant action as defined by the $p[B_{50}]$ ($r = 0.95$, $t = 7.45$, d.f. = 6) (see Table 1).

The effects of the peptide analogues on the AII E/log[A] curves were reversed following washout (washes at 15 min intervals for between 60 and 120 min, data not shown). Under all conditions, the α depression and the extent of rightward shift of the AII E/log[A] cuves were linked with respect to the concentration of antagonist.

Combined dose-ratio analysis of peptide analogues using DuP 753 as a reference antagonist

Cumulative AII E/log[A] curves were obtained in the absence and presence of [Sar^1,Ala^8]AII, 30 nM, (log dose-ratio, log

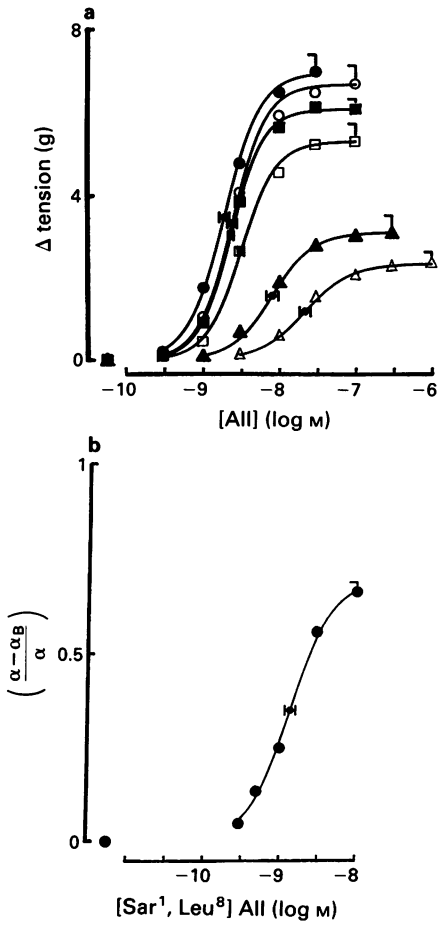


Figure 4 Analysis of the relationship between peptide ligand ([Sar¹,Leu⁸]AII) concentration and the depression of the angiotensin II (AII) E/[A] curve maxima. (α). (a) AII E/[A] curves ($n = 6$) obtained in the absence (●) and presence (nM) of [Sar¹,Leu⁸]AII, 0.3 (○), 0.5 (■), 1 (□), 3 (▲) and 10 (△). (b) The change in α has been expressed with reference to the control curve value α_B in the presence of the peptide ligand. The curve shown superimposed on the experimental data was obtained by logistic curve-fitting (see text for details).

$r_B = 1.43$) or DuP 753, 100 nM, (log $r_C = 1.46$) or both (log $r_{B+C} = 1.52$) (Figure 5). When these data were inserted in equations (2) and (3), the S_A value ($S_A = 0.02 \pm 0.10$, d.f. = 23) but not the S_M value ($S_M = -0.88 \pm 0.02$, d.f. = 23), was not significantly different from zero, indicating a syntopic interaction. Not only did DuP 753 act syntopically with [Sar¹,Ala⁸]AII in dose-ratio terms but also partially antagonized [Sar¹,Ala⁸]AII-induced depression of the AII maximum. Therefore the antagonism of AII by [Sar¹,Ala⁸]AII must be regarded, by this test, as not only syntopic but competitive as well. No significant differences were found by repeating the combined dose-ratio analysis using different orders of incubation. Similar results were obtained with [Sar¹,Ile⁸]AII (10 nM) and DuP 753 (100 nM). In addition, DuP 753 (100 nM) was found to antagonize totally the depression of the AII curve maxima by a lower concentration (1 nM) of [Sar¹,Ile⁸]AII.

Development and application of an explanatory model

To what extent could the features of the antagonism of AII by the peptide analogues be accounted for, qualitatively and quantitatively, within the framework of pharmacological concepts of ligand-receptor interactions? We have used the modelling process previously described by Black & Leff (1983). The AII E/[A] curves, in the absence and presence of all the

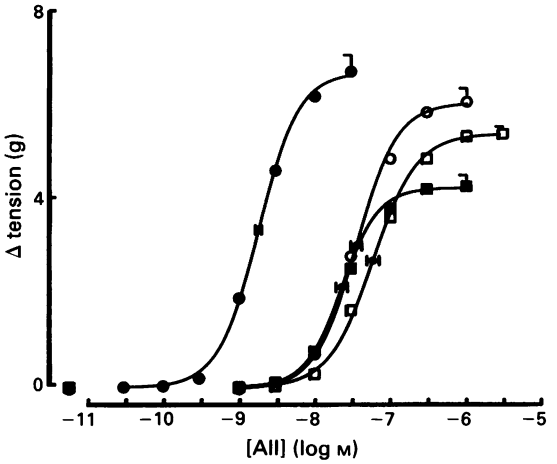


Figure 5 Combined dose-ratio analysis of angiotensin II (AII)-receptor antagonism in rabbit aorta. AII E/[A] curves obtained in the absence (●) and presence of DuP 753 (100 nM) (○), [Sar¹, Ala⁸]AII (30 nM) (■) and a combination of DuP 753 (100 nM) with [Sar¹,Ala⁸]AII (30 nM) (□).

antagonists could be adequately described by the logistic function (equation (1)). Therefore, the starting point was the form of the model of agonism (Black & Leff, 1983) which accounts for logistic E/[A] curves,

$$E = \frac{E_M[A]^{n\tau n}}{(K_A + [A])^n + [A]^{n\tau n}} \quad (5)$$

where E_M is the maximum effect achievable in the system; K_A is the equilibrium dissociation constant of an agonist A; τ is the transducer ratio which provides a measure of the efficiency of A in the system; n is the midpoint slope parameter of the transducer function. The problem now reduced to determining which of the four parameters needed to be changed to account for the observed profiles of AII antagonism.

Because all the effects of the ligands were concluded to be a consequence of reversible and syntopic receptor binding which had attained equilibrium, this component of the antagonism could be expressed in the model by multiplying K_A by the factor $(1 + [B]/K_B)$ in the usual way,

$$E = \frac{E_M[A]^{n\tau n}}{(K_A(1 + [B]/K_B) + [A])^n + [A]^{n\tau n}} \quad (6)$$

where [B] is the concentration of a peptide analogue and K_B, its equilibrium dissociation constant. This competition at the receptor level can only account for parallel rightward shift of E/log[A] curves with no change in the upper asymptote or slope index.

The experimental parameters α, p and $[A_{50}]$, obtained by logistic curve-fitting, are related to the model parameters as follows:

$$\alpha = \frac{E_M.\tau^n}{1 + \tau^n} \quad (7)$$

$$p = \frac{n(2 + \tau^n)\ ((2 + \tau^n)^{1/n} - 1)}{(2 + \tau^n)^{1/n}(1 + \tau^n)} \quad (8)$$

$$[A_{50}] = \frac{K_A}{(2 + \tau^n)^{1/n} - 1} \quad (9)$$

Changes in all three of these logistic parameters were detected (see Table 1). Therefore, because K_A and E_M only appear in two of the three equations above, an effect of the antagonist on either of these model parameters alone was not sufficient to account for the data. Only the model parameters

τ and n, because they appear in all three equations, could theoretically and singularly account for the data. In practice, only changes in τ could account for the data; changes in n can be ruled out because the midpoint slope index of the E/log[A] curves, p, is more sensitive than α to changes in n (equations (7) and (8)) yet large changes in α were detected for only small changes of p in the analysis.

The ligands appeared to be equipotent in terms of both reduction of α and the rightward shift, that is $p[B_{50}] \simeq pA_2$ (Table 1). Therefore, we assumed that these two events are both related to receptor occupancy. Further, because the reduction in α was saturable, whatever the mechanism, a simple relationship between the receptor occupancy and a change in τ could be expressed as,

$$\tau' = \tau \left(1 - \frac{\beta[B]}{K_B + [B]}\right) \quad (10)$$

where, τ' is τ in the presence of a peptide analogue, β is a proportionality constant included to define the maximum reduction in τ that each ligand can produce.

The model may then be represented as follows, where τ' is defined by equation (10),

$$E = \frac{E_M \tau'^n [A]^n}{(K_A(1 + [B]/K_B) + [A])^n + [A]^n \tau'^n} \quad (11)$$

In this new model, the upper asymptote of the E/log[A] curve, when $[A] \rightarrow \infty$ is given by,

$$\alpha = \frac{E_M \tau'^n}{1 + \tau'^n} \quad (12)$$

and the midpoint location parameter, [A] for 0.5α is given by,

$$[A_{50}] = \frac{K_A(1 + [B]/K_B)}{(2 + \tau'^n)^{1/n} - 1} \quad (13)$$

where τ' remains as defined in equation (10).

The model expectation for changes in τ could then be deduced. The effects of a reduction in the value of τ on the expression of competitive antagonism are dependent on the initial value of τ, that is the efficacy of the agonist; the system behaves as though irreversible receptor antagonism occurs with concurrent, competitive antagonism (Black *et al.*, 1985b). For high efficacy agonists and when the maximum τ reduction is not sufficient to reduce significantly the value of α, the midpoint location of the E/log[A] curve approximates to,

$$[A_{50}] = \frac{K_A(1 + [B]/K_B)}{\tau'} \quad (14)$$

Under these conditions, dose-ratios will be greater than expected. For low efficacy systems, as the value of τ is reduced so α will be reduced according to equations (10) and (12), but the effect on the $[A_{50}]$ is diminished as the value of the control $[A_{50}]$ approaches asymptotically $K_A(2^{1/n} - 1)$ (see Black *et al.*, 1985a). The behaviour of the model with a high and low efficacy agonist is illustrated in Figure 6.

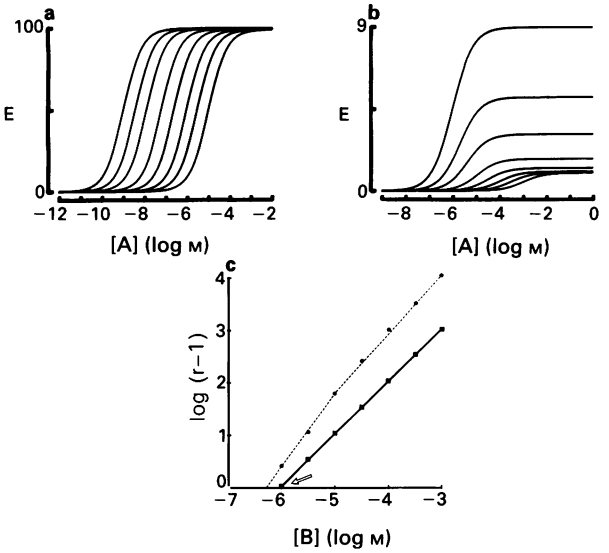


Figure 6 Simulation showing the effect of concurrent affinity and efficacy reduction in the operational model (equations (10) and (11)) for both (a) high and (b) low efficacy values. Parameters were set as follows: $E_M = 100$, $pK_A = 6$, $\beta = 0.9$, $n = 1$. (a) $\tau = 1000$ (b) $\tau = 0.1$. (c) Schild plots corresponding to this simulated data shown in panels (a, ●....●) and (b, ■—■).

Model fitting: rabbit aorta

One of the peptide analogues, [Sar^1,Tyr(Me)^4]AII, expressed significant agonist activity and, for completeness, this was accounted for in the model-fitting programme by adding the following system of equations to equations (10) and (11),

$$E_B = \frac{E_M \tau_B^n [B]^n}{(K_B(1 + [A]/K_A) + [B])^n + [B]^n \tau_B^n} \quad (15)$$

$$E_T = E + E_B \quad (16)$$

where E_T is the total effect produced by [Sar^1,Tyr(Me)^4]AII (still given as B) and AII (A). These equations, with equation (11), simultaneously describe the competitive interaction between one agonist (AII) and another with significantly lower efficacy ([Sar^1,Tyr(Me)^4]AII) such that the expression of efficacy by the latter compound does not significantly perturb the expression of its competitive antagonism (Jenkinson, 1978; Kenakin, 1987). The addition of these equations adds one extra estimable parameter, τ_B, the efficacy of [Sar^1,Tyr(Me)^4]AII.

The model assumes a reduction in τ. Therefore, the same principles apply for model-fitting of these data as they do when agonist K_A value estimates are made using irreversible antagonist pretreatment (Black *et al.*, 1985b). The model fit was set up to provide single values of the AII-dependent parameters, K_A and τ, single values of the tissue-dependent parameters, E_M and n, and individual values of the peptide analogue-dependent parameters, β and K_B. In this way, β was the only peptide analogue-dependent parameter allowed to vary between compounds to account for the reduction in τ and hence the depression of α, the changes in E/log[A] slope index and the steep Schild plots. In practice, log values of the equilibrium dissociation constants (K_B and K_A) were estimated as these are assumed to be normally-distributed.

Ideally, we would have liked to fit all the data simultaneously to the model. However, regardless of other problems, with such a large data set (circa 6000 E/[A] values) and large number of parameters to be estimated (26), we had insufficient computer capacity to perform the task. However, we were able to fit the data obtained with each peptide separately (Figure 7). In addition, two tests of the model were made by allocating the eight peptides into two groups based on the behaviour of the ligands. In particular, the data were divided to investigate the effects of different degrees of depression and the expression of efficacy by [Sar^1,Tyr(Me)^4]AII on the quality of the fit. The mean experimental data from each of these groups were fitted to the model (equations (10), (11), (15) and (16)). It was found that consistent estimates of the ligand and tissue dependent-parameters (K_A, τ, E_M and n) could be obtained regardless of the grouping, indicating the robustness of the model. The estimates of the peptide-analogue-dependent parameters (β and K_B) and their variance obtained from fitting the mean data in two groups (a, c, g, h and a, d, e, f referring to Figure 3 legend) are presented in Table 1.

The values of τ (1.42 ± 0.20), n (2.20 ± 0.30) and E_m (9.3 ± 1.0 g) were consistent with AII behaving as a partial agonist at the AII-receptor ($\alpha = 68\%$ E_m) in rabbit aorta and a pK_A value of 8.94 ± 0.10 was obtained. The effects of the small changes observed in τ are amplified in the system

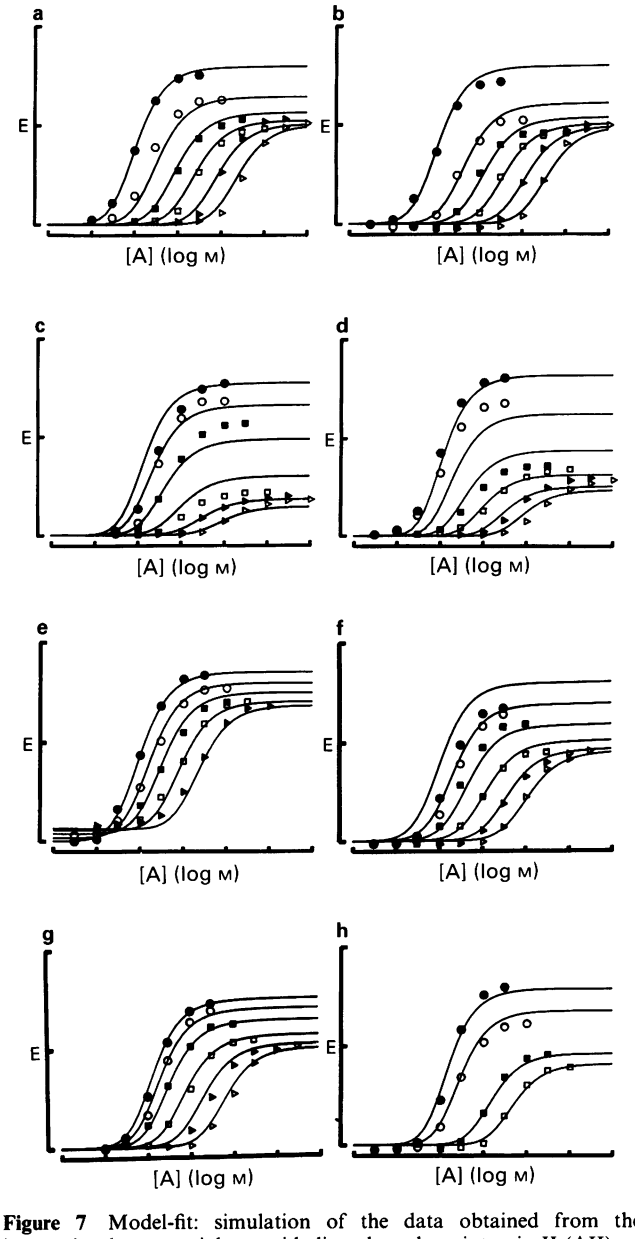


Figure 7 Model-fit: simulation of the data obtained from the interaction between eight peptide ligands and angiotensin II (AII) on rabbit aorta. The curves shown superimposed on the mean experimental data points were obtained using the parameter estimates, presented in Table 1 and the text, inserted into equations (10), (11), (15) and (16). (See Figure 3 legend for peptide analogue identification and concentrations).

because AII is a partial agonist and, in particular, because of the steep transducer function slope, n, in the model (see Black *et al.*, 1985a). The low value of τ accounts for the results that the model-fit derived pK_B values were close to the pA_2 values estimated by the Schild method (also see Figure 6).

Model fitting: cross-tissue analysis

Having established that the model could account for all the data obtained on the rabbit aorta assay, a useful test was established by attempting to fit the data obtained with one of the most frequently-quoted peptide analogues ([Sar^1,Ala^8]AII) on the guinea-pig aorta, ileum longitudinal and stomach smooth muscle assays. The location of the AII control E/log[A] curve varied significantly across these assays and although, qualitatively, a similar complex profile of antagonism was obtained, the degree of depression of α also varied across the assays. The non-peptide antagonist, DuP 753, behaved as simple competitive antagonist in all the assays with no significant difference between the pK_B values estimated, and acted syntopically with [Sar^1,Ala^8]AII as judged by combined dose-ratio analysis (data not shown).

For the model to have utility it would be expected that satisfactory fits could be obtained by only allowing the tissue-dependent parameters E_M, τ and n to vary between assays. Therefore in the model-fits the ligand-dependent parameters, pK_B, β and pK_A, were constrained to those values obtained on the rabbit aorta assay.

The model could apparently account for the data as judged by inspection of Figure 8. However, the estimates of the model parameters E_M and τ in each tissue were associated with extremely high variance (Figure 8 legend). This follows from the nature of the data set. The control curve log[A_{50}] values on the three guinea-pig assays are greater than the $\log K_A$ estimated in rabbit aorta. In the operational model of agonism this can only occur when n is significantly greater than unity and for relatively low τ values (Black *et al.*, 1985a). Reliable estimations of E_M and τ requires knowledge of how, experimentally, [A_{50}] changes with α to define the underlying transducer function. When the competitive element, $(1 + [B]/K_B)$, due to the peptide analogues is removed then in the three guinea-pig assays the [A_{50}] values are indistinguishable from the control [A_{50}] values. In terms of the model, this indicates that AII is operating through the pseudolinear region of the transducer function. Under this condition, therefore, there is minimal information available to define the transducer function asymptote, E_M and the absolute value of τ. However, the uncertainties in the parameter estimates do not invalidate the model test which is that satisfactory fitting can occur with fixed values of β, pK_A and pK_B.

Discussion

Previously, several explanations have been offered for the complex profile of antagonism observed with AII-derived peptide antagonists. Only non-equilibrium conditions and/or allotopic actions, have been proposed. Mechanistically, Koziarz & Moore (1989) suggested that [Sar^1,Ile^8]AII binds to a secondary site quite distinct from the primary AII-receptor binding site. Similarly, Timmermans *et al.* (1991) invoked an allosteric model to account for the depression. This latter model, first used to explain the pharmacological behaviour of 'non-competitive' 5-HT-S_2-receptor-ligand interactions (de Chaffoy de Courcelles *et al.*, 1986), was derived by analogy to enzymological models of allosteric interactions.

In contrast to the above, the current analysis suggests that the behaviour of all the peptide analogues could be accounted for by a reversible and syntopic action at the AII-receptor which results in a decrease in both the apparent affinity and efficacy of AII. The model is useful in allowing us to estimate pK_B values from otherwise uninterpretable data and also to account for cross-tissue differences. However, this model begs the question of how it can be that an antagonist could combine these properties by a single site action.

Speculation about the molecular basis for these effects is possible from the nature of the efficacy parameters and the known biochemical properties of AII-receptor interactions. In the operational model of agonism (Black & Leff, 1983), the efficacy parameter, τ, is given by the ratio of the concentration of receptors ([R_0]) and the midpoint location parameter of the transducer function (K_E). Therefore, changes in either of these parameters could account for the data. However, because the interaction with DuP 753 pointed to the occupancy-determined nature of the reduction of efficacy, we prefer to ascribe the effects to changes in [R_0]. Furthermore,

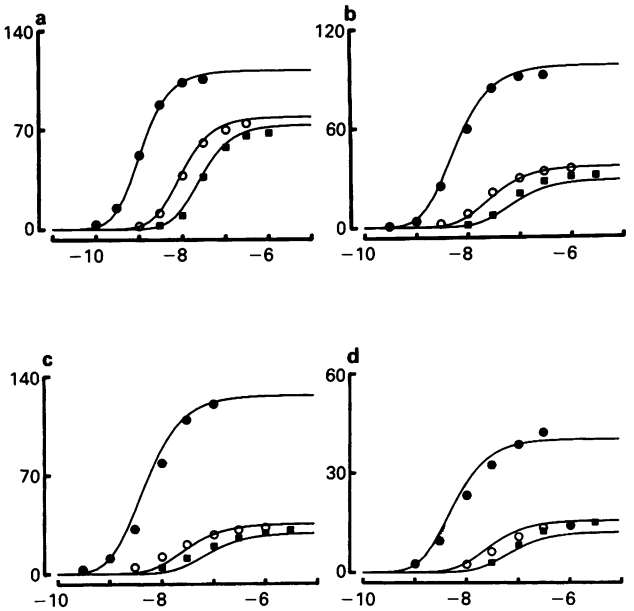


Figure 8 The interaction between [Sar^1,Ala^8]AII (10 and 30 nM) and angiotensin II (AII) on the rabbit aorta (a) and guinea-pig aorta (b), ileum (c) and stomach (d). The curves shown superimposed on the mean experimental data points, with (●) 0, (○) 10 and (■) 30 nM [Sar^1,Ala^8]AII, were obtained by model-fitting of the data to the system of equations (10) and (11). Abscissae: [AII]: log M. Ordinates: % of sighter (response to AII 30 nM (a), 300 nM (b, c, d) before the experiments). In the fits, the parameters pK_B (8.74), pK_A (8.94) and β (0.43) were fixed. The other parameters (± s.e.) were estimated as follows:

Assay	τ	n	E_M
Rabbit aorta	1.83 ± 0.24	2.22 ± 0.29	7.76 ± 1.02
Guinea-pig ileum	0.31 ± 1.65	2.88 ± 0.60	3480 ± 137000
Guinea-pig stomach	0.34 ± 1.58	2.41 ± 0.94	521 ± 4830
Guinea-pig aorta	0.31 ± 1.12	2.49−0.67	2270 ± 10800

biochemically, AII-receptors have been reported to be internalised after occupation (Crozat *et al.*, 1986; Bianchi *et al.*, 1986; Griendling *et al.*, 1987; Delafontaine *et al.*, 1987). Internalisation has the modelling effect of producing an irreversible reduction in the value of $[R_0]$. Physiologically, this is imagined to be offset by the expression of unoccupied receptors into the cell membrane. The regulation of, and the relationship between, the processes of receptor internalisation and expression is unknown to us. Nevertheless, without that knowledge, we may imagine that a ligand-generated imbalance between the rates of internalisation and expression would produce a reduction in $[R_0]$.

If our assumption is correct that the interaction between AII and DuP 753 was simply competitive, internalisation of AII-receptors, following receptor occupation by AII cannot, alone, account for the complex interaction with the peptide analogues. The complexity has to be related to an intrinsic property of the peptide analogues. Any explanation has to account for the saturability of the depression and its reversal by both DuP 753 and 'washout' but not by AII itself. An answer to all of these is suggested by assuming that the peptide analogues, although lacking contractile efficacy, can also internalise the AII-receptor (Ullian & Linas, 1990). The saturability and the reversing effects of both DuP 753 and 'washout' could be explained by the receptor expression assumed to be occurring concomitantly. The failure of AII to surmount the antagonism by the peptide analogues would be due to the replacement of peptide analogue receptor occupancy by AII-receptor occupancy. Unlike DuP 753, AII can effectively maintain the receptor deficit by internalisation.

The feature of the results is the ligand-dependent nature of the complexity. The extent of depression was independent of the level of receptor occupancy and therefore it is as though rates of internalisation can be influenced by an 'efficacy-like' property of the ligands themselves. This property appears quite independent of the efficacy for the contractile process. The greater the capacity of the internalisation process, the longer it would be expected to take for the system to come into equilibrium. The compounds that produced the greatest depression required the longest periods of incubation (see Table 1).

Of particular interest was the model-derived finding that AII behaves as a partial agonist in all four of the assays investigated. This interpretation is reinforced by the fact that the pK_A value estimated for AII was within the same nM range as the pK_B values estimated for the structurally-related antagonists. It may not be a coincidence that in the rabbit aorta assay the intrinsic activity (α) of AII was the same, approximately 60%, with respect to both the model-fit-derived system maximum effect (E_M) and the maximum response obtained with noradrenaline.

Although there is aesthetic satisfaction in producing a model which provides a simplifying and unifying explanation for the complex behaviour of the peptide ligands, it does not follow that the new explanatory model is more likely to be correct. However, because the model can be specified and, therefore, expressed formally, it does allow it to be objectively tested and rejected or refined, as appropriate. In this case, one test of the model was carried out by applying it to the results of the cross-tissue analysis of the interaction between AII and [Sar^1,Ala^8]AII on the guinea-pig aorta, ileum and stomach assays. The outcome was consistent with the presence of a homogeneous AII-receptor population within and between these tissues.

The derived model and the molecular mechanism may be general for peptide receptor systems. It also remains to be seen whether the complexity reported and the receptor internalisation inferred will be restricted to peptide ligands.

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Vasoconstriction of guinea-pig submucosal arterioles following sympathetic nerve stimulation is mediated by the release of ATP

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1 The nature of the transmitter mediating vasoconstriction of guinea-pig submucosal arterioles following sympathetic nerve stimulation was studied.

2 Prazosin (0.1 μM) abolished the response to exogenously applied phenylephrine (1 μM) but had no effect on constrictions of submucosal arterioles evoked by nerve stimulation (100 pulses at 10 Hz).

3 Vasoconstrictions and excitatory junction potentials elicited by nerve stimulation were potentiated by idazoxan (0.1 μM).

4 Following reserpine treatment, catecholamine fluorescence was absent in submucosal arterioles but nerve-evoked vasoconstrictions were unaltered.

5 Vasoconstrictions and excitatory junction potentials recorded in response to sympathetic nerve stimulation, as well as constrictions evoked by exogenously applied ATP (3 μM), were abolished by the P_2-purinoceptor antagonist, suramin (100 μM). Suramin had no effect on the vasoconstriction in response to noradrenaline (3 μM), or the nicotinic excitatory postsynaptic potentials (e.p.s.ps) and noradrenergic inhibitory postsynaptic potentials (i.p.s.ps) recorded from submucosal neurones.

6 We conclude that postjunctional responses of submucosal arterioles following sympathetic nerve stimulation are mediated solely through the activation of P_{2X}-purinoceptors by ATP or a related purine nucelotide. The function of neurally released noradrenaline is to act through prejunctional α_2-adrenoceptors to depress transmitter release.

Keywords: P_{2X}-purinoceptors; enteric nervous system; suramin; α, β-methylene ATP; NANC transmission

Introduction

Although noradrenaline (NA) is present in high concentrations in sympathetic nerve terminals, NA does not mediate all the postjunctional responses elicited by sympathetic nerve stimulation (Ambache & Zar, 1971; Burnstock, 1986). Studies on guinea-pig submucosal arterioles have shown that both the underlying changes in membrane potential (excitatory junction potentials, e.j.ps) and vasoconstrictions following short trains of low or high frequency stimulation of the sympathetic nerves are unaffected by α_1-adrenoceptor antagonists (Hirst & Neild, 1980; 1981; Shen *et al.*, 1990). Paradoxically these vessels constrict when low concentrations of NA are applied exogenously, and NA released from the sympathetic nerves following electrical stimulation acts through prejunctional α_2-adrenoceptors to depress transmitter release. Hirst & Neild (1980; 1981) suggested that the α-adrenoceptor resistant responses in this vessel were due to the action of NA through a novel γ-adrenoceptor. In a number of other arteries α-adrenoceptor antagonist resistant contractions following sympathetic nerve stimulation have been recorded (Muramatsu *et al.*, 1989; Sjoblom-Widfeldt *et al.*, 1990; Brizzolara & Burnstock, 1990). These are thought to be due to ATP, which is co-released with NA, acting through P_{2X}-purinoceptors because they are abolished following desensitization of P_{2X}-purinoceptors with the stable adenosine 5′-triphosphate (ATP) analogue α,β-methylene ATP (Ramme *et al.*, 1987; Burnstock & Warland, 1987; Hirst & Jobling, 1989; Evans & Cunnane, 1992). Recently the trypanocide, suramin, has been demonstrated to be an antagonist at the P_2-purinoceptor in the rodent vas deferens (Dunn & Blakeley, 1988; von Kugelgen & Starke, 1989) and the guinea-pig taenia caeci (Den Hertog *et al.*, 1989; Hoyle *et al.*, 1990).

The aims of the present study were three fold: (1) to determine if an α_1-adrenoceptor antagonist sensitive contraction can be recorded in this vessel in response to nerve stimulation using 100 pulses at 10 Hz, parameters that to our knowledge have revealed an α_1-adrenoceptor-mediated contraction in all arteries studied; (2) to establish if the prazosin-resistant contraction is due to the activation of γ-adrenoceptors by NA or the activation of P_{2X}-purinoceptors by ATP; (3) to test the specificity of suramin for antagonizing P_2-purinoceptors.

Methods

Submucosal plexus preparations were obtained from the small intestine of guinea-pigs (150–250 g); methods of tissue preparation and intracellular recording from submucosal neurones were as detailed previously (Surprenant, 1984). Tissues were superfused at 3–4 ml min^{-1} with a physiological solution of the following composition (mM): NaCl 126, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5, KCl 5, $NaHCO_3$ 25, glucose 11; gassed with 95% O_2 and 5% CO_2. Temperature was maintained at 34–36°C.

The perivascular nerves on the surface of the arterioles were stimulated electrically through blunt glass microelectrodes (tip diameter 20–50 μm) filled with physiological saline and placed lightly on the surface of the arteriole about 1 mm away from the recording site. In contractility experiments, magnesium was omitted from the solution because constrictions in response to nerve stimulation or exogenously applied agonists were greater; similar observations have been reported in a variety of other blood vessels (Ramme *et al.*, 1987; Evans & Cunnane, 1991). Test agents were added to

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the superfusion medium to give the required final concentration.

Nerve stimulation (100 pulses at 10 Hz, 0.2 ms pulse width at 40 V from a Grass S88 stimulator) was delivered at 5 min intervals with 5–8 sets of these stimuli being given before any drug application; the amplitudes of successive control responses varied by less than ± 5%. Vasoconstrictor substances were applied for a duration of 2–5 min every 20 or 30 min with 2–4 such applications being given before any drug application. Data are expressed as a percentage of the response before drug application; all values are mean ± s.e.mean. Tests of significance were by Student's t test; $P<0.05$ was considered significant, n = number of animals except where stated otherwise.

Vessel diameter recordings

Changes in the outside diameter of the arterioles following nerve stimulation or the application of agonists were monitored by the Diamtrak system (Neild, 1989). This system can track the diameter of small vessels by on-line computer analysis of TV images with an Imaging Technology PCVision-Plus frame grabber board (Imaging Technology Inc., Woburn, Mass., U.S.A.) in an IBM PC/AT computer. The result was converted to an analog signal which was displayed on a conventional chart recorder. The data were sampled at 10–20 Hz and the resolution of the system was less than 1 μm.

Electrophysiological recordings

Changes in membrane potential of submucosal neurones and from the smooth muscle cells of submucosal arterioles were recorded with glass microelectrodes, filled with 1 M KCl with tip resistances of 130–180 MΩ. Signals were recorded with an Axoclamp 2A amplifier and displayed on a Gould 2400S chart recorder. Changes in membrane potential of arterioles were measured following nerve stimulation with short trains of stimuli (10–15 pulses) at 1 or 2 Hz from the stimulating electrode used for contraction studies. The same stimulating electrode was also used to evoke nicotinic excitatory postsynaptic potentials (e.p.s.ps; 0.1 Hz, pulse width 0.1 ms, 5–10 V) and noradrenergic inhibitory postsynaptic potentials (i.p.s.ps; 6 pulses 20 Hz, pulse width, 0.2 ms, 50 V) from the submucosal neurones following the stimulation of adjacent ganglia.

Reserpine pretreatment

Animals were pretreated with reserpine (5 mg kg^{-1}) i.p. 18 h before the experiment. The extent of the depletion of tissue NA was estimated histologically by the glyoxylic acid staining technique for catecholamines (Lindvall & Bjorklund, 1974; Furness & Costa, 1975).

Drugs

Adenosine 5′-triphosphate, α,β-methylene ATP, (+)-amphetamine, (−)-noradrenaline bitartrate, (−)-phenylephrine hydrochloride, reserpine, and tetrodotoxin were obtained from Sigma. P^1,P^5-di-(adenosine-5′)pentaphosphate (Boehringer), guanethidine sulphate (Ciba Geigy), idazoxan (Reckitt & Coleman), prazosin hydrochloride (Research Biochemicals) and suramin hexasodium salt (Bayer) were obtained from the sources given in parentheses.

Results

Contractility studies

Electrical stimulation of guinea-pig submucosal arterioles with trains of 100 pulses at 10 Hz every 5 min evoked reproducible vasoconstrictions measured as changes in the outside diameter of the vessel. The diameter of the arteriole quickly returned to its control value on cessation of stimulation. In the majority of experiments the vasoconstriction could be resolved into a rapidly rising transient peak within the first 30–40 s followed by a smaller amplitude maintained constriction (Figure 1). Electrically evoked responses were abolished by tetrodotoxin (0.3 μM, $n = 8$).

Effects of α-adrenoceptor antagonists The α_1-adrenoceptor antagonist, prazosin (0.1 μM), had no effect on either the resting tone of the vessel, or the nerve-evoked vasoconstriction measured either at the initial peak amplitude or during the maintained plateau phase (Figure 1). Contractions elicited by the selective α_1-adrenoceptor agonist, phenylephrine (1 μM), of similar amplitude to the nerve-evoked constriction were abolished by 0.1 μM prazosin ($n = 5$) (see also Vanner *et al.*, 1990).

The α_2-adrenoceptor antagonist, idazoxan (0.1 μM), significantly increased both the initial peak amplitude of the neurally evoked vasoconstriction as well as the maintained plateau phase (Figure 2). The potentiation of the nerve-evoked constriction by idazoxan was unaffected by prazosin (0.1 μM) (peak height, idazoxan 165 ± 22%, idazoxan + prazosin 184 ± 36%; maintained vasoconstriction, idazoxan 242 ± 21%, idazoxan + prazosin 204 ± 35%, $n = 4$).

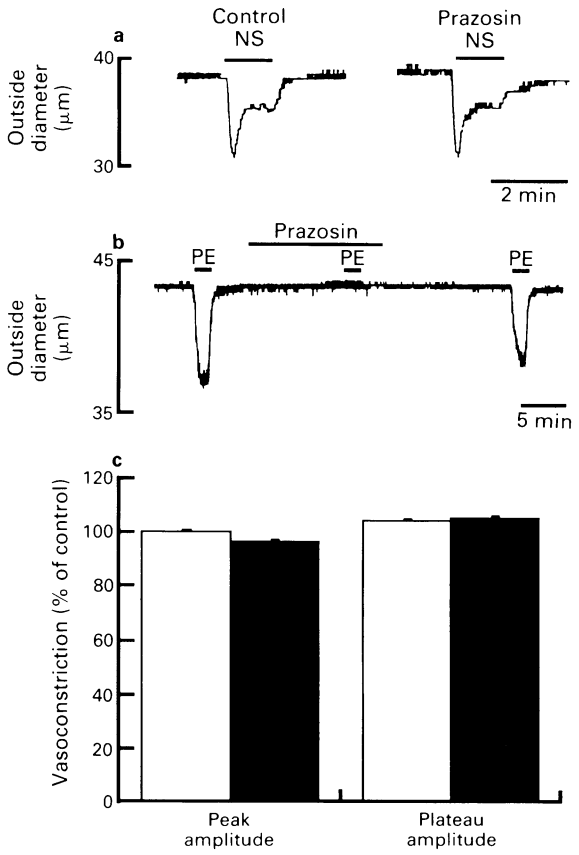


Figure 1 Constrictions of submucosal arterioles following nerve stimulation are not mediated by the activation of α_1-adrenoceptors by noradrenaline. (a) Nerve evoked constrictions (100 pulses at 10 Hz) were unaffected by the α_1-adrenoceptor antagonist, prazosin (0.1 μM). (b) Constrictions elicited by the exogenous application of the α_1-adrenoceptor agonist, phenylephrine (PE, 1 μM) were abolished by prazosin (0.1 μM), an effect reversed on washout. (c) Summary of the effects of prazosin (0.1 μM) on the initial peak and plateau amplitude of nerve-evoked constrictions. Data are plotted as mean (± s.e.mean shown by vertical bars) % of the nerve-evoked constriction before prazosin application ($n = 5$; control, open columns, prazosin, solid columns). Periods of nerve stimulation (NS) and phenylephrine applications are indicated by bars.

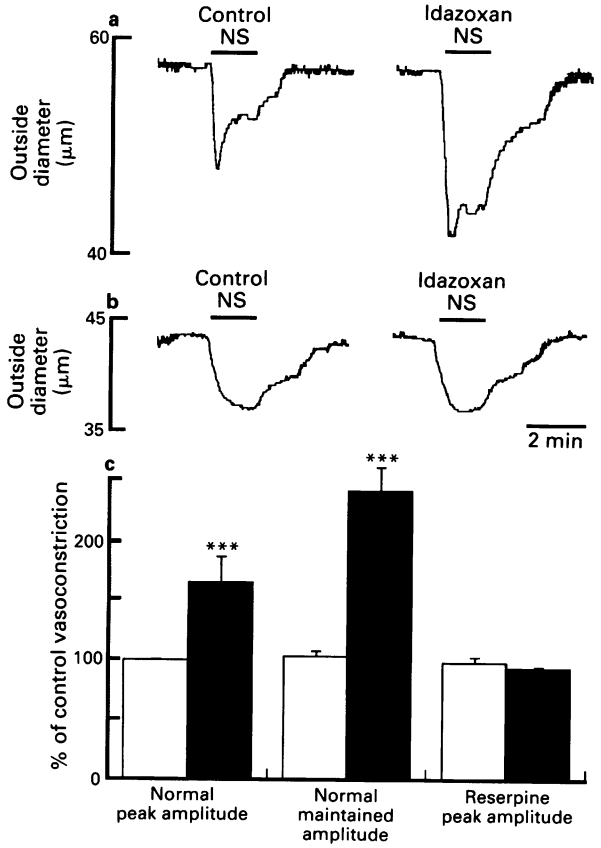


Figure 2 Noradrenaline released following nerve stimulation acts through pre-junctional α_2-adrenoceptors. (a) Constrictions of submucosal arterioles were potentiated in the presence of the α_2-adrenoceptor antagonist, idazoxan (0.1 μM). (b) Idazoxan (0.1 μM) had no effect on the amplitude of nerve-evoked constrictions after reserpine treatment. (c) Summary of the effects of idazoxan (0.1 μM) on nerve-evoked contractions from normal and reserpinized guinea-pigs. Results from normal animals ($n = 5$) were quantified both as initial peak amplitude and the amplitude of the maintained response. Peak amplitude only was measured in arterioles from reserpinized animals because these preparations did not show two distinct phases of constriction ($n = 5$). Data are plotted as mean (± s.e.mean, vertical bars) of the % of the nerve evoked constriction before idazoxan application (control, open columns; idazaxon, solid columns). Periods of nerve stimulation (NS) are marked by bars. $^{***}P < 0.001$.

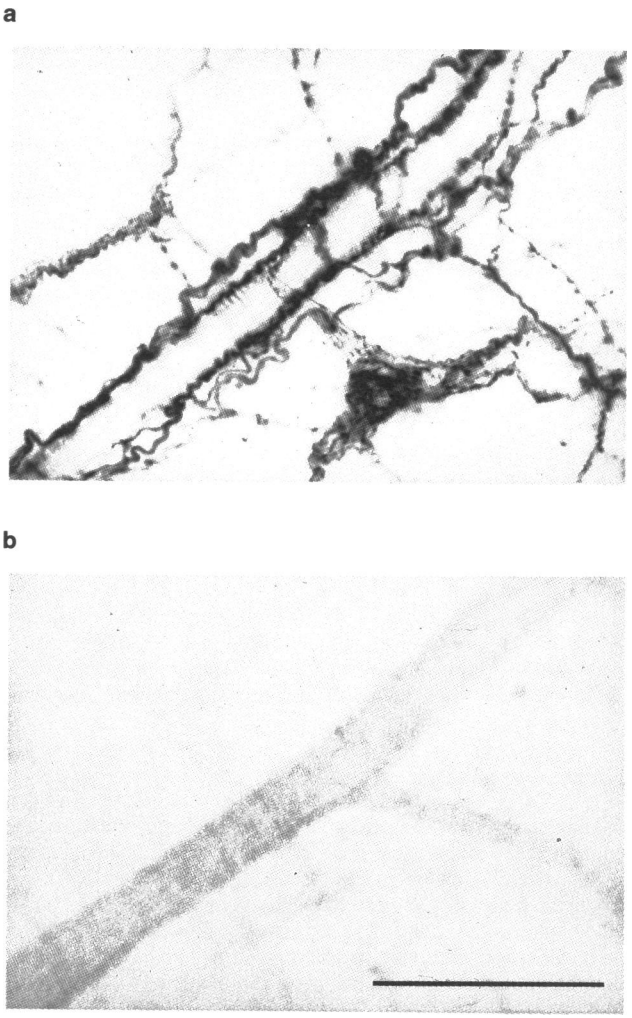


Figure 3 Reserpine pretreatment abolishes catecholamine fluorescence in guinea-pig submucosal plexus following glyoxylic acid staining. (a) In control preparations dense catecholamine containing nerve fibres are associated with submucosal arterioles as well as adjacent ganglia. (b) No fluorescence was observed after reserpine treatment. Calibration bar = 100 μm.

Reserpinization Dense catecholamine fluorescence was present in all control preparations of the submucosal plexus (Figure 3a) (see also Furness & Costa, 1987). No catecholamine fluorescence was observed following glyoxylic acid staining of submucosal plexus preparations from reserpine pretreated guinea-pigs (Figure 3b) indicating an apparent absence of tissue noradrenaline. However, nerve evoked constrictions of similar magnitude to control were still recorded following reserpine treatment. Control vasoconstrictions were 21.2 ± 2.4 μm and vasoconstrictions in reserpinized animals were 28 ± 3.2 μm ($n = 8$ arterioles from 4 animals). In contrast to control tissues, these responses were not potentiated by idazoxan (0.1 μM, $n = 6$ arterioles from 4 animals, Figure 2b).

Effects of the adrenergic neurone blocker guanethidine Guanethidine (3 μM) abolished vasoconstrictions evoked by nerve stimulation, an effect reversed by the concomitant application of amphetamine (3 μM, $n = 4$, Figure 4). A similar reversal of the effects of guanethidine by amphetamine has been shown in the guinea-pig vas deferens (Brock & Cunnane, 1988) and rabbit jejunal artery (Evans, 1990). The mechanism of action of amphetamine was not examined in this study; however, Brock & Cunnane (1988) suggest that amphetamine probably acts by displacing guanethidine from sympathetic nerve terminals. The abolition of the response to electrical stimulation with the adrenergic neurone blocker, guanethidine, further demonstrates that the nerve-evoked, α-adrenoceptor antagonist-resistant vasoconstriction was generated by the release of transmitter from sympathetic nerves.

Determination of the α-adrenoceptor antagonist resistant component of constriction In a number of arteries where α-adrenoceptor antagonist resistant contractions have been reported the residual contraction appears to be due to the activation of P_{2X}-purinoceptors by ATP (for review, see von Kugelgen & Starke, 1991). Application of exogenous ATP (3 μM) to the submucosal arterioles produced transient vasoconstrictions which reached peak amplitude in the first 30 s of application and returned towards the control diameter on maintained application. Reproducible responses to exogenously applied ATP were obtained if the interval between successive applications was greater than 30 min. The stable ATP analogues α,β-methylene ATP (mATP) (0.1–3 μM) and P^1,P^5-di-(adenosine-5')pentaphosphate (DADPP) (0.1–3 μM) produced transient constrictions, which peaked in the first 2 min of application. The vasoconstriction did not return to

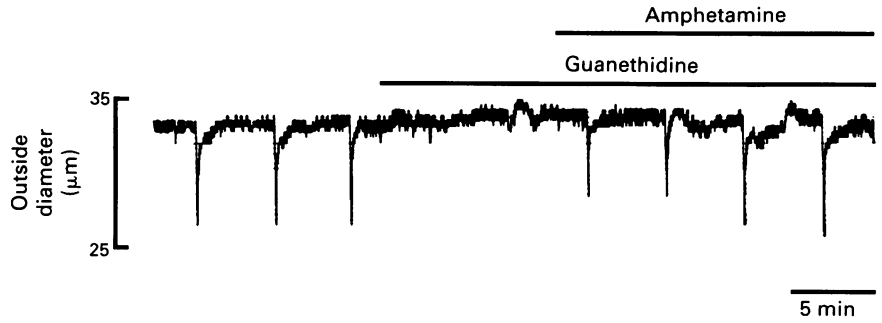


Figure 4 Vasoconstrictions of the guinea-pig submucosal arterioles elicited by electrical stimulation are mediated by transmitter released from sympathetic nerves. Vasoconstrictions to nerve stimulation (100 pulses at 10 Hz) were abolished by guanethidine (3 μM), an effect reversed by the indirectly acting sympathomimetic, amphetamine (3 μM).

baseline on prolonged application but declined to a maintained level; the amplitude of the maintained vasoconstriction elictied by mATP (1 μM) or DADPP (1 μM) was 30.6 ± 7.3% and 30.7 ± 1.9% of the peak response respectively ($n = 3$, Figure 5). The diameter of the vessel returned to control values within 3–5 min of washout of mATP or DADPP.

The putative P_2-purinoceptor antagonist, suramin (100 μM), had no effect on the resting diameter of the vessel or the vasoconstriction to exogenously applied NA (3 μM), but abolished both the nerve-evoked constriction and the response to exogenously applied ATP (3 μM, Figure 6). Constrictions evoked by nerve stimulation or the exogenous application of ATP were partially reversed following a 20 min washout period. Suramin also abolished the potentiated vasoconstrictions obtained following the removal of the α_2-autoinhibitory input with idazoxan ($n = 3$), as well as the neurally evoked constrictions recorded in reserpine-treated tissues ($n = 2$). These results strongly suggest that sympathetic vasoconstriction in submucosal arterioles is mediated solely by the action of ATP.

Intracellular recordings from submucosal neurones

Following ganglionic stimulation nicotinic fast e.p.s.ps and noradrenergic i.p.s.ps were recorded (see North & Surpre-

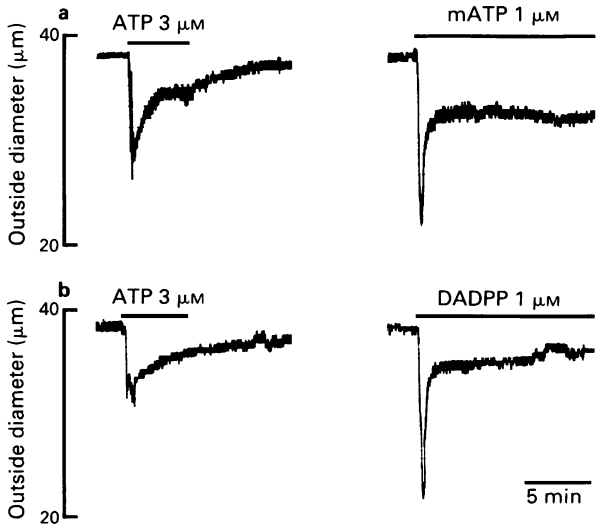


Figure 5 Vasoconstrictions of submucosal arterioles in response to superfusion with P_2-purinoceptor agonists. (a) ATP (3 μM) and α,β-methylene ATP (mATP, 1 μM) were perfused for the durations indicated by the bars; (b) ATP (3 μM) and P^1,P^5-di-(adenosine-5′) pentaphosphate (DADPP, 1 μM) were applied as indicated. Responses shown in (a) and (b) are from different preparations.

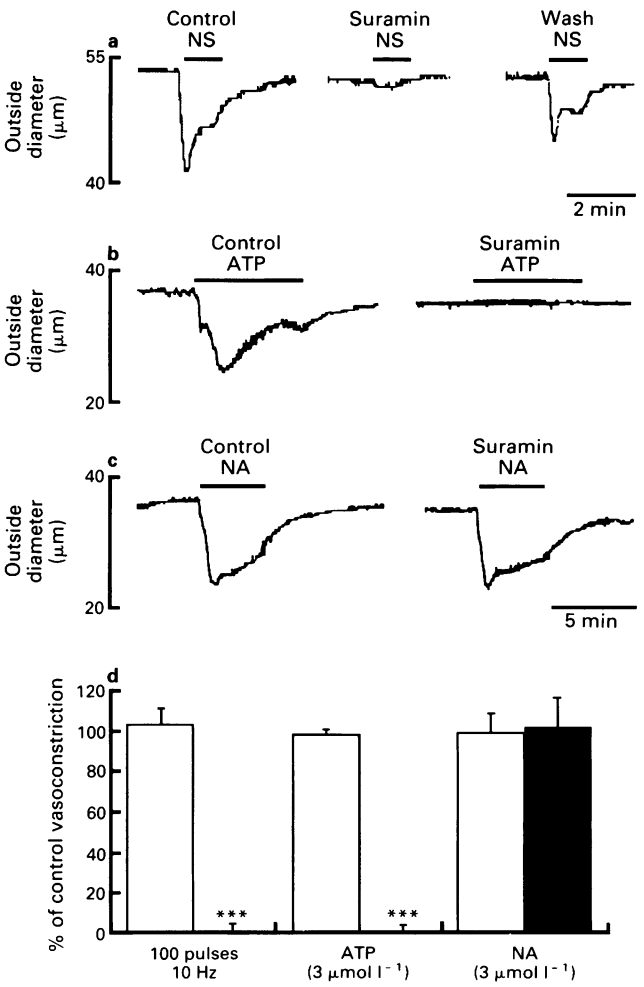


Figure 6 Constrictions of submucosal arterioles in response to nerve stimulation are mediated through the activation of P_2-purinoceptors. (a) Nerve-evoked constrictions (100 pulses at 10 Hz) were abolished by the putative P_2-purinoceptor antagonist, suramin (100 μM); the response is partially reversed on washout (20 min). (b) Constrictions to exogenously applied ATP (3 μM) were abolished by suramin (100 μM). (c) Suramin (100 μM) had no effect on the contraction evoked by the exogenous application of noradrenaline (NA, 3 μM). (d) Summary of the effects of suramin, data are expressed as mean (± s.e.mean shown by vertical bar) of the % of constriction before suramin application in response to nerve stimulation ($n = 6$), and the responses to exogenously applied ATP ($n = 4$) or NA ($n = 4$). Open columns = control responses; solid = suramin. Periods of nerve stimulation (NS) or the superfusion of ATP or NA are indicated by bars. $^{***}P < 0.001$.

nant, 1985). These two events were used to assess further the specificity of suramin as an antagonist at the P_{2X}-purinoceptor.

Suramin had no effect on the amplitude (control 18.7 ± 1.6 mV, suramin 18.9 ± 3.2 mV, $P = 0.94$, $n = 4$) or the time constant of decay of nicotinic fast e.p.s.ps (control 21.4 ± 5.1 ms, suramin 17.2 ± 4.1 ms, $P = 0.49$) evoked by nerve stimulation (0.1 Hz) when the membrane potential was held at −90 mV so that action potential initiation was prevented. At the resting membrane potential (−45 to −65 mV) many e.p.s.ps were suprathreshold for initiation of action potentials; suramin had no effect on action potential threshold, amplitude or duration (Figure 7a).

Previous studies have characterized the i.p.s.p. recorded in normal preparations as being due to the action of NA released from sympathetic nerves onto α_2-adrenoceptors on the submucosal neurones (North & Surprenant, 1985), although a non-adrenergic i.p.s.p. has also been observed (Mihara *et al.*, 1987; Bornstein *et al.*, 1988). In this study i.p.s.ps recorded in control animals were abolished by 1 μM idazoxan. Suramin had no effect on the amplitude of the i.p.s.p. (control 30 ± 4.1 mV, suramin 30 ± 3 mV, $n = 4$), or the time taken for the i.p.s.p. to decay by 50% (control 750 ± 330 ms, suramin 5 min 900 ± 380 ms, $P = 0.73$; suramin 10 min 1000 ± 350 ms, $P = 0.51$, $n = 4$; Figure 7b). After reserpine treatment noradrenergic (ie. idazoxan-sensitive) i.p.s.ps were not recorded in neurones which were hyperpolarized by superfusion with the α_2-adrenoceptor agonist, UK14304; it has been shown previously that all submucosal neurones which are hyperpolarized by α_2-adrenoceptor agonists also exhibt noradrenergic i.p.s.ps in response to nerve stimulation (Surprenant & North, 1988). These observations further strengthen the conclusion that reserpine treatment does produce complete functional depletion of NA from sympathetic nerves in the submucosal plexus.

Intracellular recordings from submucosal arterioles

Idazoxan (0.1 μM) increased the steady-state amplitude of e.j.ps in a train ($n = 3$, Figure 8a). Suramin (100 μM) abolished all e.j.ps recorded in control solution ($n = 2$) or in the presence of idazoxan ($n = 2$, Figure 8c). The blockade of the e.j.ps by suramin was only partially reversed (to approximately 50% of control amplitude) after 15–20 min of wash (Figure 8d).

Discussion

The vasoconstriction of guinea-pig submucosal arterioles evoked by 100 pulses at 10 Hz was unaffected by the α_1-adrenoceptor antagonist, prazosin. This is despite the fact that both in the present and a previous study (Shen *et al.*, 1990) NA has been demonstrated to be released from sympathetic nerves in the submucosal plexus following nerve stimulation and activates inhibitory prejunctional α_2-adrenoceptors on both submucosal and sympathetic neurones. Moreover, vasoconstrictions of similar amplitude to the neurogenic response can be evoked by the exogenous application of low concentrations of α-adrenoceptor agonists (Shen *et al.*, 1990; Vanner *et al.*, 1990). Resistance to α-adrenoceptor blockade is not a new phenomenon and has been described in many vascular tissues (for review, see Burnstock, 1990). However it is usually only a component that is resistant. Purely α-resistant contractions in response to short trains of low frequency stimuli have been reported in two vessels, the rabbit jejunal artery (Ramme *et al.*, 1987; Evans & Cunnane, 1992) and the rabbit saphenous artery (Burnstock & Warland, 1987); even in these vessels a substantial component of the contraction can be demonstrated to be noradrenergic in origin following prolonged high frequency stimulation. The present study on the guinea-pig submucosal arterioles has failed to reveal an α-adrenoceptor-sensitive component of the vasoconstriction at stimulation parameters that have demonstrated a substantial noradrenergic contraction in all arteries studied to date. It would thus seem that neurally released NA fails to reach a concentration sufficient to activate postjunctional α-adrenoceptors and mediate vasoconstriction of submucosal arterioles. These results also support the idea (Hirst & Edwards, 1989) that classical α_1-adrenoceptors are

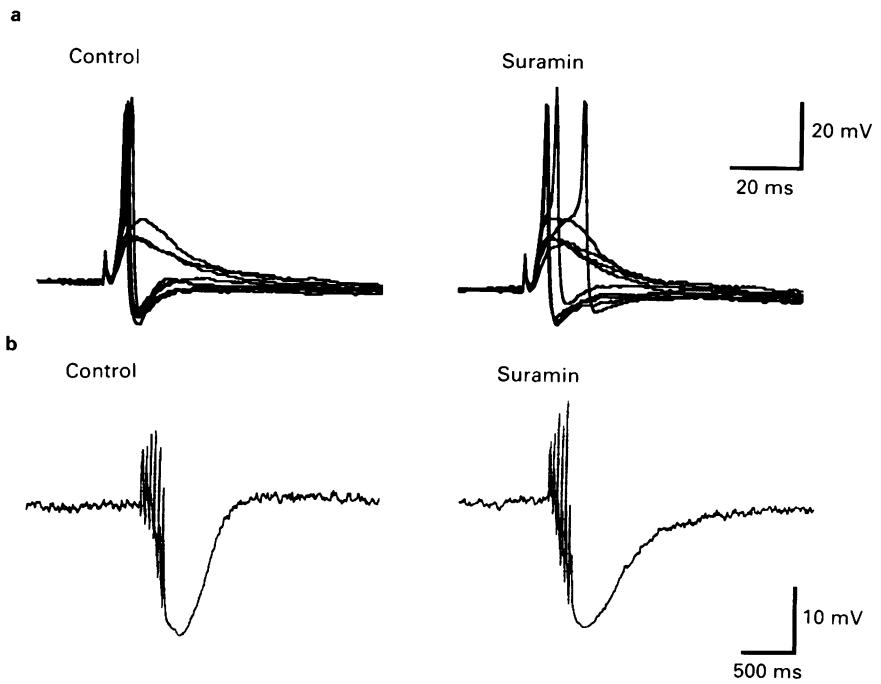


Figure 7 Effects of suramin (100 μM) on synaptic potentials recorded from guinea-pig submucosal neurones following electrical stimulation of adjacent ganglia. (a) Nicotinic e.p.s.ps evoked by 10 pulses at 0.1 Hz. Suramin had no effect on the amplitude or time course of e.p.s.ps, or the initiation of action potential firing. (b) Noradrenergic i.p.s.ps evoked by 6 pulses at 20 Hz were unaffected by the application of suramin.

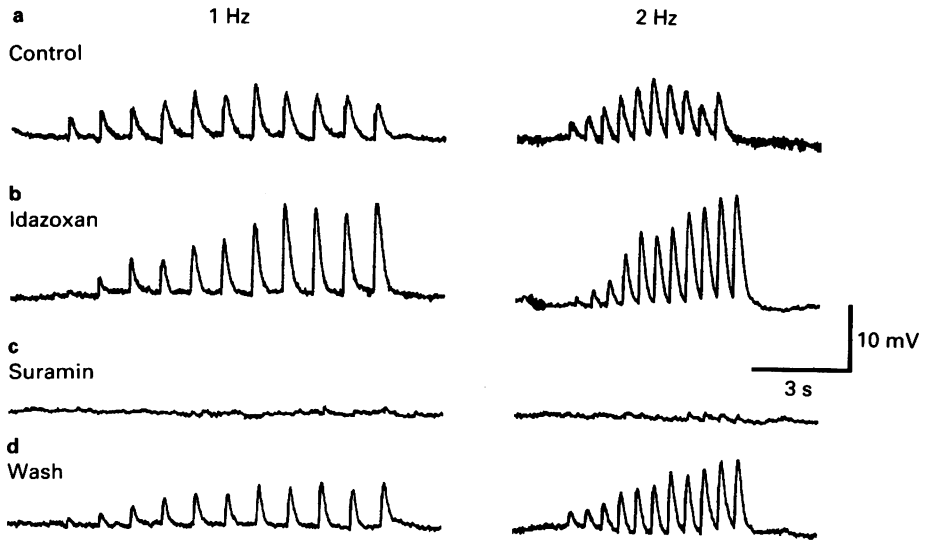


Figure 8 Effects of idazoxon (0.1 μM) and suramin (100 μM) on e.j.ps recorded following nerve stimulation from guinea-pig submucosal arterioles. E.j.ps were recorded in response to 11 pulses at 1 or 2 Hz. (a) Control, e.j.ps increased in amplitude over the first 5–6 pulses before declining on continued stimulation. (b) In the presence of idazoxan (0.1 μM) the maximum e.j.p. amplitude was potentiated. (c) E.j.ps were abolished by suramin (100 μM); in this experiment suramin was added in the continued presence of idazoxan. (d) The blockade of e.j.ps was partially reversed 15 min after the washout; in this experiment idazoxan was present in the washout solution.

found extrajunctionally and that another receptor at the sympathetic neuroeffector junction mediates the response to sympathetic nerve stimulation.

An important functional role for NA following sympathetic nerve stimulation was demonstrated with the competitive α_2-adrenoceptor antagonist, idazoxan. Contractile responses and e.j.ps were potentiated in the presence of idazoxan indicating that NA acts through prejunctional α_2-adrenoceptors to depress transmitter release. Potentiation of nerve-evoked transmitter release by α_2-adrenoceptor antagonists has been reported for a number of blood vessels and other tissues (Starke, 1977; 1987; Mishima *et al.*, 1984). The potentiation of the contractile response, in particular the maintained component, suggests that the decline of the nerve-evoked vasoconstriction following the transient peak response can be explained at least in part by α_2-adrenoceptor activation.

Results obtained with reserpine provide considerable evidence that a transmitter other than NA is responsible for mediating vasoconstriction of submucosal arterioles following sympathetic nerve stimulation. Following reserpine treatment, vasoconstrictions elicited by nerve stimulation were of similar magnitude to those recorded in control tissues. Our results are similar to those in the guinea-pig vas deferens (Kirkpatrick & Burnstock, 1987) and rabbit saphenous artery (Warland & Burnstock, 1987), in which reserpine did not significantly alter the magnitude of the prazosin-resistant (presumably purinergic) nerve-evoked contraction. The functional depletion of NA from the sympathetic nerves innervating the submucosal plexus was demonstrated both by the absence of catecholamine fluorescence and by the failure to record idazoxan-sensitive noradrenergic i.p.s.ps in submucosal neurones following reserpine treatment. Previous studies in the guinea-pig have shown that the reserpine pretreatment regime used in the present study depletes the NA content of the vas deferens, a tissue with a dense sympathetic innervation, by more than 95% (Wakade & Krusz, 1972; Kirkpatrick & Burnstock, 1987), and abolishes the potentiating effects of the α_2-adrenoceptor antagonist, yohimbine (Brock *et al.*, 1990). Similarly, NA-mediated electrophysiological responses recorded from the rat brain slice preparation are abolished following reserpine treatment (Pan & Williams, 1989).

In a number of vessels where α-antagonist resistant responses have been recorded, they have been shown to be due to the activation of P_{2X}-purinoceptors by ATP co-released with NA from sympathetic nerves (von Kugelgen & Starke, 1985; Burnstock & Warland, 1987; Brizzolara & Burnstock, 1990; Sjoblom-Widfeldt *et al.*, 1990). In the present study, arteriolar constrictions were evoked in response to the exogenous application of ATP, mATP and DADPP. The finding that mATP and DADPP were more potent than ATP is in keeping with the pharmacological profile of the P_{2X}-purinoceptor (see Burnstock, 1991).

In other studies (MacKenzie *et al.*, 1988; Evans & Cunnane, 1992) mATP and DADPP produced contractions that faded completely in the continued presence of the agonist. This full desensitization of the response has been used to quantitate the degree to which the neurally mediated contraction is due to the activation of P_{2X}-purinoceptors. In the present study neither mATP nor DADPP produced a vasoconstriction that fully desensitized during the maintained presence of these agonists. Therefore mATP and DADPP were considered unsuitable for adquately characterizing the P_{2X}-purinoceptor mediated response in submucosal arterioles.

Suramin abolished nerve evoked e.j.ps and constrictions of submucosal arterioles elicited by either exogenously applied ATP or sympathetic nerve stimulation, but had no effects on the arteriolar constriction to exogenously applied NA, nor on the noradrenergic i.p.s.ps or nicotinic e.p.s.ps recorded from submucosal neurones. A similar specificity of suramin in antagonizing P_2-purinoceptors has been reported in the rodent vas deferens and guinea-pig taenia caeci (Dunn & Blakeley, 1988; Den Hertog *et al.*, 1989; von Kugelgen *et al.*, 1989a). The finding that suramin has no effect on the noradrenergic i.p.s.p. taken with the lack of effect on the constriction to exogenoulsy applied NA, implies not only that suramin does not affect the postjunctional response to NA but also has no effect on transmitter release from sympathetic nerves. The lack of prejunctional actions of suramin on transmitter release from sympathetic nerves has been reported from studies of the overflow of tritiated NA following nerve stimulation in the mouse vas deferens (von Kugelgen *et al.*, 1989b). In the present study responses to ATP (3 μM) and nerve stimulation were both abolished, suggesting that in submucosal arterioles, suramin is an effective antagonist of

the constrictor response mediated through the activation of P_2-purinoceptors.

The results obtained with suramin, taken together with the agonist potencies of ATP, mATP and DADPP, are consistent with the view that vasoconstrictions and the underlying changes in membrane potential, the e.j.ps, following nerve stimulation are mediated through the action of ATP or a structurally related purine nucleotide through P_{2X}-purinoceptors. These data, particularly those from reserpinized tissues, provide no evidence to suggest that the α-adrenoceptor-resistant vasoconstriction and underlying changes in membrane potential are due to the activation of γ-adrenoceptors by NA as suggested by Hirst & Neild (1980, 1981).

In summary we have described for the first time a blood vessel in which all the postjunctional responses evoked by stimulation of the sympathetic nerves are mediated by the activation of P_{2X}-purinoceptors by ATP or a related purine nucleotide. Submucosal arterioles comprise the resistance vessels of the gastrointestinal microcirculation and have been estimated to contribute up to 40% of the total mesenteric-splanchnic resistance (Lundgren, 1984; Parks & Jacobsen, 1987; Granger *et al.*, 1987), as well as contributing significantly to the maintenance of systemic blood pressure. Thus purinergic transmission may play a major role in the neural regulation of the gastrointestinal microcirculation. The functional role of neurally released NA in submucosal arterioles appears to be restricted to its prejunctional modulation of transmitter release.

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Effect of the hypoglycaemic drug (−)-AZ-DF-265 on ATP-sensitive potassium channels in rat pancreatic β-cells

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1 It has previously been shown that the hypoglycaemic drug (−)-AZ-DF-265 stimulates insulin release from mouse islets; in the presence of 3 mM glucose it also inhibits ^{86}Rb-efflux from ^{86}Rb-loaded islets. Based on these data we tested the hypothesis that (−)-AZ-DF-265 inhibits ATP-sensitive potassium channels.

2 We voltage-clamped the plasma membrane of single rat pancreatic β-cells in the whole-cell configuration and measured the current flowing through ATP-sensitive potassium channels. (−)-AZ-DF-265 was applied to the outside of the cell; it inhibited the current half-maximally at a concentration of 1.2 ± 0.2 nM with a Hill coefficient of 0.7 ± 0.1. The inhibition was reversible on washing.

3 In intact RINm5F cells, the sulphonylurea [^{3}H]-glibenclamide bound with an affinity of 0.24 ± 0.01 nM and a Hill coefficient of 2.1 ± 0.4. Treatment with (−)-AZ-DF-265 led to the displacement of [^{3}H]-glibenclamide; this effect was half-maximal at 8.6 ± 1.7 nM and displayed a Hill coefficient of 0.53 ± 0.01. Meglitinide and tolbutamide, which represent, respectively, the benzamido and sulphonylurea moieties of glibenclamide, showed Hill coefficients of 0.8 ± 0.1 and 0.9 ± 0.1, respectively.

4 At pH 7.4 and with phosphate/borate buffer, (−)-AZ-DF-265 had an apparent octanol/water partition coefficient of about 53, which is intermediate between the partition coefficients of glibenclamide and glipizide. Nevertheless, (−)-AZ-DF-265 bound only to a minor degree to glass and plastic.

5 In conclusion, (−)-AZ-DF-265 inhibits ATP-sensitive potassium channels and displaces [^{3}H]-glibenclamide from the sulphonylurea receptor.

Keywords: AZ-DF-265; pancreatic β cells; RINm5F cells; ATP-sensitive potassium channel; glibenclamide; meglitinide; tolbutamide; sulphonylurea receptor

Introduction

Several new hypoglycaemic drugs have been developed in efforts to circumvent the problem of a gradual loss of responsiveness which is common with type II diabetic patients who use sulphonylurea hypoglycaemic drugs (Jackson & Bressler, 1981; Asmal & Marble, 1984; Henquin, 1990). The mechanism of action of these new drugs remains to be explained.

Stimulus-secretion coupling in pancreatic β-cells is thought to proceed as follows: an elevated concentration of glucose leads to a more pronounced inhibition of adenosine 5′-triphosphate (ATP)-sensitive potassium channels, which in turn leads to a depolarization of the plasma membrane, the opening of voltage-sensitive calcium channels, and the activation by increased intracellular calcium of the exocytotic machinery which moves secretory granules containing insulin to the plasma membrane surface (Prentki & Matschinsky, 1987; Ashcroft, 1988; Cook *et al.*, 1988).

Sulphonylureas inhibit ATP-sensitive potassium channels both in intact cells and in excised membrane patches (Sturgess *et al.*, 1985; Trube *et al.*, 1986). It is not known whether the sulphonylurea binding site is on the channel itself or on a protein that affects channel activity. We have shown previously that the non-sulphonylurea drug, linogliride, also leads to the inhibition of ATP-sensitive potassium channels (Ronner *et al.*, 1991). Garrino & Henquin (1988) reported that another new drug, (−)-AZ-DF-265 (4-{N-(α-phenyl-2-piperidino-benzyl) carbamoyl] methyl} benzoic acid) stimulates insulin release and electrical activity, and also inhibits ^{86}Rb-efflux from ^{86}Rb-preloaded mouse islets in the presence of 3 mM glucose. Since the substituted carbamoyl group of (−)-AZ-DF-265 may be homologous with the sulphonylurea group, we tested the hypotheses that (−)-AZ-DF-265 inhibits ATP-sensitive K^+-channels and displaces [^{3}H]-glibenclamide from its binding site.

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Methods

Animals and isolation of islets

Pathogen-free male Wistar rats were obtained from Charles River Laboratories, Inc. (Kingston, N.Y., U.S.A.). Islets were isolated in sterile fashion by a procedure which was modified from that of Lacy & Kostianovsky (1967). After hand-picking, the islets were further freed of exocrine tissue by centrifugation in a discontinuous Ficoll gradient (Lindall *et al.*, 1969). One hundred islets were then dissociated into single cells with trypsin in the absence of calcium, but with the addition of DNase, and the cells purified and washed as described by Pipeleers *et al.* (1985). The cells were then allowed to attach to poly-D-lysine-coated glass cover-slips and maintained for 1–2 days in Eagle's minimum essential medium (MEM) containing glucose (10 mM), penicillin (67,000 u l^{-1}), streptomycin (67 mg l^{-1}), and amphotericin (1.7 mg l^{-1}). A detailed account of these procedures has been published (Ronner *et al.*, 1991).

Electrophysiological experiments

The cells were placed in a thermostated chamber containing the following medium (mM): KCl 5.6, NaCl 140, $MgCl_2$ 1.2, $CaCl_2$ 2.6, HEPES-KOH 10, pH 7.4. The chamber had an inside diameter of 22 mm and was filled with 0.3–1 ml medium. The chamber was perfused at a flow rate (driven by a peristaltic pump) of 2 to 3 times the chamber volume per

min. The concentration of drug in the chamber was calculated from the equation $d = D(e^{tf/V} - 1)/e^{tf/V}$, whereby d denotes the average concentration of (−)-AZ-DF-265 in the chamber, D is the concentration of (−)-AZ-DF-265 in the incoming perifusion medium, e is the basis of the natural logarithm (i.e., 2.718), t is the time since switching to the new solution, f is the perifusate flow-rate, and V is the chamber volume. The traces (continuous lines) in Figure 1 are the result of these calculations. Control experiments with bromphenol blue showed that new medium distributed homogeneously over the entire volume of the chamber within the expected time. In addition, perifusion of cells with twice the normal concentration of K^+ led to a drop in holding current following the predicted, calculated time-course. All experiments were performed at 21–23°C. The following materials were in contact with (−)-AZ-DF-265: borosilicate glass, plastic pipette tips, PVC tubing, and in some instances silicone rubber tubing.

Electrodes were pulled from Corning 7052 glass (i.d. 1.15 mm, o.d. 1.75 mm; Garner Glass Co., Claremont, CA, U.S.A.), coated with Sylgard 184 (Dow Corning, Midland, MI, U.S.A.), and fire-polished to tip openings with 'bubble numbers' of 5.0–5.2 (Corey & Stevens, 1983). The electrodes were filled with the following solution (mM): K^+ 157 (as KCl 125), $MgCl_2$ 1, $CaCl_2$ 2, EGTA 10, and HEPES-KOH 5, pH 7.15 (free $[Ca^{2+}] = \sim 53$ nM); equal concentrations of ATP and additional $MgCl_2$ were added as noted for individual experiments.

β-Cells were selected for their large size (Pipeleers & Pipeleers-Marichal, 1981; our own immunocytochemical experiments, not shown); we chose cells which had an apparent diameter of 15–17 μm when observed in phase contrast. The plasma membrane potential was held constant at −70 mV in the whole-cell configuration. Alternating hyperpolarizing and depolarizing pulses of 10 mV amplitude and 200 ms duration were applied every 2 s (Trube *et al.*, 1986). The membrane current was filtered through a low-pass eight-pole Bessel filter set to a corner frequency of 1,000 Hz (−3dB). The current was then digitized at a rate of 500 Hz and recorded onto the hard-disc of a computer with 'pCLAMP' software (versions 5.0 and 5.5; Axon Instruments, Foster City, CA, U.S.A.). This recording set-up has been described in greater detail in an earlier publication (Ronner *et al.*, 1991). Average current excursions from the holding current in response to the voltage pulses were calculated from the average integrated response to sets of 10 stimuli, as described previously (Ronner *et al.*, 1991). Outward currents were given a positive sign. Current responses in the negative direction are not shown in the semi-logarithmic plots; within 5% error they had the same magnitude as the positive current deflections. Whenever the holding current (at −70 mV) was more negative than −10pA, the seal between the cell and the electrode was assumed to have deteriorated and recordings were terminated. Even at a more negative membrane potential of −80 mV the current was always more positive than −50 pA.

(−)-AZ-DF-265 was dissolved in 30 mM NaOH to a concentration of 2 mM. This stock solution was stored in separate aliquots at −20°C. Final concentrations in the bath medium of 1–1,000 nM were obtained by appropriate dilution of the stock solution.

Octanol water partition experiments

The aqueous phase consisted of 50 mM boric acid and 50 mM mono- or dipotassium phosphate titrated to pH 7–9 with KOH. (−)-AZ-DF-265 was added to the aqueous phase to final concentrations of 46–182 μM. We accounted for the small change in pH (i.e., 0.09–0.13) caused by the addition of (−)-AZ-DF-265, which had been dissolved in 30 mM NaOH. The aqueous phase and 1-octanol, at a ratio of 5:1, were vortexed vigorously. After centrifugation (15 min, 450 *g*) the absorbance of the lower, aqueous phase was determined at 240 nm; for this measurement aqueous phases of pH 8 to 9 were diluted 2 to 8 fold with pure phosphate-borate buffer of the same pH. The extinction coefficient of (−)-AZ-DF-265 was determined by adding 25–37 μM (−)-AZ-DF-265 to octanol-pretreated buffer of the appropriate dilution. Over the pH range 7–9, the weighted mean extinction coefficient was 16,400 ± 500 (means ± s.e.mean, 13 data pairs). The concentration of (−)-AZ-DF-265 in the octanol phase was calculated from the difference in concentration in the aqueous phase before and after equilibration with octanol; thereby, we took into account that octanol is soluble in water to 4.5 mM and that water is soluble in octanol to 2.3 M (Leo *et al.*, 1971).

[^{3}H]-glibenclamide binding to intact RINm5F cells

RINm5F cells were grown to confluence in tissue culture medium RPMI 1640 (Moore *et al.*, 1967) supplemented with 10% (vol/vol) foetal calf serum, 100,000 u l^{-1} penicillin, and 100 mg l^{-1} streptomycin. The cells were scraped with a plastic policeman into medium of the same composition as the one used in electrophysiological experiments except that it also contained 10 mM glucose (i.e. (mM): KCl 5.6, NaCl 140, $MgCl_2$ 1.2, $CaCl_2$ 2.6, glucose 10, HEPES-KOH 10, pH 7.4). The cells were sedimented and resuspended. Samples of $2–5 \times 10^6$ cells in 1 ml final volume were incubated at room temperature for 90 min in plastic Eppendorf centrifuge tubes in the presence of 0.33 nM [^{3}H]-glibenclamide (about 2,400 c.p.m. ml^{-1}; 8.3 Ci $mmol^{-1}$; manufactured by Chemsyn Science Laboratories, Lenexa, Kansas, U.S.A.) and various concentrations of non-radioactive glibenclamide, (−)-AZ-DF-265, meglitimide or tolbutamide. Coating of the plastic tubes with Sigmacote (Sigma) was necessary to reduce non-specific binding of [^{3}H]-glibenclamide from about 150 c.p.m. to less than 3 c.p.m. The tubes were vortexed briefly every 20 min to counteract the settling of the cells. At the end of the incubation, the cells were sedimented by centrifugation for 2 s at 15,800 *g* in a Beckman microfuge E. The pellets were resuspended in 0.8 ml of drug-free saline incubation medium and resedimented within less than 30 s. The final pellets were suspended in 0.4 ml incubation medium. The tubes with the cell suspension were then placed in scintillation vials containing 10 ml EcoLite (+) scintillation fluid (ICN Biomedicals, Costa Mesa, CA, U.S.A.). Radioactivity was determined by counting for 10–60 min; the counting efficiency was about 28%.

Reagents

Trypsin and DNase were obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.). (−)-AZ-DF-265 (i.e., 4-{[N-(α-phenyl-2-piperidino-benzyl) carbamoyl] methyl} benzoic acid) of 99.7% enantiomeric purity was a generous gift of the Dr Karl Thomae GmbH (Biberach an der Riss, Germany). Glipizide was a gift of Pfizer (Groton, CT, U.S.A.) and meglitinide of Hoechst AG (Frankfurt am Main, Germany). Inorganic salts and 1-octanol were of the 'analyzed' grade and were obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.). Eagle's MEM, RPMI 1640 culture medium, antibiotics, and all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Statistical analysis and curve fitting

Current responses to the 10 mV pulses were plotted semi-logarithmically against time. Based on the decline of the current amplitude in an individual experiment and in control experiments carried out on the same day without application of (−)-AZ-DF-265, estimates of channel run-down were made. The average of these two values was then used as the probable rate of current decline (run-down) observed in an individual experiment. The maximal inhibition of the current response by (−)-AZ-DF-265 was then determined relative to

the estimate for run-down. For curve fitting, means ± s.e.means were calculated for all experiments carried out at a similar concentration of (−)-AZ-DF-265. The following curve, which describes drug-receptor interactions, was then fitted to these means:

$$I/I_c = 1 - \{a/[1 + (IC_{50}/d)^n]\} \quad \text{(Eq. 1)}$$

Thereby, I, is the current measured in the presence of drug; I_c, is an estimate of the current in the absence of drug; a, is the fraction of the current that is sensitive to the drug; IC_{50}, is the concentration of the drug which produces a half-maximal effect; d, is the test concentration of the drug; and n is a measure of the concentration-dependence of the interaction between drug and target. The data were fitted to equation 1 using a Marquardt-Levenberg algorithm which is available in the graphing programme Sigmaplot (version 4.0, Jandel Scientific, Corte Madera, CA, U.S.A.). The means were given weights of 1/s.e.mean. Errors stated for the fitted parameters are asymptotic standard deviations obtained from the fitting programme.

For each preparation of RINm5F cells we measured the displacement of [^{3}H]-glibenclamide by cold glibenclamide (control) and by one of the test drugs (i.e., (−)-AZ-DF-265, meglitinide, or tolbutamide). Since the displacement of [^{3}H]-glibenclamide by one of the test drugs depends on the affinities of both glibenclamide and the test drug, we combined the data for the displacement of [^{3}H]-glibenclamide by cold glibenclamide and by the test drug, and we fitted the aggregate data to the equation:

$$c = a + \{b/[1 + (IC_{50(Glib)}/d)^n(1 + (I/IC_{50(test)})^m)]\}\{e/d\} \quad \text{(Eq. 2)}$$

with the Marquardt-Levenberg algorithm described above. Equation 2 is derived from equation 1 by inserting the term $1 + (I/IC_{50(test)})^m$, for the competition of the test drug for the binding site of [^{3}H]-glibenclamide, and the correction factor e/d for the dilution of [^{3}H]-glibenclamide by cold glibenclamide. In equation 2, c reflects the measured amount of [^{3}H] in the final sample; the variable, a, is a measure of non-specific binding of [^{3}H]-glibenclamide; the variable, b, is a measure of the number of glibenclamide binding sites; the variable, $IC_{50(Glib)}$, is the concentration of glibenclamide at which half of the binding sites are saturated; d, is the total concentration of glibenclamide (radioactive and non-radioactive); I, is the concentration of test drug; the variable, $IC_{50(test)}$, is the concentration of the test drug at which half of the binding sites are saturated; e, is the concentration of [^{3}H]-labelled glibenclamide, and both variables n and m are Hill coefficients.

Results

We have shown previously (Ronner *et al.*, 1991) that the peak potassium current as measured from pancreatic β-cells via the whole-cell configuration is inhibited by more than 95% by increasing the concentration of ATP from 0.3 mM to 3 mM. With 0.3 mM ATP in the electrode, tolbutamide (1 mM) inhibited the current by 97%. These data are in good agreement with published data (Trube *et al.*, 1986; Ohno-Shosaku *et al.*, 1987; Ashcroft, 1988; Sturgess *et al.*, 1988; Zünkler *et al.*, 1988; Ashcroft *et al.*, 1989) and suggest that most of the current observed under our recording conditions is carried through ATP- and tolbutamide-sensitive K^+-channels.

Figure 1 shows the effect of (−)-AZ-DF-265 on the β-cell K^+-current. With 3 nM (−)-AZ-DF-265 (Figure 1b), the time course for the onset of the inhibition was slower than the rate of exchange of the bath solution (as calculated from the volume of the bath and the rate of perfusion; illustrated by the almost vertical line). A few minutes were thus required for (−)-AZ-DF-265 to reach equilibrium binding to its target. The wash-out of the drug and recovery of the current response were much slower than the onset of drug action. With low concentrations of (−)-AZ-DF-265 (i.e., 1–3 nM) the current stayed at a near constant level after removal of the drug (Figure 1b). A more pronounced recovery of the current was observed with levels of (−)-AZ-DF-265 of ⩾ 30 nM which produced almost complete inhibition of the current (Figure 1c).

Figure 2 shows a concentration-response curve for the inhibition of the whole-cell current by (−)-AZ-DF-265. When fitted to equation 1 (see Methods) the best fit was obtained for $IC_{50} = 1.19 \pm 0.24$ nM, $a = 0.98 \pm 0.02$, and $n = 0.72 \pm 0.11$.

In the presence of bovine serum albumin (BSA) 1 mg ml^{-1}, 30 and 100 nM (−)-AZ-DF-265 inhibited the current by

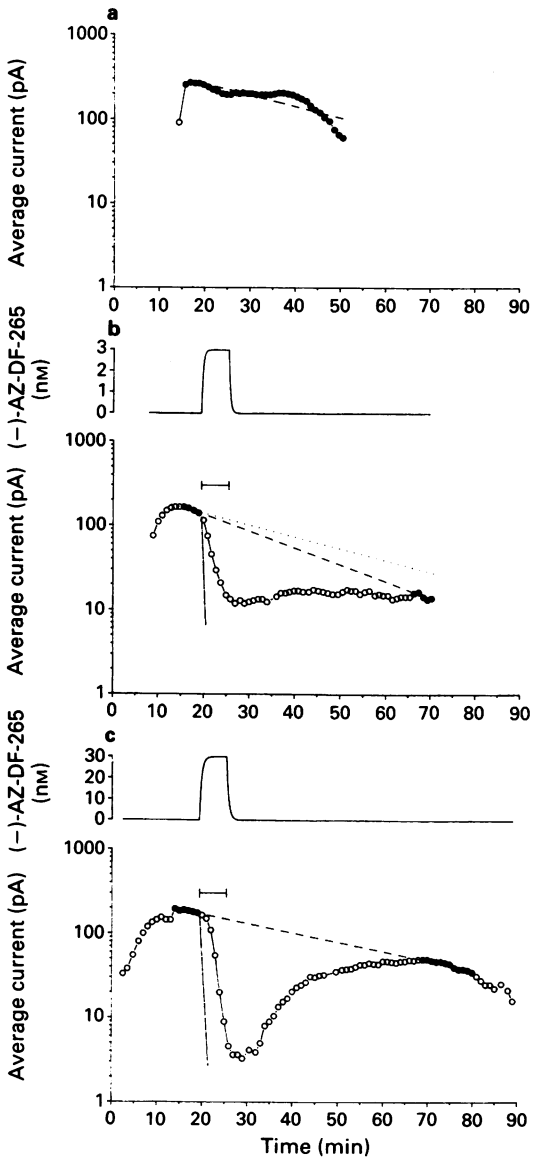


Figure 1 Effect of (−)-AZ-DF-265 on rat β-cell potassium current. Recordings from single cells in the whole-cell voltage-clamp configuration with 0.3 mM ATP in the electrode solution. Panels show average current levels in response to 10 mV de- and hyperpolarizing pulses (cell polarized to −70 mV). The chamber was perfused with bath medium (a); and medium contained 3 and 30 nM (−)-AZ-DF-265 during times indicated in panels (b) and (c), respectively. The concentration of (−)-AZ-DF-265 in the chamber was calculated mathematically (see Methods). Dashed lines show the rate of current run-down based on selected data points from individual experiments (indicated by ●). The dotted line in (b) shows the run-down observed on the same day in control experiments without the application of (−)-AZ-DF-265. The near-vertical lines show rate of exchange of bath medium.

79 ± 1 and 90 ± 2%, respectively (mean ± s.e.mean; n = 2). When equation 1 was fitted to these means, and with n set to 0.72, the values of IC_{50} and a were 4.8 nM and 1.0, respectively. By comparing these data to the inhibition obtained in the absence of BSA one may conclude that in the presence of BSA (1 mg ml^{-1}) only about one fourth of the (−)-AZ-DF-265 is free while the rest is bound to BSA.

We examined the competition of (−)-AZ-DF-265 and glibenclamide for glibenclamide binding sites. To circumvent the need for large quantities of islet cells, we used intact RINm5F cells for our [^{3}H]-glibenclamide binding experiments. We incubated the cells in the same medium as in our electrophysiological experiments, except that 10 mM glucose was added. Figure 3a shows a summary graph derived from a total of 13 such binding experiments in which each concentration of drug had been tested in duplicate; error bars illustrate the s.e.mean with the number of observations set to 4 or 5, respectively. Equation 2 was fitted to means which were given weights of 1/s.e. Glibenclamide and (−)-AZ-DF-265 bound half-maximally at concentrations of 0.25 ± 0.02 and 8.6 ± 1.7 nM, respectively, while the associated slope factors n and m had values of 2.0 ± 0.8 and 0.53 ± 0.01, respectively. If n was set to 1, $IC_{50(Glib)}$, $IC_{50(AZDF)}$, and m had values of 0.30 ± 0.06, 8.1 ± 1.6, and 0.51 ± 0.04, respectively. Thus, (−)-AZ-DF-265 convincingly displays negative cooperativity.

Glibenclamide may consist of two pharmacologically effective moieties built around an amide and a sulphonylurea group, respectively (groups 'C' and 'E-F' in Gutsche *et al.*, 1974; see also: Rufer & Losert, 1979; Garrino *et al.*, 1986; Henquin *et al.*, 1987). The hypoglycaemic drug, meglitinide, carries the substituted amide moiety of glibenclamide, while tolbutamide carries the sulphonylurea moiety. Thus, the presence of two moieties could be responsible for the observed positive cooperativity of glibenclamide binding. As Figure 3 (b,c) shows the Hill coefficients for tolbutamide and meglitinide were 0.9 ± 0.1 and 0.8 ± 0.1, respectively while the Hill coefficients for glibenclamide were 3.4 ± 2.3 and 3.0 ± 2.4, respectively, in these two sets of experiments. When data on the displacement of [^{3}H]-glibenclamide by cold glibenclamide were pooled from 15 different experiments, equation 2 produced a Hill coefficient of 2.1 ± 0.4 (graph not shown).

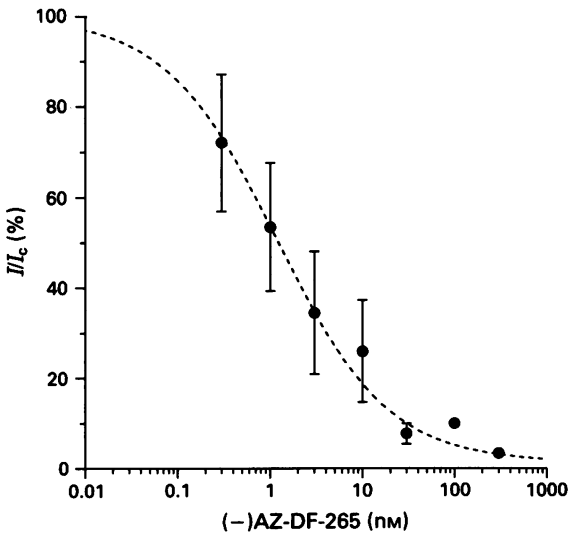


Figure 2 Concentration-response curve for inhibition of β-cell potassium current by (−)-AZ-DF-265. Means were fitted to equation 1 (see Methods). The number of experiments at each concentration of (−)-AZ-DF-265 was as follows: 5 (0.3 nM), 4 (1 nM), 6 (3 nM), 5 (10 nM), 4 (30 nM), 1 (100 nM), 2 (300 nM). The fitted curve is drawn according to equation 1 with a = 0.987, IC_{50} = 1.19 nM, and n = 0.72. Data are plotted as the ratio of the current in the presence of (−)-AZ-DF-265 to that in the absence of the drug (I_c).

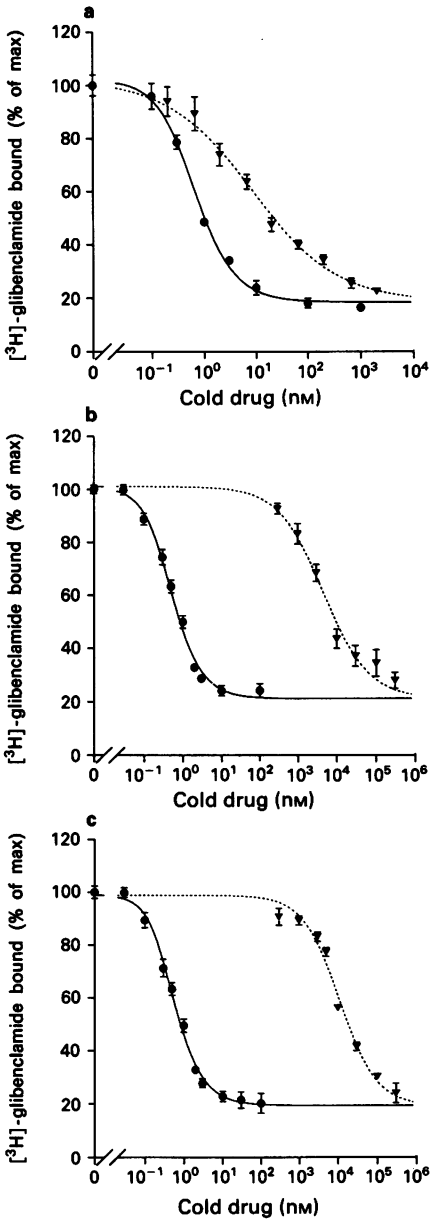


Figure 3 Displacement of [^{3}H]-glibenclamide from intact RINm5F cells by cold glibenclamide and by (−)-AZ-DF-265, meglitinide, and tolbutamide. Cells were incubated in the same medium as in electrophysiological experiments, except that 10 mM glucose was added. The data were fitted to equation 2 (see Methods). (a) Displacement of [^{3}H]-glibenclamide by cold glibenclamide (●) and by (−)-AZ-DF-265 (▼). Mean of 4 experiments. Fitted curves are drawn to a = 0.18, b = 1.33, $IC_{50(Glib)}$ = 0.25 nM, n = 2.0, $IC_{50(AZDF)}$ = 8.6 nM, and m = 0.53. (b) Displacement of [^{3}H]-glibenclamide by cold glibenclamide (●) and by meglitinide (▼). Mean of 4 experiments. Fitted curves are drawn to a = 0.21, b = 1.03, $IC_{50(Glib)}$ = 0.22 nM, n = 2.98, $IC_{50(Megl)}$ = 4.5 μM, and m = 0.76. (c) Displacement of [^{3}H]-glibenclamide by cold glibenclamide (●) and by tolbutamide (▼). Mean of 5 experiments. Fitted curves are drawn to a = 0.19, b = 1.07 $IC_{50(Glib)}$ = 0.24 nM, n = 3.36, $IC_{50(Tolb)}$ = 10.9 μM, and m = 0.93. In (a), (b) and (c), s.e.means shown by vertical bars.

In two experiments, RINm5F cells were incubated with 0.33 nM [^{3}H]-glibenclamide for 40 or 60 min, after which an excess of cold glibenclamide (0.1 μM) or (−)-AZ-DF-265 (2 μM) was added. Within the next 90 min, 39 or 34%, respectively, of the [^{3}H]-glibenclamide dissociated from the cells with no significant difference between cold glibenclamide and (−)-AZ-DF-265. Hence, the displacement of [^{3}H]-glibenclamide by cold (−)-AZ-DF-265 in the competition

experiments shown in Figure 3a is not due to an (−)-AZ-DF-265-induced allosteric increase of the rate of dissociation of glibenclamide.

We determined the lipid solubility of (−)-AZ-DF-265 as it influences the ease with which the drug can be used experimentally. We chose 1-octanol as a membrane-lipid-like phase since data are already available on the partitioning of sulphonylureas between water and octanol (Panten et al., 1989). The data in Figure 4 show that (−)-AZ-DF-265 predominantly partitioned into the octanol phase at physiological pH. Compared to two high-affinity second generation sulphonylureas, glibenclamide and glipizide (apparent partition coefficients at pH 7.5 estimated to be 71 and 2.0, respectively, based on data of Panten et al., 1989), (−)-AZ-DF-265 at pH 7.5 had an intermediate octanol/water partition coefficient of 44.

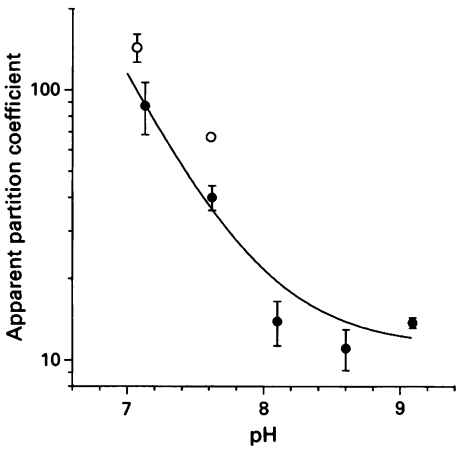


Figure 4 The octanol/water partitioning of (−)-AZ-DF-265 as a function of pH. The water phase contained either 50 mM K-borate and 50 mM K-phosphate (●) or 100 mM Na/K-phosphate (○) at the indicated pH. (−)-AZ-DF-265 was dissolved in the water phase at 46–182 μM. The concentration remaining in the water phase after equilibration with 1-octanol was determined by measuring the absorbance at 240 nm against controls without drug. The points show mean (s.e.mean indicated by vertical bars) of 3–5 experiments at each pH.

Discussion

The hypoglycaemic drug (−)-AZ-DF-265 potently inhibits the pancreatic β-cell ATP-sensitive K^+ current. The residual K^+ current after maximal block by (−)-AZ-DF-265 was similar to that observed after maximal block by tolbutamide or linogliride (Ronner et al., 1991).

(−)-AZ-DF-265 was as potent in inhibiting β-cell ATP-sensitive potassium currents as the second generation sulphonylureas. Thus, 1.2 nM (−)-AZ-DF-265 inhibited the current half-maximally in our experiments. By comparison, as much as 27 nM glibenclamide was needed to inhibit the ATP-sensitive K^+-current of CRI-G1 insulinoma cells half-maximally (Sturgess et al., 1988). However, in mouse β-cells, in the presence of 0.2% (w/v) serum albumin, 4 and 6.4 nM glibenclamide and glipizide, respectively, half-maximally inhibited the ATP-sensitive K^+-current (Zünkler et al., 1988). Furthermore, taking into account that only 17 and 57% of glibenclamide and glipizide, respectively, were free, half-maximal inhibition was achieved at free concentrations of 0.7 and 3.6 nM, respectively (Panten et al., 1989).

(−)-AZ-DF-265 was about 9 to 80 fold more potent in our electrophysiological assay ($IC_{50} \approx 1.2$ nM) than in the insulin secretory studies of Garrino & Henquin (1988). (IC_{50} in their study estimated by us to be about 100 and 11 nM at 3 and 10 mM glucose, respectively). The higher apparent potency in our assay compared to the secretion studies can be explained in part by the omission of serum albumin. Thus, in the presence of 0.1% (w/v) BSA, we estimate that half-maximal inhibition of the β-cell K^+-current was achieved at about 5 nM (−)-AZ-DF-265. The effects of (−)-AZ-DF-265 on ATP-sensitive K^+channels were only slowly reversible on wash-out of the drug. Similarly, the effects of (−)-AZ-DF-265 on insulin release from perifused mouse islets disappeared only after a 20–30 min wash-out (Garrino & Henquin, 1988).

The octanol-water partition experiments with the phosphate-borate buffer are directly comparable to those of Panten et al. (1989) with sulphonylureas. At pH 7.4 we observed an apparent distribution coefficient of 53. Our data with pure phosphate buffer (distribution coefficient of 97 at pH 7.4; see Figure 4) compare favourably with the distribution coefficient of 132 at pH 7.4 which was reported to us by Dr K. Noll (Dr Karl Thomae GmbH, Biberach an der Riss, Germany; personal communication, 1990). A detailed mathematical analysis of the pH-dependence of the apparent partition coefficient was not performed because the pK values of (−)-AZ-DF-265 are, as yet, unknown. We used (−)-AZ-DF-265 in nanomolar concentrations in aqueous solutions in the absence of carrier protein. (−)-AZ-DF-265 did not noticeably adsorb to plastic tubing or glass.

We demonstrate that (−)-AZ-DF-265 displaces [^{3}H]-glibenclamide from its binding site. The simplest explanation is that (−)-AZ-DF-265 and glibenclamide compete for the same binding sites. This view is supported by the perceived homology between the substituted carbamoyl group of (−)-AZ-DF-265 and the benzamido as well as possibly the sulphonylurea group of glibenclamide. In addition, (−)-AZ-DF-265 did not affect the rate of dissociation of bound [^{3}H]-glibenclamide. Hence, an alternative situation, in which (−)-AZ-DF-265 influences sulphonylurea binding either allosterically or via another protein, appears less probable.

Glibenclamide with its sulphonylurea and benzamido moieties may be bifunctional (Gutsche et al., 1974) and bind to two closely spaced subunits of its receptor. Indeed, glibenclamide consistently showed positive cooperativity in our individual assays and also in pooled data from 15 different experiments. On the other hand, (−)-AZ-DF-265 is most likely mono-functional. (−)-AZ-DF-265 showed marked negative cooperativity ($m = 0.53 \pm 0.01$) in its displacement of [^{3}H]-glibenclamide. This could be due to steric hindrance between neighbouring bound (−)-AZ-DF-265 molecules. Indeed, meglitinide and tolbutamide, which represent essentially the benzamido and sulphonylurea moieties of glibenclamide respectively, had Hill coefficients of about 1.

Recently, Verspohl et al. (1990) showed that AG-EE-86 and AG-EE-388 displace [^{3}H]-glibenclamide from a particulate fraction of RINm5F cells. The AG-EE drugs differ from AZ-DF-265 in one substituent of the central carbamoyl group. Verspohl et al. (1990) fitted their data to a model with two types of receptors with a 2,000 fold difference in affinity. Our data did not permit us to determine the affinity of the low affinity receptors. However, visual inspection and comparison of our data with those of Verspohl suggests that low affinity binding sites were much less abundant in our preparation of intact cells than in their membrane preparation. In addition, our model which presumes exclusively high affinity glibenclamide binding sites fits our data well.

Compared to a 98.6% pure (−)-enantiomer, a 94.4% pure (+)-enantiomer of AZ-DF-265 is at least 10 fold less potent in stimulating insulin release from mouse islets (Garrino & Henquin, 1988). We have not yet studied the effect of (+)-AZ-DF-265 on the activity of ATP-sensitive K^+-channels.

In conclusion, (−)-AZ-DF-265 leads to the inhibition of an ATP-sensitive potassium current in the whole-cell configuration and it also prevents the binding of [^{3}H]-glibenclamide to the sulphonylurea receptor. We therefore suggest that (−)-AZ-DF-265 binds to the sulphonylurea receptor.

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Involvement of substance P present in primary afferent neurones in modulation of cutaneous blood flow in the instep of rat hind paw

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1 The participation of small-diameter afferent fibres in the microcirculatory haemodynamics of cutaneous tissue was examined by studies on the effects of antidromic stimulation of primary afferent neurones on cutaneous blood flow (CBF) and tachykinin release into the subcutaneous space in the instep of the hind paw of rats.

2 Antidromic stimulation of the sectioned sciatic nerve induced a biphasic flow response, an initial transient decrease followed by an increase, with no alteration in the blood pressure.

3 Neither phase was affected by pretreatment with phentolamine (0.1 mg kg^{-1}, i.a.), propranolol (0.5 mg kg^{-1}, i.a.), atropine (0.5 mg kg^{-1}, i.a.), methysergide (0.5 mg kg^{-1}, i.a.) or mepyramine (10 mg kg^{-1}, i.a.) plus cimetidine (10 mg kg^{-1}, i.a.), but both were significantly inhibited by pretreatment with capsaicin (50 mg kg^{-1}, s.c.).

4 Spantide (1–2 μmol kg^{-1}, i.a.), a substance P (SP) antagonist, reduced the basal CBF, and also inhibited both phases of the biphasic flow response evoked by antidromic stimulation of the sectioned sciatic nerve.

5 Intra-arterial infusion of SP (0.5 μmol kg^{-1}, i.a.) induced a biphasic flow response similar to that elicited by antidromic stimulation of the sectioned sciatic nerve.

6 Antidromic stimulation of the sectioned sciatic nerve caused a marked increase in SP release into the subcutaneous perfusate of the instep of the rat hind paw, but no detectable increase in neurokinin A release.

7 We suggest that SP and its receptors are mainly responsible for the vascular response induced by stimulation of the sectioned sciatic nerve, and that small-diameter afferent fibres containing SP tonically regulate vascular tone in cutaneous microvessels.

Keywords: Antidromic stimulation of the sciatic nerve; cutaneous blood flow; substance P; rat hind paw; primary afferent neurones

Introduction

Vasodilatation (heat, redness), swelling (oedema), and pain, which are the principal signs of inflammation, can all be produced by activity in unmyelinated primary afferent neurones. In a sense, then, primary afferent neurones are not merely pathways for passive signals indicating that tissue damage has occurred, but may play an active role in body defences by participating in the inflammatory process. Recent pathophysiological studies in man and laboratory animals have indicated that unmyelinated sensory neurones containing substance P (SP) may contribute to the pathogeneses of thermal injury (Saria, 1984; Yonehara *et al.*, 1987), polyarthritis (Lembeck & Gamse, 1982), respiratory-tract irritation (Lundberg & Saria, 1983), etc. For example, in rats with experimental arthritis, an increase in the concentration of SP was observed in peripheral nerves with branches innervating inflamed joints (Levine *et al.*, 1984), and administration of capsaicin to rats before or after the onset of arthritis diminished paw swelling and tenderness (Colpaert *et al.*, 1983). Furthermore, thermal injury produced by heat stimulation at 45–55°C was found not only to excite pain afferents (Fleischer *et al.*, 1983) but also to cause vascular protein leakage in the skin through release of SP from the peripheral endings of capsaicin-sensitivie, primary afferent neurones (Saria & Lundberg, 1983; Yonehara *et al.*, 1987). In addition to these pathophysiological findings, which indicate involvement of a neurogenic component in the inflammatory response, recent immunohistochemical studies have shown that SP-positive activity is present in nerve axons and terminals in most peripheral organs including skin (Hökfelt *et al.*, 1975; Cuello *et al.*, 1978). SP immunofluorescent fibres in the skin were found in unmyelinated primary sensory neurones terminating around the small blood vessels in the dermis and epithelium. Moreover, electrical stimulation of the small-diameter afferent fibres was shown to induce release of SP in the periphery (Olgart *et al.*, 1977; White & Helme, 1985), and to produce the same cutaneous vasodilatation and plasma extravasation (Lembeck *et al.*, 1982), as after intradermal or intra-arterial administration of SP (Hagermark *et al.*, 1978; Lembeck & Holzer, 1979). These findings suggest that afferent impulses from nerve terminals of small-diameter afferent fibres generated by noxious stimuli may cause release of SP, which may finally control the inflammatory response through regulation of the microcirculation.

The aim of this study was to demonstrate more clearly the participation of small-diameter afferent fibres in microcirculatory haemodynamics in cutaneous tissue. For this, we examined the effects of antidromic stimulation of primary afferent neurones on cutaneous blood flow (CBF) by the noninvasive technique of laser Doppler flowmetry and on SP release into the subcutaneous (s.c.) space in the instep of the hind paw of rats. In addition, since recently another tachykinin, neurokinin A (NKA), which is chemically releated to SP, was discovered in the peripheral nervous system, especially in small-diameter afferent fibres containing SP, we also determined the release of NKA into the subcutaneous space.

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Methods

Measurement of blood flow

Male Sprague-Dawley rats (140–160 g body weight) were anaesthetized with urethane (780 mg kg^{-1}, i.p.) and then paralyzed by an injection of alcuronium (1 mg kg^{-1}, s.c.) with supplementary injections of the same dose when necessary. The trachea was cannulated and artificial ventilation was carried out during experiments. In most experiments the sciatic nerve was cut centrally and placed on bipolar stimulating electrodes insulated with enamel. The blood pressure was monitored continuously through a cannula inserted into the common carotid artery by means of a pressure transducer. The cutaneous blood flow in the instep of the hind paw was measured with a laser Doppler flowmeter (ALF 2100, ADVANCE). The instep of the hind paw was depilated with commercial depilatory cream, and then the laser probe was placed at a site 5 mm from the caput of the os metatarsal tertium to basis. The experiment was started 1 h later to allow time for the effects of surgery and the depilatory cream to wear off.

The change of blood flow was calculated according to the following formula: $(C-B)/A \times 100$, where A and B represent the blood flows just before intra-arterial infusion of drugs or saline and just before electrical stimulation of the sciatic nerve, respectively and C represents the minimal or maximal blood flow after electrical stimulation.

Release of substance P and neurokinin A into the subcutaneous space

Male Sprague-Dawley rats (140–160 g body weight) were anaesthetized with urethane (780 mg kg^{-1}, i.p.). A double polyethylene tube about 5 cm in length (the inner tube of 0.5 mm diameter was 5 mm longer than the external one of 3 mm diameter) was introduced into the s.c. space of the instep. Perfusion was carried out through this double coaxial tube with a peristaltic pump at a rate of 0.1 ml min^{-1} with saline containing the aminopeptidase inhibitor bestatin (3 mg/100 ml) and the dipeptidyl carboxypeptidase inhibitor, captopril (0.1 mM). Fractions of perfusate were collected through the outer tube for periods of 10 min with a fraction collector and the tubes were placed in an ice bath. Each 10-min sample (1 ml volume) was lyophilized, and then radioimmunoassayed for SP and NKA with the methods described by Yonehara *et al.* (1986) and Takano *et al.* (1986), respectively. The antiserum of SP cross-reacted with neurokinin A (1%) and neurokinin B (NKB) (0.2%). The detection limit of SP was 1 fmol. On the other hand, the detection limit of NKA was 3 fmol. The NKA antiserum showed no cross-reactivity with SP, but cross-reacted with kassinin (1.5%) and NKB (5.4%).

Electrical stimulation

The sciatic nerve was sectioned proximally, and the end was placed on bipolar, enamel-insulated, stimulating electrodes under liquid paraffin. The sciatic nerve was stimulated for a period of 30 s or 10 min with a 10 V square wave (frequency 2 Hz, duration 1 ms).

Drug administration

Drugs were dissolved in saline and administered by the intra-arterial (i.a.) or s.c. route. Solutions were infused i.a. on the side of measurement, via an arterial cannula inserted into the contralateral common iliac artery. Infusion was carried out continuously for a period of 5 min with the microinjection pump (CMA/100, Carnegie Medicin, Sweden) from 10 min before electrical stimulation. The i.a. infusion rate was 12 µl min^{-1}, and the dead volume of the inserted arterial cannula was 10 µl.

Denervation of the hind paw

A solution of 1% capsaicin solution in Tween 80 was prepared by dissolving 50 mg of crystalline capsaicin in 0.5 ml ethanol, adding 0.5 ml Tween 80, and adjusting the final volume to 5 ml with saline. Capsaicin at a dose of 50 mg kg^{-1} was injected s.c. into rats within a week after their birth, as described by Jancsó *et al.* (1967). Control rats were treated with the solvent only. Two months later, animals showing at least 40% increase in latency in response to noxious thermal stimulation (52°C) compared with control rats, were used for experiments.

Substances used

The following compounds were used: atropine sulphate (Nakarai Chem., Ltd., Japan); phentolamine mesylate (Ciba-Geigy, Japan); propranolol (Sumitomo Chem. Ind., Japan); mepyramine maleate, cimetidine and capsaicin (Sigma, U.S.A.); methysergide bimaleate (Sandoz, Switzerland); SP, NKA and spantide (Peptide Institute, Inc., Japan); [^{125}I]-[Tyr^{8}]SP (New England Nuclear Co., U.S.A.); N-[propionyl-^{3}H]propionylated NKA (Amersham, U.K.); NKA antiserum (donation by Prof. H. Kamiya and Dr Y. Takano, Fukuoka University, Japan).

Results

Effect of electrical stimulation of the sciatic nerve on cutaneous blood flow

Antidromic stimulation of the intact sciatic nerve for 30 s caused an immediate increase in CBF which reached a peak ($256.0 \pm 1.1\%$, $n = 50$) 30 to 60 s after the beginning of stimulation and lasted for 9–12 min. The blood pressure also increased. On the other hand, stimulation of the distal end of the sectioned sciatic nerve led to a biphasic flow response with no alteration of the blood pressure. This biphasic response consisted of an initial transient decrease, followed by an increase, that is, CBF decreased immediately after the start of stimulation ($-28.1 \pm 1.1\%$, $n = 171$) and then increased near the end of stimulation. The increase was maximal 1 to 2 min after the start of stimulation ($84.8 \pm 13.5\%$, $n = 172$) and lasted for 10–12 min (Figure 1). Therefore, in all subsequent experiments, the sciatic nerve was sectioned to avoid a systemic influence on the effect of sciatic nerve stimulation as far as possible. On repeated stimulations, reproducible responses of the CBF to electrical stimulation of the sectioned sciatic nerve were obtained until, at least, the seventh stimulation when a resting period of 1 h was allowed between stimulations.

Involvement of the autonomic nervous system, histamine and 5-hydroxytryptamine in the changes in CBF induced by electrical stimulation of the sectioned sciatic nerve

Intra-arterial infusion of phentolamine (0.1 mg kg^{-1}), propranolol (0.5 mg kg^{-1}) or atropine (0.5 mg kg^{-1}) did not modify the basal CBF, or the changes of the CBF induced by antidromic stimulation of the sectioned sciatic nerve (Table 1). Similar results were obtained when mepryamine (10 mg kg^{-1}) together with cimetidine (10 mg kg^{-1}) or methysergide (0.5 mg kg^{-1}) was administered in the same way (Table 2). The size of the antidromic vasodilatation was not affected by systemic administration of mepyramine plus cimetidine (each 10 mg kg^{-1}, s.c.) 30 min previously. In all these experiments, the blood pressure was unaffected by i.a. infusion of drugs alone or in combination with antidromic stimulation of the sectioned sciatic nerve.

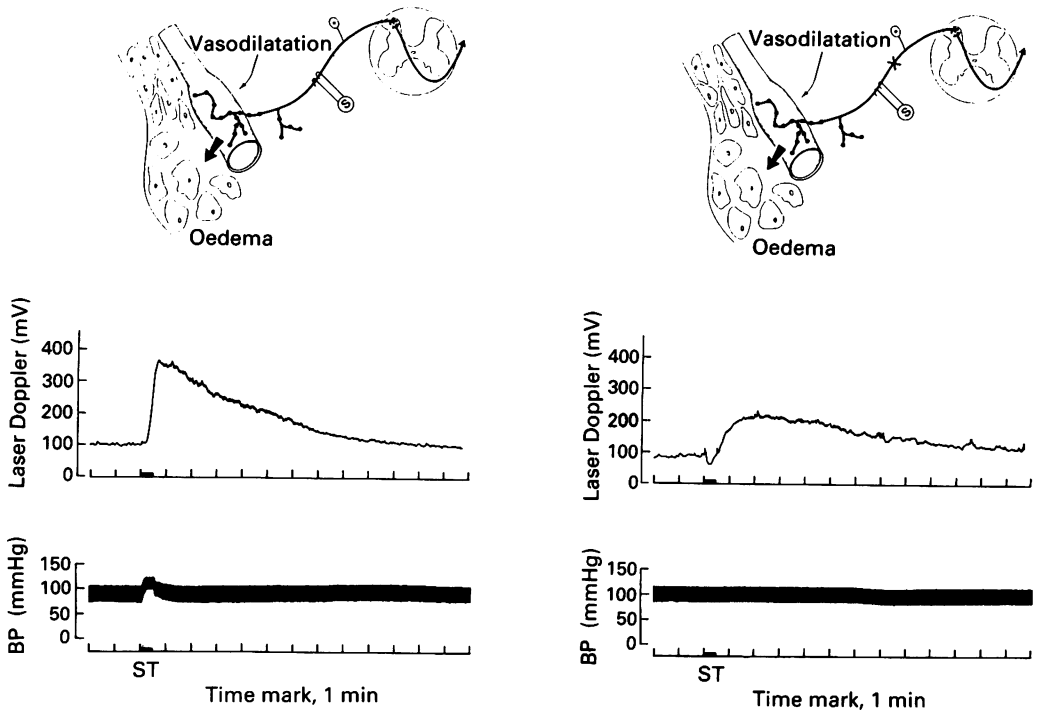


Figure 1 Representative laser Doppler flowmetry traces (upper) and systemic blood pressure traces (lower) following antidromic stimulation of the intact (left) or the sectioned (right) sciatic nerve. Electrical stimulation of the sciatic nerve was performed with a 10 V square wave for 30 s at the time indicated by the solid line (ST).

Table 1 Involvement of the autonomic nervous system in changes in cutaneous blood flow (CBF) induced in the rat hind paw by electrical stimulation of the sectioned sciatic nerve for 30 s

Test compound	*Dose* (i.a.)	*First phase (%)* (minimum CBF)	*Second phase (%)* (maximum CBF)
Saline		−33.2 ± 4.7	88.3 ± 18.5
Phentolamine	0.1 mg kg^{-1}	−25.7 ± 10.2	79.1 ± 18.1
Saline		−27.0 ± 2.0	81.2 ± 12.4
Propranolol	0.5 mg kg^{-1}	−27.7 ± 5.7	87.0 ± 18.2
Saline		−29.0 ± 7.4	83.4 ± 21.2
Atropine	0.5 mg kg^{-1}	−35.2 ± 7.7	78.5 ± 22.7

CBF just before i.a. infusion of saline or test compounds was considered as 0% (see Methods). Ten animals were used for each compound. Values are mean ± s.e.mean.

Table 2 Effect of mepyramine plus cimetidine and methysergide on changes in cutaneous blood flow (CBF) induced in the rat hind paw by electrical stimulation of the sectioned sciatic nerve for 30 s

Test compound	*Dose* (i.a.)	*First phase (%)* (minimum CBF)	*Second phase (%)* (maximum CBF)
Saline		−15.6 ± 4.9	79.1 ± 11.5
Cimetidine	10 mg kg^{-1}		
+ Mepyramine	10 mg kg^{-1}	−16.6 ± 6.3	93.6 ± 20.1
Saline		−22.9 ± 3.3	81.2 ± 19.1
Methysergide	0.5 mg kg^{-1}	−21.7 ± 4.0	76.0 ± 18.4

CBF just before i.a. infusion of saline or test compounds was considered as 0% (see Methods). Ten animals were used for each compound. Values are mean ± s.e.mean.

Effects of capsaicin and spantide

Pretreatment of newborn rats with capsaicin (50 mg kg^{-1}, s.c.) and i.a. infusion of spantide (1–2 μmol kg^{-1}) reduced the basal blood flow, and significantly inhibited both phases of change in the CBF; the phase of decrease and the phase of increase of the CBF induced by electrical nerve stimulation (Figures 2 and 3, and Table 3). On the other hand, as shown in Figure 3, blood pressure, particularly systolic pressure, gradually decreased following i.a. infusion of spantide. The stimulation of the sectioned sciatic nerve subsequent to administration of spantide, however, produced very little alteration of the blood pressure.

Effect of i.a. infusion of substance P

Infusion of SP into the common iliac artery induced a similar biphasic response to that induced by antidromic stimulation

of the sectioned sciatic nerve; that is, the CBF decreased, immediately after the start of infusion of SP, to a minimum within 30 s, and then increased to a maximum about 1 min after the start of infusion. Subsequently, the CBF decreased even when SP infusion was continued for 5 min (Figure 4).

Effect of electrical stimulation of the sectioned sciatic nerve on the substance P and neurokinin A levels in the subcutaneous perfusates

The SP concentration in the perfusate was initially high, but gradually decreased with time to a steady level after 30 min, which was maintained for at least 4 h. The SP level in the resting state was 1.24 ± 0.1 fmol $10\,min^{-1}$ ($n = 28$). Antidromic stimulation of the sectioned sciatic nerve caused marked increase in SP release (Figure 5). On the other hand, both NKA levels in the resting state and during antidromic stimulation of the sectioned sciatic nerve were below the detection limit of the assay.

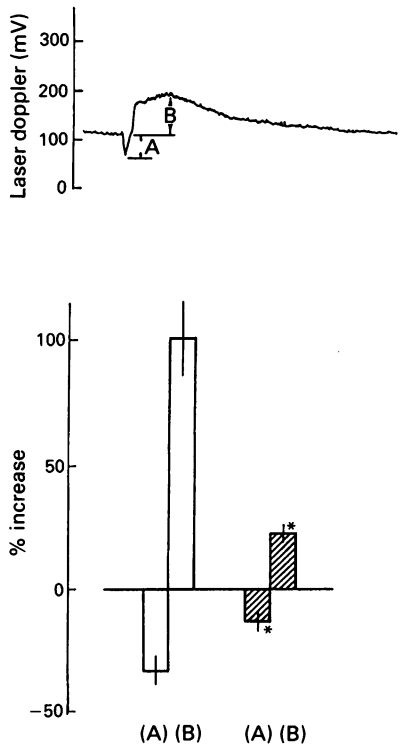


Figure 2 Effect of pretreatment with capsaicin on changes in cutaneous blood flow (CBF) induced in the rat hind paw by electrical stimulation of the sectioned sciatic nerve for 30 s. Capsaicin (50 mg kg^{-1}, s.c.) was administered within a week after birth, i.e., 2 months before the experiment. Changes are shown in the CBF induced by antidromic stimulation in vehicle- (open columns) and capsaicin-treated (hatched columns) animals, at times A and B in the upper trace. CBF just before antidromic stimulation was taken as 0% (see Methods). Columns and vertical lines are means ± s.e. of values for 10 rats. ★$P < 0.05$ vs. change in CBF in vehicle-treated animals in each phase.

Discussion

Changes in tissue blood flow observed by laser Doppler flowmetry are dependent on the blood pressure and the

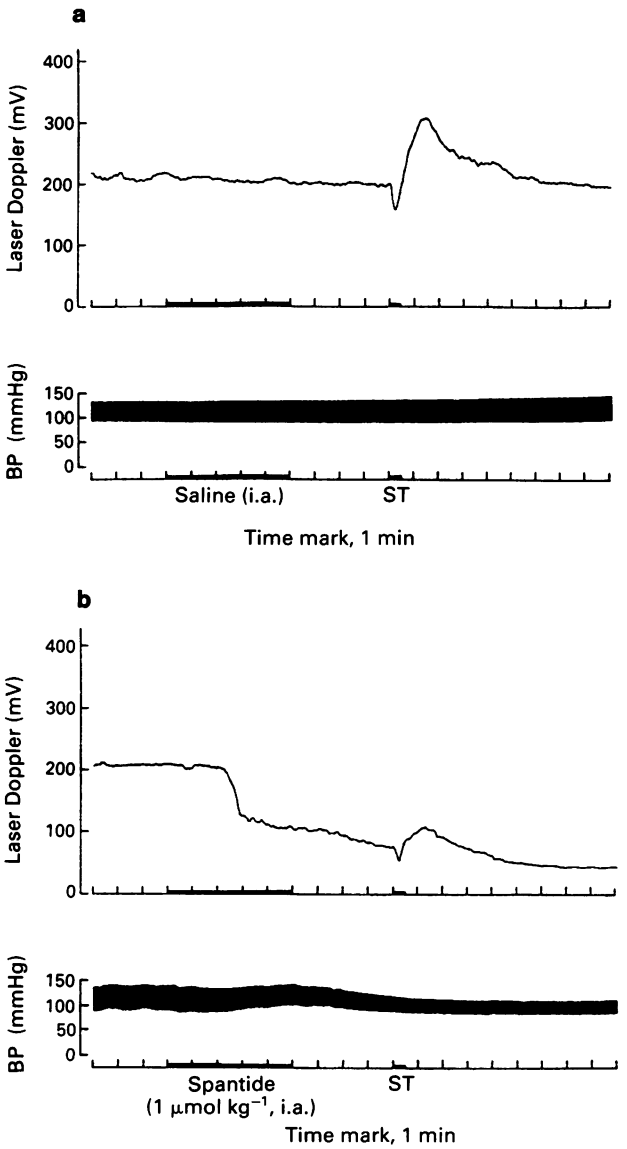


Figure 3 Representative laser Doppler flowmetry traces (upper) and systemic blood pressure traces (lower) on antidromic stimulation of the sectioned sciatic nerve after i.a. infusion of saline (a) or spantide (b).

Table 3 Effect of spantide on changes in cutaneous blood flow (CBF) induced in the rat hind paw by electrical stimulation of the sectioned sciatic nerve for 30 s

Test compound	*Dose* (i.a.)	*First phase (%)* (minimum CBF)	*Second phase (%)* (maximum CBF)
Saline		−27.0 ± 3.7	72.5 ± 11.2
Spantide	1 μmol kg^{-1}	−11.0 ± 2.5*	41.6 ± 8.3*
Saline		−22.3 ± 3.1	71.0 ± 5.3
Spantide	2 μmol kg^{-1}	−5.3 ± 1.7*	12.5 ± 6.4*

CBF just before i.a. infusion of saline or spantide was taken as 0% (see Methods). Ten animals were used for each dose of spantide. Values are mean ± s.e.mean. *$P < 0.05$ vs. change in CBF in saline-treated animals in each phase (Student's t test for paired values).

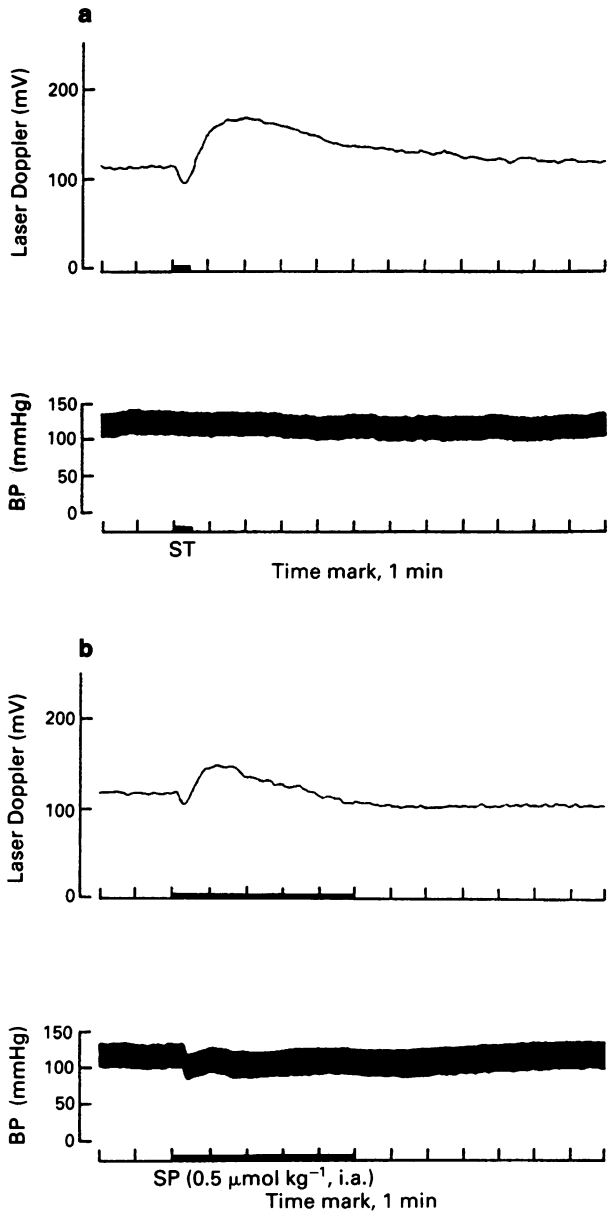


Figure 4 Representative laser Doppler flowmetry traces (upper) and systemic blood pressure traces (lower) following antidromic stimulation of the sectioned sciatic nerve (a) and i.a. infusion of substance P (SP) (b).

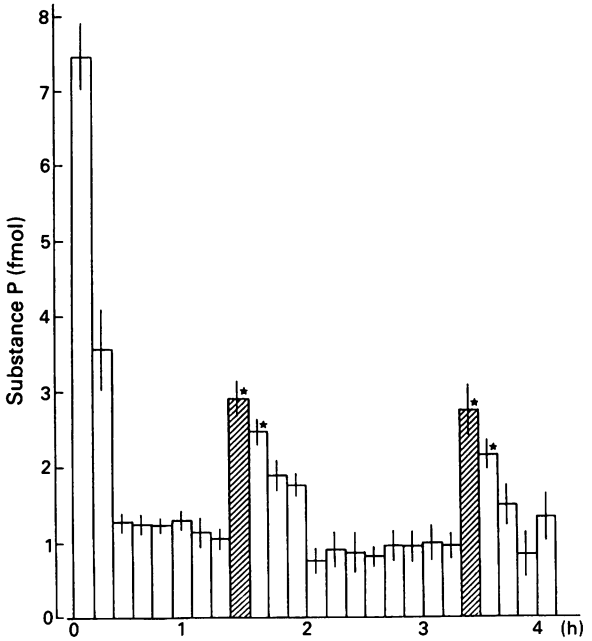


Figure 5 Effect of electrical stimulation of the sectioned sciatic nerve on the release of substance P into the subcutaneous perfusate. The nerve was stimulated with a 10 V square wave at the times indicated by the hatched columns. Columns and vertical lines represent means ± s.e. of values for 14 rats. ★$P<0.05$ vs. the mean value for the last three fractions before stimulation (Student's t test for paired values).

diameter of the blood vessels. In the present study, an increase in blood pressure was observed following electrical stimulation of the intact sciatic nerve, but on stimulation of the cut end of the sciatic nerve sectioned centrally, the increase in blood pressure disappeared without a change in the increase of CBF. A reproducible response of the CBF to electrical stimulation of the sectioned sciatic nerve was obtained until, at least, the seventh stimulation when a resting period of 1 h was allowed between stimulations. These findings suggest that vasoactive substances involved in regulation of blood flow in cutaneous microvessels are present in the sciatic nerve. Cutaneous nerves are known to have two distinct functional categories of axons: primary afferents with cell bodies in the dorsal root ganglion and sympathetic postganglionic fibres with cell bodies in sympathetic ganglia (Fields, 1987). Furthermore, experiments have shown that vasoconstriction and vasodilatation in peripheral tissues are mediated by sympathetic α-adrenoceptors, and sympathetic β-adrenoceptors and parasympathetic nerves, respectively. In the present study, if the reduction of CBF observed immediately after the start of stimulation of the cut sciatic nerve was mediated through sympathetic α-adrenoceptors, pretreatment with phentolamine, an α-adrenoceptor blocker, should have inhibited this reduction of CBF. So our finding that phentolamine did not block the response indicates that the mechanism of the effect on cutting the sciatic nerve did not involve activation of sympathetic α-adrenoceptors. On the other hand, the increase in the CBF after its decrease was not affected by pretreatment with propranolol (β-adrenoceptor blocker) or atropine (antimuscarinic receptor drug), indicating that the autonomic nervous system is not involved in this vasodilatation on antidromic stimulation. A similar phenomenon has been observed in the dental pulp of cats; namely, pretreatment with propranolol or atropine was found not to influence the increase in pulpal blood flow produced by electrical stimulation of the inferior alveolar nerve (Gazelius & Olgart, 1980). Couture & Cuello (1984), however, demonstrated that the vasodilatation induced in the rat inferior lip by electrical stimulation of the trigeminal nerve was unaffected by pretreatment with guanethidine plus phentolamine and propranolol, but was significantly reduced by atropine. These observation indicate that the influence of the autonomic nervous system on vasodilatation induced by antidromic stimulation may vary in different species or tissues.

The laser Doppler flow meter used in the present study emits light of a 2mW He-Ne laser (wavelength 632.8 nm) which penetrates approximately 1 mm into the skin. Thus the measured changes in blood flow occur in the microvasculature. In the skin, small diameter nerve fibres containing SP are found in the dermis and are sometimes seen to be in association with small blood vessels (Hökfelt *et al.*, 1975; Cuello *et al.*, 1978). The nerve fibres can also be seen to branch from the dermal fibres into the epithelium. Electrical stimulation of the sciatic nerve resulted in a significant increase in SP-like immunoreactivity in the perfusate of the subcutaneous space in the instep of the rat hind paw, as

shown in Figure 5, or in blister fluid of the rat hind paw, and this increase was abolished by neonatal pretreatment of rats with capsaicin (White & Helme, 1985). Treatment of newborn rats with capsaicin results in selective and permanent degeneration of small-diameter afferent fibres and significant decrease in the SP content (Jancsó *et al.*, 1980) and capsaicin-treated animals show significantly increased latencies in response to a noxious thermal stimulus in either the tail-flick or hot-plate test (Nagy *et al.*, 1980). In the present study, the changes in the blood flow in the microvessels of the skin induced by electrical stimulation of the sectioned sciatic nerve were significantly inhibited by capsacin and spantide. The latter compound has been shown to be one of the best SP antagonists in peripheral tissues (Håkanson & Sundler, 1985; Rosell, 1985). In this connection, Rosell *et al.* (1983, 1985) have observed that, when tested on the hamster urinary bladder, which contracts at low concentrations of NKA and eledoisin but is rather insensitive to SP and physalaemin, spantide did not block the actions of NKA and eledoisin. On the guinea-pig ileum preparation, the contractions elicited by NKA were blocked in a noncompetitive manner. These findings indicated that spantide has a high affinity for SP receptors but not for NKA and eledoisin receptors. However, it has recently been found in experiments on the isolated spinal cord-tail preparation of the newborn rat that spantide depressed the actions of not only SP but also NKA (Nussbaumer *et al.*, 1989). Therefore, a hypothesis emerges that an antagonistic action of spantide for tachykinin receptors existing in peripheral organs may differ from that existing in the central nervous system. In the present study, however, involvement of NKA in the changes in CBF observed on antidromic stimulation cannot be completely neglected because it is known that NKA coexists with SP in primary afferent neurones (Maggio & Hunter, 1984; Dalsgaard *et al.*, 1985) and is synthesized via the same precursor (Nawa *et al.*, 1983). According to Ogawa *et al.* (1985), the amount of NKA in the dorsal roots and the dorsal root ganglion in the rat spinal cord was about one-third of that of SP. In our perfusion experiment, moreover, an increase in NKA release evoked by antidromic stimulation of the sectioned sciatic nerve was not observed. These observations including our results suggest that SP released from the peripheral endings of capsaicin-sensitive small-diameter, afferent fibres present in the sciatic nerve is mainly involved in the changes in CBF observed following electrical stimulation of the sectioned sciatic nerve. This suggestion is also supported by the fact that the responses evoked by electrical stimulation were mimicked by infusion of SP into the side of measurement via an arterial cannula inserted into the contralateral common iliac artery; this is, i.a. infusion of SP induced by biphasic flow response with an initial transient decrease, followed by an increase. D'Orleans-Juste *et al.* (1985) demonstrated that arterial relaxation *in vitro* was endothelium-dependent. According to Furchgott (1983), moreover, SP is a potent vasodilator whose action depends on the presence of endothelial cells. If these findings also apply to skin blood vessels, SP should be administered directly to its receptor by intra-arterial infusion, whereas SP released by antidromic stimulation should have to diffuse to its receptor sites. Concerning the participation of SP in microcirculatory haemodynamics, Galligan *et al.* (1990) reported that with an optical method in preparations of guinea-pig submucosal plexus *in vitro*, intrinsic intestinal neurones containing SP but not calcitonin gene-related peptide (CGRP) and vasoactive intestinal polypeptide supply submucosal arterioles after sympathetic efferents and sensory afferents are removed, and stimulation of these neurones releases SP to produce arteriolar dilatation.

The reason for the initial phase of reduction of CBF observed on antidromic stimulation or i.a. infusion of SP is unknown. If this reduction reflects vasoconstriction, SP may initially induce contraction of smooth muscle by activating SP receptors located on smooth muscle cells (Mastrangelo *et al.*, 1983) or by promoting the release of an endothelium-derived contracting factor such as endothelin (De May & Vanhoutte, 1982; Yanagisawa *et al.*, 1988) through activating SP receptors on the endothelium. In this connection, it is noteworthy that SP receptors were found on the endothelium of dog renal arteries (Stephenson *et al.*, 1987; Stephenson & Summers, 1987). Another possibility is that the initial decrease in CBF, could occur in compensation for rapid transient vasodilatation induced by SP released locally around the skin microvessels. Intra-arterial infusion of spantide induced decrease in the basal CBF compared with that in the saline-treated group (Figure 4), so in the normal basal state, the small-diameter afferent fibres probably maintain some vasodilator tone through spontaneous release of SP from peripheral nerve terminals.

There is evidence that SP can release histamine from mast cells (Johnson & Erdos, 1973; Erjavee *et al.*, 1981) and it has been suggested that mast cell histamine plays a part in vasodilatation following electrical stimulation of cutaneous nerves (Lembeck & Holzer, 1979). However, in this study, vasodilatation induced by antidromic stimulation was not influenced by mepyramine plus cimetidine, indicating that the vascular response was not mediated by the release of histamine from mast cells. This is similar to findings in the dental pulp of cats, where antihistamines did not influence vasodilatation produced by inferior alveolar nerve stimulation (Gazelius & Olgart 1980). It is difficult to explain the discrepancy between our results and the observations of others indicating a role of histamine. Perhaps the link between the SP system and the release of histamine from mast cells is less essential for vasodilatation induced by antidromic stimulation in the instep of the hind paw of rats than in other tissue, but more research is needed on this problem.

In the present study, we did not exclude the possibility of participation of other peptides present in capsaicin-sensitive, small-diameter afferent fibres and autonomic neurones, and other endogenous inflammatory substances. CGRP, for instance, has been shown to be localized with SP in many sensory neurones and tissues including skin (Gibbins *et al.*, 1985; Wallengren *et al.*, 1987), and this peptide is a potent vasodilator in several species such as rats (Gamse & Saria, 1985) and man (Wallengren & Håkanson, 1987). Bradykinin, a chemical mediator of inflammation, may also act on the peripheral sensory nerve endings to elicit the release of SP (White & Zimmermann, 1988).

In conclusion, our study provides further evidence that endogenous SP and its receptors are mainly responsible for the vasodilator response induced by sciatic nerve stimulation, and suggests that SP-containing nerves, capsaicin-sensitive, small-diameter afferent fibres, tonically regulate vascular tone in cutaneous microvessels.

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Platelet activating factor and systemic anaphylaxis in *Nippostrongylus brasiliensis*-sensitized rats: differential effects of PAF antagonists

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1 The effects of two platelet-activating factor (PAF) antagonists, WEB 2086 and BN 52021, in reducing the changes in extravasation (Evans blue technique) and blood flow (radiolabelled microsphere method) to various organs and tissues following anaphylactic shock in the *Nippostrongylus brasiliensis*-sensitized rat were investigated.

2 Both antagonists attenuated anaphylaxis-induced increases in plasma protein leak in the trachea, stomach and small intestine, although they did not block extravasation in the colon and kidneys.

3 Anaphylaxis-induced decreases in blood flow to the adrenals were effectively antagonized by WEB 2086, although this antagonist did not reverse blood flow decreases to any other tissues. BN 52021, on the other hand, did not alter anaphylaxis-induced decreases in blood flow to the adrenals, but effectively prevented dramatic decreases in blood flow to the large and small bowel and spleen.

4 Anaphylactic shock produced marked reduction in blood pressure that was partly reversed by WEB 2086, whereas BN 52021 effectively blocked the decreases in cardiac output.

5 Thus, PAF is responsible for some of the haemodynamic and extravasation of protein changes associated with systemic anaphylaxis in the rat, although the differential inhibition observed with the two antagonists suggests that PAF alters vascular responsiveness through different mechanisms in selected tissues.

Keywords: *Nippostrongylus brasiliensis*; blood flow; extravasation; parasite; antagonists; anaphylaxis; PAF

Introduction

Animal models of helminth infections, such as *Nippostrongylus brasiliensis* (Nb) in the rat, frequently develop type I hypersensitivity reactions especially following antigen challenge in sensitized animals (Keller, 1970; Jarrett & Miller, 1982). These reactions are characterised by profound depression of cardiovascular function which lasts at least 1 h after challenge (Mathison *et al.*, 1990a). Some of the mediators potentially involved in precipitating the cardiovascular changes during anaphylaxis are histamine (Church, 1975), prostaglandins and leukotrienes (Moqbel *et al.*, 1986; Heavey *et al.*, 1988) and platelet activating factor (PAF; Fesus *et al.*, 1977; Casals-Stenzel, 1987; Vargaftig & Braquet, 1987).

A role for PAF in the early phase of anaphylaxis is not apparent in the guinea-pig (Felix *et al.*, 1990) or rabbit (Lohman & Holonen, 1990), whereas in the rat Fesus and coworkers (1977) have proposed that PAF may participate in the initial haemodynamic alterations due to analphylaxis. An understanding of the role of PAF in pathophysiological processes is further complicated by differential inhibition by various antagonists of PAF-induced oedema (Hellewell & Williams, 1989) and bronchoconstriction (Pretolani *et al.*, 1989). In this study we compared the inhibitory effects of two PAF antagonists (WEB 2086 and BN 52021) on the haemodynamic changes and leakage of plasma protein associated with acute anaphylaxis in the Nb-sensitized rat. Not only did we wish to evaluate the role of PAF in mediating the pathophysiological effects of anaphylaxis in the Nb-sensitized rat, but we also wished to determine if the two PAF antagonists had comparable inhibitory effects.

Methods

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Canada) of an initial weight of 200–250 g were maintained in filter top cages (2 to 3 per cage) to minimize the possibility of other infections. Animals were infected by s.c. injection in the scruff of the neck with 3000 third stage infective larvae of *Nippostrongylus brasiliensis*. The larvae were collected from faecal cultures and extensively washed prior to injection. Studies were performed 30 to 35 days after the initial infection on anaesthetized animals (intraperitoneal, pentobarbitone sodium, 55 mg kg^{-1} body wt) and maintained with supplemental injections as required.

Measurement of plasma extravasation

Changes in permeability to serum albumin were analyzed with Evans blue (Saria & Lundberg, 1983). The right jugular vein was cannulated for injection of Evans blue (20 mg kg^{-1}) in saline (20 mg ml^{-1}) several minutes before administration of antigen (150 worm equivalents (WE)). Five min after antigen injection, via the jugular vein, the thorax was opened and the animals were perfused via the left ventricle with 50 ml of saline. The right atrium was opened to wash out blood and perfusate. Small fragments, weighing 20 to 100 mg, of various tissues were excised, weighed and placed in 2 ml of formamide and incubated without homogenization for 72 h at room temperature (20°C). Colorimetric measurements were performed with a Beckman DU-50 Spectrophotometer at the absorbance maximum for Evans blue (620 nm). Calculations of dye content in the tissues were based on external standards in formamide.

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Blood flow measurments with radiolabelled microspheres

To assess haemodynamic values and collect the reference blood sample the right femoral artery was cannulated (PE50). The left ventricle was cannulated through the right carotid artery with PE50 tubing. Heart rate (HR), mean arterial blood pressure (MABP) and mean left ventricular pressure were recorded with a Statham P23Db pressure transducer connected to a Beckman Dynograph.

Regional blood flow and cardiac output were determined with radioactive microspheres (^{57}Co, 15 ± 3 μm, sp. act. 10 mCi g^{-1}; New England Nuclear). The microspheres were suspended in 0.9% NaCl containing 0.05% Tween-80. Prior to use, the stock solution of microspheres was shaken vigorously for 15 s and then an aliquot was withdrawn and diluted in 0.9% saline containing 0.05% Tween-80. Immediately before the microspheres were injected the solution was passed repeatedly through a 27 gauge needle connected to a 1 ml syringe. The microspheres (approximately 80,000 to 100,000) were injected in a total volume of 0.5 ml with 0.8 ml of saline flush into the left ventricle. Starting 15 s before the microsphere injection, the reference blood sample was drawn into a motor-driven syringe at a rate of 0.68 ml min^{-1} for 75 s.

The microspheres were injected into the left ventricle 5 min after the administration of antigen via the jugular vein. Following collection of the reference blood sample the animals were killed with an overdose of urethane. Individual tissues were removed, packed into tared tubes, their wet weights recorded and counted on a dual channel LKB gamma counter. Data reductions were performed with the programme described by Flaim and coworkers (1984), adapted and modified to run on a VAX Series 7000 computer.

Several requirements, as defined by Hadengue *et al.* (1989), were met before using the data from an individual animal: (a) a difference of less than 10% between left and right kidneys, indicative of adequate microsphere mixing; (b) a stability in mean arterial blood pressure and heart rate between the pre- and post-injection periods.

Blood flow was expressed as net flow g^{-1} of wet tissue (ml min^{-1} g^{-1}). Cardiac output (CO) was calculated as follows: CO (ml min^{-1}) = radioactivity injected (c.p.m.)/ reference sample readioactivity (c.p.m.) × 0.68 (ml min^{-1}). Total peripheral resistance (TPR) was calculated according to the formula where:

TPR (dyn.s.cm^{-5}) = MABP (mmHg) × 80/CO (ml min^{-1}).

Treatment with platelet-activating factor antagonists

The PAF antagonists, which were soluble in saline, were administered via the jugular vein. Control animals received comparable volumes of saline. Either 3 mg kg^{-1} of the antagonist, WEB 2086 (3-(4-(2-chlorophenyl)-9-methyl-6H-thieno(3,2-f) (1,2,4)-thiazolo-(4,3-α) (1,4)-diazepine-2-yl)-1-(4-morpholinyl)-1-propanone; Boehringer-Ingelheim, Germany), or 20 mg kg^{-1} of BN 52021 (9H-1,7α-{epoxymethano}-1H,6αH-cyclopenta (c) furo-{2,3-β} furo-{3',2':3,4) cyclopenta {1,2-d} furan-5,9,12-{4H}-trione, 3-tert-butyl hexahydro-8-methyl; Institut Henri Beaufour, France) were administered 10 min before the determination of blood flows. Wallace and coworkers (1990) have shown that these concentrations of WEB 2086 and BN 52021 inhibit haemoconcentration induced by PAF (10 min infusion of 100 ng kg^{-1} min^{-1}).

Statistical analysis

Results are expressed as mean ± s.e.mean. To compare the variability between the three groups of animals, one-way analysis of variance was applied and the Student-Neuman-Keul procedure (Ott, 1989) was used to determined significance at the $P < 0.05$ level.

Results

Administration of allergen to sensitized rats produced increases in leakage of plasma proteins of up to 20 fold in the trachea, 5 fold in the jejunum or of only 30 to 50% in the pancreas and stomach (Figure 1). Both WEB 2086 and BN 52021 completely antagonized increases in plasma protein leak in the stomach, but only reduced the extravasation by 30 to 40% in the trachea and the small bowel. WEB 2086 blocked the permeability changes induced by anaphylaxis completely in the pancreas, although BN 52021 was ineffective.

The administration of WEB 2086 and BN 52021 themselves did not alter baseline MABP, HR, CO and TPR (Table 1) as measured prior to antigen challenge. Five min after induction of anaphylaxis MABP, HR and CO had decreased substantially whereas TPR (Table 2) was not affected to a statistically significant extent. Interestingly these two antagonists had contrasting effects on these cardiovascular parameters. WEB 2086 protected significantly against the drop in MABP and HR, but was ineffective in preventing the decrease in CO. The other antagonist, BN 52021, protected against the drop in CO but was ineffective in preventing the decrease in MABP or HR. No significant changes in TPR were noticed with the four groups of rats.

Anaphylactic shock produced marked and rapid reductions in blood flow to numerous tissues and organs with the decreases ranging from 50% in the thymus (not shown) to 75% in the adrenals and greater than 90% in the small bowel (e.g. jejunum in Figure 2). Whereas the pre-administration of WEB 2086 maintained adrenal blood flow at 75% of normal after anaphylaxis, this antagonist was ineffective in altering the marked reductions in blood flow to all other tissues. BN 52021, on the other hand, was effective in maintaining blood flow at a higher rate in the spleen and the gastrointestinal tract, although this antagonist did not affect the reduced blood flow to the adrenals. In a previous study (Mathison *et al.*, 1990b) we have shown that sensitization to the nematode parasite *per se* decreases blood flow to the stomach, jejunum and duodenum, and that this effect of sensitization was reversed by both WEB 2086 and BN 52021. The modification of blood flow to various organs induced by anaphylaxis appeared to be unrelated to changes in basal blood flow induced by the PAF antagonists.

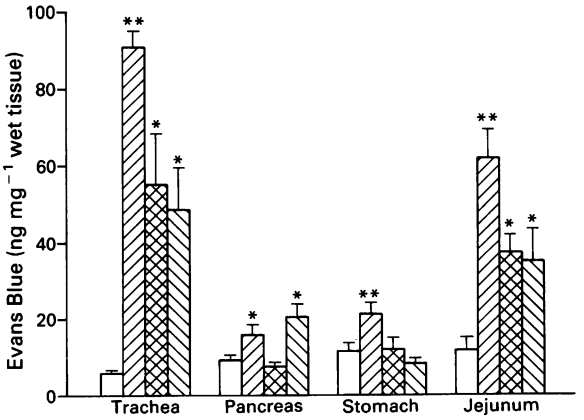


Figure 1 Effects of antigen challenge on the extravasation of Evans Blue in unsensitized (□; $n = 5$), and three groups of rats sensitized to *N. brasiliensis*; untreated (▨; $n = 6$), 3 mg kg^{-1} of WEB 2086 (▩; $n = 5$) and 20 mg kg^{-1} of BN 52021 (▧; $n = 5$). *Greater than unsensitized: $P < 0.05$; **greater than unsensitized, WEB 2086 and BN 52021; $P < 0.05$. Values are presented as means with s.e.mean shown by vertical bars.

Table 1 Effects of WEB 2086 (3mg kg^{-1}) and BN 52021 (20 mg kg^{-1}) on cardiovascular parameters of rats sensitized to *N. brasiliensis* before challenge with antigen

	MABP	*HR*	*CO*	*TPR*
Unsensitized	114.0 ± 6.0	345 ± 16	134.2 ± 25.4	86.5 ± 16.3
Sensitized	117.7 ± 3.2	352 ± 13	139.9 ± 22.6	77.9 ± 11.2
WEB 2086	114.0 ± 7.2	330 ± 6	133.7 ± 13.1	70.7 ± 6.5
BN 52021	110.0 ± 3.9	336 ± 8	149.4 ± 12.8	68.5 ± 4.7

Values are presented as means ± s.e.mean for 6 to 8 rats.
MABP: mean arterial blood pressure; HR: heart rate; CO: cardiac output; TPR: total peripheral resistance.

Table 2 Cardiovascular parameters measured 5 min after i.v. administration of worm antigen to unsensitized ($n = 8$), and three groups of rats sensitized to *N. brasiliensis*; (1) untreated ($n = 8$); (2) WEB 2086 3 mg kg^{-1} ($n = 6$) and (3) BN 52021 20 mg kg^{-1} ($n = 6$)

	MABP	*HR*	*CO*	*TPR*
Unsensitized	112.2 ± 6.7*	365 ± 15*	111.4 ± 12.6*	85.4 ± 8.9
Sensitized	75.5 ± 4.6	285 ± 12	53.4 ± 9.1	141.3 ± 24.0
WEB 2086	96.7 ± 9.5	390 ± 17*	76.0 ± 11.7	108.7 ± 12.9
BN 52021	68.2 ± 6.5	316 ± 16	98.1 ± 20.0*	68.5 ± 4.6

Values are presented as means ± s.e.mean.
Levels of significance: *$P < 0.05$, different from sensitized rats.
MABP: mean arterial blood pressure; HR: heart rate; CO: cardiac output; TPR: total peripheral resistance.

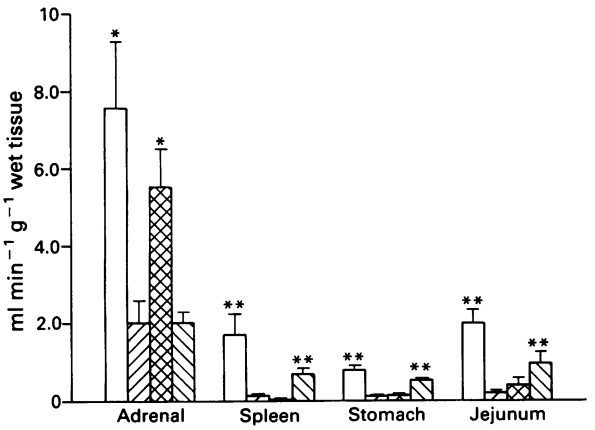


Figure 2 Effects of antigen challenge on blood flow in unsensitized (□; $n = 8$), and three groups of rats sensitized to *N. brasiliensis*; untreated (▨; $n = 8$), 3 mg kg^{-1} of WEB 2086 (▩; $n = 6$) and 20 mg kg^{-1} of BN 52021 (▧; $n = 6$).
*Greater than sensitized, untreated and BN 52021; $P < 0.05$; **greater than untreated and WEB 2086.
Values are presented as means with s.e.mean shown by vertical bars.

Discussion

The marked hypotensive actions of PAF in the rat (Feuerstein *et al.*, 1982; Siren & Feuerstein, 1989) suggest that this lipid is a probable candidate for mediator of systemic anaphylaxis in the rat. This suggestion was corroborated by the present findings in that two PAF antagonists (BN 52021 and WEB 2086), albeit somewhat differentially (see below), attenuated the permeability and cardiovascular changes associated with acute anaphylaxis in the Nb-sensitized rat.

The doses of PAF released early in the anaphylaxis in the rat appear to be substantial as low doses of PAF (1 nmol kg^{-1} or less), although hypotensive in action, decreased TPR consequent to a peripheral vasodilatation in the rat (Siren & Feuerstein, 1989). In the hands of these investigators a higher dose of PAF (3 nmol kg^{-1}) produced an increase in TPR and a decrease in cardiac index; characteristics similar to those seen during acute anaphylaxis in the Nb rat model.

Although exogenously administered PAF induces a rapid (within 5 min) increase in extravasation in the rat (Sanchez-Crespo *et al.*, 1982), the permeability changes that occur with anaphylaxis would appear to be mediated, at least in part, by agents other than PAF (possibly histamine or arachidonic acid metabolites) in some organs (e.g. trachea and jejunum), whereas in others (e.g. stomach) this lipid mediator accounted exclusively for the smaller increase in extravasation. The first indication of differential effects of WEB 2086 and BN 52021 on anaphylaxis-induced vascular responses was apparent in the pancreas, where WEB 2086 effectively blocked any increase in extravasation, whereas the other antagonist was without effect.

BN 52021, which modified neither blood flow to the adrenals nor affected MABP, re-established to a significant extent blood flow to the splanchnic organs. Associated with this increase in splanchnic blood flow an apparent decrease in TPR was also noted, although only CO was increased significantly. Siren & Feuerstein (1989) have reported that BN 52021 blocked the decrease in cardiac index induced by PAF and attenuated the mesenteric constrictor effect of 3 nmol kg^{-1} of PAF. Thus, BN 52021 may exert its protective effects against the haemodynamic changes associated with anaphylaxis by acting predominately on the splanchnic vascular beds. Although Siren & Feuerstein (1989) showed that BN 52021 at 15 mg kg^{-1} abolished the cardiac depressant effects of exogenous PAF, this antagonist at a slightly higher dose (20 mg kg^{-1}) did not affect anaphylaxis induced decreases in MABP or HR. These discrepancies cannot be readily explained.

It is tempting to speculate that the preservation of adrenal blood flow to the adrenals by WEB 2086, with a possible maintenance effect on the release of catecholamines into the circulation may have contributed to the protective effect of this antagonist against the anaphylactic decrease in MABP and HR. Zukowska-Grojec and coworkers (1985) have shown that the β-adrenoceptors play an important role in antagonizing PAF-induced hypotension, since propranolol attenuated the hypotensive effects of PAF. The increased cardiac contractility, possibly arising from increased circulating catecholamines, would contribute to the prevention of PAF-induced decreases in blood pressure. Nevertheless, WEB 2086 was unable to maintain splanchnic blood flow and an inevitable consequence of vascular pooling in this region is a

reduced cardiac output.

In a previous study (Mathison *et al.*, 1990b) we have shown that WEB 2086 and BN 52021 were equally effective in preventing decreases in blood flow to the stomach, duodenum and jejunum due to sensitization of the rats to the nematode parasite. The apparent dissociation of the effects of these antagonists on basal blood flow in sensitized animals compared with the differential inhibitory effects seen with them following antigen challenge suggest that complex mechanisms are responsible for haemodynamic alterations during anaphylaxis. Further studies are required to examine possible interactions between PAF and other vasoactive mediators during acute anaphylaxis. Anaphylaxis undoubtedly induces an efflux of water into the extravascular space with a consequent increase in tissue wet weight, which could be reflected in an apparent decrease in blood flow to the affected tissues. This consideration, nevertheless, would not contribute substantially to the differential effects of PAF antagonists noted in this study, since an absence of a correlation between the changes in plasma extravasation and blood flow was apparent.

In summary, the results indicate that the systemic haemodynamic changes induced by IgE-mediated systemic anaphylaxis in the rat are in part due to the actions of PAF. Although both WEB 2086 and BN 52021 are potent PAF antagonists in certain experimental systems, the dissociation of the antagonistic actions of these agents on cardiovascular haemodynamics and regional blood flow in an early phase of anaphylaxis suggest tht PAF mediates its cardiovascular actions by more than one mechanism.

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Biological activity and metabolism of 20-hydroxyeicosatetraenoic acid in the human platelet

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1 The cytochrome P-450 metabolite of arachidonic acid, 20-hydroxyeicosatetraenoic acid (20-HETE), was found to be a potent, dose-dependent inhibitor of platelet aggregation and inhibitor of thromboxane biosynthesis induced by arachidonic acid (IC_{50} 5.2 ± 1.5 μM), A23187 (IC_{50} 16.2 ± 5.4 μM), and U46619 (IC_{50} 7.8 ± 2.4 μM). 20-HETE did not inhibit thrombin-induced aggregation.

2 The human platelet metabolized 20-HETE to a series of novel metabolites formed by cyclo-oxygenase as well as lipoxygenase pathways. The structures of the metabolites were identified by mass spectrometry as 20-hydroxy-thromboxane B_2, 12,17-dihydroxyheptadecatrienoic acid, 12,20-dihydroxy-eicosatetraenoic acid, and 11,20-dihydroxyeicosatetraenoic acid.

3 The identification of the 11-hydroxy metabolite of 20-HETE suggests that 20-HETE is less efficiently cyclized to an endoperoxide intermediate by cyclo-oxygenase than is arachidonate.

4 Although some biological activity of 20-HETE may be related to competition with endogenous arachidonate for cyclo-oxygenase metabolism, the predominant mechanism of action of 20-HETE appears to be through antagonism of the prostaglandin H_2/thromboxane A_2 receptor.

Keywords: 20-Hydroxyeicosatetraenoic acid (20-HETE); cytochrome P450; mass spectrometry, platelets; cyclo-oxygenase metabolism; lipoxygenase metabolism; platelet aggregation

Introduction

It is now widely appreciated that arachidonic acid can be oxidatively converted into biologically active eicosanoids by three distinct metabolic pathways. The cyclo-oxygenase pathway yields prostaglandins and thromboxanes (Smith & Marnett, 1991). Various lipoxygenase pathways yield 5-, 12-, and 15-hydroxyeicosatetraenoic acids (HETEs) with the 5-lipoxygenase also catalyzing the formation of leukotriene A_4 which is subsequently converted into the biologically active leukotrienes (Samuelsson *et al.*, 1987). The cytochrome P-450 mono-oxygenase pathway yields regioisomeric-*cis*-epoxides as well as lipoxygenase-like products such as 12(**R**)-HETE and also ω-oxidized metabolites of arachidonic acid (Fitzpatrick & Murphy, 1989). Hepatocytes (Capdevila *et al.*, 1990), renal cortical, and medulla preparations (Morrison & Pascoe, 1981; Oliw *et al.*, 1981; Schwartzman *et al.*, 1986; Takahashi *et al.*, 1990) contain an active cytochrome P-450 which readily catalyzes the formation of 20-hydroxyeicosatetraenoic acid (20-HETE) as well as 19-hydroxyeicosatetraenoic acid (19-HETE). In addition, 20-HETE is a major metabolite of arachidonic acid formed within the resting polymorphonuclear leukocyte (PMN) following the addition of exogenous arachidonic acid (Hatzelmann & Ullrich, 1988). Recently, 20-HETE has been found to possess diverse biological properties, including the ability to inhibit rabbit renal medulla microsomal Na^+/K^+-ATPase (Carroll *et al.*, 1991), stimulate erythropoietin-dependent stem cell growth in human bone marrow (Abraham *et al.*, 1991), and constrict rat aortic rings (Escalante *et al.*, 1989). Interestingly, the vasoconstrictor properties of 20-HETE were found to be dependent upon further metabolic action of endothelial cyclo-oxygenase to yield 20-hydroxy-prostaglandin H_2 (PGH_2) (Schwartzman *et al.*, 1989). Furthermore, the endoperoxides/thromboxane receptor antagonist, SQ29548, was found to block the vascular reactivity of 20-HETE. These results suggest that the thromboxane receptor as well as metabolism of 20-HETE may play an important role in biological activities of this molecule. However, little is known about the metabolism and biological effects of 20-HETE on cells which are found in the blood, in particular the platelet which is known to have a thromboxane receptor as well as specific enzymes which can metabolize arachidonic acid including cyclo-oxygenase, thromboxane synthase, and 12-lipoxygenase (Marcus, 1978). We show here that 20-HETE can interact with platelets in three ways: (i) it can inhibit platelet aggregation induced by several, but not all agonists; (ii) platelets can metabolically transform 20-HETE via 12-lipoxygenase- as well as cyclo-oxygenase-dependent mechanisms into platelet inactive products; and (iii) it inhibits formation of thromboxane A_2 (TxA_2) during platelet activation.

Methods

Preparation of human platelets

Venous blood was obtained from adult human volunteers who had not received medication for a period of at least one week before collection. The blood was anticoagulated with acid citrate-dextrose anticoagulant (ACD; National Institutes of Health [NIH] formula A) and centrifuged for 15 min at 120 *g* at 25°C. The platelet-rich plasma (PRP) was separated and platelets isolated and washed in citrate buffer containing 100 nM PGE_1 and resuspended in Hank's balanced salt solution (HBSS) at $2-4 \times 10^8$ platelets ml^{-1} as previously described (Maclouf & Murphy, 1988).

Platelet aggregation measurements

Platelet suspensions (0.6 ml) were incubated for 2 min at 37°C with 20-HETE prior to application of stimuli which included arachidonic acid (1 μM), ionophore A23187 (0.1 μM), thrombin (0.1 u ml^{-1}) or U46619 (11α, 9α-epoxymethano-

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PGH_2, 0.5 μM), a prostaglandin H_2/thromboxane A_2 (PGH_2/TxA_2)-mimetic. For certain experiments PRP was incubated with aspirin (1 mM, 30 min) before the platelets were washed. For all experiments organic solvents were less than 0.1% final concentration in the platelet suspensions. Aggregation was monitored photometrically and was quantitated by measurement of light transmittance 3 min after the addition of the stimulus. In some experiments the platelet suspensions were centrifuged (1000 *g*) after aggregation for 3 min and supernatants frozen (− 20°C) for subsequent analysis of TxB_2 by enzyme immunoassay (Pradelles *et al.*, 1985).

To determine whether 20-HETE inhibition of platelet aggregation was dependent on lipoxygenase metabolism, aspirin-treated platelets were incubated with nordihydroguaiaretic acid (NDGA, 10 μM, 2 min) followed by 20-HETE (0.1 μCi [^{3}H]-20-HETE diluted to 30 μM with cold 20-HETE, 2 min) followed by U46619 (0.5 μM). Aggregation was monitored for 3 min after U46619 addition and then platelet incubations were stopped with 1 vol methanol and the metabolites analyzed by reverse phase high performance liquid chromatography (r.p.h.p.l.c.) as described below.

Adenosine 3':5'-cyclic monophosphate

(Cyclic AMP) measurements Washed platelets (2×10^9/ml) were incubated with 0.2 mM isobutylmethylxanthine (a phosphodiesterase inhibitor) and either PGE_1 (30 μM) or 20-HETE (30 μM) at 37°C for 30 s. Platelets were centrifuged at 250 *g* for 5 min and the cell pellet extracted twice with 1 ml ethanol. The ethanol was evaporated and residue was assayed for cyclic AMP by immunoassay (Gilman, 1970).

Identification of 20-HETE metabolites in platelets

Platelets (20 ml) were incubated with 0.02 μCi [^{3}H]-20-HETE diluted with cold 20-HETE (1 μM) for 2 min at 37°C. The incubation was stopped with 2 vol of ice cold methanol and the cells centrifuged at 2000 *g* for 10 min. The organic solvent was removed from the supernatant by rotary evaporation and the aqueous residue diluted with 2 vol of sodium acetate buffer (pH 4.0) and applied to a prewashed C_{18} solid phase extraction column (Analytichem International, CA, U.S.A.). The column was washed with distilled water and eicosanoids eluted with pure methanol. The methanol solvent was removed under reduced pressure and the eicosanoids redissolved in 1 ml 20% methanol/water for analysis by r.p.h.p.l.c.

R.p.h.p.l.c. was performed on an Ultramex 5 μm C_{18} column (250 × 4.6 mm) (Phenomenex, Torrance, CA, U.S.A.) by use of methanol/water/acetic acid (55:45:0.05 pH 5.7 adjusted with NH_4OH) at 1.0 ml min^{-1}. Gradient elution was carried out from 55–70% methanol (20 min), 70–80% methanol (15 min), and 80–100% methanol (5 min). Fractions were collected at 0.5 min intervals. In some experiments, the h.p.l.c. effluent was monitored for u.v. absorbance and ^{3}H with an online radioactive detector (Radiomatic, Tampa, FL, U.S.A.), with a Flow-Scint II (Packard Radiomatic, Tampa, FL) and a scintillation mixture in the ratio of 3:1 h.p.l.c. effluent to scintillation cocktail.

An aliquot of the radioactive fractions was derivatized to the pentafluorobenzyl ester, trimethylsilyl ether and analyzed by electron capture gas chromatograph-mass spectrometry (g.c.-m.s.) using methane as moderating gas (Strife & Murphy, 1984). Another aliquot of the radioactive fraction was derivatized with ethereal diazomethane, then *bis*(trimethylsilyl)trifluoroacetamide (BSTFA) and analyzed by electron ionization g.c.-m.s. The remainder of the radioactive fraction was reduced by use of 5% rhodium on alumina at room temperature for 3 min (Murphy & Sala, 1990) prior to conversion into the pentafluorobenzyl esters or methyl esters, and trimethylsilyl ethers for analysis by g.c.-m.s. The methyl esters of a radioactive dihydroxy metabolite fraction were further purified by normal phase (n.p.)-h.p.l.c. with a 5 μm silica column (250 × 4.6 mm, Ultramex, Phenomenox, CA, U.S.A.) with 2% isopropanol in hexane, 2 ml min^{-1} before analysis by electron ionization g.c.-m.s. G.c.-m.s. was performed on a Finnigan-MAT SSQ70 mass spectrometer with a 10 m DB-1 capillary column and injector held at 280°C. The column temperature was programmed from 100°C–300°C at 15°C min^{-1} using helium as carrier gas with a linear velocity of 10 m s^{-1}.

Materials

20-HETE (5,6,8,9,11,12,14,15-[^{3}H]20-HETE) (240 Ci $mmol^{-1}$) was obtained from Dupont-New England Nuclear. Synthetic 20-HETE and 19(S)-HETE were gifts from Dr J.R. Falck, University of Texas, Southwest Medical Center, Dallas, TX, U.S.A. All solvents employed were h.p.l.c. grade (Fisher) and other reagents were the highest grade commercially available. *Bis*(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Supelco. Rhodium (5%) on alumina powder was obtained from Fluka AG, Switzerland and pentafluorobenzyl bromide was obtained from Aldrich Chemical Co.

Results

Platelet-antiaggregatory properties of 20-HETE

At concentrations ⩽ 30 μM, 20-HETE had no detectable agonist activity on human platelets. However, it inhibited arachidonic acid-induced aggregation of platelets in a dose-dependent manner with an IC_{50} of 5.2 ± 1.5 μM (Figure 1a). 20-HETE also inhibited TxB_2 formation in a dose-dependent manner, reducing it from a control level of 308 ± 16 ng

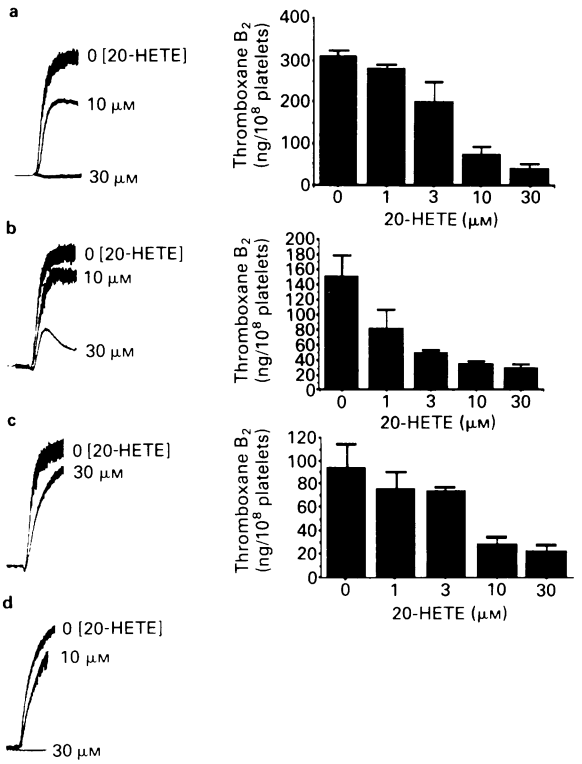


Figure 1 Effect of 20-HETE on aggregation and thromboxane B_2 (TxB_2) fraction by platelets stimulated by (a) 1 μM arachidonic acid; (b) 0.1 μM A23187; (c) 0.1 u ml^{-1} thrombin; (d) 0.5 μM U46619 (aspirin-treated platelets). Aggregation trace are from a single representative experiment. TxB_2 concentrations are a mean of three experiments; s.e. shown by vertical bars.

$TxB_2/10^8$ platelets to 41 ± 10 ng $TxB_2/10^8$ with 30 μM 20-HETE. The positional isomer, 19(S)-HETE was less potent, inhibiting platelet aggregation by only 23% at this concentration.

The A23187-induced aggregation was also inhibited by 20-HETE with an IC_{50} of 16.2 ± 5.4 μM with a parallel decline in TxB_2 production from 147 ± 27 to 28 ± 5 ng/10^8 cells (Figure 1b). Aggregation by the PGH_2/TxA_2 agonist, U46619, was inhibited by 20-HETE with an IC_{50} concentration of 7.8 ± 2.4 μM in aspirin-treated platelets (Figure 1d). In contrast to other agonists, thrombin-induced aggregation was only inhibited by 14% in the presence of 30 μM 20-HETE despite a 4 fold decrease in TxB_2 formation (Figure 1c).

There was no significant difference between the levels of cyclic AMP observed in resting and 20-HETE (30 μM)-treated platelets (Table 1) suggesting that elevation of platelet cyclic AMP was not the primary mechanism for its effects.

When aspirin-treated platelets were incubated with NDGA (10 μM) for 2 min before 20-HETE incubation (30 μM), U46619-induced platelet aggregation was completely inhibited (Figure 2). However, NDGA decreased production of the 20-HETE metabolite 12,20-diHETE by 80% (data not shown). This suggested that a 12-hydroperoxy intermediate was not responsible for 20-HETE inhibition of platelet aggregation. In contrast to platelets, 20-HETE had no effects on the neutrophil activation. At 30 μM it did not induce shape change, cause Ca^{2+} mobilization, or inhibit leukotriene B_4 (LTB_4)-induced calcium mobilization (Scanlon *et al.*, 1987).

Identification of platelet 20-HETE metabolites

When [^{3}H]-20-HETE (1 μM) was incubated with platelets for 2 min at 37°C, several metabolites were formed (Figure 3). Their relative concentration and extent of 20-HETE metabolism was dependent upon the initial concentration of 20-HETE, the number of platelets, and the time of incubation. In all incubations less than 2% of the added 20-HETE remained associated with the pellet after methanol extraction suggesting that it was not readily incorporated into platelet phospholipids.

Metabolite I (Figure 3) displayed no u.v. absorption above 210 nm. Electron capture g.c.-m.s. of the pentafluorobenzyl (PFB) ester trimethylsilyl (TMS) ether derivative of metabolite I (equivalent carbon value of 25.6) produced an abundant ion at m/z 673 (M-181) with additional ions at m/z 583 (M-181-90), m/z 493 (M-181-2(90)), and m/z 403 (M-181-3(90)). These results were consistent with a molecule having at least three hydroxyl groups and molecular weight of 386 daltons. The electron ionization of the methyl ester, trimethylsilyl ether derivative of metabolite I yielded a mass spectrum similar to that previously described for 20-hydroxy-TxB_2 (Powell, 1979), but with an equivalent carbon value of 26.6 This was lower than that previously measured (equivalent carbon value of 27.5) (Powell, 1979). Prominent ions were observed at m/z 598 (M-90), 508 (M-2(90)), 439 (loss of $\bullet(CH_2)_5OSiMe_3$ and $SiMe_3OH$), 389 ($Me_3SiO^+ = CH$-$CH = CH$-CH ($OSiMe_3$)-$(CH_2)_4$-CH_2OSiMe_3) 323, 313, 256 ($Me_3SiO^+ = CH = CH$-CH_2-$CH = CH$-$(CH_2)_3$-CO_2Me), 217, 129 and a base peak at m/z 73. It was concluded that metabolite I was 20-hydroxy-TxB_2 formed by the action of platelet cyclo-oxygenase on 20-HETE and subsequent action of thromboxane synthase on the intermediate 20-hydroxy-PGH_2.

Metabolite II displayed an ultraviolet spectrum with a λ_{max} 232 nm typical of a conjugated diene. Electron capture g.c.-m.s. of the PFB ester, TMS ether of metabolite II produced ions at m/z 439 (M-181), 349 (M-181-90) and 259 (M-181-2(90)) which were consistent with two hydroxy groups in the metabolite and a molecular weight of 296 daltons. Reduction of the metabolite increased the equivalent carbon value from 21.6 to 21.8 for the PFB ester TMS ether and resulted in an electron capture mass spectrum with prominent ions at m/z 445 (M-181), 355 (M-181-90), and 265 (M-181-2(90)) consistent with the reduction of three double bonds and the presence of two hydroxy groups within a C17 carboxylic acid. Electron ionization of the PFB ester TMS ether of

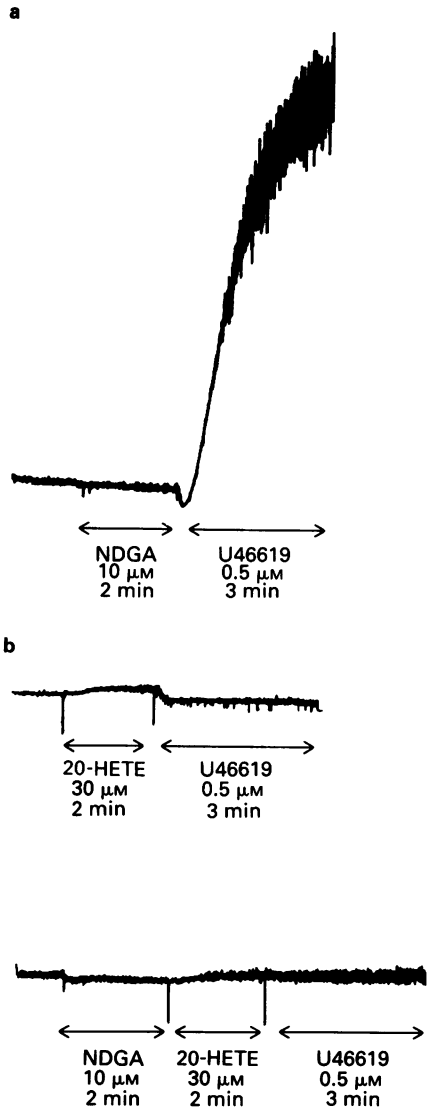


Figure 2 (a) Nordihydroguaiaretic acid (NDGA, 10 μM, 2 min) by itself has no effect on platelet aggregation induced by 0.5 μM U46619 as seen by the increase transmittance in light in the aggregometer during the 3 min incubation. (b) 20-HETE blocks U46619-induced aggregation. (c) NDGA does not block the antiaggregatory effect of 20-HETE although extraction of this incubation revealed inhibition of 12-lipoxygenase.

Table 1 Effect of 20-HETE on cyclic AMP levels in platelets[1]

Treatment	*Cyclic AMP (pmol/10^9 platelets)*
Control	4.8 ± 0.7
20-HETE (30 μM)	3.8 ± 0.1
PGE_1 (30 μM)	55.2 ± 7.7

[1]Platelets were incubated with 0.2 μM isobutylmethylxanthine and either prostaglandin E_1 (PGE_1) or 20-hydroxy-5,8,11,14-eicosatetraenoic acid (20-HETE) for 30 s at 37°C. Platelets were centrifuged and the cell pellet extracted with ethanol. Values are mean ± s.e. of 4 experiments.

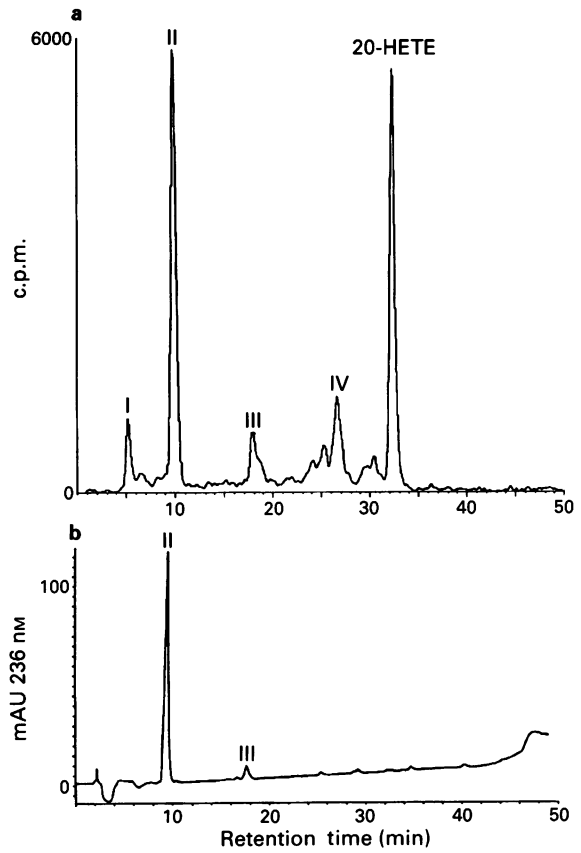


Figure 3 Reverse phase high performance liquid chromatography analysis of metabolites formed from the incubation of 20-HETE with human platelets. Platelets (20 ml) were incubated with 0.02 μCi [^{3}H]-20-HETE (1 μM) for 2 min at 37°C.

metabolite II produced ions at m/z 461 (which were observed at m/z 467 following reduction) consistent with α-cleavage at C-12 and loss of $\bullet(CH_2)_4CH_2OSiMe_3$, consistent with a C_{17} carboxylic acid. Electron ionization mass spectrometry of the trimethylsilyl ether methyl ester of metabolite II (equivalent carbon value 22.3) produced ions at m/z 454 ($M^{+\cdot}$), 295 (α-cleavage at C-12, loss of $\bullet(CH_2)_4CH_2OSiMe_3$) and m/z 313 (Figure 4a). Trimethylsilylation with [$^2H_9$]-BSTFA shifted m/z 313 to m/z 331 indicating that this ion contained two intact trimethylsilyl groups. Thus m/z 313 could correspond to a rearrangement ion suggested to be a stabilized 6-membered ring structure illustrated in Figure 5.

The electron ionization of the reduced molecule did not yield a corresponding ion (Figure 4b). However, abundant ions were observed for the methyl ester TMS derivative of reduced metabolite II (equivalent carbon value 22.6) at m/z 445 (M-15), at 429 (M-31), 413 (M-15-32), 355 (M-15-90), 301 (α-cleavage at C-12, loss of $\bullet(CH_2)_4CH_2OSiMe_3$), 261 (α-cleavage at C-12, loss of $\bullet(CH_2)_{10}COOCH_3$), and m/z 272 (rearrangement of the trimethylsilyl group to the carboxyl group (VanRollins & Murphy, 1984)). These data were consistent with the structure of metabolite II as 12,17-dihydroxy-5,8,10-heptadecatrienoic acid formed by the action of platelet cyclo-oxygenase on 20-HETE, followed by decomposition and loss of malonyldialdehyde from the cyclic endoperoxide intermediate compatible with the established mechanism for arachidonic acid (Hamberg & Samuelsson, 1974).

Electron capture g.c.-m.s. of PFB ester, TMS ether of metabolite III (equivalent carbon value of 23.7) resulted in abundant ions at m/z 479 (M-181) and 389 (M-181-90). The electron capture mass spectrum of the reduced molecule (equivalent carbon value of 24.9) produced ions at m/z 487 (M-181), 397 (M-181-90), and 307 (M-181-2(90)). These results are consistent with a dihydroxy metabolite having a molecular weight of 336 daltons. The methyl ester of metabolite III was further purified on n.p.-h.p.l.c. and resulted in the separation of two components with retention times of 30.9 (IIIA) and 32.4 min (IIIB) and u.v. absorption maxima at 234 and 232 nm, respectively. The retention times and u.v. spectra of these metabolites were identical to that

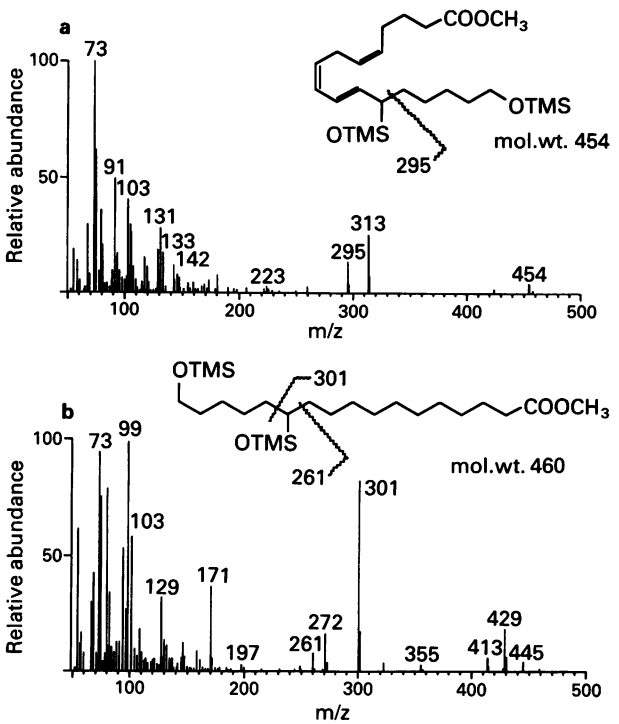


Figure 4 (a) Electron ionization (70 eV) mass spectrum of the methyl ester trimethylsilyl (TMS) ether of metabolite II. (b) Electron ionization (70 eV) mass spectrum of metabolite II following catalytic hydrogenation and derivatization as the methyl ester TMS ether.

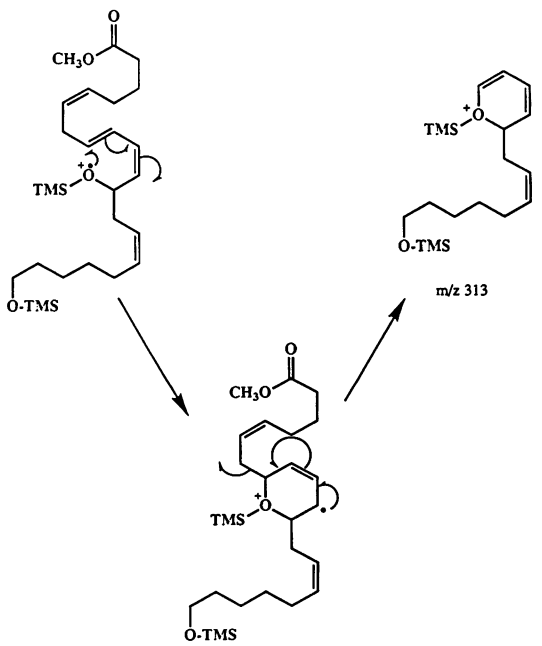


Figure 5 Mechanism of formation of ion m/z 313 in the electron ionization mass spectrum of methyl 12,17-trimethylsiloxyheptadecatrienoate (metabolite II).

for 12,20-diHETE and 11,20-diHETE respectively (authentic standards were synthesized from the incubation of 12-HETE and 11-HETE with human neutrophils).

Electron ionization g.c.-m.s. of the methyl ester TMS ether of IIIA (equivalent carbon value of 24.6) revealed ions at m/z 479 (M-15), 404 (M-90), 353 (M-141, loss of ($\bullet C_6H_{10}$-$COOCH_3$) and an abundant ions at m/z 295 (α-cleavage at C-12, loss of $\bullet CH_{12}CH_2OSiMe_3$, with a base peak at m/z 73. Reduction of IIIA (equivalent carbon value 25.4) resulted in major ions at m/z 303 (α-cleavage at C-12, loss of $\bullet(CH_2)_{10}COOCH_3$), 301 (α-cleavage at C-12, loss of $\bullet(CH_2)_7CH_2OSiMe_3$), and 272 (trimethylsilyl rearrangement to the carboxyl moiety). Both the electron ionization mass spectra of the native and reduced metabolite IIIA were identical to those described previously for 12,20-dihydroxy-eicosatetraenoic acid by Marcus *et al.* (1984), and had the same gas chromatographic retention parameter and u.v. spectra as 12,20-diHETE prepared by the incubation of 12-HETE with neutrophils in this laboratory.

Electron ionization of the methyl ester, trimethylsilyl ether of metabolite IIIB revealed ions at m/z 479 (M-15), 313 α-cleavage at C-11, loss of $\bullet C_9H_{14}COOCH_3$) 295 and 223 (313-90) (Figure 6a). The mass spectrum and the gas chromatographic retention value (equivalent carbon value 24.6) was exactly the same as that for the 11,20-diHETE standards prepared by the incubation of 11-HETE with neutrophils with the exception of the ion at m/z 295. This was most likely to be a residual contaminant of 12,20-diHETE identified as metabolite IIIA which was incompletely separated on the n.p.-h.p.l.c. system.

Reduction of IIIB (equivalent carbon value 25.4) followed by mass spectrometric analysis of the methyl ester trimethylsilyl ether derivative (Figure 6b) revealed ions at m/z 487 (M-15), 365 (M-90-47), 317 (α-cleavage at C-11, loss of $\bullet C_9H_{18}COOCH_3$) and a prominent ion at m/z 387 (α-cleavage at C-11, loss of $\bullet(CH_2)_7CH_2OSiMe_3$). An ion at m/z 258 would correspond to the rearrangement of trimethylsilyl group to carboxyl moiety (VanRollins & Murphy, 1984). With the exception of the contaminant ions at m/z 301 and 303 from the reduced 12,20-diHETE, this mass spectrum was identical to that of derivatized 11,20-diHETE. From h.p.l.c. and g.c. retention data, u.v. data, and mass spectral data, metabolite IIIB corresponds to 11,20-dihydroxy-5,8,12,14-eicosatetraenoic acid. Selected ion monitoring of the α-cleavage ions produced by the electron ionization of the methyl ester trimethylsilyl ether of the native diHETEs (m/z 295, 12,20-diHETE, m/z 11,20-diHETE) showed that both diHETEs were produced in similar concentrations in platelets. However, only 12,20-diHETE was produced in aspirin-treated platelets (data not shown) which confirmed 11,20-diHETE was a product of cyclo-oxygenase metabolism.

Insufficient material was available for further structural characterization of metabolite IV. None of the four metabolites had stimulatory or inhibitory effects on platelet aggregation.

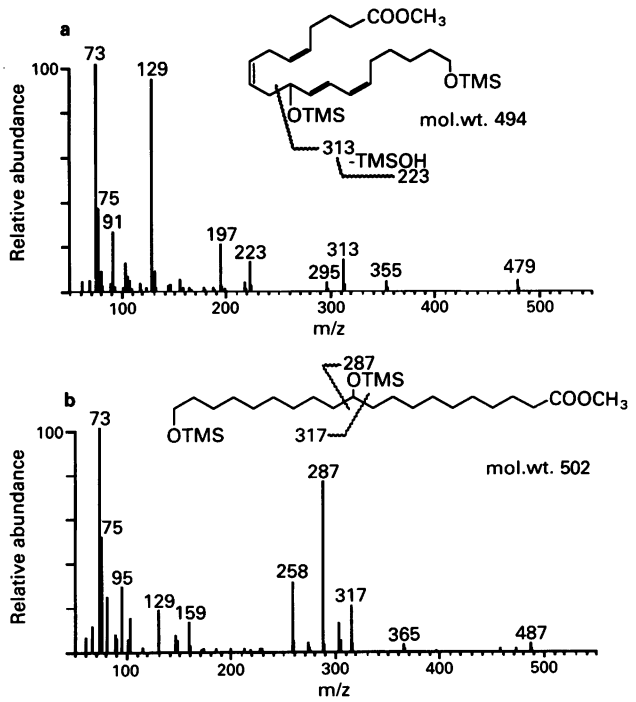


Figure 6 (a) Electron ionization (70 eV) mass spectrum of the methyl ester trimethylsilyl (TMS) ether of metabolite IIIB. (b) Electron ionization (70 eV) mass spectra of metabolite IIIB following catalytic hydrogenation and derivatization as the methyl ester TMS ether.

Discussion

Interest in the cytochrome P-450 pathway of arachidonic acid metabolism and specifically the formation of the ω-hydroxylation product, 20-HETE, stems from several reports of biological activities, especially in the renal system (McGiff, 1991). Reported renal activities for 20-HETE include inhibition of (Na^+/K^+)ATPase (Carroll *et al.*, 1991), effect on ion transport in the loop of Henle (Escalante *et al.*, 1991), and effects on natriuresis without affecting renal hemodynamics or glomerular filtration rate (Takahashi *et al.*, 1990). This metabolite also has potent vascular effects causing contractions of the aorta that are dependent upon metabolism of 20-HETE by cyclo-oxygenase (Escalante *et al.*, 1989). Interestingly 20-HETE has been found to be a stimulator of erythropoietin-dependent stem cell clonal growth in human bone marrow even at 10^{-11} M (Abraham *et al.*, 1991).

Biological effects of 20-HETE on the human platelet have not been previously reported. Human platelet aggregation induced by arachidonic acid, ionophore (A23187), and U46619 could be inhibited by 20-HETE in a dose-dependent manner. Importantly, thrombin-induced platelet aggregation was not inhibited. With each agonist, TxB_2 formation significantly declined in a dose-dependent manner to the amount of added 20-HETE, suggesting that one action of 20-HETE was to inhibit cyclo-oxygenase. However, cyclo-oxygenase inhibition by 20-HETE cannot completely account for platelet antiaggregatory activity since A23187-induced aggregation is independent of cyclo-oxygenase-catalyzed metabolism and phospholipase C (PLC)-catalyzed phosphoinositide hydrolysis (Levy-Toledano *et al.*, 1982). Inhibition of platelet aggregation by 20-HETE could not be blocked by aspirin even though cyclo-oxygenase was inhibited, further suggesting an independent mechanism. Inhibition of A23187-induced aggregation suggests that 20-HETE directly inibits [Ca^{2+}] increase in the platelet. The lack of inhibition of thrombin-induced aggregation suggests that phospholipase C is not inhibited because platelet activation by thrombin is associated with activation of phospholipase C and subsequent activation of phospholipase A_2. 20-HETE also inhibits U46619-induced aggregation in platelets which were treated with aspirin to block cyclo-oxygenase. Thus, antagonism of the PGH_2/TxA_2 receptor by 2-HETE could account for the inhibition of arachidonic acid and U46619-induced aggregation. Agonists that stimulate adenylate cyclase, such as PGE_1, inhibit aggregation induced by thrombin or ionophore. In this study, thrombin-induced aggregation was not inhibited by 20-HETE and 20-HETE did not increase cyclic AMP which argues against stimulation of adenylate cyclase as a mechanism for antiaggregatory action of this eicosanoid.

This platelet effect of 20-HETE resembles that of another group of cytochrome P-450 metabolites, the epoxyeicosatetraenoic acids (EETs) (Bernstrom *et al.*, 1991). However, inhibition of platelet aggregation by 20-HETE appears to be specific for this positional HETE isomer, in that no

significant antiaggregatory effect has been reported for other HETEs including 19(S)-HETE described here. In addition, 12,20-diHETE did not inhibit arachidonic acid-induced aggregation of platelets. It is important to note that 12-HPETE has been previously reported to inhibit platelet aggregation (Aharony *et al.*, 1982; Siegel *et al.*, 1979; Brune & Ullrich, 1991) and this effect has been found to involve an elevation of cyclic GMP (Grune & Ullrich, 1991). Our failure to block 20-HETE inhibition of platelet aggregation while inhibiting 12-lipoxygenase in the platelet with NDGA suggests that 20-HETE is not exerting its effect through the formation of a 12-hydroperoxide intermediate. However, such considerations did lead to a study of the metabolism of 20-HETE in the platelet described here.

Platelet metabolism of 20-HETE was rapid and extensive as outlined in Figure 7. Within 2 min over 50% of 20-HETE (1 μM) could be metabolized to cyclo-oxygenase (and thromboxane synthase) products such as 20-hydroxy-TxB_2, 12,17-dihydroxyheptadecatrienoic acid (12,17-diHHTrE) and 11,20-diHETE as well as the 12-lipoxygenase product 12,20-diHETE. The rapid metabolism of 20-HETE by cyclo-oxygenase and 12-lipoxygenase suggests that 20-HETE may effectively compete for metabolism with released arachidonic acid as substrates. This would explain, in part, the significant decrease in TxB_2 formation with increasing concentrations of 20-HETE when platelets were stimulated with arachidonic acid, ionophore, and thrombin. It is not known whether the unstable metabolites, 20-hydroxy-TxA_2 or 20-hydoxy-PGH_2, inhibit the pharmacological activity of TxA_2 and PGH_2 either at the TxA_2/PGH_2 receptor or by receptor desensitization associated with the mobilization of the intracellular stores of Ca^{2+}.

The 20-HETE metabolite, 11,20-diHETE, is likely to be formed as an initial intermediate in the cyclo-oxygenase catalyzed addition of oxygen to 20-HETE before the formation of a cyclic endoperoxide (Figure 7). Hamberg & Samuelsson (1967) showed that the initial step in the formation of prostaglandins from arachidonic acid was the steroespecific removal of a hydrogen at C-13 followed by the introduction of oxygen at C-11 in a radical centred process. The 11-hydroperoxy radical of 8,12,14-eicosatetraenoic acid can then be cyclized into an endoperoxide by attack of the C-11 hydroperoxy radical and cyclization to form the 9,11-dioxo bridge (Samuelsson, 1972). This leaves a carbon-centred radical at C-8 which can attack C-12 to generate a free radical at C-15 to which a second oxygen molecule attaches to give a hydroperoxy radical at C-15. There have been data to support an intermediate 11-hydroperoxy radical as shown by a peroxidase reduction product, 11-HETE, which has been found as a minor metabolite in some studies (Morrison & Crowley, 1984; Hemler *et al.*, 1978; Bailey *et al.*, 1983). However, it would appear that the rate of cyclization of the 11-hydroperoxy radical intermediate by cyclo-oxygenase may be compromised when 20-HETE is substrate, leading to

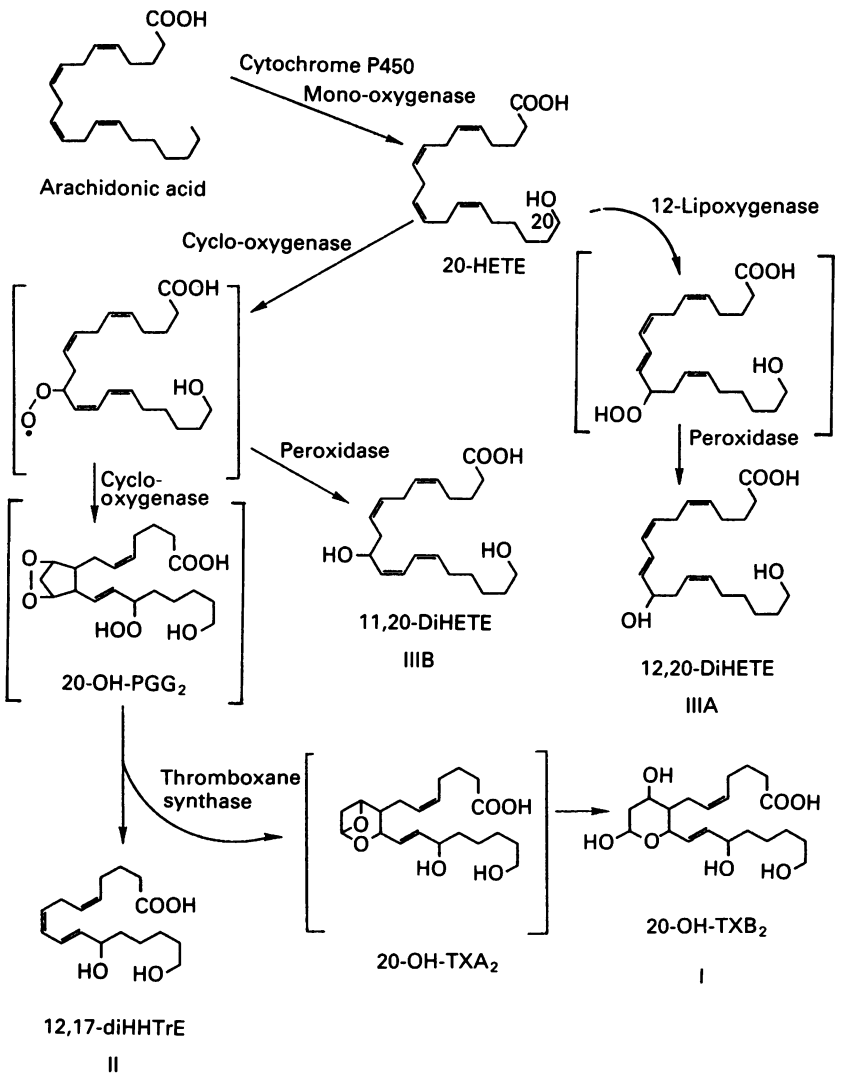


Figure 7 Metabolism of 20-HETE by platelet cyclo-oxygenase and 12-lipoxygenase pathways.

accumulation of a 11-hydroperoxy radical intermediate. This intermediate would be reduced by peroxidase to 11,20-diHETE.

In our laboratory, studies have shown that 20-HETE has no effect on neutrophil function as measured by shape change of neutrophil or alteration in intracellular free calcium concentrations. However, it is interesting to note that in the ionophore-stimulated neutrophil, 20-HETE serves as a substrate for 5-lipoxygenase and is rapidly metabolized to 5,20-dihydroxyeicosatetraenoic acid and to a lesser extent, 20-hydroxy-LTB_4 (data not shown).

Recent investigations have revealed 20-HETE to be a significant metabolite of arachidonic acid produced by various cell types. Isolated cells from the thick ascending loop of Henle in the kidney release 20-HETE following stimulation with arginine vasopressin (10^{-8} to 10^{-7} M) and calcitonin (10^{-9} to 10^{-8} M) (Schwartzman *et al.*, 1985). Hepatocytes as well as unstimulated neutrophils have also been found to transform exogenous free arachiconic acid into this metabolite (Hatzelmann & Ullrich, 1988; Capdevila *et al.*, 1990). Recently, 20-HETE biosynthesis was found to be elevated in the kidneys of young spontaneous hypertensive rats (Sacerdoti *et al.*, 1988). Collectively, these experiments suggest that this cytochrome P450 metabolite of arachidonic acid is produced by various cells and may escape into the circulation to exert a biological effect. The results of the experiments described here show that 20-HETE can be rapidly metabolized by the platelet but, if the platelet has been stimulated by certain agonists, 20-HETE may exert anti-aggregatory properties if present in sufficient quantities.

In summary, 20-HETE but not 19-HETE inhibits platelet aggregation, most probably by antagonism of the PGH_2/TxA_2 receptor. 20-HETE competes with endogenous arachidonic acid for cyclo-oxygenase and 12-lipoxygenase metabolism within the platelet. It is rapidly converted into 20-hydroxy-TxA_2 and the 12,17-dihydroxyheptadecatrienoic acid via 20-hydroxyendoperoxide intermediates which previously had been shown to be potent vasoconstrictors (Schwartzman *et al.*, 1989). Further investigations are under way to determine the amount of endogenous 20-HETE and metabolites produced during inflammatory reactions.

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Functional comparisons of gastrin/cholecystokinin receptors in isolated preparations of gastric mucosa and ileum

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1 The gastrin cholecystokinin (CCK) receptors mediating stimulation of acid secretion in rat isolated gastric mucosa (RGM) and contraction in guinea-pig isolated ileum longitudinal muscle-myenteric plexus (GPI) have been characterized by use of peptide agonists and the non-peptide antagonists, lorglumide, devazepide and L-365,260.

2 In RGM, gastrin peptides (sulphated gastrin heptadecapeptide (G-17), non-sulphated (ns) G-17 and pentagastrin) were potent agonists of acid secretion (EC_{50} values of 4.3, 16 and 27 nM respectively). Sulphated CCK octapeptide (CCK-8) was also a potent agonist, ($EC_{50} = 0.9$ nM), but was less efficacious, producing a lower maximal response. In contrast, in GPI, CCK-8 was a potent full agonist ($EC_{50} = 1.4$ nM) and was more than 1000 times more potent than the gastrin peptides in producing a sustained contractile response.

3 In GPI, CCK-8 (0.1 to 100 nM) produced sustained contractile responses, whilst CCK-4 (3 to 1000 nM) produced transient responses. These responses had different sensitivities to atropine (1 μM), suggesting that more than one receptor may mediate contraction in this tissue.

4 In RGM, L-365,260 was the most potent antagonist of pentagastrin-stimulated acid secretion ($pA_2 = 7.6$). This functional affinity estimate was similar to that for L-365,260 as an antagonist of excitatory responses in rat ventromedial hypothalamic slices (Kemp *et al.*, 1989) but differed from binding affinity estimates in guinea-pig cortex and gastric glands (Freidinger, 1989).

5 In GPI, devazepide, L-365,260 and lorglumide yielded different affinity estimates when compared against CCK-8 and CCK-4 or pentagastrin respectively. These studies were consistent with the view that the sustained response produced by CCK-8 was mediated by CCK_A receptors and the transient response produced by CCK-4 and pentagastrin was mediated by CCK_B receptors.

6 Affinity estimates for L-365,260 and lorglumide against CCK-4 or pentagastrin in GPI were significantly different from corresponding estimates against pentagastrin in RGM. These studies are consistent with the view that gastrin/CCK_B receptors in GPI may differ from those in RGM.

Keywords: Gastrin/CCK receptor subtypes; rat gastric mucosa; guinea-pig ileum; devazepide; L-365,260; lorglumide; pentagastrin; CCK-8; CCK-4

Introduction

Gastrin and cholecystokinin (CCK) share a high degree of structural homology and have identical pentapeptide sequences at their biologically relevant carboxyl terminal (Rehfeld, 1981). In the gastrointestinal tract, gastrin and CCK have distinct physiological roles. Gastrin stimulates gastric acid secretion and gastrointestinal mucosal cell growth, whilst CCK stimulates pancreatic amylase secretion, gallbladder contraction and modifies gastrointestinal motility (Walsh, 1987).

The different physiological actions of gastrin and CCK suggest that this peptide family mediates its effects via more than one receptor. Binding studies based on the affinities of gastrin/CCK peptides in pancreas and cortex have suggested the existence of two receptor types (Innis & Snyder, 1980); according to the nomenclature proposed by Moran *et al.* (1986), these are termed CCK_A and CCK_B receptors. CCK_A receptors have been demonstrated in pancreas (Innis & Snyder, 1980), gastrointestinal tissue and in discrete regions of the central nervous system (CNS) (Hill *et al.*, 1987). Functional studies of CCK_A receptors demonstrated marked differences in agonist activity, with sulphated CCK octapeptide (CCK-8) being more than 1000 times more potent than other gastrin/CCK peptides, including non-sulphated (ns) CCK-8 (Jensen *et al.*, 1982). Selective antagonists of CCK_A receptors have been identified and include devazepide (Chang & Lotti, 1986) and lorglumide (Makovec *et al.*, 1985).

CCK_B receptors are more widely distributed in the CNS (Innis & Snyder, 1980; Hill *et al.*, 1987) and recent studies in small cell lung cancer cell lines suggest that CCK_B receptors may also be located peripherally (Lin *et al.*, 1990). Comparisons of agonist potency ratios differ for CCK_A and CCK_B receptors with CCK-8 being less than 10 times more potent than other gastrin/CCK peptides at CCK_B receptors (Bohme *et al.*, 1988; Boden & Hill, 1988; Lin *et al.*, 1990). The benzodiazepine, L-365,260 (Lotti & Chang, 1989) and more recently, the dipeptoid PD134308 (Hughes *et al.*, 1990) and the pyrazolidinone LY26684 (Howbert *et al.*, 1991) have been described as potent and selective CCK_B receptor antagonists. Functional studies of acid secretion in dog parietal cells (Soll *et al.*, 1980) and smooth muscle contraction in guinea-pig dispersed fundus (Bitar *et al.*, 1982; Menozzi *et al.*, 1989) provide evidence for the existence of a third subtype, the gastrin receptor. In these studies, gastrin heptadecapeptide (G-17) and CCK-8 are equipotent, but in contrast to studies of CCK_B receptors (Bohme *et al.*, 1988; Boden & Hill, 1988; Lin *et al.*, 1900), CCK-4 is at least 300 times weaker than CCK-8 (Bitar *et al.*, 1982; Menozzi *et al.*, 1989). Binding studies using guinea-pig dispersed gastric glands yield affinity estimates for antagonists similar to those obtained for CCK_B receptor binding (Bock *et al.*, 1989) and this has led to the conclusion that gastrin and CCK_B receptors do not differ (Woodruff & Hughes, 1991).

Gastrin/CCK receptor classification has been decided lar-

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gely on the results of binding studies using homogenates of pancreas (CCK_A receptors), cerebral cortex (CCK_B receptors) and dispersed gastric glands (gastrin/CCK_B receptors) (Bock *et al.*, 1989; Hughes *et al.*, 1990). The purpose of the present study was to evaluate the activity of gastrin/CCK agonists and antagonists by use of functional responses in isolated preparations. The preparations used were chosen to reflect some of the actions of gastrin and CCK in the gastrointestinal tract. These were rat isolated gastric mucosa (RGM), where gastrin stimulates acid secretion and the longitudinal muscle-myenteric plexus preparation of guinea-pig isolated ileum (GPI) where CCK-8 induces contraction (Yau *et al.*, 1974).

Some of this work has been presented at meetings of the British Pharmacological Society (Spraggs & Patel, 1989; 1990). The results obtained in these studies with gastrin/CCK agonists and antagonists are consistent with the view that both CCK_A and CCK_B receptors mediate contraction in GPI. In addition, comparisons of antagonist affinities suggest that gastrin/CCK_B receptors in GPI may differ from those in RGM.

Methods

Rat isolated gastric mucosa

Acid secretion was measured from rat isolated gastric mucosa (RGM) prepared as described by Reeves & Stables (1985). Sprague-Dawley rats weighing 90–110 g were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.). Following laparotomy, the stomach was freed from connective tissue and the rumen was removed. The muscle layers overlying the fundic region of the stomach were separated from the mucosa by injection of saline (0.9% NaCl). The muscle layer was then cut and peeled back to expose the mucosa. A perfusion chamber (area = 1.54 cm^2) was placed in the stomach and the exposed mucosa was tied over the chamber. The chamber with attached mucosa was then removed and placed in a 20 ml organ bath which contained Krebs-Henseleit solution at 37°C gassed with 95% O_2 and 5% CO_2 (serosal bathing solution). The mucosal surface which faced into the perfusion chamber was superfused by means of a peristaltic pump (Watson Marlow Ltd) at a rate of 0.5 ml min^{-1} with an unbuffered solution of similar ionic composition at 37°C gassed with 100% CO_2 (mucosal bathing solution). The mucosal effluent was passed over a combination pH electrode (Russell) and pH recorded via a pH meter (Radiometer). All acid output values were converted from pH to nmol min^{-1} of hydrogen ion. The ionic compositions (mM) of the solutions were:- serosal: NaCl 118.5, $NaHCO_3$ 25.0, KCl 4.7, $MgSO_4$ 0.6, KH_2PO_4 1.2, $CaCl_2$ 1.3 and glucose 11.1. Indomethacin has been shown previously to enhance the responsiveness to secretagogues of the RGM (Reeves & Stables, 1985) and consequently was included in all experiments at a concentration of 3 μM. The composition of the mucosal solution was (mM) NaCl 144.5, KCl 4.7, $MgSO_4$ 0.6, $CaCl_2$ 1.3 and glucose 11.1

Responses to pentagastrin and other secretagogues were obtained by cumulative addition. In each preparation, a standard concentration-response curve to pentagastrin (1 to 300 nM) was constructed. For agonist studies this was followed by a concentration-response curve to another gastrin/CCK peptide. For antagonist studies this was followed by incubation of antagonist for 30 min, before a second concentration-response curve to pentagastrin was obtained. Preliminary experiments established that concentration-response curves to pentagastrin were repeatable, with no significant change in EC_{50} or maximal response.

Guinea-pig isolated ileum longitudinal muscle-myenteric plexus (GPI)

Male Dunkin-Hartley guinea-pigs weighing approximately 250 g were killed by cervical dislocation and 10 cm of terminal ileum was rapidly removed and placed in Krebs-Henseleit solution. A 3 cm length of ileum was placed over a glass rod and the longitudinal muscle with adherent myenteric plexus was removed with a cotton tip. This muscle strip was attached to an isometric force transducer and bathed in Krebs-Henseleit solution (serosal, see above). The tissue was washed, and adjusted to a resting tension of 0.8 g. Responses to sulphated cholecystokinin octapeptide (CCK-8), cholecystokinin tetrapeptide (CCK-4), pentagastrin and dimethylphenylpiperazinium (DMPP) were obtained by sequential addition with a contact time of 1 min and a cycle time of 5 min. Preliminary experiments established that concentration-response curves to these agonists could be reproduced. Therefore studies to investigate the effects of agonists and antagonists employed a protocol identical to that used in RGM.

Analysis of data

Responses in these preparations were normalised by expressing the data as a percentage of the maximum response in the control curve. EC_{50} values were determined graphically from individual experiments and are expressed as geometric mean with 95% confidence limits. For agonists, potency ratios (APR) were determined from the ratio of EC_{50} values for test and standard agonist (pentagastrin or CCK-8) in individual experiments. For antagonist studies, equieffective concentration-ratios (CR) were determined from EC_{50} values in the absence and following incubation of antagonist for 30 min. These concentration ratios were subjected to Schild analysis by linear regression to determine pA_2 and Schild slope values for antagonists.

Compounds

The various gastrin/CCK peptide fragments used in these studies were purchased from Peninsula Laboratories Ltd, Bachem Inc or Research Plus Inc. Pentagastrin was dissolved in dimethylsulphoxide (DMSO) at a concentration of 0.01 M. The other peptides were dissolved in 0.05 M NH_4HCO_3 at either 0.01 or 0.001 M. Aliquots of peptides were stored at −20°C and discarded after use.

The non-peptidic antagonists lorglumide, devazepide and L-365,260 ((R)-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-N'-(3-methylphenyl)-urea) were synthesised by the Chemistry Division, Glaxo Group Research Ltd. These compounds were dissolved in DMSO at concentrations of 0.1 M or less and subsequently diluted in Krebs-Henseleit solution or DMSO. The bath concentration of DMSO in all experiments did not exceed 1% and did not affect the responses to agonists in RGM or GPI. Histamine, dimethylphenylpiperazinium (DMPP), atropine and tetrodotoxin (TTX) were obtained from Sigma Chemical Co. and dissolved in saline. The peptidase inhibitors, bestatin and phosphoramidon, were obtained from Peninsula Laboratories Ltd and dissolved in saline. All solutions were added to organ baths in volumes of 1% or less.

Results

Agonist studies

Rat isolated gastric mucosa Rat isolated gastric mucosa (RGM) spontaneously secreted acid and reached a stable basal acid secretion rate of approximately 50 nmol H^+ min^{-1} after 90 min.

Following a priming concentration of pentagastrin (100

nM), subsequent concentration-response curves were reproducible with first and second curves producing EC_{50} values of 27 (15–49) and 21 (9–46) nM and maxima of 133 ± 34 and 136 ± 29 nmol H^+ min^{-1} ($n = 4$) respectively. The effects of gastrin/CCK peptides on acid secretion were determined in the presence of the peptidase inhibitors phosphoramidon (1 μM) and bestatin (100 μM) (Stephens-Smith *et al.*, 1988). These inhibitors did not modify responses to pentagastrin, producing a CR of 1.7 (0.9–3.3) and maximum responses of 137 ± 12 and 125 ± 8 nmol H^+ min^{-1} ($n = 4$) before and after peptidase inhibition. Figure 1 shows the effects of gastrin/CCK peptides on acid secretion and these data are summarized in Table 1. The human gastrins (G-17 and non-sulphated (ns) G-17) and pentagastrin were potent stimulants of acid secretion, whilst nsCCK-8 was weaker. CCK-8 had threshold effects similar to the gastrins but was a partial agonist with a maximum response of 29 ± 13% ($n = 4$) at a concentration of 10 nM. In addition, CCK-8 (100 nM) antagonized subsequent responses to pentagastrin at concentrations up to 1 μM (data not shown). CCK-4 was a weak partial agonist, with a maximum response similar to that observed with CCK-8.

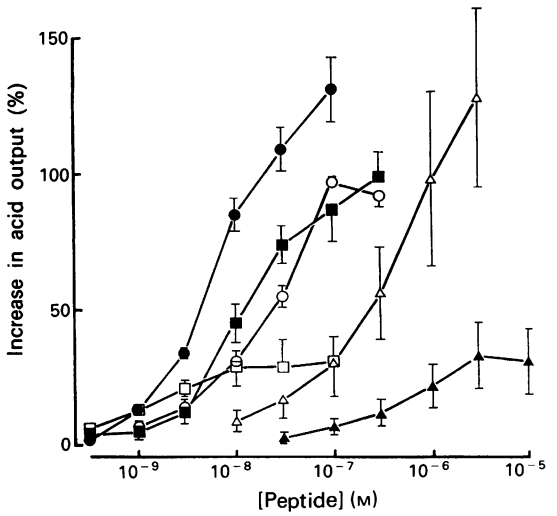


Figure 1 Effects of gastrin/CCK peptides on acid secretion in rat isolated gastric mucosa. Increases in acid secretion are expressed as percentages of the maximal response to pentagastrin in each tissue. For clarity, only one of the concentration-response curves for pentagastrin is shown and is representative of the others in this series of experiments. Values are mean (vertical bars show s.e.mean), $n = 4$ for the following peptides; G-17 (●), nsG-17 (■), pentagastrin (○), CCK-8 (□), nsCCK-8 (△) and CCK-4 (▲). For abbreviations, see text.

Table 1 Potencies of gastrin/CCK peptides in rat gastric mucosa (RGM) and guinea-pig ileum (GPI)

Agonist	*Rat gastric mucosa*	*Guinea-pig ileum*
CCK-8	0.9 (0.2–5)*	1.4 (0.9–2.1)
nsCCK-8	384 (34–4337)	>2000
CCK-4	613 (280–1341)*	31200 (17100–56900)
G-17	4.3 (3.6–5.1)	1170
nsG-17	16 (5–34)	>10000
Pentagastrin	27 (15–49)	>10000

Values are EC_{50} (in nM) expressed as geometric mean with 95% confidence limits ($n > 4$).
In RGM, some peptides produced maxima that were clearly smaller than the standard agonist (pentagastrin) and are denoted by *. In these cases the EC_{50} value is determined from individual 50% responses for that peptide.
In GPI, some agonists produced a biphasic contractile response (see Figure 2). The EC_{50} values for all the peptides in GPI were determined from the sustained component of the contractile response.
For abbreviations, see text.

Guinea-pig isolated ileum At all concentrations tested, CCK-8 produced a biphasic contractile response consisting of a transient 'spike' increase in tension followed by a sustained contraction which was maintained until washout of the compound (Figure 2). In contrast the contractile response profile to CCK-4 differed from that observed for CCK-8 (Figure 2). CCK-4 (3–1000 nM) stimulated a monophasic contraction which was not sustained, unlike the biphasic contractile response of CCK-8. At higher concentrations of CCK-4 (> 10 μM) a biphasic contraction, similar to that produced by CCK-8 was observed. The mechanism of responses to both of these agonists were investigated further by use of tetrodotoxin (TTX) and atropine. CCK-8 (0.1–100 nM) produced sustained contractions of GPI with an EC_{50} value of 1.4 (0.9–2.1) nM and a maximum increase in tension of 1.28 ± 0.06 g at 100 nM ($n = 6$). These responses were reproducible with a second CCK-8 curve producing an EC_{50} value of 1.9 (1.4–2.6) nM and a maximal increase in tension of 1.45 ± 0.10 g at 100 nM. Tetrodotoxin (300 nM) inhibited the sustained response to CCK-8 by 93%, whilst atropine (1 and 10 μM) reduced this response by only 50% (Figure 3a). These results suggested that CCK-8 induced a sustained contraction indirectly, via a neuronal mechanism, which was partly mediated by release of acetylcholine acting at muscarinic receptors. CCK-4 (1 nM–3 μM) produced transient concentration related increases in tension with an EC_{50} of 49 (40–61) nM and a maximal response of 0.67 ± 0.07 g. Concentration-response curves for CCK-4 were reproducible in individual tissues (CR = 1.29, E_{max} for curve 2 = 0.65 ± 0.05g). Both TTX (300 nM) and atropine (1 μM) abolished responses to CCK-4 (Figure 3b) suggesting that neuronally released acetylcholine was the sole mediator of the effect of CCK-4 in GPI. Since this mechanism differed from that for CCK-8, it was possible that the gastrin/CCK receptor responsible for the contractile effect of CCK-4 was different and this was investigated further with the antagonists lorglumide, devazepide and L-365,260 (see later).

CCK-8, nsCCK-8, CCK-4 and the gastrin peptides were tested as agonists in GPI by measuring their effects on the sustained portion of the response in the presence of the peptidase inhibitors phosphoramidon (1 μM) and bestatin (100 μM). Preliminary studies in 6 preparations showed that these inhibitors did not modify concentration-response curves to CCK-8, (CR = 1.1 (0.6–1.8) $n = 6$). A striking difference in agonist potencies was observed, with CCK-8 being more than 1000 times more potent than the other agonists (Figure 4, Table 1).

Antagonist studies

Rat isolated gastric mucosa The effects of gastrin/CCK receptor antagonists on pentagastrin stimulated acid secre-

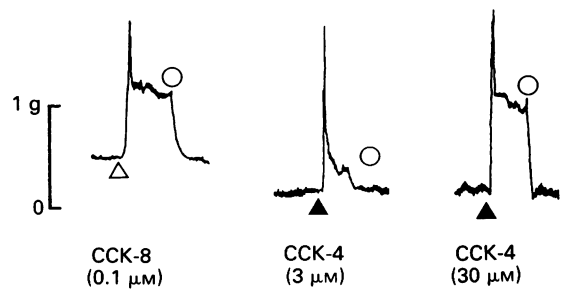


Figure 2 Tracings of representative responses to CCK-8 (10 nM) and CCK-4 (3 μM and 30 μM) in guinea-pig ileum. Peptides were added at time points (△ and ▲) and were removed from the bath following a 60 s contact time (o). CCK-8 produced a biphasic contraction with a sustained phase that was maintained until washout. In contrast, CCK-4 (3 μM) produced only a 'spike' contraction which returned to basal levels before washout. Higher concentrations of CCK-4 (30 μM) produced a response that resembled CCK-8.

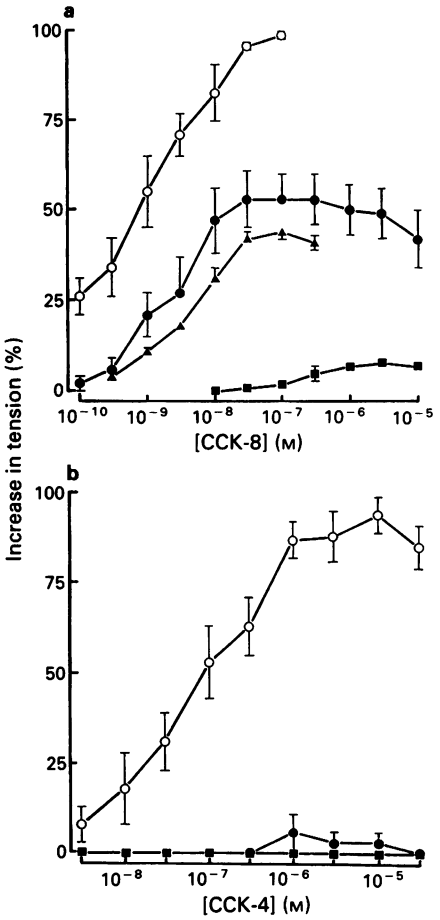


Figure 3 Effects of tetrodotoxin and atropine on contractile responses to CCK-8 sustained response (a) and CCK-4 transient response (b) in guinea-pig ileum. The format of these graphs is similar to Figure 3. Values are mean (vertical bars shown s.e.mean), $n = 4$ for the following treatments; CCK-8 or CCK-4 alone (o,). CCK-8 or CCK-4 in the presence of tetrodotoxin (300 nM, ■) or atropine (1 μM, ● and 10 μM, ▲). For abbreviations, see text.

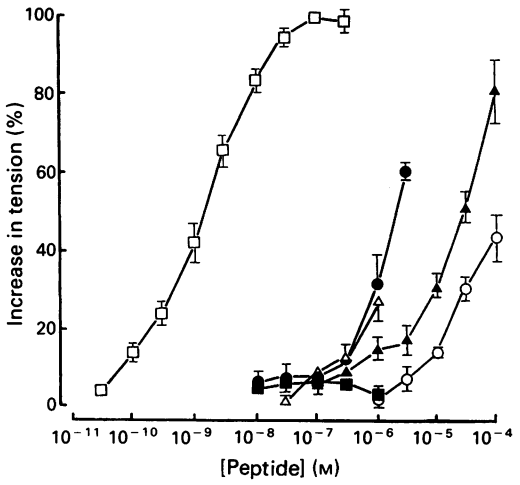


Figure 4 Contractile responses to gastrin/CCK peptides in guinea-pig isolated ileum. Sustained increases in tension were expressed as percentages of the maximal response to CCK-8 in each tissue. For clarity, only one of the concentration-response curves for CCK-8 is shown and is representative of others in this series of experiments. Values are mean (vertical bars show s.e.mean), $n = 4$ to 6, for the following peptides: CCK-8 (□), CCK-4 (▲), G-17 (●), nsCCK-8 (△), nsG-17 (■) and pentagastrin (○). For abbreviations, see text.

tion in RGM are summarised in Table 2. Lorglumide (10–100 μM) produced concentration-related rightward displacements of pentagastrin responses (Figure 5a). At a concentration of 10 μM, lorglumide increased the maximal response to pentagastrin (154 ± 28%, $n = 4$), but at the higher concentration of 100 μM a reduction in the maximum response was observed. Schild analysis yielded a slope value which was significantly greater than unity (Table 2). The steep Schild slopes encountered with this antagonist in RGM did not appear to be due to loss of antagonist from the biophase by enzymic degradation since repeating these experiments in the presence of phosphoramidon (1 μM) and bestatin (100 μM) and in silane-coated organ baths did not modify the antagonist profile or potency of lorglumide (data not shown).

The benzodiazepine analogue, L-365,260 (0.1–3 μM) was the most potent antagonist of pentagastrin encountered in these studies (Figure 5b) and, unlike lorglumide, was a competitive antagonist with a Schild slope value not significantly different from unity (Table 2). In contrast, the apparent affinity of the CCK_A receptor antagonist devazepide was approximately 50 times less potent than L-365,260 (Figure 5c, Table 2).

Secretory responses to pentagastrin in RGM were inhibited by ranitidine (0.1–10 μM) in an insurmountable manner (data not shown), suggesting that these responses were indirect, mediated via the release of histamine. Therefore, the selectivity of gastrin receptor antagonists in RGM was investigated with histamine used as a secretagogue. Histamine (1–300 μM) stimulated large increases in acid secretion

Table 2 Estimates of apparent affinity for antagonists in rat gastric mucosa (RGM) and guinea-pig ileum (GPI)

		Antagonist affinities[a]		
Preparation	*Agonist*	*Devazepide*	*L-365,260*	*Lorglumide*
RGM	Pentagastrin	5.77 ± 0.30	7.62 ± 0.10	5.47 ± 0.21
			(1.11 ± 0.11)	(1.39 ± 0.28*)
GPI	CCK-8	10.40 ± 0.30	7.72 ± 0.33	7.50 ± 0.13
	(sustained)	(0.94 ± 0.13)	(1.14 ± 0.24)	(1.50 ± 0.13*)
GPI[b]	CCK-4	<7.3	8.35 ± 0.26	6.47 ± 0.20
	(transient)		(1.17 ± 0.22)	(1.16 ± 0.21)
GPI	Pentagastrin	<7	8.84 ± 0.24	6.39 ± 0.08
	(transient)		(1.29 ± 0.21)	(1.71 ± 0.15*)

[a]pA_2 and Schild slope values (in parentheses), shown as mean ± s.e.mean, were derived from linear regression of the respective Schild plots, $n = 12$ to 15. Slope values marked * were significantly greater than unity. The values without slope values are pK_B estimates derived from a single concentration using the Gaddum equation ($n = 4$ to 12).
[b]Except for devazepide itself, these experiments were performed in the presence of devazepide (100 nM).

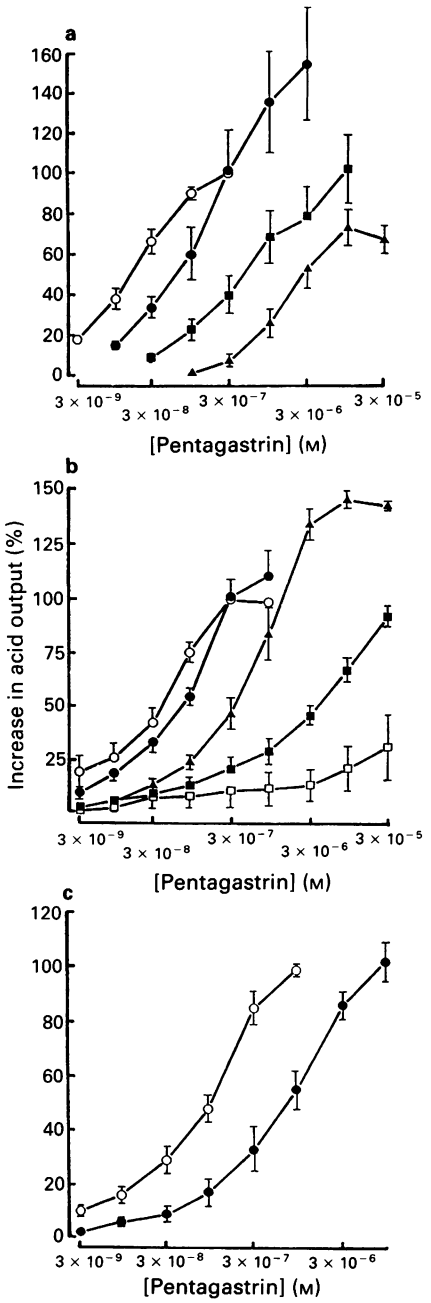


Figure 5 Effects of gastrin/CCK receptor antagonists as inhibitors of pentagastrin-stimulated acid secretion in rat isolated gastric mucosa. The format of these graphs is similar to Figure 1. Values are mean (vertical bars s.e.mean), $n = 4$. In (a) are shown the effects of lorglumide at 10 μM (●), 30 μM (■) and 100 μM (▲); (b) shows the effects of L-365,260 at 0.01 μM (●), 0.1 μM (▲), 1 μM (■) and 10 μM (□); (c) shows the effect of devazepide at 10 μM (●). Representative examples of the pentagastrin concentration – response curves from each of these series of experiments are shown (○).

with an EC_{50} value of 29 (20–44) μM and a maximum of 355 ± 83 nmol H^+ min^{-1} ($n = 4$). These responses to histamine could be repeated in single tissues with no significant change in EC_{50} (CR = 1.4 (0.5–3.8)) or maximum. Lorglumide (100 μM) produced a small displacement of the histamine responses (concentration-ratio = 8) and some depression of maximum ($E_{max} = 68 \pm 17\%$, $n = 4$) which may account for its insurmountable antagonist activity when pentagastrin was used as agonist. A high concentration of L-365,260 (10 μM) produced a nine fold displacement and a reduction in the maximum response to histamine ($E_{max} = 58 \pm 12\%$, $n = 4$).

Guinea-pig isolated ileum The effects of the gastrin/CCK antagonists against CCK-8 induced sustained contractions in GPI are summarized in Table 2. Lorglumide produced concentration-related displacements in concentration-response curves for CCK-8 with a pA_2 value of 7.50 and a Schild slope value of 1.50 which was significantly greater than unity (Figure 6a, Table 2). Devazepide (0.3–30 nM) was extremely potent and competitive antagonist of CCK-8 in GPI (Figure 6c, Table 2). L-365,260 (0.1–3 μM) was also a competitive antagonist of CCK-8, but was approximately 300 times less potent than devazepide (Figure 6d, Table 2).

In contrast to its activity against CCK-8 in GPI, the CCK_A receptor antagonist, devazepide (100 nM) was a weak inhibitor of CCK-4-induced transient contractions (Table 2, Figure 7b). Preliminary experiments demonstrated that concentrations of CCK-4 greater than 30 μM produced a res-

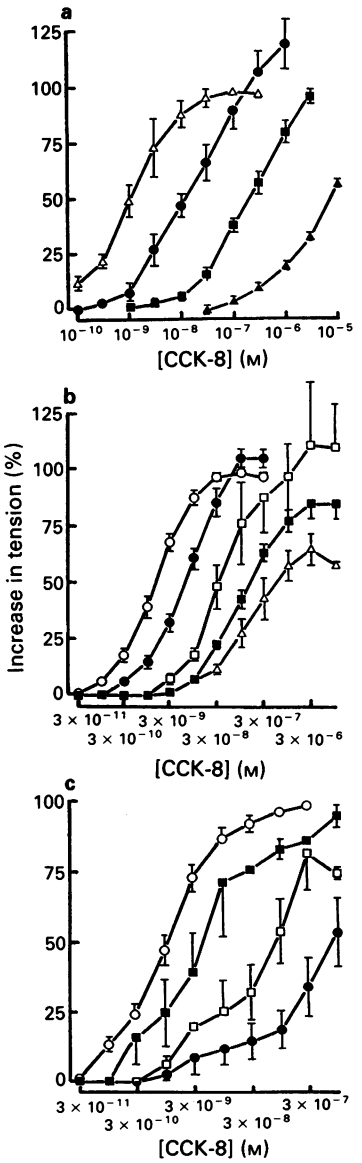


Figure 6 Effects of gastrin/CCK receptor antagonists as inhibitors of CCK-8-induced sustained contractions in guinea-pig isolated ileum. The format of these graphs is similar to Figure 3. Values are mean (vertical bars show s.e.mean), $n = 4$ to 6. In (a) is shown the effects of lorglumide at 0.1 μM (●), 1 μM (■) and 10 μM (▲); (b) shows the effects of L-365,260 at 0.1 μM (●), 0.3 μM (□), 1 μM (■) and 10 μM (△); (c) shows the effects of devazepide at 0.3 nM (■), 3 nM (□) and 30 nM (●). Representative examples of control CCK-8 concentration-response curves are shown for each series of experiments (○, △).

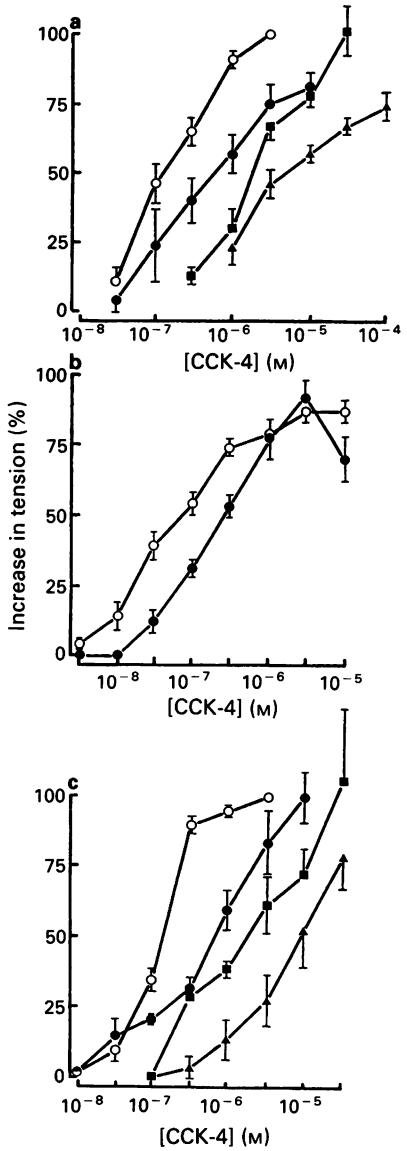


Figure 7 Effects of lorglumide, devazepide and L-365,260 as inhibitors of CCK-4-induced contraction in guinea-pig isolated ileum. The format of these graphs is similar to Figure 3. Values are mean (vertical bars show s.e.mean), $n = 3$ in (a), $n = 12$ in (b) and $n = 5$ in (c). In (a) is shown the effects of lorglumide at 1 μM (●), 3 μM (■) and 10 μM (▲); (b) shows the effects of devazepide (0.1 nM, ●); (c) shows the effects of L-365,260 at 30 nM (●) 100 nM (■) and 300 nM (▲). Experiments with lorglumide and L-365,260 were performed in the presence of devazepide (100 nM) to exclude interference from CCK_A-mediated contractions. Control CCK-4 curves are shown (○).

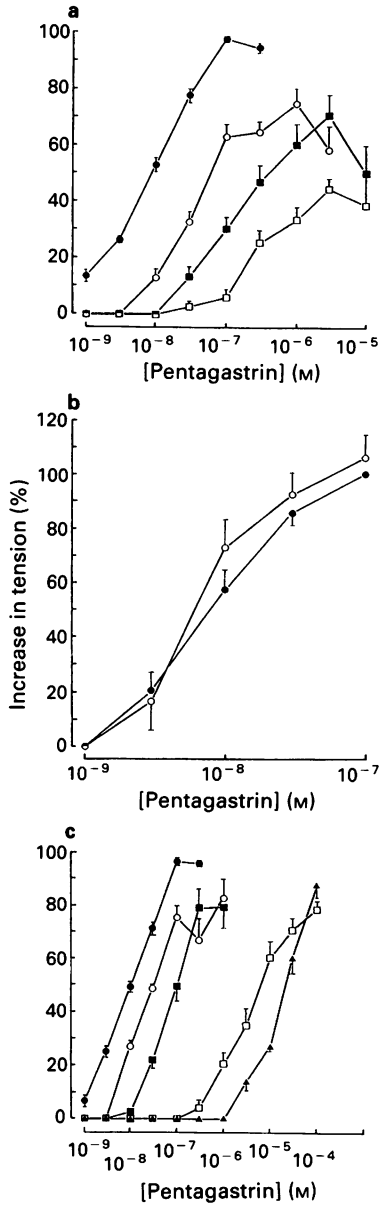


Figure 8 Effects of lorglumide, devazepide and L-365,260 as inhibitors of pentagastrin-induced transient contraction in guinea-pig isolated ileum. The format of these graphs is similar to Figure 3. Values are mean (vertical bars show s.e.mean), $n = 4$. In (a) is shown the effects of lorglumide at 1 μM (○), 3 μM (■) and 10 μM (□); (b) shows the effects of devazepide at 0.1 μM (○); (c) shows the effects of L-365,260 at 3 nM (○), 10 nM (■), 30 nM (□) and 100 nM (▲). Control pentagastrin curves are shown (●).

ponse profile that resembled CCK-8 (Figure 2). Therefore the potential for interference of CCK_A receptor-mediated effects on the transient responses to CCK-4 was excluded by inclusion of devazepide (100 nM) in the bathing medium. In the presence of devazepide, L-365,260 (30–300 nM) was a potent antagonist of CCK-4 (Figure 7c, Table 2) and Schild analysis of these data yielded a pA_2 value of 8.35 and a Schild slope of 1.17 which was not significantly different from unity. Under these conditions lorglumide was also a competitive antagonist of CCK-4 (Figure 7a, Table 2) with a pA_2 value of 6.47 and a Schild slope of 1.16.

In contrast to CCK-4, pentagastrin (1 nM to 100 μM) produced only transient contractile responses, the sustained response being absent throughout the concentration-range. Therefore antagonist affinities of devazepide, L-365,260 and lorglumide (without pretreatment of devazepide) were also determined in GPI against pentagastrin as agonist (Figure 8, Table 2). In these studies, affinity estimates not significantly different from those using CCK-4 (in the presence of devazepide, 100 nM) were obtained.

The specificity of antagonists in GPI was determined by use of the nicotinic agonist, DMPP. Contractile responses to DMPP were inhibited by TTX and atropine by greater than 80% (data not shown) suggesting that the majority of the effect of DMPP was mediated via neuronal release of acetylcholine. At a high concentration of 100 μM, lorglumide abolished the contractile effects of DMPP (data not shown). This non-selective action may account for the increasing antagonist activity and maximal depression seen with lorglumide as an antagonist of CCK-8 (Table 2). Devazepide and L-365,260 appeared selective antagonists of CCK-8 and CCK-4 responses in GPI since they did not modify responses to DMPP at a concentration 300 times their respective pA_2 values (data not shown).

Discussion

Gastrin/CCK receptors have largely been characterized by the affinities of agonists and antagonists in binding studies using parietal cells, gastric glands, and homogenates of brain cortex or pancreas (Makovec *et al.*, 1986; Bock *et al.*, 1989; Hughes *et al.*, 1990). However, different estimates of receptor binding affinity for these antagonists have been observed in other studies (Hill & Woodruff, 1989; Huang *et al.*, 1989). In the present studies we have attempted to characterize gastrin/CCK receptors by determining the apparent affinities of gastrin/CCK receptor antagonists in functional studies using isolated preparations of rat gastric mucosa (RGM) and guinea-pig ileum longitudinal muscle myenteric plexus (GPI).

Prior to investigating the actions of antagonists in these preparations, information on the gastrin/CCK receptors that may mediate the responses was gained by comparing the agonist potencies of a range of peptides from the gastrin/CCK family and these studies are summarized in Table 1. In RGM, CCK-8 and CCK-4 were partial agonists, with a lower maximal response than the gastrin peptides. The differences in maximal responses encountered in RGM make direct comparisons of agonist potency-ratios for gastrin/CCK peptides in other tissues difficult and such comparisons should be interpreted with caution. With this caveat, the difference in potency in RGM between CCK-8 and CCK-4 (680 fold, Table 1) suggests that this response resembled those mediated by gastrin-like receptors (Soll *et al.*, 1981; Bitar *et al.*, 1982; Menozzi *et al.*, 1989) more than those mediated by CCK_B receptors, where CCK-8 is less than 10 times more potent than CCK-4.

The rank order of potency for agonists obtained in GPI differed from RGM, with sustained contractions to CCK-8 being greater than 1000 times more potent than the other peptides. The rank order of agonist potency for this response in GPI resembled that described for stimulation of amylase release in guinea-pig dispersed pancreatic acini (Jensen *et al.*, 1980; 1982) and is generally consistent with the sustained contraction in GPI being mediated by CCK_A receptors.

Non-peptidic antagonists with reported affinity for gastrin/CCK receptors (Makovec *et al.*, 1985; Bock *et al.*, 1988) were used to characterize further the actions of this peptide family in RGM and GPI (Table 2). In GPI, where sustained contractile responses to CCK-8 were measured, devazepide was the most potent antagonist with an apparent affinity estimate similar to that reported previously in this preparation (Chang & Lotti, 1986). L-365,260 was also a competitive antagonist, but was approximately 300 times less potent than devazepide. These effects were consistent with the view that CCK-8-induced sustained contractions of GPI are mediated by CCK_A receptors. The pA_2 value for lorglumide further supported this view (Makovec *et al.*, 1985).

Although CCK-4 was approximately 3000 times less potent than CCK-8 in producing a sustained contraction in GPI, the tetrapeptide was only approximately 50 times less potent than CCK-8 in producing a transient contraction. This latter relative potency in GPI was not consistent with an action solely at CCK_A receptors (Jensen *et al.*, 1982) and the effects of CCK-4 were investigated further. The present studies confirmed previous observations that the responses to CCK-8 in GPI were complex and mediated only in part by neuronal acetylcholine release (Hutchison & Dockray, 1981; Chang *et al.*, 1984). In contrast, transient responses to CCK-4 appeared to be mediated solely by neuronal acetylcholine release, possibly suggesting activity at a different receptor. When CCK-4 or pentagastrin were used as agonists instead of CCK-8 and transient contractions measured, lorglumide, devazepide and L-365,260 produced different antagonist affinity estimates. The most striking difference was a loss of potency of more than 3000 fold when devazepide was used as an antagonist against CCK-4 and pentagastrin. This suggested that the contractile effects of CCK-4 and pentagastrin were not mediated by CCK_A receptors and were more consistent with activity at gastrin/CCK_B receptors (Chang & Lotti, 1986; Freidinger, 1989). Similar observations have recently been made by others (D.G. Trist, personal communication; Lucaites *et al.*, 1991).

In RGM, L-365,260 was the most potent antagonist of pentagastrin-stimulated acid secretion. In agreement with previous studies (Black *et al.*, 1985) pentagastrin-stimulated responses were sensitive to blockade by histamine H_2 receptor antagonists suggesting that pentagastrin stimulates acid secretion indirectly, via release of histamine. In the present studies, L-365,260 was a less potent antagonist of histamine-induced acid secretion, possessing a 100 fold selectivity for gastrin over histamine H_2 receptors. L-365,260 was approximately 50 times less potent in RGM than would have been predicted by its binding affinity in guinea-pig gastric glands and at CCK_B receptors in guinea-pig cortex (Bock *et al.*, 1989). Furthermore, the affinity estimates for L-365,260 and lorglumide versus pentagastrin in RGM were significantly different from corresponding estimates against pentagastrin in GPI ($P<0.05$ by no overlap of confidence limits) consistent with the view that responses in RGM are mediated by a receptor different from the gastrin/CCK_B receptor type mediating transient contraction in GPI.

Agonist potency-ratios have supported the view that gastrin and CCK_B receptors are distinct (e.g. Bohme *et al.*, 1988; Menozzi *et al.*, 1989). However, binding studies comparing affinity estimates of gastrin/CCK antagonists in guinea-pig cortex and gastric glands have been unable to disclose differences in affinity in these two preparations (Bock *et al.*, 1989; Freidinger, 1989; Hughes *et al.*, 1990). This is clearly different from the present studies where affinity estimates for L-365,260 and lorglumide in RGM and GPI (using CCK-4 and pentagastrin as agonists) differ significantly. Differences in apparent affinity of antagonists in RGM and GPI may be due to species differences in the gastrin/CCK_B receptor. For example, affinity estimates for L-365,260 derived from gastrin/CCK_B binding studies in rat spinal cord (Hill & Woodruff, 1989) and functional studies in rat ventromedial hypothalamus (Kemp *et al.*, 1989) correspond to the present value for L-365,260 in RGM. In addition, the present studies demonstrate that L-365,260 has a 50 fold lower affinity for gastrin/CCK_B receptors than is apparent from estimates in guinea-pig gastric gland binding studies (Bock *et al.*, 1989). However, it is possible that binding studies in gastric glands do not reflect interaction with receptors controlling acid secretion. In this context, recent studies have suggested that CCK may interact with more than one receptor type in gastric glands, one of which resembles the CCK_B receptor (Praissman & Brand, 1990). Corresponding functional studies of acid secretion in guinea-pig gastric mucosa are required for comparison with binding studies from this species to help resolve this issue.

The present studies using gastrin/CCK agonists and antagonists in functional isolated preparations are consistent with the view that the actions of this peptide family may be mediated by three receptor subtypes. Furthermore, the present studies suggest it may be possible to distinguish gastrin/CCK_B receptors in preparations from different species. Isolated preparations with which to study functional responses mediated by gastrin/CCK_B receptors are not widely available and additional information in other systems is now required to confirm the view that these receptor subtypes differ.

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Structure-activity relations of amiloride and its analogues in blocking the mechanosensitive channel in *Xenopus* oocytes

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1 Patch clamp recording techniques have been used to compare the block caused by amiloride and some of its structural analogues of the mechanosensitive (MS) cation selective channel in frog (*Xenopus laevis*) oocytes.

2 Like amiloride, the amiloride analogues dimethylamiloride (DMA), benzamil and bromohexamethyleneamiloride (BrHMA) block the MS channel in a highly voltage-dependent manner.

3 All analogues tested were more potent blockers than amiloride with IC_{50}'s of 500 μM (amiloride), 370 μM (DMA), 95 μM (benzamil) and 34 μM (BrHMA).

4 Hill plots gave Hill coefficients of 2 (amiloride), 1.8 (DMA), 1 (benzamil) and 1.2 (BrHMA) indicating that the binding of two ligand molecules may be necessary for the block caused by amiloride, DMA and possibly BrHMA whereas only a single ligand molecule may be required for the block by benzamil.

5 The potential use of BrHMA as a light-activated, covalent label of the MS channel protein is discussed.

6 The amiloride analogue 'fingerprinting' of the blocking site on the MS channel indicates it is structurally different from previously described amiloride-sensitive ion transport pathways but may be related to the amiloride binding site on outer hair cells of the ear.

Keywords: Mechanosensitive channel; amiloride analogues; structure-activity; *Xenopus* oocytes

Introduction

The mechanosensitive (MS) channel remains the only major class of membrane ion channel molecule for which there is no structural information. It has been proposed that due to its critical role in cell volume regulation (Hamill, 1983; Christensen, 1987), the MS channel was the first channel molecule to have evolved (Hille, 1989). Furthermore, since the MS channel is the only channel class shared by prokaryotes and eukaryotes (Martinac *et al.*, 1987), Hille (1989) has speculated that it may be the ancestor or 'channel prototype' for other classes of membrane ion channels, namely the ligand-gated and voltage-gated channels. For these reasons, there is major interest in obtaining structural/sequence information on the MS channel molecule. However, a major problem for both protein purification and physiological studies is the absence of specific, high affinity ligands for this molecule. Such ligands proved crucial in the isolation and identification of other membrane ion channels, such as tetrodotoxin for the voltage-gated Na^+ channel (Agnew *et al.*, 1978), α-bungarotoxin for the acetylcholine receptor channel (Changeux *et al.*, 1970) and a light-activated strychnine analogue for the glycine receptor channel (Graham *et al.*, 1981).

Recently, we have demonstrated that amiloride blocks a MS cation selective channel in *Xenopus* oocytes (Lane *et al.*, 1991). Our original interest in this compound was based on previous reports that amiloride blocked mechanotransduction in a variety of systems including the lateral line organ of *Necturus*, the frog skin and the chick ear hair cell (reviewed in Jorgensen & Ohmori, 1988). Our studies indicated that amiloride blocks the MS channel in a cooperative, voltage-dependent manner with an IC_{50} of 500 μM. Although amiloride does block the channel, the IC_{50} is too high for its use in purification studies. In an attempt to find higher affinity blockers we have screened a number of structurally related amiloride analogues (Kleyman & Cragoe, 1988; 1990) for their effectiveness in blocking the MS channel.

Amiloride is known to block at least three other distinct ion transport pathways, namely, the epithelial Na^+ channel, the Na^+-Ca^{2+} exchanger and the Na^+-H^+ exchanger (Kleyman & Cragoe, 1988; 1990). Therefore, at the same time as screening for a high affinity blocker of the MS channel we were also interested in 'fingerprinting' this channel with respect to block by different amiloride analogues and thus determine to which, if any, of the three amiloride blockable pathways the MS channel is most related. For this reason we initially chose benzamil, dimethylamiloride (DMA) and hexamethyleneamiloride (HMA) because these compounds are known to be potent blockers of either one or more of the above ion transport pathways.

Methods

The methods used here to study amiloride block of MS channels in *Xenopus* oocytes were similar to those described previously (Lane *et al.*, 1991). Frogs were anaesthetized by being placed for approximately 20 min in a beaker containing 300 mg ethyl 3-aminobenzoate methanesulphonic acid (Aldrich) in 200 ml of distilled water. Sterile surgical procedures were used to remove the oocytes. Standard patch clamp techniques (Hamill *et al.*, 1981) were used to record single MS channel currents from *Xenopus laevis* oocytes on cell-attached patches (Methfessel *et al.*, 1986). Single MS channels were activated by brief mouth applied suction (10–50 mmHg). The pipette solution contained (in mM): KF 80, KCl 20, EGTA (KOH), 10 and HEPES (KOH), 10; pH 7.2. The amiloride analogues were dissolved in the pipette solution in concentrations ranging from 10 μM to 1 mM. Previous control experiments (Lane *et al.*, 1991) in which KCl was used instead of KF gave identical results. The bath solution for all recordings contained (in mM): NaCl 115, $CaCl_2$ 1.8,

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KCl 1.5, HEPES (NaOH) 10, pH 7.2. All recordings were carried out at room temperature (19–22°C). Single MS channel currents were initially recorded on a digital video recorder (Neuro-corder, DR484, Neuro Data Instrument Corp. NY) and amplitudes were determined during subsequent playback directly from the oscilloscope screen.

Figure 1 illustrates the structure of amiloride and the three analogues that were studied. Amiloride was obtained from Sigma and the three analogues were generous gifts provided by Dr Thomas Kleyman. Due to availability, bromohexamethyleneamiloride (BrHMA) was used in these studies instead of HMA. Kleyman & Cragoe (1990) have shown bromo- substitution does not significantly alter potency of HMA at sites previously tested.

Results

Figure 2 describes the single MS channel current-voltage relationship at several analogue concentrations for each of the analogues tested. The data shown in Figures 2a and 3a are taken from our previous study (Lane *et al.*, 1991) and are presented for comparison. As a general observation all three analogues were more potent than amiloride in blocking the MS channel and showed the same voltage-dependence in that the block was relieved at positive potentials. The solid lines in Figure 2a, b and d are the predicted relationship based on a previously described model which assumes a voltage-dependent conformational change of the MS channel followed by cooperative, voltage-independent binding of two blocking molecules (Lane *et al.*, 1991). However, the data in Figure 2c were adequately described by assuming a single binding site. Equation 1 represents these models mathematically (see also equation A3 in Lane *et al.*, 1991. However, note that a minus sign in the voltage term of equations A2 and A3 was inadvertently omitted).

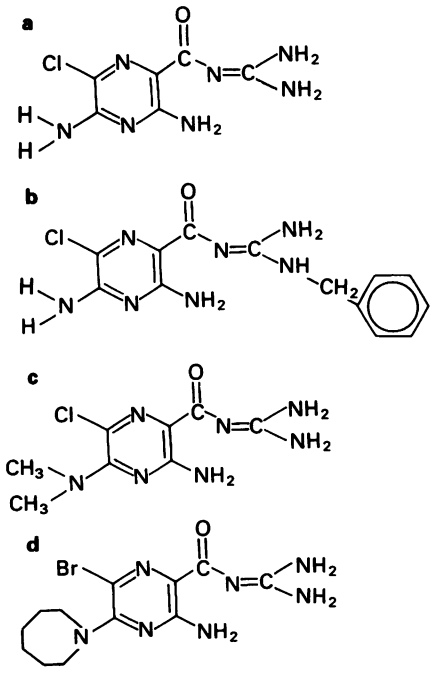


Figure 1 The structure of amiloride (a) and three amiloride analogues: (b) benzamil; (c) dimethylamiloride and (d) bromohexamethyleneamiloride. Amiloride is a pyrazinoylguandine bearing amino groups in the 3- and 5- positions and a chloro group in the 6-position of the pyrazine ring.

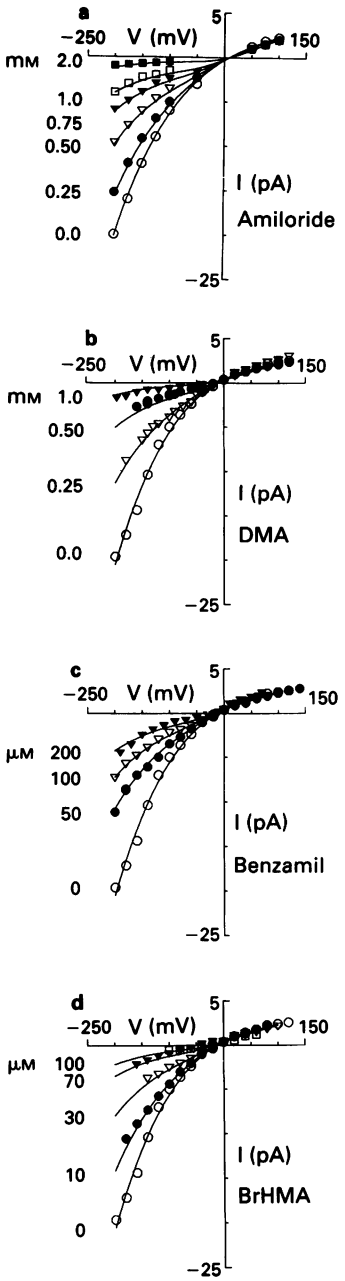


Figure 2 Single channel current-voltage relationship for amiloride and the three analogues. All measurements were from cell-attached patches and represent pooled data for each drug concentration from 2–4 patches. The solid lines fitted to the data were derived from the model described in equation 1. The model assumes a voltage-dependent conformational change with two voltage-independent, cooperative binding sites for amiloride, dimethylamiloride (DMA) and bromohexamethyleneamiloride (BrHMA) and a single voltage-independent site for benzamil and predicts the voltage- and concentration-dependent block of single MS channel currents by extracellular blocker. For all fits, $K_{oc} = 0.1$ and $\delta = 0.65$. (a) For amiloride $K_1 = 5$ mM (the binding of the first molecule); and $K_2 = 50$ μM (the binding of the second molecule). (b) DMA, K_1 and $K_2 = 1.6$ mM and 57 μM, respectively. (c) Benzamil, $K_1 = 56$ μM. (d) BrHMA, K_1 and $K_2 = 23$ μM and 186 μM, respectively.

$$I_B = I_0 \frac{1}{1 + \left(\frac{[B]}{K_1} + \frac{[B]^2}{K_1 K_2}\right)\left(\frac{K_c}{1 + K_c}\right)} \qquad \text{Eqn.(1)}$$

$$K_c = K_{oc} \exp\left\{\frac{-zF}{RT}\,\delta\,V\right\}$$

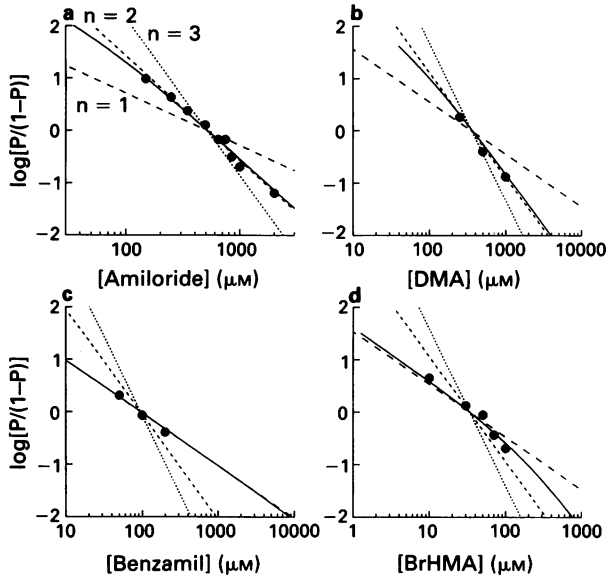


Figure 3 Hill plots calculated from MS channel currents measured at −100 mV with Hill coefficients of 2 (amiloride), 1.8 (dimethylamiloride, DMA), 1 (benzamil) and 1.2 (bromohexamethyleneamiloride, BrHMA). $P = I/I_0$, where I and I_0 are the single MS channel currents in the presence and absence of blocker, respectively. The solid line is the prediction of the conformational model drawn using eqn (1), parameters listed in Figure 2 legend. The interrupted lines show the slopes predicted for Hill coefficients (n) of 1, 2 and 3.

In this equation, I_B and I_0 are the MS currents in the presence and absence of blocker, respectively. [B] is the blocker concentration, K_1 and K_2 are the voltage-independent dissociation constants for the first and second ligand binding, respectively. K_c and K_{oc} are the equilibrium constants at V and at V = 0, respectively, for the conformational change and δ describes the voltage-dependence of this change. z, F, R, T and V have their usual meaning. When applying the model to benzamil, the term in the denominator containing K_2 is omitted. In cases where two sites are necessary, they are not assumed to be identical. The model does not distinguish between the possibilities that (a) the second binding site is always accessible but with negligible binding until the first site is occupied and (b) the second site is inaccessible or nonexistent until the first site is occupied at which time the second site becomes available.

Figure 3 shows the Hill plots for amiloride and the three analogues. The Hill coefficients were 2.0 (amiloride), 1.8 (DMA), 1.0 (benzamil) and 1.2 (BrHMA). The Hill coefficients indicate that there are probably two binding sites for amiloride and DMA and possibly BrHMA while a single binding site is sufficient for the benzamil block. From the Hill plots the IC_{50} of the block was determined for each compound. Based on these IC_{50}'s (included in parentheses) the relative potency is BrHMA (34 μM) > benzamil (95 μM) > DMA (370 μM) > alimoride (500 μM).

Discussion

The main goal of this study was to find a MS channel blocking ligand of higher affinity than amiloride. Our results demonstrate that the photoactivatable amiloride analogue, BrHMA, blocks the channel with an IC_{50} of 34 μM which is an order of magnitude more potent that amiloride. This IC_{50} value is comparable to or better than other compounds such as streptomycin and quinidine, which have been shown to block mechanotransduction in other systems (Martinac, 1992). Although the lanthanide gadolinium is a more potent blocker of the oocyte MS channel (Yang & Sachs, 1989), BrHMA may prove more useful in isolation and purification procedures because of its potential application, when photoactivated and radiolabelled, as a covalently bound label. Photolysis of 6-bromo amiloride analogues has been used previously to identify putative subunits of the epithelial Na^+ channel (Kleyman *et al.*, 1986; Benos *et al.*, 1987) and the Na^+/H^+ exchanger (Friedrich *et al.*, 1986). The relative high affinity of BrHMA for the MS channel may make it a good candidate for this technique.

Comparison of the order of blocking potency of a series of analogues can serve to classify the binding site of the receptor and enable comparison with the binding sites on other amiloride-sensitive ion transport pathways. Table 1 summarizes the results of the analogues we tested and gives their relative potencies in blocking three other amiloride-sensitive ion transport pathways (Kleyman & Cragoe, 1990). Also included in the table are recent results, reported in abstract form, of the analogue block of the transducer currents recorded from mouse outer hair cells (Rüsch *et al.*, 1991). In contrast to the qualitative differences observed between MS channels and other transport pathways, which would suggest structurally different binding sites, the MS channels in the oocyte and the outer hair cell display an identical order of potencies and differ only in their IC_{50}s. A similar quantitative difference in amiloride block at the two sites has been noted previously (cf, Jorgensen & Ohmori, 1988; Lane *et al.*, 1991). Our ability to 'fingerprint' (i.e., determine the relative potency of specific analogues) the amiloride receptor of the MS channel should prove particularly useful in identifying the role of MS channels in different physiological functions. For example, amiloride blockable pathways have been implicated in a variety of physiological functions including volume regulation, fertilization and cell proliferation (Benos, 1982). However, the exact pathway or step which amiloride blocks in these processes is not known. By fingerprinting a particular function with respect to its block by amiloride analogues and comparing it to the MS channel fingerprint it may be possible to determine the involvement of mechanosensitivity in that particular function.

Table 1 Relative potency of block by amiloride and its analogues of various ion transport pathways

Transport pathway	*Amiloride* [μM]	*Benzamil*	*DMA*	*BrHMA or HMA**
MS channel *Xenopus* oocyte	1 [500.0]	5.3	1.4	14.7
MS channel hair cell	1 [53.0]	6.0	1.3	11.0*
Epithelial Na^+ channel	1 [0.34]	9.0	< 0.04	<0.04*
Na^+-Ca^{2+} exchanger	1 [1100.0]	11.0	2.0	11.0*
Na^+-H^+ exchanger	1 [84.0]	<0.08	12	524.0* 566.0

The relative IC_{50} (IC_{50} amiloride/IC_{50} analogue) for amiloride and analogue block of MS channel and other ion transport pathways. The actual IC_{50} of the amiloride block for each pathway is listed in parentheses (in μM). The oocyte MS channel data are from this study, the outer hair cell data from Rüsch *et al.* (1991) and the other data from Kleyman & Cragoe, 1988; 1990. The data with an * are for HMA, otherwise the data are for BrHMA. In the case of the Na^+/H^+ exchanger, data for both analogues were available.
DMA: dimethylamoride; BrHMA: bromohexamethyleneamiloride; HMA: hexamethyleneamiloride.

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The role of CD18 in IL-8 induced dermal and synovial inflammation

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1 The intradermal administration of endothelial IL-8 ($IL\text{-}8_{1\text{-}77}$) or monocyte derived IL-8 ($IL\text{-}8_{1\text{-}72}$) to rabbits produced a concentration-dependent increase in plasma extravasation and an accumulation of polymorphonuclear leukocytes (PMNs) when measured over a 3 h time period. When plasma extravasation and PMN accumulation were measured over a 30 min time period no significant increases in PMN accumulation or plasma extravasation were observed in response to IL-8 alone. However, under these conditions, the addition of prostaglandin E_2 (100 pmol) produced a significant potentiation of IL-8-induced plasma extravasation. There was no significant difference between the biological activities of $IL\text{-}8_{1\text{-}77}$ and $IL\text{-}8_{1\text{-}72}$.

2 Plasma extravasation and PMN accumulation induced by IL-8 were inhibited in rabbits pretreated with the monoclonal antibody designated IB4 (1 mg kg^{-1}, i.v.) directed against the common β chain (CD18) of the leukocyte integrins.

3 The intra-articular administration to rabbits of $IL\text{-}8_{1\text{-}77}$ (1 nmol) resulted 24 h later in the appearance of a mixed population of leukocytes (PMNs and mononuclear cells) in synovial lavage fluid. Biochemical analyses revealed the presence of an increased level of sulphated proteoglycans (sPG) and of the metalloproteinase stromelysin. Pretreatment of rabbits with IB4 (3 mg kg^{-1}, i.v.) inhibited the accumulation of PMNs but had no effect on the mononuclear infiltrate nor on the levels of sPG or stromelysin.

4 The intradermal or intra-articular injection of *E. coli*-derived endotoxin induced similar inflammatory changes to those observed with IL-8. The possibility that the biological activities of IL-8 were attributable to minor contamination with endotoxin is unlikely for two reasons. Firstly, biological effects of endotoxin were observed at levels greater than that contained in the IL-8 preparation. Secondly, reduction of the endotoxin content of the IL-8 preparation by a factor of 10 did not produce a concomitant reduction in the observed biological activity of the IL-8.

Keywords: Inflammation; interleukin 8; leukocyte integrins; PMNs; plasma extravasation; cartilage degradation; stromelysin; proteoglycan

Introduction

The local accumulation of polymorphonuclear leukocytes (PMNs) is a characteristic feature of the inflammatory response to both infectious and non-infectious stimuli. This process is mediated by the elaboration of molecules which exhibit chemotactic activity for PMNs. Many compounds, of both humoral and cellular origin, have been identified which have chemotactic activity for PMNs. In the last few years, a novel PMN chemotactic activity, referred to variously as monocyte derived neutrophil chemotactic factor (MDNCF: Yoshimura *et al.*, 1987a, b), monocyte-derived neutrophil-activating peptide (MONAP: Schroder *et al.*, 1987), neutrophil activating factor (NAF: Walz *et al.*, 1987) and interleukin 8 (IL-8: Westwick *et al.*, 1989) has been identified and characterized. Monocytes are not the only source of IL-8, as similar material has also been identified, either functionally or at the gene level, from endothelial cells (Strieter *et al.*, 1988; 1989a; Gimbrone *et al.*, 1989; Schroder & Christophers, 1989), fibroblasts (Strieter *et al.*, 1989b; Van Damme *et al.*, 1989; Schroder *et al.*, 1990), human T-lymphocytes (Maestrelli *et al.*, 1988; Schroder *et al.*, 1988), hepatocytes (Thornton *et al.*, 1990) and epithelial cells (Elner *et al.*, 1990).

IL-8 is generated initially as a precursor of 99 amino acids (Matsushima *et al.*, 1988) which includes a signal peptide of 22 amino acids. Different mature forms of IL-8 have been identified (Yoshimura *et al.*, 1989) which suggests that the precursor of IL-8 undergoes multiple forms of processing. Human peripheral blood monocytes stimulated with lipopolysaccharide (LPS) have been reported to generate either equivalent amounts of the 72 and 77 amino acid peptides (Yoshimura *et al.*, 1989) or predominantly the 72 amino acid variant (Lindley *et al.*, 1988). However, the predominant IL-8 peptide generated by both endothelial cells (Gimbrone *et al.*, 1989) and fibroblasts (Schroder *et al.*, 1990) is the 77 amino acid variant containing an N-terminal pentapeptide extension.

In addition to its PMN chemotactic activity *in vitro*, IL-8 elicits a range of biological activities. These include induction of PMN degranulation (Schroder *et al.*, 1987; Peveri *et al.*, 1988), stimulation of PMN superoxide anion formation (Thelen *et al.*, 1988), elevation of PMN cytosolic free calcium (Thelen *et al.*, 1988), enhancement of PMN-mediated *Candida* killing (Djeu *et al.*, 1990), stimulation of the 5-lipoxygenase enzyme (Schroder, 1989), stimulation of PMN adherence to normal endothelial cells (Carveth *et al.*, 1989), inhibition of PMN adhesion to IL-1-stimulated endothelial cells (Gimbrone *et al.*, 1989), histamine release (White *et al.*, 1989) and chemotactic activity *in vitro* for T-lymphocytes (Larsen *et al.*, 1989).

The sequelae of IL-8 administration to animals include a series of biological responses consistent with its profile of activity *in vitro*. For example, in rabbits the local intradermal injection of IL-8 induces PMN accumulation and plasma extravasation (Colditz *et al.*, 1989; Rampart *et al.*, 1989; Colditz *et al.*, 1990). Furthermore, the intra-articular injection of IL-8 reslts in PMN accumulation in synovial lavage fluid (Akahoshi *et al.*, 1990). PMN accumulation in tissues,

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in response to locally generated or administered mediators, is preceded by an adhesive interaction between the PMN and the vascular endothelium. This interaction is dependent, at least in part, on the recognition of ligands (counter-receptors) on the endothelial surface (e.g. ICAM-1) by the leukocyte β_2 integrin CD11/CD18 complex on the PMN plasma membrane (Carlos & Harlan, 1990). IL-8 increases the surface expression of the CD11/CD18 complex on PMNs (Detmers *et al.*, 1990) and antibodies directed against the CD11/CD18 complex inhibit the IL-8 stimulated increase in PMN adherence to normal vascular endothelial monolayers (Carveth *et al.*, 1989).

The majority of data available to date, concerning the biological activities of IL-8, *in vivo* and *in vitro*, have been obtained with the 72 amino acid monocyte derived peptide ($IL\text{-}8_{1\text{-}72}$). In order to define further the relative activities of $IL\text{-}8_{1\text{-}72}$ and $IL\text{-}8_{1\text{-}77}$ we compared the effects of the two peptides on dermal PMN accumulation and plasma extravasation in the rabbit. We also examined the ability of $IL\text{-}8_{1\text{-}77}$ to elicit joint inflammation in the rabbit knee. Furthermore, we examined the extent to which IL-8-induced dermal and joint inflammation is dependent on the CD11/CD18 complex by utilising a monoclonal antibody, designated IB4, directed against the common β chain (CD18) of the leukocyte integrins, which effectively blocks the interaction of CD18 with its counter-receptor on endothelial cells (Lundberg & Wright, 1990).

Methods

Animals

Female New Zealand White rabbits (2.5–3.0 kg) were purchased from Hazelton Research Products (Denver, PA, USA). All experimental procedures involving the use of animals were approved by the Merck Institutional Animal Care and Use Committee. Animals were handled and cared for in accordance with guidelines set forth in the National Institutes of Health guide for the care and use of laboratory animals.

Measurement of plasma extravasation and PMN accumulation in rabbit skin

The dorsal hair of female New Zealand White rabbits (2–2.5 kg) was shaved at least 24 h before experimentation. Rabbits were anaesthetized by an intramuscular injection of ketamine HCl (25 mg kg^{-1}) and xylazine (2 mg kg^{-1}) and [^{125}I]-bovine serum albumin (10 μCi) was injected into the marginal ear vein, as a marker for plasma extravasation. Groups of animals were then administered the anti-CD18 monoclonal antibody, IB4 (1 mg kg^{-1}) or an equivalent volume of saline. IL-8 (1–100 pmol in a volume of 50 μl) or saline were injected intradermally into 4 replicate sites in the dorsum 15 min after the injection of the antibody. Three hours later the animals were re-anaesthetized by an intramuscular injection of ketamine HCl (25 mg kg^{-1}) and xylazine (2 mg kg^{-1}), and a blood sample (1 ml) was taken by cardiac puncture. The blood was centrifuged (800 *g*; 3 min; 20°C) to prepare plasma which was aspirated and retained. Animals were then killed with approximately 750 μl Socumb (sodium pentobarbitone, 389 mg ml^{-1} in 40% isopropyl alcohol) and injection sites were excised with a 6 mm biopsy punch. Radioactivity (^{125}I) present in skin samples and cell-free plasma (50 μl) was measured in a gamma counter. By reference to the specific radioactivity of the cell-free plasma, the extent of plasma extravasation was expressed as μl plasma equivalents per 6 mm biopsy. The skin biopsy was then homogenized in 5 ml of 0.5% hexadecyltrimethyl ammonium bromide (HTAB) with a polytron homogenizer. Chloroform (1 ml) was added to the sample, which was then vortexed and centrifuged (1600 *g*; 15 min; 20°C). Four aliquots (50 μl) of the aqueous supernatant were added to wells in a 96 well plate for measurement of myeloperoxidase (MPO) activity, as an index of PMN content. Duplicate wells of the 96 well plate received buffer (KH_2PO_4 44 mM; K_2HPO_4 6 mM; H_2O_2 0.0015%; pH 6.0) alone and duplicate wells received buffer containing MPO substrate (3',3-dimethoxybenzidine dihydrochloride, 1.1 mM). Reactions were allowed to proceed for 15 min at room temperature and MPO activity was measured as the change in absorbance at 450 nm measured in a plate reading spectrophotometer. By reference to a standard curve constructed with known quantities of rabbit PMNs in HTAB, the extent of PMN accumulation in each skin biopsy was estimated.

Induction of joint inflammation

Female NZW rabbits (3.0–3.5 kg) were anaesthetized with an intramuscular injection of ketamine HCl (30 mg kg^{-1}) plus xylazine (3 mg kg^{-1}). Groups of animals were administered either the anti-CD18 monoclonal antibody, IB4 (3 mg kg^{-1}), a dose known to prevent agonist-induced PMN accumulation in skin and synovial cavity for 24 h (data not shown), or an equivalent volume of saline intravenously via the marginal ear vein. After 15 min the rear knees were shaved, swabbed with isopropyl alcohol and injected intra-articularly with IL-8 (1 nmol in a volume of 500 μl) or 500 μl saline into the contralateral knee. The animals were allowed to regain consciousness and 24 h later were killed with approximately 750 μl Socumb. The knee joints were lavaged with 2 ml of sterile PBS containing EDTA (5 mM). Lavage fluids were immediately placed on ice. Samples (100 μl) of lavage fluid were removed for the determination of leukocytes, the remainder was centrifuged (1600 *g*; 15 min; 4°C) and the cell-free supernatant frozen at −20°C pending further biochemical analyses.

Measurement of cell accumulation in synovial lavage fluid

Total and differential cell counts of synovial lavage fluid were determined by light microscopy. Leukocytes were counted in an improved Neubauer counting chamber following staining of cells with gentian violet (0.1% crystal violet, 1% acetic acid in distilled water).

Measurement of sulphated proteoglycans (sPG) in synovial lavage fluid

The concentration of sPG in cell-free synovial lavage fluid was measured by the 1,9-dimethylmethylene blue binding assay (Farndale *et al.*, 1982). Keratan sulphate (Sigma, St. Louis, MO., U.S.A.) was used to generate the standard curve. Data are expressed as the concentration of sulphated PG in $\mu g\ ml^{-1}$ of synovial lavage fluid.

Measurement of stromelysin in synovial lavage fluid

Stromelysin levels in synovial lavage fluid were measured by a double antibody trapping ELISA based on a similar assay for the measurement of human stromelysin (Walakovits *et al.*, 1991). The modification of the assay for measuring rabbit stromelysin requires the use of sheep anti-rabbit prostromeylsin. The assay has a half maximum of detection of ~50 ng ml^{-1} with a log-linear range of 0.5–250 ng ml^{-1}. The assay detects both rabbit prostromelysin and active stromelysin with equivalent sensitivity. Rabbit stromelysin complexed with rabbit tissue inhibitor of metalloproteinase (TIMP) is also recognised by the assay but with approximately 2 fold lower sensitivity than free activated enzyme. The assay does not recognise the closely related metalloproteinase, rabbit collagenase, even at concentrations up to 1 $\mu g\ ml^{-1}$.

Materials

Monocyte and endothelial-derived IL-8 were supplied as freeze dried preparations from Biosource International (Westlake Village, CA, U.S.A.). The material was reconstituted in pyrogen-free water and diluted for use in endotoxin-free bovine serum albumin (1 mg ml^{-1}) in pyrogen-free saline. Endotoxin levels of IL-8 were determined by a colorimetric *Limulus* amoebocyte lysate assay (Whittaker Bioproducts, Walkersville, MD, U.S.A.). Where indicated in the results section, certain IL-8 preparations were passed through a 'Detoxi-Gel' column (Pierce, Rockford, IL, U.S.A.) for removal of endotoxin.

The IB4 monoclonal anti-CD18 antibody was purified by protein A chromatography from serum-free culture medium supernatants of IB4 murine hybridoma cells (Wright *et al.*, 1983) grown in a hollow fibre bioreactor. The antibody was prepared as a stock solution at 1 mg ml^{-1} in PBS (pH 7.2) and had an endotoxin level of <100 pg mg^{-1} protein.

Statistical analyses

Dose-response data were analysed by analysis of variance and other comparisons were made by Student's *t* test as indicated in the text or figure legends.

Results

IL-8 induced dermal inflammation

The intradermal injection of either IL-8_{1-77} or IL-8_{1-72} produced a concentration-dependent increase in plasma extravasation (Figure 1a) and PMN accumulation (Figure 1b). Whilst both peptides induced significant increases in plasma extravasation and PMN accumulation compared with saline-injected skin sites there were no significant differences in response betwen the two peptides at any concentration tested. *In vitro*, IL-8_{1-72} is more active than IL-8_{1-77} in assays of PMN activation (Herbert *et al.*, 1990; Schroder *et al.*, 1990). It is possible that a difference in *in vivo* activity of the two peptides exists but was masked in the experiments described here by instability of the peptides during the 3 h measurement period of PMN accumulation and plasma extravasation. Therefore, an additional series of experiments were performed in the presence and absence of the vasodilator prostaglandin E_2 (PGE_2) which potentiates the activity of permeability increasing agents (Williams & Jose, 1981) to allow the measurement of responses over a 30 min period. Neither IL-8_{1-72} nor IL-8_{1-77} when injected alone into rabbit skin induced increases in plasma extravasation or PMN accumulation (Table 1). The addition of PGE_2 (100 pmol) to either IL-8_{1-72} or IL-8_{1-77} produced a marked increase in plasma extravasation compared with either agent alone but did not significantly increase PMN accumulation. Furthermore, no significant difference existed in the activity of IL-8_{1-72} and IL-8_{1-77} in the presence of PGE_2.

The effect of the anti-CD18 monoclonal antibody IB4 (1 mg kg^{-1}, i.v.) on IL-8-induced dermal inflammation was examined. Plasma extravasation (Figure 2a) and PMN accumulation (Figure 2b) induced by either IL-8_{1-72} or IL-8_{1-77} were significantly inhibited in IB4 compared with vehicle-injected control animals.

IL-8-induced joint inflammation

Because no difference in activity between IL-8_{1-72} and IL-8_{1-77} was apparent in studies on dermal inflammation only one of the peptides (IL-8_{1-77}) was used to investigate the effects of IL-8 on joint inflammation in the rabbit knee. The intra-articular injection of IL-8_{1-77} (Figure 3) induced a significant ($P<0.001$) increase in total leukocytes, PMNs and mononuclear leukocytes in synovial lavage fluid compared with the

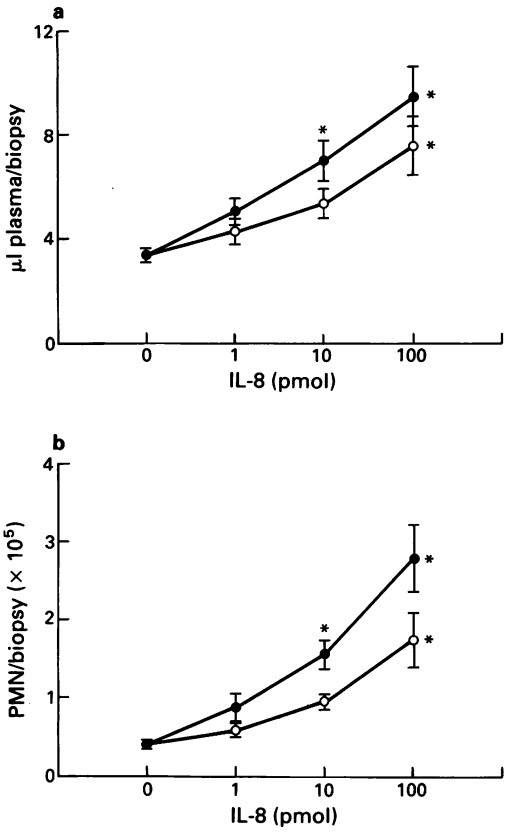


Figure 1 The effects of IL-8_{1-72} (1–100 pmol: ○) and IL-8_{1-77} (1–100 pmol: ●) on dermal plasma extravasation (a) and dermal PMN accumulation (b) were determined. Plasma extravasation was measured 3 h after the intradermal injection of stimuli as the local accumulation of intravenously injected ^{125}I-labelled human serum albumin and PMN accumulation was measured as the tissue content of myeloperoxidase (MPO). MPO levels were converted to numbers of cells from a standard curve constructed from known numbers of rabbit peripheral blood PMNs. Each point represents the mean and vertical lines the standard error for triplicate determinations in 6–12 animals. *$P<0.05$ (ANOVA) compared with saline-injected skin.

Table 1 The effects of IL-8_{1-72} and IL-8_{1-77} on plasma extravasation and PMN accumulation in rabbit skin

Treatment	*Plasma* (µl/site)	*PMNs* (× 10^{-5}/site
Saline	3.6 ± 0.4	0.3 ± 0.1
PGE_2	5.1 ± 0.8	0.3 ± 0.1
IL-8_{1-72}	3.8 ± 0.5	0.4 ± 0.2
IL-8_{1-77}	5.2 ± 0.6	0.5 ± 0.2
IL-8_{1-72} + PGE_2	13.4 ± 1.1*	0.7 ± 0.2
IL-8_{1-77} + PGE_2	12.7 ± 0.5*	0.7 ± 0.2

The effects of IL-8_{1-72} (100 pmol) and IL-8_{1-77} (100 pmol), in the presence and absence of prostaglandin E_2 (PGE_2; 100 pmol), on plasma extravasation and PMN accumulation were measured in rabbit skin 30 min following the intradermal injection of stimuli. Plasma extravasation was measured as the local accumulation of intravenously injectd ^{125}I-labelled bovine serum albumin and PMN accumulation as the myeloperoxidase activity of skin homogenates. Data are presented as the mean ± s.e.mean of duplicate determinations in 3 animals. *$P<0.001$ (Student's *t* test) compared with peptide alone.

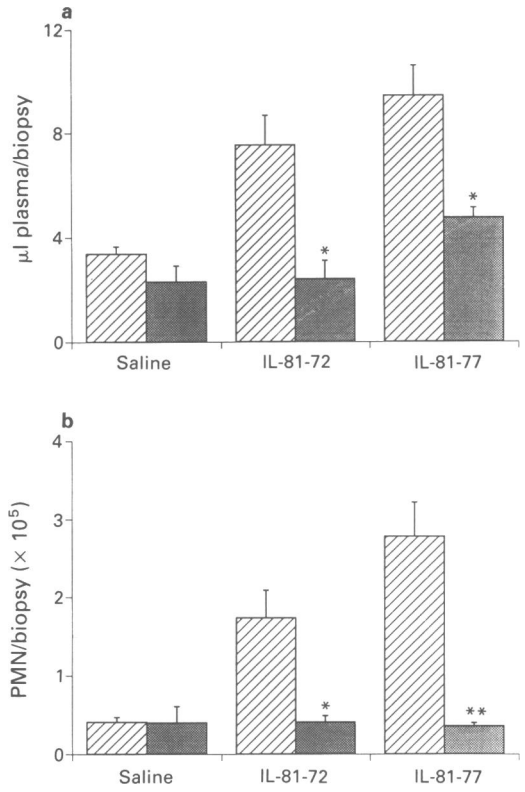


Figure 2 The effects of IB4 (1 mg kg^{-1}, i.v.) on IL-8_{1-72} (100 pmol) and IL-8_{1-77} (100 pmol)-induced dermal plasma extravasation (a) and dermal PMN accumulation (b) were determined. Plasma extravasation was measured 3 h after the intradermal injection of stimuli as the local accumulation of intravenously injected [^{125}I]-labelled human serum albumin and PMN accumulation was measured as the tissue content of myeloperoxidase (MPO). MPO levels were converted to numbers of cells from a standard curve constructed from known numbers of rabbit peripheral blood PMNs. Each column represents the mean and vertical bars the standard error for triplicate determinations in 3–12 animals: control, hatched columns; IB4, stippled columns; $*P<0.05$, $**P<0.01$ (Student's t-test) compared with control animals.

number of cells detected in the contralateral joints injected with saline, which was always below 5×10^3 cells ml^{-1} (data not shown). The pretreatment of rabbits (Figure 3) with IB4 (3 mg kg^{-1}) significantly ($P<0.01$) inhibited the infiltration of total leukocytes and PMNs. Although the number of mononuclear leukocytes was reduced by 54% this difference did not achieve statistical significance.

Biochemical analyses of the synovial lavage fluid revealed a significant ($P<0.0001$) increase in the amount of sPG (Figure 4) and immunoreactive stromelysin ($P<0.05$; Figure 5) detected in synovial lavage fluid from IL-8_{1-77}-injected knees compared with contralateral knees. Neither the increase in proteoglycans nor stromelysin was reduced by pretreatment with IB4.

Endotoxin contamination of IL-8

Examination of the IL-8 preparations with a colorimetric *Limulus* amoebocyte lysate (LAL) assay revealed the presence of low but measurable amounts of endotoxin. The values were 500 and 5 fmol of endotoxin mg^{-1} for IL-8_{1-77} and IL-8_{1-72} respectively. This concentration of endotoxin would

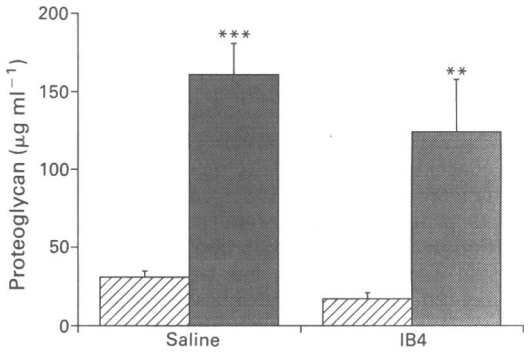


Figure 4 The appearance of sulphated proteoglycans (sPG) in the synovial lavage fluid obtained 24 h after the intra-articular injection of IL-8_{1-77} (1 nmol) was determined in two groups of rabbits treated with either saline or IB4 (3 mg kg^{-1}, i.v.): hatched columns, contralateral; stippled columns, challenge. Sulphated proteoglycans were determined by the dimethylmethylene blue binding assay. Each column represents the mean and vertical bars the standard deviation of 4 animals. $***P<0.0001$; $**P<0.001$ (Student's t test) compared with control animals.

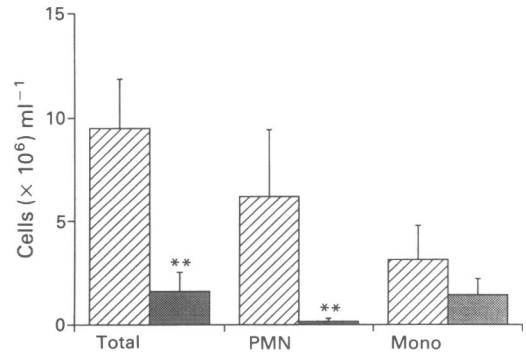


Figure 3 The effect of IL-8_{1-77} (1 nmol) on leukocyte accumulation in the knee joints of rabbits treated with saline (hatched columns) or IB4 (3 mg kg^{-1}, i.v.) (stippled columns). Leukocytes were measured by light microscopy 24 h after the intra-articular injection of IL-8. Each column represents the mean and vertical bars the standard deviation of 4 animals: Mono = mononuclear leukocytes. $**P<0.01$ (Student's t test) compared with control animals.

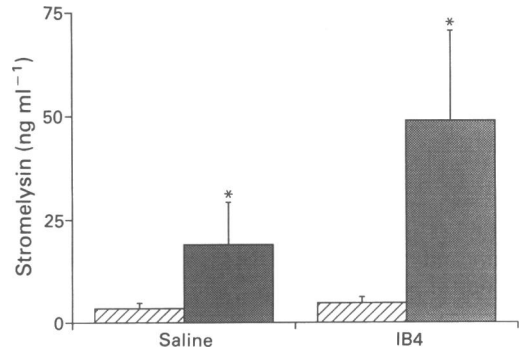


Figure 5 The appearance of stromelysin in the synovial lavage fluid obtained 24 h after the intra-articular injection of IL-8_{1-77} (1 nmol) was determined in two groups of rabbits treated with either saline or IB4 (3 mg kg^{-1}; i.v.): hatched columns, contralateral; stippled columns, challenge. Stromelysin was determined by a specific enzyme linked immunoassay. Each column represents the mean and vertical bars the standard deviation of 4 animals. $*P<0.05$ (Student's t test) compared with control animals.

have resulted in the injection of 5 fmol of endotoxin into the knee joints and a maximum of 0.5 fmol of endotoxin injected intradermally with IL-8_{1-77} or 5 amol of endotoxin with IL-8_{1-72}. Because of the pro-inflamamtory activity of endotoxin, we assessed the potential contribution of contaminant endotoxin to the inflammatory responses evoked by IL-8.

The intradermal injection of authentic *E. coli*-derived endotoxin produced a dose-related increase in dermal PMN accumulation and plasma extravasation (Figure 6). However, the maximum amount of endotoxin which would have been injected intradermally with IL-8_{1-77} (0.5 fmol) would not be sufficient to account for the inflammatory potential of IL-8 in the skin. Similar experiments were performed in the joint (Figure 7) where it can be seen that at levels of endotoxin equivalent to those injected into the joint with IL-8_{1-77} (5 fmol), endotoxin produces little PMN accumulation but did cause substantial release of sPG. With respect to PMN accumulation, it is unlikely that endotoxin contamination is contributing to the biological effect of IL-8. However, a contribution to the release of sPG by endotoxin could not be ruled out. To investigate further the effect of endotoxin, we passed the IL-8_{1-77} solution over a 'Detoxi-Gel' column to reduce the endotoxin content. This resulted in a reduction of endotoxin from 500 fmol mg^{-1} to 50 fmol mg^{-1} but did not result in a loss of IL-8_{1-77} peptide, determined by an IL-8 binding assay to human PMN membranes (data not shown). From our dose-response data for endotoxin in the knee joint we would predict that a 10 fold reduction in the endotoxin content would abolish the ability of the IL-8_{1-77} preparation to release proteoglycans from cartilage, were endotoxin to be the biologically active principal. However the 'low endotoxin' IL-8_{1-77} was as effective as the 'high endotoxin' IL-8_{1-77} in producing PMN accumulation, release of sPG and stromelysin (Table 2).

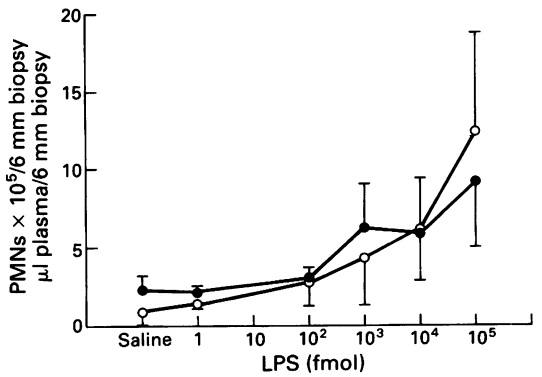


Figure 6 The pro-inflammatory activity of *E. coli*-derived endotoxin injected intradermally in rabbits was determined. Plasma extravasation (●) was measured 3 h after the intradermal injection of stimuli as the local accumulation of intravenously injected ^{125}I-labelled human serum albumin. PMN accumulation (○) was measured as the tissue content of myeloperoxidase (MPO). MPO levels were converted to numbers of cells from a standard curve constructed from known numbers of rabbit peripheral blood PMNs. Each point represents the mean and vertical bars the standard deviation of 5 animals.

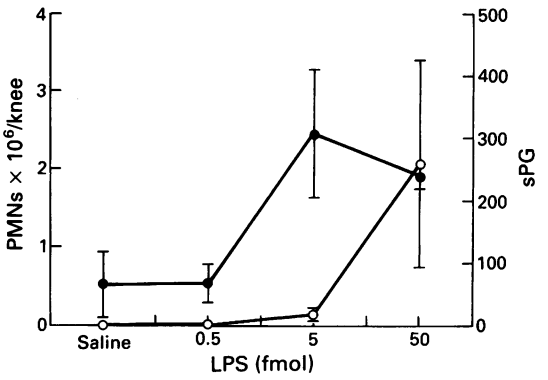


Figure 7 The pro-inflammatory activity of *E. coli*-derived endotoxin injected intra-articularly in rabbits was determined. PMNs (○) were measured by light microscopy 24 h after the intra-articular injection of IL-8_{1-77}. Sulphated proteoglycans (●) were determined by the dimethylmethylene blue binding assay. Each point represents the mean and vertical bars the standard deviation of 5 animals.

Discussion

Several mature forms of the recently described cytokine, interleukin-8, have been described (Lindley *et al.*, 1988; Yoshimura *et al.*, 1989; Schroder *et al.*, 1990). Monocytes have been reported to produce either predominantly the 72 amino acid (IL-8_{1-72}) peptide (Lindley *et al.*, 1988) or equivalent amounts of both peptides (Yoshimura *et al.*, 1989) whereas fibroblasts (Schroder *et al.*, 1990) and endothelial cells (Gimbrone *et al.*, 1989) generate the 77 amino acid (IL-8_{1-77}) variant. Both forms of IL-8 are reported to induce the degranulation of cytochalasin B-treated human PMNs with IL-8_{1-72} being the more potent form (Hebert *et al.*, 1990; Schroder *et al.*, 1990).

In contrast to the documented greater activity of IL-8_{1-72} compared with IL-8_{1-77} *in vitro*, we found the two peptides to have equivalent activity as mediators of plasma extravasation and PMN accumulation in rabbit skin. These data are in agreement with those of Hechtman *et al.* (1991) who found that both peptides were equiactive at inducing dermal PMN accumulation following their intradermal administration to rabbits and were also equiactive as inhibitors of agonist-

Table 2 The effects of IL-8_{1-77} on joint inflammation before (high endotoxin) and after (low endotoxin) passage through a 'Detoxi Gel' column

Treatment	*PMNs* ($\times 10^{-6}$ ml^{-1})	*sPG* (µg ml^{-1})	*Stromelysin* (ng ml^{-1})
Saline	0.003 ± 0.007	25 ± 13	4.6 ± 3.6
IL-8_{1-77} (High endotoxin)	6.2 ± 3.2***	161 ± 40***	19.1 ± 21*
IL-8_{1-77} (Low endotoxin)	4.2 ± 3.5***	144 ± 61**	62.8 ± 39*

IL-8_{1-77} was passed through a column of 'Detoxi Gel' to reduce the endotoxin concentration from 500 fmol mg^{-1} to 50 fmol mg^{-1}. The IL-8_{1-77} preparations (1 nmol in 500 ml) or saline were injected into the knees of rabbits. The animals were killed 24 h later and measurements of PMN accumulation, stromelysin and sulphated proteoglycans (sPG) performed on synovial lavage fluid. Values are mean ± s.d. of 4 animals.

*$P<0.05$; **$P<0.001$; ***$P<0.0001$ vs saline. Differences between high and low endotoxin all non-significant, Student's *t* test.

induced dermal PMN accumulation when the peptides were administered intravenously. It is conceivable that a difference in activity does exist but that this is masked by differential metabolism of the peptides. For example, it has been clearly demonstrated that IL-8_{1-77} is rapidly converted to IL-8_{1-72} by the addition of thrombin (Hebert *et al.*, 1990). In experiments where we measured dermal inflammation over a 30 min as opposed to 3 h period, we did not detect a difference in activity between the peptides. However, without knowing the stability of the peptides *in vivo* under the experimental conditions reported here it is impossible to conclude absolutely that the two IL-8 variants have equivalent activity.

The ability of vasodilator prostaglandins to potentiate plasma extravasation (Williams & Jose, 1981; Wedmore & Williams, 1981) and PMN accumulation (Issekutz, 1981) induced by a range of inflammatory mediators is well established. Our data demonstrating a potentiation of IL-8-induced plasma extravasation by PGE_2 confirm those previously reported by Rampart *et al.* (1989). However, whilst PMN accumulation was marginally greater in the presence of PGE_2, this was not a statistically significant finding and contrasts with the data of Rampart *et al.* (1989). A difference in methods of quantitation of tissue PMNs (enzymatic activity of skin homogenates versus accumulation of radiolabelled leukocytes) may account for this discrepancy.

The ability of IL-8 to induce PMN accumulation in the rabbit knee joint has been described previously in abstract form (Akahoshi *et al.*, 1990). We have confirmed and extended this report by showing that IL-8_{1-77} induces the accumulation of mononuclear leukocytes, and the appearance of sulphated proteoglycans and of stromelysin in the synovial lavage fluid. The accumulation of mononuclear leukocytes in response to IL-8 has not previously been reported. Following the intradermal injection of IL-8, the leukocytic infiltrate determined by light microscopy is reported to be exclusively PMNs with no evidence for monocytes, eosinophils, basophils or lymphocytes (Colditz *et al.*, 1989). Our observation of a mononuclear leukocyte infiltrate, in synovial lavage fluid in response to IL-8_{1-77}, may be explained on the basis of a different anatomical site, the use of IL-8_{1-77} in contrast to IL-8_{1-72} or perhaps the use of a 24 h response in the knee compared with 4 h in the skin.

The mechanism by which IL-8 induces monocyte accumulation is not clear. A direct chemotactic action for monocytes is unlikely considering that monocytes do not exhibit chemotaxis for IL-8 *in vitro* (Yoshimura *et al.*, 1987b). Specific receptors for IL-8 have been demonstrated on PMNs (Besemer *et al.*, 1989; Matsushima & Oppenheim, 1989; Samanta *et al.*, 1989; Grob *et al.*, 1990; Leonard *et al.*, 1990), whilst the evidence for receptors on monocytes is less convincing. Specific binding of IL-8 to peripheral blood monocytes has been reported (Besemer *et al.*, 1989; Matsushima & Oppenheim, 1989; Grob *et al.*, 1990; Leonard *et al.*, 1990). However, the calculated number of receptors per monocyte is approximately one tenth of that on PMNs and may well be attributed to contamination of monocyte preparations with PMNs. An alternative explanation for the accumulation of monocytes *in vivo* in response to IL-8_{1-77} may be an indirect action consequent upon the evoked release of biologically active autacoids from accumulating PMNs or from tissue cells. For example, IL-8 can stimulate PMN 5-lipoxygenase (Schroder, 1989) which, in the presence of arachidonic acid, will result in the formation of leukotriene B_4 and monohydroxyeicosatetraenoic acids with chemotactic activity for monocytes (Bray, 1983).

In comparison to control animals, the synovial lavage fluids from IL-8_{1-77} injected rabbits contained an increased concentration of proteoglycan, indicating that proteoglycan loss from articular cartilage (Pettipher *et al.*, 1989) has taken place in response to IL-8. Cartilage degradation induced by IL-8 stimulated neutrophils *in vitro* has recently been described (Elford & Cooper, 1991). In these experiments the coculture of ^{35}S-labelled cartilage with PMNs and human IL-8 caused a rapid loss of radioactivity from the cartilage. This phenomenon was believed to be mediated via PMN degranulation and was not a direct effect of IL-8 on the cartilage. It is unlikely that the loss of sPG from cartilage observed *in vivo* following the intra-articular injection of IL-8 is mediated via PMN degranulation. This contention is made on the basis that pre-treatment of rabbits with IB4 inhibited IL-8-induced PMN accumulation in synovial lavage fluid, but had no effect on the levels of sPG. Although a role for PMNs in mediating cartilage degradation induced by IL-8 *in vivo* is unlikely, the mechanism by which IL-8 induces cartilage degradation is not clear. The presence of elevated levels of the metalloproteinase stromelysin, an enzyme with the capacity to degrade cartilage (Okada *et al.*, 1986), suggests that the release of sPG is via this enzyme. However, the cellular source of stromelysin and the mechanisms whereby IL-8 stimulates the production of stromelysin, are not known.

The contribution of contaminating endotoxin to the biological effects of IL-8 appears to be minimal. In studies on the dermal effects of IL-8 the amounts of the cytokine injected, and hence of endotoxin, were low and clearly were not sufficient to account for the observed cellular accumulation and plasma extravasation. With respect to studies in the knee joint, larger amounts of IL-8 were injected and the amount of endotoxin in preliminary studies was sufficient to produce release of sPG but not to induce PMN accumulation. Our observation that reducing the amount of endotoxin present in the IL-8_{1-77} by a factor of 10 had no effect on the pro-inflammatory properties of IL-8_{1-77} argues strongly against endotoxin being the biologically active principal. However, we cannot rule out completely a contribution from the endotoxin. We have used an *E. coli* derived endotoxin as an indicator of the biological activity for an endotoxin contaminant of unknown aetiology. It is possible that the endotoxin in our IL-8 preparation is derived from an organism other than *E. coli* or even an alternative strain of the bacterium and may exhibit greater, or lesser, biological activity. Furthermore we cannot exclude the possibility of a synergy between IL-8 and low doses of endotoxin which accounts for the biological phenomena observed.

The results of this study demonstrate that the distinct forms of the recently described cytokine, IL-8, exhibit a similar spectrum of biological activity. We also document novel activities for IL-8 in terms of monocyte accumulation, generation of stromelysin and cartilage degradation. The extent to which IL-8 may contribute to the pathogenesis of inflammatory disease clearly warrants further investigation, particularly in the light of recent reports demonstrating the presence of biologically relevant levels of IL-8 in synovial fluid from patients with rheumatoid arthritis (Akahoshi *et al.*, 1990; Brennan *et al.*, 1990).

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Effect of MgATP on pinacidil-induced displacement of glibenclamide from the sulphonylurea receptor in a pancreatic β-cell line and rat cerebral cortex

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1 The effects of blockers and openers of K^+ channels on binding of [^{3}H]-glibenclamide to microsomes obtained from a pancreatic β-cell line (HIT-T15) or rat cerebral cortex were examined.

2 The blockers quinine, chlorpromazine and thiopentone and the openers cromakalim [(±) 6-cyano-3,4-dihydro-2,2-dimethyl-trans-4-(2-oxo-1-pyrrolidyl)-2H-benzo[b]pyran-3-ol] and minoxidil sulphate did not significantly interact with the sulphonylurea receptor of HIT-cells both at phosphorylating (presence of MgATP) and dephosphorylating (absence of MgATP) conditions.

3 In the absence of MgATP, pinacidil (200– 500 μM) did not significantly displace [^{3}H]-glibenclamide binding to microsomes from HIT-cells. The displacement of [^{3}H]-glibenclamide binding was strongly enhanced by MgATP and was due to a decrease in the number of high affinity binding sites for glibenclamide.

4 MgATP enhanced pinacidil-induced inhibition of [^{3}H]-glibenclamide binding to microsomes from rat cerebral cortex.

5 The effect of MgATP on pinacidil-induced inhibition of [^{3}H]-glibenclamide binding was maintained after solubilization of the membranes from HIT-cells or rat cerebral cortex.

6 It is concluded that the sulphonylurea receptor is regulated not only by sulphonylureas but also by the K^+ channel openers, diazoxide and pinacidil, and by protein phosphorylation. The binding sites for sulphonylureas and these K^+ channel openers are not identical, but appear to be located at a single protein or at tightly associated proteins.

Keywords: Pinacidil; glibenclamide; MgATP; sulphonylurea receptor; pancreatic β-cells; cerebral cortex

Introduction

Pinacidil is a vasodilator which hyperpolarizes smooth muscle cells via opening of K^+-channels in the plasma membrane (for reviews Edwards & Weston, 1990; Robertson & Steinberg, 1990). In addition to its effects on smooth muscle cells, pinacidil activates K^+-channels in ventricular myocytes (Fan *et al.*, 1990), insulin-secreting cells (Garrino *et al.*, 1989; Dunne, 1990) and oocytes (Honoré & Lazdunski, 1991). In these cells the ATP-dependent K^+-channel (K-ATP-channel) appears to be the major site of action of pinacidil. The K-ATP-channel is blocked by sulphonylureas (for review see Ashcroft & Ashcroft, 1990). In insulin-secreting cells this block initiates a chain of events eventually leading to the release of insulin, whereas opening of K-ATP-channels by diazoxide or pinacidil inhibits insulin release due to hyperpolarization of the cells (Trube *et al.*, 1986; Lebrun *et al.*, 1988; Garrino *et al.*, 1989; Dunne, 1990).

In microsomal membranes from insulin-secreting cells, a high affinity binding site has been identified which represents the sulphonylurea receptor (Geisen *et al.*, 1985; Schmid-Antomarchi *et al.*, 1987; Gaines *et al.*, 1988; Panten *et al.*, 1989; Niki *et al.*, 1989). In the absence of nucleotides, diazoxide did not significantly displace [^{3}H]-glibenclamide from the sulphonylurea receptor (Schwanstecher *et al.*, 1991). However, in the presence of the Mg complex of ATP (MgATP), [^{3}H]-glibenclamide binding was decreased and diazoxide-induced displacement of [^{3}H]-glibenclamide binding was increased. These findings are consistent with the view that sulphonylureas and diazoxide exert their effects by interaction with the same binding site at the sulphonylurea receptor, the binding properties of which are modulated by protein phosphorylation (Schwanstecher *et al.*, 1991). However, due to the difficulty in dissolving high concentrations of diazoxide, a more detailed analysis of the interaction between sulphonylureas and diazoxide was not feasible.

Pinacidil inhibited [^{3}H]-glibenclamide binding to cardiac and cortex membranes only at concentrations higher than 100 μM (Gopalakrishnan *et al.*, 1991; Angel & Bidet, 1991). These experiments were carried out in the absence of nucleotides. Therefore, the structural similarity of pinacidil to diazoxide (Figure 1) and the better solubility of pinacidil led us to test the hypothesis that sulphonylureas and pinacidil compete for the same binding site in the presence of MgATP. The results do not support this hypothesis.

Methods

Cell culture and preparation of microsomes from HIT-cells

HIT cells were grown as monolayers in RPMI 1640 containing 10% foetal bovine serum, 100 nM sodium selenite, 100 μg ml^{-1} streptomycin and 100 u ml^{-1} penicillin G. Cells were fed two times weekly and subcultured once per ten days as described by others (Hill & Boyd, 1984). Microsomes were prepared from monolayers on 94 mm petri dishes (passage 77–90). The plates were cooled on ice, washed twice with phosphate-buffered saline, and then harvested with a cell scraper. The cells were pelleted (50 *g*, 5 min, 20°C) and used to prepare crude membranes as follows. Cells were resuspended in ice-cold Tris buffer (5 mM, pH 8.0, 100 μM phenylmethylsulphonyl fluoride), left for 40 min on ice and then homogenized with 15 strokes of a Teflon-glass homogenizer (300 r.p.m.). The homogenate was centrifuged

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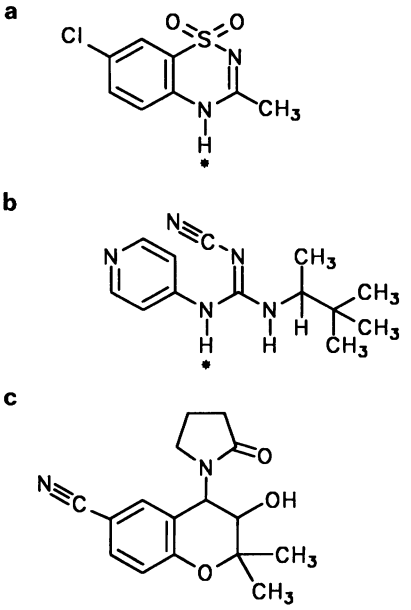


Figure 1 Structures of diazoxide (a), pinacidil (b) and cromakalim (c). For diazoxide and pinacidil the structures predominant in tautomeric equilibrium are shown (Wohl, 1970; Petersen *et al.*, 1978). Asterisks indicate dissociating protons ($pK_a = 8.6$ or 10.8 for diazoxide or pinacidil, respectively).

at 100,000 *g* and 4°C for 30 min, rehomogenized in Tris-buffer (50 mM, pH 7.4, 100 μM phenylmethylsulphonyl fluoride) and centrifuged again at 100,000 *g* and 4°C for 30 min. Microsomes were rehomogenized in ice-cold Tris buffer (50 mM, pH 7.4, 100 μM phenylmethylsulphonyl fluoride) and immediately frozen in liquid nitrogen until binding studies or solubilization was performed.

Preparation of microsomes from rat cerebral cortex

Male Wistar rats (300–400 g) fed *ad libitum* were stunned, decapitated, the cerebral cortex was prepared on ice and immediately immersed in ice-cold Tris buffer (290 mM sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 100 μM phenylmethylsulphonyl fluoride, 10 mM Tris, pH 7.4). The tissue was homogenized in 10 volumes of this buffer with 10 strokes of a teflon-glass homogenizer (300 r.p.m.), and then centrifuged at 600 *g* and 4°C for 15 min. The supernatant was centrifuged at 100,000 *g* and 4°C for 30 min, rehomogenized in Tris-buffer (50 mM, pH 7.4, 100 μM phenylmethylsulphonyl fluoride) and centrifuged again at 100,000 *g* and 4°C for 30 min. Microsomes were rehomogenized in ice-cold Tris buffer (50 mM, pH 7.4, 100 μM phenylmethylsulphonyl fluoride) and immediately frozen in liquid nitrogen until binding studies or solubilization was performed.

Measurement of [^{3}H]-glibenclamide binding to microsomes

Measurement of [^{3}H]-glibenclamide (51 Ci mmol^{-1}) binding to microsomes (at 22°C) was performed as previously described (Schwanstecher *et al.*, 1991).

Solubilization of microsomes

Microsomes from HIT-cells or rat cerebral cortex were thawed, rehomogenized and mixed with ice-cold solubilization buffer to give the final concentrations: 10 mg ml^{-1} microsomal protein; 1% Triton X-100 or 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS); 0.2% (w/v) phosphatidylcholine (egg); 10% (w/v) glycerole; 115 mM KCl; 1 mM EDTA; 100 μM phenylmethylsulphonyl fluoride; 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), pH 7.4. The solution was gently stirred for 1 h at 4°C and centrifuged at 120,000 *g* and 4°C for 60 min. The supernatant was then collected and stored at − 80°C until binding studies were performed. [^{3}H]-glibenclamide binding of the supernatant was not diminished after centrifugation at 120,000 *g* for 4 h or filtration through a Millex GS-filter (0.22 μm). The protein content of the microsomal suspensions or the detergent extracts was determined according to Peterson (1977) with bovine albumin used as reference. The amounts of solubilized protein were determined by use of controls with appropriate concentrations of detergent.

Measurement of [^{3}H]-glibenclamide binding to the solubilized receptor

The incubation mixture consisted of 0.7 ml Tris-buffer (50 mM, pH 7.4), 100 μl [^{3}H]-glibenclamide (3 nM), 200 μl detergent extract (final protein concentration 400 μg ml^{-1}) and other additions as described in the Results section. Unless stated otherwise, incubations were carried out for 2 h at room temperature and were terminated as described by Glossmann & Ferry (1983) with minor modifications. Incubations were terminated by addition of 1 ml of an ice-cold mixture containing 0.4% (w/v) bovine γ-globulin, 20% (w/v) polyethylene glycol, 0.4% (w/v) Triton X-100 and 50 mM Tris (pH 7.4). The incubation tubes were vortexed immediately after the addition of this mixture and were kept on ice for 10–20 min. Aliquots were filtered (Whatman GF/C filters, 25 mm diameter, soaked in ice-cold wash buffer before use) under vacuum and washed rapidly three times with 4 ml of a Tris-buffer (50 mM Tris, 8% (w/v) polyethylene glycol, pH 7.4). The ^{3}H content of the filters was determined in a liquid scintillation counter after addition of 4 ml of scintillation fluid (Quickszint 402, Zinsser, Frankfurt, Germany) and equilibration for 24 h at room temperature. Associations were started by addition of microsomes or detergent extract. Nonspecific binding was determined by incubations in the additional presence of 100 nM unlabelled glibenclamide. Specific binding was determined by subtracting nonspecific from total binding.

Treatment of results

Data analysis was performed as previously described (Schwanstecher *et al.*, 1991). Results are presented as mean ± s.e. mean for independent experiments using separate membrane preparations. Significances were calculated by the two-tailed U-test of Wilcoxon and of Mann and Whitney with the Bonferroni correction for multiple comparisons (Wallenstein *et al.*, 1980). $P<0.05$ was considered significant.

Relations between drug concentrations and specific binding were analyzed by fitting the function

$$B = 100 \times \left(1 - \frac{[A]^n}{[IC_{50}]^n + [A]^n}\right)$$

or

$$B = B_1 \times \left(1 - \frac{[A]}{[IC_{50(1)}] + [A]}\right) + B_2 \times \left(1 - \frac{[A]}{[IC_{50(2)}] + [A]}\right)$$

to the experimental data by a non-linear least squares routine. B is specific binding, B_1 or B_2 the amount of binding sites for high or low affinity binding, respectively, [A] the drug concentration, IC_{50} the half-maximally inhibitory concentration (one-site model), $IC_{50(1)}$ or $IC_{50(2)}$ the half-maximally inhibitory concentration for high or low affinity binding, respectively (two-site model), n the slope parameter (Hill coefficient). The improvement of the fit assuming a model with two binding sites instead of one binding site was tested by an F-test. The model for two binding sites was

considered to be appropriate when the fit was significantly better ($P<0.01$) than the one for the single binding site. In no case could the fit be improved by assuming more than two sites. K_D and K'_D values were calculated from half-maximally displacing concentrations (IC_{50}) according to Cheng & Prusoff (1973).

Solutions

Concentrations of the Mg^{2+}-complex of nucleotide and free Mg^{2+} are indicated in the Results section. The free Mg^{2+} concentration was held close to 1 mM by adding appropriate amounts of $MgCl_2$ to the nucleotide containing solutions. The concentrations of free ATP or ADP amounted to 1.9 or 27% of total concentrations, respectively. The required amounts of $MgCl_2$ and the composition of the solutions were calculated for pH 7.4 with a computer programme (Fabiato, 1988). The logarithms of the absolute stability constants (log K) for binding of H^+ and Mg^{2+} to ATP and ADP were taken from Sigel *et al.* (1987) or Taqui Khan & Martell (1962), respectively. The log K values were corrected for the ionic strength of the incubation buffer (40 mM) (Harrison & Bers, 1987). The log K values for all proton-ligand complexes were corrected by 0.08 in order to include the proton activity coefficient appropriate for the incubation solution (Harrison & Bers, 1989).

Materials

[^{3}H]-glibenclamide (51 Ci mmol^{-1}, 96% radiochemical purity) was from NEN (Dreieich, Germany). Minoxidil sulphate was from Upjohn Co. (Kalamazoo, MI, U.S.A.). Cromakalim [(±)-6-cyano-3,4-dihydro-2,2-dimethyl-trans-4-(2-oxo-1-pyrrolidyl)-2H-benzo[b]pyran-3-ol] was from Smith-Kline-Beecham (Harlow, GB). Pinacidil was from Leo Pharmaceutical Products (Ballerup, Denmark). Thiopentone was from Byk Gulden (Konstanz, Germany). Chlorpromazine, quinine, polyethylene glycol and phosphatidylcholine (egg) were obtained from Sigma (St. Louis, MO, U.S.A.). Bovine γ-globulin was obtained from Serva (Heidelberg, Germany). Triton X-100 and CHAPS were obtained from Boehringer (Mannheim, Germany). All other chemicals and drugs were obtained from the sources described elsewhere (Schwanstecher *et al.*, 1991; Panten *et al.*, 1989).

Results

Table 1 shows that the K^+ channel blockers, quinine (20–500 μM), chlorpromazine (10–100 μM), thiopentone (100–200 μM) and the K^+ channel openers, cromakalim (200 μM) and minoxidil sulphate (100–500 μM), did not significantly alter [^{3}H]-glibenclamide binding to microsomes from HIT-cells in the absence or presence of MgATP (300 μM). In the absence of MgATP pinacidil concentrations up to 500 μM did not significantly displace [^{3}H]-glibenclamide binding to microsomes from HIT-cells (Table 1, Figure 2). Pinacidil, 1 mM displaced [^{3}H]-glibenclamide binding slightly by 15 ± 2% ($n = 4$, $P<0.05$; Figure 2). This displacement of [^{3}H]-glibenclamide binding was strongly enhanced by MgATP (Table 1, Figure 2). In the presence of MgATP (300 μM) pinacidil displaced [^{3}H]-glibenclamide binding to microsomes from HIT-cells half-maximally at 490 ± 25 μM (Hill coefficient = 0.83; Figure 2). The competitive inhibition assays with unlabelled glibenclamide revealed high ($IC_{50(1)} = 0.88$ nM, $B_1 = 91\%$) and low ($IC_{50(2)} = 0.6$ μM, $B_2 = 9\%$) affinity binding of glibenclamide to microsomes from HIT-cells (Figure 2). The $IC_{50(1)}$ value corresponded to a dissociation constant of 0.58 nM (Cheng & Prusoff, 1973). MgATP (300 μM) inhibited binding of [^{3}H]-glibenclamide (0.3 nM) to HIT-cell microsomes by 35 ± 4% ($n = 4$).

As shown in Figure 3, pinacidil did not increase the half-maximal inhibitory concentration (IC_{50}) for displacement of [^{3}H]-glibenclamide binding by unlabelled glibenclamide in the presence of MgATP. Analysis of these data (Figure 3) assuming a model with two binding sites revealed the following values for $IC_{50(1)}$, B_1, $IC_{50(2)}$ and B_2:1.5 nM, 90%, 1.2 μM, 10% (0 mM pinacidil); 1.4 nM, 62%, 0.7 μM, 9% (0.2 mM pinacidil); 1.5 nM, 36%, 1.2 μM, 6% (1 mM pinacidil). From these experiments the following apparent dissociation constants (K'_D) for high affinity binding of glibenclamide were calculated (Cheng & Prusoff, 1973): 1.2 nM (0 mM pinacidil), 1.1 nM (0.2 mM pinacidil), 1.2 nM (1 mM pinacidil). Calculation of B_{max} values from these data according to DeBlasi *et al.* (1989) indicated that 0.2 or 1 mM pinacidil reduced the B_{max} value for high affinity binding to 63 ± 3% ($n = 4$, $P<0.05$) or 36 ± 4% of the B_{max} value in the absence of pinacidil, respectively.

Table 2 shows that pinacidil-induced displacement of [^{3}H]-glibenclamide binding to microsomes from HIT-cells was not

Table 1 Effects of potassium channel modulators on binding of [^{3}H]-glibenclamide to microsomes from HIT-cells

Drug	*Concentration* (μM)	*[^{3}H]-glibenclamide binding (%)* *no MgATP*	*MgATP*
Pinacidil	200	98 ± 2	71 ± 2[a]
	500	89 ± 3	47 ± 2[a]
Cromakalim	200	97 ± 7	98 ± 4
Minoxidil sulphate	100	100 ± 3	97 ± 2
	500	91 ± 3	88 ± 7
Quinine	20	97 ± 5	98 ± 3
	100	105 ± 3	106 ± 2
	500	106 ± 3	102 ± 4
Chlorpromazine	10	106 ± 3	112 ± 6
	100	109 ± 7	100 ± 4
Thiopentone	100	95 ± 3	89 ± 5
	200	87 ± 3	83 ± 6

[^{3}H]-glibenclamide binding was measured in 1 ml of buffer containing (final concentrations) 0.3 nM [^{3}H]-glibenclamide, 1 mM free Mg^{2+}, the indicated concentration of test-drug and no MgATP or 300 μM MgATP. Incubations were started by addition of microsomal protein from HIT-cells (final concentration 200 μg ml^{-1}) and were terminated after 60 min by filtration. Results are presented as percentages of specific binding measured in parallel control incubations (test-drug-free media with or without 300 μM MgATP). The listed values are means ± s.e.mean for results from 4 separate experiments using separate microsomal preparations. [a]$P<0.05$ for comparison with the corresponding value in the absence of MgATP.

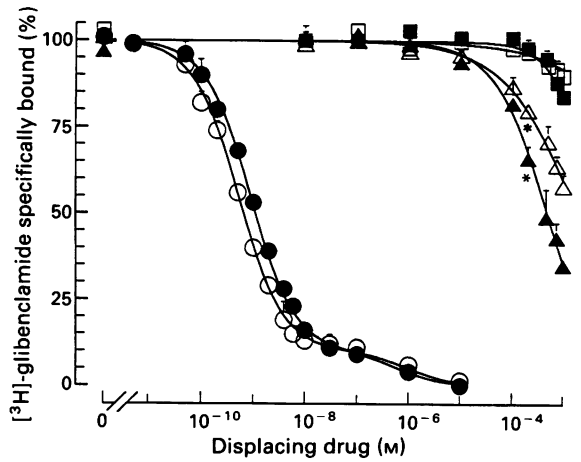


Figure 2 Effect of pinacidil on binding of [^{3}H]-glibenclamide to microsomes from HIT-cells (closed symbols) or rat cerebral cortex (open symbols). Inhibition of [^{3}H]-glibenclamide binding by unlabelled glibenclamide (circles), pinacidil (squares) or pinacidil in the presence of MgATP (triangles) was measured in 1 ml of buffer containing (final concentrations) 0.3 nM [^{3}H]-glibenclamide, 1 mM free Mg^{2+}, the indicated concentrations of displacing drug (logarithmic scale) and no MgATP (circles and squares) or 300 μM MgATP (triangles). Incubations were started by addition of microsomal protein from HIT-cells or rat cerebral cortex (200 or 400 μg ml^{-1} final concentration, respectively) and were terminated after 60 min by filtration. Results are presented as percentages of specific binding of [^{3}H]-glibenclamide in the absence of unlabelled drugs and in the absence or presence of MgATP. Values are given as means (with s.e. mean shown by vertical bars when larger than symbols) for results from 4 separate experiments using separate membrane preparations. *$P<0.05$ for comparison with the corresponding value for inhibition of [^{3}H]-glibenclamide binding by pinacidil in the absence of MgATP.

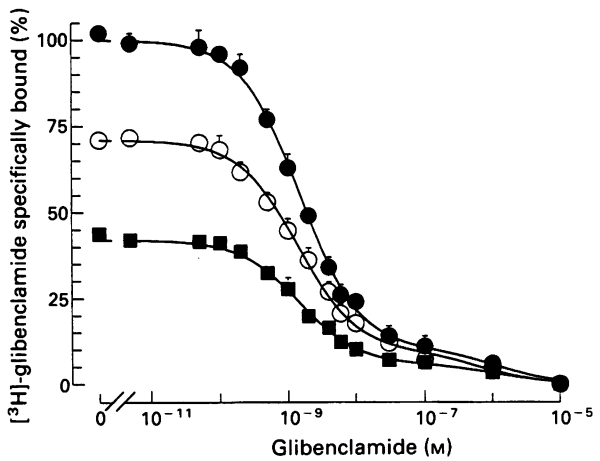


Figure 3 Effect of pinacidil on the apparent dissociation constant (K'_D) for glibenclamide in microsomes from HIT-cells. Inhibition of [^{3}H]-glibenclamide binding by unlabelled glibenclamide in the presence of 0 (●), 0.2 mM (○) or 1 mM (■) pinacidil was measured in 1 ml of buffer containing (final concentrations) 0.3 nM [^{3}H]-glibenclamide, 300 μM MgATP, 1 mM free Mg^{2+} and the indicated concentrations of unlabelled glibenclamide (logarithmic scale). Incubations were started by addition of microsomal protein (200 μg ml^{-1} final concentration) and were terminated after 60 min by filtration. Results are presented as percentages of specific binding of [^{3}H]-glibenclamide in the absence of pinacidil and unlabelled glibenclamide. Values are given as means (with s.e. mean shown by vertical bars when larger than symbols) for results from 4 separate experiments using separate membrane preparations.

Table 2 Effect of hexokinase and glucose on pinacidil-induced displacement of [^{3}H]-glibenclamide binding to microsomes from HIT-cells exposed to MgADP

	[^{3}H]-glibenclamide binding (%)			
Nucleotide	*No HK No Gluc*	*+ HK*	*+ Gluc*	*+ HK + Gluc*
None	100	98 ± 3	89 ± 3	95 ± 5
MgADP	40 ± 3[a]	47 ± 4[a]	43 ± 5[a]	89 ± 5[b]

Incubations in media containing (final concentrations) 1 mM pinacidil, 0.3 nM [^{3}H]-glibenclamide, 1 mM free Mg^{2+} and the indicated additions [0.5 mM MgADP; 15 u ml^{-1} hexokinase (HK); 20 mM glucose (Gluc)] were started by addition of microsomal protein (200 μg ml^{-1} final concentration) and were terminated after 60 min by filtration. Each single experiment consisted of 8 parallel incubations in different media as indicated. Results are given as percentages of specific binding in the absence of nucleotide, HK and Gluc. All listed values are means ± s.e.mean for results of 4 separate experiments using separate microsomal preparations. Pinacidil (1 mM) inhibited [^{3}H]-glibenclamide binding in the absence of MgADP by 24%.
[a]$P<0.05$ for comparison with the corresponding value in the absence of MgADP.
[b]$P<0.05$ for comparison with specific binding in the presence of MgADP and HK (but absence of Gluc).

only enhanced by MgATP but also by MgADP. However, this effect of MgADP was not observed in the simultaneous presence of hexokinase and glucose (Table 2). This finding indicates that the effect of MgADP requires the formation of MgATP.

To characterize further the mechanism by which pinacidil interacts with the sulphonylurea receptor, the receptor was solubilized from HIT-cell microsomes using nonionic (Triton X-100) or zwitterionic (CHAPS) detergents. In the absence of both MgATP and pinacidil, competitive inhibition assay with unlabelled glibenclamide indicated only high affinity binding of glibenclamide to sites solubilized with Triton X-100 from HIT-cells (Figure 4). Analysis of these data (one-site model) revealed a half-maximal inhibitory concentration of 0.72 nM (corresponding to a K_D of 0.42 nM) and a Hill coefficient of 0.96. Addition of MgATP (300 μM) inhibited binding of [^{3}H]-glibenclamide (0.3 nM) to solubilized binding sites from HIT-cells by 77 ± 4% ($n = 4$, Triton-extract) or 65% ($n = 2$, CHAPS-extract). In the absence of MgATP, 200 μM pinacidil inhibited [^{3}H]-glibenclamide (0.3 nM) binding to sites solubilized with Triton X-100 (Figure 4) or CHAPS (results not shown in a figure) by 14 ± 5% ($n = 4$, $P>0.05$) or 5% ($n = 2$), respectively. In the presence of MgATP (300 μM), however, 200 μM pinacidil decreased specific binding of [^{3}H]-glibenclamide (0.3 nM) by 57 ± 6% (Triton-extract; Figure 4) or 31% ($n = 2$, CHAPS-extract; results not shown in a figure), respectively. In the presence of MgATP (300 μM) pinacidil displaced [^{3}H]-glibenclamide binding to solubilized binding sites from HIT-cells half-maximally at 140 ± 8 μM (Hill coefficient = 0.60, Triton-extract; Figure 4) or 790 μM ($n = 2$, CHAPS-extract; results not shown in a figure).

High affinity binding sites for sulphonylureas similar to those in insulin-secreting cells have been found in mammalian brain. To test whether pinacidil-induced inhibition of [^{3}H]-glibenclamide binding to cerebral sites, was also enhanced by MgATP, membrane-bound and solubilized binding sites were examined. The following values were obtained for inhibition of [^{3}H]-glibenclamide binding to cerebral sites by unlabelled glibenclamide: $IC_{50(1)} = 0.52$ nM, $B_1 = 90\%$, $IC_{50(2)} = 0.9$ μM, $B_2 = 10\%$ (microsomal binding site; Figure

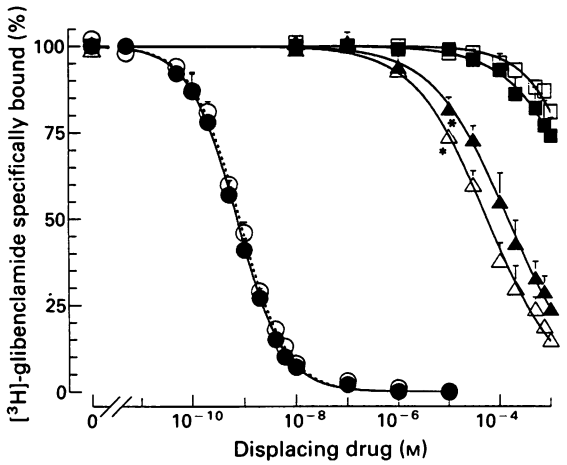


Figure 4 Effect of pinacidil on binding of [^{3}H]-glibenclamide to solubilized binding sites from HIT-cells (closed symbols) or rat cerebral cortex (open symbols). Inhibition of [^{3}H]-glibenclamide binding by unlabelled glibenclamide (circles), pinacidil (squares) or pinacidil in the presence of MgATP (triangles) was measured in 1 ml of buffer containing (final concentrations) 0.3 nM [^{3}H]-glibenclamide, 1 mM free Mg^{2+}, the indicated concentrations of displacing drug (logarithmic scale) and no MgATP (circles and squares) or 300 μM MgATP (triangles). Incubations were started by addition of solubilized protein (400 μg ml^{-1} final concentration) and terminated after 120 min by polyethylene glycol precipitation. Results are presented as percentages of specific binding of [^{3}H]-glibenclamide in the absence of unlabelled drugs and in the absence or presence of mgATP. Values are given as means (with s.e. mean shown by vertical bars when larger than symbols) for results from 4 separate experiments using separate membrane preparations. *$P<0.05$ for comparison with the corresponding value for inhibition of [^{3}H]-glibenclamide binding by pinacidil in the absence of MgATP.

2); $IC_{50} = 0.81$ nM, Hill coefficient = 0.96 (Triton-extract; Figure 4, dashed line). The IC_{50} values for high affinity binding corresponded to dissociation constants of 0.22 nM (microsomal site) or 0.51 nM (Triton-extract). Addition of MgATP (300 μM) inhibited binding of [^{3}H]-glibenclamide (0.3 nM) to cerebral sites by 38 ± 3% ($n = 4$, microsomes) or 81 ± 4% ($n = 4$, Triton-extract). MgATP (300 μM) significantly enhanced inhibition of [^{3}H]-glibenclamide binding to microsomes from rat cerebral cortex by 1 mM pinacidil from 10 ± 2% to 42 ± 3% ($n = 4$, $P<0.05$; Figure 2). In the presence of MgATP (300 μM) pinacidil displaced [^{3}H]-glibenclamide binding to sites solubilized with Triton X-100 half-maximally at 56 ± 6 μM (Hill coefficient = 0.61; Figure 4).

Discussion

In the present studies, it was found that blockers of the K-ATP-channel which are structurally unrelated to sulphonylureas (quinine, chlorpromazine, thiopentone) do not significantly interact with the sulphonylurea receptor both in phosphorylating and dephosphorylating conditions. The tested concentrations of quinine (20–500 μM), chlorpromazine (10–100 μM) and thiopentone (100–200 μM) inhibit the K-ATP-channels in insulin-secreting cells almost completely (Bokvist *et al.*, 1990; Kozlowski & Ashford, 1991; Müller *et al.*, 1991). Evidence was previously presented that MgATP-induced inhibition of binding reflects a general phenomenon, being valid for the interaction of all sulphonylureas with their receptor in pancreatic islets (Schwanstecher *et al.*, 1992). This effect of MgATP is accompanied by enhanced displacement of [^{3}H]-glibenclamide from the sulphonylurea receptor by diazoxide (Schwanstecher *et al.*, 1991). We now show that MgATP also enhances pinacidil-induced displacement of [^{3}H]-glibenclamide from the sulphonylurea receptor in insulin-secreting cells. Similar effects are not observed for cromakalim (200 μM) or minoxidil sulphate (100–500 μM) which have been suggested to open K^+-channels in smooth muscle or insulin-secreting cells (Edwards & Weston, 1990; Robertson & Steinberg, 1990; Newgreen *et al.*, 1990; Dunne *et al.*, 1990). These findings support the idea that MgATP-dependent interaction with sulphonylurea binding is characteristic of K^+ channel openers structurally related to diazoxide and pinacidil.

Pinacidil-induced displacement of [^{3}H]-glibenclamide from the sulphonylurea receptor in insulin-secreting cells was not only enhanced by MgATP but also by MgADP (Table 2). However, this effect of MgADP probably requires formation of MgATP by transphosphorylation reactions in the microsomal incubations. This is suggested by the finding that MgADP does not enhance pinacidil-induced displacement of [^{3}H]-glibenclamide binding when the MgATP concentration is kept low by the hexokinase reaction (Table 2).

In the presence of MgATP, pinacidil decreases the number of binding sites but not the affinity for binding of glibenclamide to its receptor (Figure 3). This observation shows that the inhibitory effect of pinacidil cannot be explained by a competitive interaction of pinacidil and sulphonylureas at the high affinity binding site. Functional studies with vascular smooth muscle also argue against a competitive antagonism between pinacidil and sulphonylureas (Steinberg *et al.*, 1991). The present results do not allow a definite conclusion on the mechanism of the inhibitory effect of pinacidil. However, irreversible binding of pinacidil to the binding site for sulphonylureas is unlikely, since pinacidil-induced opening of the K-ATP-channels in insulin-secreting cells is reversible within 2 min (Garrino *et al.*, 1989). One possible explanation for the interaction between pinacidil and sulphonylureas could be that binding of pinacidil to a distinct receptor allosterically decreases the number of sulphonylurea receptors. In analogy to the suggestion for the morphine-enkephalin receptors (Rothman & Westfall, 1982), pinacidil could allosterically cause K'_D values for sulphonylureas of such magnitude that sulphonylurea binding to the pinacidil-induced conformation of the sulphonylurea receptor would not be detectable.

The inhibitory effect of pinacidil on glibenclamide binding is maintained after solubilization of the sulphonylurea receptor with nonionic (Triton X-100) and zwitterionic (CHAPS) detergents, which differ in their efficiency in preventing aggregation of solubilized proteins. This finding suggests a location of the pinacidil and sulphonylurea binding sites at the same protein or at closely associated proteins.

High affinity binding sites for sulphonylureas similar to those of insulin-secreting cells have been shown to occur in brain (Kaubisch *et al.*, 1982; Geisen *et al.*, 1985; Lupo & Bataille, 1987; Bernardi *et al.*, 1988; Mourre *et al.*, 1990; Gehlert *et al.*, 1990; Treherne & Ashford, 1991; Zini *et al.*, 1991; Angel & Bidet, 1991; Gopalakrishnan *et al.*, 1991). The present study confirms that pinacidil concentrations higher than 100 μM are required for significant inhibition of [^{3}H]-glibenclamide binding to brain membranes incubated in the absence of MgATP (Angel & Bidet, 1991; Gopalakrishnan *et al.*, 1991). In high affinity binding sites from cerebral cortex, MgATP caused an enhancement of pinacidil-induced displacement of [^{3}H]-glibenclamide binding, similar to that described for the sulphonylurea receptor in insulin-secreting cells (Figures 2 and 4). This observation supports the view that the cerebral site represents a sulphonylurea receptor resembling the pancreatic receptor.

The dissociation constants for high and low affinity binding of glibenclamide to membranes from HIT-cells and cerebral cortex are in accordance with those found in earlier investigations (Kaubisch *et al.*, 1982; Geisen *et al.*, 1985; Lupo & Bataille, 1987; Aguilar-Bryan *et al.*, 1990; Zini *et al.*, 1991). However, low affinity binding is missing in detergent

extracts from HIT-cell or brain microsomes (Figure 4). This might be due to selective denaturation of binding proteins with low affinity during solubilization in the presence of Triton X-100. Alternatively, it is conceivable that low affinity binding reflects binding of glibenclamide to phospholipids in the microsomal membranes (Deleers & Malaisse, 1984). After solubilization these phospholipids probably escape precipitation by polyethylene glycol.

In pancreatic β-cells, the potency of diazoxide for activation of K^+-channels and inhibition of insulin release is about 10 fold higher than the potency of pinacidil (Garrino *et al.*, 1989). In microsomal membranes from pancreatic islets incubated in the presence of MgATP, 140 μM diazoxide reduced [^{3}H]-glibenclamide binding by 50% (Schwanstecher *et al.*, 1991). Inhibition by 50% of [^{3}H]-glibenclamide binding to HIT-cell microsomes was caused by 490 μM pinacidil in the presence of MgATP (this study). The more pronounced difference between the potencies of diazoxide and pinacidil in intact β-cells as compared to microsomal membranes might be due to destruction of the normal environment for the membrane receptors when preparing microsomes. This view is supported by the increase in potency of pinacidil induced by solubilization of the membrane proteins.

The total concentration of cytosolic ATP in islet cells ranges above 0.5 mM (Malaisse & Sener, 1987) and more than 90% of this concentration probably exists as MgATP complex (Corkey *et al.*, 1986). Therefore, the states of the binding sites for pinacidil and sulphonylureas observed in the presence of MgATP might predominate in the intact β-cell. Even in the presence of MgATP the pinacidil concentration giving 50% displacement of [^{3}H]-glibenclamide binding from HIT-cell microsomes is high (490 μM). However, hyperpolarization of the pancreatic β-cells is induced by opening of much less than 10% of all K-ATP-channels in the plasma membrane (Cook *et al.*, 1988; Panten *et al.*, 1990). Hence occupation of a small percentage of the pinacidil receptors would be sufficient for inhibition of insulin release.

Inhibition of the opening activity of the K-ATP-channel in β-cells by ATP does not require the presence of Mg^{2+} (Ashcroft & Ashcroft, 1990). Therefore, MgATP appears to modulate the binding properties of the sulphonylurea receptor via a site not identical with the site mediating ATP-induced channel closure. Moreover, sulphonylureas do not compete with free ATP or MgATP for binding to β-cell microsomes (Schwanstecher *et al.*, 1992). These findings are in favour of the view that MgATP-induced modulation of binding, ATP-induced channel closure and sulphonylurea-induced channel closure are mediated by three separate binding sites. The present study suggests an additional binding site for the K^+ channel openers, diazoxide and pinacidil. The binding sites for these K^+ channel openers on the one hand and for sulphonylureas on the other hand seem to be located at a single protein or at tightly associated proteins. The Mg complex of ADP (MgADP) in the β-cell cytosol increases the opening activity of the K-ATP-channel by interaction with a site probably not identical with the sites mediating ATP- or sulphonylurea-induced channel inhibition (Ashcroft & Ashcroft, 1990; Schwanstecher *et al.*, 1992). In the presence of MgATP, pinacidil (this study) and diazoxide (Schwanstecher *et al.*, 1991), but not free ADP + MgADP (Schwanstecher *et al.*, 1992), diminished [^{3}H]-glibenclamide binding. Hence, a completely separate binding site for MgADP might exist. It is therefore suggested that channel-opening and channel-closing ligands control the K-ATP-channel via four separate binding sites. A protein kinase tightly associated with the K-ATP-channel and/or regulatory proteins appears to exert additional control. MgATP serves as substrate for protein phosphorylation which could modulate the binding properties of the sulphonylurea receptor.

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The contribution of α-adrenoceptors to neurally-mediated contractions of the rabbit urethral smooth muscle

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1 The nature of the nerve-mediated contractions in the urethral smooth muscle from the rabbit was studied *in vitro*. Field stimulation caused smaller contractile responses than in the detrusor of the rabbit.

2 There was no significant difference in response to field stimulation or exogenous agents acting on adrenoceptors between longitudinal and circular strips from the rabbit urethra. Histological studies showed that the urethral muscle is arranged in three layers, which run circularly and longitudinally.

3 Atropine had very little effect on the response to field stimulation, phentolamine almost abolished the contractile response to nerve stimulation and sometimes unmasked a relaxation.

4 The α_1-adrenoceptor blocking agent, prazosin, blocked both the contractile response to the α_1-receptor agonist phenylephrine and that to intrinsic nerve stimulation, with similar potencies. The α_2-blocking agent yohimbine shifted the dose-response curve of the contractile response to the α_2-agonist, clonidine, in a dose-dependent manner, 10^{-7} M causing a 10 fold shift. This concentration had no effect on the response to intrinsic nerve stimulation, suggesting that α_2-receptors are not involved in the response. Higher concentrations of yohimbine caused a suppression of the nerve-evoked response which is assumed to be non-specific.

5 Noradrenaline, phenylephrine, and clonidine caused dose-dependent contractile responses in the rabbit urethral strips. The contractions induced by clonidine developed more slowly than those induced by noradrenaline and phenylephrine.

6 These results demonstrate that the rabbit urethral smooth muscle contains both α_1- and α_2-adrenoceptors, and the nerve-mediated contraction of the rabbit urethra is adrenergic in nature and mediated mainly via α_1-adrenoceptors.

Keywords: α_1-Adrenoceptor; α_2-adrenoceptor; rabbit urethra; adrenergic innervation; field stimulation

Introduction

Adrenergic innervation of the bladder and urethra smooth musculature has been extensively demonstrated in animals by various investigators (Levin & Wein, 1979; Andersson *et al.*, 1984; Gosling, 1986). There is general agreement, however, that the smooth muscle of the bladder and proximal urethra in both a variety of animals and in humans contains both α- and β-adrenoceptors. These receptors play an important role in the filling/storage phase of the micturition cycle (Wein, 1987). In the urethra, smooth muscle tone is believed to be controlled by the sympathetic nervous system via α-adrenoceptors (Donker *et al.*, 1972; Awad & Downie, 1976). α-Adrenoceptor-mediated responses predominate in the bladder base and proximal urethra, whereas β-adrenoceptor-mediated responses predominate in the bladder body (Edvardsen & Setekleiv, 1968; Edvardsen, 1968; Anderson *et al.*, 1971; Elmer, 1974; Benson *et al.*, 1976; Levin *et al.*, 1980).

It is generally accepted that there are two major α-adrenoceptor subtypes, the α_1- and the α_2-adrenoceptors (Rang & Dale, 1987). By means of selective agonists and antagonists these α-adrenoceptor subtypes have been characterized in different tissues and species (Wikberg, 1979; Davey, 1986).

Andersson & Sjögren (1982) have established that both α-subtypes are present and stimulation of these receptors increases, and blockade decreases, the intraurethral pressure, and that these effects can be used therapeutically in functional disorders of the lower urinary tract.

The purpose of the present study was to examine the nature of the nerve-mediated contractions in the urethral smooth muscle from the rabbit *in vitro* by field stimulation of muscle strips, and recording the isometric tension generated. To investigate α-adrenoceptor subtypes in the urethra, the nature of the responses to the exogenously applied selective adrenoceptor agonists isoprenaline, phenylephrine and clonidine were examined. The inhibition of the responses to adrenoceptor agonists by their antagonists was also examined.

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Methods

Preparation of specimens

Rabbit urethra were obtained from New Zealand White rabbits the organs of which were being used for experiments by other research groups in the University. They were of either sex, weighing from 600 g to 2500 g. The animals were stunned by a blow to the neck and exsanguinated. The specimens were placed in oxygenated Krebs solution. The mucosa was then dissected free from the urethral muscles. The urethral strips were made in either a longitudinal or transverse direction. An operating microscope was used to ensure that there was good longitudinal alignment of the muscle bundles within a strip. Strips of lower urinary tract smooth muscle measuring approximately 8 mm × 1 mm × 1 mm were prepared.

Tension recording and stimulation

Fine silk ligatures were tied to each end of a strip which was then mounted between platinum ring electrodes 1 cm apart in a specially constructed Perspex organ bath. The organ bath had a capacity of 0.2 ml and was continuously perfused with warmed (35–37°C) Krebs solution at a flow rate of 1 ml min^{-1} (Brading & Sibley, 1983). Initially, the strips were

allowed to equilibrate for at least 1 h, after a resting tension of 1 g had been applied. Tension was measured isometrically with Pioden UF1 transducers and recorded on a Watanabe multichannel pen recorder after amplification.

Activation of intrinsic nerves was achieved by electrical field stimulation by pulses with the following parameters: 50 V, 0.05 ms width, 5 s trains at varying frequency. Successive trains of stimuli were given at least 5 min after the previous contraction had returned to baseline. After each drug-induced response, recovery periods of 10–30 min were allowed before further drug application. Drugs and solutions were applied by dipping the ends of the feeder tubes for the perfusion system into the appropriate solutions. This allowed accurately timed exposure of the tissues to different solutions, and by following the bubbles introduced when the solution was changed the instant of tissue contract was recorded.

At the start of each experiment, the contractile response of the strips to a 2-min application of 126 mM KCl was obtained and subsequent responses were recorded as a percentage of the control response. This dose of KCl produced a near maximal contraction.

Drugs

The following drugs were used: hexamethonium bromide, tetrodotoxin (TTX), α,β-methylene adenosine 5′-triphosphate (α,β-methylene ATP), prazosin hydrochloride, clonidine hydrochloride, (−)-phenylephrine (all these drugs were obtained from Sigma); atropine sulphate and (−)-noradrenaline (obtained from B.D.H.); propranolol hydrochloride (I.C.I), isoprenaline sulphate (Burroughs Wellcome), phentolamine mesylate (Ciba). Drugs were, where possible, dissolved in distilled water to make a concentrated stock solution; these were refrigerated until needed. α,β-methylene ATP was stored in the deep freeze, and stock solution made up freshly for each experiment. Prazosin was dissolved in 1:9 of DMA (N N-dimethylacetamide, B.D.H.): distilled water, and subsequent dilution of the drug was made with Krebs solution. (−)-Phenylephrine or noradrenaline stock was prepared with added ascorbic acid (0.1 mg) to prevent oxidization. Drug concentrations are given as the final bath values. The vehicle was checked and had no effect. The Krebs solution used had the following composition (mM): NaCl 120.0, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 15.4, NaH_2PO_4 1.0 and glucose 11.5. All solutions were equilibrated with 97% O_2: 3% CO_2, pH 7.4 at 35–37°C. High K^+ solution (126 mM) was prepared by replacing NaCl with an equimolar amount of KCl in normal Krebs solution.

Statistical analysis

Student's *t* test and analysis of variance were used to compare differences in responses between the control and experimental curves. A probability level of $P < 0.05$ was accepted as significant. When appropriate, results are presented as means ± standard error of the mean (s.e.mean).

Results

The rabbit urethra is approximately 1 cm in length, in both male and female. Microscopically, the urethra comprises an outer muscle coat and an innner transitional epithelium. Transverse sections through the proximal and distal portion of the urethra and longitudinal sections through the whole urethra showed that muscle bundles are arranged in three ill-defined layers. Longitudinally-orientated muscle bundles predominate on the inner and outer aspects of a substantial middle layer composed of more circularly-disposed bundles.

Small contractile responses to field stimulation (approximately 30% of the response in the anterior detrusor) were obtained in urethral strips. Figure 1 shows the frequency-dependency of the response in comparison with the detrusor

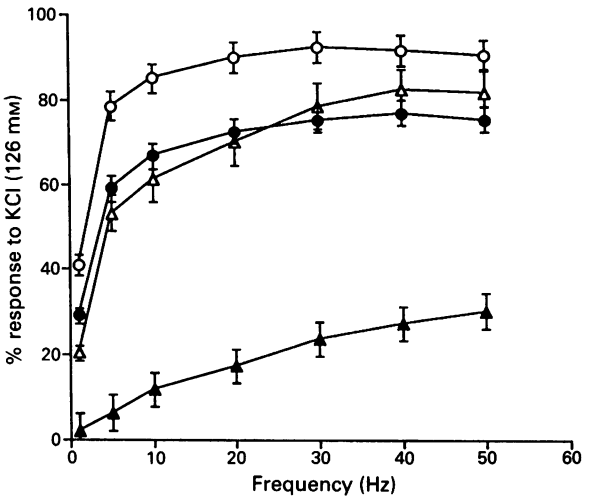


Figure 1 Frequency-response curves of the anterior detrusor (○, $n = 45$), posterior detrusor (●, $n = 48$), trigone (△, $n = 27$) and longitudinal urethral strips (▲, $n = 13$) from male rabbits. Stimulus parameters: 50 V, 0.05 ms width, 5 s trains. Vertical bars indicate s.e.mean.

and trigone. Occasionally, a slight relaxation followed by contraction was observed in the urethral strips. There were no significant differences in the response to field stimulation between longitudinal and circular strips from the rabbit urethra ($n = 8$).

Unlike rabbit urinary bladder, in the urethral strips 10^{-7} M atropine had very little effect on the response to field stimulation; the non-selective α-adrenoceptor blocker phentolamine (2×10^{-7} M), however, almost abolished the contractile response to nerve stimulation and sometimes unmasked a relaxation (Figure 2). To investigate the subtypes of the adrenergic nervous influence, selective α_1- and α_2-blockers were used. The results revealed that the α_2-blocker yohimbine (10^{-7} M) had no effect on the contractile responses to field stimulation ($n = 8$), and the α_1-blocker prazosin (10^{-7} M and 10^{-6} M) caused dose-dependent inhibition of the contractile response to field stimulation. The inhibition of field stimulation by the combination of prazosin and yohimbine (both 10^{-7} M, $n = 13$) was no different from that due to 10^{-7} M prazosin alone ($n = 7$). Interestingly, 10^{-6} M yohimbine produced a large reduction in the contractile response at all stimulation frequencies ($n = 6$). This reduction was similar to that caused by 10^{-6} M prazosin ($n = 6$) (Figure 3). All responses to transmural stimulation were blocked completely by TTX (0.1 μg ml^{-1}).

In experiments on strips from rabbit urethra, noradrenaline caused a dose-dependent contraction, the maximum response being reached after a 2 min application of 5×10^{-5} M noradrenaline. There was no difference in the contractile response to noradrenaline between circular and longitudinal muscle strips. This response could be inhibited by the α-blocking agent phentolamine (10^{-6} M), which unmasked a small relaxation response in some strips (Figure 4). To characterize the subtypes of α-receptor in the urethral strips, the α_1-receptor agonist phenylephrine, α_2-receptor agonist clonidine, selective α_1-blocking agent prazosin and α_2-blocking agent yohimbine were used. Dose-dependent contractile response to phenylephrine, noradrenaline and clonidine in the rabbit urethra strips were obtained (Figure 5), the maximum response induced by phenylephrine was reached with a 2 min application of 3×10^{-4} M, and for clonidine with a 10 s application in a concentration of 10^{-5} M. The contractions induced by clonidine developed more slowly than those induced by noradrenaline and phenylephrine, and the relaxation after wash-out of the agonist was also slower (Figure 4).

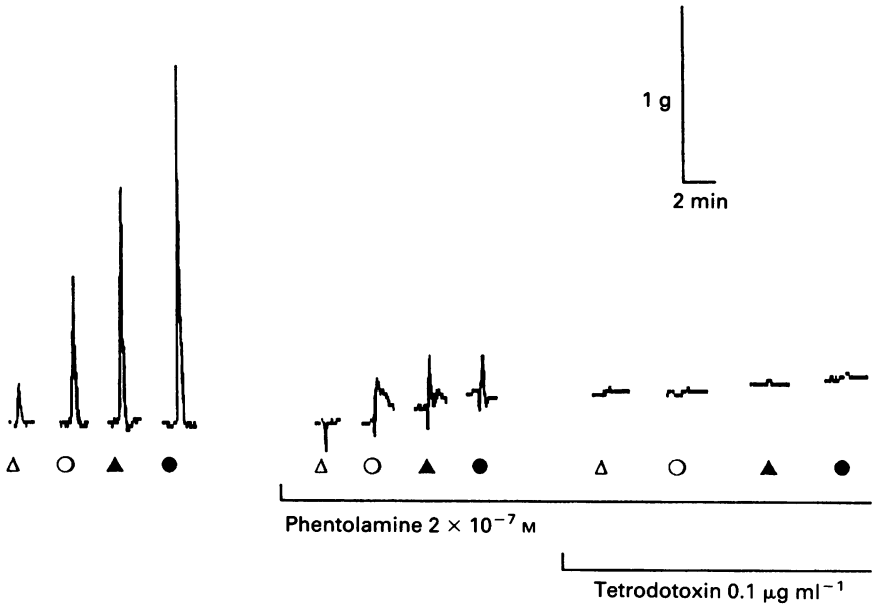


Figure 2 Field stimulation-induced contraction at 5 Hz (Δ), 10 Hz (○), 20 Hz (▲) and 40 Hz (●) in male rabbit urethra before and after exposure to phentolamine 2×10^{-7} M for 20 min. The effects of adding tetrodotoxin 0.1 μg ml^{-1} on the response is shown. Stimulus strength 50 V; stimulus duration 0.05 ms; stimulus train 5 s.

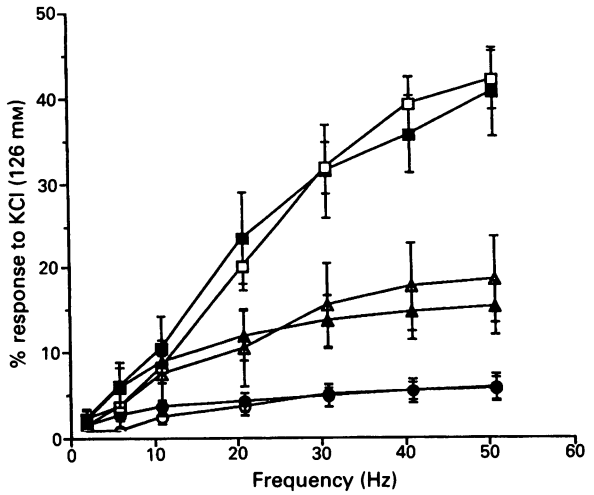


Figure 3 Effect of prazosin and yohimbine on the contractile responses to field stimulation in the male rabbit urethra. Yohimbine 10^{-7} M (■, $n = 8$) had no effect on the control (□) contractile responses to field stimulation; prazosin (10^{-7} M, Δ; 10^{-6}, ●) caused dose-dependent inhibition of the contractile response to field stimulation. The inhibition of field stimulation by the combination of prazosin and yohimbine (both 10^{-7} M, ▲, $n = 13$) was no different from that due to 10^{-7} M prazosin ($n = 7$) alone. Yohimbine 10^{-6} M (○, $n = 6$) produces a large reduction of contraction which is similar to that caused by 10^{-6} M prazosin ($n = 6$). Vertical bars represent s.e.mean.

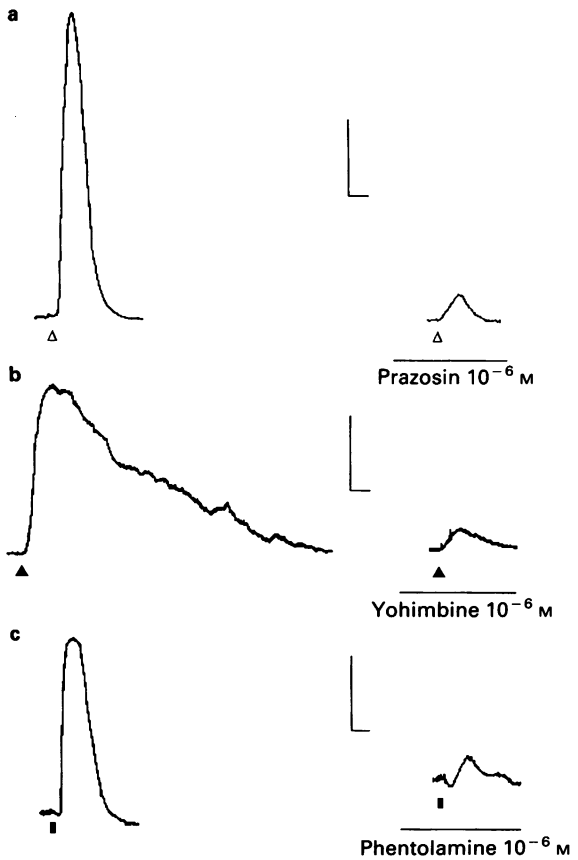


Figure 4 Contractile responses to noradrenaline (■, c), phenylephrine (Δ, a) and clonidine (▲, b) at the same concentration of 10^{-5} M in rabbit urethral strips. The contractions induced by clonidine (10 s application) developed more slowly than those induced by noradrenaline (2 min application) and phenylephrine (2 min application) and relaxation after contraction was also slower. The contraction was blocked after a 30 min exposure to the blockers. Horizontal bar represents 2 min. Vertical bar represents 0.5 g of tension.

The EC_{50} value for noradrenaline was 8.3×10^{-7} M ($n = 12$), for phenylephrine it was 4.5×10^{-6} M ($n = 16$) and for clonidine it was 3.0×10^{-6} M ($n = 6$). The contractile response to 3×10^{-4} M phenylephrine was larger than the contractile response due to 5×10^{-5} M noradrenaline (118.6 ± 2.2% of NA response; $n = 12$), whereas the contractile response to clonidine 5×10^{-5} M was smaller (60.2 ± 2.4% of NA response; $n = 9$) than that due to noradrenaline.

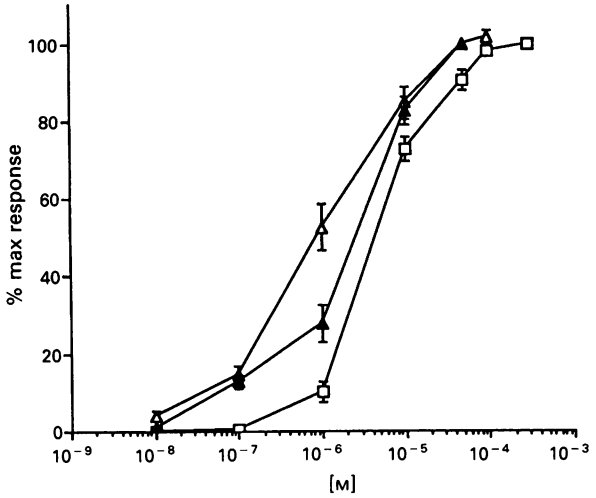


Figure 5 Dose-dependent contractile responses to noradrenaline (△, $n = 12$), clonidine (▲, $n = 6$) and phenylephrine (□, $n = 16$) in the rabbit urethral strips. The EC_{50} value for noradrenaline is $8.3 \pm 0.7 \times 10^{-7}$ M, for clonidine, $3.0 \pm 0.8 \times 10^{-6}$ M and for phenylephrine, $4.5 \pm 0.1 \times 10^{-6}$ M. 100% denotes the maximum response elicited by each agonist. Vertical bars indicate s.e.mean.

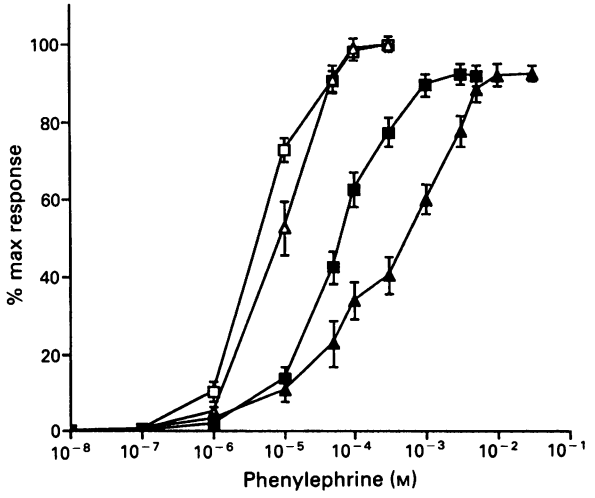


Figure 6 Effect of prazosin on the contractile response to phenylephrine in the rabbit urethra. Results showed that prazosin (10^{-8} M, △, $n = 10$; 10^{-7} M, ■, $n = 12$; 10^{-6} M, ▲, $n = 10$) cause dose-dependent inhibition, the response curves shifted to right of the control (□). The K_B value from the Schild plot was 7.2×10^{-9} M. Vertical bars show s.e.mean.

Prazosin caused a dose-dependent inhibition of the phenylephrine-induced contraction (Figure 6). Prazosin (10^{-6} M) inhibited the contractile response to phenylephrine (5×10^{-5} M) by $74.7 \pm 4.3\%$ and inhibited that to phenylephrine (3×10^{-4} M) by $60 \pm 5\%$ ($n = 10$). The drug shifted the dose-response curve to the right with little change in the maximum indicating a competitive antagonist ($K_B = 7.2 \times 10^{-9}$ M). The α_2-blocker yohimbine also caused a dose-dependent inhibition of the contractile response to clonidine (Figure 7). It was not possible from the results to tell if the antagonism was competitive, since high enough concentrations of clonidine were not used. Yohimbine (10^{-6} M) inhibited the contractile response to clonidine (5×10^{-5} M) by $91 \pm 2\%$ ($n = 6$). It was found that there was no significant effect of prazosin on the clonidine-induced contraction ($n = 4$), or yohimbine on the phenylephrine-induced contraction ($n = 4$) in the urethral strips.

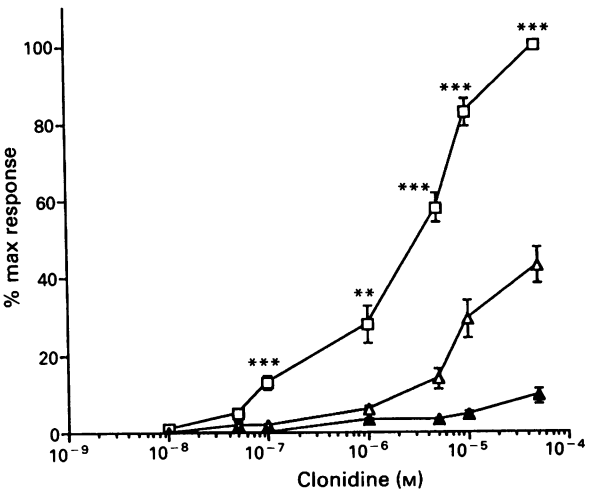


Figure 7 Effects of yohimbine 10^{-7} M (△, $n = 6$) and 10^{-6} M (▲, $n = 6$) on the contractile response to clonidine in the male rabbit urethral strips. It showed irreversible competitive antagonism on the clonidine concentration-effect curves. Control (□). Vertical bar represent s.e.mean. $^{**}P < 0.01$; $^{***}P < 0.001$.

Discussion

Studies of the effects of adrenergic nerves on the smooth muscle of rabbit urethra have been performed by isometric tension recording using electrical field stimulation and application of exogenous agents. Field stimulation caused smaller contractile responses than in the detrusor from the rabbit. There was no significant difference in response to field stimulation between longitudinal and circular strips from the rabbit urethra. This is in agreement with the findings of other studies (Mattiasson *et al.*, 1989). It is interesting to note that despite the histological evidence that the muscle is arranged in three layers, which run circularly and longitudinally, the pharmacological findings, such as responses to field stimulation, clonidine and phenylephrine showed no significant differences in behaviour between longitudinal and circular strips. This finding agrees with that of Andersson *et al.* (1984).

Phentolamine inhibited the nerve-mediated contraction in the majority of preparations and unmasked relaxation in some urethral strips, which was abolished by adding propranolol. However, atropine and α,β-methylene ATP had no effect on the contractile response to field stimulation in the rabbit urethra. This suggests that nerve-mediated contraction in the rabbit urethra is induced by α-adrenoceptor activation, but some effect via β-adrenoceptors may also be present in this tissue. It cannot be excluded that some other non-adrenergic non-cholinergic neurotransmitters are involved in the response of the urethra. Mattiasson *et al.* (1989) found that vasoactive intestinal polypeptide (VIP) completely relaxed both circular and longitudinal preparations from the female rabbit urethra which had been contracted by noradrenaline.

To study the postjunctional sub-types of α-adrenoceptors, we examined the responses of the rabbit urethral smooth muscle using selective α_1- and α_2-adrenoceptor agents and non-selective adrenoceptor agents. Noradrenaline, as well as the α_1-adrenoceptor selective agonist phenylephrine and the α_2-adrenoceptor selective agonist clonidine induced a dose-dependent contraction of the urethral smooth muscle. The α_1-blocker, prazosin, not only inhibited the contractile response to phenylephrine but also blocked nerve-mediated contraction in the urethra. A different result was noted with the

α_2-blocker, yohimbine. At 10^{-7} M, yohimbine had no effect on the contractile response to field stimulation, although at this concentration it markedly inhibited the contractile response to the α_2-agonist, clonidine. On the other hand, the inhibition of field stimulation by a combination of prazosin and yohimbine (both 10^{-7} M) was no different from that due to 10^{-7} M prazosin alone, suggesting the contractile response to nerve stimulation in the rabbit urethral preparations is mainly due to α_1-stimulation. Higher concentrations of yohimbine (10^{-6} M), which had no effect on the phenylephrine-induced contraction, produced clear inhibition of nerve-evoked contraction at all frequencies. This reduction was very similar to that caused by 10^{-6} M prazosin, suggesting that yohimbine has a non-selective effect on the response to intrinsic nerve stimulation at this concentration. However, these results demonstrated that the rabbit urethral smooth muscle contains both sub-types of α–adrenoceptors (Andersson *et al.*, 1984).

In common with cholinergic transmission, there is evidence for autoinhibition at adrenergic terminals, where NA acts on prejunctional α_2-adrenoceptors leading to negative feed back inhibition of the release of neurotransmitter (Langer, 1977; Burnstock, 1983). This is unlikely to be occurring in the present study because yohimbine had no specific effect on the contractile response to nerve stimulation as previously described. Nevertheless, the present result is in agreement with the finding of other studies that a significant part of the nerve-mediated contraction of full thickness preparations of the female rabbit urethra is adrenergic in nature and mediated mainly via α_1-adrenoceptors even if the muscle tissue contains a considerable number of α_2-adrenoceptors. The adrenoceptor population is reported to consist of 25% α_1- and 75% α_2-adrenoceptors (Andersson *et al.*, 1984; Mattiasson *et al.*, 1989).

In conclusion, the present results demonstrated that the rabbit urethral smooth muscle contains both sub-types of α-adrenoceptors, and the nerve-mediated contraction of the rabbit urethra is adrenergic nature and mediated mainly via α_1-adrenoceptors.

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The action of palytoxin on the isolated detrusor muscle of the rat

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1 The effects of a coelenterate toxin, palytoxin (PTX) have been studied in the isolated detrusor muscle of the rat.

2 PTX (1–100 nM) initiated concentration-dependent contractions of the detrusor; the contraction led to an irreversible tachyphylaxis. Muscle desensitized to PTX continued to respond to acetylcholine (ACh) and excess K^+ but the contractions were reduced compared to pre-PTX contractions.

3 Contractions evoked by PTX were not affected by the presence of atropine (10 μM), indomethacin (10 μM) or tetrodotoxin (0.5 μM) but were greatly reduced by nifedipine (3 μM) and by the absence of K^+. PTX could not evoke contractions in the absence of Ca^{2+} or in tissues depolarized by exposure to excess K^+.

4 PTX abolished the neurogenic contractile responses to electrical field stimulation (EFS).

5 Combined treatment with atropine (10 μM) plus nifedipine (3 μM) abolished contractile responses to EFS and greatly reduced the contractile response to PTX.

6 The contractile response to PTX (100 nM) was reduced following exposure of the muscle to α,β-methylene ATP.

7 Exposure to PTX (100 nM) for 1–3 h reduced both the ACh content of the detrusor (by more than 80%), and the immunoreactivity of neuropeptide Y-containing nerve fibres compared to control.

8 It is concluded that the primary effect of PTX is to promote the release of endogenous motor transmitters, leading to their eventual depletion.

Keywords: Palytoxin; bladder; detrusor muscle

Introduction

Extracts of corals of the genus *Palythoa* have long been known to be toxic, and inhabitants of the Indo-Pacific have, in the past, used them as arrow- and spear-tip poisons. Palytoxin (PTX) appears to be the major active ingredient of these extracts (Moore & Scheuer, 1971; Beress *et al.*, 1983). It is a complex non-peptide with a calculated mol. wt. of approximately 3,300 and it has an LD_{50} in most species of 10–1000 ng kg^{-1}. Death is usually said to be caused by vasoconstriction of peripheral origin (Habermann, 1989).

Excitable cells exposed to the toxin are depolarized. In most cells the depolarization is not specifically Na^+-dependent, but seems instead to reflect a rather general increase in permeability to cations (Tesseraux *et al.*, 1983; Castle & Strichartz, 1988; Ikeda *et al.*, 1988; Muramatsu *et al.*, 1988; Rouzaire-Dubois & Dubois, 1990). In smooth muscle, PTX is spasmogenic. The origin of the activity is unclear and may vary between tissues; for example Shibata *et al.* (1986) have suggested that the contraction of the rabbit urinary bladder is mainly caused by the toxin-mediated release of metabolites of arachidonic acid; Ishida *et al.* (1985a) suggested that in the guinea-pig vas deferens, contractions are caused by the combination of a direct action of the toxin on the smooth muscle and the toxin-mediated release of noradrenaline; Ozaki *et al.* (1983) suggested that the PTX-induced contraction of the guinea-pig aorta is due to a Na^+-dependent depolarization of the smooth muscle, but Nagase & Karaki (1987) suggest that the contraction of the aorta of the rat and rabbit is caused by a combination of the release of prostaglandins from endothelium and smooth muscle, noradrenaline from nerve terminals and a direct depolarization of the smooth muscle.

Because PTX is known to have entered the food chain and given rise to human poisoning (Alcala *et al.*, 1988), it is important that its pharmacological activity is clarified. This paper describes our observations on the rat detrusor muscle.

Methods

Animals

All experiments were performed on tissues removed from adult male Wistar rats weighing 200–350 g and male guinea-pigs weighing 400–500 g. The animals were killed by concussion and decapitation.

Experimental procedures

Preparation for organ bath studies The lower abdomen was opened. The urinary bladder was held at its apex and the coat and connective tissue were cut away. The bladder was then removed and washed in Krebs-Henseleit solution (see below). Two lateral incisions were made in the bladder wall, and the tissue was opened into a rectangular sheet. Strips 1–1.5 × 0.2 cm were prepared with a pair of fine scissors. Single strips were suspended between parallel platinum electrodes in a 2 ml jacketed organ bath containing Krebs-Henseleit solution (composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 11) aerated with 95% O_2 + 5% CO_2 at 37°C, attached to an isometric transducer and a potentiometric recorder, at a resting tension of 0.5 g and allowed to equilibrate for 30 min before the start of further experimentation; during this period the preparation was repeatedly washed with fresh Krebs-Henseleit solution. The preparation of the strips has been described in detail by Luheshi & Zar (1990).

At the end of equilibration period, each strip was exposed to acetylcholine (ACh) 1 mM. The period of exposure lasted until the resulting contractile response had peaked (usually less than 1 min). The ACh-evoked tension was used as the

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standard against which the contractile response evoked by exposure to PTX was assessed.

Electrical field stimulation Electrical field stimulation (EFS) was delivered through a Grass S88 electronic stimulator using trains of 5 pulses of 0.4 ms pulse duration at a frequency of 10 Hz, and at supramaximal voltage (50–70 V) repeated once every min.

Exposure to antagonists (atropine, indomethacin and nifedipine) was for at least 30 min, before their effect on the tissue response to ACh, PTX or EFS was determined. The only exception to this was exposure to tetrodotoxin (TTX) which lasted for 10 min before its effects were ascertained.

Immunohistochemistry The immunochemical technique used for the detection of neuropeptide Y (NPY) was similar to that described in detail by Iravani (1989). Tissues were fixed by immersion in 0.4% w/v *p*-benzoquinone in 0.01 M phosphate buffered saline (PBS) pH 7.4 for 2 h at 4°C and were then washed for at least 24 h with several changes of 0.01 M PBS containing 10% w/v sucrose and 0.01% w/v sodium azide (NaN_3). The tissues were then frozen in isopentane cooled with liquid nitrogen and frozen sections, 10 μm thick, were cut. The sections were placed on glass slides coated with a fine film of chrome-gelatin, to prevent detachment during further processing and were allowed 60 min to dry in air. The first layer antibody was rabbit anti-NPY IgG, diluted 1:200 with 0.01 M PBS containing 0.1% w/v bovine serum albumin (BSA) and 0.01% w/v NaN_3. This was applied to the sections for 16 h at room temperature in a covered Petri-dish, containing a moist filter paper in order to maintain humidity and thus prevent drying. Before the second layer antibody was applied, the preparations were washed in three changes of 0.01 M PBS at 10 min intervals. The second layer antibody, goat anti-rabbit IgG, conjugated with fluorescein isothiocyanate, was applied at a dilution of 1:50 in 0.01 M PBS containing 0.1% w/v BSA and 0.01% w/v NaN_3 for 2 h at room temperature. The preparations were washed in three changes of 0.01 M PBS every 10 min and mounted in DPX. The sections were then examined with a Nikon abophot microscope, equipped with an Incident Light Source (100 W) and V-2a filter block. Photographs were taken with a Nikon HFX-II camera, using Ilford PH5 film rated at 320 ASA and developed in Ilford Microphen for 8.5 min at 20°C.

Extraction of acetylcholine The method used for extraction of ACh was based on that of Paton & Zar (1968). After excision (see above), the rat urinary bladder was opened to form a rectangle and divided into two equal symmetrical pieces. On half was reserved for treatment with PTX and the other served as the control. Each half was cut into four strips to facilitate both the access of PTX to the deeper layers of the tissue and its subsequent removal from the tissue. The strips from each bladder-half were placed in a 2 ml bath containing Krebs-Henseleit solution aerated with 95% O_2 + 5% CO_2 at 37°C and washed repeatedly for 30 min. PTX was then added to one bath at a final concentration 100 nM. After 90 min incubation, both sets of tissue slices were washed for 60 min with changes of Krebs-Henseleit solution at 37°C every 10 min. The tissues from each bath were then carefully blotted to remove excess moisture, weighed, chopped into small pieces and placed in 2 ml of 10% trichloroacetic acid (TCA) at room temperature to extract ACh. The tissues were gently agitated for 90 min in TCA, and filtered through filter papers moistened with distilled water. The filtrates were shaken with 6 volumes of water-saturated ether in a separating funnel four times to remove the TCA. The ether was removed by bubbling the extracts with air at 60°C for 20 min and the residual acidity was neutralized to pH 7.0 with sodium bicarbonate. The final volume of the extract was determined.

Bioassay of acetylcholine The bioassay for ACh was carried out on a guinea-pig ileum preparation by comparison of the responses to various volumes of extract with those to known amounts of ACh. A 3.5–4 cm segment of ileum was removed from a humanely killed guinea-pig, flushed clean with Krebs-Henseleit solution, and suspended in a 5 ml organ bath. The bathing fluid contained morphine sulphate 13 μM and physostigmine sulphate 3 nM and was aerated with 95% O_2 plus 5% CO_2 at 30°C. The preparation was attached to an isometric transducer and a potentiometric recorder at a resting tension of 0.5 g and was allowed to equilibrate for 1–2 h before the assay was started. During this period the preparation was repeatedly washed with fresh Krebs-Henseleit solution containing morphine sulphate and physostigmine. The specificity of the assay for ACh was confirmed by demonstrating that atropine (10 μM) would completely block the response of the ileum to the extract, and that heating the extract to 100°C in NaOH (50 mM) for 2 min would also inactivate it.

Drugs

The drugs used and their sources were: acetylcholine chloride, atropine sulphate, indomethacin, α,β-methylene ATP (all from Sigma), morphine sulphate, EDTA, DPX mountant (BDH), nifedipine (Bayer), palytoxin (Prof L. Beress, University of Kiel, Germany), tetrodotoxin (Sankyo), physostigmine sulphate (Burroughs Wellcome & Co), rabbit anti-porcine neuropeptide serum and goat anti-rabbit IgG, (FITC) (Peninsula Laboratories Inc.). Solutions of drugs were usually made fresh on the day of their use. Stock solutions of tetrodotoxin and PTX were stored at − 20°C. All drugs except indomethacin and nifedipine were dissolved in distilled water. Nifedipine and indomethacin were dissolved in absolute ethanol to make stock solutions of 1 mM and 10 mM respectively. Because nifedipine is highly unstable if exposed to daylight or to ordinary laboratory light, all experiments involving the use of nifedipine were conducted in a laboratory illuminated solely with sodium light. Solutions of PTX were prepared with due regard to its toxicity; it was assumed that its molecular weight was precisely 3,300.

Statistics

All values are expressed as mean ± s.e.mean. Differences between means were compared by Student's *t* test. If the probability that a difference arose by chance was lower than 5% the difference was considered statistically significant. *n* refers to the number of strips of detrusor used, and only one strip from a bladder was used. Thus number of strips = number of bladders.

Results

Effect of palytoxin on muscle tone

At concentrations below 1 nM, PTX had no apparent effect on the tone of the rat isolated detrusor preparation, but concentrations greater than 1 nM evoked contractions. The contractile response to PTX was concentration-dependent and was maximal at 100 nM (Figure 1). A representative contraction to a single maximally effective dose of PTX (100 nM) is shown in Figure 2. The response had a latency of about 10 s and reached its peak in about 2 min. Once the peak was reached, the contraction was not maintained and despite the continued presence of the toxin in the bath, the preparation relaxed virtually to its original base-line in about 15 min. Persistent tachyphylaxis was a striking feature of the contractile action of PTX, and this is also illustrated in Figure 2. Following the decline of the initial contraction, the preparation was washed repeatedly with fresh Krebs-Henseleit solution; a second exposure to PTX, 100 nM failed to

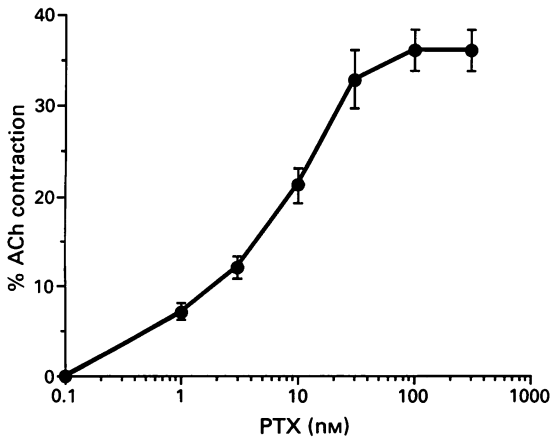


Figure 1 Dose-response curve showing the contractile action of palytoxin (PTX) on the rat isolated detrusor. The PTX-contractions are calculated as a percentage of contraction evoked by acetylcholine (ACh) 1 mM and each point represents mean of experiments on 5 strips of detrusor from 5 animals; s.e.mean shown by vertical bars.

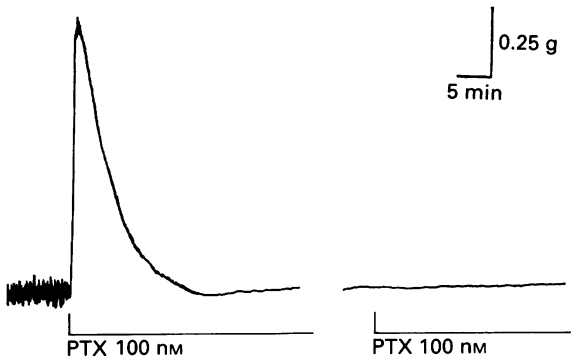


Figure 2 Contractile effect of palytoxin (PTX) (100 nM) on the rat isolated detrusor. The interruption in the record represents a 20 min interval, during which the preparation was washed repeatedly with fresh Krebs-Henseleit solution. Note the decay of the initial contractile response to PTX and the total insensitivity of the preparation to a repeat exposure to PTX.

evoke any contraction (Figure 2). In other experiments, repeated washes with Krebs-Henseleit solution lasting for periods up to 2 h, following an initial exposure to PTX 100 nM, failed to reverse the tachyphylaxis.

The tension generated in response to exposure to a fixed concentration of PTX was significantly greater when the concentration was achieved by a single bolus rather than by cumulatively-rising doses (mean response to PTX, 100 nM, as % of the response to ACh 1 mM, $= 58 \pm 3$, $n = 8$, and 36 ± 2, $n = 5$, with single and cumulative applications respectively).

Effect of palytoxin on spontaneous activity

The isolated detrusor preparations invariably exhibited a modest degree of spontaneous activity. PTX enhanced the spontaneous activity in a concentration-dependent manner by increasing both the force and frequency of spontaneous contractions (Figure 3). At a concentration of 100 nM or more, it caused an increase in resting tension, and spontaneous activity slowly ceased (Figure 3a,b). The presence of the cyclo-oxygenase inhibitor, indomethacin (10 μM), in the bathing medium prevented neither spontaneous activity nor the effect of PTX.

Effect of palytoxin on contractions evoked by electrical field stimulation

Electrical field stimulation (EFS) evoked consistent and reproducible contractile responses. These contractile responses were blocked by exposure to TTX (0.5 μM) and were therefore neurogenic. Figure 4a,b shows parts of the records from two experiments demonstrating the effect of the PTX on motor transmission. Preparation (a) was exposed to PTX in cumulatively increasing concentrations of 0.1 nM, 1 nM and 10 nM. PTX, 0.1 nM, had no effect on the contractile response to EFS; at 1 nM the response was slightly reduced and at 10 nM, PTX totally blocked the response to EFS. Preparation (b) was exposed to PTX, 100 nM, by a single application; it abolished transmission as with PTX 10 nM, but the effect was achieved more rapidly.

Effect of palytoxin on acetylcholine-evoked contractions

A typical experiment demonstrating the effect of PTX on ACh-evoked contractions is shown in Figure 5. After obtaining a control response to ACh (1 mM), the preparation was washed (5 min; 3 changes of bathing fluid) and then exposed to PTX (100 nM). The contractile response to PTX was allowed to subside fully. The preparation was exposed once

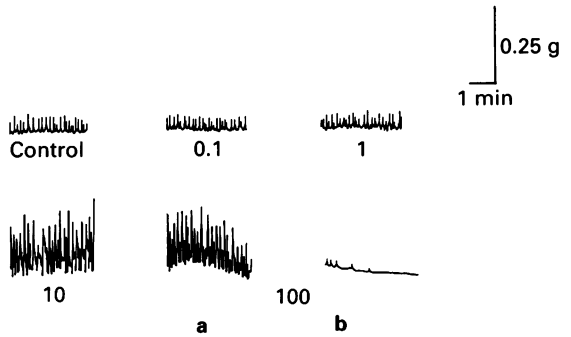


Figure 3 Effect of palytoxin (PTX) on the spontaneous activity of the rat isolated detrusor. The different panels show the spontaneous activity of a detrusor strip before (control) and during exposure to PTX 0.1, 1, 10, and 100 nM. Exposure to PTX, 100 nM, produced a rapid contraction and the spontaneous activity during the contracted state is shown in (a); subsequently during the continued presence of PTX, the preparation relaxed with gradual, but complete extinction of spontaneous activity (b).

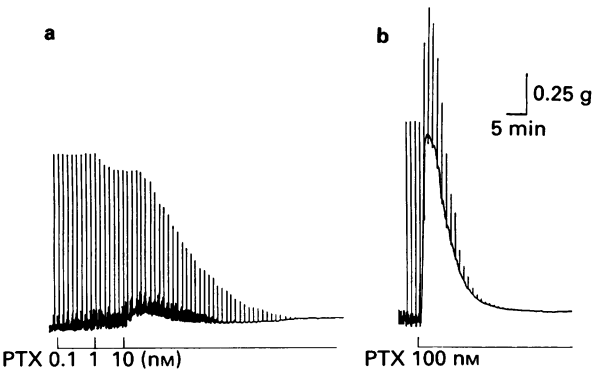


Figure 4 Effect of palytoxin (PTX) on the neurogenic response of rat isolated detrusor to electrical field stimulaton. The nerves were stimulated by trains of 5 pulses of supramaximal voltage, 0.4 ms duration at 10 Hz once every min. Preparation (a) was exposed to cumulatively increasing concentrations of PTX (0.1, 1 and 10 nM) while preparation (b) was treated with a bolus of 100 nM PTX. Note the concentration-dependence of the action of PTX on neurogenic contractions.

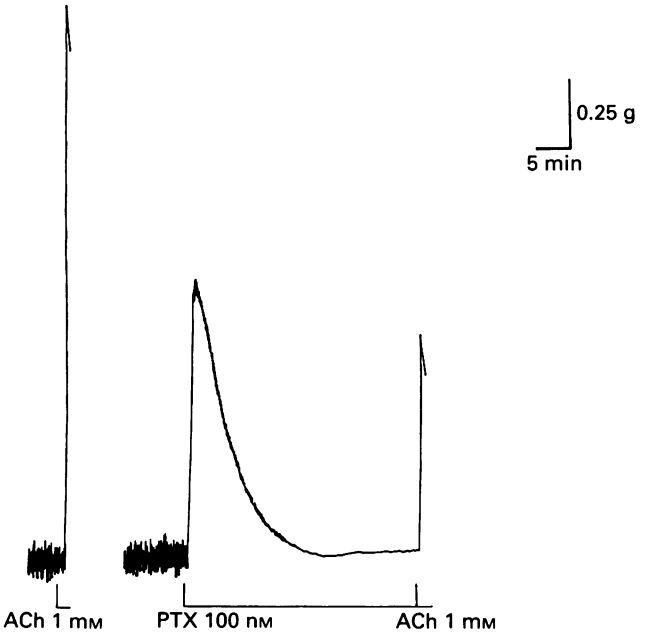


Figure 5 Effect of palytoxin (PTX) on acetylcholine (ACh)-evoked contractions of rat isolated detrusor. The preparation was exposed to ACh, 1 mM, and after obtaining the peak contraction, ACh was washed out and the preparation allowed to relax to its pre-ACh resting tension. The preparation was then exposed to PTX, 100 nM, and when the tone was stable, it was exposed to ACh 1 mM once again.

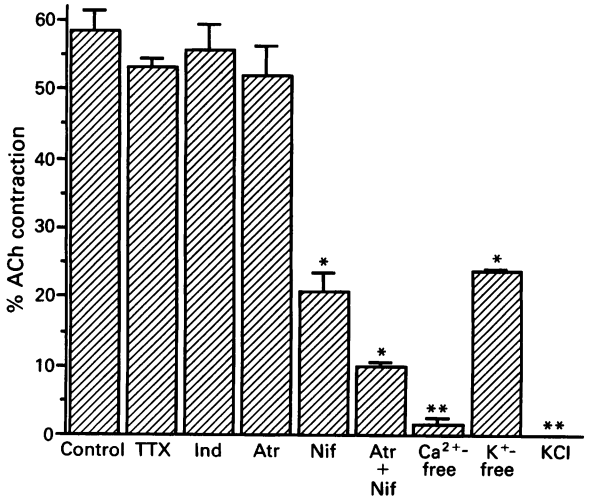


Figure 6 The effect of tetrodotoxin (TTX) 0.5 μM ($n = 4$), indomethacin (Ind) 10 μM ($n = 4$), atropine (Atr) 10 μM ($n = 4$), nifedipine (Nif) 3 μM ($n = 4$), Atr 10 μM + Nif 3 μM ($n = 5$), absence of Ca^{2+} ($n = 5$), absence of K^+ ($n = 3$) and excess K^+ 250 mM ($n = 3$) on palytoxin (PTX)-evoked contractions of rat isolated detrusor. PTX-evoked contractions have been expressed as percentage (mean with s.e.mean shown by vertical bars) of the acetylcholine (ACh)-evoked contraction. Control ($n = 8$) represents experiments in which contractions to PTX were evoked without any pretreatment. Significant differences from the control are depicted by $^*P < 0.01$ and $^{**}P < 0.001$.

more to ACh (1 mM) in the continued presence of PTX (100 nM). ACh still evoked a contraction, but in a series of 5 experiments the amplitude of contraction was reduced by 56 ± 2%. A similar result was obtained when PTX was washed out of the tissue bath before the second exposure to ACh. Control experiments, in which no PTX was used, showed that there was no change in the amplitude of the response to ACh during three successive exposures.

Effect of pharmacological agents on palytoxin-evoked contractions

TTX 0.5 μM, indomethacin 10 μM or atropine 10 μM all failed to affect PTX-evoked contractions compared to PTX-evoked contractions of untreated controls (Figure 6). The Ca^{2+}-channel antagonist, nifedipine (3 μM), reduced the size of the PTX-evoked contraction by 60% (Figure 6). The time course of the PTX-contraction was also changed in nifedipine-treated preparations. This is shown in Figure 7, which illustrates an experiment on two detrusor strips from the same rat. Strip (a) was treated with nifedipine (3 μM) for 30 min, before being exposed to PTX (100 nM); the second strip, (b), was not treated with nifedipine and served as the control. Strip (b) responded to PTX with a brisk contraction, which reached its peak in under 2 min and then subsided by about 90% in approximately 15 min. Strip (a) responded sluggishly to PTX; the initial large, brisk contraction was absent and it was replaced with a smaller slower, but sustained contraction which reached its peak in about 40 min.

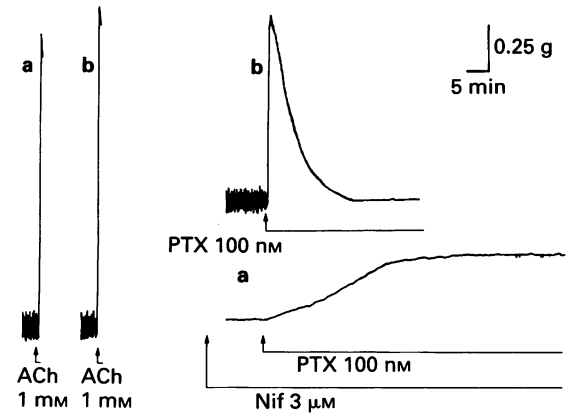


Figure 7 Effect of nifedipine (Nif) on palytoxin (PTX) evoked contraction of rat isolated detrusor. Strips (a) and (b) were exposed to acetylcholine (ACh) 1 mM for 30 s. Strip (a) was then treated with nifedipine for 30 min before exposing it to PTX. Strip (b) was exposed to PTX alone without any nifedipine treatment. Note the absence of phasic contraction by PTX in the nifedipine-treated strip (a).

Effect of absence of Ca^{2+}

Preparations incubated in Ca^{2+}-free Krebs-Henseleit solution (no added Ca^{2+}; 1.2 mM EDTA), lacked spontaneous activity and were virtually unresponsive to PTX (100 nM) (Figure 6).

Effect of absence of K^+

PTX (100 nM) still caused a contraction of the detrusor muscle when incubated in K^+-free Krebs Henseleit solution but the contractions were 60% smaller than those obtained by PTX 100 nM in the presence of K^+ (Figure 6).

Effect of excess K^+

Preparations 'desensitized' to PTX contracted when exposed to excess K^+ (KCl 250 mM) but the K^+-evoked contraction was smaller than that produced in preparations not previously exposed to PTX (Figure 8a). Depolarization of the detrusor strip by excess K^+ (KCl 250 mM) evoked a large, brisk contraction which gradually subsided fully in about 30 min during the continued presence of excess K^+. The addition of PTX (100 nM) following the high K^+ failed to evoke any contractile response (Figures 8b and 6).

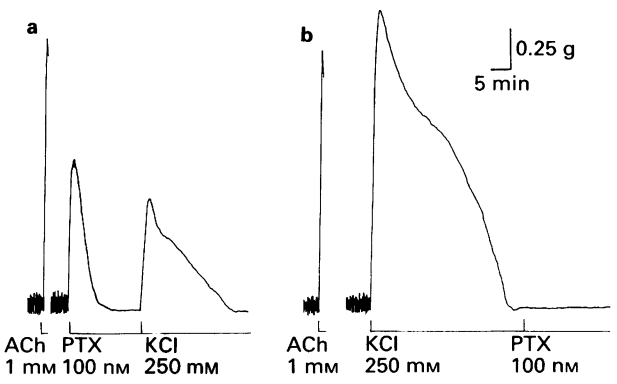


Figure 8 Effect of palytoxin (PTX) treatment on a K^+-evoked contraction (a) and of K^+-induced depolarization on PTX-evoked contraction (b) in rat isolated detrusor.

Effect of palytoxin following postsynaptic block of motor transmission

It has been reported recently that motor transmission in the detrusor muscle of the rat can be fully blocked by a combination of atropine (10 μM) plus nifedipine (3 μM) (Zar *et al.*, 1990). A total of five experiments were done in which the action of PTX (100 nM) was examined in the combined presence of atropine (10 μM) plus nifedipine (3 μM). Records from typical experiments are shown in Figure 9; electrically-evoked motor transmission was abolished and PTX (100 nM) caused a sluggish and a much smaller contraction than in the absence of nifedipine and atropine (compare Figure 9a,b).

The effect of desensitization of purinoceptors on the response to palytoxin

There is considerable evidence that ATP or an ATP-like substance functions as a non-cholinergic motor transmitter in the rodent urinary bladder (Burnstock *et al.*, 1978). In five preparations purinoceptors were desensitized by exposing the tissue to α,β-methylene ATP (α,β-MeATP) (2 × 10 μM). After densensitization (Figure 10), the contraction caused by PTX (100 nM), was 12 ± 3% of the contractile response evoked by ACh (1 mM) compared to a typical PTX-induced response of 58 ± 3% ($n = 8$) of that evoked by ACh (1 mM).

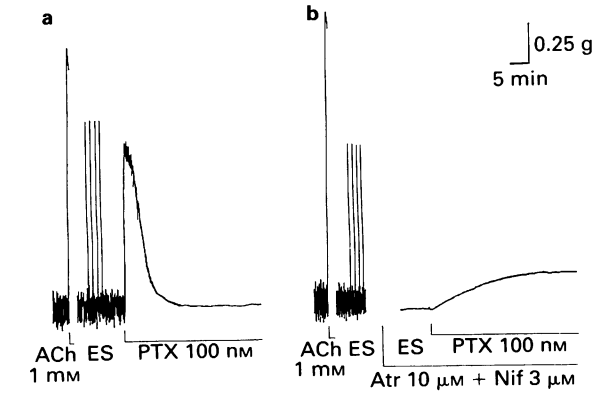


Figure 9 Effect of atropine + nifedipine on contractions of rat isolated detrusor evoked by electrical field stimulation (ES) and by palytoxin (PTX). Both preparations, (a) and (b) from the same animal. After obtaining a contractile response to acetylcholine (ACh) and ES, strip (b) was treated with atropine (Atr) 10 μM plus nifedipine (Nif) 3 μM for 30 min before repeating ES and exposure to PTX. Note the loss of phasic responses to both ES and the phasic contractile response to PTX. Parameters of ES; trains of 5 pulses, supramaximal voltage, 0.4 ms duration, 10 Hz once every min.

Effect of palytoxin on neuropeptide Y-containing innervation

Control preparations of rat detrusor muscle incubated for 3 h in normal Krebs-Henseleit solution were used for the immunocytochemical demonstration of NPY-containing axons. Fluorescent images demonstrated the dense plexus of varicose nerves extending throughout the muscle. After exposure to PTX (100 nM) for 1 h, NPY-reactivity was greatly reduced, and by 3 h, NPY-reactivity was virtually non-existent (Figure 11).

Palytoxin-induced release of acetylcholine

The acetylcholine content of detrusor muscles was reducd by 82 ± 6% (range 74–98%, $n = 4$) following exposure to PTX (100 nM) for 90 min compared to control.

Discussion

The observation that palytoxin (PTX) was a powerful spasmogen in the detrusor muscle of the rat urinary bladder was not unexpected since a wide range of other smooth muscles contract when exposed to PTX, including the urinary bladder of the rabbit (Ito *et al.*, 1976; 1977; Ohizimu & Shibata, 1980; Ozaki *et al.*, 1983; 1987; Ishida *et al.*, 1985a,b; Shibata *et al.*, 1986). The response of the rat muscle was Ca^{2+}-dependent, resistant to TTX and exhibited profound tachyphylaxis.

There is considerable evidence that PTX impairs the physiological integrity of excitable tissues, primarily by increasing the permeability of the plasma membranes to a range of small cations and, in some cases, compounds such as inulin (Habermann, 1989). This increase in permeability results from the opening of cation selective channels (Rouzaire-Dubois & Dubois, 1990 for example) and leads to non-specific depolarization (Dubois & Cohen, 1977; Pichon, 1982; Muramatsu *et al.*, 1984). It is superficially attractive to suggest, therefore, that PTX is a general cytotoxin, and to interpret its wide range of pharmacological actions in this light. It is clear, however, that the toxin is not a non-specific

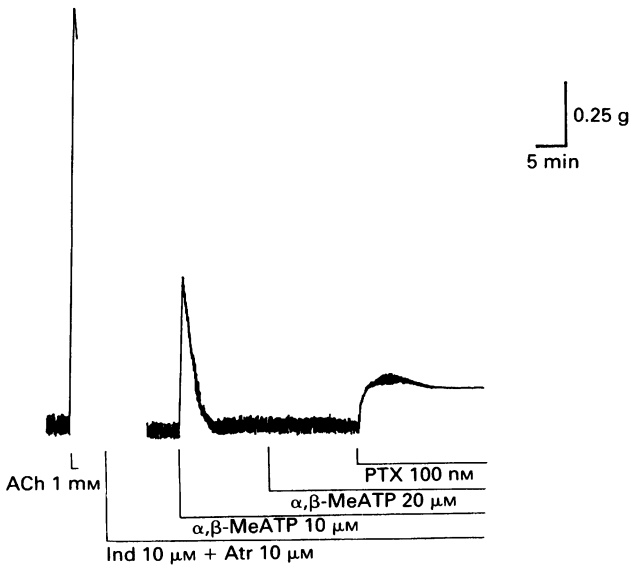


Figure 10 Effect of α,β-methylene ATP (α,β-MeATP) desensitization on palytoxin (PTX)-evoked contraction in rat detrusor. After obtaining a control acetylcholine (ACh) response, the preparation was treated with indomethacin (Ind) + atropine (Atr) for 30 min before exposure to α,β-MeATP first at 10 μM and then at 20 μM. Note the successful induction of α,β-MeATP desensitization and the small contractile response to PTX following α,β-MeATP-densensitization.

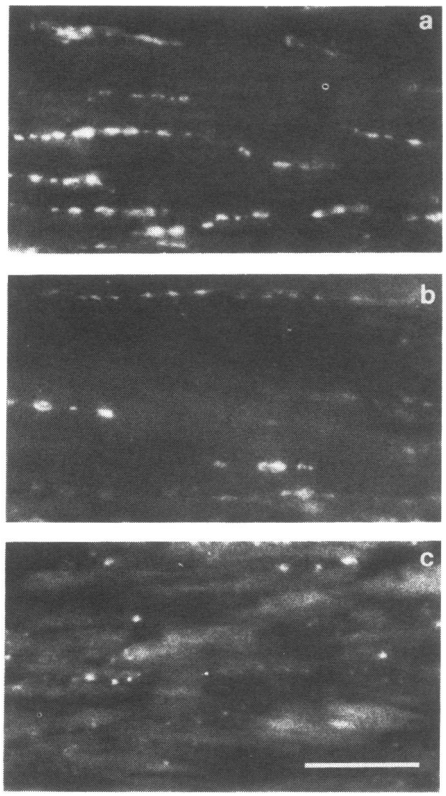


Figure 11 Fluorescence micrographs of neuropeptide Y (NPY)-immunoreactive nerve fibres in longitudinal sections of rat detrusor after treatment with palytoxin (PTX) (100 nM) for 60 min (b) and 180 min (c). Section (a) was not treated with PTX and served as control. Note the decrease of NPY-immunoreactivity after PTX-treatment. The scale bar represents 25 μm for all three plates.

cytotoxin. In our own experiments, for example, when tachyphylaxis rendered the preparations totally unresponsive to PTX, exposure to ACh (1 mM) and high K^+ still evoked a contraction even though the amplitude of the responses was reduced by 40–50% in each case compared to pre-PTX treatment. Moreover, PTX does not affect the growth of bacteria and yeasts in culture (unpublished observations reported in Habermann, 1989).

Motor transmission in the rodent detrusor muscle is now widely recognised to be partly cholinergic and partly non-cholinergic in nature. The precise identity of the non-cholinergic component is unclear, but neuropeptide Y (NPY) and ATP or an ATP-like compound are the most likely candidates. It is possible that both are involved (Ambache & Zar, 1970; Burnstock *et al.*, 1972; Brading & Williams, 1990; Luheshi & Zar, 1990). In the rabbit detrusor, there is some evidence that metabolites of arachidonic acid play a role in motor transmission (Johns & Paton, 1976; Andersson *et al.*, 1980), but this is not the case in the rat (Ambache & Zar, 1970). PTX was shown to deplete the rat detrusor of ACh and NPY. We believe the transmitter-depleting effect of PTX represents its primary mode of action. This accounts for the ability of PTX to block motor transmission in the detrusor of the rat. It also explains the contractile effect of PTX on first exposure and the subsequent tachyphylaxis; it is consistent with the observation that the contraction of the tissue in response to PTX can be largely prevented by the presence of atropine and nifedipine, a combination of drugs known to block the motor response of the rat detrusor to electrical field stimulation. Indomethacin partially blocks the response of the rabbit detrusor muscle to PTX (Shibata *et al.*, 1986), but had no effect on the response of the rat detrusor muscle. This difference is consistent with the postulated role played by metabolites of arachidonic acid in the rabbit, but not the rat detrusor (see above). In our investigation, the drastic reduction in the ability of PTX to evoke a contraction of detrusor following APT-desensitization is consistent with the postulated role of ATP as a major non-cholinergic motor neurotransmitter in this tissue (Burnstock *et al.*, 1978; Brading & Williams, 1990).

The effect of PTX on the force and frequency of spontaneous mechanical activity in the rat detrusor is also consistent with the view that the primary action of the toxin is to initiate the release of transmitter from motor nerve terminals. The spontaneous activity in this tissue is thought to be initiated by the spontaneous low-level release of transmitter (Kock & Pompeius, 1963). We suggest that exposure to PTX increases the amount of transmitter released, thus enhancing spontaneous activity and initiating an increase in resting tension. This initial phase of enhanced activity and increase in tone would be expected to subside as the available transmitter is depleted from stores in the nerve terminal, and to cease when the amount released falls below the threshold required to initiate mechanical activity in the muscle.

The effect of the toxin was not blocked by TTX, so it may be concluded that fast, depolarization-dependent Na^+ channels are not a necessary component involved in PTX-initiated transmitter release. The reduction of Ca^{2+} from 2 mM to zero blocked the motor response of the muscle to PTX, but preliminary experiments on the mechanism by which PTX induces transmitter release (Posangi, Harris & Zar, unpublished) suggest that Ca^{2+} is not an essential requirement for PTX-induced transmitter release. We would suggest therefore, that it is the mechanical response initiated by the liberated transmitter that is Ca^{2+}-sensitive.

Preparations desensitized to PTX contracted when exposed to K^+ and to ACh. The contractions elicited by the two antagonists were smaller than before exposure to PTX. The observations show that desensitization to PTX does not reflect an inability of the muscle to contract and it is reasonable to infer that the acute exposure of smooth muscle to PTX does not result in the destruction of the tissue. The reduction in amplitude of K^+- and ACh-induced contractions may indicate, however, that the toxin causes a limited degree of damage. No ultrastructural studies have yet been made on smooth muscle exposed to PTX but it is probably relevant that Tesseraux *et al.* (1983) have shown that the toxin damages skeletal muscle after exposure *in vitro*. An alternative explanation is that part of the motor response of the detrusor muscle to K^+ and ACh is indirect and results from the depolarization of motor-nerve terminals. If transmitter stores have already been depleted by exposure to PTX (see above) it might be expected that the indirect component of the response to K^+ and ACh would be impaired.

The transmitters responsible for mediating motor transmission in the detrusor muscle of the rat have not been formally identified. ACh is a known transmitter but there is good evidence that both ATP (or an ATP-like substance) and NPY are also involved in motor transmission. Nifedipine inhibits the non-cholinergic components of transmission and a proportion of the cholinergic component (Iravani *et al.*, 1988; Bo & Burnstock, 1990). In our experiments nifedipine blocked the effects of PTX by 60% and the PTX-induced contraction could be further inhibited by the combined application of nifedipine and atropine. The data may be interpreted as suggesting that atropine blocked the nifedipine-insensitive component of cholinergic transmission. Atropine alone failed to affect PTX-induced contractions. Why this was so is difficult to understand, because PTX clearly caused the release of ACh from the tissue. It is possible that the three candidate transmitters in the detrusor (ACh, ATP, NPY) interact in a subtle fashion and that the physiological significance of the individual transmitters in an experimental environment varies according to the conditions of the experiment. Thus, PTX may cause the rapid release of the non-cholinergic transmitters, but cause only the very slow release of ACh. It may be notable in this regard that the PTX-

induced contraction of the detrusor was initiated after a delay of only 10 s and reached its peak by 2 min. This very brisk response was selectively blocked by nifedipine. The nifedipine-insensitive component rose slowly to reach its peak after 10–15 min. It is attractive to suppose that this latter response was caused by ACh. Until more is known of the details of the neuromuscular physiology of the detrusor muscle of the rat, it is not profitable to speculate further.

Our observations on the pharmacological activity of PTX do not address directly its mechanism of action. Recently, however, Rouzaire-Dubois & Dubois (1990) have studied the activity of PTX on neuroblastoma-glioma NG108-15 hybrid cells using patch clamping. It was shown that PTX activated cation-selective (but non-specific) channels in the plasma membrane. It was also shown that the current consequent upon the opening of the channels was maintained with low doses (<1 nM) of PTX but declined in the continued presence of high doses (>10 nM) of the toxin. After high doses of PTX, the cells were refractory to further doses, even after prolonged washing. These data (and those of others who have also shown that PTX opens cation selective channels in excitable cells; see Rouzaire-Dubois & Dubois, 1990 for a summary) are not inconsistent with our own suggestions that the release of transmitter from motor nerve terminals is the primary mechanism of action, but they do suggest an alternative explanation for the onset of tachyphylaxis. Rouzaire-Dubois & Dubois (1990) have also noted that the rate of onset of channel opening and the size of the total current were dose-dependent. They interpreted this as suggesting that the binding of PTX to its 'receptor' on the plasma membrane was probably co-operative. They then interpreted the fall in the current with time, in the presence of high doses of toxin, and the tachyphylaxis as indicating that the toxin-receptor complex leaves the membrane and is either internalised or enters the external medium. If re-incorporation of the receptor is low, there will be a continuing down-regulation of the system. Clearly, such a mechanism would also explain all of our experimental data. The mechanism would not necessarily be expected to lead to the release of all available transmitter, although our data clearly indicate that, within the limitations of the techniques we have used, all transmitter stores are depleted.

The cell surface receptor that binds PTX has not been identified, but Na^+-K^+ ATPase is a strong candidate (Habermann, 1989). Whether PTX renders the ATPase an ion 'channel' is less clear. It is of interest, however, that PTX is a powerful inhibitor of Na^+-K^+ ATPase activity (Haberman, 1989), and inhibition of Na^+-K^+ ATPase is considered a primary step involved in the physiology of transmitter release in the autonomic nervous system (Paton *et al.*, 1971).

So far, discussions on the mechanism of action of PTX have concentrated on the role of ATPase and the opening of ion-channels. It is striking that very little work has been done on the morphology of tissue exposed to PTX. Tesseraux *et al.* (1983) showed that PTX caused severe damage to skeletal muscle exposed to concentrations as low as 1 nM and that the damage involved the sarcoplasmic reticulum, mitochondria and contractile elements. It seems highly probable that one explanation for the total depletion of transmitter and the irreversibility of the effects of high doses of PTX is that in addition to its specific activity, PTX causes significant structural damage to neuronal tissue.

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Effects of BRL 38227, sodium nitroprusside and verapamil on collateral perfusion following acute arterial occlusion in the rabbit isolated ear

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1 We have used an isolated, buffer-perfused, rabbit ear model of acute arterial occlusion to investigate the effects of the nitrovasodilator sodium nitroprusside, the potassium channel activator BRL 38227 (the active (−)-enantiomer of cromakalim) and the calcium antagonist, verapamil, on collateral perfusion in the absence of pharmacological tone.

2 Verapamil was the most potent vasodilator ($EC_{50} = 72.6 \pm 32.0$ nM) of 5-hydroxytryptamine/histamine-induced tone in the rabbit isolated perfused ear. Sodium nitroprusside and BRL 38227 were less potent with respective EC_{50} values of 488 ± 75 nM and 296 ± 40 nM. Following inhibition of endothelium-derived relaxing factor (EDRF) synthesis, the potency of BRL 38227 was significantly ($P < 0.001$) increased with an EC_{50} of 55.6 ± 5.0 nM.

3 BRL 38227 at 500 nM and 3 μM induced substantial increases in collateral perfusion following arterial ligation in the absence of pharmacological tone compared to control. Furthermore 3 μM BRL 38227 completely reversed the attenuation of collateral perfusion which followed inhibition of EDRF synthesis with 100 μM N^G-nitro-L-arginine methyl ester (L-NAME).

4 Sodium nitroprusside (500 nM and 3 μM) induced modest improvements in collateral perfusion in the early stages after arterial occlusion.

5 Verapamil did not influence collateral perfusion at either of the concentrations used (50 nM and 3 μM), even though it was a potent vasodilator.

6 The results of this study indicate that BRL 38227, and to a much lesser extent sodium nitroprusside, selectively improve collateral perfusion following arterial occlusion, even in the presence of effects of EDRF on acute collateralization, while verapamil has no effect. Furthermore, BRL 38227 also improves collateral perfusion following inhibition of EDRF synthesis. It remains to be established whether BRL 38227 has beneficial actions in acute arterial occlusion *in vivo*.

Keywords: Rabbit ear; microangiography; collateral flow; sodium nitroprusside; BRL 38227; verapamil; N^G-nitro-L-arginine methyl ester; endothelium-derived relaxing factor

Introduction

Pre-existing collateral vessels provide an alternative pathway to flow following arterial occlusion and thus have an important role in limiting tissue ischaemia and infarction. The function of these vessels is therefore critical to the maintenance of tissue perfusion following acute arterial occlusion. It is well established in both the canine and feline hindquarter circulations that acute arterial occlusion is accompanied by the rapid diversion of flow (within minutes) through pre-existing collateral vessels, and can thus maintain adequate tissue perfusion distal to the occlusion (Winblad *et al.*, 1959; John & Warren, 1961; Rosenthal & Guyton, 1968; Schaub *et al.*, 1976). Despite the importance of collateral flow, little is known of the precise mechanisms governing the recruitment and opening of collateral vessels, although it appears that the pressure gradient across the occlusion may act as a driving force for collateral perfusion (Holman, 1949; Holman & Taylor, 1952). This is supported by the observations that the development of such flow in the canine femoral artery varies directly with the pressure distal to the ligation (Winblad *et al.*, 1959) and regression of collateral perfusion is seen when distal pressure is elevated by shunting hypoxic blood from the contralateral femoral artery to the occluded area (Winblad *et al.*, 1959). Furthermore, the conductance of collateral circulation is reduced by lowering proximal arterial pressure (Rosenthal & Guyton, 1968).

Possible candidates for stimulation of collateral perfusion include chemical mediators originating from the ischaemic region (Lewis, 1940). In a recent study with a newly-developed rabbit isolated ear model of arterial occlusion we have shown that endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980), which is thought to be nitric oxide or closely related chemical species (Palmer *et al.*, 1987), plays a central role in both the initiation and maintenance of collateral perfusion (Randall & Griffith, 1992a,b). Indeed, when EDRF synthesis is inhibited by N^G-nitro-L-arginine methyl ester (L-NAME) the time-dependent development of collateral perfusion is severely impaired, and this inhibitory effect may be reversed by an excess of L-arginine. We have now investigated the effects of three pharmacologically different classes of vasodilators on collateral perfusion in this model. We have also investigated whether BRL 38227 influences collateral perfusion in the absence of EDRF activity to determine whether it might be effective in promoting collateral perfusion in pathological states in which EDRF activity has been reported to be impaired (e.g. hypertension: (Lüscher & Vanhoutte, 1986; Mayhan *et al.*, 1987, atherosclerosis: Harrison *et al.*, 1987 or hypercholesterolemia: Verbeuren *et al.*, 1986).

Methods

Preparation of the rabbit ear vascular bed

Male New Zealand White rabbits (2–2.5 kg) were killed by cervical dislocation. An ear was removed and the central

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artery cannulated and perfused wtih Holman's solution (composition, mM: NaCl 120, KCl 5, $CaCl_2$ 2.5, NaH_2PO_4 1.3, $NaHCO_3$ 25, sucrose 10 and D-glucose 11) at a flow rate of 3.5 ml min^{-1}. The physiological buffer also contained 5% (w/v) dextran (mol. wt.: 80,000) to increase its viscosity (to ca. 2.3 mPaS) and 10 μM indomethacin to eliminate prostanoid activity. The buffer was gassed with 95% O_2/5% CO_2 and maintained at 35°C.

Experimental protocol

The ear was positioned vertically in front of an X-ray microscope (4 μm focal spot diameter) and loose sutures placed around the central artery at its midpoint and apex (Figure 1). Following an equilibration period (1 h), microangiograms were taken of the resting preparations. The exposure time was 15 s at 30 kV. Radiographic contrast was achieved by a 35 s pulse of iohexol diluted 3 fold in the perfusion fluid (final iodine concentration 100 mg ml^{-1}). Use of this agent is without significant influence on either vasomotor tone or vascular responses during the period of X-ray exposure (Griffith *et al.*, 1988). A segment of central ear artery (24.5 ± 0.5 mm; $n = 52$) was then isolated by tightening the sutures and microangiograms were then taken at 2, 5, 10, 15, 30, 60 and 90 min after ligation. Preliminary experiments, in which only the midpoint of the central ear artery was ligated, indicated that there was rapid and complete retrograde filling of the central artery distal to the ligation due to anastomotic supply arising from the daughter branches of its apical bifurcation. This phenomenon has also been reported in the rabbit ear *in vivo* by other workers (Bellman *et al.*, 1959).

The perfusion pressure of the intact preparation was continuously monitored by means of a pressure transducer placed close to the inflow cannula. The pressure drop across the cannula was determined at the end of each experiment and subtracted from the recorded pressure.

The effects of vasodilators were assessed by addition to the perfusion fluid 30 min prior to ligation and the level of collateral perfusion was then determined. The concentrations of the vasodilators were chosen in each case to approximate to the EC_{50} for causing relaxation of established tone and also a concentration that gave maximal dilatation. In order to determine these concentrations the vasodilator properties of the agents were determined in different ear preparations in which submaximal tone (ca. 60%, Randall & Griffith, 1991) was established by combination of 1 μM 5-hydroxytryptamine and 1 μM histamine. In the case of BRL 38227 the effects of 10 μM glibenclamide were examined by addition of this agent in the presence of the maximum concentration of BRL 38227 used.

In the case of BRL 38227 the effects on collateral perfusion were also assessed following inhibition of EDRF synthesis with N^G-nitro-L-arginine methyl ester (Randall & Griffith, 1991). In these experiments 100 μM N^G-nitro-L-arginine methyl ester (L-NAME) was added to the perfusion fluid 60 min before ligation); 30 min after this addition; BRL 38227 was added to the perfusion fluid and after 30 min of preincubation the preparation was ligated and collateral perfusion assessed. The vasorelaxant properties of BRL 38227 were also assessed against pharmacological tone in preparations pretreated with 100 μM L-NAME 30 min before addition of the vasoconstrictor agents. The concentrations of 5-hydroxytryptamine and histamine were reduced to 100 nM to give a level of tone equivalent to that found in the absence of L-NAME (Randall & Griffith, 1991).

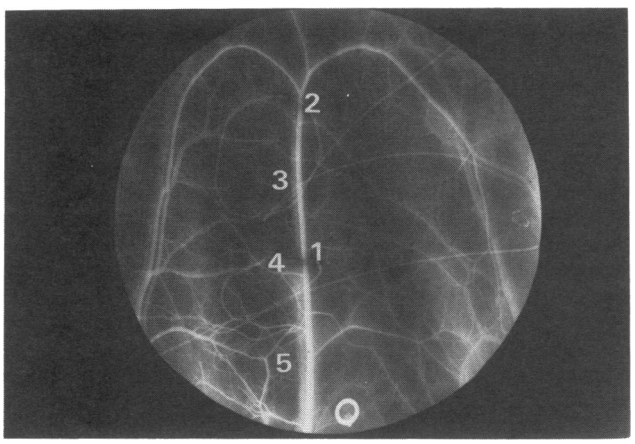


Figure 1 An angiogram of the rabbit isolated perfused ear preparation before arterial occlusion: (1) indicates the position of the midpoint suture, (2) the apical suture, (3) the segment of the central artery, (4) a first order branch generation proximal (pG1) to the midpoint suture and (5) the central artery proximal (pG0) to the segment. The disc at the bottom of the angiogram is the gold calibration grid with a diameter of 3 mm.

Quantitation

The microangiograms were analysed by a semi-interactive image analysis system as previously described (Griffith *et al.*, 1988). A gold calibration grid was placed on the surface of the ear to determine the magnification factor and the radiographs were further magnified by means of an electronic image analysis system. The diameter of each vessel studied was measured 4 times and the results averaged. Filling of the segment isolated between ligatures was assessed as the area of the segment on the angiogram which had filled with radiographic contrast, relative to its preocclusion value. Angiography carried out in the absence of radiographic contrast, but following previous pulses, showed that there was no stasis of contrast medium in the segment. Similarly, real-time video imaging indicated that clearance of contrast from the segment via the low resistance pathways provided by arteriovenous shunts and arterial branches was rapid (< 30 s). The filling of the segment with contrast medium therefore reflects perfusion via collateral vessels and not a time-dependent accumulation of radiographic contrast.

As an estimate of collateral flow and also as a marker for the vascular effects of the vasodilators and L-NAME, the diameter of a first order branch artery (ca. 200 μm) situated close to but proximal (< 3 mm) to the midpoint ligation (Figure 1) was measured as a function of time. This vessel was termed pG1 (i.e. proximal first order generation). These vessels are close to the isolated segment and constitute the main feed vessels to the collateral circulation (Randall & Griffith, 1982b). We have previously shown that the diversion of flow through these vessels to supply a collateral circulation is accompanied by dilatation while the diameter of more proximal first order arteries remains unaltered. The diameter of the central ear artery proximal to the midpoint ligation (pG0) (Figure 1) was also measured. We have previously shown that the diameter of this artery can increase as a consequence of passive distension by an increase in intravascular pressure (Griffith *et al.*, 1987; Randall *et al.*, 1990).

Data and statistical analysis

All data are given as the mean ± s.e.mean and were compared by either paired or unpaired Student's t tests or analysis of variance as appropriate. EC_{50} for vasodilator responses were obtained from individual concentration-response curves as the concentration at which half maximal reduction in established tone occurred.

Drugs

All solutions were prepared on the day of the experiment. N^G-nitro-L-arginine methyl ester, 5-hydroxytryptamine as creatinine sulphate complex, histamine dihydrochloride, and

sodium nitroprusside (all from Sigma Chemical Company, Poole), were dissolved in saline. Verapamil hydrochloride (Sigma) and glibenclamide (Hoechst, Hounslow) were dissolved in absolute ethanol and BRL 38227 (a generous gift from Smith Kline Beecham, Surrey) was dissolved in 70% (v/v) ethanol. Iohexol (Omnipaque, Nycomed, U.K.) was diluted in the perfusion fluid. Indomethacin (Sigma) was dissolved in 5% (w/v) $NaHCO_3$. All drugs were then diluted to the required concentrations in the Holman's solution.

Results

Effects of isolating a segment of the central ear artery

Perfusion pressure in the resting ear preparation was 37.9 ± 4.6 mmHg ($n = 16$). Figure 2a shows that 2 min after ligation, filling was 18.2 ± 3.7% of its preocclusion value ($n = 18$). Filling of the segment increased progressively with time and was 26.1 ± 3.6% at 15 min ($n = 18$), 31.1 ± 4.1% at 30 min ($n = 16$), 39.8 ± 4.6% at 60 min ($n = 16$) and 42.7 ± 5.9% at 90 min ($n = 9$) (Figure 2a). Perfusion pressure increased modestly with time after ligation, becoming significantly ($P < 0.05$) greater than preocclusion levels after 5 min. This change persisted throughout the experiment (Figure 2c).

Figure 2b shows that the diameter of pG1 increased significantly ($P < 0.05$) from a control value of 201 ± 13 μm ($n = 17$) to a value of 241 ± 21 μm ($n = 16$) 2 min after ligation. This increase in diameter persisted throughout the period of the experiment peaking at 274 ± 18 μm ($n = 11$) 60 min after ligation (Figure 2b). There was also a significant ($P < 0.001$) increase in the diameter of pG0 from 705 ± 26 μm to 817 ± 51 μm post ligation (both $n = 18$), followed by a slow decline to values not significantly different from the pre-occlusion value (Figure 2d). In all subsequent experiments investigating the effects of vasodilators almost identical changes in the diameter of pG0 were observed so, for brevity, these data have been omitted.

Vasodilator properties of sodium nitroprusside, BRL 38227 and verapamil

In 5 preparations resting perfusion pressure was 18.8 ± 2.6 mmHg and following addition of 1 μM 5-hydroxytryptamine and 1 μM histamine, perfusion pressure rose by 91.4 ± 15.4 mmHg to 110 ± 16 mmHg. In these preparations sodium nitroprusside (1 nM–10 μM) brought about concentration-related reductions in established tone with an EC_{50} of 488 ± 75 nM and a maximum reduction in established tone of 81.9 ± 5.6% (Figure 3a).

In 5 different preparations perfusion pressure increased from 26.0 ± 3.7 mmHg to 113 ± 22 mmHg following addition of the combination of vasoconstrictors. In these preparations BRL 38227 (10 nM–30 μM) similarly induced concentration-dependent relaxations of established tone with an EC_{50} of 296 ± 40 nM, and a maximum reduction in tone of 80.0 ± 1.8% (Figure 3a). These parameters do not differ significantly from those obtained for sodium nitroprusside. In

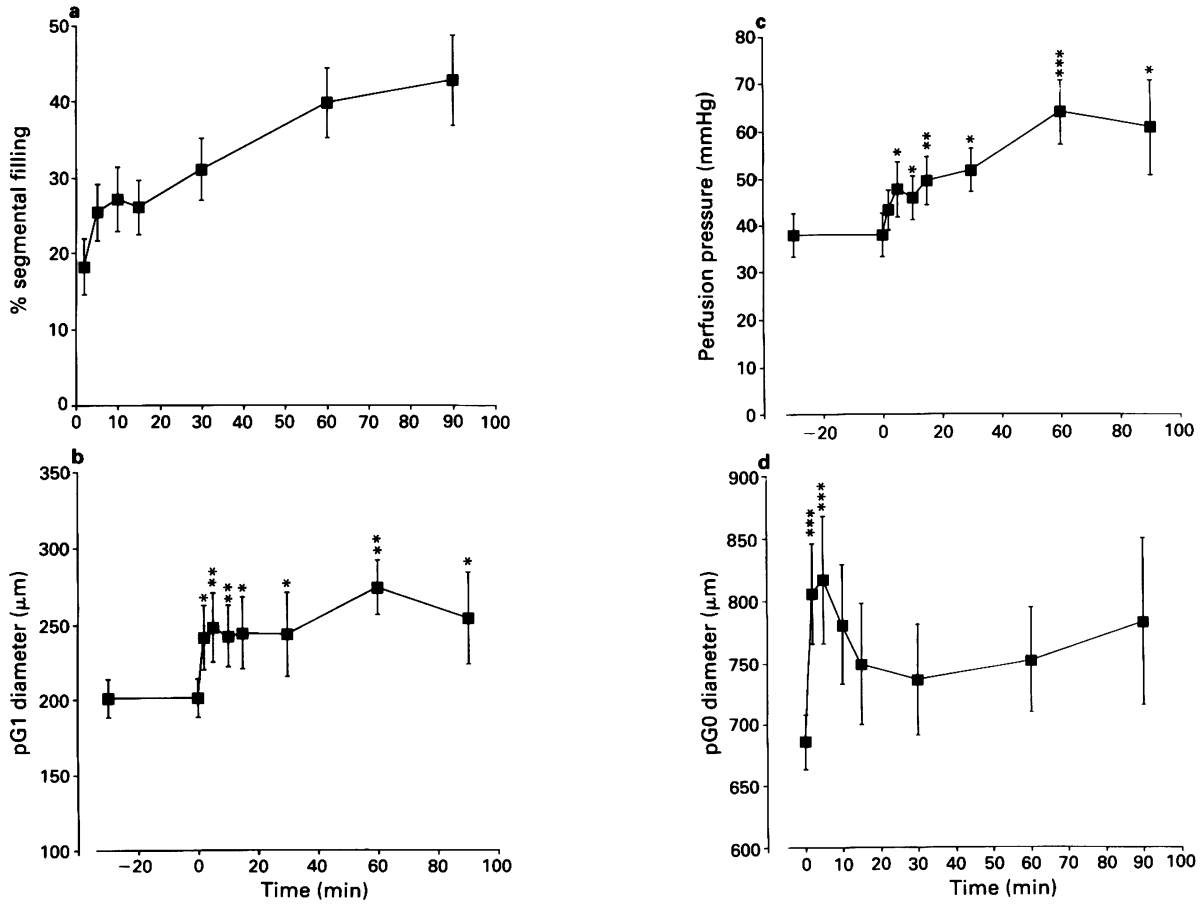


Figure 2 (a) The level of filling (%) of the isolated segment of the central artery normalized with respect to the preocclusion control value; (b) the time course of changes in diameter of pG1; (c) changes in perfusion pressure with respect to time and (d) time-course of changes in diameter of pG0 in the isolated ear preparations following arterial occlusion. Ligation was performed at 0 min. In (b), (c) and (d) statistical differences between baseline preligation values and those obtained after ligation are indicated by $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. Data are given as the mean with bars representing ± s.e.mean.

3 experiments 10 μM glibenclamide completely reversed the reductions in tone evoked by 30 μM BRL 38227, returning the level of tone to 118 ± 14% of that before the additions of BRL 38227. When added alone, glibenclamide did not influence perfusion pressure in the constricted preparations ($n = 3$).

In order to examine whether the vasodilator properties of BRL 38227 were influenced by EDRF activity, 7 preparations were pretreated with 100 μM L-NAME prior to preconstriction and construction of the concentration-response curves. In these preparations resting perfusion pressure was 20.7 ± 1.9 mmHg and following addition of L-NAME was unchanged with a value of 26.4 ± 5.2 mmHg. Addition of 5-hydroxytryptamine and histamine (both 100 nM) increased perfusion pressure to 140 ± 10 mmHg, which was not significantly different from that obtained in preparations treated with 1 μM of the constrictors in the absence of L-NAME. Lemakalim (10 nM–10 μM), in the presence of L-NAME, caused relaxations of tone with an EC_{50} of 55.6 ± 5.0 nM which was significantly ($P < 0.001$) less than that obtained in the absence of L-NAME (Figure 3b). The maximum relaxation was 86.3 ± 4.6% and did not differ from that obtained in the absence of L-NAME reported above.

In a further 5 preparations, basal perfusion pressure was 36.7 ± 12.1 mmHg and was increased by 109 ± 31 mmHg to 146 ± 35 mmHg following the addition of 5-hydroxytryptamine and histamine (both 1 μM). In these preparations verapamil (1 nM-1 μM) caused relaxations of established tone with an EC_{50} of 72.6 ± 32.0 nM and a maximum reduction in tone of 63.8 ± 11.4% (Figure 3a). The EC_{50} was significantly less than that for sodium nitroprusside ($P < 0.001$) and BRL 38227 ($P < 0.01$) making verapamil the most potent of the vasodilators studied. The maximum vasodilator response to verapamil did not differ significantly from those found for either sodium nitroprusside or BRL 38227.

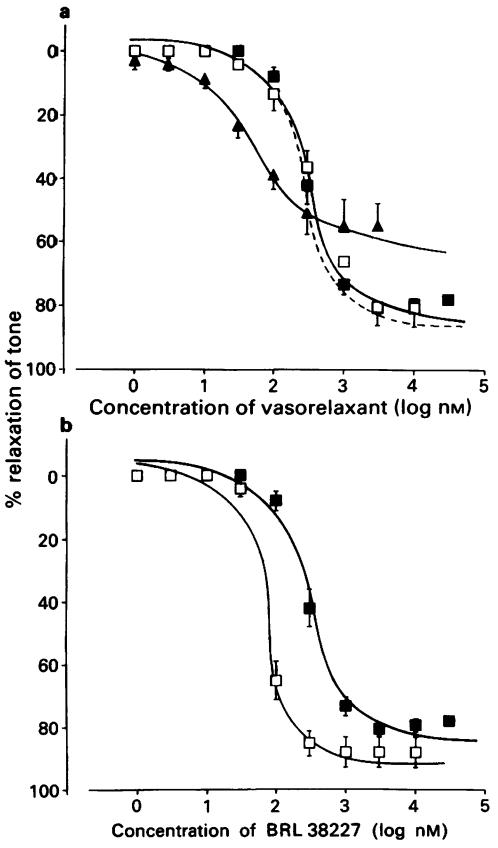


Figure 3 (a) The vasodilator effects of sodium nitroprusside (□), BRL 38227 (■) and verapamil (▲) in ear preparations preconstricted with 5-hydroxytryptamine and histamine (both 1 μM). (b) The vasodilator properties of BRL 38227 in the absence (■; data from a) and the presence of 100 μM N^G-nitro-L-arginine methyl ester (L-NAME), □) in preparations preconstricted with 5-hydroxytryptamine and histamine. Data are given as the mean with bars representing ± s.e.mean.

Effect of sodium nitroprusside on collateral perfusion following arterial occlusion

In 11 preparations, resting perfusion pressure was 50.9 ± 8.4 mmHg and following addition of 500 nM sodium nitroprusside perfusion pressure was unaltered with a value of 44.1 ± 8.5 mmHg; 2 min after ligation, filling of the isolated segment was 33.2 ± 5.3% of its preocclusion value and was significantly ($P < 0.05$) greater than that seen in the absence of the vasodilator (Figure 4). The degree of filling increased progressively with time being 42.7 ± 3.2% at 15 min, 49.2 ± 4.0% at 30 min reaching a maximum of 53.0 ± 4.8% at 90 min. For the first 15 min the level of filling was significantly greater than in the control preparations. Immediately following ligation, perfusion pressure was 57.7 ± 9.7 mmHg and not significantly different from the preocclusion value. Perfusion pressure increased significantly ($P < 0.05$) 5 min after ligation and reached 89.0 ± 14.6 mmHg 90 min after occlusion. Prior to ligation the diameter of pG1 was 253 ± 16 μm and this was unaffected by addition of sodium nitroprusside (252 ± 16 μm). After ligation the diameter of pG1 increased progressively and was significantly ($P < 0.05$) greater than resting 15 min after ligation (284 ± 20 μm); 90 min after ligation the diameter was 354 ± 28 μm.

In 9 different preparations, perfusion pressure was 44.2 ± 12.7 mmHg and was not significantly affected by addition of 3 μM sodium nitroprusside (36.7 ± 12.0 mmHg). Following isolation of the segment by ligation, filling was 25.0 ± 6.5% and increased to 41.9 ± 4.9% after 5 min of occlusion. This level of filling was maintained throughout the remainder of the experiment being 46.1 ± 4.6% (15 min), 44.8 ± 9.5 (60 min) and 40.9 ± 10.0% at 60 min (Figure 4). In the 5–30 min after ligation the level of filling was significantly ($P < 0.05$) greater than that in control preparations. In preparations receiving 3 μM sodium nitroprusside,

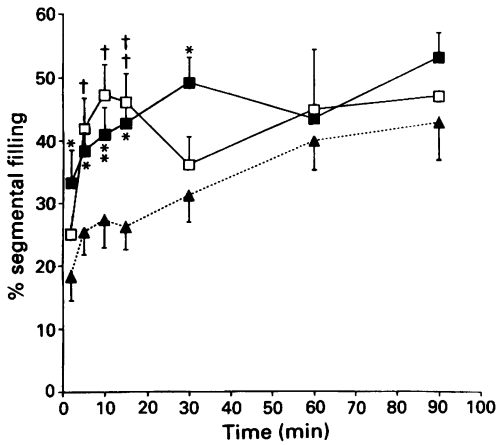


Figure 4 The effects of sodium nitroprusside (SNP) on collateral perfusion in the rabbit ear model of arterial occlusion. The variation of filling (normalized with respect to preocclusion controls) of the isolated segment of central artery wtih time is indicated by (■) and (□) for perfusion with 500 nM and 3 μM sodium nitroprusside respectively. Control data (taken from Figure 2a) are indicated by ▲ and are included for comparison. Statistical differences between control and preparations perfused with sodium nitroprusside are indicated by: †$P < 0.05$ and ††$P < 0.01$ for preparations receiving 3 μM SNP and *$P < 0.05$ and **$P < 0.01$ for those perfused with 500 nM. Data are given as the mean with bars representing ± s.e.mean.

perfusion pressure remained at a constant level following addition of the agent (36.9 ± 12.0 mmHg) and also following ligation such that after 90 min it was 50.0 ± 12.8 mmHg. The preligation diameter of pG1 was similarly not significantly affected by sodium nitroprusside being 202 ± 21 μm in its absence and 235 ± 21 μm in its presence. However, immediately following ligation the diameter of these vessels increased to 274 ± 25 μm and remained at this level throughout the experiment.

Effect of BRL 38227 on collateral perfusion following arterial occlusion

BRL 38227 (500 nM) did not affect perfusion pressure which was 44.3 ± 9.1 mmHg in its absence and 39.5 ± 7.6 mmHg in its presence (both $n = 6$); 2 min after ligation, filling of the isolated segment was 42.2 ± 7.6% and this increased to 71.6 ± 9.0% at 10 min after which time the level of filling remained at this level (Figure 5a). Throughout the experiment the level of filling was significantly ($P < 0.01$) greater than corresponding values obtained from the control preparations not treated with the vasodilator. In preparations receiving 500 nM BRL 38227, perfusion pressure remained similar to the preocclusion values for the entirety of the ligation period (Figure 5c). Figure 5b indicates that prior to ligation, BRL 38227 did not affect the diameter of pG1, while after ligation there was significant ($P < 0.05$) dilatation which peaked at 30 min with a value of 297 ± 36 μm.

In the preparations which received 3 μM BRL 38227, perfusion pressure was 46.9 ± 14.0 mmHg in its absence and 43.4 ± 14.0 mmHg in its presence (both $n = 6$). In these preparations ligation was followed by substantial improvements in segmental filling compared to preparations not receiving the vasodilator, being 46.4 ± 9.8% after 2 min and increasing progressively with time reaching a maximum of 77.2 ± 7.4% at 90 min (Figure 5a). Throughout the 90 min of occlusion, the level of filling was significantly ($P < 0.001$) greater than that found in control preparations not receiving the drug but not significantly different from those perfused with 500 nM of the agent. Following ligation, perfusion pressure remained at a level not significantly different from the preocclusion value (Figure 5c). The diameter of pG1 (178 ± 24 μm) was also unaffected by BRL 38227 alone (178 ± 28 μm) but increased significantly ($P < 0.05$) to 233 ± 29 μm within 2 min of ligation and reached a plateau around 250 μm after 10 min (Figure 5b).

Effects of BRL 38227 on collateral perfusion in the presence of L-NAME

In the 9 preparations treated with 100 μM L-NAME perfusion pressure was 29.3 ± 1.8 mmHg before addition and 42.1 ± 7.0 mmHg afterwards and these values were not significantly different. Following subsequent addition of BRL 38227 to the physiological salt solution, perfusion pressure was 33.1 ± 3.3 mmHg. In these preparations, filling of the isolated segment was significantly ($P < 0.05$) greater than that seen in the presence of L-NAME alone (Randall & Griffith, 1992a,b) for 15–60 min after ligation (Figure 6a). There were no significant differences in segmental filling between preparations treated with 3 μM BRL 38227 alone and those receiving both BRL 38227 and L-NAME. Addition of L-NAME to preparations prior to BRL 38227 did not significantly alter the diameter of pG1 (224 ± 41 μm cf. 231 ± 38 μm). However, after ligation there was dilatation of pG1 comparable to that seen in the presence of BRL 38227 alone (Figure 6b). This contrasts the lack of effect on pG1 seen in the presence of L-NAME alone (Randall & Griffith, 1992a,b). Throughout the ligation period in the presence of L-NAME and BRL 38227, perfusion pressure was unaltered and this contrasts the post-ligation pressor effect seen in the presence of L-NAME alone (Figure 6c).

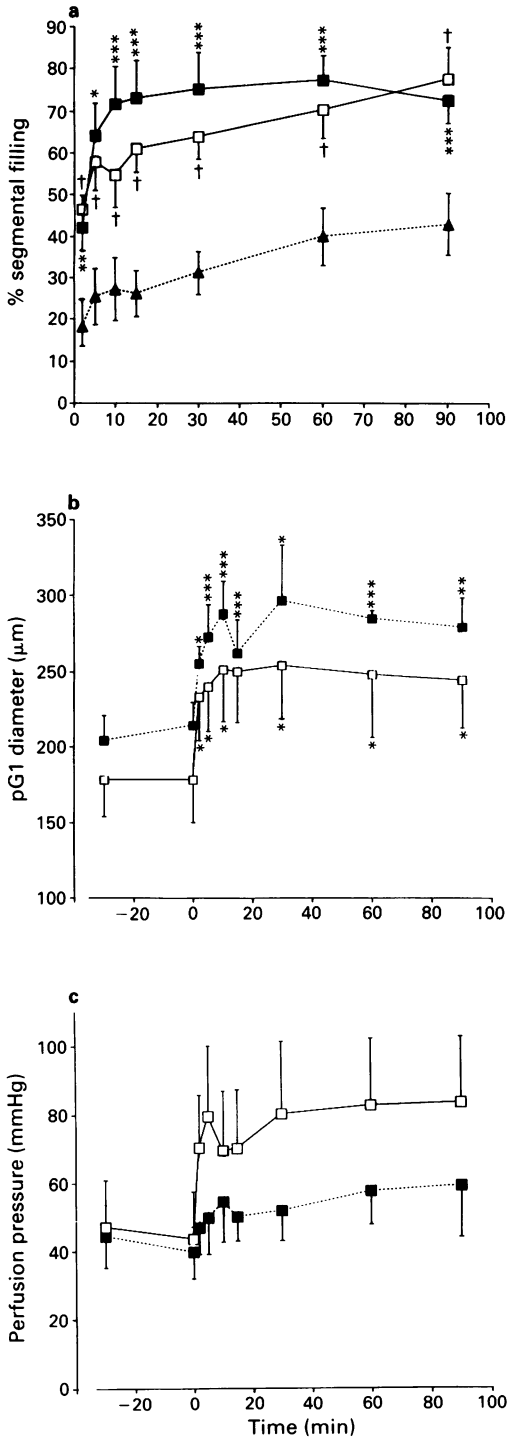


Figure 5 The effects of BRL 38227 on collateral perfusion and perfusion pressure in the rabbit ear model of arterial occlusion. (a) The variation of filling (normalized with respect to preocclusion controls) of the isolated segment of central artery with time in preparations perfused with 500 nM (■) and 3 μM (□) BRL 38227. The (▲) indicates control data taken from Figure 2a. Statistical differences between control and drug treated preparations are indicated by †$P < 0.001$ for perfusion with 3 μM and *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ for 500 nM. (b) The diameter changes in pG1 during arterial ligation in preparations perfused with 500 nM (■) and 3 μM (□) BRL 38227. Statistical differences between baseline diameters and those obtained after ligation are indicated by *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. (c) Variation in perfusion pressure in preparations perfused with 500 nM (■) and (□) 3 μM BRL 38227. In (b) and (c) perfusion with the vasodilator was started 30 min before ligation and the preparations were ligated at 0 min. Data are given as the mean, with bars representing ± s.e.mean.

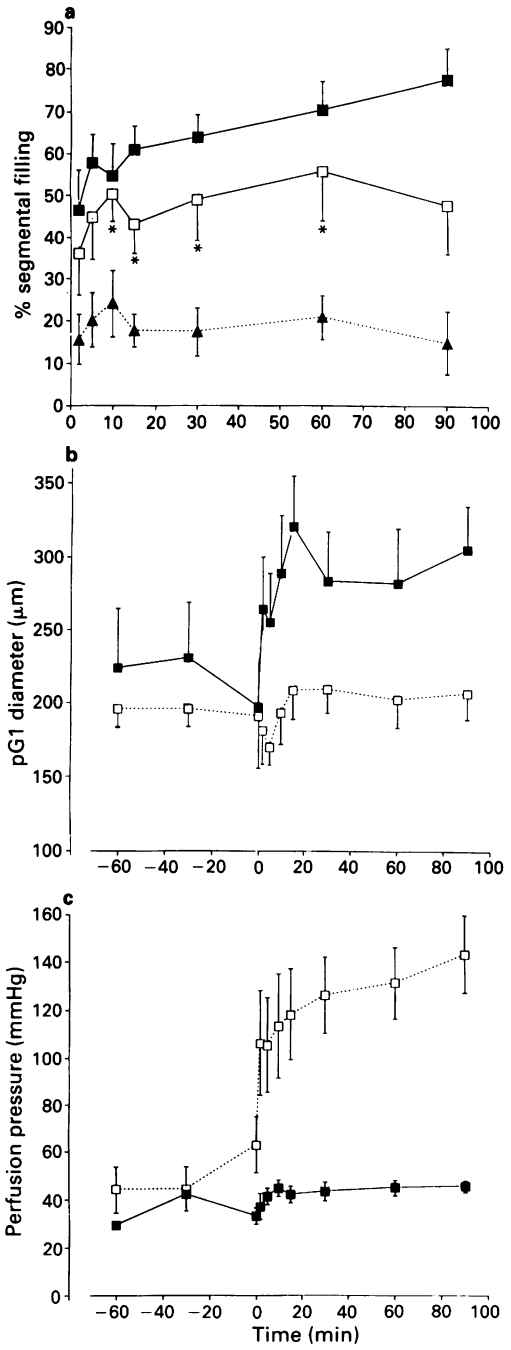


Figure 6 The effects of BRL 38227 on collateral perfusion and perfuson pressure in the rabbit ear model of arterial occlusion in the presence of 100 μM N^G-nitro-L-arginine methyl ester (L-NAME). (a) The variation of filling (normalized with respect to preocclusion controls) of the isolated segment of central artery with time in preparations perfused with 3 μM (■) BRL 38227 alone (data taken from Figure 5a), in the presence of L-NAME (▲) alone (data taken from Randall & Griffith, 1992b) and preparations receiving 3 μM BRL 38227 and 100 μM L-NAME (□). Statistical differences between preparations treated with L-NAME and those treated with L-NAME plus BRL 38227 are indicated by $*P<0.05$. (b) The variation of the diameter of pG1 with time in preparations perfused with 100 μM L-NAME (□) alone (data taken from Randall & Griffith, 1992b) and preparations receiving 3 μM BRL 38227 and 100 μM L-NAME (■). (c) The variation of perfusion pressure with time in preparations perfused in the presence of L-NAME (□) alone (data taken from Randall & Griffith, 1992b) and preparations receiving 3 μM BRL 38227 and 100 μM L-NAME (■). In (b) and (c) perfusion with L-NAME occurred 60 min before ligation, addition of the vasodilator was started 30 min before ligation and the preparations were ligated at 0 min. Data are given as the mean, with bars representing ± s.e.mean.

Effect of verapamil on collateral perfusion following arterial occlusion

Verapamil (50 nM) did not affect perfusion pressure which was 29.1 ± 8.8 mmHg before and 30.0 ± 9.1 mmHg after its addition (both $n = 5$); 2 min after ligation, filling of the isolated segment was 13.0 ± 6.1% and this increased to 20.0 ± 8.7% at 15 min reaching a maximum of 42.7 ± 10.5% at 90 min. The level of segmental filling was not significantly different from that in control preparations (Figure 7). In preparations perfused with verapamil, perfusion pressure remained similar to the preocclusion values following ligation. Verapamil did not affect the baseline diameter of pG1 (238 ± 15 μm before and 245 ± 14 μm after addition), while after ligation there was only significant ($P<0.05$) dilatation between 60–90 min, with a maximum value of 311 ± 35 μm at 60 min.

In the preparations which received 3 μM verapamil, perfusion pressure was 32.2 ± 4.5 mmHg in its absence and 33.3 ± 4.6 mmHg in its presence (both $n = 6$). Two minutes after ligation filling was 30.7 ± 3.8% and reached a maximum of 39.4 ± 8.6% after 15 min (Figure 7). The level of filling was statistically similar to that found in the control preparations for the entirety of the ligation period. Perfusion pressure remained significantly ($P<0.05$) greater than the resting value for the 90 min after ligation. The diameter of pG1 was also unaffected by verapamil (209 ± 19 μm cf. 227 ± 15 μm) but increased to 273 ± 329 μm within 2 min of ligation and remained elevated throughout the experiment.

Discussion

We have recently demonstrated that EDRF plays a central role in collateral perfusion after arterial occlusion (Randall & Griffith, 1992a,b). In the present study we have investigated the effects of exogenous vasodilators on collateral perfusion and show that BRL 38227 substantially enhances collateral perfusion over a period of 90 min, that sodium nitroprusside causes improvement in the early stages after occlusion (ca. 15 min) but that verapamil, which was the most potent vasodilator in pharmacologically-constricted preparations, was without significant effect.

The rabbit ear model of arterial occlusion is well suited for investigating the role of purely vascular responses in acute

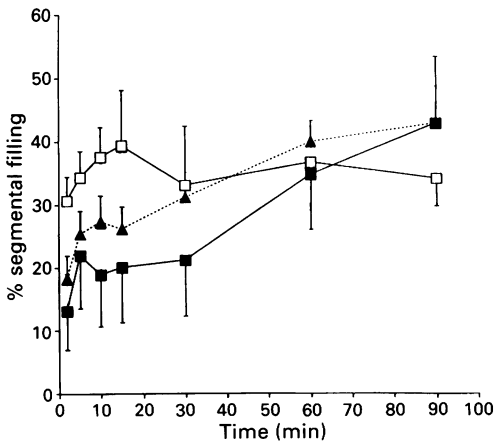


Figure 7 The effects of verapamil on collateral perfusion in the rabbit ear model of arterial occlusion. The variation of filling (normalized with respect to preocclusion controls) of the isolated segment of central artery with time is indicated by (■) and (□) for perfusion with 50 nM and 3 μM verapamil respectively. The (▲) indicates control data from Figure 2a and has been included for comparison. Data are given as the mean, with bars representing ± s.e.mean.

collateralization since there is little complication from humoral, nervous or metabolic regulation. It was considered appropriate to carry out these occlusion experiments in the absence of pharmacological constriction, since acutely ischaemic organs are generally at minimal tone. Previous studies have indicated that filling of a segment of central ear artery isolated between ligatures is a reliable indicator of collateral perfusion (Randall & Griffith, 1992a,b). In addition to filling of the isolated segment we have also reported on the selective diameter changes of primary arterial branches (pG1) proximally close to the occlusion which feed the collateral circulation. Indeed in the present study there was a similar correlation between the dilatation of pG1 and segmental filling as noted previously (Randall & Griffith, 1992a,b). In our previous study we also noted that G1 vessels located proximally further away (10–30 mm) from the midpoint ligation did not undergo diameter changes after ligation indicating that only the pG1 vessels adjacent to the ligation feed the collateral circulation (Randall & Griffith, 1992b).

Arterial occlusion in control preparations was followed by time-dependent filling of the isolated segment, approaching a plateau after 60 min such that pre-existing collateral vessels restored filling of the isolated segment to nearly half of the preocclusion level. These observations are consistent with previous *in vivo* studies in which occlusion of the canine superficial femoral artery was followed by restoration of blood flow (between one third and one half control levels) within 5 to 8 min (John & Warren, 1961). Collateral flow then reaches a plateau at approximately 70% of the control level at 30 min (John & Warren, 1961; Rosenthal & Guyton, 1968). In the current study, segmented filling was accompanied by diameter changes of pG1 which increased by approximately 20% representing a ca. 80% increase in conductance and this change persisted throughout the time course of the experiment.

We have also considered the variations of perfusion pressure and diameter of pG0 with time which give insight into the effects of arterial occlusion on the distribution of flow. Ligation of the control preparations resulted in only a modest increase in perfusion pressure, despite the main arterial supply to the distal half of the ear having been occluded. These findings are analogous to those reported following acute occlusion of the canine superficial femoral artery *in vivo* (Winblad *et al.*, 1959) and are consistent with the diversion of flow limiting the rise in pressure proximal to the occlusion. In preparations receiving BRL 38227, which had the greatest influence on collateral perfusion, perfusion pressure was unaltered compared to preocclusion values. This is consistent with the vasodilator opening collateral pathways and facilitating the redistribution of flow. Conversely, in the presence of 3 μM verapamil, which did not influence collateral function, perfusion pressure increased in a slow, time-dependent fashion similar to that observed in the control experiments. Occlusion of all preparations was accompanied by an immediate increase in the diameter of pG0 which probably reflects the previously described phenomenon of 'paradoxical dilatation' in response to increased distal resistance (Griffith *et al.*, 1987; Randall *et al.*, 1990).

The net effect of any vasodilator on collateral perfusion will be governed by the actions of the agent on collateral vessels and alternative arterial pathways not providing collateral perfusion. Selectivity may be influenced by alterations of tissue responsiveness occurring as a result of hypoxia/ischaemia following occlusion and these changes may be specific for a particular agent. For vasodilators which act on ion channels, the relative distribution of channels between collateral and non-collateral pathways may also govern selectivity. Furthermore changes in hypoxia may alter the activity of these channels and this may also influence activity. Selective vasodilatation or vascular steal of collateral or non-collateral pathways will therefore determine the particular effects of a vasodilator on collateral perfusion.

BRL 38227 significantly increasd collateral perfusion compared to control preparations in our model. Furthermore, in the pharmacologically-constricted preparations the vasodilator actions of BRL 38227 were fully reversed by glibenclamide, confirming that this vasodilator action occurs through activation of ATP-sensitive potassium channels. Improvement in collateral perfusion in the presence of BRL 38227 was indicated by the substantial increases in segmental filling coupled with increases in diameter of pG1. Thus 3 μM BRL 38227 caused a 50% increase in the diameter of pG1 which is substantially greater than that seen in control preparations (ca. 20%). The actions of BRL 38227 would appear specific for the collateral vessels since there was dilatation of the feed vessels (pG1) and increased segmental filling. An action on the low resistance outflow pathways from the segment may be excluded since dilatation of these vessels would have increased exit of perfusate from the segment leading to reduced filling and this was not the case. Interestingly, BRL 38227 was equieffective at improving collateral perfusion at both the supramaximal concentration and the EC_{50} for relaxation of induced tone. This implies that the EC_{50} for relaxation of tone has maximal effects on collateral perfusion so that BRL 38227 is more potent as a collateral vasodilator than as a net relaxant of tone in the whole ear preparation. Heterogeneity in responses to cromakalim are known to occur in other species such as the rat where the agent is a potent vasodilator of small mesenteric arteries but is without action on small cerebral arteries (McCarron *et al.*, 1991). Similarly the *in vivo* vasodilator responses to BRL 38227 in the rat (Gardiner *et al.*, 1991) and cromakalim in the rat and rabbit (Cook & Hof, 1988) show marked regional differences.

That ATP-sensitive potassium channel activators may selectively dilate pre-existing collateral vessels has already been shown by Lamping & Gross (1984) in the canine coronary bed. In their study Lamping & Gross demonstrated that nicorandil improved collateral blood flow following acute coronary occlusion but only when aortic pressure was maintained artificially. The actions of nicorandil must be viewed with caution since this agent contains a nitroxy substituent group and may have nitrovasodilator activity (Edwards & Weston, 1990) dependent on guanosine 3':5'-cyclic monophosphate (cyclic GMP) elevation (Endoh & Taira, 1983). A selective action of potassium channel activators on pre-existing collateral vessels may therefore account for protection of the myocardium in ischaemia (Lampint *et al.*, 1984) and improved postreperfusion function (Grover *et al.*, 1989) which are known to occur in response to these agents. A physiological role for ATP-sensitive potassium channels in coronary vasodilatation has been demonstrated by Komaru *et al.* (1991) who showed that glibenclamide opposes arteriolar dilatations in response to coronary occlusion in the dog. Furthermore Angersbach & Nicholson (1988) have previously reported that cromakalim, nicorandil and pinacidil selectively increase blood flow to hypoxic regions of skeletal muscle in chronic tissue ischaemia in the rat hindlimb.

Sodium nitroprusside, which acts via the generation of nitric oxide, brought about improvements in collateral perfusion in the early stages after arterial occlusion. Both concentrations of the nitrovasodilator afforded an increase in filling above control levels for at least the first 15 min after occlusion. In common with BRL 38227, both concentrations of sodium nitroprusside had maximal effects on collateral perfusion which implies that this agent is also more potent on collateral vessels compared to its vasodilator effects of pharmacologically induced tone in the whole ear preparation. Nitroglycerin has been shown to increase collateral flow in the canine coronary circulation after occlusion (Capurro *et al.*, 1976) but this action is selective for newly developed collateral vessels, generated as a consequence of chronic arterial stenosis, as Lamping & Gross (1984) failed to detect any increase in true collateral flow following acute coronary occlusion in the dog.

The final agent investigated was the calcium antagonist,

verapamil. Although this agent acted as a potent vasodilator in pharmacologically constricted preparations it did not affect collateral perfusion and compared with the other vasodilators studied, had minimal effects on the diameter of pG1. Since there was no deterioration in collateral perfusion compared to control, this may suggest that the vasodilator is equipotent on collateral and non-collateral pathways since steal did not appear to occur or that the agent did not affect either pathway.

Our investigations have also considered whether BRL 38227 was effective following inhibition of EDRF activity, since this factor appears to be essential for normal collateral perfusion (Randall & Griffith, 1992a,b). In the vasodilator experiments, BRL 38227 was more potent (5 fold) in the presence of L-NAME compared to control. This was somewhat suprising since *in vivo*, the vasodilator properties of BRL 38227 in the rat are not, except in the case of the mesenteric circulation, influenced by L-NAME (Gardiner *et al.*, 1991). However, it is well established that responses to nitrovasodilators are augmented after inhibition of EDRF activity (Shirasaki & Su, 1985) and this is thought to be due to supersensitivity of the guanylyl cyclase (Moncada *et al.*, 1991). In the present context the increased potency of BRL 38227 cannot be explained through interactions at the level of guanylyl cyclase as the agent acts independently of cyclic GMP (Taylor *et al.*, 1988), and we have shown that the vasodilator responses were completely blocked by glibenclamide. Enhanced activity of vasodilators on loss of basal EDRF activity is not confined to nitrovasodilators, as vasodilator responses to an endothelium-independent agent isoprenaline are augmented after loss of the endothelium in rabbit aorta (White *et al.*, 1986). This interaction is explained by basal EDRF activity having a tonic vasodilator input which is abolished by endothelial removal, thus enabling other vasodilators to have greater impact. In the presence of L-NAME and BRL 38227, collateral perfusion was generally greater than that seen in the presence of L-NAME. Furthermore the level of perfusion was not significantly different from that seen in the presence of both EDRF activity and BRL 38227. Interestingly, BRL 38227 abolished the post-ligation increase in perfusion pressure (ca. 100 mmHg) seen in the presence of L-NAME alone (Randall & Griffith, 1992a, b). The level of collateral perfusion seen in the present experiments indicates that BRL 38227 can act independently of EDRF activity and may therefore be effective at increasing collateral flow in states where EDRF activity has been reported to be impaired (e.g. hypertension, Lüscher & Vanhoutte, 1986; Mayhan *et al.*, 1987; atherosclerosis, Harrison *et al.*, 1987, or hypercholesterolemia, Verbeuren *et al.*, 1986). This observation may be of considerable therapeutic relevance since these states may predispose towards arterial occlusion.

The selective action of BRL 38227 on pre-existing collateral vessels requires explanation. Changes occurring during hypoxia or ischaemia may reduce the level of oxidative phosphorylation leading to a reduction in ATP production. This may result in membrane depolarization, suggesting that potassium channel activators would have a greater hyperpolarizing effect, and lead to selective dilatation of such vessels. A reduction in intracellular ATP concentrations would also potentiate the actions of drugs such as BRL 38227 which act on potassium channels sensitive to ATP. The impact of calcium channel blockers will be relatively unaffected by ATP levels and so verapamil will be without selective action on these vessels. An alternative explanation might be that the apparently selective action of potassium channel activators over other vasodilators reflects differences in ion channel populations between collateral and non-collateral vessels.

In conclusion, the present findings indicate a selective action for BRL 38227 on collateral perfusion and to a lesser extent for sodium nitroprusside. It remains to be established whether BRL 38227 may prove of therapeutic importance in acute occlusive arterial disease.

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Developmental changes in endothelium-dependent pulmonary vasodilatation in pigs

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1 We compared *in vitro* endothelium-dependent vasorelaxant responses to acetylcholine (ACh) and the endothelium-independent vasodilator response to sodium nitroprusside (SNP) in prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-precontracted muscular pulmonary arteries (PA) from pigs aged 5 min to 2 h (neonatal), 3–10 days, 3–8 weeks and adults.

2 In the pulmonary artery (PA) rings from neonatal animals, the vasodilator response to ACh was negligible. However, responses to ACh were present in all PA rings from older animals, being greatest at 3–10 days and then decreasing with age ($P<0.001$, ANOVA). ACh (30 μM) induced a $1 \pm 1\%$, $92 \pm 9\%$, $62 \pm 5\%$ and $51 \pm 6\%$ reduction of the $PGF_{2\alpha}$-generated tension in neonatal, 3–10 days, 3–8 weeks and adult groups, respectively.

3 The relaxant response to SNP was present in the PA rings from all age groups and increased with age ($P<0.001$, ANOVA). SNP (1 μM)-induced relaxation was $55 \pm 9\%$, $73 \pm 7\%$, $97 \pm 5\%$ and $93 \pm 6\%$ in neonatal, 3–10 days, 3–8 week and adult groups, respectively.

4 Removal of the vascular endothelium abolished the relaxant response to ACh but had no effect on the response to SNP in any groups.

5 N^G-monomethyl-L-arginine (30 μM), a nitric oxide synthesis inhibitor, inhibited the response to ACh but not to SNP. The lipoxygenase inhibitor, nordihydroguaiaretic acid, had no significant effect on responses to ACh or SNP in any group.

6 These findings suggest that the nitric oxide pathway may not play a part in dilating the pig pulmonary arteries at birth, but may be important during the transitional period of establishing a stable post-natal pulmonary circulation. The increase in response to SNP with age parallels the increase in smooth muscle cell myofilaments to which it may be related.

Keywords: Endothelium-derived relaxing factor; pulmonary artery; endothelium; vascular development

Introduction

Since Furchgott & Zawadzki (1980) first demonstrated that the vasodilator action of acetylcholine (ACh) was dependent on the presence of an intact endothelium, the vascular endothelium has proved to play a crucial role in mediating the dilator response to a variety of stimuli in the systemic and pulmonary circulations (Furchgott, 1984; Greenberg *et al.*, 1987). In response to these stimuli, endothelial cells release a labile endothelium-derived relaxing factor (EDRF), which causes vascular smooth muscle relaxation (Furchgott & Vanhoutte, 1989). EDRF has been identified as nitric oxide (NO) or a related compound (Palmer *et al.*, 1987; 1988; Moncada *et al.*, 1989; Ignarro, 1989), and inhibition of NO synthesis by compounds such as N^G-monomethyl-L-arginine (L-NMMA) inhibits the vasodilator response to ACh in both systemic (Amezcua *et al.*, 1988; Rees *et al.*, 1989a) and pulmonary circulations (Davidson & Eldermerdash, 1990; Crawley *et al.*, 1990; Liu *et al.*, 1991). L-NMMA induces dose-dependent increase in mean systemic blood pressure (Rees *et al.*, 1989b; Gardiner *et al.*, 1990) and decreases regional blood flow *in vivo* (Gardiner *et al.*, 1990).

The pulmonary circulation undergoes significant changes, both structurally (Haworth & Hislop, 1981; Hall & Haworth, 1986; 1987), and functionally (Su *et al.*, 1977; Davidson, 1987; Dunn *et al.*, 1989; Teitel *et al.*, 1990), at birth. The mechanisms responsible for this pulmonary adaptation are incompletely understood. Vasodilators such as prostacyclin (Leffler *et al.*, 1978; Leffler & Hessler, 1981; Davidson, 1988) have been implicated. Endothelium-derived NO may also play a role in the sheep (Abman *et al.*, 1990), but not in the rabbit (Zubrow *et al.*, 1989). The role of the vascular endothelium in regulating or modulating perinatal pulmonary adaptation and reactivity has not been studied in detail.

EDRF activity has been shown to be present in pulmonary vessels at 1–3 days in guinea-pigs (Davidson & Eldemerdash, 1990) but is absent or greatly reduced in foetal pulmonary arteries of rabbit and sheep (Zubrow *et al.*, 1989; Abman *et al.*, 1991). These results suggest that, in addition to a developmental change after birth (Abman *et al.*, 1991; Zellers & Vanhoutte, 1991), the endothelium-dependent response may change rapidly at birth with the dramatic pulmonary haemodynamic changes.

We have, therefore, examined the endothelium-mediated dilator response to ACh and the endothelium-independent dilator responses to sodium nitroprusside (SNP) in the pulmonary arteries from pigs immediately after birth (aged 5 min to 2 h: neonatal), compared those with the responses in the pulmonary arteries from 3–10 days, 3–8 weeks and young adults, in order to characterize further the developmental change in the endothelium-dependent response. To study the mechanism(s) involved, we used L-NMMA to inhibit NO synthesis and indomethacin to inhibit the cyclooxygenase production. Lipoxygenase products may play a role in mediating endothelium-dependent relaxation in rabbit aorta (Singer & Peach, 1983; Forstermann & Neufang, 1984; Forstermann *et al.*, 1986), therefore, we also studied the effect of nordihydroguaiaretic acid (NDGA, a lipoxygenase inhibitor) on the endothelium-dependent relaxation in these vessels.

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Methods

Twenty-five large white pigs were killed by exsanguination. Immediately after death the heart and lungs were removed and pulmonary arteries were carefully dissected. The arteries were put in oxygenated Krebs-Henseleit (K-H) solution kept at 4°C and transported to the laboratory for use. The transport time and conditions were standardized for all groups. Intrapulmonary arteries were examined in 4 groups of pigs aged less than 2 h (neonatal, $n = 6$), 3–10 days ($n = 6$), 3–8 weeks ($n = 8$) and 6–9 months (adult, $n = 5$). In all animals the distal 2/3 of both lower lobe pulmonary arteries were cleaned of surrounding tissues and cut into rings of 2.5 or 3.5 mm in length depending upon the size of the lung. A total of 4 rings (neonatal and 3–10 day groups) or 6 rings (3–8 week and adult groups) were studied in each animal. In each animal at least one ring was a control and two rings were for L-NMMA or NDGA treatment. In the remaining ring(s), vascular endothelium was removed by gently rubbing the intimal surface with a steel probe or a piece of fine sand paper.

Organ chamber studies

Vessel rings from pigs aged less than 10 days were positioned in 2 ml organ baths by a pair of stainless steel wires inserted horizontally in the lumen of the vessel rings. One wire was fixed and the other attached to a force transducer (FT. 03 Grass, Quincy, MA, U.S.A.). Rings from large pigs were fixed vertically between two hooks in 5 ml organ chambers. One hook was fixed and the other attached to a force transducer. The organ chambers contained K-H solution composed of: NaCl 118, KCl 5.9, $MgSO_4.7H_2O$ 1.2, $CaCl_2.6H_2O$ 2.5, NaH_2PO_4 1.2, glucose 5.6 and $NaHCO_3$ 25.5 mM. They were maintained at 37°C and aerated with a gas mixture of 95% O_2 and 5% CO_2. Indomethacin (10 μM) was present in all experiments. The optimal resting tension of each vessel was determined initially by increasing resting tension gradually in 200 mg increments and challenging repeatedly with 40 mM KCl, until the response to KCl was not further increased. The optimal resting tension for each vessel was calculated from the tension-response curve (see Results section). The vessel ring was allowed to equilibrate at its optimal resting tension for 30 min and a concentration-response curve to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was obtained. Each individual concentration-response curve to $PGF_{2\alpha}$ was fitted and the EC_{50} value estimated by a computer programme (Graph PAD InPlot, GraphPAD software, San Diego, CA, U.S.A.). After a further 1 h equilibration, relaxation studies were started. In each animal one ring was incubated with 30 μM L-NMMA and one with 50 μM NDGA for 20 min before precontraction with $PGF_{2\alpha}$. All vessel rings were precontracted with the EC_{50} concentration of $PGF_{2\alpha}$ and after a stable contraction had been achieved, cumulative concentration-response curves to ACh and then SNP were obtained. At the end of each experiment the vessel rings were fixed with 2.5% glutaraldehyde for histological examination.

Drugs

The following drugs were used: prostaglandin $F_{2\alpha}$ (Upjohn Ltd, Crawley, Sussex), acetylcholine chloride, sodium nitroprusside, indomethacin, nordihydroguaiaretic acid, L-N^G-monomethyl arginine (Sigma, Poole, Dorset). All drugs were freshly prepared on the day of experiment.

Calculation and analysis of results

Contractions were measured from the vertical distance between the baseline and the stable tone induced by $PGF_{2\alpha}$ and are presented in absolute tension (mg). Relaxations were measured from a common reference line denoting the stable contractions elicited by EC_{50} concentrations of $PGF_{2\alpha}$ before adding ACh or SNP. The vertical distance between this reference line and the largest downward deflection in the tracing at each concentration of ACh or SNP was recorded and expressed as the percentage of the $PGF_{2\alpha}$-induced contraction. The mean values at each concentration point were used to compare the relaxant response to ACh and then SNP in vessel rings of different ages, and the same vessels in the presence and absence of an intact endothelium, L-NMMA, or NDGA. All values in the text are presented as mean ± s.e.means. n refers to the numbers of animals from which the PA rings were taken. Results were compared by using paired Student's t test or one-way analysis of variance followed by Bonferroni corrected t test for stepwise comparison, when multiple comparison was made (Wallenstein *et al.*, 1981). P values <0.05 were considered to be significant.

Morphological examination

Vascular rings were processed for electronmicroscopic examination. One μm sections were cut and stained with toluidine blue. From these it was possible to confirm the presence or absence of endothelial cells. Transmission electronmicrographs of endothelial cells were also studied.

Results

The optimal resting tension increased significantly with maturation and was 933 ± 10 ($n = 6$ animals), 1060 ± 40 ($n = 6$ animals), 1138 ± 11 ($n = 8$ animals) and 1124 ± 35 ($n = 5$ animals) mg for neonatal, 3–10 day, 3–8 week and adult groups respectively ($P < 0.03$ between any two adjacent groups, except between 3–8 week and adult group). $PGF_{2\alpha}$-generated contraction also increased significantly with age in rings with and without endothelium (Table 1). Light microscope examination confirmed that an intact endothelial lining was consistently present in unrubbed vessel rings and absent in rubbed vessel rings (Figure 1). Examination by transmission electron microscopy showed that the endothelial morphological appearances were identical among all age groups.

Table 1 Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced tensions (mg, pre-acetylcholine) in developing and adult pig pulmonary arteries

	n	*Neonatal*	n	*3–10 day*	n	*3–8 week*	n	*Adult*
E^+	6	544 ± 52*	6	761 ± 155†	8	1238 ± 141	5	1641 ± 310
E^-	6	516 ± 105*	6	796 ± 182†	8	1223 ± 170	5	1884 ± 309
L-NMMA	6	540 ± 102*	6	870 ± 144†	8	1255 ± 160	5	2009 ± 271
NDGA	6	442 ± 102*	6	720 ± 107†	8	965 ± 154	5	1525 ± 238

EC_{50} concentration of $PGF_{2\alpha}$-induced contractions are shown.
E^+: endothelium-intact rings; E^-: endothelium-denuded rings. L-NMMA or NDGA: endothelium intact rings pretreated with L-N^G-monomethyl arginine (30 μM) or nordihydroguaiaretic acid (50 μM).
*$P < 0.01$, compared with 3–8 week and adult groups.
†$P < 0.02$, compared with adult group.
Data show means ± s.e.mean.

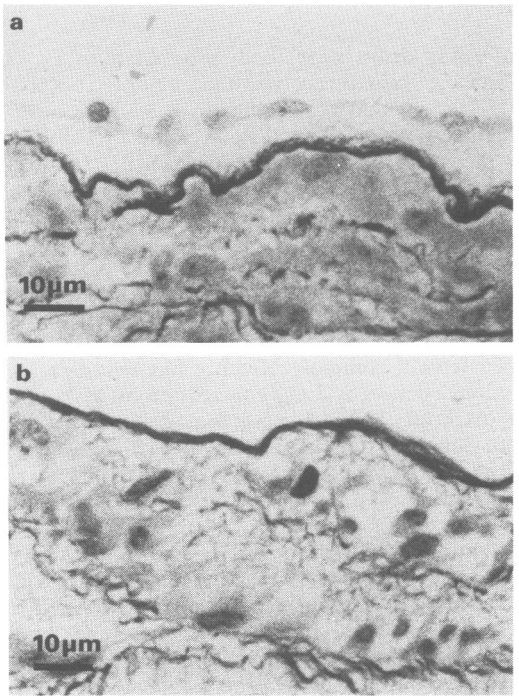


Figure 1 Photomicrographs from two sections of adjacent rings of pulmonary artery from a neonatal pig (5 min after delivery), showing the presence of an intact endothelial lining on the unrubbed ring (a) and the absence of the endothelium on the rubbed ring (b). Toluidine blue stain, magnification × 1000.

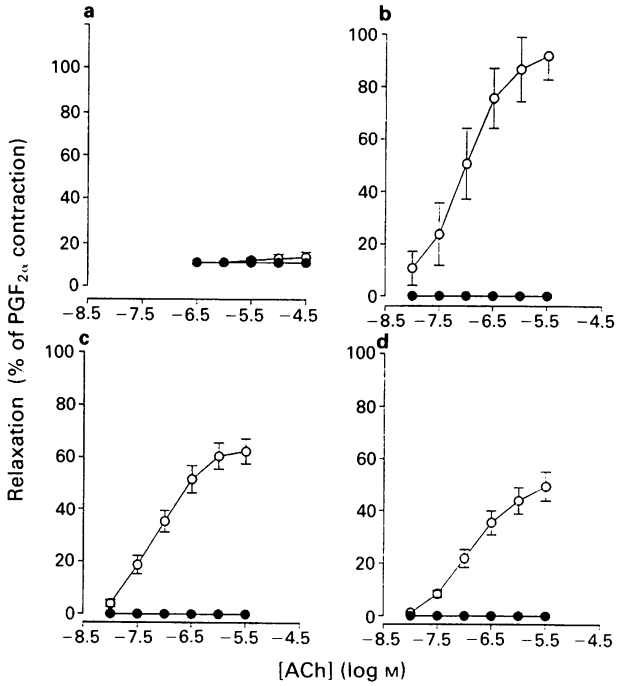


Figure 2 The vasorelaxant response to acetylcholine (ACh) in the precontracted pulmonary artery rings from neonatal (a), 3–10 days (b), 3–8 week (c) and adult (d) pigs. Relaxations were expressed as a percentage of the EC_{50} concentration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced contractions which are shown in Table 1. (○) Rings with endothelium; (●) rings without endothelium; means ± s.e.mean (vertical bars) of 5–8 animals.

Endothelium-dependent relaxation

There was no significant difference in $PGF_{2\alpha}$ (EC_{50} concentration)–induced contractions in vessel rings with or without endothelium at any age (Table 1). ACh had little relaxant effect on the endothelium intact vessel rings in the neonatal group (Figure 2a). The maximal relaxation was of 2.3 ± 2.3% (at 100 μM, $n = 6$) reduction of the $PGF_{2\alpha}$-induced contraction. ACh induced a concentration-dependent relaxation in the endothelium intact precontracted vessel rings from pigs aged 3 days onwards (Figure 2b-d). This relaxation was greatest at 3–10 days, less at 3–8 weeks and still less in adult group (Figure 4a). The relaxant responses to ACh were abolished by removal of endothelium in all age groups (Figure 2a-d). Higher concentrations of ACh (above 30 μM) caused a further contraction in the precontracted neonatal vessel rings with or without endothelium. In rings from older animals, higher concentrations of ACh produced a contraction of the already dilated vessel rings. The precontracted endothelium intact neonatal PA rings which relax poorly in response to ACh, had little relaxant response to bradykinin. The maximal relaxation at 1 μM was 9.7 ± 3.7 ($n = 2$) of the $PGF_{2\alpha}$-induced contraction.

The tensions generated by a second administration of $PGF_{2\alpha}$ (EC_{50} concentration) before adding SNP were not significantly different in the endothelium intact or denuded rings in any age group (Table 2). In all age groups, SNP induced concentration-dependent relaxation of the $PGF_{2\alpha}$ precontracted vessel rings, which was unaffected by endothelial denudation (Figure 3a-d). The vasorelaxant response to SNP increased significantly with age (Figure 4b).

Effects of L-NMMA and NDGA

The tensions induced by EC_{50} concentration of $PGF_{2\alpha}$ in the endothelium-intact vessel rings in the absence and presence of 30 μM L-NMMA or 50 μM NDGA before the administration of ACh or SNP are shown in Table 1 and Table 2. Neither

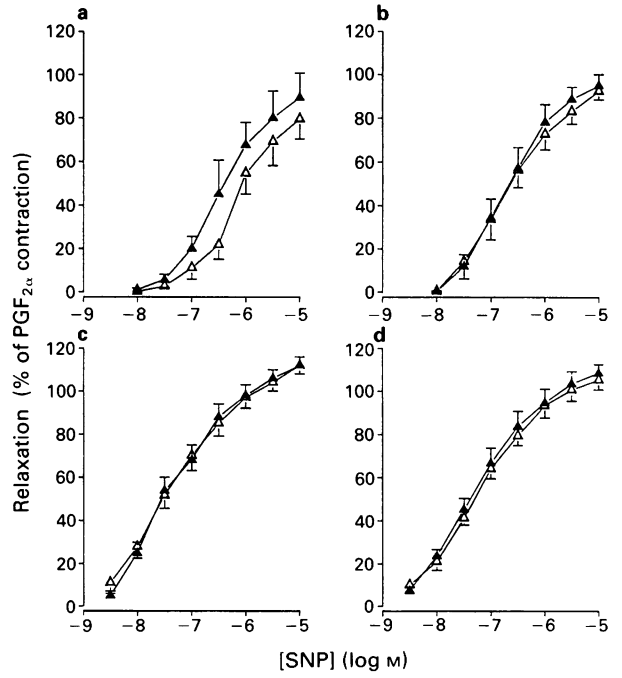


Figure 3 The vasorelaxant response to sodium nitroprusside (SNP) in precontracted pulmonary artery rings from neonatal (a), 3–10 days (b), 3–8 week (c) and adult (d) pigs. Relaxations were expressed as a percentage of the EC_{50} concentration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced contractions which are shown in Table 2. (△) Rings with endothelium and (▲) rings without endothelium; means ± s.e.mean (vertical bars) of 5–7 animals.

Table 2 Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-elicited contractions (mg, pre-sodium nitroprusside) in developing and adult pig pulmonary arteries

	n	*Neonatal*	n	*3–10 day*	n	*3–8 week*	n	*Adult*
E^+	6	544 ± 77*	6	862 ± 126†	7	1342 ± 187	5	1535 ± 318
E^-	6	504 ± 73*	6	857 ± 155†	7	1329 ± 119	5	2047 ± 513
L-NMMA	6	663 ± 111*	6	886 ± 152†	7	1427 ± 293	5	1954 ± 540
NDGA	5	587 ± 49*	5	923 ± 152†	6	1404 ± 245	5	1439 ± 100

Symbols and abbreviations used here are the same as in Table 1.

L-NMMA nor NDGA had a significant effect on the $PGF_{2\alpha}$-induced contraction in any age group. L-NMMA had no effect on the baseline tension in the neonatal group, but increased the baseline tension by 149 ± 49 ($n = 6$), 137 ± 51 ($n = 8$) and 197 ± 96 ($n = 5$) mg in 3–10 day, 3–8 week and adult groups respectively ($P < 0.05$, compared with control rings in each group). L-NMMA incubation significantly

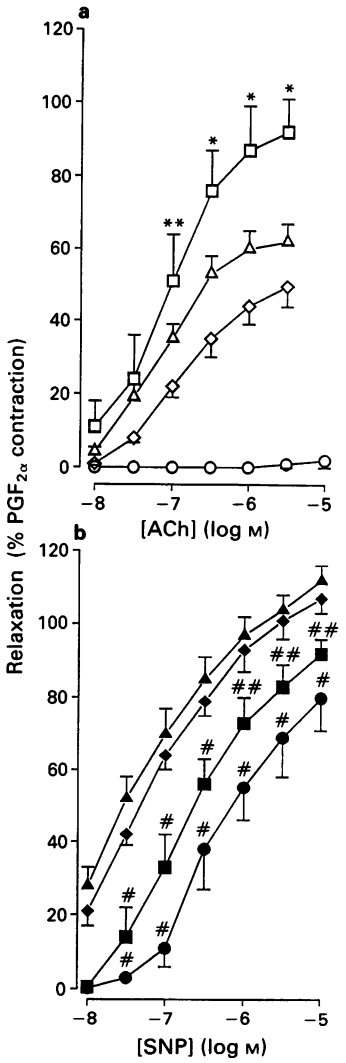


Figure 4 Comparison of the endothelium-dependent relaxant responses to acetylcholine (ACh) (a) and of the endothelium-independent response to sodium nitroprusside (SNP) (b) in the endothelium-intact, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-precontracted, pulmonary artery rings from neonatal (○, ●), 3–10 days (□, ■), 3–8 weeks (△, ▲) and adult (◇, ◆) pigs. Relaxations were expressed as a percentage of EC_{50} concentration of $PGF_{2\alpha}$-generated tensions which are shown in Tables 1 and 2. $*P < 0.05$, compared with other 3 groups; $**P < 0.05$, compared with neonatal and adult groups; $\#P < 0.05$, compared with 3–8 week and adult groups; $\#\#P < 0.05$, compared with 3–8 week group; means ± s.e.mean (vertical bars) of 5–8 animals.

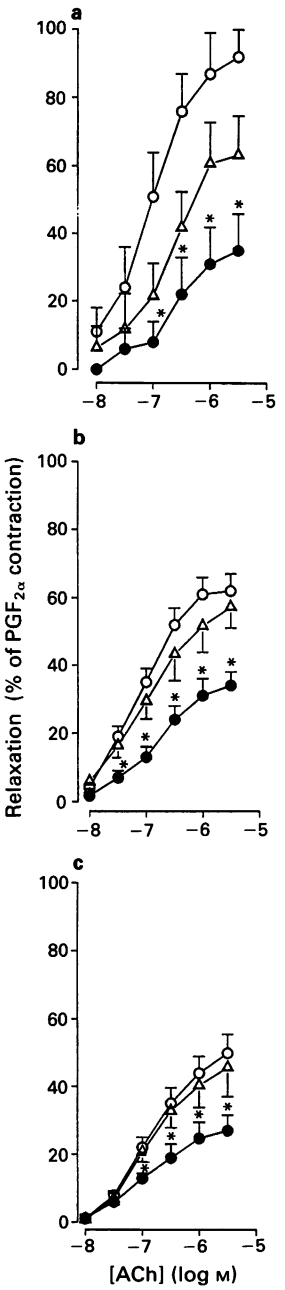


Figure 5 Effects of L-N^G-monomethyl arginine (L-NMMA, 30 μM) and nordihydroguaiaretic acid (NDGA, 50 μM) on acetylcholine (ACh)-induced relaxation in the endothelium intact prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-precontracted pulmonary artery rings from 3–10 day (a), 3–8 week (b) and adult (c) pigs. Relaxations were expressed as percentage of EC_{50} concentration of $PGF_{2\alpha}$-induced contractions shown in Table 1. (○) Control rings; (●) in the presence of L-NMMA and (△) in the presence of nordihydroguaiaretic acid. $*P < 0.05$, compared with control rings; means ± s.e.mean (bars) of 5–8 animals.

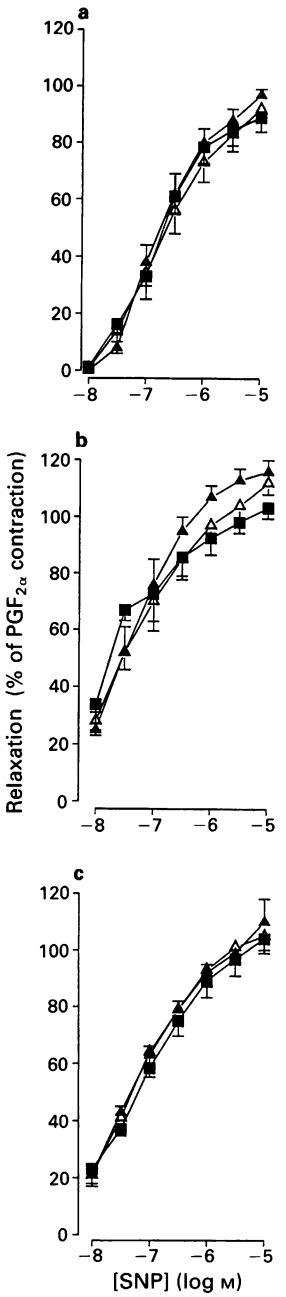


Figure 6 Effects of N^G-monomethyl-L-arginine (L-NMMA, 30 μM) and nordihydroguaiaretic acid (NDGA, 50 μM) on sodium nitroprusside (SNP)-induced relaxation in the endothelium intact prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-precontracted pulmonary artery rings from 3–10 day (a), 3–8 week (b) and adult (c). Relaxations were expressed as percentage of EC_{50} concentration of $PGF_{2\alpha}$-elicited contractions shown in Table 2. (Δ) Control rings; (▲) in the presence of L-NMMA and (■) in the presence of NDGA; means ± s.e.mean (bars) of 5–6 animals.

reduced the relaxation induced by ACh (Figure 5a–c). The inhibition of ACh induced relaxation by L-NMMA was greater at 3–10 days than at 3–8 weeks and adults. L-NMMA had no effect on SNP-induced relaxations (Figure 6a-c). NDGA had no detectable effect on the baseline tension and did not affect either ACh (Figure 5a-c) or SNP (Figure 6a-c)-induced relaxations in the endothelium-intact vessel rings of any group.

Discussion

Our results demonstrated a two phase change in the endothelium-dependent response to ACh in the pig pulmonary arteries. The response to ACh is negligible at birth, but develops rapidly after birth with a maximal effect at 3–10 days, and then declines gradually towards adulthood. This confirms findings of other investigators who showed absent, or greatly reduced, endothelium-dependent dilator response to ACh in foetal pulmonary arteries of rabbit and sheep (Zubrow *et al.*, 1989; Abman *et al.*, 1991). The vasodilator response is present by 1–3 days in isolated pulmonary arteries of guinea-pig (Davidson & Eldermerdash, 1990) and pig (Zellers & Vanhoutte, 1991). Our results extend the initial observation that showed a maturational change in the endothelium-dependent response to ACh in the perinatal pulmonary arteries (Abman *et al.*, 1991; Zellers & Vanhoutte, 1991).

Dilatation of small pulmonary arteries and reduction in pulmonary artery pressure occur minutes after birth, with continuing decrease in pulmonary artery pressure and resistance for the first 24 h in the pig (Haworth & Hislop, 1981). In normal full-term lambs, pulmonary arterial blood pressure falls to near adult levels within 2–4 h (Heymann & Soifer, 1989). After the initial rapid fall in pulmonary vascular resistance and pulmonary arterial blood pressure, there is a slow progressive fall, reaching adult levels within 2–6 weeks after birth (Heymann & Soifer, 1989; Haworth & Hislop, 1981). A previous study (Zellers & Vanhoutte, 1991) showed a greater dilator response to ACh and bradykinin in 10 day pig pulmonary arteries compared with pulmonary artery rings from 3 day old pigs, indicating that endothelium-dependent relaxation mediated by ACh and bradykinin may play a role in the slow reduction of pulmonary artery pressure. The time course for the change in the endothelium-dependent response to ACh in the early stages of adaptation remains unclear. We therefore compared the endothelium-dependent relaxant response to ACh in the pulmonary arteries from pigs aged 5 min to 2 h, 3–10 days, 3–8 weeks and adults. The virtual absence of an endothelium-dependent relaxant response to ACh in the pig pulmonary artery rings 5 min to 2 h after birth indicates that the endothelium-dependent response develops after birth. Thus endothelium-dependent relaxation does not appear to play a part in the initial pulmonary vasodilatation which occurs at birth in the pig. However, the greater response to ACh at 3–10 days of age suggests that EDRF may play an important role in the transitional phase of adapation to extra-uterine life.

There appears to be a difference in the EDRF activity *in vitro* and *in vivo* in the foetal and neonatal pulmonary vessels. ACh dilates late-gestation foetal sheep pulmonary arterioles *in vivo*. Infusion of nitro-L-arginine, an inhibitor of NO synthesis, inhibits both basal and stimulated EDRF activities, and attenuates the rise in pulmonary blood flow at delivery (Abman *et al.*, 1990). On the other hand, the same authors have reported a greatly reduced basal and stimulated EDRF activity in foetal PA rings compared with newborn and adult in the same specie *in vitro* (Abman *et al.*, 1991). EDRF production is absent in the foetal rabbit PA rings (Zubrow *et al.*, 1989). Conditions *in vivo* and *in vitro* differ significantly. Other factors, such as species-dependent variation and difference in the responses to these vasodilators between large conductive and small resistance vessels may also contribute to this discrepancy. Nevertheless, all the *in vitro* results indicate that EDRF activity in the foetal and neonatal pulmonary arteries is less than in pulmonary arteries of other age groups. The relevance of these *in vitro* results to *in vivo* control of pulmonary vascular tone needs to be further investigated.

EDRF has been characterized as NO or a related substance (Palmer *et al.*, 1987; 1988; Moncada *et al.*, 1989; Ignarro, 1989). L-NMMA is a selective inhibitor of NO synthesis (Moncada *et al.*, 1989; Johns *et al.*, 1990). Our results showed that L-NMMA treatment markedly reduced the relaxant response to ACh, which strongly suggests that the ACh-induced vasorelaxant response in the immature pulmonary arteries (in animals over 3 days) is mediated predominantly by endothelium-derived NO, as in the adult

pulmonary arteries. The much greater relaxation in 3–10 day pulmonary arteries than in the 3–8 week and adult pulmonary arteries in response to ACh indicates that either more EDRF is released from pulmonary vascular endothelium or that pulmonary vascular smooth muscle cells of 3–10 day animals are more sensitive to EDRF than those in 3–8 week and adult vessels. The latter possibility is unlikely, since the vasodilator response to SNP which relaxes pulmonary vessels via the same intracellular mechanism as NO, is less in the younger animals. Moreover, in the presence of L-NMMA the response to ACh was similar at all ages, indicating that L-NMMA had a greater inhibitory effect on 3–10 day animals. This result suggests that more NO is produced in young animals. Another possibility is that 3–10 day pulmonary arteries have more endothelial muscarinic receptors or alternatively these receptors have a higher affinity for ACh. The latter possibility is unlikely, since the maturation-dependent change in the response to ACh is very similar to the response to bradykinin and A23187, a non-receptor-mediated, endothelium-dependent vasodilator, in pulmonary arteries from pigs aged 3 to 30 days (Zellers & Vanhoutte, 1991). Moreover, we found that neonatal PA rings had little relaxant response to both ACh and bradykinin. It is unlikely that the endothelium-smooth muscle barrier is more accessible for NO in 3–10 days PA rings than that of other age groups, since our transmission electronmicrographs showed no morphological difference in the endothelium layer among these four groups of PA rings. Zellers & Vanhoutte's (1991) report of an increase in the response to ACh and BK up to 10 days is in agreement with our results, which showed that the ACh-induced relaxation peaked at 3–10 days.

The lack of a significant vasodilator response to ACh in neonatal pulmonary arteries cannot be explained by a defect in vascular smooth muscle relaxation, because the relaxant response to SNP was present in these vessels. Other investigators have found that the response to SNP was similar in foetal and adult pulmonary arteries, although the relaxant response to ACh was absent or greatly reduced in the foetal vessels (Zubrow *et al.*, 1989; Abman *et al.*, 1991). Several possibilities, such as lack of endothelial muscarinic receptor or receptor coupling mechanism, or an inability of endothelial cells to synthesize EDRF could explain the failure to respond to ACh in the neonatal pulmonary artery. Other factors such as the availability of L-arginine and a defect of signal transduction may also contribute to the lack of response to ACh in the neonatal pulmonary artery. All these possibilities remain to be explored. The fact that L-NMMA had no effect on the baseline tension in the neonatal pulmonary arteries suggests a lack of EDRF activity in these vessels. Abman *et al.* (1991) have also shown that foetal sheep pulmonary artery rings were virtually unresponsive to increasing concentrations of haemoglobin, whereas haemaglobin induced a concentration-related increase in the vascular tensions in 1–4 week and adult pulmonary arteries.

In the present study the possible effect of the cyclo-oxygenase products on the endothelium-dependent relaxation to ACh was prevented by use of indomethacin. Although ACh-induced EDRF is not a cyclo-oxygenase product, ACh can stimulate prostacyclin (PGI_2) release from vascular endothelial cells (Fedderson *et al.*, 1986). PGI_2 is involved in the adjustment of the pulmonary circulation at birth (Leffler & Hessler, 1981; Davidson, 1988). With respect to the lipoxygenase products, the failure of NDGA to inhibit ACh-induced relaxation in the precontracted endoethelium intact vessel rings indicates that lipoxygenase products were not involved in mediating the ACh relaxation in the immature pulmonary arteries. NDGA has been shown to inhibit ACh- or methacholine-induced relaxation in the rabbit aorta (Singer & Peach, 1983; Forstermann & Neufang, 1984; Forstermann *et al.*, 1986). These differences could be explained by region and/or species-dependent variation.

The present functional studies have shown that both the $PGF_{2\alpha}$-generated tension and the vasorelaxant response to SNP increased progressively with age. These results are in agreement with the earlier report showing a maturational change in the vasorelaxant response to SNP in guinea-pig aorta (Balaraman *et al.*, 1990), but inconsistent with others who reported that the vasorelaxant response to SNP is similar within these age ranges in sheep and pig (Abman *et al.*, 1991; Zellers & Vanhoutte, 1991). Our findings are compatible with our previous morphological studies showing that the number of contractile myofilaments in pig pulmonary artery smooth muscle increased with age during the developmental period (Hall & Haworth, 1987). A similar increase in myofilaments has also been described in man (Allen & Haworth, 1988). Developmental changes have also been found in pulmonary arterial innervation (Wharton *et al.*, 1988) and in the responses to other vasoactive agents such as histamine, noradrenaline and 5-hydroxytryptamine (Newman *et al.*, 1979; Dunn *et al.*, 1989). All these observations suggest that the response to many therapeutic agents may be different in neonates from that seen in adult life.

In summary, our results show a two phase change in the endothelium-dependent response to ACh in the immature pig pulmonary artery, being absent at birth, reaching a maximum at 3–10 days and then declining with age. The vasorelaxant response to SNP increases progressively with age. These results suggest that the NO pathway may not play a role in the earlier pulmonary vasodilatation which occurs at birth, but be particularly important in the transitional pulmonary adaptation process to establish a stable pulmonary circulation after birth.

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Pituitary adenylate cyclase activating polypeptide is a potent vasodilator and oedema potentiator in rabbit skin *in vivo*

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1 The effects of pituitary adenylate cyclase activating polypeptide (PACAP) on microvascular blood flow and plasma protein leakage were investigated in rabbit skin *in vivo*.

2 Intradermal injection of PACAP38, the 38 amino acid form of the peptide, caused a dose-dependent increase in blood flow measured by a ^{133}Xe clearance technique. An equivalent increase in blood flow was induced by 10^{-12} mol per site of PACAP38, 10^{-12} mol per site of human α-calcitonin gene-related peptide (CGRP) and 10^{-10} mol per site of vasoactive intestinal polypeptide (VIP).

3 The vasodilator activity of PACAP38 was not significantly different from that of the 27 amino acid form of the peptide, PACAP27, when measured with a laser Doppler flow meter, causing a 104 ± 14% compared with 110 ± 18% increase above basal blood flow at 10^{-12} mol per site respectively.

4 At 10^{-12} mol per site the effect of PACAP38 was longer lasting than that of CGRP. Blood flow remained significantly increased above control at 2 h with PACAP38 ($P<0.05$) whereas blood flow after intradermal CGRP had returned to control values by this time.

5 PACAP38 injected alone had no significant effect on microvascular leakage of ^{125}I-labelled albumin. However, PACAP38 significantly potentiated bradykinin-induced oedema where it was approximately 100 fold more potent than VIP.

6 Oedema potentiation induced by PACAP38 was not inhibited by indomethacin at a dose which did inhibit potentiation of bradykinin-induced oedema by arachidonic acid.

7 PACAP38 is at least as potent as other peptides which have been postulated to be involved in the inflammatory response when tested in rabbit skin *in vivo*. PACAP may contribute to both the hyperaemia and oedema components of inflammation.

Keywords: Pituitary adenylate cyclase activating polypeptide; vasodilatation; artery; vasoactive intestinal polypeptide; calcitonin gene-related peptide; adenylate cyclase

Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) was first isolated from ovine hypothalami. Its name derives from the observation that it is a powerful stimulant of adenylate cyclase in anterior pituitary cells in culture, being over 1000 times more potent than vasoactive intestinal polypeptide (VIP) in this system (Miyata *et al.*, 1989). The amino acid sequence of rat, ovine and human PACAP are identical (Ogi *et al.*, 1990).

A 38 amino acid and a 27 amino acid form of PACAP have been described (Miyata *et al.*, 1990). Both cause long lasting, endothelium-independent, relaxation of pre-contracted aortic rings and are more potent than VIP in stimulating adenylate cyclase in isolated vascular smooth muscle cells (Warren *et al.*, 1991). When injected intravenously in rats, both forms cause short lasting hypotension (Miyata *et al.*, 1990; Nandha *et al.*, 1991). The vasoactivity of the peptide appears to be incorporated within the first 27 amino acid sequence which is common to both PACAP38 and PACAP-27.

PACAP mRNA is expressed in several mammalian tissues (Kimura *et al.*, 1990; Koves *et al.*, 1990) indicating that its distribution is extensive. The cDNA's encoding the precursor of PACAP from the human testis and ovine hypothalamus are identical (Kimura *et al.*, 1990). Receptor binding studies show high levels of binding in rat lung and brain with low levels in the aorta (Lam *et al.*, 1990).

The N-terminal sequence of PACAP27 shows 68% homology with VIP (Miyata *et al.*, 1990) and there is evidence that both peptides can bind to the same receptor in the periphery (Gottschall *et al.*, 1990; Nandha *et al.*, 1991).

Specific receptors for PACAP exist in bovine and rat brain (Gottschall *et al.*, 1990; Lam *et al.*, 1990; Ohtaki *et al.*, 1990), a pancreatic cell line (Buscail *et al.*, 1990) and astrocytes (Tatsuno *et al.*, 1990). In human tissue, specific sites have also been demonstrated in the hypothalamus, brain stem, cerebellum, cortex and basal ganglia (Suda *et al.*, 1991) suggesting that it is likely to be a central neurotransmitter. Although PACAP binding to the sites mentioned above is not displaced by VIP, these two peptides probably share the same receptor in some blood vessels (Nandha *et al.*, 1991) and lung tissues (Gottschall *et al.*, 1990).

The clearance of ^{133}Xe from rabbit skin has been used to study a number of peptides with potential vasodilator activity in the microcirculation *in vivo*. With this method the most potent vasodilator peptides tested to date have been calcitonin gene-related peptide (CGRP, Brain *et al.*, 1985; Brain & Williams, 1989) and VIP (Williams, 1982). In this paper the vasodilator activity of PACAP in rabbit skin has been compared with VIP and CGRP. The ability of PACAP to potentiate bradykinin-induced oedema formation has also been tested by measuring the intradermal leakage of intravenously injected ^{125}I-labelled albumin.

Methods

All experiments were carried out in a room where the temperature was thermostatically controlled at 24–25°C. Intradermal injections were given into the clipped dorsal skin of anaesthetized (sodium pentobarbitone, 30 mg kg^{-1}, i.v.) male New Zealand White rabbits (3–4.5 kg).

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Blood flow experiments

Blood flow was measured as the clearance of intradermally injected ^{133}Xe as previously described (Williams, 1979). VIP, CGRP or PACAP38 were added to ^{133}Xe in solution (activity 5–10 μCi per 100 μl) and injected intradermally in 100 μl volumes. In all experiments test agents were dissolved in phosphate buffered saline containing 0.3% albumin (PBS-albumin). Injections were given rapidly in a randomized block order according to a balanced site pattern. Six replicates were given per dose. Each animal was killed 15 min after the intradermal injections by an overdose of pentobarbitone. The dorsal skin was removed and the injection sites excised with a 17 mm diameter punch. Skin samples were placed under paraffin oil in sealed tubes and counted, together with samples of injection fluids, in an automatic gamma counter. The clearance per site was calculated as a percentage of the clearance from sites where PBS-albumin had been injected as control, as previously described (Williams, 1979).

The vasodilator effect of PACAP27 and PACAP38 was compared by measuring local blood flow in the dorsal skin with a Perimed II laser-Doppler flow meter (Brain *et al.*, 1986; Brain & Williams, 1989) connected to a computer recorder (Maclab, Apple Computer Inc, Cupertino, California). After clipping, the skin was depilated with a commercial depilatory cream (Immac) and the rabbit left for 1 h before starting the experiment. Each peptide was injected in a balanced site pattern at 10^{-14}, 10^{-13}, 10^{-12} and 10^{-11} mol per site and readings taken 30 min later. This was repeated 4 times in each of 5 rabbits. The laser-Doppler flow meter was used to compare the two peptides to confirm the ^{133}Xe clearance technique findings obtained with PACAP38. The laser-Doppler flow probe has the limitation of measuring red cell flux at only one depth but has the advantage that multiple measurements can be made at the same site allowing the determination of the duration of action.

To determine the duration of action, the laser-Doppler flow meter was used to compare PACAP38 and CGRP injected at 10^{-12} mol per site. The rabbit was prepared as above and the skin marked out with four sites per test agent in a balanced site pattern. Blood flow at each site was measured and then peptide or control (100 μl PBS-albumin) were injected at 1.5 min intervals. Measurements were repeated at 15 min intervals for 1 h and again at 2 h after injection. Each measurement was taken as the mean of three readings of 15 s each with 10 s between readings. The laser probe was held at right angles to the skin by a plastic guide. Results are presented as the percentage change in red cell flux above the pre-injection value for each site.

Oedema formation experiments

Local oedema formation was measured as the intradermal accumulation of intravenously injected ^{125}I-human serum albumin (5 μCi kg^{-1}) as previously described (Brain & Williams, 1989). Test agents were dissolved in 100 μl of PBS-albumin and injected intradermally in a balanced site pattern, 6 site replicates per rabbit. After 30 min, the animal was killed with an overdose of pentobarbitone and a 5 ml blood sample taken by cardiac puncture into heparin (10 uml^{-1} final concentration). The dorsal skin was removed and the injection sites punched out and counted as above. Oedema formation was expressed by dividing each skin ^{125}I count by the radioactivity in 1 μl of plasma at death.

Statistical analysis

Statistical analysis was performed by ANOVA and results termed significant if $P<0.05$.

Drugs and chemicals

PACAP27 and PACAP38 were obtained from Penninsula (Merseyside, UK). ^{133}Xe and ^{125}I-human serum albumin were from Amersham International, pentobarbitone sodium from May & Baker. Human αCGRP was from Bachem Feinchemikalien AG, (Bubendorf, Switzerland). All other drugs and chemicals were from the Sigma Chemical Co., (Poole, Dorset).

Results

PACAP38 caused a dose-related increase in skin blood flow as measured by ^{133}Xe clearance (Figure 1). The potency of the peptide was not significantly different from that of CGRP in this model but both peptides were approximately 100 fold more potent than VIP at both 10^{-12} and 10^{-11} mol per site ($P<0.05$ in each case). PACAP38 induced a significant response at a dose as low as 10^{-14} mol per site ($P<0.05$ compared with control).

The multiple site ^{133}Xe clearance technique enables the comparison of the quantitative effects of vasoactive substances on the microcirculation at the site of injection in the dermis. The laser-Doppler flow probe measures red cell flux in the superficial skin tissue at a depth of approximately 1 mm. The dose-response curve to PACAP38 measured by the laser-Doppler technique is shown in Figure 2. A significant change was induced by 10^{-13} mol per site by this method ($P<0.05$ compared with control). PACAP27 had similar effects on red cell flux and the dose-response to this peptide was not significantly different to that of PACAP38 (Figure 2).

With the laser-Doppler system, serial measurements can be made at the same site and thus the time course of action of vasoactive agents compared. Figure 3 shows a comparison of the effects of PACAP38 and CGRP given at the same dose (10^{-12} mol per site). Both peptides increased red cell flux, which was maximal at approximately 30 min. However, responses to PACAP38 were of longer duration; at 120 min PACAP38 still caused increased red cell flux whereas the response to CGRP had returned to baseline by this time. The

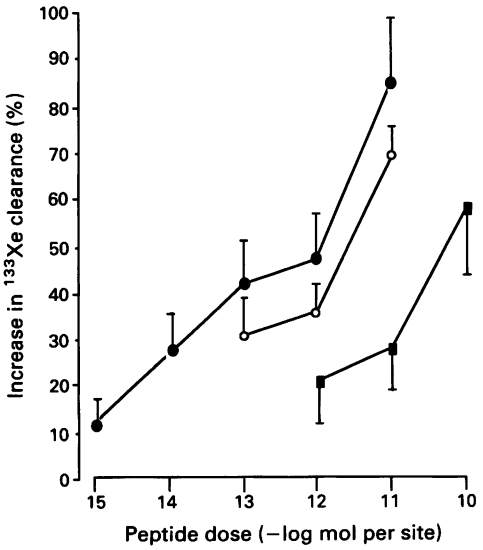


Figure 1 Effect of intradermal injections of the 38 amino acid form of pituitary adenylate cyclase activating polypeptide (PACAP38, ●), calcitonin gene-related peptide (CGRP, ○) and vasoactive intestinal polypeptide (VIP, ■) on rabbit skin blood flow measured by ^{133}Xe clearance. The dose-response to PACAP38 was performed 6 times in each rabbit and the data are the mean (± s.e.mean shown by vertical bars) of 5 rabbits. In a further 5 rabbits, 2 doses of PACAP38, 3 doses of CGRP and 3 doses of VIP were given to compare potency, each dose being repeated 5 times per rabbit. At 10^{-12} and 10^{-11} mol per site VIP was significantly less potent than the comparable dose of PACAP38 ($P<0.05$).

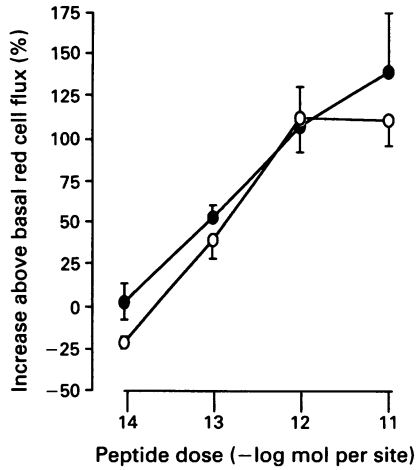


Figure 2 A comparison of the vasodilator effects of the 27 and 38 amino acid forms of pituitary adenylate cyclase activating polypeptide (PACAP). The peptides were injected in 100 µl volumes and measurements taken before and 30 min after injection. Blood flow was measured by laser Doppler flow meter and results are the mean ± s.e.mean of 5 rabbits, each experiment was repeated 4 times in each rabbit; PACAP38, (●); PACAP27 (○).

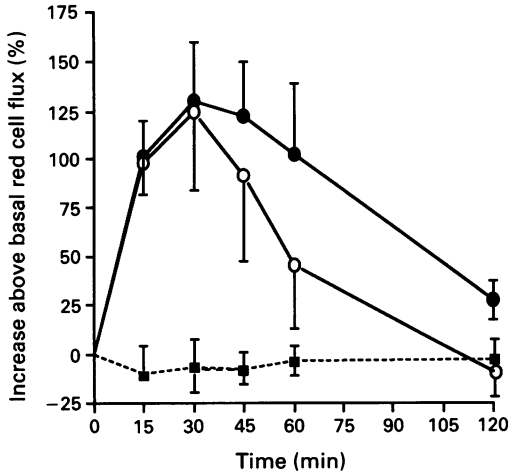


Figure 3 A comparison of the duration of action of pituitary adenylate cyclase activating polypeptide, 38 amino acid form (PACAP38, 10^{-12} mol per site) and calcitonin gene-related peptide (CGRP, 10^{-12} mol per site) in causing vasodilatation in rabbit skin. Peptides were injected intradermally in 100 µl volumes. Blood flow was measured by laser-Doppler flow meter which allows serial measurements at the same site. Results are the mean (s.e.mean shown by vertical bars) for 5 rabbits, each experiment was repeated 4 times in each rabbit. PACAP38: (●); CGRP (○); control (PBS + 0.3% albumin) (■). PACAP38 was significantly longer lasting than CGRP if the results from 45–120 min for each peptide were pooled, or if the slopes of the results from 30–120 min were compared.

duration of action of PACAP38 was significantly longer than CGRP ($P<0.05$) if the results were analyzed by testing for a significant difference between the slopes of the data, approximated to a straight line, from 30 to 120 min, or by comparing the pooled CGRP data from 45 to 120 min with that of the PACAP38 data over the same time course.

Figure 4 shows the potentiation by PACAP38 of bradykinin-induced microvascular plasma protein leakage in the skin. Bradykinin at 10^{-10} mol per site induced a response in this test that was approximately four fold greater than the PBS-albumin control value. PACAP38 alone induced little plasma protein leakage; skin sample plasma volumes were not significantly different from the PBS-albumin controls. However, PACAP38 did cause a marked dose-related potentiation of plasma protein leakage when injected in combination with bradykinin.

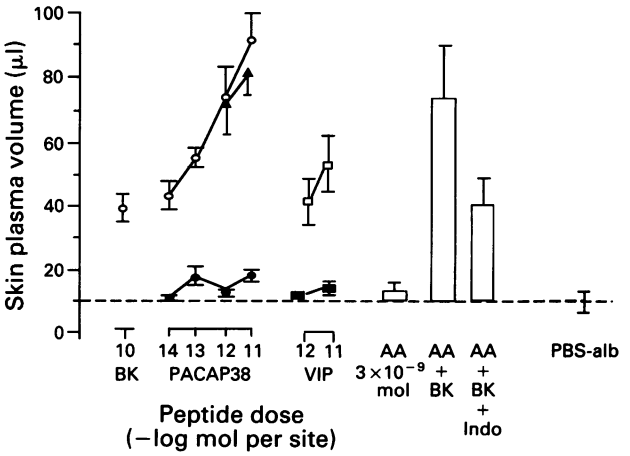


Figure 4 Potentiation of bradykinin-induced oedema formation in rabbit skin by pituitary adenylate cyclase activating polypeptide, 38 amino acid form (PACAP38). Local oedema formation was measured as the intradermal accumulation of intravenously injected ^{125}I-albumin. Test agents were dissolved in 100 µl of PBS-albumin and injected intradermally in a balanced site pattern. Each experiment was repeated in 6 sites per rabbit, each datum point is the mean of 6–10 rabbits. Bradykinin alone (BK, 10^{-10} mol per site) increased oedema formation ($P<0.05$ compared with control). This was potentiated by the co-injection of PACAP (○), ($P<0.05$ for 10^{-12} and 10^{-11} mol per site compared with bradykinin alone) in a dose-dependent manner whereas PACAP alone had no significant effect (●). The potentiation of bradykinin by PACAP at two doses was not significantly altered by the presence of indomethacin (3×10^{-9} mol/site, ▲). Intradermal injection of vasoactive intestinal polypeptide (VIP, ■) did not cause oedema formation whereas it potentiated bradykinin-induced oedema (□, $P<0.05$), where 10^{-11} mol per site had an equivalent effect to PACAP 10^{-13} mol per site. Arachidonic acid (3×10^{-9} mol per site, AA) had no significant effect on oedema formation but did significantly potentiate the effect of bradykinn (AA + BK, $P<0.05$, $n=7$ rabbits), an effect that was significantly inhibited by the presence of indomethacin (AA + BK + Indo, $P<0.05$, $n=7$ rabbits).

The potentiation of responses to bradykinin by PACAP38 was not affected significantly by indomethacin, suggesting cyclo-oxygenase was not involved in the response. In contrast, potentiation of responses to bradykinin by arachidonic acid were reduced by indomethacin to the level of the response to bradykinin alone ($P<0.05$, Figure 4).

VIP also potentiated plasma protein leakage induced by bradykinin ($P<0.05$ at each dose) but was 100 fold less potent than PACAP38 (Figure 4), the same potency difference as in the blood flow model (Figure 1).

Discussion

PACAP is a vasodilator neuropeptide with an amino acid sequence which shows similarities to that of VIP. The present experiments show PACAP to be approximately 100 fold more potent than VIP as a vasodilator in rabbit skin *in vivo* and of similar potency to CGRP. The 27 amino acid and the 38 amino acid forms of PACAP had similar activity. The observed effects of PACAP are in keeping with its ability to relax pre-contracted isolated rabbit aortic rings where it is also approximately 100 fold more potent than VIP (Warren *et al.*, 1991).

Although of similar potency to CGRP, the effect of PACAP38 was significantly longer lasting; vasodilatation was still measurable 2 h after the intradermal injection of 1 picomol. This long duration of action is in keeping with its prolonged effect in isolated aortic rings (Warren *et al.*, 1991).

However, when injected intravenously in whole animals, it is short acting. The hypotensive response to a bolus injection of PACAP in the rat lasts about 5 min (Nandha *et al.*, 1991), possibly because of clearance by organs such as the liver or lungs.

Although PACAP binding sites in the central nervous system are specific and cannot be displaced by VIP, it has been proposed that these two peptides share the same receptor in large blood vessels (Nandha *et al.*, 1991). Both are approximately equipotent at causing hypotension in the rat although PACAP was 100 fold more potent both in the present experiment and in isolated aortic rings (Warren *et al.*, 1991).

It is clear that PACAP is a potent activator of adenylate cyclase in both aortic smooth muscle and cultured neural cells, yet its intracellular vasodilator mechanism has not been confirmed. If PACAP relaxes vascular smooth muscle via adenylate cyclase, this is probably by triggering the sequestration of calcium ions into the sarcoplasmic reticulum; the subsequent lowering of intracellular calcium concentration reducing the formation of the calcium-calmodulin complex hence reducing actin-myosin interaction (MacDermot, 1990). An alternative explanation, that adenosine 3':5'-cyclic monophosphate (cyclic-AMP)-dependent phosphorylation of myosin light chain kinase causes relaxation, may not occur *in vivo* (Kamm & Stull, 1985). Interestingly, rabbit isolated aortic rings do not relax with iloprost, an adenylate cyclase-dependent vasodilator, whereas they do respond to PACAP (Gryglewski *et al.*, 1989; Warren *et al.*, 1991) suggesting that PACAP could act by a mechanism independent of adenylate cyclase.

Several vasodilators, such as CGRP, VIP and prostaglandins have been shown to potentiate oedema formation induced by bradykinin and other mediators of vascular permeability. In the present experiments PACAP potentiated bradykinin-induced oedema formation in a dose-dependent fashion, although it did not induce oedema formation alone. This effect was independent of cyclo-oxygenase as it was not inhibited by indomethacin. PACAP was again approximately 100 fold more potent than VIP in this system.

PACAP is a potent vasodilator in the rabbit skin. Binding sites are present at low levels in rat aorta (Lam *et al.*, 1990) and PACAP is found in close association with some blood vessels in the brain (Koves *et al.*, 1990). It is not known if PACAP is found in association with peripheral blood vessels *in vivo* and it remains to be ascertained whether sufficient endogenous peptide is present to affect vascular tone. Specific antagonists will be needed to determine if PACAP contributes to blood vessel tone *in vivo*. Our results suggest that picomolar amounts may have significant effects on both the hyperaemia and oedema response of inflammation.

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Selective and full β_1-adrenoceptor agonist action of a catechol derivative of denopamine (T-0509) in the guinea-pig cardiac muscle and trachea: comparison with denopamine, xamoterol and isoprenaline

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1 The pharmacological actions of T-0509, a 3-hydroxy derivative of denopamine, were studied in various guinea-pig tissues; these effects were compared with those of isoprenaline, denopamine and xamoterol.

2 The intrinsic activities of the positive inotropic actions of T-0509, denopamine and xamoterol compared with isoprenaline (= 100%) in the papillary muscle were 99%, 83% and 28%, respectively, while their relative potencies (EC_{50} agonist EC_{50} isoprenaline) were 0.23, 33 and 1.4, respectively. The intrinsic activities of T-0509, denopamine and xamoterol as positive chronotropic agents in the right atria were 98%, 69% and 48%, respectively, and their equipotent concentrations (isoprenaline = 1) were 0.24, 50 and 4, respectively.

3 The positive chronotropic actions of T-0509 and denopamine were antagonized by bisoprolol (3×10^{-8} M), but not by ICI 118,551 (3×10^{-8} M).

4 The intrinsic activity of T-0509 in histamine-contracted tracheae was similar to that of isoprenaline, but its equipotent concentration was 38; the effects of both agents were antagonized by ICI 118,551 (3×10^{-8} M), but not by bisoprolol (3×10^{-8} M). Denopamine and xamoterol did not show any agonist activity on guinea-pig trachea.

5 Denopamine and xamoterol antagonized the positive chronotropic (pA_2, denopamine: 6.98, xamoterol: 7.75) and tracheal relaxant (pA_2, denopamine: 5.39, xamoterol: 6.25) effects of isoprenaline.

6 Isoprenaline, T-0509 and denopamine, but not xamoterol, contracted the guinea-pig aorta in a decreasing order in the presence of propranolol (10^{-6} M).

7 Based on the above studies, T-0509 appears to be a highly selective β_1-adrenoceptor agonist with full agonist properties, while denopamine and xamoterol appear to be selective, but partial β_1-adrenoceptor agonists.

Keywords: T-0509; denopamine derivative; selective β_1-adrenoceptor with full agonist action; cardiac muscle; trachea; aorta

Introduction

In mammalian hearts, β-adrenoceptors are predominantly of the β_1-subtype (Stiles *et al.*, 1984). β_1-Adrenoceptor stimulation is known to cause positive inotropic action through activation of adenylate cyclase (Lemoine *et al.*, 1989a,c). The orally-active cardiotonic agents, denopamine and xamoterol, are highly selective β_1-adrenoceptor agonists with partial agonist properties (Nagao *et al.*, 1984; Ikeo *et al.*, 1985; Narita *et al.*, 1986; Yokoyama *et al.*, 1988; Lemoine *et al.*, 1989a; Gurden *et al.*, 1989) and have been introduced recently for the treatment of patients with congestive heart failure (Kino *et al.*, 1983; Ikeda *et al.*, 1987; Furlong & Brogden, 1988). Denopamine causes positive inotropic effects with small increases in heart rate and in myocardial oxygen consumption in both dogs (Ikeo & Nagao, 1985) and man (Thormann *et al.*, 1985).

Partial β_1-adrenoceptor agonists such as denopamine, xamoterol, prenalterol, ICI 89,963 and ICI 119,033 have marked positive inotropic action and only limited stimulatory action on adenylate cyclase (Franklin & Twose, 1979; Hedberg & Mattson, 1981; Main, 1982; Bing *et al.*, 1984; Inamasu *et al.*, 1987; Lemoine *et al.*, 1989a). These agonists have been thought to be less prone to cause arrhythmogenic side-effects and β-adrenoceptor desensitization than full agonists (Pouleur *et al.*, 1984; Chemnitius & Bing, 1985; Yabana *et al.*, 1986; Benovic *et al.*, 1988; Narita *et al.*, 1988). However, it is not known whether either β_1/β_2-adenoceptor selectivity or partial agonist activity is responsible for the favourable properties of partial β_1-adrenoceptor agonists in the heart. Therefore, the advent of a highly selective β_1-adenoceptor agonist having full agonist activity aids in the understanding of the favourable properties of partial β_1-adrenoceptor agonists.

T-0509 is a catechol derivative of denopamine (Figure 1), and has been reported to produce full β_1-adrenoceptor agonist activity with a small increase in adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels in the dog isolated ventricular muscle (Kurosawa *et al.*, 1990). However, details of β_1/β_2-adrenoceptor selectivity and α-adrenoceptor agonist activity of T-0509 have not been reported.

In the present paper, we have studied the pharmacological properties of T-0509 by using guinea-pig cardiac muscle, trachea and aorta as systems with functions of β_1-, β_2- and α-adrenoceptors (Buckner & Patil, 1971; Patil *et al.*, 1971; 1972; Malta *et al.*, 1985; Hattori *et al.*, 1987), respectively. We have compared T-0509 with a β-adrenoceptor full agonist, isoprenaline, and the partial agonists, denopamine and xamoterol. Furthermore, to evaluate β_1/β_2-adrenoceptor selectivity of T-0509, the effects of the highly selective β_1-

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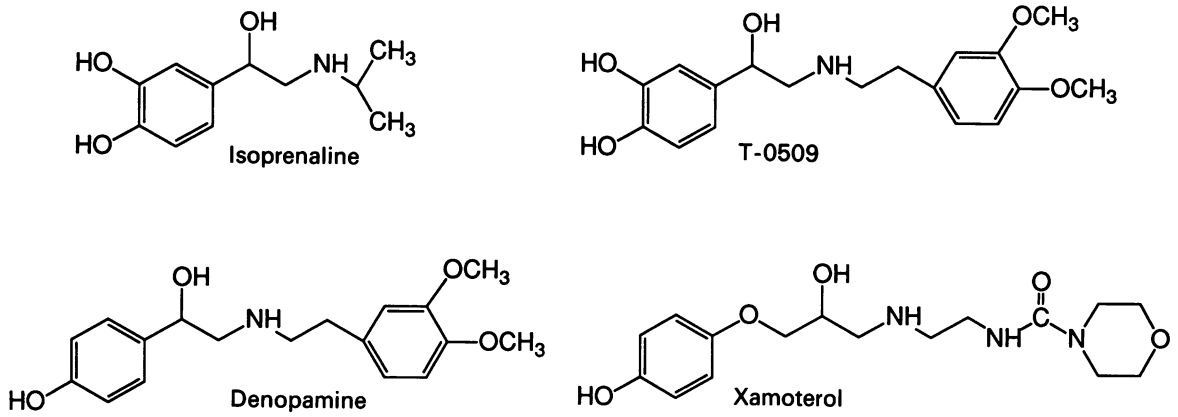


Figure 1 Chemical structures of denopamine, T-0509, xamoterol and isoprenaline.

adrenoceptor antagonist, bisoprolol (Schliep & Harting, 1984; Klockow *et al.*, 1986) and the highly selective β_2-adrenoceptor antagonsit, ICI 118,551 (O'Donnell & Wanstall, 1980; Bilski *et al.*, 1983), have also been examined. A preliminary account of this work has previously been presented to The Japanese Pharmacological Society (Yabana *et al.*, 1989).

Methods

Male guinea-pigs weighing 210–550 g (4–10 weeks old, Hartley strain, Shizuoka Laboratory Center (SLC), Shizuoka, Japan) were treated with reserpine (5 mg kg^{-1}, i.p.), 16 h before the experiments in order to deplete thenoradrenaline content of the cardiac muscle. Animals were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and killed by exsanguination from the carotid artery. The heart, trachea or descending aorta was rapidly removed and placed in Krebs-Henseleit solution (composition mM: Na^+ 143.3, K^+ 5.9, Ca^{2+} 2.6, Mg^{2+} 1.2, Cl^- 128.3, $H_2PO_4^-$ 2.2, HCO_3^- 24.9, SO_4^{2-} 1.2 glucose 5.6 and pH 7.4).

The left ventricular papillary muscle and the right atrium from the heart were suspended under a resting tension of 0.3–0.6 g in the Krebs-Henseleit solution at 30°C. The tracheal chain (5 mm in length, four preparations per trachea) and the descending aorta (ring segments of 5 mm in length) were suspended under a resting tension of 1.0–1.5 g in Krebs-Henseleit solution at 37°C. The Krebs-Henseleit solution was aerated with 95% O_2:5% CO_2. The isometric tension was measured with a strain gauge transducer (UL-10GR, Minebea, Tokyo) and recorded on a pen recorder (WR-3701, Graphtec, Tokyo). The left ventricular papillary muscle was driven by a field stimulation of 1.3 Hz frequency and 0.5 ms duration with 1.5 times greater voltage than the threshold. The spontaneous beating rate of the right atria was counted with a heart rate meter (AT-601G, Nihon Kohden, Tokyo). The preparations were equilibrated for 60 min before starting the experiments.

Tracheae were contracted with histamine (10^{-5} M), which produced submaximal contraction, and agonists were added on the plateau phase of the histamine contraction. In the cardiac muscle and trachea, a single cumulative concentration-response curve (CCRC) to isoprenaline, T-0509, denopamine or xamoterol was obtained with each preparation. Intrinsic activities of T-0509, denopamine and xamoterol in the cardiac muscle were obtained from the maximum response induced by addition of 10^{-4} M isoprenaline at the end of the experiment. Maximum relaxation of the trachea was obtained with papaverine (10^{-4} M) at the end of the experiment.

The CCRCs to isoprenaline or T-0509 in the cardiac muscle and trachea were also obtained in the presence of the extraneuronal uptake inhibitor, deoxycorticosterone acetate (DOCA, 2.5×10^{-5} M) (Henry & Goldie, 1990) in order to evaluate the influence of extraneuronal uptake on agonist potencies in two preparations.

When the β-adrenoceptor antagonistic actions of the drugs in the right atria and trachea were studied, the CCRC to isoprenaline was constructed on each preparation after a 20 min pretreatment period in the presence or absence of the drug (three different concentrations). Each preparation was exposed to only one concentration of the drug.

In other experiments, a single CCRC to T-0509 or denopamine in the right atria and trachea was constructed on each preparation in the presence or absence of the highly selective β_1-adrenoceptor antagonist, bisoprolol, after a 20 min pretreatment period. The influence of the highly selective β_2-adrenoceptor antagonist, ICI 118,551, on the positive chronotropic and relaxant actions of T-0509 or denopamine were also investigated.

In the guinea-pig aorta, 1 μM propranolol was applied in all experiments to inhibit the β-adrenoceptor agonist actions to enable α-adrenoceptor agonist actions to be evaluated. Initially, aortae in the presence of DOCA (2.5×10^{-5} M) were contracted by adding noradrenaline (10^{-5} M). Following a 30 min washout period, the CCRC to the agonist in the absence or presence of 10^{-6} M phentolamine was obtained. The responses are expressed as percentage of the noradrenaline (10^{-3} M)-induced maximum response obtained at the end of experiments.

Data analysis

Agonist potencies were expressed in terms of pD_2 values and intrinsic activities. pD_2 is defined as the negative logarithm of EC_{50}, where EC_{50} is the concentration of the agonist producing half maximum response determined from graphical plots of the CCRC of each preparation. The relative potency of the agonist was represented as EC_{50} ratio (EC_{50} for agonist/EC_{50} for isoprenaline). Intrinsic activities of agonists were represented as percentage of the maximum response to isoprenaline or papaverine.

Antagonist potencies were expressed in terms of pA_2 values. The pA_2 values were estimated from the regression lines obtained in the Schild plot analysis (Arunlakshana & Schild, 1959). If antagonism is competitive, a plot of the logarithm of (concentration ratio − 1) against the negative logarithm of the concentration of antagonist yields a straight line with a slope of unity. The point of intercept of this line with the abscissa scale is the pA_2 value. The concentration-ratio is the ratio of the EC_{50} for isoprenaline in the presence

and absence of the antagonist. It should be pointed out that in the right atria, in which denopamine increased basal rate, concentration-ratios were not calculated from equiactive isoprenaline concentrations (Stephenson, 1956), but rather from the half-maximal concentration of isoprenaline (Kenakin & Black, 1978).

Experimental values are given as a mean ± s.e.mean. Results were analysed by unpaired Student's t test or Scheffe's test after one way analysis of variance (ANOVA). Probability values less than 0.05 were considered to be significant.

Drugs

The following drugs were used in this study; denopamine hydrochloride [(−)-(**R**)-1-(p-hydroxyphenyl)-2-[(3,4-dimethoxyphenethyl) amino]ethanol]-hydrochloride (Tanabe Seiyaku, Osaka, Japan), T-0509 [(−)-(**R**)-1-(3,4-dihydroxyphenyl)-2-[(3,4-dimethoxyphenethyl) amino]ethanol] -hydrochloride (Tanabe Seiyaku, Osaka, Japan), (±)-isoprenaline hydrochloride (Nacalai tesque, Tokyo, Japan), xamoterol (synthesized at Organic Chemistry Research Laboratory of Tanabe Seiyaku), (−)-bisoprolol hemifumarate (E. Merck, Darmstadt, Germany), (±)-ICI 118,551 hydrochloride ([erythro-(±)-1- (7-methylindan-4-yloxy) -3-isopropylaminobutan-2ol] hydrochloride; synthesized at Organic Chemistry Research Laboratory of Tanabe Seiyaku), propranolol hydrochloride (Nacalai tesque, Tokyo, Japan), reserpine (Apoplon, Daiiti Seiyaku, Tokyo, Japan), deoxycorticosterone acetate (Nacalai tesque, Tokyo, Japan), noradrenaline (Nacalai tesque, Tokyo, Japan) and phentolamine mesylate (Regitin, Ciba-Geigy, Tokyo, Japan). All drugs except reserpine and deoxycorticosterone acetate were dissolved and diluted with Krebs-Henseleit solution containing 0.1 mg ml^{-1} ascorbic acid. Deoxycorticosterone acetate was dissolved in propylene glycol to prepare a 25 mM stock stolution. Other chemicals were purchased from commercial sources.

Results

β-Agonist actions in the isolated cardiac muscle

T-0509, denopamine, xamoterol and isoprenaline all exhibited concentration-dependent positive inotropic actions in the isolated ventricular papillary muscle, as shown in Figure 2a. The pD_2 values determined for these four agonists are summarized in Table 1. The maximum responses to T-0509 (99.1 ± 0.5%) did not differ significantly from those to isoprenaline (100%), although it was four times more potent than isoprenaline ($P < 0.01$, difference between pD_2s). The slopes of the CCRCs for denopamine and xamoterol were less steep than that for isoprenaline, and maximum responses to denopamine (82.8 ± 3.7%) and xamoterol (29.3 ± 5.6%) were significantly lower than that to

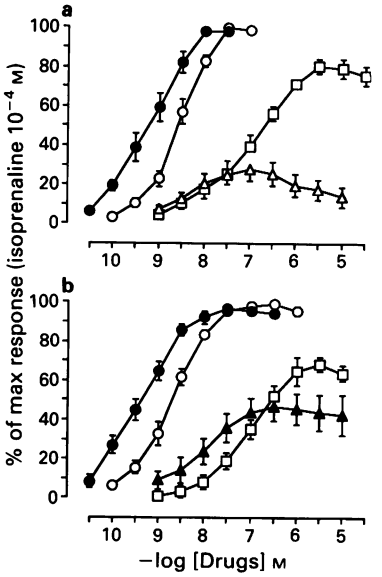


Figure 2 Concentration-response curves for the effects of denopamine, T-0509, xamoterol and isoprenaline on contractile force of the left ventricular papillary muscle (a) and spontaneous beating rate of the right atria (b) of the guinea-pig. Initial contractile force and spontaneous beating rate were 396 ± 66 mg and 150 ± 5 beats min^{-1}, respectively. The data are expressed as percentages of the maximum response to 10^{-4} M isoprenaline. (○) Isoprenaline ($n = 9$);(●) T-0509 ($n = 7$); (□) denopamine ($n = 10$); (△) xamoterol ($n = 10$). The points and vertical bars represent means ± s.e.mean.

Table 1 Potencies of denopamine, T-0509, isoprenaline and xamoterol in isolated tissue preparations of guinea-pigs

	n	pD_2 (− logEC_{50})	EC_{50} agonist/ EC_{50} isoprenaline	Intrinsic activity
Papillary muscle (force)				
Isoprenaline	9	8.59 ± 0.06	1	100
T-0509	7	9.22 ± 0.15*	0.23	
Denopamine	10	7.08 ± 0.19**	33	82.8 ± 3.7*
Xamoterol	10	8.43 ± 0.07	1.4	28.3 ± 5.6**
Right atria (rate)				
Isoprenaline	10	8.74 ± 0.08	1	100
T-0509	8	9.35 ± 0.11*	0.24	
Denopamine	13	7.07 ± 0.12**	50	68.7 ± 3.5*,[a]
Xamoterol	8	8.14 ± 0.24	4	48.3 ± 7.3**,[b]
Trachea (relaxation)				
Isoprenaline	5	9.01 ± 0.07	1	95.6 ± 0.5
T-0509	5	7.43 ± 0.06††	38	95.2 ± 0.2
Denopamine	9	NC		NC
Xamoterol	6	NC		NC

pD_2 values and intrinsic activities (% of the maximum response to isoprenaline (cardiac muscles) or papaverine (trachea) in the papillary muscle, right atria and trachea were calculated from the experiments of Figures 2a, b and 3, respectively. The relative potencies of agonists were defined as EC_{50} agonist/EC_{50} isoprenaline. Values are expressed as mean ± s.e.mean from n preparations. NC: not calculated because the maximum response could not be observed even at highest concentration of 3×10^{-3} M. *$P < 0.05$; **$P < 0.01$ as compared with all agonists by one way analysis of variance and Scheffe's test. ††$P < 0.01$ as compared with isoprenaline by unpaired Student's t test. [a]$P < 0.05$ as compared with the intrinsic activity of denopamine by unpaired Student's t test. [b]$P < 0.05$ as compared with the intrinsic activity of xamoterol by unpaired Student's t test.

isoprenaline. The maximum response to denopamine was significantly higher than that to xamoterol, and its pD_2 value was significantly lower.

In isolated right atria, isoprenaline, T-0509, denopamine and xamoterol all caused concentration-dependent positive chronotropic actions (Figure 2b and Table 1). The maximum response to T-0509 (98.4 ± 0.5%) was again similar to that to isoprenaline, and it was four times more potent than isoprenaline. The maximum responses to denopamine (68.6 ± 3.5%) and xamoterol (48.3 ± 7.3%) were lower than that to isoprenaline ($P < 0.01$). The maximum response to denopamine was again higher than that to xamoterol ($P < 0.01$), and its pD_2 was smaller ($P < 0.01$). Furthermore, the maximum positive inotropic response to denopamine was larger than its maximum positive chronotropic response ($P < 0.05$), but for xamoterol, the opposite was apparent ($P < 0.05$).

The potencies of isoprenaline (papillary muscles: $pD_2 = 8.59 \pm 0.08$, $n = 5$; atria: $pD_2 = 8.63 \pm 0.04$, $n = 4$) and T-0509 (papillary muscles: $pD_2 = 9.29 \pm 0.09$, $n = 4$; atria: $pD_2 = 9.22 \pm 0.10$, $n = 4$) in the presence of DOCA (2.5×10^{-5} M) did not differ from those in the absence of DOCA listed in Table 1.

β-Agonist actions in the isolated trachea

T-0509 and isoprenaline demonstrated concentration-dependent relaxant actions in the histamine-contracted trachea (Figure 3). Although T-0509 was less potent than isoprenaline ($P < 0.01$) (see Table 1), the maximum response to T-0509 was comparable to that to isoprenaline. Denopamine and xamoterol caused only about 30% relaxation even at the highest concentration of 3×10^{-3} M. Furthermore, the tracheal relaxant actions of denopamine and xamoterol were not influenced by pretreatment with 10^{-6} M propranolol.

As in the case of the positive inotropic action, the potencies of isoprenaline ($pD_2 = 9.03 \pm 0.09$, $n = 4$) and T-0509 ($pD_2 = 7.45 \pm 0.08$, $n = 4$) in the presence of DOCA (2.5×10^{-5} M) did not differ from those in the absence of DOCA listed in Table 1.

Influences of bisoprolol and ICI 118,551 on responses to T-0509 and denopamine on right atria

The CCRCs for positive chronotropic action of T-0509 or denopamine were shifted to the right in a parallel way in the presence of 3×10^{-8} M bisoprolol, but not 3×10^{-8} M ICI 118,551 (data not shown). The pD_2s of T-0509 and denopamine were reduced with bisoprolol from 9.23 ± 0.02 to 8.04 ± 0.11 ($P < 0.01$) and from 7.08 ± 0.08 to 5.99 ± 0.13, ($P < 0.01$), respectively. Conversely, the CCRC for tracheal relaxant action of T-0509 was shifted to the right in the presence of 3×10^{-8} M ICI 118,551, but not 3×10^{-8} M bisoprolol (data not shown). The pD_2s of isoprenaline and T-0509 were reduced with ICI 118,551 from 8.03 ± 0.09 to 6.42 ± 0.14 ($P < 0.01$) and from 6.56 ± 0.06 to 5.17 ± 0.05 ($P < 0.01$), respectively.

Influences of denopamine and xamoterol on the response to isoprenaline on right atria

Pretreatment with denopamine itself increased the beating rate of isolated right atria, but caused a parallel rightward shift of the CCRC to isoprenaline after normalization (Figure 4). The Schild plot of the data yielded a linear regression with a slope of 1.06 ± 0.14, which was not signficantly different from unity, and the pA_2 value for denopamine was 6.98 ± 0.2 (Table 2). This pA_2 value was close to its pD_2 value in the right atria. Denopamine also shifted the CCRC of isoprenaline to the right in a parallel fasion in the histamine contracted trachea (data not shown). The Schild plot had a slope of 1.02 ± 0.14, which was not significantly different from unity, and the pA_2 value for denopamine was 5.39 ± 0.13 (Table 2). Xamoterol also exhibited β-antagonist actions in the right atria ($pA_2 = 7.75 \pm 0.14$, slope = 0.94 ± 0.09) and trachea ($pA_2 = 6.25 \pm 0.11$, slope = 1.06 ± 0.13), respectively (Table 2).

α-Adrenoceptor agonist actions in isolated aorta

In the presence of 10^{-6} M propranolol, isoprenaline, T-0509 and denopamine contracted the aorta, although none of these agonists caused the maximum response even at the highest concentration used (3×10^{-3} M) (Figure 5). T-0509 at concentrations of 3×10^{-5} to 3×10^{-3} M was weaker than isoprenaline ($P < 0.01$). On the other hand, xamoterol (10^{-5}

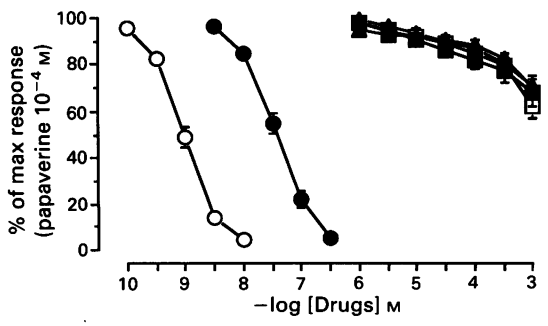


Figure 3 Concentration-response curves for the relaxant actions of β-adrenoceptor agonists in the absence (○, ●, △, □) and the presence (▲, ■) of 10^{-6} M propranolol on the histamine-induced contraction in the guinea-pig trachea. The data are expressed as percentages of the maximum response to 10^{-4} M papaverine: (○) isoprenaline ($n = 5$); (●) T-0509 ($n = 5$), (△) denopamine ($n = 9$); (▲) denopamine + propranolol ($n = 4$); (□) xamoterol ($n = 6$); and (■) xamoterol + propranolol ($n = 4$). The points and vertical bars represent means ± s.e.mean.

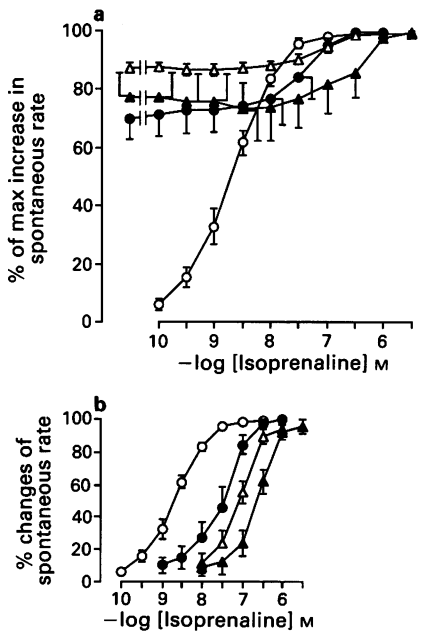


Figure 4 Influence of denopamine on concentration-response curves for the positive chronotropic action of isoprenaline in the guinea-pig right atria. (a) Concentration-response curves of isoprenaline in the absence and the presence of denopamine expressed as percentages of the maximum response to isoprenaline. (b) Normalized concentration-response curves of isoprenaline. (○) Control ($n = 15$); (●) 10^{-6} M denopamine ($n = 5$); (△) 3×10^{-6} M denopamine ($n = 5$); (▲) 10^{-5} M denopamine ($n = 5$). The points and vertical bars represent means ± s.e.mean.

Table 2 Antagonistic actions of denopamine and xamoterol in the isolated right atria and trachea of guinea-pig

	n	pA_2	Slope	r
Right atria (rate)				
Denopamine	15	6.98 ± 0.20	1.06 ± 0.14	0.89
Xamoterol	12	7.75 ± 0.14	0.94 ± 0.09	0.94
Trachea (relaxation)				
Denopamine	21	5.39 ± 0.13	1.02 ± 0.14	0.87
Xamoterol	15	6.25 ± 0.11	1.06 ± 0.13	0.91

pA_2 value, slope and r (correlation coefficient of regression line) were obtained from Schild plots according to Arunlakshana & Schild as described in Methods. Values are expressed as the mean ± s.e.mean from n experiments.

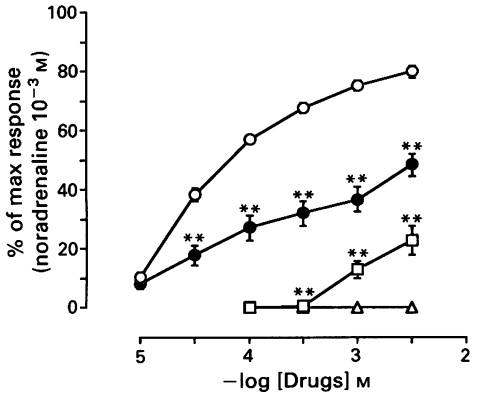


Figure 5 Contractile effects of denopamine, T-0509 and isoprenaline in the guinea-pig aorta pretreated with 10^{-6} M propranolol ($n = 6$). The data are expressed as percentages of the response to 10^{-3} M noradrenaline. (○) Isoprenaline; (●) T-0509; (□) denopamine; (△) xamoterol. The points represent means of 6 experiments and vertical bars show s.e.mean. $^{**}P < 0.01$ as compared with isoprenaline by one way analysis of variance and Scheffe's test.

to 3×10^{-3} M) did not produce any contraction. In the presence of phentolamine (10^{-6} M), isprenaline, T-0509 and denopamine induced no contractile activity ($n = 4$).

Discussion

The results of the present experiments show that T-0509 is a potent and highly selective β_1-adrenoceptor agonist with full agonist activity, whereas denopamine and xamoterol are both selective β_1-adrenoceptor agonists with partial agonist activity. The intrinsic activity of denopamine is higher than that of xamoterol.

The results obtained from the DOCA-treated and non-treated preparations suggest that extraneuronal uptake of catecholamines in the guinea-pig cardiac muscle and trachea (<10 weeks old) is negligible. The extraneuronal uptake inhibitor, hydrocortisone, has been reported not to potentiate either the positive inotropic and chronotropic actions of isoprenaline on cardiac muscle (Goldie, 1975), or its relaxant action on the trachea from young (<12 weeks old) guinea-pigs (Takayanagi *et al.*, 1990). We have obtained similar results with T-0509 and isoprenaline in the present study by using guinea-pigs of 10 weeks old. However, extraneuronal uptake has been reported to play an important role in the β-adrenoceptor action of isoprenaline in the trachea from older (40 and 100 weeks) guinea-pigs (Takayanagi *et al.*, 1990). Therefore, extraneuronal uptake in the guinea-pig trachea appears to be dependent on the age of the guinea-pigs.

Guinea-pig ventricular muscle has been reported to have only β_1-adrenoceptors (Engel *et al.*, 1980; Hedberg *et al.*, 1980; Freissmuth *et al.*, 1986), and guinea-pig right atria to have predominantly β_1-adrenoceptors (Hedberg *et al.*, 1980; Johansson & Persson, 1983). Furthermore, β_1-adrenoceptors have been reported to couple more efficiently to the positive inotropic and chronotropic responses than β_2-adrenoceptors (Molenaar & Summers, 1987). In the present paper, T-0509 was demonstrated to be four times more potent as a positive inotropic and chronotropic agent than isoprenaline in the papillary muscle and right atria, and to have similar intrinsic activities to those of isoprenaline. Moreover, T-0509 caused positive chronotropic action through β_1-adrenoceptors. This finding was consistent with that in the canine ventricular muscle reported by Kurosawa *et al.* (1990) and with that in the guinea-pig right atria reported by Molenaar & Summers (1987).

On the other hand, guinea-pig trachea has been reported to have predominantly β_2-adrenoceptors (Engel *et al.*, 1980; Carswell & Nahorski, 1983). However, β_1-adrenoceptor-stimulation has been reported to relax spontaneous tone in the guinea-pig trachea (Zaagsma *et al.*, 1983; Lemoine *et al.*, 1989b). In the present study, T-0509, in contrast to the cardiac muscle, exhibited a relaxant action with a potency 38 times less than isoprenaline through β_2-adrenoceptors in the histamine-contracted trachea, but with a similar intrinsic activity to isoprenaline. These results indicate that T-0509, like denopamine and xamoterol, did not produce tracheal relaxant action through β_1-adrenoceptors. Therefore, it is suggested that β-adrenoceptors in the guinea-pig trachea are predominantly of the β_2-subtype. Thus, T-0509 is considered to be a full agonist at β_2-adrenoceptors, but to exhibit at least a 150 fold selectivity towards β_1-adrenoceptors. As an α-adrenoceptor agonist, T-0509 was also less potent than isoprenaline and did not cause maximum responses even at the highest concentration used (3×10^{-3} M). Thus, these results indicate that T-0509 is a highly selective agonist at β_1-adrenoceptors, having weak β_2-adrenoceptor agonist and minimal α-adrenoceptor agonist activity. Furthermore, T-0509 exhibits full β-adrenoceptor agonist activity

Xamoterol exhibited lower intrinsic activity than did denopamine. Xamoterol has been reported either to cause positive inotropic action with low intrinsic activity (Hattori *et al.*, 1987) or not to cause any inotropic activity (Malta *et al.*, 1985). Our results with xamoterol agree with the former, and also with those in the cat papillary muscle reported by Lemoine *et al.* (1989a). Low intrinsic activity of xamoterol seems to result in its acting as an agonist at rest, and as an antagonist during exercise in man (Sato *et al.*, 1987; McCaffrey *et al.*, 1988). On the other hand, denopamine has been reported to produce partial agonist action with high intrinsic activity in the dog ventricular muscle (Yokoyama *et al.*, 1988), but to have full agonist action in the guinea-pig right atria (sinoatrial rate) (Lemoine *et al.*, 1989c) and in the cat papillary muscle and right atria (sinoatrial rate) (Hoshiyama *et al.*, 1985; Lemoine *et al.*, 1989c). Our results have confirmed that denopamine has a high intrinsic activity on β_1-adrenoceptors, even if it is not a full agonist. Thus, the high intrinsic activity of denopamine seems to be responsible for it acting as an agonist in man (Hori *et al.*, 1991).

Denopamine activated only β_1-adrenoceptors in the right atria and neither denopamine nor xamoterol showed any tracheal relaxant activity. Xamoterol has been reported to lack agonistic property in the guinea-pig trachea (Hicks *et*

al., 1987), uterus and ileum (Malta et al., 1985) and in the dog femoral artery (Nuttall & Snow, 1982) which are all β_2-adrenoceptor containing preparations. Moreover, denopamine and xamoterol, when tested for antagonist activity in the right atria and trachea, exhibited different pA_2 values, indicating that both agonists show β_1- and β_2-adrenoceptor antagonist actions. The pA_2 values of xamoterol in both tissues were consistent with those reported by Hicks et al. (1987) (guinea-pig right atria, $pA_2 = 7.88$; trachea, $pA_2 = 6.57$). These results indicate that denopamine is a partial β_1-adrenoceptor agonist, and suggest that, like xamoterol, the β_2-adrenoceptor antagonist action of denopamine contributes to its β_1-adrenoceptor selectivity. Therefore, we could not confirm the full agonistic action of denopamine in the guinea-pig right atria reported by Lemoine et al. (1989c).

The intrinsic activity of xamoterol, like that of another partial agonist prenalterol (Kaumann et al., 1980a,b), has been reported to be slightly higher for positive chronotropy than for positive inotropy (Lemoine et al., 1989b). In contrast, the intrinsic activity of denopamine was reported to be higher in positive inotropy than in positive chronotropy (Hoshiyama et al., 1985; Narita et al., 1986). Our results have confirmed these findings, and may reflect the distinct characteristics of the β_1-adrenoceptors in guinea-pig right atria and left ventricular papillary muscle.

To date, noradrenaline, isoprenaline and selective β_2-adrenoceptor agonists such as procaterol (Yabuuti et al., 1977; Yamashita et al., 1978) are frequently used to evaluate β-adrenoceptor-adenylate cyclase coupling, and to characterize β-adrenoceptor subtypes. Noradrenaline is a β_1-selective agonist with potent α-adrenoceptor agonist activity, and this activity has been reported to induce positive inotropic action (Schumann et al., 1978; Bruchner et al., 1984) and cardiac hypertrophy (Simpson, 1985). Recently, β_2-adrenoceptors in the human heart have been found to couple to adenylate cyclase and to mediate positive inotropic and chronotropic actions (Gille et al., 1985; Kaumann & Lemoine, 1987). Isoprenaline in human right atrium has been reported to produce positive inotropic action via stimulation of β_1- and β_2-adrenoceptors (Zerkowski et al., 1986). Since T-0509 was a highly selective and full β_1-adrenoceptor agonist as mentioned above, like procaterol it may be useful in the study of β-adrenoceptor-adenylate cyclase coupling and in characterizing β-adrenoceptor subtypes in experimental animals and man.

In summary, T-0509 appears to be a highly selective and full β_1-adrenoceptor agonist, having weak β_2-adrenoceptor agonist and minimum α-adrenoceptor agonist actions. T-0509 may be useful as a standard tool in the study of β-adrenoceptors. Moreover, denopamine and xamoterol are also both selective β_1-adrenoceptor partial agonists, but the intrinsic activity of denopamine is higher than that of xamoterol.

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The effects of combined angiotensin converting enzyme inhibition and β-adrenoceptor blockade on plasma renin activity in anaesthetized dogs

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1 The effects of β-adrenoceptor blockade on the changes in plasma renin activity (PRA) following angiotensin enzyme (ACE) inhibition were investigated in pentobarbitone-chloralose anaesthetized dogs.

2 ACE-inhibition, with enalapril (2 mg kg^{-1}), caused a significant reduction in systemic arterial blood pressure (BP) with little or no effect on cardiac function, and a significant elevation of plasma renin activity (PRA). By contrast β-adrenoceptor blockade with atenolol (1 mg kg^{-1}), caused a similar reduction in BP but in addition, significantly reduced cardiac function and PRA.

3 A combination of enalapril with atenolol, caused a significant reduction in BP, cardiac function and PRA, hence there was no elevation of PRA, as was seen following ACE-inhibition with enalapril alone.

4 The observations with β-adrenoceptor blockade alone, show that there is an important homeostatic role for the renal sympathetic innervation, mediated by β-adrenoceptors, in controlling basal renin levels. Furthermore, the renal sympathetic innervation appears to be an important contributor to the renin release caused by an ACE-inhibitor as the additional presence of a β-adrenoceptor blocking agent will prevent this release.

5 BW B385C (2 mg kg^{-1}), which combines both ACE-inhibition and β-adrenoceptor blocking properties, also produced reductions in BP and cardiac function similar to those seen with the enalapril/atenolol combination. In addition, for an equivalent degree of ACE-inhibition by BW 385C, to that seen with enalapril alone, the elevation of PRA was attenuated.

6 A combination of ACE-inhibition and β-adrenoceptor blocking activity in a single entity, such as BW B385C, therefore also produces a reduced renin release when compared with an ACE-inhibitor, such as enalapril. This provides further confirmation of the importance of the renal sympathetic innervation in the renin response to ACE-inhibition, and supports the concept of combining ACE-inhibition with β-adrenoceptor blockade.

Keywords: BW B385C; ACE-inhibition/β-adrenoceptor blockade; plasma renin activity

Introduction

The inhibition of angiotensin converting enzyme (ACE) prevents the conversion of the decapeptide, angiotensin I, to the octapeptide, angiotensin II, a potent vasoconstrictor, but consistently elevates plasma renin activity (PRA) and plasma angiotensin I in both animals and man (Johnston, 1984). Although angiotensin I is a less potent vasoconstrictor, its elevated levels, in conjunction with the reduced levels of angiotensin II, may conceivably allow angiotensin I to replace angiotensin II as the functional component of the renin-angiotensin system within the cardiovascular system. It may therefore be argued that a more complete control of the renin-angiotensin system might be achieved by preventing the PRA increase associated with ACE-inhibition. Although the exact mechanism(s) by which ACE-inhibition evokes this renin response is not clear, there is evidence to show that it is sensitive to β-adrenoceptor blockade in animals (Antonaccio *et al.*, 1979; Schriffin *et al.*, 1981; 1984; Noble & Parker, 1986) and in man (Pickering *et al.*, 1982; Macgregor *et al.*, 1985; Belz *et al.*, 1988).

We have therefore carried out a series of experiments in anaesthetized dogs to investigate the effect of β-adrenoceptor blockade upon the renin response to ACE-inhibition. In these experiments we have compared the effects of enalapril (an ACE-inhibitor) alone, with atenolol (a β-adrenoceptor antagonist) alone, and with a combination of enalapril and atenolol. In addition we have examined the effects of BW B385C (N- [(S)-1-carboxy-5-(4- [(S)-2-hydroxy-3-isopropyl-aminoproproxy] -1H-indole-2-carboxamido) pentyl] -(S)-alanyl -(S)-proline), as shown in Figure 1, a novel compound shown to possess both ACE-inhibition and β-adrenoceptor blocking properties *in vitro* and *in vivo* (Allan *et al.*, 1987; 1988). Preliminary accounts of the results described here have been presented to the British Pharmacological Society at its meeting in Oxford (Cambridge *et al.*, 1987) and to the International Hypertension Society at its meeting in Montreal (Cambridge *et al.*, 1990).

Methods

Normotensive beagle dogs (Campbell Farm, Interfauna) of either sex (9–10 kg) were anaesthetized initially with sodium thiopentone (30 mg kg^{-1}; May and Baker) via a cephalic vein. Polythene cannulae were then placed in the left femoral vein, for administration of anaesthetic and drugs, and subsequent anaesthesia maintained by intravenous injection of a mixture of sodium pentobarbitone (6 mg kg^{-1}; Sagatal, May and Baker) and α-chloralose (15 mg kg^{-1}; Koch-Light). Further cannulae were then placed in the abdominal aorta via the left femoral artery, for the measurement of systemic blood pressure via a blood pressure transducer (P23, Statham), and the right femoral artery, for arterial blood samples. A 'pig-tail' catheter (paediatric, Cordis) was then passed down the left carotid artery into the left ventricular

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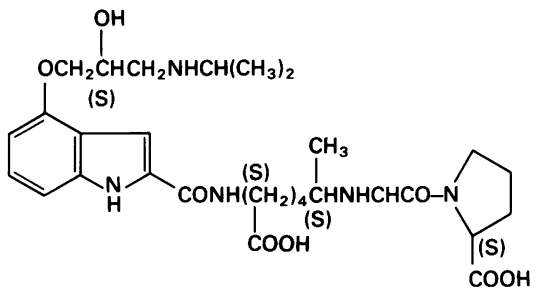


Figure 1 The chemical structure of BW B385C.

chamber, for the measurement of left ventricular pressure via a blood pressure transducer (P23, Statham). Finally, each animal was tracheotomized and pump-ventilated with room air (Palmer large animal respirator, 20 breaths min^{-1}, 20 ml kg^{-1} tidal volume), and allowed to equilibrate for approximately 60 min after completion of the surgery.

Each animal was maintained at 37°C (body/rectal temperature) by a heated underblanket. Arterial blood samples were taken before beginning each experiment to ensure that blood gases (analysed by ABL-3, Radiometer Blood Gas Analyser) were maintained within acceptable limits (Green, 1979). All measured cardiovascular parameters (systemic blood pressure, left ventricular pressure (and its first derivative LV*dP/dt*), and heart rate) were continuously recorded on a polygraph (Grass Model 7 or Gould 2800S), throughout the experiment.

At the start of the experimental period, each animal received a bolus i.v. injection of angiotensin I (0.6 μg kg^{-1}) and isoprenaline (0.1 μg kg^{-1}). These doses were derived from preliminary experiments where a 0.6 μg kg^{-1} dose of angiotensin I was found to produce a consistent sub-maximal pressor response and a 0.1 μg kg^{-1} dose of isoprenaline was found to produce a consistent sub-maximal tachycardic response. Thirty minutes after administration of angiotensin I and isoprenaline, a control period of 40 min was begun, before starting a 100 min infusion period. Arterial blood samples (3.5 ml) were taken before, and at the end of, the infusion period and this blood volume replaced by saline. Each blood sample was divided into two portions (1.5 and 2 ml) and collected into plastic tubes containing heparin or EDTA respectively. They were then centrifuged at 4°C for 15 min at 3000 r.p.m. (Chilspin), the plasma removed into fresh plastic tubes, and immediately frozen (− 20°C) for subsequent assay. The heparin-treated samples were assayed by a spectrophotometric assay (Holmqvist *et al.*, 1979) to measure plasma angiotensin converting enzyme activity (PAA, expressed as units per litres [$u.l^{-1}$]). The EDTA-treated samples were assayed by radioimmunoassay (Baxter-Travenol Diagnostics Inc,) to measure PRA (expressed as nanograms of angiotensin I formed per millilitre of plasma per hour [ng AI $ml^{-1}h^{-1}$]).

During the test infusion period, each animal received a continuous i.v. infusion of saline ($n = 6$ animals), enalapril (0.02 mg kg^{-1} min^{-1}; $n = 9$ animals), atenolol 0.01 mg kg^{-1} min^{-1}; $n = 6$ animals), enalapril/atenolol combination (0.02/0.01 mg kg^{-1} min^{-1}; $n = 6$ animals) or BW B385C (0.02 mg kg^{-1} min^{-1}; $n = 8$ animals) delivered at 0.2 ml min^{-1} with a Braun Perfusor pump. These doses were derived from preliminary experiments and chosen to provide equivalent ACE-inhibition, based on inhibition of an angiotensin I pressor response and reduction in PAA, and/or β-adrenoceptor blockade, based on inhibition of an isoprenaline tachycardic response. At the end of the infusion period, the i.v. infusion was stopped and each animal then received a further bolus i.v. injection of angiotensin I and isoprenaline (doses as before).

Drugs and solutions used

Enalapril was a gift from Merck, Sharp and Dohme (Hoddesdon, U.K.). Atenolol, angiotensin I and isoprenaline were obtained from Sigma Chemicals. BW B385C (N-[S)-1-carboxy-5-(4-[(S)-2-hydroxy-3-isopropylaminoproproxy]-1H-indole-2 carboxamido) pentyl]-(S)-alanyl-(S)-proline) is the S-enantiomer of BW A575C (see Cambridge *et al.*, 1988) and was synthesized by Dr G.W. Hardy in the Department of Medicinal Chemistry, Wellcome Research Laboratories (see Figure 1). All drugs used were dissolved in 0.9% w/v NaCl (saline) and when administered by i.v. bolus, were injected in volumes of 0.1 ml kg^{-1} followed by a suitable volume of saline to wash in the drug solution.

Analysis of data

All data are presented as means ± s.e.mean for both absolute values and percentage changes, and statistical analyses of this data were carried out with the Instat statistical software package (Graphpad Software Inc.). These analyses utilised the paired (within groups) and unpaired (between groups) Student's *t* test. However, for the analysis of the PRA data the uneven distribution of the data necessitated the use of the non-parametric, two-tailed Willcoxon test (within groups) and the two-tailed Mann-Whitney test (between groups). For all of these analyses, $P<0.05$ was taken as statistically significant.

Results

Control group

Statistical analysis of the data from the control group of animals ($n = 6$) which received only a saline infusion, showed there was no significant change in diastolic blood pressure (113 ± 4 to 115 ± 3 mmHg), heart rate (184 ± 7 to 187 ± 8 beat min^{-1}) and LV*dP/dt* (5250 ± 382 to 4875 ± 301 mmHg s^{-1}) when comparing pre-infusion values with those at the end of the infusion period. Similarly, there was no significant change in PRA (5.4 ± 1.7 to 4.5 ± 0.8 ng AI $ml^{-1}h^{-1}$), or PAA (29.7 ± 3.6 to 32.5 ± 3.6 u l^{-1}) or in the tachycardic response due to i.v. isoprenaline and the pressor response to i.v. angiotensin I.

Treatment groups

Although there were some minor differences between the treatment groups, the mean preinfusion values for each measured parameter were broadly similar (see Table 1). However, the mean value for PRA for the atenolol-treated group was significantly ($P<0.05$) lower than that for the other treatment groups. There was no apparent reason for this difference, although it was noted that this group had the lowest mean values for heart rate and LV*dP/dt*, which could suggest that the animals in this group had a lower resting sympathetic drive to the kidneys and the heart.

Enalapril treatment In the group of animals ($n = 9$) which received an infusion of enalapril (to a total dose of 2 mg kg^{-1}) there was a significant reduction in diastolic blood pressure (by 21.4 ± 4.1 mmHg, $P<0.05$) but no significant change in either heart rate or LV*dP/dt* (see Figure 2). The infusion of enalapril also caused a significant decrease in PAA (by 86.4 ± 3.7%, $P<0.05$), and a significant reduction in the pressor response to angiotensin I (by 81 ± 3%, $P<0.05$), but had no effect upon the tachycardic response to isoprenaline (see Figures 3 and 4). In 7/9 animals there was also a marked increase in PRA (by 442 ± 191%) resulting in a significant increase in group mean PRA (by 342 ± 160%, $P<0.05$) as shown in Figures 4 and 5.

Table 1 The pre-infusion values of the measured parameters in the control ($n = 6$) and enalapril (E, $n = 9$), atenolol (A, $n = 6$), enalapril/atenolol (EA, $n = 6$) and BW B385C (BW, $n = 8$) treatment groups

Measured parameter	*Control*	*E*	*A*	*EA*	*BW*
Diastolic blood pressure (mmHg)	113 ± 4	114 ± 7	123 ± 3	114 ± 5	121 ± 3
Heart rate (beat min^{-1})	184 ± 7	183 ± 10	167 ± 9	179 ± 6	187 ± 11
LV*dP/dt* (mmHg s^{-1})	5250 ± 382	5028 ± 569	3642 ± 455	4500 ± 403	5078 ± 430
Plasma ACE activity (u l^{-1})	29.7 ± 3.6	38.8 ± 2.9	39.8 ± 6.7	31.3 ± 3.1	35.5 ± 2.6
Plasma renin activity (ng AI ml^{-1} h^{-1})	5.4 ± 1.7	6.6 ± 1.3	2.8 ± 0.4	8.2 ± 2.6	5.3 ± 1.3

Values are mean ± s.e.mean.

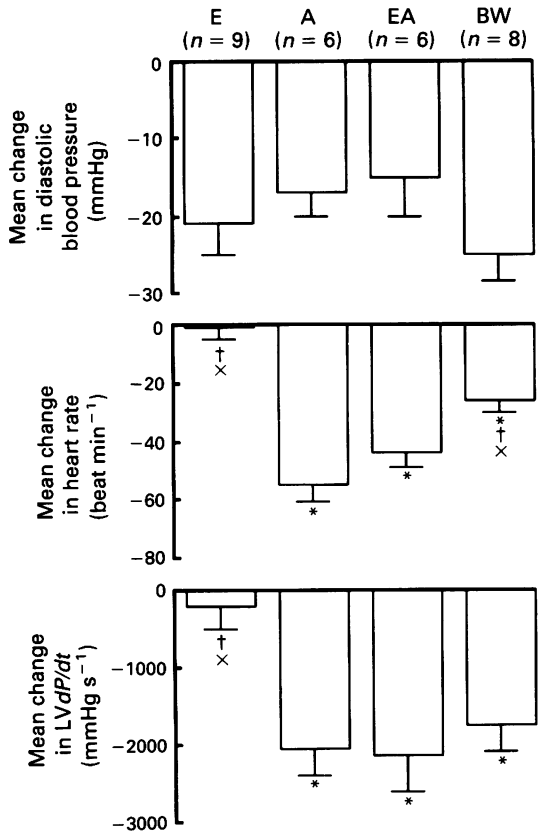


Figure 2 The cardiovascular effects of enalapril (E), atenolol (A), enalapril + atenolol (EA), and BW B385C (BW) treatment in anaesthetized dogs. The doses for each treatment are enalapril and BW B385C (2 mg kg^{-1}, i.v.) and atenolol (1 mg kg^{-1}, i.v.) The effects of each treatment are shown as the mean (± s.e.mean shown by vertical bars) changes from pretreatment values from 6–9 animals. * Statistically significant difference *vs.* E ($P < 0.05$). † Statistically significant difference *vs.* A ($P < 0.05$). X Statistically significant difference *vs.* EA ($P < 0.05$).

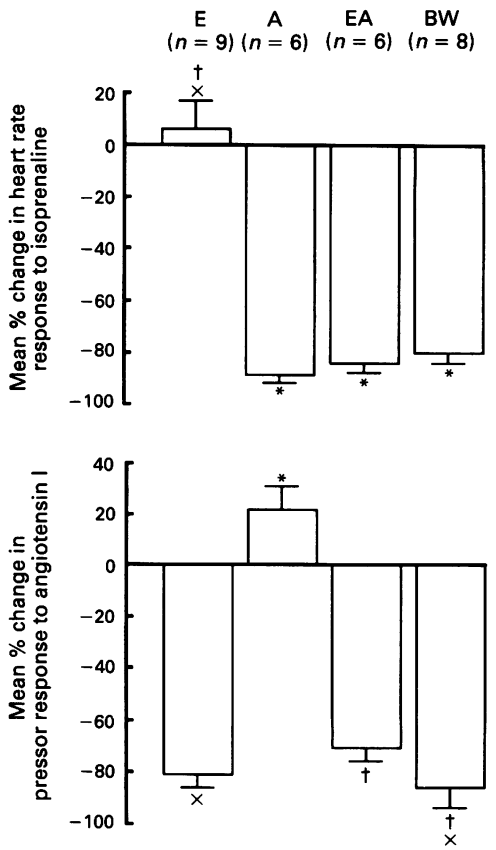


Figure 3 The angiotensin converting enzyme (ACE)-inhibition and β-adrenoceptor blocking effects of enalapril (E), and atenolol (A), enalapril + atenolol (EA) and BW B385C (BW) treatment in anaesthetized dogs. The doses for each treatment are enalapril and BW B385C (2 mg kg^{-1}, i.v.) and atenolol (1 mg kg^{-1}, i.v.). The effects of each treatment are shown as the mean (± s.e.mean shown by vertical bars) percentage inhibition of a pretreatment response from 6–9 animals. * Statistically significant difference *vs.* E ($P < 0.05$). † Statistically significant difference *vs.* A ($P < 0.05$). X Statistically significant difference *vs.* EA ($P < 0.05$).

Atenolol treatment In the group of animals ($n = 6$) which received an infusion of atenolol (to a total dose of 1 mg kg^{-1}), there was a significant ($P < 0.05$) reduction in diastolic blood pressure (by 17 ± 3 mmHg), heart rate (by 55 ± 6 beat min^{-1}) and LV*dP/dt* (by 2050 ± 485 mmHg s^{-1}) as shown in Figure 2. This infusion had no effect upon PAA, or the pressor response to angiotensin I, but significantly reduced the tachycardic response to isoprenaline (by 89 ± 2%, $P < 0.05$) as shown in Figures 3 and 4. In addition, in all the animals in this group, there was a marked decrease in PRA, resulting in a significant group mean reduction in PRA (by 58 ± 9%, $P < 0.05$) as shown in Figures 4 and 5.

Enalapril and atenolol treatment In the group of animals ($n = 6$) which received an infusion of enalapril (to a total dose of 2 mg kg^{-1}) in combination with atenolol (to a total dose of 1 mg kg^{-1}) there was a significant ($P < 0.05$) reduction in diastolic blood pressure (by 15 ± 5 mmHg), heart rate (by 43 ± 5 beat min^{-1}) and LV*dP/dt* (by 2125 ± 287 mmHg s^{-1}) as shown in Figure 2. This enalapril/atenolol combination also caused a significant reduction in PAA (by 78 ± 4%, $P < 0.05$), and a significant reduction in the tachycardic res-

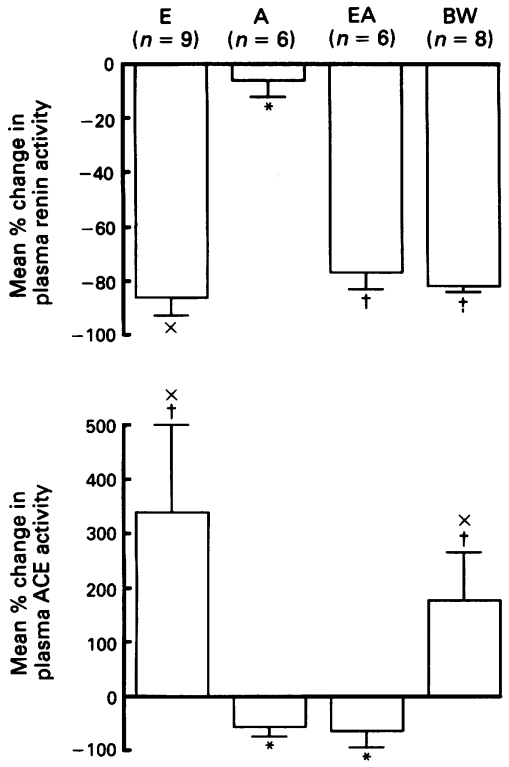


Figure 4 The plasma angiotensin converting enzyme (ACE) and renin activity changes due to enalapril (E), atenolol (A), enalapril- + atenolol (EA) and BW B385C (BW) treatment in anaesthetized dogs. The doses for each treatment are enalapril and BW B385C (2 mg kg^{-1}, i.v.) and atenolol (1 mg kg^{-1}, i.v.). The effects of each treatment are shown as the mean (± s.e.mean shown by vertical bars) percentage change from pretreatment values from 6–9 animals. * Statistically significant difference *vs.* E ($P<0.05$). † Statistically significant difference *vs.* A ($P<0.05$). X Statistically significant difference *vs.* EA ($P<0.05$).

ponse due to isoprenaline (by 84 ± 3%, $P<0.05$) and the pressor response to angiotensin I (by 72 ± 4%, $P<0.05$) as shown in Figures 3 and 4. In 5/6 animals these effects were accompanied by a marked decrease in PRA (by 73 ± 14%), resulting in a significant group mean reduction in PRA (by 61 ± 16%, $P<0.05$) as shown in Figures 4 and 5.

BW B385C treatment In the group of animals ($n = 8$) which received an infusion of BW B385C (to a total dose of 2 mg kg^{-1}) there was a significant ($P<0.05$) reduction in diastolic blood pressure (by 24 ± 3 mmHg), heart rate (by 26 ± 4 beat min^{-1}) and LV$dP/dt$ (by 1703 ± 331 mmHg s^{-1}) as shown in Figure 2. Infusion of BW B385C also caused a significant reduction in PAA (by 81 ± 2%, $P<0.05$), and the tachycardic response to isoprenaline (by 80 ± 4%, $P<0.05$) and the pressor response to angiotensin I (by 86 ± 4%, $P<0.05$) as shown in Figures 3 and 4. These effects were accompanied by an apparent increase in group mean PRA (by 179 ± 92%) but this did not achieve statistical significance (see Figure 4). However, there was a variable response of PRA to BW B385C, for in 4/8 animals in this group there was a mean reduction in PRA (by 18 ± 11%), a small increase (55%) in one animal, whilst in the remaining three animals there was a marked increase (by 484 ± 64%) as shown in Figure 5.

Statistical comparisons between the various treatment groups showed some significant differences. On diastolic blood pressure, there was no significant difference between the effects of the enalapril, atenolol, enalapril/atenolol combination, or BW B385C treatment (see Figure 2). However, unlike enalapril, the atenolol, enalapril/atenolol combination and BW B385C treatments caused a significant ($P<0.05$) reduction in heart rate and LV$dP/dt$ (see Figure 2). On heart rate, the effects of the atenolol and enalapril/atenolol combination were similar, but significantly greater ($P<0.05$) than those of BW B385C (see Figure 2). However, there were no significant differences between these three treatments on LV$dP/dt$. Similarly, with the exception of the atenolol treatment, there were no important differences between these treatments on the reduction of PAA (see Figure 4), and the inhibition of the pressor response to angiotensin I, although there was a minor but significant difference in the latter between the enalapril/atenolol combination and BW B385C treatments (see Figure 3). On PRA, the mean increase produced by enalapril was double that produced by BW B385C, although as a consequence of the inter-animal variation, this difference was not statistically significant. By contrast, the effects of both enalapril and BW B385C were significantly ($P<0.05$) different from the mean decrease produced by atenolol and the enalapril/atenolol combination (see Figure 4). However, examination of the individual responses indicated that there were further qualitative differences between these different treatments. In the enalapril-treated group, 7/9 animals showed an increase in PRA of between 56 and 1219%; in the BW B385C-treated group, 4/8 animals showed an increase in PRA of between 55 and 595% and the remaining 4 animals showed no change or a decrease in PRA of up to 42%; in the enalapril/atenolol-treated group, 1/6 animals showed no change in PRA and the remaining 5 animals showed a decrease in PRA of between 19 and 89%; and in the atenolol group, 6/6 animals showed a decrease in PRA of between 30 and 83% (see Figure 5).

Discussion

In the present studies, using groups of anaesthetized dogs, we have demonstrated that ACE-inhibition, due to enalapril, significantly increases renin release, although there is considerable inter-animal variation in the magnitude of this increase. By contrast, β-adrenoceptor blockade, due to atenolol, significantly reduces renin release. These observations are consistent with the now well-documented reports from other laboratories. However, an equivalent degree of ACE-inhibition in the presence of β-adrenoceptor blockade, achieved by combining enalapril with atenolol, causes no increase in plasma renin, and is instead associated with an overall and significant reduction in renin release when compared with ACE-inhibition (enalapril) alone. Furthermore, an equivalent degree of ACE-inhibition achieved with the novel agent BW B385C, which combines both ACE-inhibition and β-adrenoceptor blocking activity, produces an attenuated, non-significant increase in renin release, when compared with ACE-inhibition (enalapril) alone. However, this apparent increase reflects considerable inter-animal variation in the response to this compound and it is interesting to note that in half of the BW B385C-treated animals there was actually a mean reduction in renin release. Thus although the overall renin response to BW B385C does not exactly mirror the response to a combination of enalapril and atenolol, nevertheless, the effect of this compound on renin release differs qualitatively from that of enalapril alone, and is more consistent with that of the enalapril/atenolol combination.

The renin response to ACE-inhibition is well-documented from studies carried out in both animals and man, but the mechanisms involved in this response are still the subject of debate (Johnston, 1984). The control of renin release itself, is extremely complex, and a number of mechanisms have now been established as having important roles in controlling the release of this proteolytic enzyme from the kidney (Keeton & Campbell, 1981). Some of these mechanisms have also been implicated in the renin response to ACE-inhibition.

One such mechanism, the renal sympathetic innervation to the juxta-glomerular apparatus, is of particular relevance to

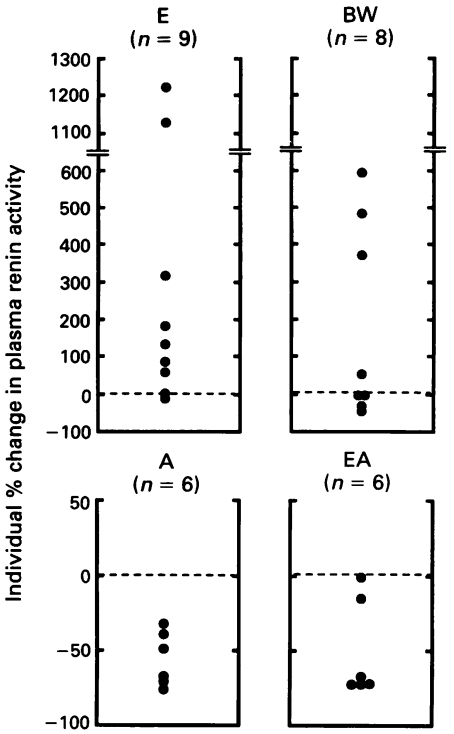


Figure 5 The individual plasma renin activity changes due to enalapril (E), atenolol (A), enalapril + atenolol (EA) and BW B385C (BW) treatment in anaesthetized dogs. The doses for each treatment are enalapril and BW B385C (2 mg kg^{-1}, i.v.) and atenolol (1 mg kg^{-1}, i.v.). The effects of each treatment are shown as the percentage change from pretreatment values for each individual animal.

the studies described here. Direct and indirect stimulation of the renal sympathetic nerves in animals causes renin release through activation of a post-junctional β-adrenoceptor, probably of the β_1 sub-type (Osborn *et al.*, 1981; Keeton & Campbell, 1981; Thames, 1984), and there is extensive documentation showing that β-adrenoceptor blocking agents reduce circulating renin levels in animals and man (Prichard & Owens, 1984). These observations have established the importance of the renal sympathetic innervation as one of the primary controls of renin release, hence it is possible that the renal sympathetic innervation also contributes to the renin response to ACE-inhibition. The cardiovascular effects that accompany ACE-inhibition, such as local and systemic blood pressure and flow changes (McCaa *et al.*, 1978; Meggs & Hollenberg, 1980; Johnston, 1984; Cambridge *et al.*, 1988), are essentially 'pro-renin release' and may therefore provide the relevant stimuli to initiate an increased sympathetic drive to the kidney. There is some circumstantial evidence from studies in anaesthetized animals which lend further support to this idea. It has been observed that anaesthesia augments the renin response to ACE-inhibition (Bing & Poulsen, 1975), and anaesthesia is known to depress the function of some central synapses, distorting the autonomic nervous response to various cardiovascular stimuli, in favour of sympathetic outflow (Korner *et al.*, 1968; Zimpfer *et al.*, 1974; Dorward *et al.*, 1985). It would therefore seem that the renal sympathetic innervation is conveniently placed to mediate the release of renin following ACE-inhibition. The observation that concomitant β-adrenoceptor blockade prevents the increase in renin release normally associated with ACE-inhibition, as described in this paper, and by other groups working in either animals (Antonaccio *et al.*, 1979; Schriffin *et al.*, 1981; 1984; Noble & Parker, 1986) or man (Pickering *et al.*, 1982; Macgregor *et al.*, 1985; Belz *et al.*, 1988), is consistent with this hypothesis.

There are other control mechanisms which may also contribute to the renin response to ACE-inhibition. The renal macula densa, which is sensitive to changes in tubular Na^+-loading (Birbari, 1972; Shade *et al.*, 1972; Freeman *et al.*, 1974; Churchill *et al.*, 1978) exerts an important control on renin release which could conceivably be influenced by the renovascular effects of ACE-inhibition (Johnston, 1984). In addition there is a negative feedback control exerted by circulating angiotensin II through a direct action upon the juxta-glomerular cells to inhibit renin release (Vander & Geelhoed, 1965; Blair-West *et al.*, 1971; Keeton *et al.*, 1976; Naftilan & Oparil, 1978). When operative, this negative feedback control can exert a powerful influence upon the release of renin. Thus it cannot be assumed that these control mechanisms do not also contribute in some way to the renin response to ACE-inhibition (Johnston, 1984).

The results of the studies described in this paper clearly show that the renin response (renin release) to ACE-inhibition can be prevented by concomitant β-adrenoceptor blockade, indicating that the renal sympathetic innervation is involved in this response. However, from careful analysis of the work of other groups, it is also apparent that the renal sympathetic innervation is unlikely to be the sole mechanism by which ACE-inhibition induces such a response. Other mechanisms, such as the renal sensing of tubular Na^+-loading or the loss of the angiotensin II-mediated negative feedback control, may also play a role depending upon the prevailing conditions. In addition, these studies also demonstrate that the combination of ACE-inhibition and β-adrenoceptor blockade found in the novel, dual-activity compound BW B385C can provide equivalent ACE-inhibition, but with an attenuated renin response, when compared with ACE-inhibition (enalapril) alone. In this respect, the attenuated renin response observed with BW B385C is more consistent with that seen with the ACE-inhibitor/β-adrenoceptor blocking agent combination of enalapril/atenolol. However, despite the pharmacological indications of equivalent ACE-inhibition and β-adrenoceptor blockade *in vivo* with these two treatments, there is a significant difference in the renin response to BW B385C compared with that to enalapril/atenolol. The reason for this difference is not clear although it is probably exaggerated by the marked variation in the individual animal response to BW B385C. However, it is possible that it reflects a difference in the intrarenal β-adrenoceptor blocking activities between BW B385C and atenolol, or their relative abilities to gain access to the juxtaglomerular β-adrenoceptors which mediate renin release. Nevertheless, it is clear that the additional β-adrenoceptor blocking activity present in BW B385C alters its ACE-inhibition profile with regard to renin release.

In conclusion therefore, our findings indicate that the renal sympathetic innervation does play an important role in the renin response to ACE-inhibition. In addition they provide further support for the concept of combining β-adrenoceptor blockade with ACE-inhibition, in order to utilise the beneficial therapeutic properties of ACE-inhibition without incurring an undesirable increase in plasma renin and angiotensin levels.

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Impaired cyclic nucleotide-mediated vasorelaxation may contribute to closure of the human umbilical artery after birth

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1 The mechanical and biochemical effects of agents that relax vascular smooth muscle either through elevation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) or adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels were compared in isolated ring preparations of human umbilical artery and rat aorta. Tone was established by preconstriction with 5-hydroxytryptamine.

2 The endothelium-dependent vasodilator calcium ionophore (A23187) (which stimulates endothelium-derived relaxing factor [EDRF] release and thus acts through soluble guanylyl cyclase), sodium nitroprusside (which stimulates soluble guanylyl cyclase directly), and atrial natriuretic peptide (which stimulates particulate guanylyl cyclase) relaxed rat aorta but not human umbilical artery.

3 Sodium nitroprusside, 10 μM, increased cyclic GMP levels from 10 to 390 pmol mg^{-1} protein at 2 min in rat aorta, as compared with a slower, relatively attenuated rise from 5 to 116 pmol mg^{-1} protein after 15 min in human umbilical artery. The rise in cyclic GMP in the umbilical artery was not significantly augmented by the cyclic GMP phosphodiesterase inhibitor, MB22948. Atrial natriuretic peptide increased cyclic GMP levels in rat aorta but not in human umbilical artery.

4 Forskolin, 10 μM, which stimulates both soluble and particulate adenylyl cyclase, maximally relaxed rat aorta and increased cyclic AMP levels from 15 to 379 pmol mg^{-1}protein at 15 min, but did not significantly relax or increase cyclic AMP levels in human umbilical artery. After preincubation with the cyclic nucleotide phosphodiesterase inhibitor, IBMX, 10 μM forskolin increased cyclic AMP levels to 1365 pmol mg^{-1} protein at 30 min in human umbilical arteries, but these high levels were not accompanied by mechanical relaxation.

5 8-Bromo-cyclic GMP and 8-bromo-cyclic AMP which are lipophilic analogues of cyclic GMP and cyclic AMP, both maximally relaxed the rat aorta at a concentration of 10 μM, but did not significantly relax the human umbilical artery.

6 The findings indicate that elevated cyclic nucleotide levels are not associated with mechanical relaxation of the post-partum human umbilical artery, as in other vessels such as rat aorta. This impaired response to cyclic nucleotides may contribute to closure of the umbilical artery after birth.

Keywords: Human umbilical artery; rat aorta; vasorelaxation; cyclic GMP; cyclic AMP

Introduction

The umbilical artery constricts rapidly after birth, thus preventing foetal blood loss (Eltherington *et al.*, 1968; Bjoro & Stray-Pedersen, 1986). The mechanisms underlying the spontaneous closure of this vessel, however, remain obscure. Neural effects are unlikely to play a role as umbilical vessels are not innervated (Spivak, 1943; Reilly & Russell, 1977). One possible stimulus to contraction may be the decrease in cord temperature from body to room temperature which occurs at delivery. Boura and coworkers (1979) attributed spasm of the umbilical vein on cooling to prostaglandin synthesis as the effect was inhibited by indomethacin. The tone of umbilical arteries is also dependent on the ambient oxygen tension. Thus, Eltherington and coworkers (1968) demonstrated that elevation of the Po_2 of the umbilical artery from the normal *in vivo* value of 15 mmHg (Bartels *et al.*, 1962) to that of air (120 mmHg) induced vasoconstriction, which was non-specifically potentiated by bradykinin, 5-hydroxytryptamine (5-HT) and adrenaline. Thromboxane A_2, prostaglandins PGE_1, PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$, bradykinin and 5-HT have all been detected in human umbilical cord blood (Karim, 1967, Tuvemo *et al.*, 1976; Melmon *et al.*, 1968; Altura *et al.*, 1972, Bjoro & Stray-Pedersen, 1986). Whether these agents actually contribute to closure of the umbilical artery post partum is, however, unknown.

In many artery types elevated cyclic nucleotide levels (guanosine 3':5'-cyclic monophosphate [cyclic GMP] and adenosine 3':5'-cyclic monophosphate [cyclic AMP]) are associated with relaxation of vascular smooth muscle (Bhalla *et al.*, 1978; Ignarro *et al.*, 1981; Lincoln, 1983; Lincoln & Fisher-Simpson, 1983; Sano *et al.*, 1983; Reid *et al.*, 1988). We have accordingly investigated whether impairment of relaxation, rather than active constriction, might be involved in the closure of the umbilical artery. Responses in the human umbilical artery were compared with those of the rat aorta.

Methods

Human umbilical cords were obtained from normal, spontaneous, full-term transvaginal deliveries, ligated at the placental and foetal ends, and within 15 min of delivery, placed in Holmans buffer at room temperature (composition, mM:NaCl 120, KCl 5, $CaCl_2$ 2.5, $NaHCO_3$ 25, NaH_2PO_4 1.3, glucose 11, sucrose 10, pH 7.2–7.4) (Griffith *et al.*, 1985; Collins *et al.*, 1986). The umbilical arteries were carefully dissected away from the cord which was bathed continuously in Holman's buffer, and were prepared either for mechanical studies or measurement of cyclic nucleotide levels.

For mechanical studies, 3 mm rings of umbilical artery were mounted in an organ bath containing Holmans' buffer

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at 37°C and changes in isometric tension measured with a force transducer. The preparations were continuously oxygenated with a mixture of 95% O_2/5% CO_2 as umbilical arteries are known to be more reactive at high oxygen concentrations than at their normal physiological Po_2 of 15 mmHg (Eltherington *et al.*, 1968; Silva de Sa, 1981). All arteries were placed under a resting tension of 1.5 g and equilibrated for 1–2 h, with frequent adjustment of baseline tension during stress relaxation. To construct concentration-response curves to vasodilators the vessels were preconstricted (by a further 1.0–4.0 g) with 5-HT at a concentration that induced maximal constriction (10 μM). In separate experiments, relaxation was also studied after constriction with submaximal concentrations of 5-HT (0.01, 0.1 and 1 μM). All experiments were performed within 4 h of delivery.

Male Wistar rats (250–300 g) were killed by a blow to the head and the aorta quickly removed and placed in Holmans' buffer. Adherent tissue was dissected away, and 3 mm rings were suspended in an organ bath as described above under a resting tension of 1.0 g. After equilibration for 1–2 h, they were preconstricted (by a further 0.5–1.0 g) with a maximal concentration of 5-HT (also 10 μM) in order to study relaxant responses.

No attempt was made to remove the endothelium from the arterial segments under study. The presence of an intact endothelium in both the umbilical artery and the rat aortic rings was confirmed by binocular microscopy after staining the luminal surface of the rings with 1% silver nitrate for 2 min, followed by a solution containing 3% cobalt bromide and 3% ammonium bromide for a further 2 min.

To determine cyclic nucleotide levels, 5 mm rings of endothelium-intact human umbilical artery or rat aorta were incubated in oxygenated (95% O_2/5% CO_2) Holmans' solution at 37°C for 2 h in the absence of 5-HT, before addition of stimulants of guanylyl or adenylyl cyclase. Additional experiments were performed with the umbilical artery to study the effects of preincubation with 10 μM MB22948 or 1 mM IBMX. The rings were subsequently immersed in liquid nitrogen and stored at − 80°C for later determination of cyclic GMP and cyclic AMP levels. The frozen arterial segments were homogenized in ice cold 6% (v/v) trichloroacetic acid, the homogenate centrifuged and the supernatant then treated with 0.5 M tri-n-octylamine dissolved in 1,1,2 trichloro-trifluoroethane, to remove the trichloroacetic acid. The samples were then centrifuged and the cyclic GMP and cyclic AMP content of the neutralised aqueous phase determined by radioimmunoassay (New England Nuclear). The residue remaining after centrifugation was treated with 1 mM NaOH and its protein content determined by the method of Bradford (1976).

Statistics

Results are expressed as mean ± s.e.mean. Comparison of data sets was performed with the unpaired Student's *t* test, $P<0.05$ being taken as significant.

Drugs

Sodium nitroprusside, calcium ionophore (A23187), 5-hydroxytryptamine, forskolin, 8-bromo-cyclic GMP, 8-bromo-cyclic AMP, 3-isobutyl-1-methylxanthine (IBMX) and atrial natriuretic peptide (ANP) were obtained from Sigma Chemical Co., Poole, Dorset. 2-0-propoxyphenyl-8-azapurin-6-one (MB22948) was a gift from Rhone-Poulenc.

Results

Preconstriction by 5-hydroxytryptamine

5-HT induced concentration-dependent constriction of the human umbilical artery ($n = 15$) and rat aorta ($n = 6$) with half-maximal concentrations (EC_{50}) of 0.007 ± 0.001 μM and 1.12 ± 0.23 μM respectively. These values were significantly different from each other ($P<0.001$), so that 5-HT was ca. 150 fold more potent in the umbilical artery than the rat aorta (Figures 1, 2). Vasoconstriction by 5-HT was completely reversible in both artery types on washout. In approximately 50% of the umbilical artery preparations, there was a slight decline in tone (pharmacological 'fade') as a function of time (up to 10% by ca. 30 min), which was independent of the presence of a vasodilator (Figure 2). Such 'fade' can account for the minor degree of relaxation of the umbilical artery preparations observed following challenge with potential relaxants and described in the following sections.

Mechanical responses

Agents acting through cyclic GMP

Endothelium-dependent relaxation in human umbilical arteries Preparations with intact endothelium and constricted by 10 μM 5-HT did not relax in response to the calcium ionophore A23187 (0.001 to 1 μM) ($n = 6$, data not shown).

Sodium nitroprusside Sodium nitroprusside (to 10 μM) did not relax human umbilical arteries constricted by 10 μM 5-HT to any significant extent (3.0 ± 1.5%, $n = 6$), and the response to 10 μM sodium nitroprusside was not affected by preincubation with 10 μM MB22948 for 20 min (10.0 ± 6.3%, $n = 6$) (Figures 2, 3). In contrast, sodium nitroprusside induced concentration-dependent relaxation of rat aortic preparations constricted by 10 μM 5-HT, which was 95.0 ± 2.1% of the established tone at the highest concentration employed (10 μM) ($n = 6$, Figure 3). This relaxation began within 30 s and was maximal within 4 min with an EC_{50} value of 0.33 ± 0.20 μM.

Atrial natriuretic peptide (ANP) Human umbilical arteries preconstricted by 10 μM 5-HT did not relax significantly in response to ANP, there being only a 9.0 ± 5.6% ($n = 6$) fall in tension after 15–20 min incubation with the highest concentration studied (1 μM) (Figure 3). In contrast, the preconstricted rat aorta relaxed by 103.8 ± 10.4% of established tone with 1 μM ANP ($n = 6$, Figure 3). This relaxation began within 1 min and was maximal by 10–15 min with an EC_{50} value of 1.8 ± 0.5 nM.

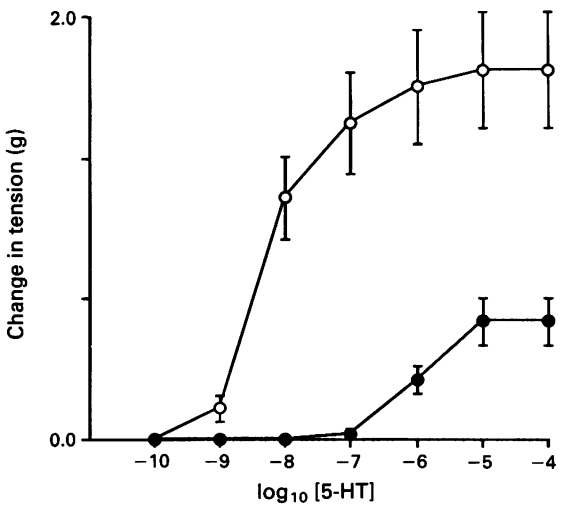
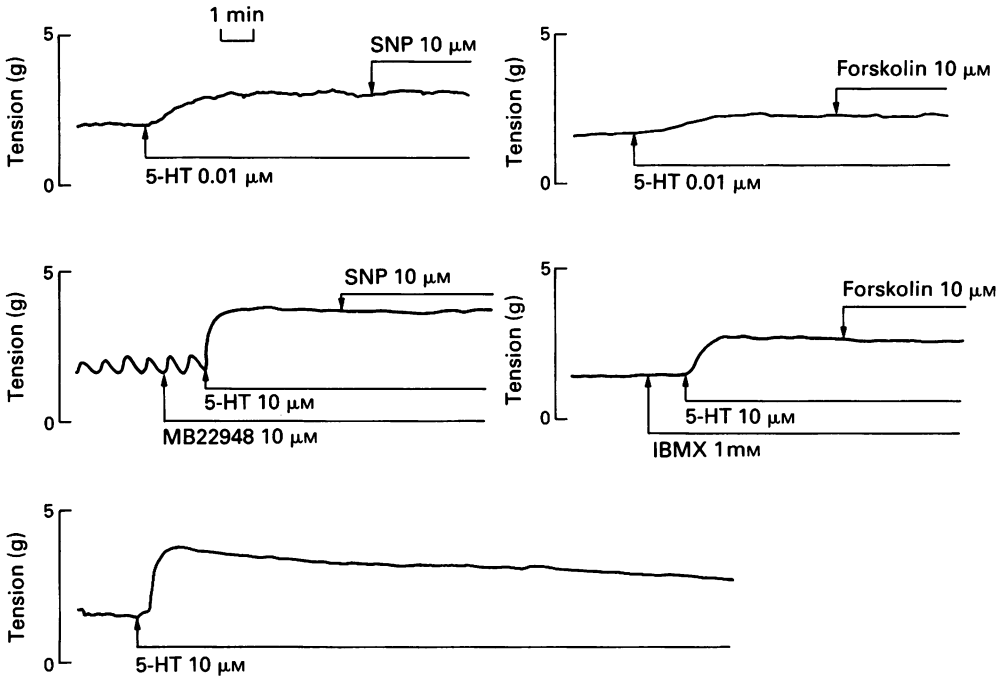


Figure 1 Concentration-response curves to 5-hydroxytryptamine (5-HT) plotting increases in tension (g) from baseline in human umbilical artery (○) and rat aorta (●). EC_{50} concentrations were 0.007 ± 0.001 and 1.12 ± 0.23 μM respectively, and were significantly different from each other ($P<0.001$). Maximal constriction to 5-HT was obtained at a concentration of 10 μM in both artery types.



Figue 2 Representative human umbilical artery traces illustrating lack of relaxation to 10 μM sodium nitroprusside (SNP) and 10 μM forskolin, in both the absence (upper) and the presence (middle) of phosphodiesterase inhibitors (10 μM MB22948 and 1 mM isobutylmethylxanthine (IMBX), respectively). This impaired response cannot be attributed to functional antagonism between 5-hydroxytryptamine (5-HT) and cyclic nucleotides as relaxation could not be elicited even when tone was established with low (i.e. submaximal) concentrations of 5-HT (0.01 μM illustrated here). In approximately 50% of umbilical artery preparations there was a slow decline in tension which was independent of the presence of a vasodilator (lower). Note that spontaneous oscillations in tone were observed in some preparations.

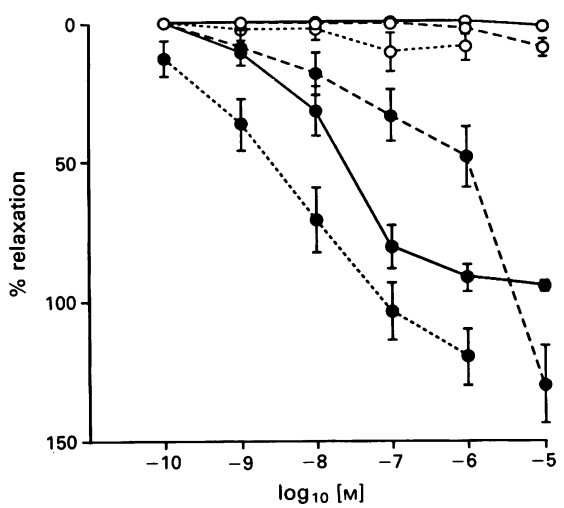


Figure 3 Effects of (a) sodium nitroprusside (continuous lines), (b) atrial natriuretic peptide (dotted lines) and (c) 8-bromo-cyclic GMP (dashed lines) in human umbilical artery (○) and rat aorta (●).

8-Bromo-cyclic GMP Human umbilical arteries preconstricted by 10 μM 5-HT were unresponsive to 8-bromo-cyclic GMP (to 10 μM), the fall in tension after 15–20 min incubation being 9.7 ± 3.2% ($n = 6$, Figure 3). In contrast, the preconstricted rat aorta relaxed by 130.3 ± 13.7% of established tone in response to 10 μM 8-bromo-cyclic GMP ($n = 6$, Figure 3). This relaxation began within 1 min and was maximal within 15–20 min with an EC_{50} value of 1.17 ± 0.25 μM.

Agents acting through cyclic AMP

Forskolin Forskolin (to 10 μM) caused no significant relaxation of human umbilical arteries preconstricted by 10 μM 5-HT (Figure 4), and the small decline in tension at 15–20 min (10.6 ± 2.5%, $n = 8$) was not significantly enhanced by preincubation with 1 mM IBMX (13.7 ± 7.4%, $n = 6$). In contrast, forskolin induced concentration-dependent relaxation of the preconstricted rat aorta which was 117.6 ± 9.7% ($n = 6$) of established tone at the highest concentration studied (10 μM) (Figure 4). This relaxation began within 1 min and was maximal within 15–20 min with an EC_{50} value of 0.29 ± 0.12 μM.

8-Bromo-cyclic AMP Human umbilical arteries preconstricted by 10 μM 5-HT were unresponsive to 8-bromo-cyclic

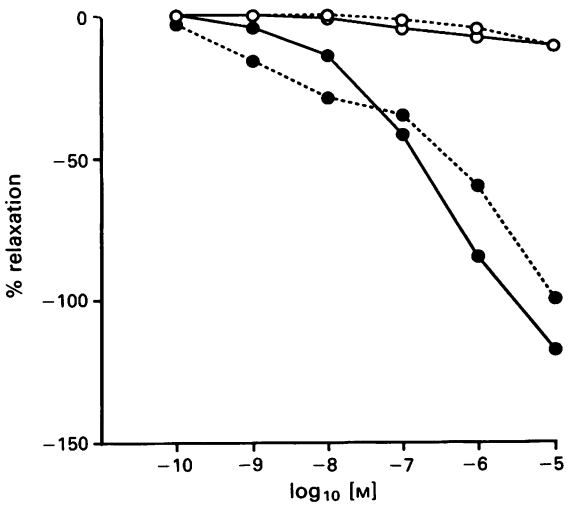


Figure 4 Effects of forskolin (continuous lines) and 8-bromo-cyclic AMP (dotted lines) in human umbilical artery (○) and rat aorta (●).

AMP (to 10 μM), the fall in tension after 15–20 min incubation being 11.2 ± 2.5% ($n = 6$) (Figure 4). In contrast, the preconstricted rat aorta relaxed by 100.0 ± 2.0% with 10 μM 8-bromo-cyclic AMP ($n = 6$, Figure 4). This relaxation began within 1 min and was maximal at 15–20 min with an EC_{50} value of 0.85 ± 0.47 μM.

Concentration of 5-HT used to establish tone

In the above experiments, 10 μM 5-HT was used to preconstrict both the umbilical artery and rat aortic preparations. This concentration induced the maximum attainable constrictor response to 5-HT in both artery types (Figure 1). In further experiments with the umbilical artery, tone was established with lower concentrations of 5-HT (0.01, 0.10 and 1 μM) to determine whether the lack of cyclic nucleotide-mediated relaxation was attributable to functional antagonism by high concentrations of 5-HT. As in the case of 10 μM 5-HT, however, sodium nitroprusside (to 10 μM) and forskolin (to 10 μM) failed to elicit mechanical relaxation at these lower concentrations of 5-HT, either in the absence or the presence of 10 μM MB22948 or 1 mM IBMX as appropriate ($n = 5$ in each case, Figure 2).

Cyclic nucleotide levels

The effects of vasodilator agents on tissue cyclic GMP and AMP levels were assessed in the absence of a constrictor agonist.

Effects of sodium nitroprusside, atrial natriuretic peptide and forskolin Basal levels of cyclic GMP were statistically similar in the umbilical artery (4.6 ± 1.4 pmol mg^{-1} protein, $n = 6$) and rat aorta (9.7 ± 2.3 pmol mg^{-1} protein, $n = 6$). In the umbilical artery, 10 μM sodium nitroprusside slowly increased cyclic GMP levels to 115.6 ± 46.4 pmol mg^{-1} protein by 15 min, after which they declined but still remained above control levels for up to 60 min (Figure 5). After preincubation with 10 μM MB22948, 10 μM sodium nitroprusside increased cyclic GMP levels to a peak of 158.8 ± 69.9 pmol mg^{-1} protein after 20 min. This was not significantly greater than the maximum induced with 10 μM sodium nitroprusside alone, but cyclic GMP levels remained elevated at 89.2 ± 25.5 pmol mg^{-1} protein after 60 min, a value ca. four-fold greater than that 60 min after administration of 10 μM sodium nitroprusside alone (Figure 5). In rat aorta, by contrast, 10 μM sodium nitroprusside stimulated a substantially greater, and considerably more rapid increase in cyclic GMP levels, with a peak of 388.3 ± 45.6 pmol mg^{-1} protein at 2 min, followed by a rapid subsequent decline (Figure 6).

ANP 1 μM did not elevate cyclic GMP levels in human umbilical artery (Figure 5). In contrast, in rat aortic cyclic GMP levels peaked after 5 min at 157.7 ± 84.7 pmol mg^{-1} protein and quickly declined thereafter (Figure 6).

Forskolin, 10 μM, had no significant effect on cylic GMP levels in either umbilical artery or rat aorta (Figures, 5, 6).

Effects of forskolin (10 μM) and sodium nitroprusside (10 μM) on cyclic AMP levels Basal cyclic AMP levels did not differ significantly between umbilical artery (9.93 ± 3.87 pmol mg^{-1} protein, $n = 6$) and rat aorta (15.1 ± 4.9 pmol mg^{-1} protein, $n = 6$). In umbilical artery, forskolin alone did not alter cyclic AMP levels over a period of 0–50 min (Figure 7), but after preincubation with 1 mM IBMX it increased cyclic AMP levels at 30 min to 1365.0 ± 427.8 pmol mg^{-1} protein after which it declined (Figure 7).

In rat aorta, forskolin increased cyclic AMP levels to a peak of 379.0 ± 62.6 pmol mg^{-1} protein at 15 min which then declined (Figure 8).

Sodium nitroprusside did not signficantly alter cyclic AMP levels in either umbilical artery or rat aorta (Figures 7 and 8).

Discussion

We have investigated the hypothesis that impaired mechanisms of vasodilatation contribute to closure of the human umbilical artery after birth, by comparing the mechanical and biochemical responses of the human umbilical artery and rat aorta to agents which normally mediate vascular smooth muscle relaxation through elevation of cyclic nucleotide levels. To study relaxation, tone was established with 5-hydroxytryptamine (5-HT) which induced reversible, concentration-dependent constriction in both vessel types. The umbilical artery preparations were found to be ca. 150 fold more sensitive to 5-HT than the rat aorta.

To elevate cyclic GMP levels, we used the calcium ionophore A23187, which has previously been shown to be a receptor-independent stimulant of EDRF release from the human umbilical artery (Van de Voorde *et al.*, 1987; Chaudhuri *et al.*, 1991); sodium nitroprusside, a direct stimulant of soluble guanylyl cyclase (Ignarro *et al.*, 1981); and atrial

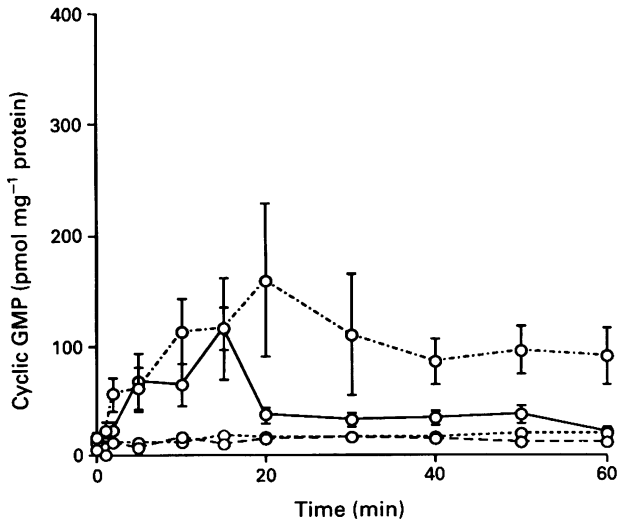


Figure 5 Time course of cyclic GMP levels in human umbilical artery following administration of 10 μM sodium nitroprusside (—), 10 μM atrial natriuretic peptide (——), 10 μM forskolin (---) and 10 μM sodium nitroprusside in the presence of 10 μM MB22948 (–·–·–).

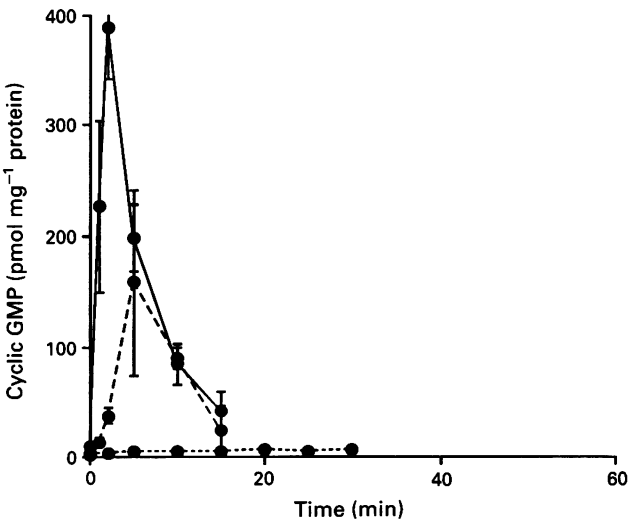


Figure 6 Time course of cyclic GMP levels in rat aorta following administration of 10 μM sodium nitroprusside (continuous lines), 10 μM forskolin (dotted lines) and 10 μM atrial natriuretic peptide (dashed lines).

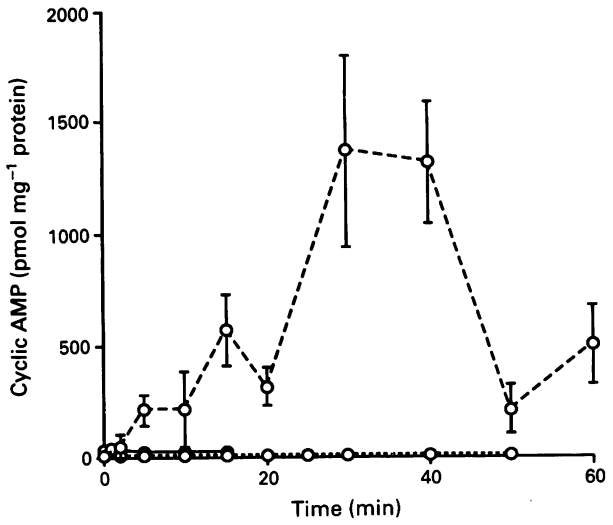


Figure 7 Time course of cyclic AMP levels in human umbilical artery following administration of 10 μM forskolin (dotted lines), 10 μM sodium nitroprusside (continuous lines) and 10 μM forskolin in the presence of 1 mM isobutyl methylxanthine (dashed lines).

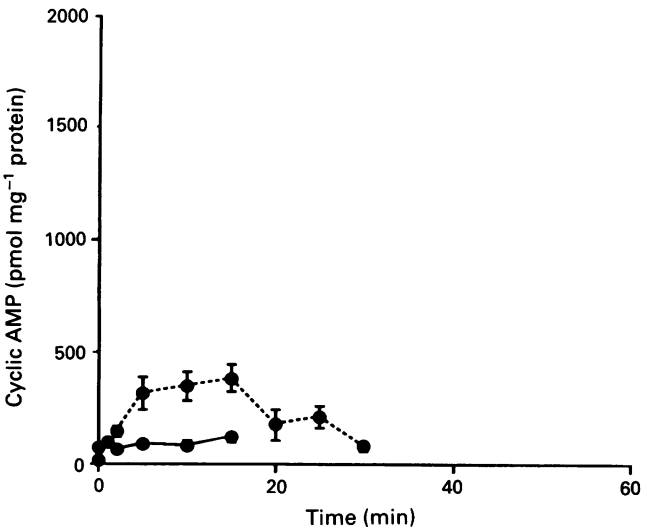


Figure 8 Time course of cyclic AMP levels in rat aorta following administration of 10 μM forskolin (dotted lines) and 10 μM sodium nitroprusside (continuous lines).

natriuretic peptide, a stimulant of particulate guanylyl (Winquist *et al.*, 1984). To elevate cyclic AMP levels, we used forskolin, a reversible stimulator of the catalytic subunit of both soluble and particulate adenylyl cyclase (Lincoln & Fisher-Simpson, 1983). In marked contrast to the rat aorta and many other blood vessels (Sano *et al.*, 1983; Lincoln & Fisher-Simpson, 1983; Reid *et al.*, 1988), the human umbilical artery did not relax to any significant extent with either of these classes of vasodilators. The findings are consistent with a previous report which demonstrated that histamine fails to induce mechanical relaxation of preconstricted endothelium-intact human umbilical artery preparations, but is nevertheless able to stimulate EDRF release from them in cascade bioassay (Van de Voorde, 1987). More recently, the ability of EDRF to relax endothelium-denuded human umbilical arteries has been shown to be markedly attenuated when compared to the response of chorionic plate and pulmonary arteries (Chaudhuri *et al.*, 1991).

The apparent lack of normal relaxation could be a consequence of impaired cyclic nucleotide formation, more rapid hydrolysis of cyclic GMP and cyclic AMP by their respective phosphodiesterases, or decreased sensitivity of the intracellular mechanisms which bring about relaxation to cyclic nucleotides. Experiments were therefore performed to measure cyclic nucleotide levels both in the absence and in the presence of a relatively specific inhibitor of cyclic GMP phosphodiesterase (MB22948) and a non-specific inhibitor of cyclic nucleotide phosphodiesterase (IBMX) (Weishaar, 1987). The mechanical effects of permeable lipophilic analogues of cyclic GMP and cyclic AMP were also examined. It has previously been shown that the sensitivity of the rat aorta to acetylcholine, a vessel in which it is an endothelium-dependent vasodilator (in contrast to the situation in the human umbilical artery; Van de Voorde *et al.*, 1987), is inversely related to the concentration of agonist used to establish tone (Dainty *et al.*, 1990). Functional antagonism between high concentrations of 5-HT and cyclic nucleotide-mediated mechanisms of vasorelaxation was excluded, however, by the demonstration that both classes of vasodilator also failed to elicit relaxation when the umbilical arteries were preconstricted by submaximal concentrations of 5-HT (0.01, 0.1 and 1 μM).

Although sodium nitroprusside did not relax the human umbilical artery, it did elevate cyclic GMP levels, albeit more slowly and to a smaller extent than in rat aorta and other artery types (Lincoln & Fisher-Simpson, 1983; Tanaka *et al.*, 1989). Previous workers have shown that nitrovasodilators (including EDRF) can elevate cyclic GMP levels in the human umbilical artery, but did not study the time course of this response (Chaudhuri *et al.*, 1991). ANP caused no rise in cyclic GMP levels in human umbilical artery. These observations suggest an attenuated soluble guanylyl cyclase response and an absence of particulate guanylyl cyclase response in the human umbilical artery. The latter finding could be explained by an absence of either the particulate enzyme or of specific ANP receptors. ANP has been reported not to act as a vasodilator in other vessels, including small arteries in the rabbit and some veins (Faison *et al.*, 1985).

MB22948, a relatively specific inhibitor of cyclic GMP phosphodiesterase (Weishaar, 1987), did not increase peak levels of cyclic GMP attained in response to sodium nitroprusside in the human umbilical artery, but significantly prolonged their elevation. This indicates that cyclic GMP phosphodiesterase is active in the human umbilical artery, and suggests that the impaired mechanical response to nitrovasodilators probably does not involve enhanced hydrolysis of cyclic GMP. Consistently, 8-bromo-cyclic GMP, a lipophilic analogue of cyclic GMP which mediates relaxation in the micromolar range in rat tail artery (Cheung & MacKay, 1985), rat aorta (Lincoln, 1983) and rabbit aorta (Schultz *et al.*, 1979; Napoli *et al.*, 1980), also failed to elicit significant relaxation of the human umbilical artery. These findings indicate that the responsiveness of the post-partum human umbilical artery to elevated levels of intracellular cyclic GMP is impaired.

Forskolin activates both soluble and particulate adenylyl cyclase (Sano *et al.*, 1983), but elevated cyclic AMP levels in the human umbilical artery only in the presence of the cyclic nucleotide phosphodiesterase inhibitor, IBMX. This suggests that there may be abnormally rapid hydrolysis of cyclic AMP in this vessel. Other mechanisms are also likely to be involved in the failure of relaxation, however, as even these high levels of cyclic AMP failed to affect tone, and there was a corresponding lack of response to the lipophilic cyclic AMP analogue, 8-bromo-cyclic AMP, which relaxes other artery types in the micromolar range (Ousterhout & Sperelakis, 1987). The findings suggest that there is generalised impairment of the intracellular response to elevated cyclic nucleotide levels in the post-partum human umbilical artery. In conclusion, we suggest that impaired mechanisms of relaxation may contribute to the closure of the human umbilical artery after birth and that this is associated both with par-

tially attenuated cyclic nucleotide responses to conventional stimulants and with impaired relaxation in response to elevated cyclic nucleotide levels. It remains to be established at what cellular level failure of relaxation occurs in view of the fact that cyclic GMP and cyclic AMP exert multiple effects on Ca^{2+} homeostasis and protein phosphorylation (Scheid & Pay, 1984; Suematsu *et al.*, 1984; Popescu *et al.*, 1985; Collins *et al.*, 1986; Rapoport, 1986; Twort & Van Breeman, 1988; Cornwall & Lincoln, 1989; Hirata *et al.*, 1990).

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Evidence for a glutamate receptor of the AMPA subtype which mediates insulin release from rat perfused pancreas

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1 The effect of L-glutamate has been studied on insulin secretion by the isolated perfused pancreas of the rat. The glutamate receptor subtype involved has been characterized.

2 In the presence of a slightly stimulating glucose concentration (8.3 mM), L-glutamate (5×10^{-5}–4×10^{-3} M) induced an immediate, transient and concentration-dependent insulin response. On the other hand, in the presence of a non stimulating glucose concentration (2.8 mM), L-glutamate (10^{-3} M) did not modify the basal insulin secretion.

3 The three non-NMDA receptor agonists, kainate (10^{-4}–10^{-3} M), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA, 5×10^{-5}–10^{-4} M) and quisqualate (5×10^{-6}–5×10^{-5} M) all provoked a transient and concentration-dependent insulin response from pancreas perfused with 8.3 mM glucose. Compared with glutamate, kainate exhibited a similar efficacy, whereas AMPA and quisqualate elicited only a 3 fold lower maximal insulin response. In contrast, NMDA (10^{-4}–10^{-3} M) was ineffective.

4 An antagonist of non-NMDA receptors, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 5×10^{-5} M) totally prevented the stimulatory effect of L-glutamate (4×10^{-4} M) and kainate (2×10^{-4} M). In contrast, the NMDA receptor antagonist, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine ((+) MK801) was without effect.

5 The insulin secretory effect of glutamate (4×10^{-4} M) was not affected by atropine (3×10^{-7} M) or tetrodotoxin (3×10^{-6} M).

6 Quisqualate at a high maximally effective concentration (4×10^{-4} M) inhibited glutamate (10^{-3} M) or kainate (4×10^{-4} M)-induced insulin release.

7 This study shows that L-glutamate stimulates insulin secretion in rat pancreas, by acting on an excitatory amino acid receptor of the AMPA subtype.

Keywords: Glutamate; AMPA receptor; insulin release; rat pancreas

Introduction

L-Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system. It plays an important role in many neuronal functions such as fast synaptic transmission (Tang *et al.*, 1989), neuronal plasticity (Collingridge & Singer, 1990) and also in neurodegenerative diseases (Meldrum & Garthwaite, 1990).

Many effects of glutamate are mediated by three major receptor subtypes termed according to their most selective agonists: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (previously termed quisqualate receptors) and kainate receptors (Watkins *et al.*, 1990). However, it is now clear that most responses to kainate are mediated by the AMPA receptor subtype where kainate acts as a full agonist, whereas AMPA (or quisqualate) gives only partial responses. These receptors are gated ionic channels permeable to Na^+, K^+ and for the NMDA subtype, to Ca^{2+} as well.

Despite extensive research on glutamate receptors in the central nervous system, little is known about the existence of peripheral glutamate receptors. A NMDA receptor subtype has been characterized in the myenteric plexus of the guinea-pig ileum (Moroni *et al.*, 1986; Shannon & Sawyer, 1989).

Much evidence shows that pancreatic islets cells, particularly insulin secreting β cells, share with neurones common characteristics; they contain tyrosine hydroxylase (Teitelman & Lee, 1987), neurone specific enolase (Polak *et al.*, 1984), Go protein (Terashima *et al.*, 1987), glutamic acid decarboxylase, high levels of γ-aminobutyric acid (GABA) (Okada, 1986) as well as synaptic-like microvesicles (Reetz *et al.*, 1991).

The present study was designed to investigate an eventual effect of L-glutamate on insulin secretion and to characterize the receptor subtype involved. This work, performed on the isolated perfused pancreas of the rat, shows that L-glutamate stimulates insulin release by activating a glutamate receptor of the AMPA subtype with similar pharmacological characteristics to that described in the central nervous system.

Methods

Our experiments were performed on male Wistar rats fed *ad libitum* and weighing 330 to 350 g. The surgical procedure for the rat isolated perfused pancreas has been previously described (Loubatières *et al.*, 1969; Bertrand *et al.*, 1986). After anaesthesia with sodium pentobarbitone (60 mg kg^{-1}, i.p.), the pancreas was totally isolated from all neighbouring tissues; it was perfused through its own arterial system with a Krebs Ringer bicarbonate buffer containing 2 g l^{-1} pure bovine serum albumin (fraction V) and glucose 8.3 mM. The Krebs buffer had the following ionic composition (mM): NaCl 108, KH_2PO_4 1.19, KCl 4.74, $CaCl_2$ 2.54, $MgSO_4$ 7, H_2O 1.19 and $NaHCO_3$ 18. A mixture of O_2 (95%) and CO_2 (5%) was continuously bubbled through this medium; the pH was about 7.4. The preparation was maintained at 37.5°C. Each organ was perfused at a constant pressure: the pressure

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was selected so as to produce a flow rate of about 2.5 ml min^{-1} at the start of the experiments.

In all the experiments, a 30 min adaptation period was allowed before taking the first sample. Glutamate or other agonists, were infused for 20 min from time 45 min in the absence or from 55 min in the presence of blockers. The blockers were added from 40 min, 15 min before the agonist infusion. Samples were collected during 1 min and were then immediately frozen for insulin radioimmunoassay. Insulin in the pancreatic effluent was assayed by the method of Herbert *et al.* (1965) using the antibody supplied by Miles Laboratories (Paris). [^{125}I]-insulin was obtained from International CIS (Gif-Sur-Yvette, France). Pure rat insulin (NOVO, Copenhagen, Denmark) was used as the reference standard, the biological activity of which was 22.3 $\mu u\ ng^{-1}$. The intra- and inter-assay variations were respectively 9 and 13%.

For the kinetics of insulin output rate the results for each point are expressed as changes in relation to the value recorded before agonist administration at time 45 or 55 min (taken as 100%). Data are expressed as mean ± standard error of the mean (s.e.mean).

In order to establish the concentration-response curves for agonists we used the increase of mean insulin output rate as a percentage. This value was obtained as follows: AUC/5 (AUC = area under the curve during the first 5 min of infusion).

The data were submitted to analysis of variance followed by the multiple comparison test (Zar, 1974).

Drugs

L-Glutamic acid (monosodium salt), kainic acid, N-methyl-D-aspartic acid, glycine, atropine and tetrodotoxin were obtained from Sigma. (**RS**)-α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were from Tocris Neuramin. Quisqualic acid was purchased from Cambridge Research Biochemicals. (+) -5- Methyl-10,11- dihydro-5H-dibenzo[a,d]cyclohepten-5, 10-imine hydrogen maleate ((+)-MK801) was from Research Biochemicals Inc.

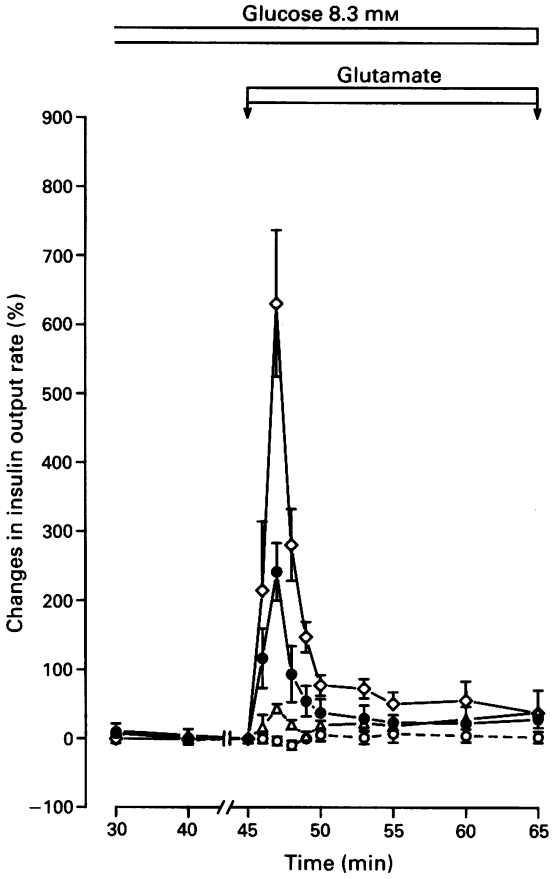


Figure 1 Effects of glutamate on insulin secretion from the isolated perfused pancreas of the rat: (△) glutamate 5×10^{-5} M ($n = 4$), (●) 2×10^{-4} M ($n = 4$), (◇) 4×10^{-3} M ($n = 5$), controls (○) ($n = 6$). The insulin output rate (ng min^{-1}) at 45 min for each set of experiments was: 14.2 ± 3.2; 14.5 ± 4.9; 12.3 ± 2.2; 15.1 ± 2.8 respectively. Each point represents the mean with s.e.mean shown by vertical lines.

Results

Effect of L-glutamate

In pancreata perfused in the presence of a slightly stimulating glucose concentration (8.3 mM), the control rate of insulin release was stable, reaching 15.1 ± 2.8 ng ml^{-1} at 45 min (the reference value).

L-Glutamate was studied at concentrations ranging between 10^{-5} and 4×10^{-3} M. At 10^{-5} M, glutamate did not significantly affect insulin release (results not shown). In the range 5×10^{-5} to 4×10^{-3} M, this amino acid provoked an immediate and transient insulin response which was concentration-dependent. The insulin response occurred in a peak form which culminated at the 2 min and lasted about 5 min although the infusion was maintained for 20 min (Figure 1). The increase of mean insulin output rate averaged a maximum of +270 ± 44% with glutamate 4×10^{-3} M (Figure 4).

On the other hand, in the presence of a non-stimulating glucose concentration (2.8 mM), L-glutamate even at 10^{-3} M, did not affect basal insulin release. The insulin output during the first 5 min of glutamate infusion was 1.2 ± 0.8 ng versus 1.1 ± 0.5 ng in controls (result not shown).

Effect of glutamate receptor agonists

Non-NMDA receptor agonists Three non-NMDA receptor agonists, kainate, AMPA and quisqualate were studied.

In the presence of 8.3 mM glucose, all three agonists elicited a concentration-dependent peak-shaped insulin response as did glutamate (Figures 2 and 3). Kainate exhibited a comparable efficacy to that of glutamate. As shown in Figure 4 the maximal increase in the mean insulin output rate obtained with kainate at 10^{-3} M (+358 ± 48%), was not significantly different from that induced by glutamate (+270 ± 44%). In contrast, quisqualate and AMPA elicited weak maximal insulin responses (+74 ± 15% and +87 ± 15% respectively), about 3 fold lower than that obtained with glutamate. However quisqualate was more potent than glutamate; it was concentration-dependently effective in the range 5×10^{-6} to 5×10^{-5} M (Figure 4).

NMDA receptor agonist NMDA, in the range 10^{-4}–10^{-3} M did not affect insulin release: the mean changes in insulin output rates during the first 5 min of NMDA infusion were +9 ± 6%; +12 ± 5% and +4 ± 2% at NMDA 10^{-4}, 4×10^{-4} and 10^{-3} M respectively versus +2 ± 5% in controls (Figure 4). These results were obtained in presence of glycine (10^{-6} M) known to potentiate NMDA effects in brain (Johnson & Asher, 1987). Mg^{2+} is known to inhibit NMDA receptor channels (Nowak *et al.*, 1984). However, in the absence of Mg^{2+}, NMDA (4×10^{-4} M) remained ineffective on insulin secretion: the mean change in insulin output rates was +2 ± 3%.

Effects of blockers of glutamate receptors on the insulin responses to glutamate and kainate (Figure 5)

In these experiments, the two most effective agonists glutamate and kainate were used at concentrations inducing about half maximal insulin response i.e. 4×10^{-4} M and 2×10^{-4} M respectively.

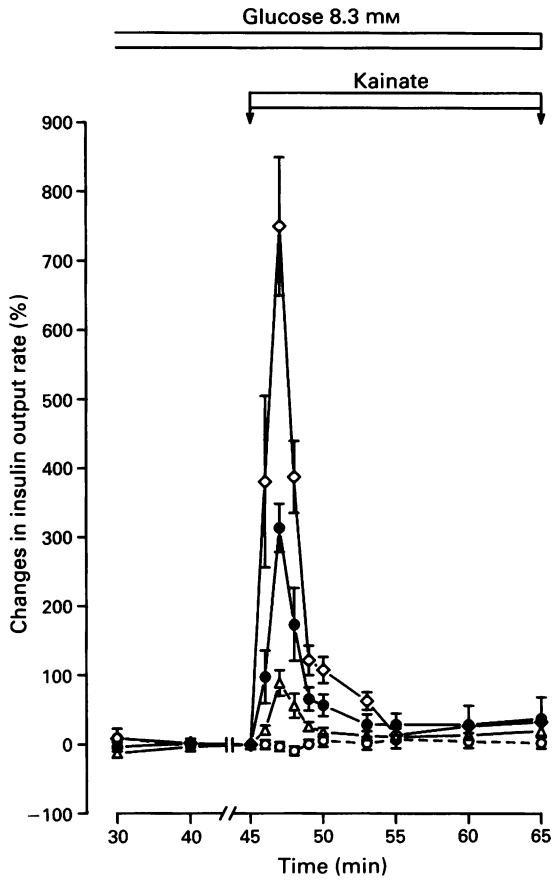


Figure 2 Effects of kainate on insulin secretion from the isolated perfused pancreas of the rat: (△) kainate 10^{-4} M ($n = 4$), (●) 2×10^{-4} M ($n = 6$), (◇) 10^{-3} M ($n = 6$). The insulin output rate (ng min^{-1}) at 45 min for each set of experiments was: 13.3 ± 6.7; 17.2 ± 1.5; 13.2 ± 1.7 respectively. Each point represents the mean with s.e.mean shown by vertical lines.

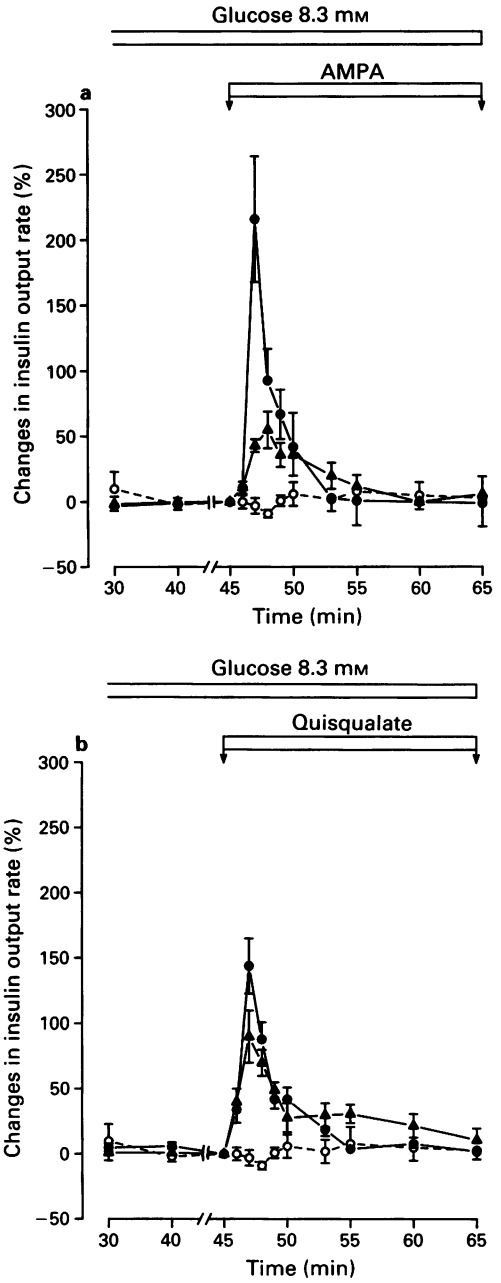


Figure 3 Effects of non-NMDA receptor agonists on insulin secretion from the isolated perfused pancreas of the rat: (a) α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA): (▲) 5×10^{-5} M ($n = 4$) and (●) 2×10^{-4} M ($n = 5$); (b) quisqualate: (▲) 10^{-5} M ($n = 4$) and (●) 5×10^{-5} M ($n = 5$). Each point represents the mean with s.e.mean shown by vertical lines.

The antagonists were introduced 15 min before glutamate or kainate and were present during the 20 min of agonist infusion.

CNQX

6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) is a competitive antagonist of non-NMDA receptors (Honoré *et al.*, 1988). CNQX at 5×10^{-5} M ineffective *per se* on insulin release during the 15 min pretreatment, totally prevented the stimulating effects of both glutamate and kainate (Figure 5a, b).

MK801

(+) -5- Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5, 10-imine ((+)-MK801) is a highly potent and non competitive blocker of NMDA receptors (Wong *et al.*, 1986). (+)-MK801 at 10^{-6} M did not affect the insulin response induced by glutamate or kainate (Figure 5a, b).

Inhibition by quisqualate of the insulin responses to glutamate and kainate (Figure 6)

In order to characterize further the non-NMDA receptor subtype involved in glutamate and kainate effects, we investigated the interactions between these agonists and quisqualate to trigger insulin release. Quisqualate was used at 4×10^{-4} M, a maximally effective concentration.

The responses to glutamate (10^{-3} M) and kainate (4×10^{-4} M) were both inhibited by the simultaneous infusion of quisqualate (Figure 6). Thus, in the presence of quisqualate, the insulin secretory responses to glutamate ($+69 \pm 10\%$) and kainate ($+100 \pm 21\%$) were not significantly different from the maximal response to quisqualate ($+72 \pm 5\%$).

Effect of atropine and tetrodotoxin (TTX) on the insulin response to glutamate and kainate (Figure 7)

We investigated a possible involvement of a nervous component in glutamate effect.

Atropine at 3×10^{-7} M, a concentration used to block acetylcholine-induced insulin release in our preparation (Loubatières *et al.*, 1973), did not significantly modify the responses to glutamate. In addition TTX at 3×10^{-6} M, a concentration used to prevent nerve action potentials (Stag-

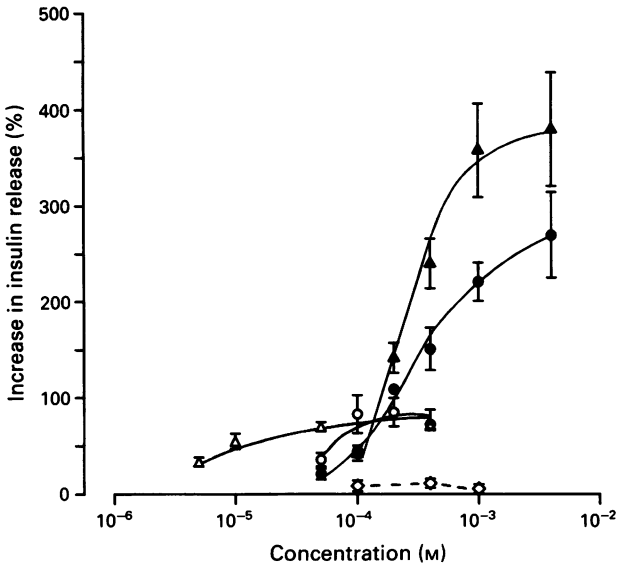


Figure 4 Concentration-response curves for the effects of glutamate (●), kainate (▲), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (○), quisqualate (△) and N-methyl-D-aspartate (NMDA) (◇) on insulin release. Each point represents the mean of 4–6 experiments and vertical lines indicate the s.e.mean.

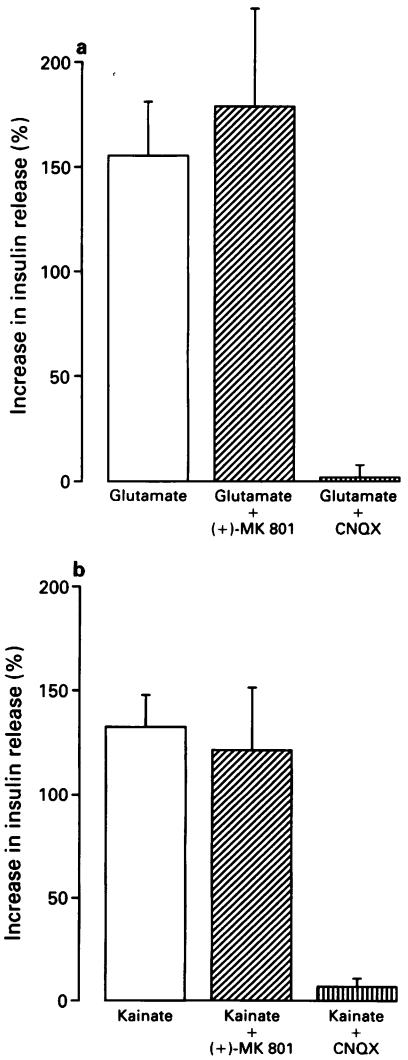


Figure 5 Effects of blockers of glutamate receptors on the insulin response induced by (a) glutamate (4×10^{-4} M) or (b) kainate (2×10^{-4} M). Each column represents the mean of 4–6 experiments and vertical lines indicate s.e.mean.

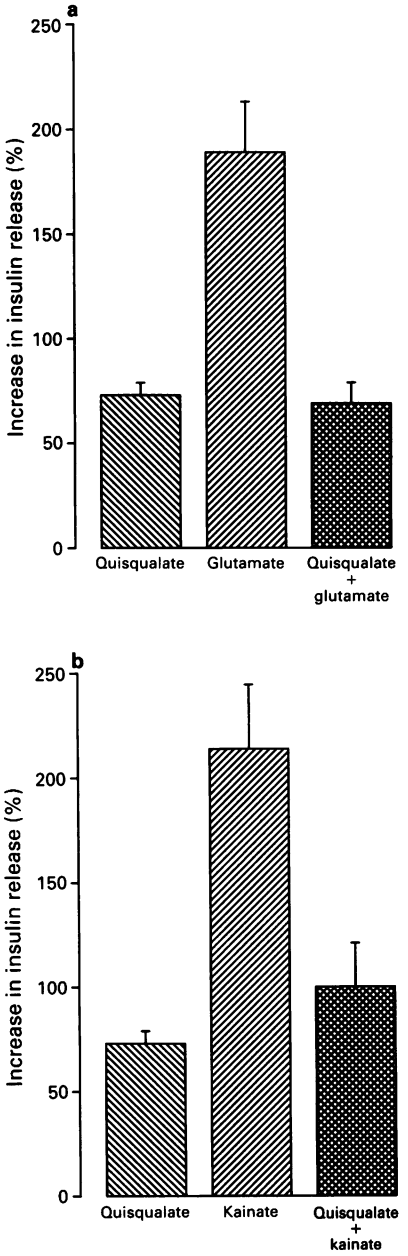


Figure 6 Inhibition by quisqualate of the insulin response induced by (a) glutamate (10^{-3} M) and (b) kainate (4×10^{-4} M). Each column represents the mean of 4–6 experiments and vertical lines indicate s.e.mean.

ner & Samols, 1985a), was ineffective *per se* on insulin release and did not alter glutamate-induced insulin release.

Discussion

The present study shows that L-glutamate transiently stimulates insulin secretion from the rat isolated pancreas. This effect appears to be mediated by a glutamate receptor of the AMPA subtype. This is the first time that the presence of such a glutamate receptor subtype has been reported in the periphery.

The stimulatory effect of glutamate was dependent on the glucose concentration: glutamate stimulated insulin secretion in the presence of a slightly stimulating concentration of glucose (8.3 mM) but not in the presence of a low concentration (2.8 mM). Thus, this amino acid is not an initiator but a potentiator of glucose-induced insulin release.

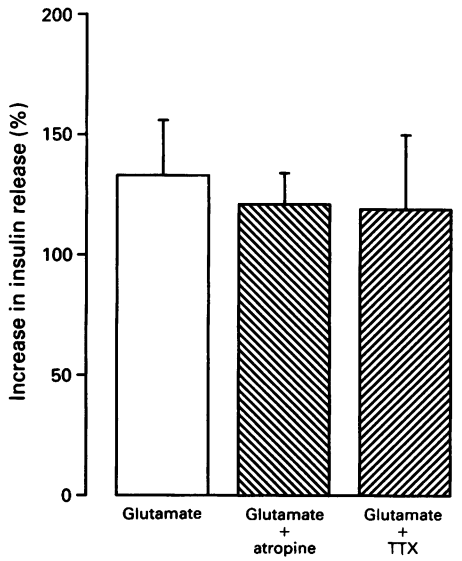


Figure 7 Effect of atropine (3×10^{-7} M) or tetrodotoxin (TTX, 3×10^{-6} M) on the insulin responses induced by glutamate (4×10^{-4} M). Each column represents the mean of 4–6 experiments and vertical lines indicate s.e.mean.

Glutamate has previously been reported not to stimulate insulin secretion, even in the presence of a high glucose concentration, in mouse pancreatic islets (Sehlin, 1972). However these studies were performed during static incubation for 60 min and therefore the transient insulin response observed in our kinetic study might have been blunted in such long-lasting static experiments.

Glutamate induced an immediate, transient insulin response in a concentration-dependent manner in the range 5×10^{-5}–4×10^{-3} M. As this effect was immediate and transient, and occurred at micromolar concentrations, a metabolic action of the amino acid is unlikely. Furthermore glutamate is very poorly taken up by islet cells (Sehlin, 1972).

Glutamate is known to exert excitatory effects in brain by acting on specific receptors classified as NMDA and non-NMDA types, including kainate and AMPA receptors (Watkins *et al.*, 1990). Our data are consistent with a non-NMDA receptor mediating glutamate-induced insulin release. Glycine is known to potentiate the action of NMDA by binding to a regulatory site of the NMDA receptor complex (Johnson & Ascher, 1987). In the presence of glycine (10^{-6} M), NMDA was ineffective whereas the response to glutamate remained unchanged (results not shown). Furthermore, even in the absence of Mg^{2+}, known to block NMDA receptor channels (Nowak *et al.*, 1984), NMDA was unable to stimulate insulin release. The involvement of a non-NMDA receptor was confirmed by the inability of (+)-MK801, a potent antagonist of NMDA receptors (Wong *et al.*, 1986), to block insulin response to glutamate. On the other hand, the three non-NMDA receptor agonists, kainate, quisqualate and AMPA, like glutamate exhibited a stimulating effect on insulin release. Furthermore the competitive and selective antagonist of non-NMDA receptors, CNQX, totally blocked the insulin response to glutamate or kainate.

The pharmacological characteristics of the insulin response induced by glutamate analogues suggest the involvement of an AMPA receptor subtype. The apparent agonist potency order on insulin secretion (quisqualate > AMPA > kainate) is in agreement with the agonist potencies reported for AMPA receptors mediating excitatory responses in brain neurones and for [^{3}H]-AMPA binding sites (see Monaghan *et al.*, 1989). In addition, the concentration-response curves show that quisqualate and AMPA induced lower maximal insulin responses than did kainate or glutamate. The response to quisqualate was not additive to those obtained with glutamate or kainate; in contrast, quisqualate inhibited insulin responses to both glutamate and kainate. Similar inhibition of the response to kainate by quisqualate, but also by AMPA, has been reported in various studies e.g. in cultures of chick motor neurones (O'Brien & Fischbach, 1986), mouse striatal neurones (Pin *et al.*, 1989; Charpentier *et al.*, 1990) and in *Xenopus* oocytes injected with rat brain mRNA (Rassendren *et al.*, 1989). These authors concluded that quisqualate and AMPA behave as apparent partial agonists at a receptor fully activated by kainate; the behaviour as full or partial agonist of kainate and AMPA (or quisqualate) would be due to the fact that they produce non desensitizing and desensitizing responses respectively.

AMPA receptors are widely distributed in mammalian brain, especially in hippocampus, cortex, striatum, septum and the molecular layer of cerebellum (Monaghan *et al.*, 1989; Young & Fagg, 1990). In contrast, AMPA receptors have never been described outside the central nervous system. A glutamate receptor displaying pharmacological characteristics of the NMDA subtype has been reported in isolated ileal longitudinal muscle-myenteric plexus of the guinea-pig (Moroni *et al.*, 1986). The activation of such receptors by glutamate or NMDA led to contraction of the ileum; this response was blocked by tetrodotoxin and a blocker of muscarinic receptors, indicating an action on the myenteric plexus. It is well known that the pancreatic islets are richly innervated by autonomic nerve fibres: parasympathetic nerve stimulation results in an increase in insulin secretion, whereas sympathetic stimulation decreases this secretion (via α_2-adrenoceptors) (see Miller, 1981). The intrapancreatic nervous system appears to be composed of a complex system of connected nerves and ganglia dispersed in the parenchyma. It has been reported that pancreatic ganglia share some functional characteristics with the myenteric plexus (Stagner & Samols, 1985b; 1986). However, unlike results obtained with the ileum, in our study glutamate does not appear to stimulate insulin release by promoting the release of acetylcholine from pancreatic cholinergic nerves, since atropine did not affect the response to the amino acid. In addition, tetrodotoxin, known to block nerve action potentials, did not prevent the insulin response to glutamate. These findings do not provide any evidence that glutamate could stimulate insulin release through an action on the intrinsic pancreatic neurones, and thus a direct action on islet cells appears more likely.

The presence in the pancreas of glutamate receptors mediating insulin release might be of physiological relevance. Indeed, it is noteworthy that glutamate is effective in circulating plasma concentrations (ranging between 30–100 μM) reported in rats (Christophe *et al.*, 1971) and man (Schmid *et al.*, 1989; Stegink *et al.*, 1991). Furthermore, monosodium glutamate is largely used to enhance food flavour. The rapidity in onset and transient nature of insulin response to glutamate in the presence of a physiological stimulating glucose concentration, suggests that this amino acid could act as an amplifier in the initiation of insulin release during food intake.

In conclusion, glutamate stimulates insulin release in rat pancreas, by acting on an excitatory amino acid receptor of the AMPA subtype similar to that described in the central nervous system.

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Cholinoceptor regulation of cyclic AMP levels in bovine adrenal medullary cells

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1 The regulation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels by cholinoceptors has been studied in cultured bovine adrenal medullary cells.

2 Acetylcholine (100 μM), nicotine (10 μM) and dimethylphenylpiperazinium (20 μM) each increased cellular cyclic AMP levels 2 to 4 fold over 5 min in the absence of phosphodiesterase inhibitors. The muscarinic agonist acetyl-β-methylcholine (100 μM) had no effect either on its own or on the response to nicotine. The responses to acetylcholine and nicotine were unaffected by atropine (1 μM) but were abolished by mecamylamine (5 μM).

3 Cellular cyclic AMP increased transiently during continuous exposure to nicotine (1– 20 μM), with the largest response seen after 5 min, a smaller response after 20 min, and no change in cyclic AMP levels seen after 90 or 180 min. The maximal response after 5 min stimulation was seen with 5– 10 μM nicotine and the EC_{50} was about 2 μM. In contrast, extracellular cyclic AMP levels did not change after 5 or 20 min stimulation with nicotine, but increased slightly after 90 min and further after 180 min.

4 The cellular cyclic AMP response to nicotine (10 μM) was unchanged or weakly enhanced in the presence of the unselective phosphodiesterase inhibitor, isobutylmethylxanthine, and was unchanged in the presence of rolipram. Nicotine did not interact synergistically with low concentrations of forskolin. The response was however completely abolished in the absence of extracellular Ca^{2+}.

5 The nicotinic cyclic AMP response was almost abolished by sphingosine (30 μM), which did not inhibit the cyclic AMP response to phorbol-12,13-dibutyrate (PDB). The nicotinic response was reduced by 55% by the calmodulin antagonist W7 at 3 μM, and by >90% at 10 μM W7. At these concentrations, W7 had no effect on the cyclic AMP response to 100 nM PDB. The nicotinic response was also selectively abolished by another calmodulin antagonist, trifluoperazine (10 μM).

6 The results indicate that nicotinic cholinoceptors can increase cyclic AMP production in chromaffin cells by a mechanism that does not directly involve G_S, that is dependent on extracellular Ca^{2+} and that is sensitive to the calmodulin antagonists W7 and trifluoperazine. We propose that a Ca^{2+}/calmodulin-sensitive adenylate cyclase may mediate the nicotinic cyclic AMP response in bovine chromaffin cells.

Keywords: Adrenal medulla; adenosine-3':5'-cyclic monophosphate; cholinoceptors; chromaffin cells; calcium; second messengers

Introduction

During stimulation of secretion from the adrenal medulla, substantial quantities of catecholamines are released; however, little depletion of adrenaline or noradrenaline occurs from the adrenal gland. These findings suggested that, during periods of increased secretion, the adrenal chromaffin cells increase their synthesis of catecholamines to replenish and maintain their secretory reserves (Bygdeman & von Euler, 1958). The coupling between secretion and catecholamine biosynthesis ('stimulation-secretion-synthesis coupling') is likely to be the result of second messengers generated in the chromaffin cells. These act via kinases to regulate the phosphorylation state, and hence the activity, of tyrosine hydroxylase, the rate-limiting enzyme for catecholamine synthesis (George *et al.*, 1989 and references therein).

A number of recent studies have indicated that tyrosine hydroxylase from bovine chromaffin cells can be phosphorylated by several different classes of endogenous kinases present in these cells, including adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent kinase, Ca^{2+}/calmodulin-dependent kinase and protein kinase C (Niggli *et al.*, 1984; Pocotte & Holz, 1986; George *et al.*, 1989). The enzyme can be phosphorylated on a number of distinct sites and several of these sites appear to play a role in regulating the activity of the enzyme (Haycock *et al.*, 1982; Meligeni *et al.*, 1982; Niggli *et al.*, 1984; Pocotte & Holz, 1986; Pocotte *et al.*, 1986; Waymire *et al.*, 1988; George *et al.*, 1989). Furthermore, the temporal pattern of phosphorylation of these different sites is not the same (Waymire *et al.*, 1988), suggesting that different kinases may regulate the activity of the enzyme at different times during the secretory response.

The splanchnic nerve transmitter acetylcholine not only evokes adrenal catecholamine secretion but also increases chromaffin cell tyrosine hydroxylase activity by affecting multiple-site phosphorylation of the enzyme (Masserano & Weiner, 1979; Amy & Kirshner, 1981; Haycock *et al.*, 1982; Meligeni, 1982; Pocotte *et al.*, 1986; Waymire, *et al.*, 1988). Cholinergic stimulation is known to increase cytosolic Ca^{2+} levels, diacylglycerol formation and cyclic AMP levels in chromaffin cells (Shima *et al.*, 1976; Eiden *et al.*, 1984; Pocotte *et al.*, 1986; TerBush & Holz, 1986; Morita *et al.*, 1987; TerBush *et al.*, 1988; Wilson, 1988). Several kinases activated by these second messengers may therefore play a role in mediating cholinergic activation of tyrosine hydroxylase.

Recently, it was observed that vasoactive intestinal polypeptide (VIP) also increases the activity of tyrosine hydroxylase in bovine chromaffin cells (Waymire *et al.*, 1991). This effect was quite distinct from cholinergic stimulation, in that the phosphorylation of only a single site in the enzyme was

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affected. The site was the same as the single site affected by cyclic AMP-dependent protein kinase (George *et al.*, 1989). Since VIP induces a cyclic AMP response in these cells (Wilson, 1988), VIP may act exclusively via cyclic AMP to regulate tyrosine hydroxylase activity.

In view of this, and to gain further insight into the possible contribution of cyclic AMP to the cholinergic regulation of adrenal tyrosine hydroxylase activity, we have investigated the mechanism of cholinoceptor regulation of cyclic AMP levels in bovine chromaffin cells.

Methods

Incubation of bovine cultured adrenal chromaffin cells

Adrenal medullary cells were isolated from bovine adrenal glands by collagenase digestion, purified by Percoll density gradient centrifugation and cultured at a density of 1.25×10^6/well in 6-well plastic culture plates, as described in detail previously (Livett *et al.*, 1987a). Cells prepared in this manner are typically 85% chromaffin cells (Marley *et al.*, 1989).

Cells were cultured for three days before use. They were then given three consecutive incubations at 37°C in 1 ml of buffer comprising (mM); NaCl 154, KCl 2.6, K_2HPO_4 2.15, KH_2PO_4 0.85, $MgSO_4$ 1.18, $CaCl_2$ 2.2 and D-glucose 10, supplemented with 0.5% bovine serum albumin and adjusted to pH 7.4. The first incubation was a 5 min wash and the second a 5, 10 or 15 min equilibration with buffer or appropriate antagonists, as indicated. The third incubation was for the times indicated in the presence of agonists together with antagonists, forskolin or phosphodiesterase inhibitors, where used.

In experiments where Ca^{2+}-free buffer was used, the 2.2 mM $CaCl_2$ in the incubation buffer was replaced with 2.2 mM $MgCl_2$, and all three incubations were performed in Ca^{2+}-free buffer.

Determination of cyclic AMP and catecholamines in cell and medium extracts

Following the third incubation, the incubation medium was removed and the cellular cyclic AMP extracted in acid ethanol. When the incubation medium was also saved for cyclic AMP assay, the medium was also extracted. Extracts were dried down in a vacuum centrifuge, reconstituted in assay buffer and their cyclic AMP content determined by radioimmunoassay. Full details of the extraction and assay of cyclic AMP are given elsewhere (Keogh & Marley, 1991; Marley *et al.*, 1991).

The cyclic AMP radioimmunoassay had an IC_{50} for cyclic AMP of 160 fmol/100 µl sample, and a detection limit of approximately 5 fmol. The assay had less than 0.05% cross-reactivity with cyclic GMP and essentially no (<0.0005%) crossreactivity with ATP, ADP, AMP, adenosine, GTP, GDP, GMP or guanosine. In addition, the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine caused no interference in the assay when used in the experiments at a concentration of 1 mM.

The release of endogenous adrenaline and noradrenaline was studied as described previously (Livett *et al.*, 1987b).

Data presentation and statistics

All results are presented as means ± s.e.mean for the stated number of observations. Due to the variability of basal cyclic AMP levels between cell preparations, results have been normalised to the basal levels for each cell preparation. Statistical significance had been assessed by Student's *t* test for two-sample comparisons, and the modified *t* statistic provided by Fisher's least significant difference (LSD) test, protected by one-way analysis of variance, for multiple comparisons. Interactions between combinations of agonists were assessed by determining whether the observed effect of the combination of drugs lay outside the 95% confidence limits for the expected effect if the two agents were simply additive (see Moeller *et al.*, 1989).

Materials

Acetyl-β-methylcholine chloride, acetylcholine chloride, atropine sulphate, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), 3-isobutyl-1-methylxanthine (IBMX), mecamylamine, nicotine, phorbol-12,13-dibutyrate, phorbol-13-monoacetate and W7 (N-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide HCl) were from Sigma Chemical Co. Forskolin and D-erythrosphingosine (bovine brain, approximately 70% erythro- and 30% threo-) were from Calbiochem. Trifluoperazine dihydrochloride was from Boehringer Mannheim. Rolipram was a generous gift from Dr G. Riisfeldt (Schering Pty. Ltd).

Forskolin, sphingosine and W7 were dissolved in ethanol and IBMX, rolipram and the phorbol esters were dissolved in dimethylsulphoxide before dilution in buffer to their working concentrations. Appropriate solvent controls were performed in every experiment.

Results

Cholinoceptor pharmacology of cyclic AMP response

As shown by others, acetylcholine (100 µM) increased cellular cyclic AMP levels in bovine chromaffin cells (Table 1). This effect was mimicked by the selective nicotinic receptor agonists dimethylphenylpiperazinium (DMPP, 20 µM) and nicotine (10 µM) but not by the muscarinic agonist acetyl-β-methylcholine (100 µM) which had no significant effect. The lack of involvement of muscarinic receptors was confirmed by the lack of effect of atropine on the response to acetylcholine and the complete antagonism of the response by mecamylamine (Figure 1a,b). In addition, the response to nicotine was unaffected by addition of acetyl-β-methylcholine (Figure 1c), indicating muscarinic receptors do not modulate the cyclic AMP response to nicotinic receptor stimulation.

The cellular cyclic AMP response to nicotine showed a steep concentration-dependence, with a maximal response at 5–10 µM and an EC_{50} of around 2 µM (Figure 2a). The response was also transient, with the cellular cyclic AMP levels declining between 5 and 20 min, and returning to basal levels by 90 min (Figure 2a).

Table 1 Effect of cholinoceptor agonists on cellular cyclic AMP levels in bovine adrenal medullary cells

Agonist	*Cellular cyclic AMP levels* (% of basal)	
Acetylcholine (100 µM)	*557 ± 13	(4)
Nicotine (10 µM)	*320 ± 10	(6)
DMPP (20 µM)	*281 ± 6	(3)
Acetyl-β-methylcholine (100 µM)	136 ± 10	(6)

Cells were incubated with the indicated drugs for 5 min. Cyclic AMP levels are expressed as a percentage of the basal levels, which were in the range 65–950 fmol/10^6 cells (mean ± s.e.mean 308 ± 52 fmol/10^6 cells for 22 cell preparations). Data presented are means ± s.e.mean for the indicated number of observations, each from single but different cell preparations and representative of similar results from at least four cell preparations for each drug. Fisher's protected LSD: *$P<0.01$ compared to basal.
DMPP: dimethylphenylpiperazinium.

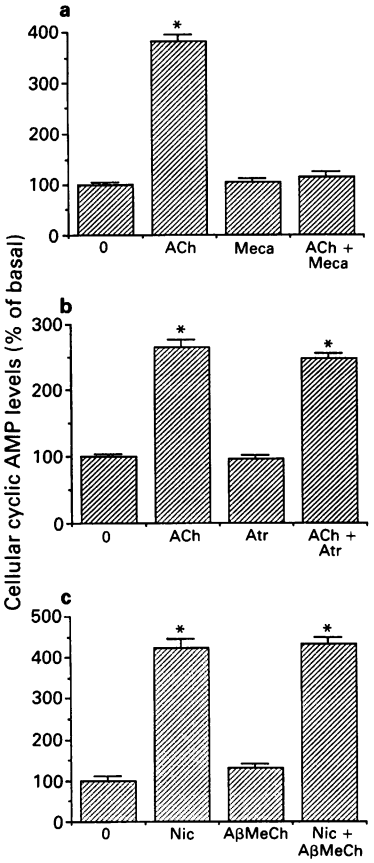


Figure 1 Pharmacology of cholinoceptor-mediated cellular cyclic AMP responses in bovine adrenal medullary cells. (a, b) Cells were pretreated for 15 min with control buffer or with mecamylamine (Meca, 5 μM) or atropine (Atr, 1 μM) before a 5 min incubation with buffer (0), acetylcholine (ACh, 100 μM) or acetylcholine together with the antagonists. (c) Cells were incubated with control buffer (0), nicotine (Nic, 10 μM), acetyl-β-methylcholine (AβMeCh, 100 μM) or both drugs for 5 min. Results are means (s.e.mean shown by vertical bars) for $n = 4-6$; data from a single cell preparation and representative of similar results on four cell preparations. Fisher's protected LSD: (a) $*P<0.01$ compared to all other treatments. (b) $*P<0.01$ compared to basal and atropine alone. (c) $*P<0.01$ compared to basal and AβMeCh alone.

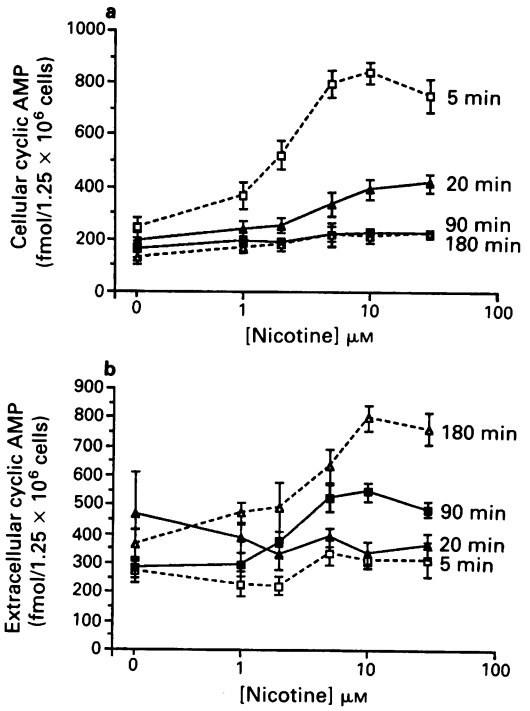


Figure 2 Concentration-dependence and time course of nicotine-induced cellular (a) and extracellular (b) cyclic AMP responses in bovine adrenal medullary cells. Cells were incubated for various times with different concentrations of nicotine. The incubation medium and cells were then extracted separately for cyclic AMP determination. Results are means (s.e.mean shown by vertical bars) for $n = 4$ from a single cell preparation. Similar results were obtained on a second cell preparation.

The mechanism of nicotinic cyclic AMP responses

Nicotine might increase cellular cyclic AMP by increasing cyclic AMP formation, reducing cyclic AMP degradation or by inhibiting cyclic AMP release from the cells.

At the early time points (5–20 min), nicotine had no effect on the export ('egress') of cyclic AMP from the cells into the incubation medium, however after 90 or 180 min significant increases in extracellular cyclic AMP were seen (Figure 2b). These observations indicate that the nicotine-induced increase in cellular cyclic AMP was not due to inhibition of cyclic AMP export from the cells.

Nicotine was still able to increase cellular cyclic AMP levels in bovine adrenal medullary cells after inhibition of all phosphodiesterase isozymes with the non-selective phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) or after inhibition of the cyclic AMP-selective phosphodiesterase isozyme with rolipram (Figure 3a). These findings suggest that nicotine increases cellular cyclic AMP levels by increasing the rate of cyclic AMP formation.

Nicotine gave additive response with IBMX in 5 of 8 cell preparations, and was enhanced by 2–3 fold in the other three. Nicotine gave additive responses with rolipram in 3 of 4 preparations, and was enhanced by only 31% in the fourth. IBMX (1 mM) and rolipram (100 μM) increased basal cyclic AMP levels in all four cell preparations on which they were both tested, by 189 ± 42% and 64 ± 20%, respectively.

To investigate the mechanism by which nicotine increased adenylate cyclase activity, cyclic AMP levels were studied in the presence of both nicotine and the diterpine, forskolin. The latter acts directly on adenylate cyclase to stimulate the enzyme, and has been reported to bind preferentially and activate adenylate cyclase when the adenylate cyclase is bound to the stimulatory G protein G_S (i.e.: during activation of adenylate cyclase by agents acting via G_S, Battaglia *et al.*, 1986; Robinson *et al.*, 1989). Consequently forskolin interacts synergistically with agents that activate adenylate cyclase via G_S (Seamon & Daly, 1983). However, nicotine gave additive responses with a low concentration of forskolin in 4 of 5 preparations and was enhanced by only 25% in the fifth (Figure 3b). In contrast, forskolin enhanced the responses of these cells to the H_2-histamine agonist, dimaprit, by more than 10 fold (Marley *et al.*, 1991) and to 1 μM prostaglandin E_1 by almost 6 fold (Marley, unpublished observations), two agents that act via G_S-coupled receptors in other tissues. This suggests that nicotine increases adenylate cyclase activity in chromaffin cells independently of G_S.

In agreement with a previous study using the non-selective cholinoceptor agonist, carbachol (Pocotte *et al.*, 1986), the cyclic AMP response to nicotine was completely abolished by omitting extracellular Ca^{2+} (Figure 3c), which as expected abolished the nicotine-induced secretion of catecholamines (data not shown). Also in agreement with the previous study, we found that incubation medium collected from cells that had been stimulated with 10 μM nicotine in the presence of Ca^{2+} failed to produce a cyclic AMP response in a second batch of cells in the presence of 5 μM mecamylamine (data not shown). Furthermore, alteration of the incubation volume from 1 ml to 0.5 ml or 2 ml, to change the dilution of any released substances, did not affect the cyclic AMP res-

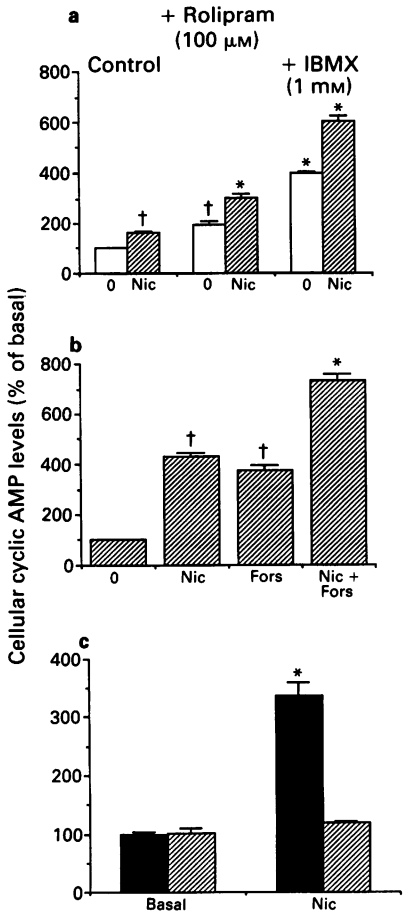


Figure 3 Effect of nicotine (Nic, 10 μM) on cellular cyclic AMP response over 5 min in bovine adrenal medullary cells in the absence and presence of (a) 3-isobutyl-1-methylxanthine (IBMX, 1 mM) or rolipram (100 μM), (b) forskolin (Fors, 0.3 μM) or (c) extracellular calcium (2.2 mM, solid columns). (a, b) Results are means (s.e.mean shown by vertical bars) for $n = 4-6$; data from a single cell preparation and representative of results on four to eight cell preparations (see text). In the results shown, nicotine gave additive responses with rolipram and forskolin and was enhanced 3.2 fold by IBMX, the maximum enhancement observed (see Moeller *et al.*, 1989). (c) In experiments in the absence of extracellular Ca^{2+} (hatched columns), cells received two 5 min washes and a 5 min stimulation in buffer in which the 2.2 mM $CaCl_2$ was replaced with $MgCl_2$. Results are means (s.e.mean shown by vertical bars) for $n = 5-6$, data from a single cell preparation and representative of results on three cell preparations. Fisher's protected LSD: $*P<0.01$ compared to all other treatments; † $P<0.01$ compared with all other conditions except each other.

ponse to nicotine (data not shown). These observations indicate that chromaffin cell products secreted during nicotinic stimulation do not contribute significantly to the cyclic AMP response.

Calcium could regulate adenylate cyclase activity by a number of mechanisms. These include direct allosteric activation via calmodulin and indirect effects mediated via protein kinase C, which is known to be activated by Ca^{2+} in chromaffin cells. The possibility that nicotinic receptors regulate chromaffin cell adenylate cyclase by Ca^{2+}-dependent activation of protein kinase C was studied by examining the effect of the tumour-promoting phorbol ester, phorbol-12,13-dibutyrate (PDB). PDB (100 nM) increased cyclic AMP in these cells to 140 ± 7% of basal ($n = 17$, data pooled from 3 cell preparations, Fisher's protected LSD: $*P<0.01$ compared with basal or phorbol-13-monoacetate), but the inactive phorbol ester phorbol-13-monoacetate (PM) had no effect (101 ± 5% of basal, $n = 19$, data pooled from 3 cell preparations).

When protein kinase C was down-regulated by chronic treatment with a high concentration of PDB (500 nM for 24 h, see Wilson, 1990), the acute cyclic AMP response to PDB was abolished whilst that to nicotine was not (data not shown). The chronic phorbol ester treatment itself, however, caused cellular cyclic AMP levels to double, which made it difficult to conclude whether protein kinase C had been effectively down-regulated by this procedure.

To investigate further a possible role for protein kinase C in the cyclic AMP response to nicotine, putative protein kinase C inhibitors were used. H7 (20 μM) did not inhibit the response to 10 μM nicotine in each of 3 cell preparations, and it also failed to inhibit the response to 100 nM PDB in four preparations (data not shown). In view of the poor selectivity of H7 for protein kinase C over other kinases *in vitro* (Hidaka *et al.*, 1984) and its ability to inhibit equally responses to forskolin and phorbol esters in chromaffin cells (Waymire *et al.*, 1991), higher concentrations were not tested here.

To investigate the possible involvement of calmodulin in the nicotinic cyclic AMP response, the selective calmodulin antagonist, W7, was used. At 100 μM, W7 had no effect on resting cellular cyclic AMP levels and completely abolished the cyclic AMP response to 10 μM nicotine (Figure 4a). Although preferentially an inhibitor of calmodulin, due to its lipophilic structure W7 can also inhibit protein kinase C (Hidaka & Hagiwara, 1987). Consequently, the concentration-dependence of the effect of W7 on nicotine- and PDB-induced cyclic AMP response was investigated (Figure 4a). The nicotinic response was inhibited by >90% at 10 μM W7 and by 55% at 3 μM W7. At these concentrations, W7 had no effect on the cyclic AMP response to PDB, which was only inhibited at much higher W7 concentrations.

Another calmodulin antagonist, trifluoperazine (10 μM), also completely blocked the cyclic AMP response to nicotine without affecting either basal cyclic AMP levels or the response to PDB (Figure 4b). Sphingosine, a kinase inhibitor with some selectivity for protein kinase C, failed at 30 μM to affect the cyclic AMP response to PDB but reduced the response to nicotine by 83% (mean of three cell preparations, Figure 4c).

Discussion

Cholinoceptor-mediated cyclic AMP responses in adrenal medullary cells

Previous studies have found conflicting evidence on the ability of cholinoceptors to increase cyclic AMP levels in chromaffin cells. A number of authors found that carbachol or acetylcholine had no effect on adenylate cyclase activity in membranes prepared from bovine adrenal medulla (Serck-Hanssen *et al.*, 1972; Hurko *et al.*, 1974; Boonyaviroj & Gutman, 1977) while others found no effect of carbachol, acetylcholine, nicotine or DMPP on cyclic AMP levels in slices or isolated cells from this tissue (Shima *et al.*, 1976; Niggli *et al.*, 1984; Chern *et al.*, 1988; Marriott *et al.*, 1988). In contrast, several reports have recorded robust cyclic AMP responses to cholinoceptor agonists in slices of bovine adrenal medulla or in cultured bovine chromaffin cells (Shima *et al.*, 1976; Eiden *et al.*, 1984; Pocotte *et al.*, 1986; Morita *et al.*, 1987; Wilson, 1988). Furthermore, cholinoceptor cyclic AMP responses have also been reported in rat, cat and dog adrenal medulla (Guidotti & Costa, 1973; Jaanus & Rubin, 1974; Shima *et al.*, 1978; Tsujimoto *et al.*, 1980). The reason for the varied results on bovine adrenal medullary preparations is not clear.

In the present study using bovine cultured chromaffin cells, we found a reproducible cellular cyclic AMP response to nicotine, DMPP and acetylcholine, although as noted by others (Tsujimoto *et al.*, 1980; Pocotte *et al.*, 1986; Morita *et al.*, 1987), this reponse was transient: at times longer than

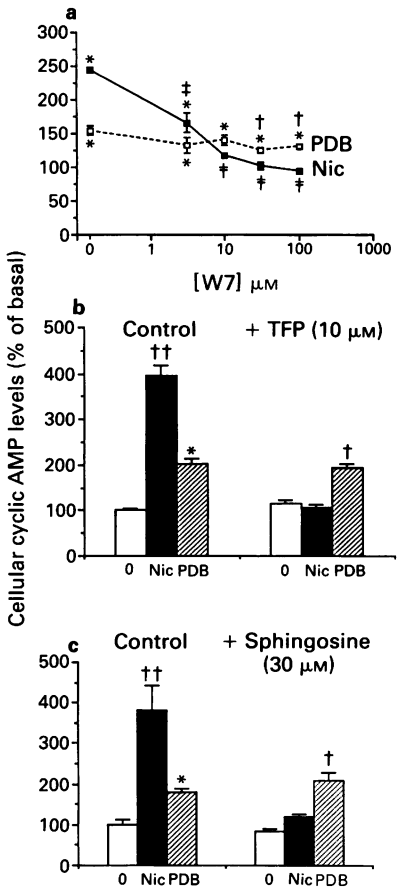


Figure 4 Effect of (a) W7 (3–100 μM), (b) trifluoperazine (TFP, 10 μM) and (c) sphingosine (30 μM) on the cellular cyclic AMP response of bovine adrenal medullary cells to nicotine (Nic, 10 μM) or phorbol-12,13-dibutyrate (PDB, 100 nM in (a), 300 nM in (b) and (c)). Cells were pretreated for 10 or 15 min with the indicated concentrations of W7, TFP or sphingosine and then incubated for 5 min with nicotine (solid symbols, solid columns) or PDB (open symbols, hatched columns) together with W7, TFP or sphingosine. Results are means (s.e.mean shown by vertical bars) for $n = 4-6$, data from a single cell preparation and representative of similar results from (a) two or (b, c) three cell preparations. Fisher's protected LSD: (a) $*P<0.05$ compared to W7 alone, † $P<0.05$ compared with PDB alone, ‡ $P<0.01$ compared to nicotine alone. W7 alone at concentrations up to 100 μM had no effect on cellular cyclic AMP levels. (b) $*P<0.01$ compared to all treatments except PDB with TFP, † $P<0.01$ compared to all treatments except PDB alone, †† $P<0.01$ compared to all other treatments. (c) $*P<0.05$ compared to basal, $P<0.01$ compared with all other conditions except PDB with sphingosine; † $P<0.01$ with all other conditions except PDB alone; †† $P<0.01$ compared to all other conditions.

20 min cholinoceptor stimulation no longer increased cellular cyclic AMP levels. Consequently, the failure to observe a cyclic AMP response in some studies may have been due to the incubation period chosen. Shima *et al.* (1976) found they needed periods longer than 20 min before they saw any cyclic AMP response. However, they used slices of bovine adrenal medulla, which may only allow slow penetration of agonists into the tissue. The transient nature of the chromaffin cell cholinoceptor cyclic AMP response is in direct contrast to that produced by VIP (Wilson, 1988) and forskolin (Marley, Thomson & Jachno, unpublished observations) which are maintained for many hours.

Our results showing no synergism between nicotine and forskolin (see below), and the complete Ca^{2+}-dependence of the nicotinic cyclic AMP response suggest strongly that the nicotinic cholinoceptors do not act on adenylate cyclase via a G_S protein, but by a mechanism involving Ca^{2+} entry. Consequently, the failure to detect any effect of cholinoceptor stimulation on adenylate cyclase activity in adrenal medullary membrane preparations is not surprising (Serck-Hanssen *et al.*, 1972; Hurko *et al.*, 1974). Acetylcholine did however increase adenylate cyclase activity in intact rat adrenal glands (Shima *et al.*, 1978). Marriott *et al.* (1988) reported that nicotine had no effect on bovine chromaffin cell cyclic AMP levels on its own, but augmented the response to low concentrations of forskolin. The reason for the difference between results of that study and the present observations is not known.

The pharmacology of cholinoceptor cyclic AMP responses in bovine chromaffin cells has not been investigated in detail previously. Although carbachol, acetylcholine and DMPP have all been reported to induce a cyclic AMP response in these cells, a possible involvement of muscarinic receptors was not excluded (Eiden *et al.*, 1984; Pocotte *et al.*, 1986; Morita *et al.*, 1987). Wilson (1988) observed that although muscarine had no effect on its own, it enhanced the cyclic AMP response to VIP. The effect of muscarinic receptors on the nicotinic cyclic AMP response was not investigated, although muscarinic receptors can modulate other responses to nicotinic receptors in these cells (see Forsberg *et al.*, 1986). In the present study, muscarinic receptors were found not to contribute to the cyclic AMP response to acetylcholine (Figure 1a,b) or to modulate the cyclic AMP response to nicotine (Figure 1c), suggesting that the cholinoceptor cyclic AMP response is solely nicotinic.

The mechanism of nicotinic receptor-mediated cyclic AMP responses in bovine adrenal medullary cells

Following the rise in cellular cyclic AMP, nicotinic stimulation was found to produce a delayed increase in cyclic AMP release suggesting that the cellular cyclic AMP response is not due to inhibition of cyclic AMP release. The phenomenon of evoked cyclic AMP release was noted over 25 years ago by Davoren & Sutherland (1963) and has been reported from rat, cat and dog adrenal glands and from bovine chromaffin cells (Jaanus & Rubin, 1974; Shima *et al.*, 1978; Tsujimoto *et al.*, 1980; 1986; Morita *et al.*, 1985; Chern *et al.*, 1988). Whether agonist-induced increases in cyclic AMP export from cells is simply a passive event secondary to the increase in cellular levels or due to a more active regulatory mechanism is not known.

The failure of phosphodiesterase inhibition with high concentrations of IBMX or rolipram to prevent the cyclic AMP response to nicotine, together with the finding that nicotine does not inhibit cyclic AMP release from the these cells, suggests that nicotine increases cellular cyclic AMP levels by increasing the rate of cyclic AMP formation (i.e. by increasing adenylate cyclase activity). It was noteworthy that the cyclic AMP response to nicotine was often not enhanced significantly by phosphodiesterase inhibition with IBMX or rolipram (Figure 3a). This may have been because the levels of cyclic AMP generated by nicotinic stimulation in some cell preparations were substantially below the K_m of the phosphodiesterases, although this appears unlikely, since IBMX and rolipram both increased basal cyclic AMP levels in all cell preparations tested. Alternatively, the cyclic AMP generated by nicotine may be in an intracellular location protected from rapid degradation by phosphodiesterases.

The lack of synergy with forskolin (Figure 3b), is in contrast with the results with histamine H_2-agonists (Marley *et al.*, 1991) and prostaglandin E_1 (agents that act through G_S in other tissues), and suggests the nicotinic activation of adenylate cyclase is indirect and not via a G_S protein (but see Marriott *et al.*, 1988). This is supported by the complete dependence of the nicotinic cyclic AMP response on extracellular Ca^{2+} (see also Pocotte *et al.*, 1986). In parallel studies we have found that Ca^{2+} entry into chromaffin cells is a sufficient and adequate stimulus to increase cyclic AMP for-

mation, whether it is induced by nicotine, histamine H_1-agonists, K^+ depolarization or veratridine (Keogh & Marley, 1991; Marley *et al.*, 1991).

Calcium is known to affect a number of second messenger transduction systems in chromaffin cells. It activates phopholipase C which generates inositol phosphates (Eberhard & Holz, 1987) together with diacylglycerol, an activator of protein kinase C. It also activates protein kinase C directly, by inducing translocation of the enzyme to the plasma membrane (TerBush & Holz, 1986; TerBush *et al.*, 1988). These effects are also produced by nicotinic stimulation in a Ca^{2+}-dependent manner (TerBush & Holz, 1986; Eberhard & Holz, 1987, TerBush *et al.*, 1988). Consequently, the Ca^{2+}-dependent nicotinic activation of adenylate cyclase could be mediated by protein kinase C. The ability of phorbol esters that activate protein kinase C to elevate chromaffin cell cyclic AMP levels supports this explanation. Protein kinase C has been reported to enhance cyclic AMP formation in a number of other cell types (see Yoshimasa *et al.*, 1987; Choi & Toscano, 1988).

Attempts to down-regulate protein kinase C by chronic treatment of the cells with high PDB concentrations (see Wilson, 1990), resulted itself in a two fold increase in cellular cyclic AMP levels. It was therefore not possible to determine whether the subsequent lack of effect of acute PDB stimulation was due to down-regulation of protein kinase C or occlusion of the PDB effect. Highly selective, cell-permeant inhibitors of protein kinase C are not yet commercially available and attempts to inhibit the cyclic AMP response to PDB with low concentrations of H7 were not successful (see Wright & Hoffman, 1980).

In contrast, the calmodulin antagonist, W7, almost completely inhibited the cyclic AMP response to nicotine at concentrations that did not reduce the response to PDB. W7 inhibits Ca^{2+}/calmodulin-dependent kinases with an IC_{50} of around 10 μM while it inhibits protein kinase C with an IC_{50} of 60 μM (Kuo *et al.*, 1984). The selective inhibition of the nicotinic cyclic AMP response over the phorbol ester response at W7 concentrations of 10 μM and below is compatible with the suggestion that nicotine is acting through a Ca^{2+}/calmodulin-dependent process. This conclusion is supported by the selective inhibition of the nicotinic cyclic AMP response by trifluoperazine, another calmodulin antagonist. Although sphingosine has been reported as an inhibitor of phorbol ester activation of protein kinase C (Hannun *et al.*, 1986), we found that at 30 μM it had no effect on the cyclic AMP response to PDB but almost completely inhibited that to nicotine (Figure 4c). Although the mechanism of inhibition of the nicotinic response by sphingosine is not clear, it is evident that it blocks the nicotinic response at concentrations at which protein kinase C is not inhibited.

Since a Ca^{2+}/calmodulin-dependent isozyme of adenylate cyclase has been identified in bovine adrenal medulla (LeDonne & Coffee, 1980), it is possible that nicotine activates this isozyme allosterically, following Ca^{2+} entry and association with calmodulin, to produce its cyclic AMP response. The present data, however, do not rule out an involvement of protein kinase C in the cyclic AMP response to nicotinic agonists, in addition to the role of Ca^{2+}/calmodulin.

The present study suggests that nicotinic receptors mediate their cyclic AMP response secondarily to inducing Ca^{2+} entry into chromaffin cells. The contribution of this secondary cyclic AMP response to the phosphorylation and activation of tyrosine hydroxylase induced by acetylcholine in chromaffin cells remains to be determined.

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Pharmacological characterization of non-NMDA subtypes of glutamate receptor in the neonatal rat hemisected spinal cord *in vitro*

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1 A grease-gap technique was used to record depolarizing responses to α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA), kainate and N-methyl-D-aspartate (NMDA) in the hemisected spinal cord of the neonatal rat. The pharmacology of non-NMDA subtypes of glutamate receptor was investigated with the novel quinoxalinedione, 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo (F)-quinoxaline (NBQX) and with a series of barbiturates.

2 NBQX antagonized AMPA- and kainate-, but not NMDA- induced depolarizations. The near parallel shifts of the major part of the dose-response curves for AMPA and kainate by NBQX gave pA_2 values (± s.e.) of 6.7 ± 0.2 and 6.8 ± 0.2 respectively, consistent with a common site of action for these two agonists.

3 Below the 50% level at which these pA_2 values were calculated, however, an NBQX-resistant plateau was seen within the kainate, but not the AMPA, dose-response curve.

4 In decreasing order of potency, methohexitone, secobarbitone, thiopentone, pentobarbitone and phenobarbitone preferentially reduced kainate-, rather than AMPA- and NMDA-, induced depolarizations. Methohexitone was also the most selective with IC_{50}s against kainate, AMPA and NMDA of 31 ± 7, 172 ± 47 and >200 μM respectively.

5 The NBQX-resistant plateau seen within the kainate dose-response curve was reduced by methohexitone. Kainate antagonism by methohexitone was not reduced by 50 μM picrotoxin.

6 We conclude that, while mixed agonist actions may hamper demonstration of antagonist selectivity, depolarizations induced by the non-NMDA ionotropic agonists, AMPA and kainate, are mediated in part via distinct receptors.

Keywords: Glutamate receptors; kainate; α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA); 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo-(F)-quinoxaline; barbiturates; rat spinal cord *in vitro*

Introduction

On the basis of agonist and antagonist studies, the post-synaptic ionotropic receptors in the mammalian central nervous system for the excitatory amino acid transmitter, L-glutamate, are now divided into N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-isoxazole-4-proprionate (AMPA, formerly quisqualate) and kainate subtypes (Watkins *et al.*, 1990).

The NMDA receptor subtype is clearly a discrete entity, with well-documented selective antagonists, modulators and channel blockers, (Watkins & Collingridge, 1989). Pharmacological differentiation between responses to kainate and AMPA has, however, proved difficult. Early electrophysiological studies supported the concept of separate receptors since glutamate diethyl ester (GDEE) inhibited AMPA but not kainate-induced responses in the cat and rat spinal cord *in vivo* (Krogsgaard Larsen *et al.*, 1980; McLennan & Liu, 1982) whereas γ-D-glutamyl-aminomethyl-sulphonate (GAMS) had the reverse selectivity (Davies & Watkins, 1985). The lack of potency of these antagonists and their poor selectivity *in vitro* have, however, both limited their usefulness and been taken as evidence for a single receptor class mediating the effects of AMPA and kainate. Initial kinetic studies showing different rates of desensitization for AMPA and kainate were also taken as evidence for separate receptors (Kiskin *et al.*, 1986; Mayer & Westbrook, 1987) but the more recent observations of cross-desensitization between these agonists indicate a common site of action (Kiskin *et al.*, 1990; Patneau & Mayer, 1991). Such results from hippocampal neurones in culture, however, do not exclude the possibility of separate subclasses of receptors at other neuronal sites.

Studies with [^{3}H]-AMPA and [^{3}H]-kainate indicate high and low affinity binding sites for each agonist with distinct structure activity requirements at the high affinity sites (Foster & Fagg, 1984; Honore & Drejer, 1988; Monaghan *et al.*, 1989). Anatomical distribution of these AMPA and kainate binding sites in brain is also quite different (Greenmayre *et al.*, 1983; Monaghan *et al.*, 1983). Despite this evidence for distinct binding sites, kainate readily displaces high affinity [^{3}H]-AMPA binding whereas the reverse is not the case (Krogsgaard-Larsen *et al.*, 1980; Honore & Drejer, 1988).

Other biochemical studies support the idea of a common receptor subtype mediating some of the actions of AMPA and kainate; for example, quisqualate and AMPA reduce kainate-induced [^{3}H]-aspartate and [^{3}H]-GABA release from cultured cerebellar granule and striatal neurones respectively (Gallo *et al.*, 1990; Pin *et al.*, 1989).

The cloning of glutamate receptor subunits and their expression in *Xenopus* oocytes initially supported the idea of a unitary non-NMDA receptor (Hollmann *et al.*, 1989). More recently, however, with the identification of more glutamate receptor genes and their expression both singly and in various combinations, AMPA- or kainate-preferring receptor channel complexes have been described (Keinanen *et al.*, 1990; Egebjerg *et al.*, 1991; for review see Dingledine, 1991).

The major breakthrough in differentiating NMDA receptors from other glutamate receptor subtypes was the elucidation of selective antagonists (Watkins & Evans, 1981). Thus

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the search for potent and selective non-NMDA antagonists assumes extreme importance in resolving the possible differentiation between AMPA and kainate receptor subtypes. The selectivity of the 6,7-dinitro- and 6-cyano-7-nitroquinoxalinediones (DNQX and CNQX respectively) for [^{3}H]-AMPA, rather than [^{3}H]-kainate, binding has parallels in some functional studies, e.g. depolarization of cortical slices (Fletcher *et al.*, 1988) and release of GABA from cortical cultures (Honore *et al.*, 1988), but not in others, e.g. excitation of spinal neurones *in vivo* (Honore *et al.*, 1988) or *in vitro* (Birch *et al.*, 1988a). Since the maximum selectivity achieved with CNQX and DNQX of approximately 5 fold is clearly insufficient to be very useful particularly in functional studies, the demonstration that a new quinoxalinedione, 2,3-dibydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline(NBQX) displaced [^{3}H]-AMPA binding 30-times more potently than that of [^{3}H]-kainate binding was thus of considerable interest (Sheardown *et al.*, 1990). In our experiments, responses to AMPA and kainate were not, however, distinguished by this antagonist when tested on rat spinal cord and brainstem neurones *in vivo*, but, on rat neocortical slices *in vitro*, NBQX was about 30 fold more potent as an antagonist of AMPA than of kainate (Lodge *et al.*, 1991). We have therefore used an *in vitro* spinal cord preparation (Birch *et al.*, 1988b) to investigate this anomaly.

In addition to their well documented facilitatory action at γ-aminobutyric acid (GABA) receptors, barbiturates are also known to reduce glutamatergic neurotransmission (for review see Simmonds & Horne, 1988). Pharmacological studies have largely focussed on barbiturate antagonism of the non-NMDA agonist, quisqualate. For example, a series of barbiturates produced rightward shifts of quisqualate dose-response curves consistent with a competitive action at low doses and non-competitive at high doses (Horne & Simmonds, 1986), although earlier studies on agonist stimulated sodium flux showed no specificity of barbiturates for quisqualate relative to kainate (Teichberg *et al.*, 1984). A report by Frandsen *et al.* (1990) that kainate-induced cytotoxicity in culture was selectively reduced by phenobarbitone indicated the possible value of reassessing the effects of barbiturates on subtypes of glutamate receptor.

Some of the data discussed here have been presented elsewhere in preliminary form (Zeman & Lodge, 1991a, b).

Methods

Dissection and tissue preparation

Following the methods of Birch *et al.* (1988b), 2–7 day-old Sprague Dawley or Wistar rats were decapitated and the spinal cords exposed from the ventral side by removal of overlying internal organs and vertebral bodies. By gentle lifting at the rostral end, while the ventral and dorsal roots were cut, the cords were freed and placed in Krebs solution (of composition (mM): NaCl 118, $NaHCO_3$ 25, glucose 11.1, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 1.25, equilibrated with 95% O_2/5% CO_2 to give pH 7.4 at room temperature) in a Petri dish, any remaining roots trimmed off and a midline hemisection made.

The hemicord was placed in a two compartment bath (Harrison & Simmonds, 1985) with a greased barrier across the thoraco-lumbar region. The rostral end of the cord was kept immersed in Krebs solution, all drug additions being made to the continuously (2 ml min^{-1}) superfused caudal end, which was less likely to have suffered damage during the excision procedure and furthermore contained a large number of neurones in the lumbar enlargement.

Pharmacological investigation and recording of responses

In preliminary experiments, unlike Birch *et al.* (1988b), we did not find that semi-cumulative dose cycles (3 min agonist pulses with 1.5 min intervals) produced repeatable responses or that their amplitudes were as large as those seen when longer intervals elapsed between agonist applications. Agonists were, therefore, applied to the lumbar cord at 20 min intervals as 5 ml aliquots of the superfusion medium. Tetrodotoxin had no effect on the nature or amplitude of responses to depolarizing agonists and so was not routinely included in the superfusate.

Agonist-induced depolarizations were detected as changes in d.c. potential across the grease-seal via Ag/AgCl electrodes in each compartment, displayed continuously on a chart recorder following amplification (× 100) and filtering (d.c.-5 Hz) and measured as peak amplitudes.

Full agonist dose-response curves (AMPA 316 nM–20 μM; kainate 316 nM–30 μM; NMDA 1–40 μM) or individual agonist doses (AMPA 10 μM; kainate 5 μM or 10 μM; NMDA 10 or 20 μM) were tested before the addition of antagonists to the superfusion medium, after which an equilibration period of at least 20 min was allowed before agonists were retested. After 1 h wash, recovery was assessed.

All studies were performed on at least two different days and four hemicords.

Source of compounds

AMPA and quisqualate were obtained from Tocris Neuramin, NBQX from Novo Nordirsk, thiopentone from C-Vet, methohexitone from Eli Lilly Co. and all other compounds from Sigma Chemical Co. Ltd.

Results

Depolarizations evoked by agonist superfusion of the hemisected cord were reproducible and of similar amplitude to those seen in the cortical wedge preparation (Harrison & Simmonds, 1985; Fletcher *et al.*, 1988). As with the cortical wedge, NMDA, AMPA and kainate were approximately equipotent and high doses of each agonist led to irreversible reductions in amplitude of all subsequent agonist responses, which is presumably a reflection of their excitotoxic properties (Olney, 1969). For this reason, maximum concentrations of agonist used in control conditions were AMPA 20 μM, kainate 30 μM and NMDA 40 μM. Depolarizing response amplitudes obtained from these doses of agonist were used as the 100% value for the normalization of dose-response curves.

Antagonism of AMPA, kainate and NMDA by NBQX

NBQX (1 μM) reduced responses to AMPA and kainate while those to NMDA were largely unaffected. Dose-dependent and reversible rightward shifts of the AMPA and kainate dose-response curves by 100 nM to 3.16 μM NBQX were seen (Figure 1). These shifts were near parallel for the AMPA curves but, for kainate, although the upper two thirds of the curve appeared to be shifted in parallel, there was also an NBQX-resistant component within the dose-response curve (Figure 1b). Schild plot slopes, calculated at the 50% maximum response level, were near unity for both AMPA (0.95 ± 0.29) and kainate (0.97 ± 0.26) and the resultant pA_2 values (± s.e.) were 6.7 ± 0.2 and 6.8 ± 0.2 for NBQX against AMPA and kainate respectively. Such results are consistent with competitive antagonism by NBQX at a single receptor subtype shared by AMPA and, in the main, by kainate.

The NBQX-resistant component of the kainate dose-response curve produced a plateau at about 33% of the normalized control response (Figure 1b). This is most obvious in the presence of 3.16 μM NBQX, the highest concentration used, when 40–320 μM kainate produced a stable level of depolarization. This part of the dose-response curve is at least 10 times less sensitive to NBQX than the

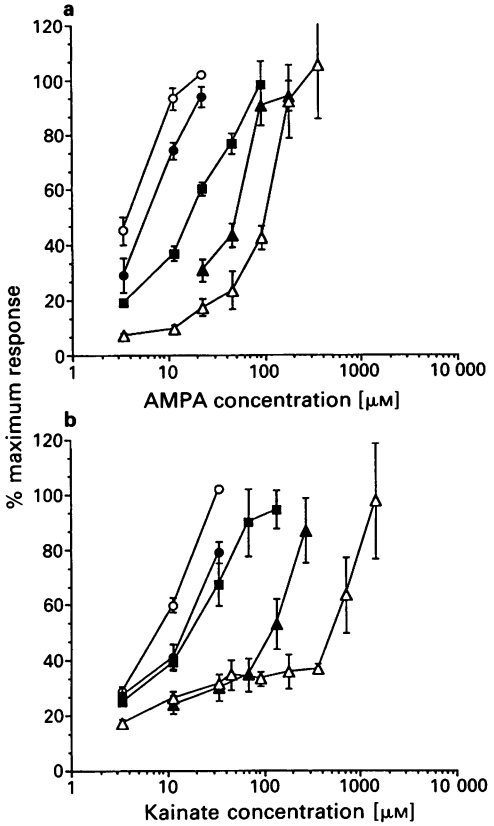


Figure 1 Effects of 100 nM to 3.16 μM 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)-quinoxalone (NBQX) on dose-response curves of the neonatal rat spinal cord to α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) and kainate. In (a) the control AMPA curve (○) is shifted to the right by NBQX at 100 nM (●), 316 nM (■), 1 μM (▲) and 3.16 μM (△). In (b) rightward shifts of the control kainate dose-response curve (○) are produced by NBQX at 100 nM (●), 316 nM (■), 1 μM (▲) and 3.16 μM (△). An NBQX-resistant component is clear in the presence of 3.16 μM NBQX (△), yielding a plateau at about 33% of the normalized maximum response. Ordinate scale: amplitude of depolarizing response expressed as a percentage of the response to (a) AMPA 20 μM and (b) kainate 30 μM. Abscissa scale: agonist concentration in μM.

remainder. No such plateau was seen within the AMPA dose-response curve (Figure 1a).

Antagonism of AMPA, kainate and NMDA by barbiturates

Following the report from Frandsen *et al.* (1990), we initially tested 10 and 100 μM phenobarbitone against single responses to NMDA (20 μM), AMPA (10 μM) and kainate (10 μM) and found a preferential reduction of that to kainate (Figure 2). The response to kainate recovered after 1 h superfusion in drug-free medium.

Five other barbiturates were tested similarly to ascertain their potency and selectivity relative to phenobarbitone. Table 1 shows the results obtained; with each barbiturate, responses to kainate were more sensitive than those to AMPA. Methohexitone was the most potent and selective, and hence was used for all further pharmacokinetic studies of responses to AMPA and kainate. It should be noted that NBQX has the reverse selectivity to barbiturates against these concentrations of AMPA and kainate.

Methohexitone, 10, 31.6 and 100 μM caused dose-dependent rightward shifts of the kainate dose-response curve, while smaller and less dose-dependent shifts of the AMPA dose-response curve were seen with 31.6 and 100 μM methohexitone (Figure 3). The antagonism was not clearly

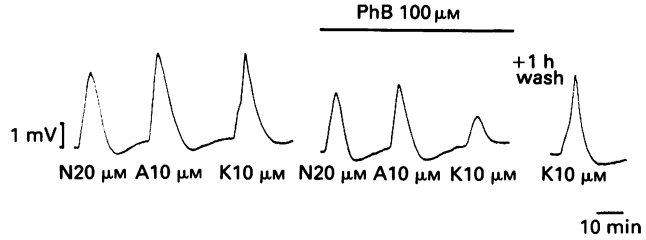


Figure 2 Selective effect of phenobarbitone (PhB, 100 μM) on responses to kainate (K, 10 μM), rather than those to α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (A, 10 μM) or N-methyl-D-aspartate (N, 20 μM) in the neonatal rat spinal cord. Phenobarbitone was superfused for 20 min before the start of the middle trace, and artificial CSF for 60 min before the right hand trace. Calibration: amplitude in mV; time in min.

competitive, with the rightward shifts being non-parallel, although the Schild plot slopes were near unity. Thus, apparent pA_2 values for methohexitone, calculated at the 25% and 50% of maximum response levels were, respectively, 4.5 and 3.3 against kainate and 2.8 and 3.1 against AMPA. Consistent with the selectivity study showing phenobarbitone to only weakly antagonize NMDA (Figure 2), methohexitone (31.6 μM) did not greatly affect the full dose-response curve to this agonist (not illustrated).

Picrotoxin (50 μM) did not reverse the effect of methohexitone on the kainate dose-response curve which, in conjunction with the observation that methohexitone application did not change the basal level of depolarization, strongly suggests the effect of methohexitone to be independent of GABA-potentiation.

Effect of methohexitone on NBQX-insensitive responses to kainate

Demonstration of an NBQX-resistant plateau within the kainate dose-response curve, coupled with the ability of methohexitone to decrease preferentially responses to this agonist, led us to assess the effect of combined application of these antagonists. Figure 4a shows that 31.6 μM methohexitone reduced the NBQX-resistant plateau of the kainate dose-response curve, while in Figure 4b, application of methohexitone to the AMPA dose-response curve already displaced to the right by NBQX did not elicit a further rightward shift.

Discussion

This study was an attempt to investigate the anomaly in our previous results between spinal cord *in vivo* and cerebral cortex *in vitro* with respect to the selectivity of NBQX as an antagonist at the non-NMDA subtypes of glutamate receptors (Lodge *et al.*, 1991). On the one hand, the present results on the spinal cord *in vitro*, showing that, for the major part of the dose-response curves, the depolarizing actions of kainate and AMPA are equally sensitive to NBQX, is in agreement with the failure of NBQX to distinguish between responses to these two agonists on spinal neurones *in vivo*. The similarity in calculated NBQX pA_2 values against kainate and AMPA suggests that the major action of these two agonists on the hemisected cord is mediated by a common receptor subtype. On the other hand, the finding that part of the kainate dose-response is more resistant to NBQX is reminiscent of the previous results from cortical slices *in vitro* and is consistant with there being a further, distinct, receptor subtype via which kainate is able to produce depolarization in both these tissues. This interpretation of our data is supported by the observation that barbiturates selectively affect that part of the kainate dose-response curve

Table 1 Barbiturate IC_{50}s *vs.* responses to α-amino-3-hydroxy-5-methyl-osoxazole-4-propionate (AMPA) 10 μM and kainate 5 μM

Barbiturate	*IC_{50} vs. AMPA*	*IC_{50} vs. kainate*	*Selectivity ratio*
Methohexitone	171.5 ± 47.4	31.1 ± 7.2	5.5
Secobarbitone	114.6 ± 25.6	54.2 ± 8.8	2.1
Thiopentone	102.5 ± 13.0	63.5 ± 11.6	1.6
Pentobarbitone	213.1 ± 38.9	80.4 ± 14.9	2.7
Phenobarbitone	882.7 ± 319	188.3 ± 42.5	4.7
NBQX	≈200 nM	≈3.16 μM	

IC_{50} values are μM ± s.d. The drugs are listed in descending order of potency against responses to kainate and the selectivity ratio indicates the degree of differential sensitivity of kainate and AMPA to antagonism. NBQX: 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)-quinoxalone.

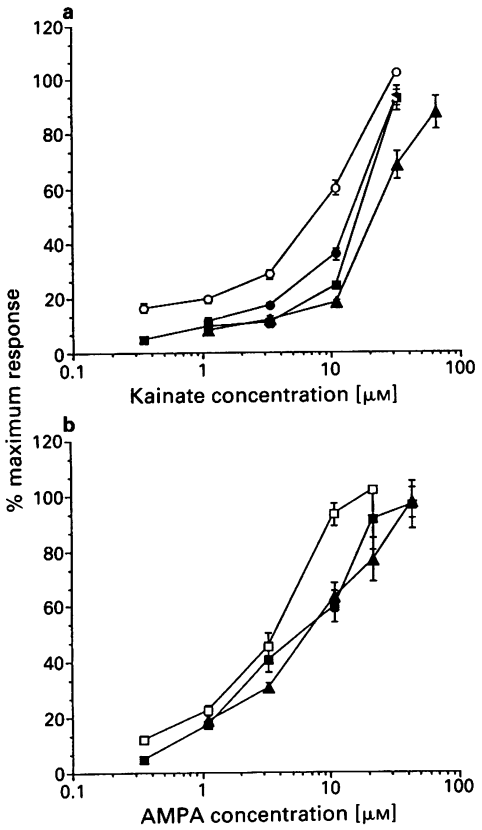


Figure 3 Effects of methohexitone (10–100 μM) on the dose-response curves to kainate and α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) in the neonatal rat spinal cord. (a) The control kainate dose-response curve (○) is dose-dependently and reversibly shifted to the right by methohexitone at 10 μM (●), 31.6 μM (■) and 100 μM (▲). (b) The shift of the control AMPA dose-response curve (○) by methohexitone at 31.6 μM (■) and 100 μM (▲) is less marked and dose-dependency less apparent. Ordinate scale: amplitude of depolarizing response expressed as a percentage of the response to kainate 30 μM or AMPA 20 μM. Abscissa scale: agonist concentration in μM.

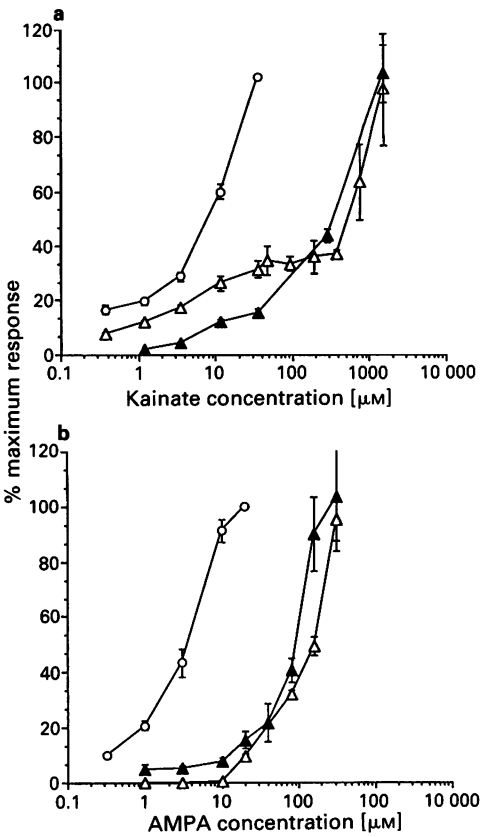


Figure 4 Effects of application of 3.16 μM 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)-quinoxalone (NBQX) alone, and in combination with 31.6 μM methohexitone, against dose-response curves to kainate and α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) in the neonatal rat spinal cord. In (a) the upper part of the control curve for kainate (○) is shifted to the right in the presence of 3.16 μM NBQX (△), with no further effect of 31.6 μM methohexitone (▲). The NBQX-resistant plateau at the lower end of the dose-response curve is uniquely sensitive to antagonism by methohexitone (31.6 μM). In (b) the control AMPA dose-response curve (○) is shifted to the right by 3.16 μM NBQX (△). Co-application of methohexitone (31.6 μM) did not elicit a marked further rightward shift (▲). Ordinate scale: amplitude of depolarizing response expressed as a percentage of that to (a) kainate 30 μM and (b) AMPA 20 μM. Abscissa scale: agonist concentration in μM.

which is resistant to NBQX and have little effect on responses in this tissue to AMPA. To account for the above data, we suggest that low doses of kainate activate a methohexitone-sensitive receptor subtype while higher doses activate an NBQX-sensitive receptor not dissimilar to that activated by AMPA.

The idea that kainate actions are mediated by receptor subtypes some of which are shared with AMPA and some of which are unique is consistent with data from binding, functional and molecular biology studies as discussed in the Introduction.

It is uncertain, however, why an NBQX-resistant component of kainate's action is not seen in most electrophysiological recordings from single neurones, thereby preventing a selective action of this antagonist being demonstrated *in vivo*. It may indicate that methohexitone-sensitive kainate receptors mediate only a small part of kainate's

action or that they are not expressed at all on the soma and proximal dendrites of the neurones being studied. Since other elements, such as glia and neuronal terminals, are also likely to play a role both in depolarizations seen in grease-seal preparations and in excitotoxic pathogenesis, the possibility of such kainate receptors being sited on these structures needs consideration. While glial cells can support both kainate and AMPA mediated conductances (Wylie *et al.* 1991), Teichberg *et al.* (1990) have reported a predominance of kainate binding proteins on some glia. It seems unlikely, however, that an action on glia makes a major contribution to the NBQX-resistant component of kainate-induced depolarization, since preliminary studies in a preparation where results are consistent with those described here (Palmer *et al.*, 1991) show that responses to AMPA and kainate decrease in parallel following exposure of neocortical slices to the gliotoxin, L-α-aminoadipate (Palmer, personal communication). It thus seems possible that some part of the signal produced by kainate is due directly to afferent terminal depolarization. The presence of kainate receptors on afferent fibres (Agrawal & Evans, 1986) and nerve terminals (see Stone, 1990). and the calcium permeability which this agonist may activate (Iino *et al.*, 1990; Egebjerg *et al.*, 1991) would allow such receptors to function as positive feedback autoreceptors, facilitating release of excitatory transmitter (Collins *et al.*, 1983; Potashner & Gerard, 1983) and contributing to excitotoxicity (Campochiaro & Coyle, 1978; Okazaki & Nadler, 1988).

The possibility that some part of the action of kainate is mediated via presynaptic receptors remains consistent with our proposal that some of the neuronal receptor complexes activated by this agonist are pharmacologically distinct from those at which AMPA acts. It will be interesting to see whether any of the recently described kainate-sensitive receptor subunit isolated from rat cerebellum, (Egebjerg *et al.*, 1991) and with a binding pattern akin to that of [^{3}H]-kainate, can eventually be resolved to presynaptic neuronal sites.

The present finding that barbiturates, and methohexitone in particular, block the NBQX-resistant component of the kainate response on the hemisected spinal cord has been repeated in rat cortical slices (Palmer *et al.*, 1991), although most previous reports of interactions between barbiturates and excitatory amino acids have largely been concerned with antagonism of quisqualate/AMPA responses (Simmonds & Horne, 1988). Since the presently described kainate-specific antagonism by methohexitone is unaffected by picrotoxin at a concentration known to inhibit GABA-mediated events (Evans, 1978), it is unlikely to be related to the well known GABA-potentiating effects of barbiturates, although some earlier reports have suggested the possibility of an interaction between GABA and kainate (e.g. Stone & Javid, 1980; Turski *et al.*, 1990). A more likely mode of action is a voltage-dependent block of channels opened by kainate (Miljkovic & MacDonald, 1986).

Consistent with the idea of barbiturate-sensitive presynaptic kainate receptors (as discussed above) are several reports of reduced release of neurotransmitters by barbiturates (e.g. Weakley, 1969; Cutler & Young, 1979) including that stimulated by kainate (Potashner & Gerard, 1983).

In summary, we propose that the profiles of AMPA and kainate antagonism by NBQX and the barbiturates indicate pharmacologically distinct sites via which these agonists act to depolarize the hemisected spinal cord preparation. The precise cellular distributions and the functions of such receptors remain to be elucidated.

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Cibacron blue stimulation of surfactant secretion in rat type II pneumocytes

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1 The effect of cibacron blue, a selective P_{2y}-purinoceptor antagonist in some systems, on the stimulatory effect of adenosine 5'-triphosphate (ATP) on [^{3}H]-phosphatidylchloine secretion was examined in primary cultures of rat type II pneumocytes prelabelled by overnight culture with [^{3}H]-choline.

2 Cibacron blue alone stimulated phosphatidylcholine secretion in a concentration-dependent manner in the range 10^{-4}–10^{-3} M. At a concentration of 10^{-4} or lower, cibacron blue had no effect on ATP-induced phosphatidylcholine secretion but at 10^{-4}–10^{-3} M it increased the effect of ATP. Enhancement of the ATP effect was apparent whether cibacron blue was added before or together with ATP. Cibacron blue also increased ATP-induced secretion in the presence of the P_1-purinoceptor antagonist, xanthine amine congener (10^{-5} M).

3 The stimulatory effect of cibacron blue on phosphatidylcholine secretion was additive to those of 5' (N-ethylcarboxyamido) adenosine (NECA) and terbutaline but less than additive to that of ATP.

4 Cibacron blue alone had no effect on formation of cyclic AMP or inositol phosphate and when added stimultaneously with ATP it did not affect the ATP-induced increase in these second messengers. Preincubation of the cells with cibacron blue before addition of ATP, however, resulted in antagonism of the ATP-induced increase in cyclic AMP and inositol phosphates. Preincubation with ATP had the same effect. The stimulatory effects of NECA and terbutaline on cyclic AMP formation were enhanced by preincubation with cibacron blue.

5 Thus, ATP-induced phosphatidylcholine secretion in type II cells is not diminished by the P_{2y}-antagonist, cibacron blue. On the contrary, cibacron blue stimulates phosphatidylcholine secretion. Cibacron blue may act as a P_2-agonist in type II pneumocytes.

Keywords: Pulmonary surfactant; lung; phosphatidylcholine secretion; P_2-purinoceptor; ATP; inositol trisphosphate; cyclic AMP; suramin

Introduction

Lung surfactant, phospholipid-rich material lining the alveolar surface, is synthesized and stored in the type II pneumocyte and secreted by exocytosis (Chander & Fisher, 1990; Haagsman & van Golde, 1991; Wright & Dobbs, 1991). Secretion of surfactant phospholipids has been shown to be influenced by a variety of physiological and pharmacological agents (Chander & Fisher, 1990; Wright & Dobbs, 1991). A role for purinoceptor agonists in the regulation of surfactant secretion is suggested by the findings that secretion of phosphatidylcholine, its major lipid component (Haagsman & van Golde, 1991), was stimulated by adenosine, adenosine analogues and adenine nucleotides in primary cultures of type II cells (Gilfillan & Rooney, 1987a,b; 1988; Gobran & Rooney, 1990; Griese *et al.*, 1991a,c; Rice & Singleton, 1986; 1987; Warburton *et al.*, 1989) while surfactant secretion *in vivo* was stimulated by adenosine (Ekelund *et al.*, 1985) and inhibited by an adenosine receptor antagonist (Rooney & Gobran, 1988).

There is functional evidence for both P_1- (adenosine) and P_2- (adenosine 5'-triphosphate, ATP) purinoceptors on the type II cell. The stimulatory effects of adenosine and its analogues on phosphatidylcholine secretion were shown to be mediated by the A_2 subtype of the P_1-receptor (Gilfillan & Rooney, 1987b; Griese *et al.*, 1991a) and there is also evidence of an inhibitory A_1-receptor on the type II cell (Gobran & Rooney, 1990). We recently found that a large part of the action of ATP on the type II cell was mediated by the P_1 receptor since as much as 75% of its effect on phosphatidylcholine secretion was antagonized by 8-[4-(2-aminoethylaminocarbonylmethyloxy)phenyl]-1,3-dipropylxanthine (xanthine amine congener, XAC) and 8-phenyltheophylline (Griese *et al.*, 1991c). However, there is evidence that ATP and ATP analogues also promote phosphatidylcholine secretion by action at a P_2-receptor (Gilfillan & Rooney, 1988; Greise *et al.*, 1991c; Rice & Singleton, 1987) that is coupled to activation of phosphoinositide-specific phospholipase C (Warburton *et al.*, 1989; Rice *et al.*, 1990; Griese *et al.*, 1991b).

P_2-receptors have been subdivided into P_{2x}- and P_{2y}-types (Burnstock & Kennedy, 1985) although at least three other subtypes have also been proposed (Gordon, 1986; Wiklund & Gustafsson, 1988; Stone, 1991). However, the receptor mediating the effect of ATP and its analogues in the type II cell does not fit the potency orders described for any of those (Rooney, 1990). Rice & Singleton (1987) proposed a classical P_{2y}-subtype for the type II cell P_2-receptor. This, however, is not supported by the potency order since 2-methylthio ATP is reported to be the most potent agonist at the P_{2y}-receptor (Burnstock & Kennedy, 1985) and it was considerably less potent than ATP in the type II cell (Gilfillan & Rooney, 1988; Griese *et al.*, 1991c). Cibacron blue, previously termed reactive blue 2 (Sigma Chemical Co., St. Louis, MO, U.S.A.), was reported to be a specific antagonist of the P_{2y}-receptor in some systems (Burnstock & Warland, 1987; Hopwood & Burnstock, 1987). Rice & Singleton (1989) found that cibacron blue antagonized the effect of ATP in the type II cell but in a preliminary study we were unable to confirm that finding (Rooney, 1990; Rooney *et al.*, 1988; 1990). We have now examined the effect of cibacron blue in more detail and show that it has unexpected actions in combination with other agents on both phosphatidylcholine secretion and second messenger formation.

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Methods

Isolation and culture to type II cells

Type II cells were isolated from the lungs of adult rats by the method of Dobbs *et al.* (1986) as described previously (Gobran & Rooney, 1990). This method involves digestion of the blood-free lungs with elastase and separation of type II pneumocytes from contaminating cells by panning on bacteriological dishes coated with IgG. The freshly isolated cells were plated at a density of $2-5 \times 10^6$ cells per dish on 35 mm i.d. plastic dishes and cultured in 1.5 ml Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal bovine serum, streptomycin (10 $\mu g\ ml^{-1}$), penicillin (100 u ml^{-1}) and, when indicated, radiochemicals for 18–20 h at 37°C in a humidified atmosphere of 90% air : 10% CO_2. At least 95% of the cells attached to the dishes after this period of culture were identifiable as type II cells (Gobran & Rooney, 1990).

Drug treatment

After culture of the cells for 18–20 h the medium was removed and the cells were rinsed with fresh DMEM without serum, antibiotics or radiochemicals. Fresh DMEM was then added and the dishes were returned to the incubator for a 30 min preincubation period. Secretagogues were then added and the incubation continued for various time periods. Antagonists were generally added at the beginning of the preincubation period. Where indicated, adenosine deaminase (3 u ml^{-1}) was also added at this stage. XAC was dissolved in dimethylsulphoxide (1% final concentration) and this solvent was added to the corresponding control dishes. The other drugs were dissolved in DMEM.

Phosphatidylcholine secretion

[^{3}H]-choline (2 $\mu Ci\ ml^{-1}$) was included in the medium during the overnight culture of the cells (2×10^6 per dish). With the exception of the time course experiments, secretion was measured 90 min after addition of the secretagogues. The medium was aspirated and centrifuged at 200 *g* for 10 min while the attached cells were lysed by addition of ice-cold water. Lipids were extracted from both cells and medium with a mixture of chloroform and methanol by the method of Bligh & Dyer (1959) and total lipid radioactivity was quantified by liquid scintillation counting (Gobran & Rooney, 1990). The total amount of lipid radioactivity ranged from 150,000 to 700,000 c.p.m. per dish. We previously reported that under these conditions phosphatidylcholine accounts for more than 95% of the lipid radioactivity in both cells and medium (Gobran & Rooney, 1990) while sphingomyelin and lysophosphatidylcholine account for the remainder. Phosphatidylcholine secretion is therefore expressed as the amount of lipid radioactivity in the medium after the 2 h incubation as a percentage of the total in cells and medium combined.

Measurement of [^{3}H]-inositol phosphates

The overnight culture of the cells ($3-5 \times 10^6$ per dish) was carried out in inositol-free DMEM to which [^{3}H]-inositol (20 $\mu Ci\ ml^{-1}$, 80–120 Ci $mmol^{-1}$) was added (Griese *et al.*, 1991b). Formation of inositol phosphates was measured in the presence of 20 mM LiCl as described previously in detail (Griese *et al.*, 1991b). Briefly, the reaction was terminated at various times after addition of the secretagogues by rapid aspiration of the medium and addition of ice-cold perchloric acid. [^{3}H]-inositol phosphates were then extracted, fractionated into individual components on ion exchange columns by a modification of the method of Berridge (1986) and quantified by liquid scintillation counting.

Adenosine 3':5'-cyclic monophosphate (cyclic AMP) assay

The medium was rapidly aspirated and 1 ml of ice-cold 0.1 M HCl was added to the dish which was placed on an ice-cooled surface. The HCl extract was evaporated to dryness at 40°C under a stream of air and the sample was stored at −70°C. After reconstitution and acetylation the cyclic AMP content was determined following the procedure described in the RIA kit.

Lactate dehydrogenase assay

The rate of lactate dehydrogenase release into the medium was determined to assess cellular integrity. The cells were cultured as in the secretion experiments but in the absence of radioactivity after which lactate dehydrogenase activity in the cells and medium was assayed by measuring disappearance of NADH at 340 nm (Wroblewski & LaDue, 1955).

Materials

Adult male Sprague-Dawley rats (200–300 g) were purchased from Charles River Breeding Laboratories (Kingston, NY, U.S.A.), rat IgG from Calbiochem (San Diego, CA, U.S.A.), porcine pancreatic elastase from Elastin Products (Owensville, MO, U.S.A.), foetal bovine serum from HyClone Laboratories (Logan, UT, U.S.A.), Dulbecco's modified Eagle's medium (DMEM) and *myo*-inositol-free DMEM from GIBCO, Grand Island, NY, U.S.A.; [*methyl*-^{3}H]-choline chloride from NEN-du Pont (Wilmington, DE, U.S.A.), *myo*-[2-^{3}H]-inositol from Amersham (Arlington Heights, IL, U.S.A.), cyclic AMP RIA kits from Biomedical Technologies (Cambridge, MA, U.S.A), XAC from Research Biochemicals (Natick, MA, U.S.A.), terbutaline sulphate (Brethine) from Geigy Pharmaceuticals (West Caldwell, NJ, U.S.A.), 5' (N-ethylcarboxyamido)adenosine (NECA), disodium ATP, α, β-methylene ATP, cibacron blue 3GA (Lot 50F-03341), calf intestinal mucosal adenosine deaminase (type VIII) and other biochemicals from Sigma (St. Louis, MO, U.S.A.). Suramin was a gift from Dr John Becher, Centers for Disease Control (Atlanta, GA, U.S.A.).

Statistics

In the secretion experiments type II cells were isolated from the pooled lungs of three rats and distributed among the various treatment groups, three dishes per group. The dishes were processed separately and the mean values calculated to yield a single data point per group per experiment. In the second messenger experiments the cells were isolated from the pooled lungs of four rats and one dish was used at each time point per experiment. Cyclic AMP extracts were analyzed in duplicate and the mean values calculated to yield a single data point per time point per experiment. All data are mean ± s.e.mean from the number of individual experiments indicated. As the cyclic AMP values were log normally distributed, they were normalized by a log transfusion prior to statistical analysis. The data were analyzed statistically by analysis of variance (ANOVA) followed by Fisher's least significance difference test or by Student's two tailed *t* test for paired samples with correction for multiple comparisons when necessary (Rohlf & Sokal, 1981).

Results

Phosphatidylcholine secretion

Cibacron blue alone at low concentrations had no effect on phosphatidylcholine secretion in type II cells but stimulated in a concentration-dependent manner at higher concentrations (Figure 1). As shown previously (Rooney *et al.*, 1988;

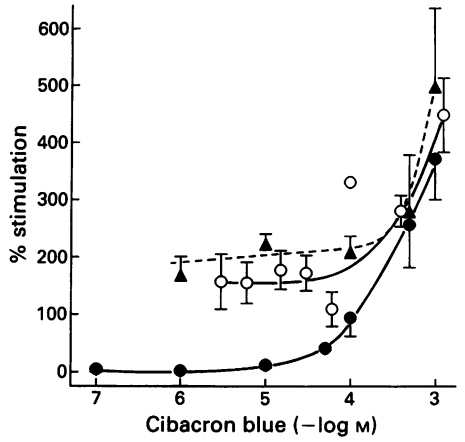


Figure 1 Phosphatidylcholine secretion in type II cells in response to various concentrations of cibacron blue alone and in combination with ATP and ATP + xanthine amine congener (XAC). The cells were cultured for 18–20 h in the presence of [^{3}H]-choline, washed with fresh medium and preincubated with the indicated concentrations of cibacron blue with and without XAC (10^{-5} M) for 30 min. ATP (10^{-5} M) was then added and the incubation continued for 90 min after which phosphatidylcholine secretion was measured. When the effect of cibacron blue alone was to be examined it was added at the end of the preincubation period: (●) cibacron blue alone; (○) cibacron blue + ATP; (▲) cibacron blue + ATP + XAC. The data are expressed as % stimulation over the basal rate of secretion in control cells incubated with vehicle only and are means ± s.e.mean (bar) from 3 experiments except for 10^{-4} M and 5×10^{-4} M cibacron blue + ATP which are from 1 and 9 experiments, respectively, and 10^{-7} M to 5×10^{-5} M cibacron blue alone, 6×10^{-5} M cibacron blue + ATP and 5×10^{-5} M cibacron blue + ATP + XAC which are means ± range from 2 experiments. The basal rate of secrtion was 1.13 ± 0.07% of cellular [^{3}H]-phosphatidylcholine secreted in 2 h ($n = 14$). The rate of secretion in cells treated with 10^{-4} M, 5×10^{-5} M and 10^{-3} M cibacron blue was significantly different from that in the controls ($P < 0.05$, ANOVA). ATP alone stimulated phosphatidylcholine secretion by 232% ± 37 (13) in the experiments without XAC while in those with XAC it stimulated by 371% ± 84 (3) and this was reduced to 171% ± 25 (3) by XAC. Some of these data were included in a preliminary publication (Rooney, 1990).

Rooney, 1990), preincubation of the cells with cibacron blue at a concentration of 10^{-4} M or less had no effect on ATP-stimulated secretion. However, at higher concentrations cibacron blue enhanced the stimulatory effect of ATP (Figure 1). Since cabacron blue has also been reported to inhibit ATP-stimulated secretion by as much as 70% (Rice & Singleton, 1989), we examined its effect in the presence of the P_1-antagonist, XAC. As previously reported (Griese *et al.*, 1991c), XAC decreased the stimulatory effect of ATP but cibacron blue at concentrations of 10^{-4} M or less caused no further inhibition (Figure 1). Again, higher concentations of cibacron blue increased secretion in the cells treated with both XAC and ATP. The stimulatory effect of cibacron blue on phosphatidylcholine secretion was observed as early as 10 min after addition of ATP and was still apparent after 150 min (Figure 2).

Cibacron blue also increased the stimulatory effect of ATP when the two agents were added simultaneously (Table 1). Whereas the effects of cibacron blue and ATP were not additive the effect of cibacron blue was additive to those of NECA and terbutaline (Table 1). Adenosine deaminase was included in the NECA and terbutaline experiments since it has been reported to enhance the stimulatory effects of these agonists on phosphatidylcholine secretion in type II cells (Gobran & Rooney, 1990).

The stimulatory effect of cibacron blue was not due to cytotoxicity. As shown in Table 2, the rate of lactate dehydrogenase release from the cells was not increased by up to 10^{-3} M cibacron blue. ATP, NECA and terbutaline were previously reported to be non-toxic to type II cells (Gilfillan & Rooney, 1987a; Gobran & Rooney, 1990) and the same was true for combinations of 5×10^{-4} M cibacron blue with 10^{-5} M ATP, 10^{-5} M NECA and 3.6×10^{-5} M terbutaline (data not shown).

The effect of a second putative P_2-antagonist, suramin (Dunn & Blakeley, 1988; Hoyle *et al.*, 1990), on ATP-stimulated phosphatidylcholine secretion is shown in Table 3. Suramin had a small but significant inhibitory effect on ATP-stimulated secretion. However, its inhibitory effect was no greater when the P_1-mediated effects of ATP were blocked by XAC. The effect of suramin was not dependent on concentration since it inhibited ATP-stimulated secretion to essentially the same extent in the concentration range 10^{-7} to 10^{-3} M. It was not cytotoxic since 10^{-4} M suramin did not increase the rate of lactate dehydrogenase release over that in control cells (Table 2).

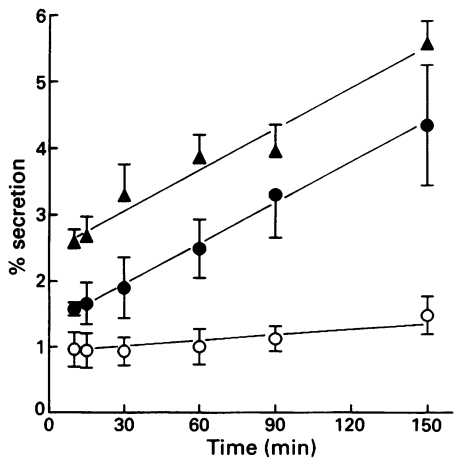


Figure 2 Time course of phosphatidylcholine secretion in type II cells. The cells were cultivated overnight with [^{3}H]-choline, transferred to fresh medium and preincubated for 30 min with or without cibacron blue (5×10^{-4} M). ATP (10^{-5} M) or vehicle was then added and the incubation continued for the indicated times after which secretion was measured: (○) control; (●) ATP; (▲) ATP + cibacron blue. The data are mean ± s.e.mean (bar) from 3 experiments.

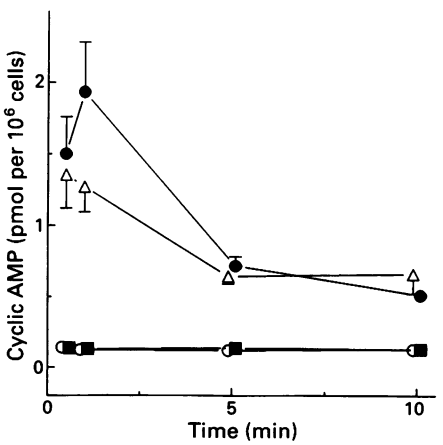


Figure 3 Time course of cyclic AMP formation in type II cells. After a 30 min preincubation period with agonists, ATP (10^{-3} M), cibacron blue (5×10^{-4} M), ATP + cibacron blue or vehicle were added and the incubation continued for the times indicated after which the cyclic AMP content was measured. The data are means ± s.e.mean (bar) from 4 experiments: (●) ATP; (■) cibacron blue; (△) ATP + cibacron blue; (○) control.

Table 1 Effect of cibacron blue in combination with surfactant secretagogues on phosphatidylcholine secretion in type II cells

Addition	*ATP* (10^{-5} M)	*NECA* (10^{-5} M)	*Terbutaline* (3.6×10^{-5} M)
	% phosphatidylcholine secretion		
Vehicle	1.35 ± 0.13	1.54 ± 0.09	1.49 ± 0.09
Cibacron blue (5×10^{-4} M)	3.71 ± 0.18	3.99 ± 0.26	4.08 ± 0.28
Secretagogue	4.69 ± 0.16	3.91 ± 0.23	3.21 ± 0.22
Cibacron blue + secretagogue	5.14 ± 0.09	6.32 ± 0.30	5.48 ± 0.26
Cibacron blue + secretagogue		*% stimulation*	
Expected	438 ± 50	313 ± 5	291 ± 12
Observed	290 ± 31	311 ± 12	271 ± 13
Observed as % of expected	66%	99%	93%
P	0.006	NS	NS

Type II cells were cultured overnight in the presence of [^{3}H]-choline, washed and preincubated in fresh medium for 30 min. Cibacron blue and secretagogues were then added and phosphatidylcholine secretion was measured 90 min later. Adenosine deaminase was added at the beginning of the preincubation when effects of 5′ (N-ethylcarboxyamido) adenosine (NECA) and terbutaline were examined. Stimulation is the amount of secretion in the presence of agonists expressed as a percentage of that in control cells incubated without agonists. The data are means ± s.e.mean from 4 experiments. Differences between % stimulation expected (calculated by addition of the individual effects) and that observed were analyzed statistically with the paired *t* test. NS, not significant ($P > 0.05$).

Table 2 Influence of cibacron blue and suramin on the rate of lactate dehydrogenase release in type II cells

Drug added	n	*Without drug* (% lactate dehydrogenase release h^{-1})	*With drug*
Cibacron blue (5×10^{-4} M)	4	1.88 ± 0.37	1.67 ± 0.38
Cibacron blue (10^{-3} M)	3	2.51 ± 0.86	2.87 ± 0.77
Suramin (10^{-4} M)	3	2.29 ± 0.17	1.97 ± 0.35

Type II cells were cultured overnight, washed and then incubated in fresh medium with and without the indicated drug concentrations for 2 h after which lactate dehydrogenase activity in cells and medium was measured. When the cells were incubated with 5×10^{-4} M and 10^{-3} M cibacron blue the media were diluted 5 fold and 10 fold, respectively, prior to enzyme assay. The media of the corresponding control cells were similarly diluted. The data are means ± s.e.mean from *n* experiments. The rate of lactate dehydrogenase release in the treated cells was not significantly different from that in the controls (paired *t* test).

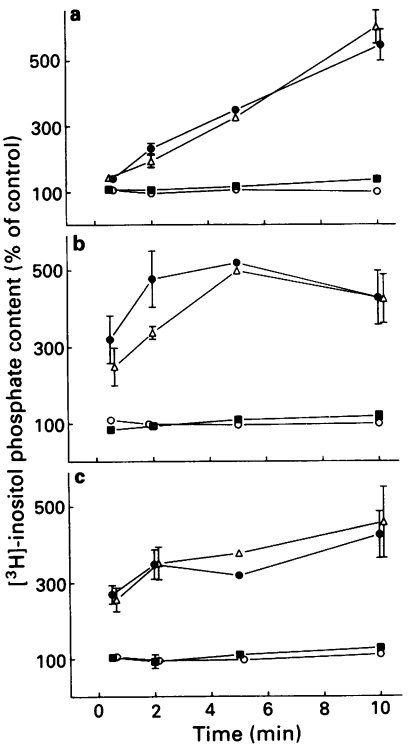


Figure 4 Time course of formation of (a) inositol phosphate (IP_1), (b) inositol bisphosphate (IP_2) and (c) inositol trisphosphate (IP_3) in type II cells. The cells were cultured overnight with [^{3}H]-inositol, washed and then incubated for 30 min in fresh medium containing 20 mM LiCl. ATP (10^{-3} M), cibacron blue (5×10^{-4} M), ATP + cibacron blue or vehicle were then added and the incubation continued for the times indicated after which [^{3}H]-inositol phosphates were measured. The data are means ± s.e.mean from 4 experiments. (●) ATP; (■) cibacron blue; (△) ATP + cibacron blue; (○) control. [^{3}H]-IP_1, [^{3}H]-IP_2 and [^{3}H]-IP_3 levels in control cells were 4230 ± 956, 823 ± 141 and 1095 ± 347 d.p.m. per dish, respectively. [^{3}H]-inositol phosphate levels in cells treated with ATP alone and with ATP + cibacron blue were not significantly different (ANOVA). Cibacron blue alone significantly increased IP_1 at 10 min over the level of control cells ($P < 0.05$, paired *t* test).

Table 3 Influence of suramin and xanthine amine congener (XAC) on ATP stimulated phosphatidylcholine secretion in type II cells

Suramin concentration	*ATP* (10^{-5} M)	*ATP + XAC* (10^{-5} M)
	% stimulation	
0	411 ± 44	115 ± 23
10^{-5} M	352 ± 35*	96 ± 14

Suramin and XAC were added 30 min prior to ATP. Other details are as described in Table 1. The data are means ± s.e.mean from 4 experiments. The basal rate of phosphatidylcholine secretion in the control cells was 1.16% ± 0.08 (7 experiments). *Significantly different from % stimulation in the absence of suramin ($P < 0.05$, paired *t* test).

Second messenger formation

To investigate the mechanism by which cibacron blue increases phosphatidylcholine secretion we examined its effect on activation of adenylate cyclase and phosphoinositide-specific phospholipase C. As reported previously (Gilfillan & Rooney, 1988; Griese *et al.*, 1991c), ATP increased cyclic AMP formation in type II cells and this effect was not significantly influenced by the simultaneous addition of cibacron blue (Figure 3). At a concentration at which it significantly increased phosphatidylcholine secretion (Figure 1) cibacron blue alone had no effect on cyclic AMP formation over a 10 min incubation period (Figure 3). Similarly, incubation of the cells with 10^{-7} M to 10^{-3} M cibacron blue for 1 min did not increase cyclic AMP formation over the level in control cells (data not shown). As also reported previously (Warburton *et al.*, 1989; Rice *et al.*, 1990; Griese *et al.*, 1991b), ATP increased the formation of inositol phosphate (IP_1), inositol bisphosphate (IP_2) and inositol trisphosphate (IP_3) and those effects were not influenced by cibacron blue (Figure 4). With the exception of a small (38%) increase in IP_1 at 10 min, cibacron blue on its own had no effect on formation of inositol phosphates (Figure 4).

In contrast to its lack of effects when added simultaneously with ATP, preincubation of the cells with cibacron blue led to reduction of the subsequent ATP-induced increase in both cyclic AMP (Figure 5) and IP_3 (Figure 6) formation. Those inhibitory effects were dependent on cibacron blue concentration with IC_{50}s of approx. 10^{-4} M (Figures 5 and 6). In contrast to its inhibition of the ATP-induced increase, cibacron blue enhanced the stimulatory effects of NECA and terbutaline on cyclic AMP formation (Table 4).

To investigate further the inhibitory effect of cibacron blue pretreatment, we preincubated the cells with P_2-agonists and then examined the effect of ATP on cyclic AMP and inositol phosphate formation. Preincubation with ATP led to the same inhibitory effect on the subsequent ATP-induced increase in cyclic AMP as did preincubation with cibacron blue (Table 5). Preincubation with cibacron blue or ATP also tended to decrease the subsequent stimulatory effects of ATP on IP_2 and IP_3 formation (Table 6). Although preincubation with α,β-methylene ATP was previously reported to diminish the effect of ATP on secretion (Gilfillan & Rooney, 1987a, 1988), it did not reduce the effects of ATP on formation of cyclic AMP (Table 5) and inositol phosphates (Table 6).

Discussion

Cibacron blue has been reported to be a relatively selective P_{2y}-antagonist (Burnstock & Warland, 1987; Hopwood & Burnstock, 1987) although at high concentrations it has also bee found to antagonize P_{2x}-mediated events (Reilly *et al.*, 1987). Nonspecific effects of cibacron blue have also been shown (Reilly *et al.*, 1987; Fedan & Lamport, 1990). The results in this paper confirm our preliminary reports (Rooney *et al.*, 1988; 1990; Rooney, 1990) that cibacron blue does not

Table 4 Effect of cibacron blue pretreatment on the stimulatory effects of 5′ (N-ethylcarboxamido) adenosine (NECA) and terbutaline on cyclic AMP formation in type II cells

Pretreatment	*NECA* (10^{-5} M)	*Terbutaline* (3.6×10^{-5} M)
	Cyclic AMP (pmol per 10^6 cells)	
Vehicle	1.32 ± 0.31	0.60 ± 0.13
Cibacron blue (5×10^{-4} M)	2.31 ± 0.42*	1.11 ± 0.28*

The cells were preincubated with vehicle or cibacron blue for 30 min. Agonists were then added and the incubation was continued for 10 min after which cyclic AMP was measured. The data are means ± s.e.mean from 4 experiments. *Significantly different form the value in the corresponding cells preincubated without cibacron blue ($P<0.05$, paired t test).

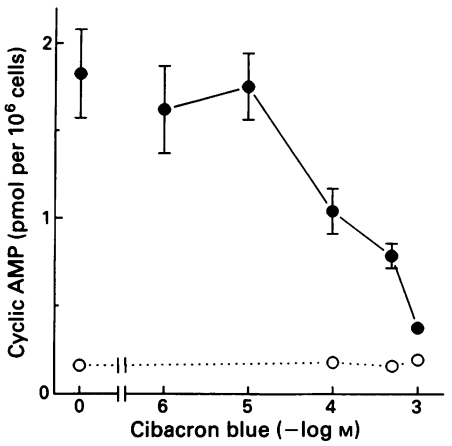


Figure 5 Concentration-response curve for the effect of cibacron blue pretreatment on ATP-induced cyclic AMP formation. The cells were preincubated with the indicated concentrations of cibacron blue for 30 min. ATP (10^{-3} M) or vehicle were then added and the incubation continued for 1 min after which cyclic AMP was measured. The data are means ± s.e.mean (bar) from 3–6 experiments without ATP (○) and 3–4 experiments with ATP (●) except for the 10^{-4} M cibacron blue without ATP value which is the mean ± range of 2 experiments.

Table 5 Effect of pretreatment with cibacron blue and P_2-agonists on ATP-induced cyclic AMP formation in type II cells

Pretreatment	*Incubation with vehicle*	*Incubation with ATP* (10^{-3} M)
	Cyclic AMP (pmol per 10^6 cells)	
Vehicle	0.12 ± 0.01 (6)	2.08 ± 0.33 (5)
Cibacron blue (5×10^{-4} M)	0.13 ± 0.02 (4)	0.58 ± 0.17 (4)*
ATP (5×10^{-4} M)	0.98 ± 0.38 (3)	0.69 ± 0.14 (3)*
α,β-methylene ATP (5×10^{-4} M)	0.20 ± 0.03 (5)	1.84 ± 0.33 (5)

After a 30 min preincubation the cells were incubated with ATP (10^{-3} M) or vehicle for 1 min and cyclic AMP was then measured. The data are means ± s.e.mean from the number of experiments indicated in parentheses. *Significantly different from the value in the cells incubated with 10^{-3} M ATP after pretreatment with vehicle ($P<0.05$, ANOVA).

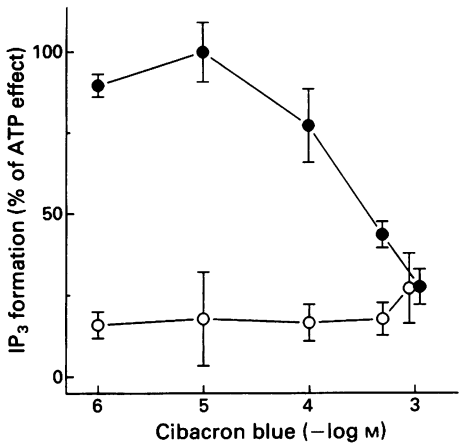


Figure 6 Concentration-response curve for the effect of cibacron blue pretreatment on ATP-induced IP_3 formation. The cells were cultured overnight in the presence of [^{3}H]-inositol, washed and preincubated for 30 min in fresh medium containing 20 mM LiCl and the indicated concentrations of cibacron blue. ATP (10^{-4} M) or vehicle were then added and the incubation continued for 2 min after which [^{3}H]-inositol trisphosphate ([^{3}H]-IP_3) was measured. The data are expressed as a percentage of the stimulatory effect of ATP in the absence of cibacron blue. The data without ATP (○) are means ± s.e.mean (bar) from 3 experiments while those with ATP (●) are from 4–5 experiments except for the 10^{-6} M value which is the mean ± range of 2 experiments. [^{3}H]-IP_3 levels in control cells incubated without cibacron blue were 1468 ± 148 d.p.m. per dish and were increased to 3646 ± 552 by 10^{-4} M ATP ($n = 5$; $P < 0.01$, paired t test).

antagonize the stimulatory effect of ATP on phosphatidylcholine secretion in type II cells. This, together with the finding that ATP is the most P_2-analogue (Rice & Singleton, 1987; Gilfillan & Rooney, 1988), suggests that the P_2-receptor mediating the effect of ATP in the type II cell is not of the P_{2y}-subtype. Suramin, another P_2-antagonist which does not discriminate between the P_{2x}- and P_{2y}-subtypes (Hoyle *et al.*, 1990), had only a small inhibitory effect on ATP-stimulated phosphatidylcholine secretion. The precise P_2-receptor subtype on the type II cell remains to be identified.

Effects of cibacron blue similar to those in this paper were recently observed in hamster tracheal epithelial cells in which mucin release was stimulated by ATP (Kim & Lee, 1991). In that system cibacron blue on its own also stimulated secretion and enhanced the effect of ATP (Kim & Lee, 1991). ATP was also the most potent P_2-analogue in the tracheal cells but whereas the potency order of other analogues was 2-methylthio ATP > α,β-methylene ATP > β,γ-methylene ATP in the tracheal cells (Kim & Lee, 1991) it was 2-methylthio ATP = β,γ-methylene ATP > α,β-methylene ATP in the type II cells (Gilfillan & Rooney, 1988). Such differences suggest that the P_2-receptors in these two different respiratory tissue cells are not identical.

Our data are different from those of Rice & Singleton (1989) who found that cibacron blue inhibited ATP-stimulated phosphatidylcholine secretion in type II cells. The inhibition was reported to be dependent on concentration with an IC_{50} of approx. 1.5×10^{-4} M. However, some inhibition was observed even at the lowest concentration examined (Rice & Singleton, 1989). Whereas we found that the stimulatory effects of cibacron blue and terbutaline were additive, Rice & Singleton (1989) reported that cibacron blue did not enhance the effect of terbutaline on phosphatidylcholine secretion. The reason for the different findings are not clear. They are not due to differences in the purity of cibacron blue since its source and lot number were the same in both studies (Rice, W.R., personal communication). Rice & Singleton (1989) did not examine the effect of cibacron blue alone or the time course of its action. Our data show that the onset of the effect of cibacron blue in combination with ATP was very rapid and that the effects of ATP and cibacron blue were not additive in contrast to those of terbutaline and cibacron blue which were. As Rice & Singleton (1989) subtracted the amount of [^{3}H]-phosphatidylcholine that was released during the 30 min preincubation from the total released at the completion of the incubation, an apparent inhibition of the effect of ATP and no enhancement of the effect of terbutaline might have been noted. Other possible differences include use of collagen-coated tissue culture dishes in the study of Rice & Singleton (1989) and our inclusion of adenosine deaminase in the terbutaline experiments.

The mechanism by which cibacron blue stimulates phosphatidylcholine secretion remains to be elucidated. That its effect on secretion was additive to those of NECA and terbutaline suggests that its action does not involve activation of adenylate cyclase. This conclusion is supported by the finding that cibacron blue did not increase cellular cyclic AMP levels. That its effect on ATP-stimulated secretion was apparent in the presence of XAC further shows that it does not act via a P_1-receptor. That the stimulatory effects of cibacron blue and ATP were not completely additive suggests that the mechanism by which these two agents stimulate phosphatidylcholine secretion are not independent of each other. ATP has been found to increase formation of cyclic AMP (Gilfillan & Rooney, 1987b; Warburton *et al.*, 1989; Griese *et al.*, 1991c), IP_3 (Warburton *et al.*, 1989; Rice *et al.*, 1990; Griese *et al.*, 1991b) and diacylglycerols (Rice *et al.*, 1990; Griese *et al.*, 1991b) as well as promote mobilization of intracellular Ca^{2+} (Rice & Singleton, 1987) in type II cells. In addition to the lack of an increase in cyclic AMP, cibacron blue also did not increase the formation of IP_2 or IP_3 but it had a small stimulatory effect on IP_1 formation. It is possible, therefore, that cibacron blue causes a small and probably delayed activation of phosphoinositide-specific phospholipase C. Cibacron blue activation of other phospholipases potentially involved in mediating the effect of ATP on phosphatidylcholine secretion (Griese *et al.*, 1991b) or action via additional mechanisms are also possible.

Although cibacron blue did not diminish the stimulatory effects of ATP on cyclic AMP and inositol phosphate formation when the two agents were added simultaneously, preincubation with cibacron blue before addition of ATP led to inhibition of the ATP effects on formation of these second messengers. Such interactions could account for the finding that the effects of ATP and cibacron blue on phosphatidylcholine secretion were less than additive. It is noteworthy

Table 6 Effect of pretreatment with cibacron blue and P_2-agonists on ATP-induced formation of inositol phosphates in type II cells

Pretreatment	*IP_1*	*IP_2*	*IP_3*
	% of control		
Vehicle	232 ± 16	477 ± 73	359 ± 30
Cibacron blue (10^{-4} M)	200 ± 21	276 ± 33	276 ± 22*
ATP (5×10^{-4} M)	257 ± 111	274 ± 93*	249 ± 75
α,β-methylene ATP (5×10^{-4} M)	319 ± 44	457 ± 69	442 ± 81

The cells were cultured for 18 h with [^{3}H]-inositol, washed and preincubated for 30 min in fresh medium containing 20 mM LiCl. ATP (10^{-3} M) was then added and the incubation continued for 2 min after which inositol phosphates were measured. The data are means ± s.e.mean from 4 experiments except for the IP_1 data from the cells pretreated with ATP which are from 3 experiments. [^{3}H]-inositol phosphate levels in control cells were as indicated in Figure 4. *Significantly different from the value in the cells pretreated with vehicle ($P < 0.05$, paired t test corrected for multiple comparisons).

that preincubation of the cells with ATP led to the same inhibition of the ATP-induced increase in cyclic AMP and inositol phosphates as did preincubation with cibacron blue. In addition, as previously reported for ATP (Griese *et al.*, 1991c), cibacron blue increased the stimulatory effect of NECA on cyclic AMP formation. Such data suggest that cibacron blue may be a P_2-agonist in the type II cell.

In summary, our data show that cibacron blue does not antagonize the stimulatory effect of ATP on phosphatidylcholine secretion in type II pneumocytes. On the contrary, 10^{-4}–10^{-3} M cibacron blue stimulated phosphatidylcholine secretion on its own and enhanced the stimulatory effect of ATP. The effect of cibacron blue was additive to those of NECA and terbutaline but less than additive to that of ATP. Cibacron blue on its own had no effect on the formation of cyclic AMP or inositol phosphates but preincubation with cibacron blue antagonized the ATP-induced increaes in these second messengers. Cibacron blue may act as a P_2-agonist in the type II cell.

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Effects of Hoe 140, a bradykinin B_2-receptor antagonist, on renal function in conscious normotensive rats

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1. The present study was designed to determine if endogenous kinins are involved in the regulation of arterial blood pressure and renal function in conscious rats given deoxycorticosterone enantate (DOC, 25 mg kg^{-1}, s.c., weekly) or vehicle for two weeks.

2. The bradykinin B_2-receptor antagonist, D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin (Hoe 140), at a dose of 300 μg kg^{-1}, s.c., blocked the hypotensive effect of 300 ng kg^{-1} bradykinin i.a., but it did not alter the blood pressure lowering action of 300 ng kg^{-1} acetylcholine or prostaglandin E_2. Inhibition of the response to bradykinin persisted up to 6 h after the administration of Hoe 140.

3. Administration of 300 μg kg^{-1} Hoe 140 s.c. four times a day did not alter mean blood pressure, renal blood flow, or renal function in rats given DOC-vehicle. However, it decreased urinary volume by 70% (from 48.2 ± 3.8 to 14.3 ± 3.7 ml 24 h^{-1}, $P<0.01$) and urinary secretion of sodium by 54% (from 1.02 ± 0.05 to 0.47 ± 0.16 mmol 24 h^{-1}, $P<0.01$) and potassium by 30% (from 2.93 ± 0.15 to 2.04 ± 0.15 mmol 24 h^{-1}, $P<0.05$) in DOC-treated rats. Mean blood pressure, glomerular filtration rate and total renal blood flow remained unchanged.

4. Our results suggest that endogenous kinins play a role in the regulation of renal excretion of water and sodium in the presence of elevated levels of DOC.

Keywords: Kinin antagonist; Hoe 140; bradykinin; kallikrein; renal blood flow; renal function; blood pressure

Introduction

Kinins are potent vasodilators, which acting as paracrine hormones, may be involved in the regulation of regional haemodynamics (Nolly *et al.*, 1990; Saed *et al.*, 1990; Wiemer *et al.*, 1991; Gardiner *et al.*, 1990; 1992) and renal excretion of water and sodium (Willis *et al.*, 1969; Carretero & Scicli, 1988). Alteration of the function of locally formed kinins, leading to predominance of vasoconstrictor tone and retention of water and salt, may contribute to the pathogenesis of arterial hypertension.

In 1985, five years after the proposal that the biological effects of kinins are mediated by two different receptor types, named B_1- and B_2-receptors (Regoli & Barabé, 1980), the first effective competitive antagonists of bradykinin were discovered (Vavrek & Stewart, 1985). B_2-receptor antagonists reportedly increase blood pressure (Carbonell *et al.*, 1988a), decrease renal blood flow (Seino *et al.*, 1988; Madeddu *et al.*, 1990a), and prevent the cardiovascular and renal effects of kininase inhibitors (Carbonell *et al.*, 1988b; Zimmerman *et al.*, 1990; Genden & Molineaux, 1991; Carretero & Scicli, 1991). Unfortunately, B_2-receptor antagonists of the first generation were characterized by low selectivity, residual agonist activity, and an ability to stimulate the release of renin, catecholamines and histamine (Mulinari *et al.*, 1987; Beierwaltes *et al.*, 1988; Rhaleb *et al.*, 1991). Thus, the effects caused by this class of antagonists might be unrelated to their kinin-blocking ability. In addition, their susceptibility to fast enzymatic degradation (Griesbacher *et al.*, 1989) limited their application to acute experiments.

Structure-activity studies, performed in order to eliminate the above described limitations, have resulted in a new series of potent, long-acting, selective, and specific B_2-receptor antagonists, devoid of agonist effect *in vitro* (Lembeck *et al.*, 1991; Hock *et al.*, 1991).

The present study was designed to evaluate the haemodynamic and renal effects caused by the administration of D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin, a newly synthesized, B_2-receptor antagonist, which reportedly inhibits bradykinin-induced decreases in blood pressure in the rat for several hours (Wirth *et al.*, 1991). Experiments were conducted in conscious rats with or without pretreatment with deoxycorticosterone, which increases renal kallikrein activity and urinary kallikrein excretion (Geller *et al.*, 1972; Nakagawa *et al.*, 1991).

Methods

Male Wistar rats (Morini, Como, Italy) weighing between 270 and 330 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. The following experimental protocols were performed in rats free to move in their own cages.

Experiment 1: Duration of action of Hoe 140 assessed by antagonism of the blood pressure response to bradykinin

With rats under light ether anaesthesia, a PE-10 tubing (Clay-Adams, Parsippany, NJ, U.S.A.) catheter was inserted into the left femoral artery 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 tunnelled under the skin and exteriorized at the back of the neck. Twenty-four hours later, mean arterial blood pressure (MBP) was measured by connecting the femoral catheter to a Statham transducer (Gould, Oxford, CA, U.S.A.) MBP was recorded on an S&W recorder (Medico Teknik, Albertslund, Denmark). The effects

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of intra-aortic bolus injections of bradykinin (300 ng kg^{-1} in 20 µl saline, via the carotid catheter) on MBP were tested before and at intervals after the administration of Hoe 140 (300 µg kg^{-1} in 0.1 ml saline, s.c.; $n = 7$) or vehicle ($n = 7$).

Experiment 2: Specificity of Hoe 140 assessed by inhibition of the blood pressure lowering effect of bradykinin, prostaglandin E_2 and acetylcholine

Rats underwent surgery as in experiment 1. Twenty-four hours after catheter implantation, Hoe 140 (300 µg kg^{-1} in 0.1 ml saline, $n = 7$) or vehicle ($n = 7$) was given subcutaneously. One hour later, the blood pressure lowering effect induced by an intra-aortic bolus injection of bradykinin (300 ng kg^{-1} in 20 µl saline) was evaluated. The same protocol was followed in rats given intra-aortic boluses of prostaglandin E_2 or acetylcholine (both at a dose of 300 ng kg^{-1} in 20 µl saline, $n = 7$ each group), instead of bradykinin.

Experiment 3: Effect of Hoe 140 on mean blood pressure in rats given either nonpressor or pressor doses of angiotensin II, phenylephrine or endothelin-1

Rats received weekly injections of DOC (25 mg kg^{-1} body weight, s.c.) or vehicle for two weeks. Then, with rats under light ether anaesthesia, two PE-10 tubing catheters (Clay-Adams, Parsippany, NJ, U.S.A.) were inserted into the left femoral artery and vein, advanced into the abdominal aorta and vena cava, respectively, tunnelled under the skin and exteriorized at the back of the neck. Experiments were conducted 24 h after catheter implantation.

First, the effect of Hoe 140 (300 µg kg^{-1} in 0.1 ml saline, s.c.) on MBP of rats with or without DOC pretreatment was tested during a 60 min infusion of saline (20 µl min^{-1}, i.v.), angiotensin II (3 ng kg^{-1} min^{-1}, i.v.), phenylephrine (7.5 ng kg^{-1} min^{-1}, i.v.) or endothelin-1 (7.5 ng kg^{-1} min^{-1}, i.v.); each group consisted of 6 rats.

In another set of experiments, rats received Hoe 140 (300 µg kg^{-1} in 0.1 ml saline, s.c.) or vehicle and 30 min later they were given bolus injections of either angiotensin II (30, 75 and 187 ng kg^{-1}, i.v.), or phenylephrine (0.6, 1.5 and 3.75 µg kg^{-1}, i.v.) or endothelin-1 (0.3, 0.75 and 3 µg kg^{-1}, i.v.). Each group ($n = 6$) received one vasoconstrictor. Each dose was given at 30 min intervals in random order, except for endothelin-1 which was injected at increasing doses. This procedure allowed return of MBP to control levels prior to subsequent injection.

Experiment 4: Effect of Hoe 140 on renal blood flow

Rats received weekly injections of DOC (25 mg kg^{-1} body weight, s.c.) or vehicle. After two weeks, they were anaesthetized with pentobarbitone sodium (50 mg kg^{-1}, i.p.) and, through a vertical mid-abdominal incision, miniaturized pulsed Doppler flowmeter probes (Crystal Biotech, Holliston, MA, U.S.A.) were placed and sutured around the left renal artery. The probe wires were tunnelled out of the back of the abdominal cavity, exteriorized and sutured on the back of the neck. One PE-10 catheter was inserted into the left femoral artery, advanced into the abdominal aorta and exteriorized at the back of the neck. Following closure of the incisions, rats were treated with ampicillin (7 mg kg^{-1}, s.c.) and allowed to recover for 5 days. After this time, the probe wires were connected to the alligator clips of Doppler cables whose terminations were plugged into a three-channel pulsed Doppler instrument (Pulse-Doppler, University of Iowa, Iowa City, IA, U.S.A.). Mean Doppler shift (electronically derived from the phasic signal) which corresponds to renal blood flow (Haywood *et al.*, 1981) was recorded on a Kipp and Zonen recorder (Delft, The Netherlands) before and at regular intervals after the injection of Hoe 140 (300 µg kg^{-1} four times a day, s.c.) or vehicle for 24 h. Zero flow was established by turning off the ultrasound signal; each group consisted of 6 rats. In our laboratory, comparison of instantaneous responses to graded doses of noradrenaline (from 0.2 to 2 nmol kg^{-1}) simultaneously detected by the pulsed Doppler system and an electromagnetic flowmeter indicates that changes in Doppler shift correlate very well with alterations in renal blood flow ($r = 0.988$, $P<0.01$) (Madeddu *et al.*, 1990b). In addition, comparison of measurements of renal blood flow by the clearance of *p*-[^{14}C]-aminohippuric acid and by the pulsed Doppler system over 6 h periods of time in conscious rats indicated that the two methods estimate changes in renal blood flow in a similar fashion ($r = 0.910$, $P<0.01$).

Experiment 5: Effect of Hoe 140 on renal function

Rats received weekly injections of DOC (25 mg kg^{-1} body weight, s.c.) or vehicle for two weeks. Then, urine collections over 24 h were obtained from rats in individual metabolic cages with free access to tap water but deprived of food. The same procedure was repeated two days later. Either vehicle- or DOC-treated rats were divided into two groups ($n = 9$ each); rats in group 1 received saline (100 µl four times a day, s.c.) on both occasions and rats in group 2 received saline on the day of the first urine collection and Hoe 140 (300 µg kg^{-1} four times a day, s.c.) on the day of the second urine collection. Blood (0.3 ml) was sampled by a small incision in the tail artery at the beginning and at the end of each urine collection to measure plasma creatinine.

Analytical procedures

MBP and Doppler renal blood flow (DRBF) are expressed in mmHg and kHz, respectively. Urinary volume (UV) was determined gravimetrically. Urinary sodium (U_{Na}) and potassium (U_K) were determine by flame photometry. Urinary osmolality was measured by determining the freezing point of urine with a Fisker Osmometer and expressed as mosmol excreted $24\ h^{-1}$. Urinary and plasma creatinine were measured by an automatic analyzer (Hitachi 704). Glomerular filtration rate was calculated as the clearance of endogenous creatinine.

All data are expressed as mean ± s.e.mean. Multivariate repeated measures analysis of variance was performed to test for interaction between time and the grouping factor. Then, univariate analysis of variance was used to test for differences among groups and over time. Differences between groups were determined by paired and unpaired *t* tests with the Bonferroni multiple comparison adjustment. Mathematical and statistical analyses were performed with a Statview II package, on a Macintosh IICX computer.

Substances

Bradykinin, angiotensin II, phenylephrine and endothelin-1 were purchasesd from Peninsula Laboratories (Belmont, CA, U.S.A.). The bradykinin antagonist, D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin was synthesized by solid phase peptide synthesis by Hoechst AG (Frankfurt, Germany). Concentrated solutions (1–10 mg ml^{-1}) of these reagents were prepared in 0.9% NaCl and stored at −20°C until use. Deoxycorticosterone enantate (DOC) was donated by Shering Company (Milan, Italy).

Results

Experiment 1: Duration of action of Hoe 140 assessed by antagonism of the blood pressure response to bradykinin

Injection of bradykinin prior to subcutaneous administration of Hoe 140 or vehicle decreased MBP from 117 ± 2 and 116 ± 2 mmHg by 13 ± 1 and 12 ± 1 mmHg, respectively. Hoe 140 at 300 µg kg^{-1} exerted a long-lasting antagonistic

effect since the response to bradykinin was inhibited completely at 1 h and by 25% at 6 h (Figure 1). Inhibition by the antagonist was dose-related since in preliminary experiments we found that the blood pressure lowering effect of bradykinin was inhibited by 55% 1 h after 30 $\mu g\,kg^{-1}$ and was restored to normal at 6 h.

Experiment 2: Specificity of Hoe 140 assessed by inhibition of the blood pressure lowering effect of bradykinin, prostaglandin E_2 and acetylcholine

Bradykinin decreased MBP by 12 ± 1 mmHg (from 113 ± 2 to 101 ± 1 mmHg, $P<0.01$) in rats given vehicle; the blood

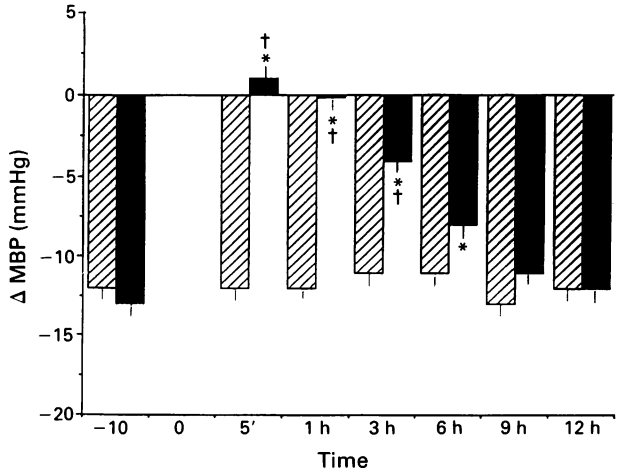


Figure 1 Inhibition of bradykinin-induced blood pressure lowering effect by 300 $\mu g\,kg^{-1}$ Hoe 140. Bradykinin (300 ng kg^{-1}) was given intra-arterially before and at intervals following the subcutaneous administration of vehicle or Hoe 140. Each column (Hoe 140 group, solid; vehicle group, hatched) represents the mean absolute change in mean blood pressure (MBP) of 7 rats; standard error of the mean is shown by vertical lines. *$P<0.05$ compared with time − 10'; † $P<0.05$ compared with vehicle group.

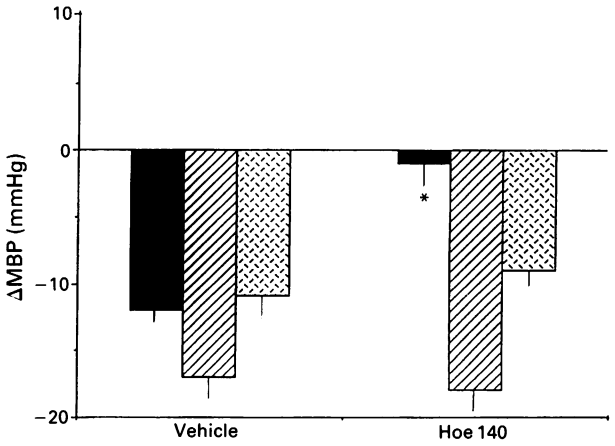


Figure 2 Specificity of Hoe 140 assessed by comparing the inhibition of the hypotensive response to bradykinin, prostaglandin E_2 or acetylcholine (300 ng kg^{-1}, i.a.). Rats received vehicle or Hoe 140 (300 $\mu g\,kg^{-1}$, s.c.) 1 h before the injection of vasodilators. Each column represents the mean absolute change in mean blood pressure (MBP) of 7 rats (solid columns, bradykinin; hatched columns, prostaglandin E_2; stippled columns, acetylcholine). Standard error of the mean is shown by vertical lines. *$P<0.05$ compared with vehicle group.

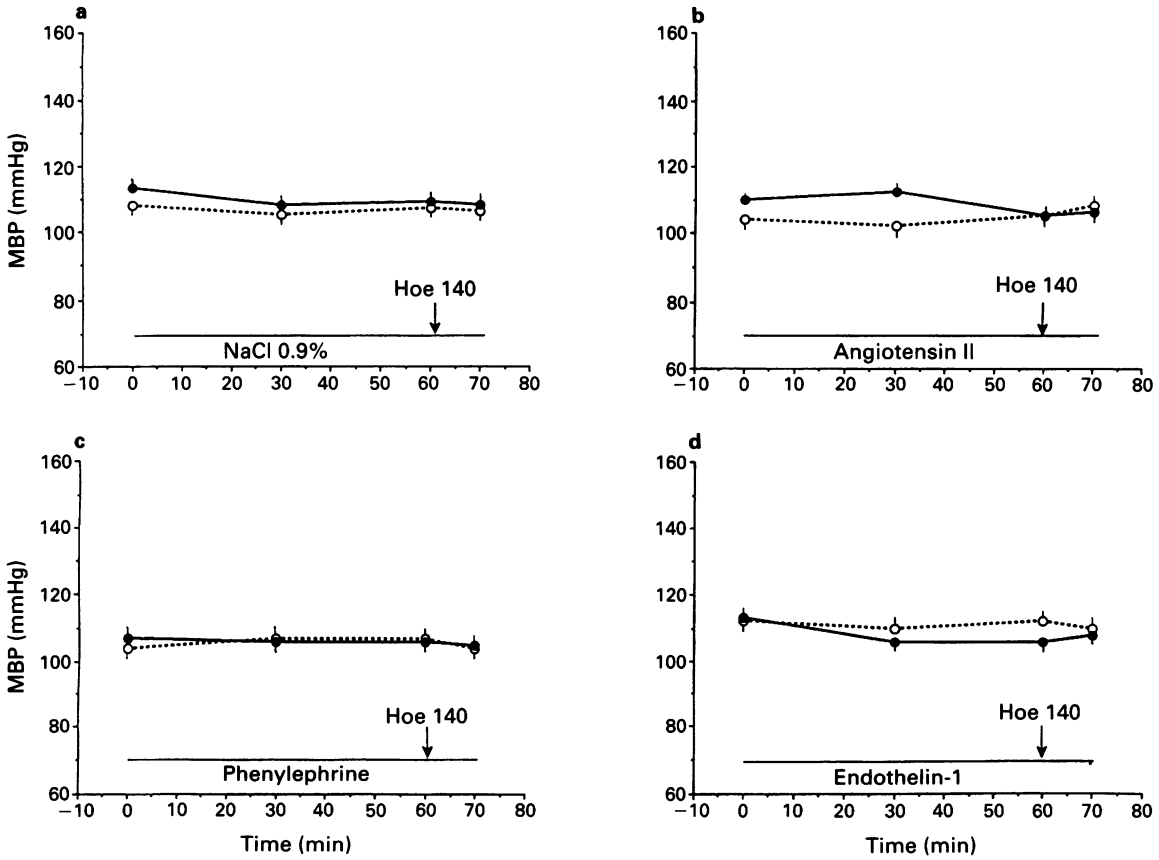


Figure 3 Effect of Hoe 140 (300 $\mu g\,kg^{-1}$, s.c.) on mean blood pressure (MBP) in rats with (solid lines) or without (broken lines) deoxycorticosterone (DOC) pretreatment during the infusion of (a) saline (20 $\mu l\,min^{-1}$, i.v.), (b) angiotensin II (3 ng $kg^{-1}\,min^{-1}$, i.v.), (c) phenylephrine (7.5 ng $kg^{-1}\,min^{-1}$, i.v.) or (d) endothelin-1 (7.5 ng $kg^{-1}\,min^{-1}$, i.v.). Each group consisted of 6 rats.

pressure lowering effect of bradykinin was abolished by Hoe 140 (before: 113 ± 1 mmHg, after bradykinin: 112 ± 1 mmHg, NS). The antagonist proved to be specific since it did not inhibit the effect of other unrelated vasodilators such as prostaglandin E_2 or acetylcholine (Figure 2).

Experiment 3: Effect of Hoe 140 on mean blood pressure in rats given either nonpressor or pressor doses of angiotensin II, phenylephrine or endothelin-1

As shown in Figure 3, Hoe 140 did not alter MBP in rats with or without deoxycorticosterone pretreatment during the infusion of vehicle or nonpressor doses of angiotensin II, phenylephrine, endothelin-1.

As shown in Figure 4, pretreatment with Hoe 140 did not alter the dose-pressor response curves to angiotensin II, phenylephrine, or endothelin-1.

Experiment 4: Effect of Hoe 140 on renal blood flow

As shown in Figure 5, administration of Hoe 140 over a period of 24 h did not alter MBP or renal blood flow in rats with or without DOC pretreatment.

Experiment 5: Effect of Hoe 140 on renal function

In rats given DOC-vehicle, no significant change in renal function was induced by Hoe 140 (Table 1).

In DOC-treated rats, Hoe 140 did not alter GFR [experimental group: vehicle (day 1), 3.16 ± 0.39 ml min^{-1}; Hoe 140 (day 2), 3.19 ± 0.13 ml min^{-1}, NS], [control group: vehicle (day 1), 2.91 ± 0.15 ml min^{-1}; vehicle (day 2), 2.87 ± 0.15 ml min^{-1}, NS]. As shown in Figure 6, Hoe 140 reduced urinary volume by 70% (from 48.2 ± 3.8 to 14.3 ± 3.7 ml $24\,h^{-1}$, $P<0.01$), Na^+ excretion by 54% (from 1.02 ± 0.05 to 0.47 ± 0.16 mmol $24\,h^{-1}$, $P<0.01$), K^+ excretion by 30% (from 2.93 ± 0.15 to 2.04 ± 0.15 mmol $24\,h^{-1}$, $P<0.05$) and osmolal excretion by 28% (from 18.32 ± 1.11 to 13.19 ± 1.18 mosmol $24\,h^{-1}$, $P<0.05$).

Discussion

Our results indicate that the compound Hoe 140 is a potent, specific and long-acting antagonist of bradykinin. When injected subcutaneously in rats that had received DOC-pretreatment, Hoe 140 caused a significant decrease in urinary volume and urinary sodium excretion without affecting MBP, total renal blood flow or glomerular filtration rate.

In the present study, we were able to demonstrate that Hoe 140 is specific *in vivo* as it did not alter the hypotensive effect caused by acetylcholine and prostaglandin E_2, thus confirming studies performed *in vitro* by Hock *et al.* (1991).

Hoe 140, at a dose which significantly inhibited the hypotensive effect of exogenous bradykinin for 24 h, did not

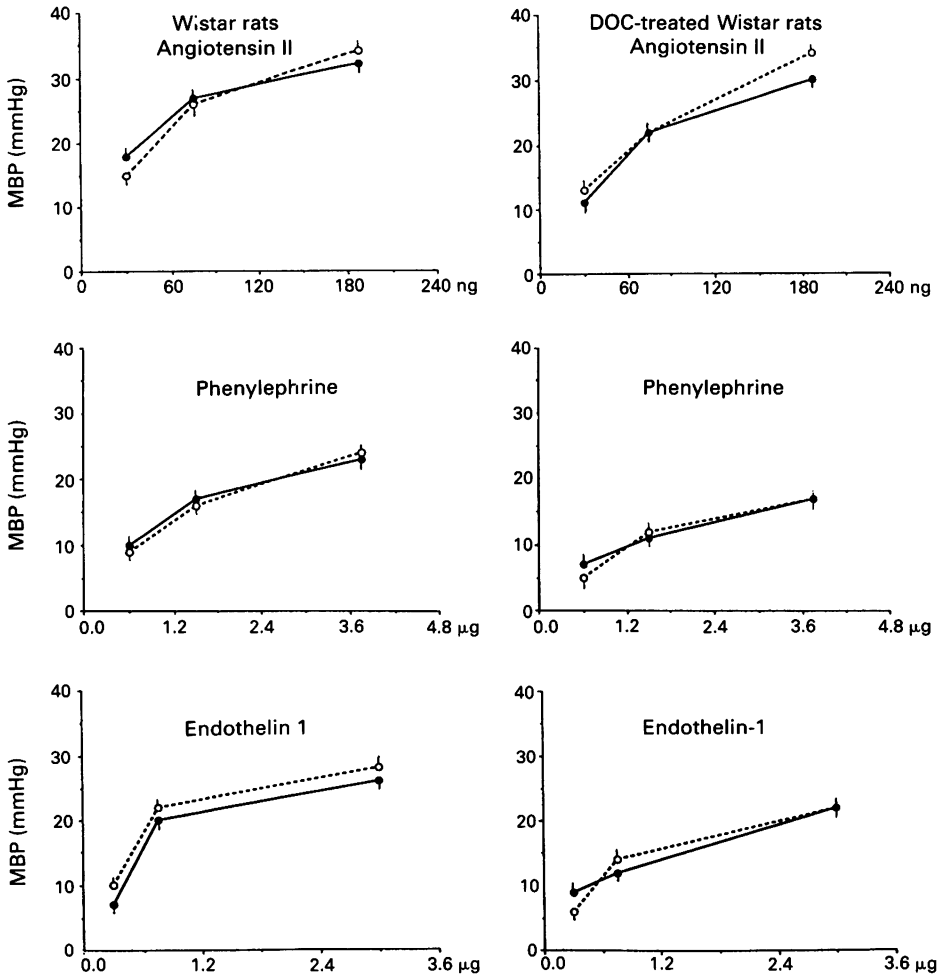


Figure 4 Dose-response curves to boluses of angiotensin II (30, 75 and 187 ng kg^{-1}, i.v.), phenylephrine (0.6, 1.5 and 3.75 µg kg^{-1}, i.v.) or endothelin-1 (0.3, 0.75 and 3 µg kg^{-1}, i.v.), in rats with (right panels) or without deoxycorticosterone (DOC) pretreatment (left panels), given 300 µg kg^{-1} Hoe 140 (solid lines) or vehicle (broken lines) subcutaneously. Each group ($n = 6$) received only one vasoconstrictor. Each dose of vasoconstrictor was given at 30 min intervals in random order except endothelin-1 which was injected at increasing doses. Mean blood pressure (MBP) returned to basal level prior to subsequent injection.

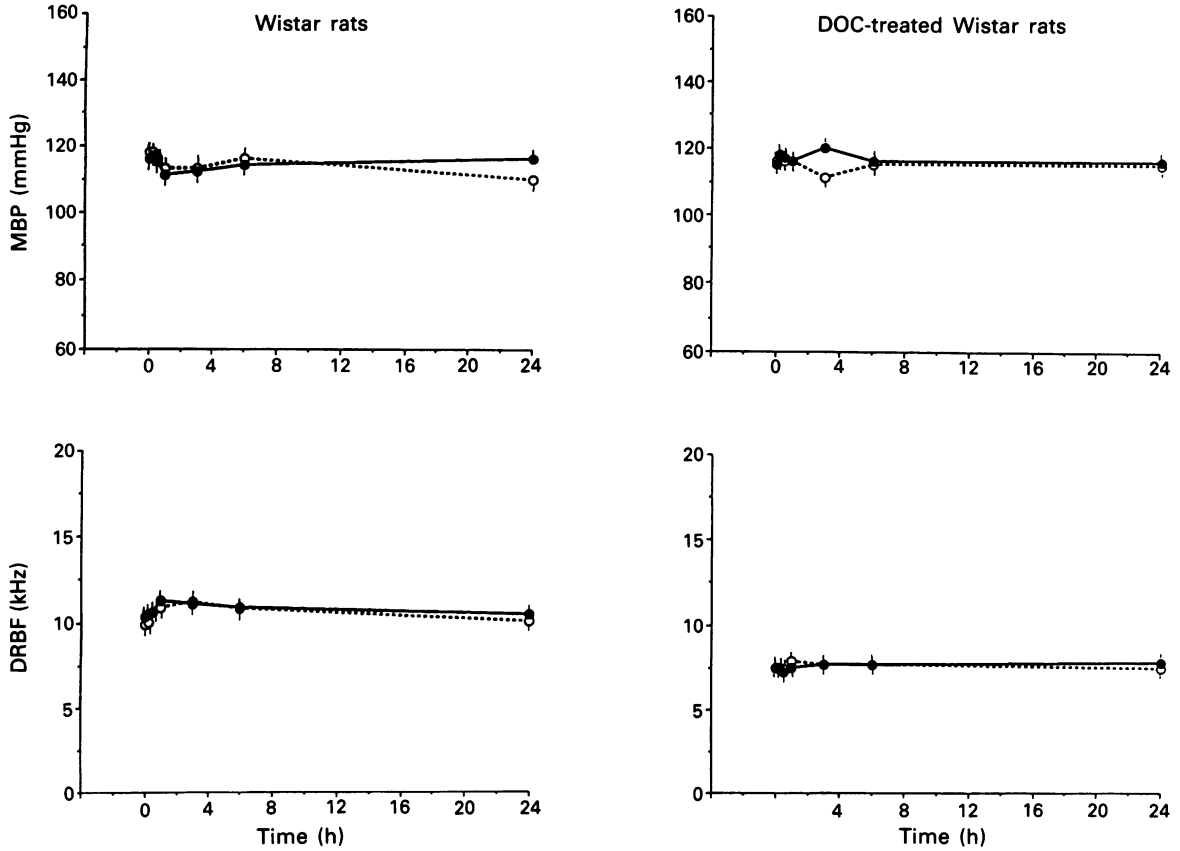


Figure 5 Effect of Hoe 140 (300 μg kg^{-1} four times a day, s.c., solid lines) or vehicle (broken lines) on mean blood pressure (MBP) and Doppler renal blood flow (DRBF) in rats with (right panels) and without (left panels) deoxycorticosterone (DOC)-pretreatment. Each group consisted of 6 rats.

Table 1 Effect of Hoe 140 on renal function in Wistar rats given deoxycorticosterone (DOC)-vehicle

	Control		*Experimental*	
Group treatment	*Vehicle*	*Vehicle*	*Vehicle*	*Hoe 140*
GFR (ml min^{-1})	2.9 ± 0.1	2.8 ± 0.1	2.8 ± 0.2	2.8 ± 0.1
UV (ml 24 h^{-1})	26.0 ± 2.1	25.2 ± 2.6	23.1 ± 1.1	22.5 ± 1.5
$U_{Na}V$ (mmol 24 h^{-1})	0.81 ± 0.13	0.75 ± 0.16	0.98 ± 0.17	1.08 ± 0.18
U_KV (mmol 24 h^{-1})	2.49 ± 0.24	2.65 ± 0.16	2.74 ± 0.16	3.17 ± 0.23
$U_{Osm}V$ (mosmol 24 h^{-1})	17.5 ± 1.6	16.8 ± 0.8	18.5 ± 0.5	18.7 ± 0.8

Two groups ($n = 9$ each) were studied: the control group received vehicle on both occasions, the experimental group received vehicle on the first occasion and Hoe 140 (300 μg kg^{-1} four times a day, s.c.) two days apart.

GFR, glomerular filtration rate; UV, urinary volume; $U_{Na}V$, urinary sodium excretion; U_kV, urinary potassium excretion; $U_{Osm}V$, urinary osmolal excretion.

alter MBP in normotensive rats. In addition, Hoe 140 did not affect MBP in rats given nonpressor or pressor doses of angiotensin II, phenylephrine or endothelin-1. Collectively, these results do not favour a major role for endogenous kinins in the regulation of arterial blood pressure in basal conditions in normotensive rats. However, kinins might modulate local and systemic vascular resistances in particular stimulated conditions (Weipert *et al.*, 1988; Berg *et al.*, 1989; Carbonell *et al.*, 1988b; Genden & Molineaux, 1991).

The present study was also undertaken to determine if endogenous kinins regulate renal haemodynamics and function in unanesthetized rats. Antagonists of the first generation reportedly decreased renal blood flow in dogs (Beierwaltes *et al.*, 1988) and rats (Madeddu *et al.*, 1990a). However, this effect was associated with stimulation of renin release (Beierwaltes *et al.*, 1988) and it was prevented by a cocktail of hormone receptor antagonists (Madeddu *et al.*, 1991), suggesting that it was caused by stimulation of neurohormonal reflexes rather than inhibition of endogenous kinins. The failure of Hoe 140 to affect renal blood flow in normotensive rats does not support a major role of kinins in the regulation of overall renal haemodynamics.

The finding that administration of Hoe 140 did not alter urinary volume, urinary sodium or potassium excretion over 24 h in normotensive, unanaesthetized rats is consistent with previous studies in which renal function was not affected by the administration of Fab fragments of kinin antibodies (Carretero & Scicli, 1991).

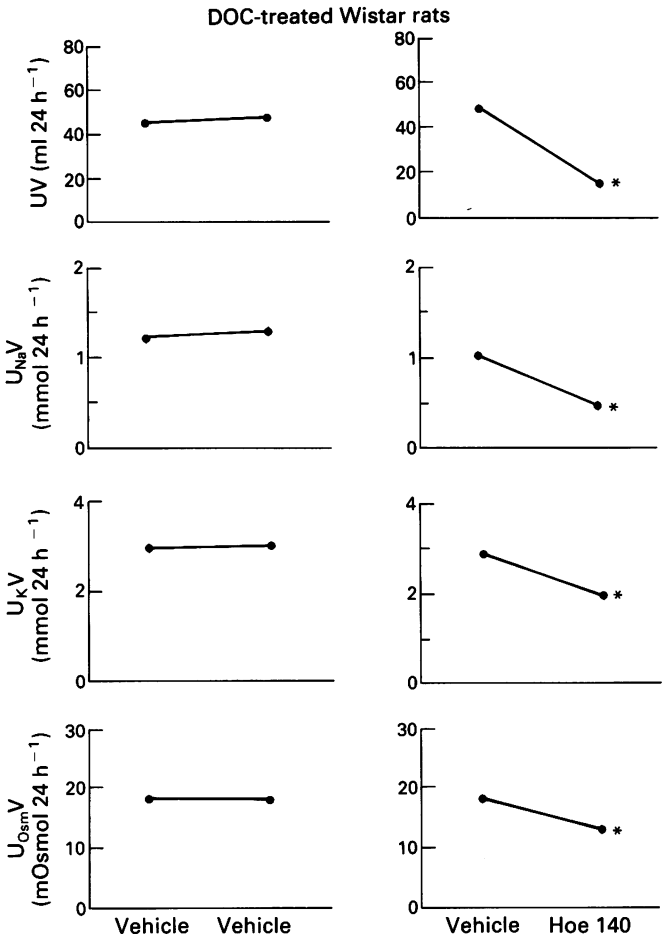


Figure 6 Effect of Hoe 140 (300 μg kg^{-1} four times a day, s.c.) or vehicle on urinary volume (UV), sodium ($U_{Na}V$), potassium (U_KV) and osmolal excretion ($U_{Osm}V$) in rats with deoxycorticosterone (DOC)-pretreatment. *$P<0.05$ *vs* vehicle.

By contrast, Hoe 140 reduced 24 h urinary volume by 70%, urinary sodium by 54%, and potassium excretion by 30% in DOC-treated rats. These results are consistent with a tubular effect of endogenous kinins since glomerular filtration rate was not modified and total renal blood flow remained unaltered. Inhibition of water and sodium reabsorption may have occurred at the level of the distal tubule where specific binding sites for bradykinin have been found (Tomita & Pisano, 1984).

Our study extends the observations of Tomiyama *et al.* (1991), who found that acute administration of the Fab fragments of kinin antibodies decreases urinary volume and sodium excretion without affecting blood pressure, renal blood flow, or glomerular filtration rate in rats given DOC acetate and salt. However, our study differs from that of Tomiyama regarding the longer duration of kinin inhibition and the fact that salt was not added to drinking water, a condition which could suppress renal kallikrein activity. By contrast, the antagonist D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin decreases urinary sodium excretion only when superimposed on the infusion of kininase inhibitors (Nakagawa *et al.*, 1990). Thus, failure of this antagonist to affect urinary sodium excretion in basal conditions may be attributable to its hydrolysis by kininases in the proximal tubule. Conversely, Hoe 140, which is excreted with urine in part as the intact compound in the rat (unpublished observations from Hoechst AG, Germany) possibly because of its resistance to kininases (Bond *et al.*, 1991), causes a decrease in urinary volume and sodium excretion. Collectively, these results suggest that kinins play a role in the regulation of electrolyte homeostasis by acting in the intratubular compartment of the kidney.

The finding that MBP of DOC-treated rats was similar to that of rats given DOC-vehicle is consistent with the observation that DOC alone does not induce sustained hypertension unless combined with chronic sodium loading and uninephrectomy. Activation of the renal kallikrein-kinin system by chronic administration of DOC (Geller *et al.*, 1972; Nakagawa *et al.*, 1991) may counteract the salt-retaining effect of DOC thus preventing the development of hypertension. Failure of Hoe 140, given over a period of 24 h, to alter MBP of DOC-treated rats does not rule out the possibility that the antidiuretic and antinatriuretic effect induced by inhibiting kinins causes fluid volume expansion leading over the long term to arterial hypertension.

In conclusion, our results indicate that endogenous kinins could play an important role in the regulation of water and sodium excretion by the kidney during chronic administration of DOC.

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Multiple actions of glaucine on cyclic nucleotide phosphodiesterases, α_1-adrenoceptor and benzothiazepine binding site at the calcium channel

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1 In the present study, the properties of glaucine (an aporphine structurally related to papaverine) were compared with those of papaverine, diltiazem, nifedipine and prazosin. The work includes functional studies on rat isolated aorta contracted with noradrenaline, caffeine or KCl, and a determination of the affinity of glaucine at calcium channel binding sites of α-adrenoceptors, by use of [^{3}H]-(+)-*cis*-diltiazem, [^{3}H]-nitrendipine and [^{3}H]-prazosin binding to cerebral cortical membranes. The effects of glaucine on the different molecular forms of cyclic nucleotide phosphodiesterases (PDE) isolated from bovine aorta were also determined.

2 Contraction evoked by noradrenaline (1 μM) or depolarizing solution (60 mM KCl) were inhibited in a concentration-dependent manner by all the compounds tested. As expected, prazosin showed a greater selectivity of action on NA-induced contraction, whereas nifedipine and diltiazem appeared more potent on KCl-induced contraction. Glaucine had a greater potency on the contraction elicited by noradrenaline whereas papaverine acted non specifically.

3 In Ca^{2+}-free solution, prazosin (0.1 μM) and glaucine (0.1 mM) inhibited the contraction evoked by NA; diltiazem (0.1 mM) diminished this contraction whereas nifedipine (1 μM) had no effect. Preincubation of tissues with glaucine, diltiazem, nifedipine and prazosin did not modify the contractile response induced by caffeine. In contrast, papaverine (0.1 mM) significantly inhibited the contractions evoked by NA or caffeine in Ca^{2+}-free medium.

4 Glaucine and papaverine show affinity at the [^{3}H]-prazosin binding site and at the benzothiazepine binding site of the Ca^{2+}-channel receptor complex, but have no effect at the dihydropyridine binding site in rat cerebral cortex. Glaucine exerts some selectivity as an inhibitor of [^{3}H]-prazosin binding as opposed to [^{3}H]-(+)-*cis*-diltiazem binding while papaverine appears to have approximately equal affinity in this respect.

5 This study confirms the presence of four phosphodiesterase (PDE) activities in bovine aorta: a calmodulin-activated PDE (CaM-PDE type I) which hydrolyzed preferentially guanosine 3':5'-cyclic monophosphate (cyclic GMP); a cyclic GMP selective form (cGMP-PDE type V); and two low K_m adenosine 3':5'-cyclic monophosphate (cyclic AMP) PDEs that are insensitive to the stimulatory effect of CaM, one of which was inhibited by cyclic GMP (CGI-PDE, type III) and the other by rolipram (cAMP-PDE, type IV). Glaucine selectively inhibits one of the two forms of Ca^{2+}-independent low K_m cAMP-PDE, the type IV. In contrast, papaverine exerts a non-selective inhibitory effect upon all PDE forms.

6 The present work provides evidence that glaucine, a benzyltetrahydroisoquinoline alkaloid, has interesting properties as an α_1-adrenoceptor antagonist, calcium entry blocker (through the benzothiazepine recognition site in the calcium channel) and as a selective inhibitor of the rolipram-sensitive cAMP-PDE, type IV PDE.

Keywords: Calcium antagonists; α_1-adrenoceptor blocking agents; rat aorta; glaucine; phosphodiesterase inhibitors

Introduction

Ca^{2+}-entry blockers can be classified into three major subgroups: dihydropyridines, phenylalkylamines and benzothiazepines. In addition, some new structural classes of compounds have recently been reported some of which related to the benzylisoquinoline structure (King *et al.*, 1988; Triggle *et al.*, 1989; D'Ocon *et al.*, 1989; 1991; Lacroix *et al.*, 1991). One of these new compounds is glaucine, an aporphine that is structurally related to papaverine (Figure 1), but shows a mechanism of uterine smooth muscle relaxant activity which is different from papaverine.

The action of papaverine is ascribed to inhibition of adenosine 3':5'-cyclic monophosphate (cyclic AMP) phosphodiesterase (Kukovetz & Pöch, 1970; Lugnier *et al.*, 1972; Bolton, 1979; Cumiskey & Feigenson, 1983), but there is evidence that it may also weakly antagonize calcium flux through specific channels (Schneider *et al.*, 1975; Reinhardt *et al.*, 1977). Glaucine, in rat uterus, showed a relaxant activity similar to that of nifedipine (Anselmi *et al.*, 1992), acting by a more specific mechanism related to inhibition of Ca^{2+}-entry through potential operated Ca^{2+} channels and has no effect on Ca^{2+}-release or Ca^{2+}-redistribution from intracellular storage sites.

In the present study, we compared the properties of glaucine with those of: diltiazem and nifedipine, established calcium antagonists; prazosin, an α_1-adrenoceptor antagonist; and papaverine. The work includes functional studies on rat

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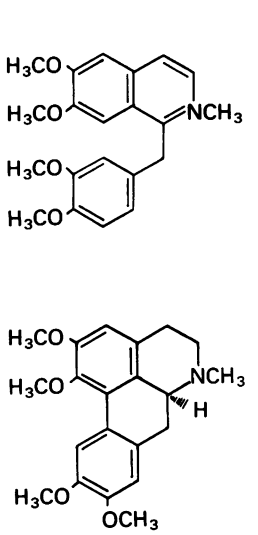


Figure 1 Chemical structures of (a) papaverine and (b) glaucine.

isolated aorta contracted by addition of noradrenaline, caffeine or KCl and in order to assess whether glaucine interacts directly with calcium channel binding sites or, α-adrenoceptors, we examined the effects of glaucine on [^{3}H]-nitrendipine, [^{3}H]-(+)-*cis*-diltiazem and [^{3}H]-prazosin binding to cerebral cortical membranes.

Since the relaxant action of papaverine is ascribed to inhibition of cyclic AMP-phosphodiesterase, we also determined if glaucine has any effects on cyclic nucleotide phosphodiesterase (PDE) activity. In mammalian cells, several distinct molecular forms of PDE exist (for review see Beavo, 1988; Beavo & Reifsnyder, 1990). Accordingly, we examined the ability of glaucine to inhibit the separated PDE enzymes isolated from bovine aorta.

Methods

Functional study

Helically cut strips of thoracic aorta of male Wistar rats (200–220 g) were prepared and mounted as described by Furchgott & Zawadzki (1980). Each preparation was suspended in a 10 ml organ bath containing Krebs-bicarbonate solution (KBS), maintained at 37°C and gassed with 95% O_2 and 5% CO_2. An initial load of 1 g was applied to each preparation and maintained throughout a 75–90 min equilibration period before agonist addition. Tension was recorded isometrically on a Phillips recorder (PM 8222) coupled to a Hewlett Packard amplifier (8805D) via force-displacement transducers (Gould Statham UC2).

The composition of KBS was as follows (mM): NaCl 118, KCl 4.75, $CaCl_2$ 1.8, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11. Ca-free solution had the same composition except that $CaCl_2$ was omitted and EDTA (0.1 mM) was added.

The absence of a relaxant response after addition of 10^{-4} M acetylcholine to preparations previously contracted with noradrenaline (1 μM) indicated the absence of a functional endothelium (Furchgott & Zawadzki, 1980).

Contractions in KBS were expressed in mg. Dose-response curves of relaxation were obtained by addition of cumulative concentrations of the compounds tested on the sustained contractions induced by NA (1 μM) or KCl (60 mM). Relaxations were expressed as a percentage of the maximum tension obtained by agonist addition. E_{max} represents the maximal relaxation obtained after addition of the highest concentration of each compound tested. A regression of response against $-\log$ [C] of test compound was performed by the least squares method for each preparation. The concentration needed to produce 50% inhibition (IC_{50}) was obtained from the linear regression plot of all points between 20–80%: of the maximal response. Contractile responses in Ca-free KBS are expressed as a percentage of the noradrenaline or caffeine-induced contraction in normal KBS.

Results are expressed as the mean ± s.e.mean of 5 or more preparations (n) obtained from different animals. Statistical significance of differences between the means was assessed by Student's t test for unpaired data. P values of less than 0.05 were considered to represent significant differences.

Radioligand binding experiments

Membranes were prepared from cerebral cortex of male Wistar rats as previously described (Schott *et al.*, 1988). Briefly, the tissue was homogenized in 10 vol (w/v) of ice-cold buffer (50 mM Tris-HCl, pH 7.5) with an ultra-turrax (twice, 15 s). The homogenate was centrifuged at 40,000 *g* at 4°C for 10 min and the resulting pellet was washed by resuspension and centrifugation under the same conditions. The final pellet was resuspended in incubation buffer to give a final protein concentration of about 1 mg ml^{-1}. Membrane aliquots (250 μl) were incubated in a final volume of 500 μl in 50 mM Tris-HCl (pH 7.5) with [^{3}H]-prazosin (0.1–0.2 nM) or [^{3}H]-nitrendipine (0.3–0.4 nM) or in 50 mM Tris-HCl pH 7.5 containing 1 mg ml^{-1} of bovine serum albumin (BSA) with [^{3}H]-(+)-*cis*-diltiazem (3–4 nM), in the absence or in the presence of drugs at various concentrations.

Incubations were carried out at 25°C for 30 min ([^{3}H]-prazosin), 90 min ([^{3}H]-nitrendipine) or 120 min ([^{3}H]-(+)-*cis*-diltiazem), after which the reaction was terminated by the addition of 3.0 ml ice-cold buffer and rapid filtration over Whatman GF/B filters that were washed with 9 ml (3 × 3 ml) of ice-cold buffer. For [^{3}H]-(+)-*cis*-diltiazem binding experiments, filters were pretreated for 120 min with polyethylenimine 0.3%. The radioactivity bound to the filters was determined by liquid scintillation counting. Non-specific binding was determined in the presence of 10^{-6} M phentolamine, 10^{-6} M nifedipine or 10^{-5} M diltiazem, respectively. All assays were performed in triplicate.

Displacement curves were analyzed by a computerized non-linear curve-fitting programme and K_i values were calculated using the formula of Cheng & Prusoff (1973).

Phosphodiesterase inhibition

Cytosolic cyclic nucleotide phosphodiesterase activities were isolated from the media layer of bovine aorta by a modification of the methods of Lugnier *et al.* (1986). Briefly, the tissue was homogenized in 10 vol (w/v) isotonic buffer A (25 mM sodium phosphate pH 6.6; 250 mM saccharose; 2 mM magnesium acetate (MgAc); 1 mM dithiothreitol; 5 mM EGTA; 2000 u ml^{-1} aprotinin; 10 μg ml^{-1} soya bean trypsin inhibitor and 10 μg ml^{-1} leupeptin) with an ultra-turrax (six times, 10 s) and a glass pestle homogenizer, and then centrifuged at 105,000 *g* for 60 min. The resulting supernatant fraction was applied to a DEAE-Sephacel ion-exchange column and first eluted by buffer B (25 mM phosphate pH 6.6; MgAc 2 mM and dithiothreitol 1 mM) until no more absorbance was detected in the eluate at 280 nm. Elution was then continued with a linear gradient of 0–0.55 M of NaCl in buffer B (flow rate 25 ml h^{-1}). Each fraction was tested for PDE activity.

Since DEAE-Sephacel did not allow the separation of CaM-PDE from cGMP-PDE as did DEAE-trisacryl chromatography (Lugnier *et al.*, 1986) a further high performance liquid chromatography (h.p.l.c.) repurification was done. The first peak was then injected into a h.p.l.c. column (TSK-DEAE-5PW), washed for 20 min with elution buffer (20 mM phosphate pH 6.6) and eluted (0.8 ml min^{-1}) by a linear NaCl gradient (0.05–0.3 M) in elution buffer. Fractions

under each PDE activity peak were pooled, dialyzed against buffer (20 mM Tris-HCl, 2 mM MgAc pH 7.5) and stored in aliquots at −80°C with bovine serum albumin (BSA).

PDE activities were measured by the two-step assay described by Wells *et al.* (1975) at a substrate ([^{3}H]-cyclic AMP or [^{3}H]-cyclic GMP) concentration of 1 μM, in the following buffer: 50 mM Tris-HCl pH 7.5, 2 mM MgAc, 1 mg ml^{-1} BSA and in the presence of 10 μM $CaCl_2$ and 18 nM calmodulin (CaM) or in the absence of calcium and CaM but with 1 mM EGTA. To prevent the influence of cross-contamination between CGI-PDE (Type III) and cAMP-PDE (Type IV), the studies performed with these forms were always carried out in the presence of 100 μM rolipram or 100 μM cyclic GMP, respectively.

The IC_{50} (concentration which produced 50% inhibition of substrate hydrolysis) for the compounds studied was determined from the concentration-response curves obtained with five concentrations of inhibitor and calculated by a non-linear regression. The apparent K_i values were obtained according to Cheng & Prusoff (1973). Results are expressed as mean ± s.e.mean of three determinations made in duplicate with three different enzymatic preparations.

Drugs and solutions

The following drugs were used: (−)-noradrenaline L-tartrate and acetylcholine (Merck); caffeine, papaverine, glaucine, nifedipine, prazosin, (+)-*cis*-diltiazem, cyclic AMP and cyclic GMP (Sigma Chemical Co); phentolamine methanesulphonate (Ciba-Geigy).

[^{3}H]-prazosin (20.2 Ci mmol^{-1}), [^{3}H]-nitrendipine (70 Ci mmol^{-1}), [8-^{3}H]-cyclic AMP (30–50 Ci mmol^{-1}) and [8-^{3}H]-cyclic GMP (5–15 Ci mmol^{-1}) were purchased from the New England Nuclear. [^{3}H]-(+)-*cis*-diltiazem (154 Ci mmol^{-1}) was obtained from Amersham.

Other reagents were of analytical grade.

Nifedipine was dissolved in ethanol (10^{-2} M) before being diluted in distilled water. Caffeine was dissolved in Ca-free KBS. The other drugs were dissolved in distilled water.

Results

Functional study

Noradrenaline at concentrations of 1 μM produced a sustained contraction (348.8 ± 31.0 mg, $n = 9$) in rat isolated thoracic aorta incubated in KBS. This concentration of noradrenaline has proved to be the maximal stimulus for noradrenaline-induced contraction in rat aorta (Noguera & D'Ocon, 1992). Addition of cumulative doses of glaucine, diltiazem, nifedipine, prazosin and papaverine induced concentration-dependent relaxation (Figure 2a) and the E_{max} and IC_{50} for all the products tested are summarized in Table 1.

Another series of experiments was performed in KBS to determine the blocking effect of each product on Ca^{2+} entry through potential-operated Ca^{2+} channels. The contractile response of rat aorta was elicited by depolarizing solution (KCl 60 mM) at 37°C and its magnitude was 225.2 ± 18.5 mg ($n = 18$). All the compounds tested produced concentration-dependent relaxations in KCl-depolarized rat aorta (Figure 2b). The E_{max}, IC_{50} values and the IC_{50} (KCl/IC_{50}(NA) ratio for each product are shown in Table 1. As expected, prazosin showed a greater selectivity of action on NA-induced contraction, whereas nifedipine and diltiazem appeared to have a more potent effect on KCl-induced contraction than on NA-induced contraction. Glaucine showed greater potency on NA-induced contraction, and papaverine showed no specific relaxant action.

Figure 3 shows the experimental procedure used to study the action of the compounds on noradrenaline-induced contraction in the absence of extracellular calcium. After 20 min incubation in Ca^{2+}-free solution, addition of noradrenaline

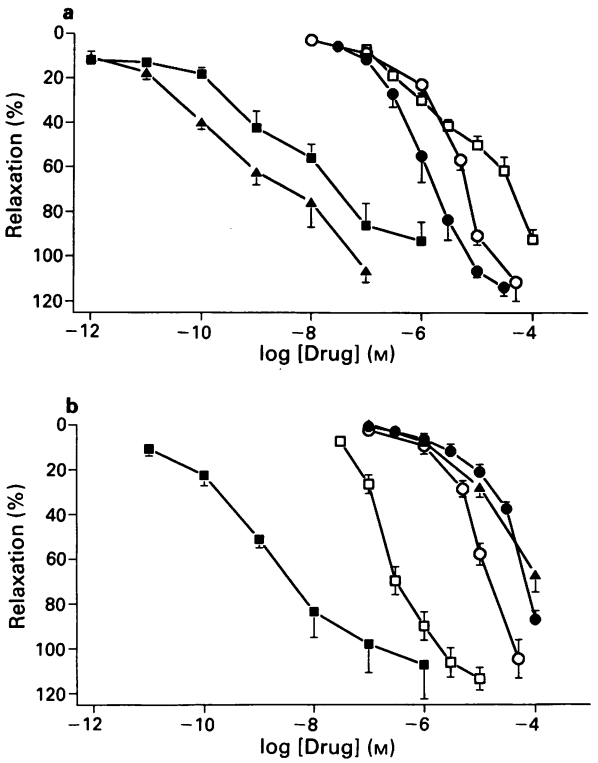


Figure 2 Relaxation dose-response curves obtained after addition of different agents in aorta previously contracted by 1 μM noradrenaline (a) or 60 mM KCl (b). Nifedipine (■); papaverine (○); prazosin (▲); glaucine (●) and diltiazem (□). Each point is the mean derived from n experiments with s.e.mean showed by vertical bars.

(1 μM) induced a phasic contraction, followed by a tonic contraction which is due to release of intracellular calcium (Karakai *et al.*, 1987; Karaki, 1989). The magnitude of this noradrenaline-induced contraction (NA_1) relative to the contraction in the presence of 1.8 mM Ca^{2+} was 24.1 ± 1.5% and 14.9 ± 2.5% ($n = 8$) for the phasic and the tonic step respectively. However the contraction was not evoked when noradrenaline 1 μM was added in Ca^{2+}-free solution a second time (NA_2). After a 20 min resting period in KBS and after 20 min in Ca^{2+}-free solution, addition of NA (NA_3) induced a contraction similar in magnitude to the first one (NA_1), thereby indicating that preincubation with 1.8 mM Ca^{2+} for 20 min restored the calcium at the intracellular storage sites. In order to study the effect of the different agents on this NA-induced contraction in Ca-free medium, the maximal concentrations of compounds that completely inhibit NA-induced contraction in KBS were added 10 min before the addition of NA (NA_3). This addition of NA in Ca^{2+}-free solution did not induce a contractile response in the presence of prazosin (10^{-7} M), papaverine (10^{-4} M) and glaucine (10^{-4} M). A lower response to NA was observed in the presence of diltiazem (10^{-4} M), whereas nifedipine (10^{-6} M) had no effect on NA_3-elicited contraction in Ca^{2+}-free solution (Table 2). These results indicate that only prazosin, papaverine and glaucine block the contractile response due to α-adrenoceptor activation in Ca^{2+}-free medium.

In order to clarify the possible action of glaucine and diltiazem on α-adrenoceptor or on the inhibition of Ca^{2+} release from the intracellular storage sites, similar experimental procedures were used but NA was replaced by caffeine (Caf) and temperature was decreased to 25°C (Figure 3b). In these conditions, Caf 10 mM induced a rapid transient contraction in Ca^{2+} 1.8 mM solution (168.6 ± 13.0 mg, $n = 11$). Addition of Caf_1 after 20 min of exposure to a Ca^{2+}-free solution yielded a phasic contraction with a magnitude that

Table 1 Inhibitory potencies of agents on contractions induced by noradrenaline (NA) and KCl in rat aorta

		NA	*KCl*	$IC_{50(NA)}/IC_{50(KCl)}$
Glaucine	E_{max} (%)	113.8 ± 3.8	87.3 ± 3.9	
		$n = 5$	$n = 11$	
	pIC_{50}	6.08 ± 0.07	4.40 ± 0.04*	0.021
Nifedipine	E_{max} (%)	93.2 ± 8.7	107.3 ± 15.2	
		$n = 5$	$n = 5$	
	pIC_{50}	8.07 ± 0.11	9.16 ± 0.10*	12.30
Diltiazem	E_{max} (%)	92.4 ± 4.5	113.6 ± 5.1	
		$n = 6$	$n = 5$	
	pIC_{50}	5.30 ± 0.12	6.78 ± 0.16*	30.19
Prazosin	E_{max} (%)	108.0 ± 4.1	67.1 ± 7.7	
		$n = 5$	$n = 4$	
	pIC_{50}	9.70 ± 0.09	4.42 ± 0.19*	5.2×10^{-6}
Papaverine	E_{max} (%)	111.5 ± 8.3	104.8 ± 8.5	
		$n = 7$	$n = 6$	
	pIC_{50}	5.51 ± 0.10	5.29 ± 0.07	0.60

$pIC_{50} = (-\log M)$
Values are means ± s.e.mean
*Significant difference ($P < 0.001$) from the corresponding values on NA-induced contraction.

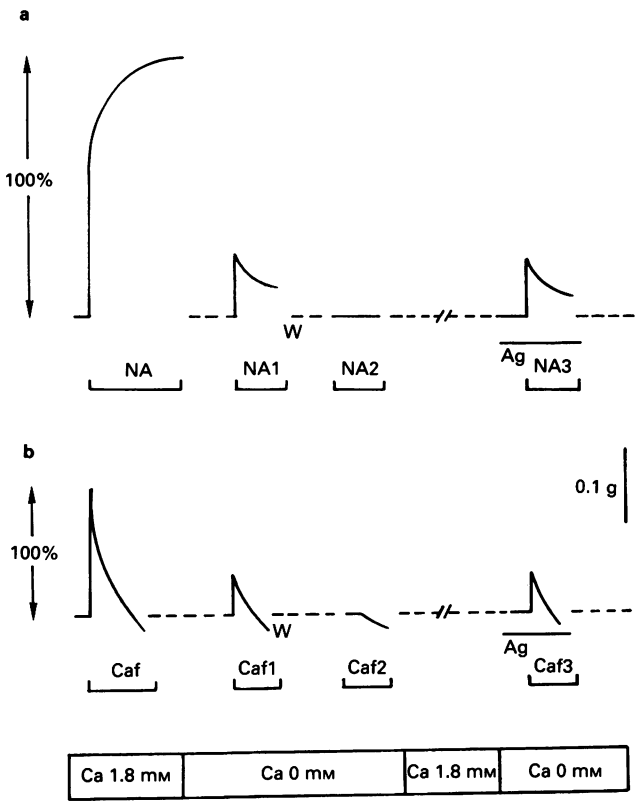


Figure 3 Schematic representation of the effect of the agents on noradrenaline (NA)-induced contraction (a) or caffeine (Caf)-induced contraction (b) in the absence of extracellular calcium. NA_1 or Caf_1: addition of the agonist after 20 min incubation in Ca^{2+}-free medium; NA_2 or Caf_2: second addition of the agonist after washing (W) in Ca^{2+}-free medium; NA_3 or Caf_3: third addition of the agonist after a 20 min resting period in KBS (Ca^{2+} 1.8 mM) and 20 min in Ca^{2+}-free solution. Ag: tested compound.

was 29.4 ± 4.4% ($n = 8$) of the value measured in the presence of Ca^{2+}. After washing, addition of Caf_2 did not induce any contractile response. After a loading period (20 min) in Krebs solution, to refill the intracellular Ca^{2+}-stores, Caf_3-induced contraction in Ca^{2+}-free medium (20 min) was restored. Pre-

Table 2 Effects of agents on noradrenaline (NA)-induced contraction of rat aorta in Ca^{2+}-free medium

Agent	*[C]*	*NA1* (%)	*NA3 + Agent* (%)	n
Glaucine	10^{-4} M	23.96 ± 8.32	—	8
Nifedipine	10^{-6} M	22.12 ± 1.36	20.30 ± 1.60	6
Diltiazem	10^{-4} M	21.76 ± 2.38	4.92 ± 1.36*	5
Prazosin	10^{-7} M	17.19 ± 3.43	—	3
Papaverine	10^{-4} M	26.97 ± 3.40	—	4

[C] Maximal concentration of agent that completely inhibits noradrenaline-induced contraction in Krebs solution.
% are calculated from the initial contraction by noradrenaline in Krebs solution.
Values are mean ± s.e.mean.
n = number of experiments.
*$P < 0.001$ from NA1.

incubation with glaucine (10^{-4} M), diltiazem (10^{-4} M), nifedipine (10^{-6} M) or prazosin (10^{-7} M) did not modify the contractile response induced by Caf_3 in Ca^{2+}-free medium after refilling the intracellular Ca^{2+} stores. Papaverine (10^{-4} M) significantly diminished the phasic contraction elicited by Caf_3 (Table 3).

Binding assays

Binding of [^{3}H]-prazosin to rat cerebral cortex was saturable, reversible and showed high affinity, with a dissociation constant K_d of 0.14 nM (Schott *et al.*, 1988). [^{3}H]-nitrendipine and [^{3}H]-(+)-*cis*-diltiazem also bound to a single class of binding site in rat cortical membrane homogenate, with K_d values of 0.7 nM and 50 nM, respectively (Schaeffer *et al.*, 1988).

The interactions of glaucine and papaverine with [^{3}H]-prazosin and [^{3}H]-(+)-*cis*-diltiazem binding are shown in Figure 4. Glaucine fully inhibited [^{3}H]-prazosin binding to cortical membranes, with an inhibition constant of 0.32 ± 0.03 μM (Figure 4a). The pseudo-Hill coefficient (slope factor) was not significantly different from unity ($n_H = 1.01 \pm 0.13$), which suggests direct competition between glaucine and the radioligand for a single common binding site. In addition, glaucine inhibited binding of [^{3}H]-(+)-*cis*-diltiazem (Figure 4b) but with a lower affinity ($IC_{50} = 10.02 \pm 2.59$ μM) and with a low pseudo-Hill slope ($n_H = 0.73 \pm 0.06$). This indi-

Table 3 Effects of agents on caffeine (Caf)-induced contraction of rat aorta in Ca^{2+}-free medium

Agent	*[C]*	*Caf1* (%)	*Caf3 + Agent* (%)	n
Glaucine	10^{-4} M	28.27 ± 4.87	24.35 ± 5.28	5
Nifedipine	10^{-6} M	30.87 ± 4.28	32.95 ± 4.50	5
Diltiazem	10^{-4} M	33.83 ± 5.50	32.32 ± 1.89	5
Prazosin	10^{-7} M	31.16 ± 6.21	25.19 ± 3.62	4
Papaverine	10^{-4} M	32.96 ± 2.13	19.32 ± 4.72*	8

[C] Maximal concentration of agent that completely inhibits noradrenaline-induced contraction in Krebs solution.
% are calculated from the initial contraction by caffeine in Krebs solution.
Values are mean ± s.e.mean
n = number of experiments.
*$P < 0.002$ from Caf1.

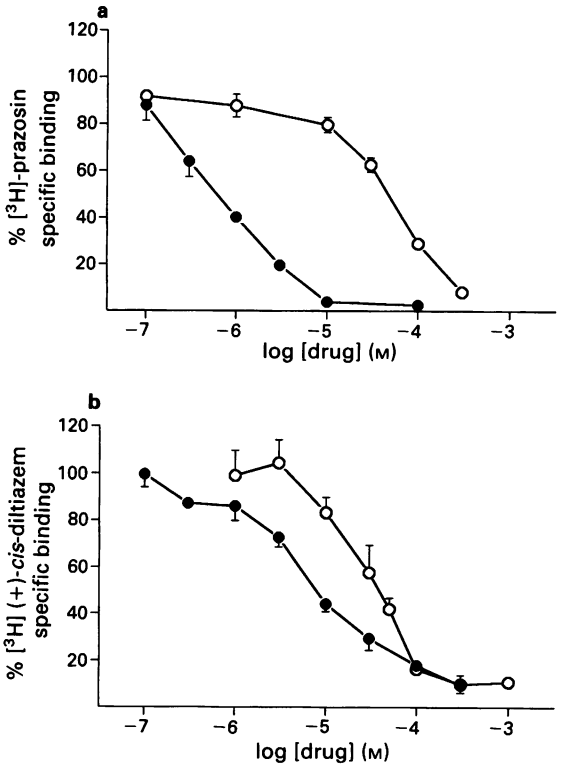


Figure 4 Displacement curves of [^{3}H]-prazosin (a) or [^{3}H]-(+)-*cis*-diltiazem specific binding (b) by papaverine (O) or glaucine (●). Each point is the mean from three experiments done in triplicate with s.e.mean showed by vertical bars.

cates that the interaction between glaucine and [^{3}H]-(+)-*cis*-diltiazem is not a simple competition for a single common binding site but a more complex interaction. Papaverine inhibited both [^{3}H]-prazosin and [^{3}H]-(+)-*cis*-diltiazem binding in a concentration-dependent manner, showing similar K_i values for both radioligands ($K_i = 33.6 \pm 8.8\,\mu M$, $n_H = 1.45 \pm 0.31$ and $K_i = 19.7 \pm 2.6\,\mu M$, $n_H = 0.83 \pm 0.16$, respectively). Glaucine appeared to have more affinity at the [^{3}H]-prazosin and [^{3}H]-(+)-*cis*-diltiazem binding site than papaverine. Moreover, glaucine was approximately 60 times more potent than papaverine in inhibiting [^{3}H]-prazosin binding, but was only 4 times more potent than papaverine in inhibiting [^{3}H]-(+)-*cis*-diltiazem binding.

[^{3}H]-nitrendipine binding was not affected by either papaverine or glaucine in concentrations up to 100 μM (results not shown).

We have also tested the effect of rolipram, a specific inhibitor of PDE type IV, on [^{3}H]-prazosin, [^{3}H]-diltiazem and [^{3}H]-nitrendipine binding to cortical membrane and have observed that rolipram did not modify the binding of these radioligands in concentrations up to 300 μM.

Inhibition of bovine aorta cyclic nucleotide phosphodiesterases

We have examined the inhibitory effect of glaucine and papaverine on the following cytosolic molecular forms of PDE isolated from bovine aorta: a PDE form called CaM-PDE (type I) which preferentially hydrolyzes cyclic GMP and is activated by calmodulin (CaM); a cyclic GMP-selective form (cGMP-PDE; type V) insensitive to the stimulatory effect of CaM; and two PDE forms that specifically hydrolyze cyclic AMP with a low K_m and which are not stimulated by the addition of Ca^{2+}-CaM; one belongs to the cyclic GMP-inhibited family (CGI-PDE, type III) and the other is a rolipram-sensitive form (cAMP-PDE, type IV) (Lugnier *et al.*, 1986; Ivorra *et al.*, 1992).

As shown in Table 4, papaverine exerted a relatively non-selective inhibitory effect on all PDE forms although the inhibition of the calmodulin-sensitive form appeared weaker than that of the other forms. In contrast, glaucine proved to be a potent and selective inhibitor of the rolipram-sensitive form of low K_m cAMP-PDE (type IV) with a K_i value of 3.3 μM, whereas it had no effect on the CGI-PDE form ($IC_{50} > 300\,\mu M$). It also inhibited hydrolysis of cyclic GMP by CaM-PDE but with a low potency, exerting similar inhibitory effects both on the basal or the activated (in the presence of calcium and CaM) state of the enzyme (K_i values of 63.3 and 44.4 μM, respectively). In addition, glaucine did not exert any inhibitory effect on the cyclic GMP hydrolytic activity of cGMP-PDE ($IC_{50} > 300\,\mu M$).

Discussion

In previous work we have shown that the relaxant effects of benzylisoquinolines in uterine smooth muscle vary, depending on whether or not an unsaturated heterocyclic ring (papaverine) or a tetrahydroisoquinoline ring (cularines, glaucine, antioquine) was present. This structural feature implies a more specific activity, similar to that of Ca^{2+}-entry blockers (D'Ocon *et al.*, 1989; 1991; Anselmi *et al.*, 1992). This selective action showed by benzyltetrahydroisoquinolines implies an inhibition of Ca^{2+}-influx from the extracellular medium without changes in the intracellular distribution of this ion. In contrast, the benzylisoquinolines with an unsaturated ring induce a relaxation mediated by both a decrease in the Ca-entry through specific channels and a decrease in the intracellular free Ca^{2+}-levels (Cortes *et al.*, 1990; Anselmi *et al.*, 1992).

These findings prompted us to examine the potential activity of the alkaloid glaucine, which shows these structural characteristics, as a Ca-channel blocker. We began the study by analyzing the relaxant action of glaucine on the contractile response of rat aorta induced by noradrenaline or KCl, in order to ascertain whether or not the action of the alkaloid was selective. A comparison of the relaxation induced by glaucine with the relaxant responses elicited by nifedipine, diltiazem, prazosin and papaverine was also made.

Analysis of the IC_{50} (NA)/IC_{50} (KCl) ratios provides information on selectivity (Table 1) and indicates that glaucine, like prazosin, exhibits greater inhibition of the contractile response induced by NA than KCl. As expected, nifedipine and diltiazem show more potent relaxant effects on responses to KCl, whereas papaverine relaxes contractile responses to NA or KCl in a non-selective way, as described in earlier papers (Bolton, 1979).

The selectivity shown by glaucine on NA-induced contraction in KBS was checked in the experiments carried out in a

Table 4 Effects of agents on cyclic nucleotide phosphodiesterases isolated from bovine aorta

PDE activity	*^{3}H-substrate*	K_i (μM) *Glaucine*	*Papaverine*
CaM-PDE type I	Cyclic GMP + EDTA	63.3 ± 20.6	8
	Cyclic GMP + CaM	44.4 ± 9.8	27.5 ± 2.2*
cGMP-PDE type V	Cyclic GMP + EGTA	> 300	1.08 ± 0.36*
CGI-PDE type III	Cyclic AMP + EGTA	> 300	0.76
cAMP-PDE type IV	Cyclic AMP + EGTA	3.3 ± 0.23	1.68

Data shown are mean ± s.e.mean from three determinations obtained in different enzymatic preparations. K_i values were calculated from IC_{50} values according to Cheng & Prusoff. PDE activity was assessed with 1 μM of [^{3}H]-cyclic GMP + 1 mM EGTA or 1 μM [^{3}H]-cyclic GMP + 10 μM $CaCl_2$ + 18 nM calmodulin for CaM-PDE; 1 μM of [^{3}H]-cyclic GMP + 1 mM EGTA for cGMP-PDE; 1 μM [^{3}H]-cyclic AMP + 1 mM EGTA and in the presence of 100 μM of rolipram for CGI-PDE or 100 μM of cyclic GMP for cAMP-PDE type IV in order to limit the cross-contamination of these forms.
*Data from Lugnier *et al.* (1986).

nominally Ca^{2+}-free medium. Under these conditions, NA and Caf induce a contractile response that is due only to the release of intracellular Ca^{2+} from the internal stores sensitive to the agonists. The mechanism whereby NA and Caf cause Ca^{2+}-release is not entirely known, but the fact that prazosin inhibits NA- but not Caf-induced contraction in Ca^{2+}-free medium indicates that the α_1-adrenoceptor is directly related to this Ca^{2+}-release induced by NA, probably by release of 1,4,5-inositol trisphosphate.

In contrast, papaverine inhibits the contractile response to both NA and Caf in these experimental procedures, again confirming its non-specific relaxant activity.

While nifedipine does not modify the contraction induced by NA or Caf in Ca^{2+}-free medium, diltiazem partially diminishes the NA-elicited contraction but produces no changes in the contractile response to Caf.

Glaucine completely inhibited NA-induced contractile responses in Ca-free medium and this effect is not attributable to direct inhibition of the smooth muscle contractile elements, or release of the intracellular calcium stores because the same concentration of alkaloid did not inhibited Caf-induced contraction. It has been suggested that, in rat aorta, the NA-induced release of intracellular Ca^{2+} is attributable to α_1-adrenoceptor activation (Chiu *et al.*, 1987; Daly *et al.*, 1990) whereas the Caf-induced Ca^{2+}-release is due to a different mechanism (Itoh *et al.*, 1983; Karaki *et al.*, 1987; Sato *et al.*, 1988). Therefore, glaucine can selectively inhibit Ca^{2+}-release mediated by α_1-adrenoceptor activation in rat aorta. This effect may be due to antagonism of α_1-adrenoceptors, or inhibition of receptor-mediated signal transduction. In order to identify the mechanism we studied the interaction of glaucine with the α_1-adrenoceptor, using radioligand binding techniques, and compared these effects with those of papaverine. The results obtained indicate that both glaucine and papaverine interact with [^{3}H]-prazosin binding to rat cerebral cortex although glaucine showed a higher affinity for the α_1-adrenoceptor binding site than did papaverine.

However, besides blocking α_1-adrenoceptors, glaucine may also have Ca^{2+}-entry blocking properties because it antagonizes contractions induced by depolarizing solution as do calcium entry blockers like nifedipine and diltiazem. For this reason, we have evaluated the effect of glaucine and papaverine on [^{3}H]-nitrendipine and [^{3}H]-(+)-*cis*-diltiazem binding sites. Both alkaloids interact with the benzothiazepine binding site of the Ca^{2+}-entry blocker receptor complex but have no effect at the dihydropyridine binding site.

Although both papaverine and glaucine inhibit [^{3}H]-(+)-*cis*-diltiazem and [^{3}H]-prazosin binding, the present results demonstrate that glaucine exerts a measure of selectivity as an inhibitor of [^{3}H]-prazosin binding compared with [^{3}H]-(+)-*cis*-diltiazem binding, while papaverine appears to show approximately equal affinity in this respect. These findings largely agree with the results obtained in the functional studies, where glaucine appeared more potent in relaxing NA-induced contraction than KCl-induced contraction, whereas papaverine relaxed both contractions in a similar manner.

On the other hand, many authors (Kukovetz & Pöch, 1970; Lugnier *et al.*, 1972; Van Inwegen *et al.*, 1979) postulated that the mechanism of action of many isoquinoline derivatives, including papaverine, involves inhibition of PDE. In order to determine to what extent inhibition of PDE may be involved in the vasorelaxant response to glaucine, we tested the effect of this compound on PDE activities isolated from bovine aorta as a model for vascular smooth muscle.

It is now apparent that there are several molecular forms of PDE identified in most mammalian tissues, including smooth muscle. In vascular smooth muscle four types of PDE have been isolated. Type I PDE (CaM-PDE) is Ca^{2+}/camodulin-dependent and preferentially hydrolyzes cyclic GMP, whereas type V (cGMP-PDE), which selectively hydrolyzes cyclic GMP, is not stimulated by Ca^{2+}/calmodulin (Lugnier *et al.*, 1986). Types III and IV are two forms of Ca^{2+}-independent PDE with a high affinity for cyclic AMP; the type III (CGI-PDE) is selectively inhibited by cyclic GMP, cilostamide and some cardiotonic agents, whereas type IV is insensitive to cyclic GMP inhibition but is selectively inhibited by rolipram (Ahn *et al.*, 1989; Lindgren *et al.*, 1990; Komas *et al.*, 1991; Ivorra *et al.*, 1992).

Our results show that glaucine selectively inhibits one of the two forms of Ca^{2+}-independent low K_m cAMP-PDE, type IV, whereas papaverine has an equipotent inhibitory effect on both forms. We have also evaluated the inhibitory effect of glaucine on the other molecular forms of PDE that preferentially hydrolyze cyclic GMP. Glaucine inhibits the CaM-PDE form but the effect is less potent compared with effects on cAMP-PDE type IV and this alkaloid has no effect on cGMP-PDE hydrolytic activity. In contrast, papaverine is a relatively non-selective inhibitor of these PDE forms (Lugnier *et al.*, 1986). The inhibitory effect of glaucine on CaM-PDE was tested both in the presence and absence of Ca^{2+}/calmodulin since the ability of a drug to inhibit the activation of the enzyme by calmodulin can be used as a test system for characterizing possible calmodulin antagonist properties. Since glaucine exerts a similar inhibitory effect on the basal or activated state of the enzyme, we can also exclude the possibility that glaucine has calmodulin-antagonist action.

These data are the first account of a benzylisoquinoline structure showing a selective inhibitory effect on one low K_m cAMP-PDE form (Type IV). There is increasing evidence for selective forms of PDE being involved in controlling different biological processes and thus selective PDE inhibition can lead to a variety of discrete pharmacological responses (Weishaar *et al.*, 1985; Beavo, 1988). In this way, inhibition of the CGI-PDE enzyme present both in cardiac (Harrison *et al.*, 1986; Reeves *et al.*, 1987; Komas *et al.*, 1989) and vascular tissues (Ahn *et al.*, 1989; Lindgren *et al.*, 1990; Komas *et al.*, 1991; Ivorra *et al.*, 1992) is important for cardiotonic/vasodilator activity (Harrison *et al.*, 1986; Kauffman *et al.*, 1987; Weishaar *et al.*, 1987; Kithas *et al.*, 1988; Ivorra *et al.*, 1992)

whereas inhibition of the cAMP-PDE, type IV, an enzyme characterized also in cerebral tissue (Strada *et al.*, 1984; Nemoz *et al.*, 1985) may be important because of its antidepressant properties (Weishaar *et al.*, 1985; Schultz & Schmidt, 1986; Sutor *et al.*, 1990).

On the basis of these findings, it seems unlikely that only the inhibitory effect of glaucine on cAMP-PDE type IV can account for the vasorelaxant properties observed in this functional study.

The different pharmacology exhibited by papaverine and glaucine might be attributed to geometry or to differences in the basicity of the heteroatom. The structural features of glaucine that define its geometry are the presence of an sp^3-like hybridized nitrogen atom, a chiral centre and also a partially flexible tetrahydroisoquinoline ring. These topological characteristics and the electronic characteristics concerning the basicity at the nitrogen site could determine a specific mode of interaction with the α-adrenoceptor, benzothiazepine receptor site at the calcium channel and inhibition of PDE IV.

In summary, the present work provides evidence that the benzyltetrahydroisoquinoline derivative, glaucine, has affinity for [^{3}H]-prazosin and [^{3}H]-diltiazem binding sites which probably account for α_1-adrenoceptor antagonist and calcium antagonist properties (through benzothiazepine receptor site in the calcium channel). The compound also shows selective inhibition of type IV PDE.

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Purinoceptors mediating relaxation and spasm in the rat gastric fundus

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1 The relaxant and spasmogenic effects of purines and analogues were studied in longitudinal strips of rat gastric fundus to characterize the purinoceptors involved. Classification was studied by use of agonist potency orders and of antagonists in circumstances where the influence of confounding factors was reduced. In general tone was raised by carbachol (0.1 μM).

2 Adenosine produced relaxation and was potentiated by nitrobenzylthioinosine (NBTI, 0.3 and 30 μM), an adenosine-uptake inhibitor. 8-Sulphophenyl-theophylline (8-SPT, 30 μM), a selective P_1-purinoceptor antagonist, antagonized adenosine and 5'-N-ethylcarboxamidoadenosine (NECA), a selective agonist at P_1-purinoceptors.

3 At resting tone, adenosine 5'-triphosphate (ATP) induced a small, phasic relaxation followed by a maintained spasm. When tone was raised by carbachol, ATP induced a larger relaxation followed by a smaller spasm. NBTI did not potentiate ATP, nor did 8-SPT antagonize ATP, suggesting that ATP does not act directly or indirectly at P_1-purinoceptors.

4 With raised tone, and in the presence of indomethacin (10 μM) and 8-SPT (30 μM), 2-methylthio ATP (2-MeSATP) and ATP produced relaxations followed by spasms while α,β-methylene ATP (α,β-MeATP) induced only relaxation; all responses were concentration-dependent. The compounds had similar slopes and maxima for relaxation and spasm. The rank orders of potency were 2-MeSATP ≫ α,β-MeATP > ATP for relaxation and 2-MeSATP ≫ ATP for spasm.

5 With raised tone, and in the presence of indomethacin and 8-SPT, desensitization to α,β-MeATP (100 μM) completely and only slightly suppressed responses to ATP and 2-MeSATP, respectively, as relaxants but had no effect on relaxant responses to adenosine. The magnitude of the spasms to ATP and 2-MeSATP was considerably increased by desensitization with α,β-MeATP but the spasm to KCl was not affected.

6 With raised tone, and in the presence of indomethacin and 8-SPT, reactive blue 2 (10 μM) non-selectively antagonized ATP, 2-MeATP, α,β-MeATP, adenosine and isoprenaline as relaxants. Reactive blue 2 prevented the spasms to ATP and 2-MeSATP but not spasm to KCl.

7 With raised tone, and in the presence of indomethacin, suramin (100 μM) antagonized ATP, but not adenosine, as relaxants and antagonized ATP, but not KCl, as spasmogens.

8 It is proposed that adenosine is susceptible to nucleoside-specific uptake and acts predominantly via a P_1-purinoceptor and also by a non-P_1-purinoceptor mechanism. ATP- and α,β-MeATP-induced relaxations probably occur via a P_{2X}-purinoceptor. The anomalous nature of the 2-MeSATP-induced relaxation suggests it acts both via a P_{2X}-purinoceptor and an additional mechanism. A P_{2Y}-purinoceptor is most likely to be involved in the spasms to ATP and 2-MeSATP. Therefore, the functional nature of the responses mediated by P_{2X}- and P_{2Y}-purinoceptors, relaxation and spasm respectively, are opposite to those seen in most smooth muscles.

Keywords: P_{2X}-purinoceptor; P_{2Y}-purinoceptor; P_1-purinoceptor; adenosine uptake; rat gastric fundus

Introduction

Adenosine, adenosine 5'-triphosphate (ATP) and analogues have long been known to induce relaxation and spasm of smooth muscles via an extracellular site of action. Burnstock (1978) suggested that these effects were elicited via two classes of receptors, designated P_1-purinoceptors and P_2-purinoceptors. At the P_1-purinoceptor the rank order of agonist potency is adenosine > ATP and methylxanthines are selective antagonists, whereas at the P_2-purinoceptor the agonist potency order is ATP > adenosine but here there are currently no potent selective antagonists. It has become clear that the P_2-purinoceptor is not homogeneous. Burnstock & Kennedy (1985) proposed a subdivision of the P_2-purinoceptor into P_{2X}- and P_{2Y}-subtypes. At the P_{2X}-purinoceptor the agonist potency order is α,β-methylene ATP (α,β-MeATP) > ATP = 2-methylthioATP (2-MeSATP), while it is 2-MeSATP ≫ ATP > α,β-MeATP at the P_{2Y}-purinoceptor. It has been suggested that α,β-MeATP is a selective desensitizing agent at P_{2X}-purinoceptors. Usually P_{2X}-purinoceptors mediate spasm while P_{2Y}-purinoceptors mediate relaxation of smooth muscle. In general, studies since 1985 have supported this subdivision of P_2-purinoceptors (Kennedy, 1990).

The identification of the purinoceptor(s) present in a particular smooth muscle is complicated by several factors; such as the presence of more than one receptor possibly mediating the same functional effect, the rapid metabolism of some agonists to other active compounds, the uptake of some agonists into the tissue by active transport processes, the lack of selectivity of many agonists for specific receptors and the lack of potent, selective antagonists. It has been suggested that reactive blue 2 is a moderately selective antagonist at P_{2Y}-purinoceptors at low but not high concentrations without

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effects at P_{2X}-purinoceptors or other receptors (Burnstock & Warland, 1987; Hopwood & Burnstock, 1987; Taylor *et al.*, 1989). Furthermore, the trypanocidal drug suramin has been reported to be a selective antagonist at P_{2X}- and P_{2Y}-purinoceptors relative to other receptors (Dunn & Blakeley, 1988; Den Hertog *et al.*, 1989; Hoyle *et al.*, 1990; Leff *et al.*, 1990; Von Kügelgen *et al.*, 1990).

There are observations in some smooth muscles of the gastrointestinal tract which are not entirely consistent with the P_{2X}-/P_{2Y}-purinoceptor classification or the proposition that P_{2X}-purinoceptors always mediate spasm and the P_{2Y}-purinoceptors always mediate relaxation. Wiklund & Gustafsson (1988) found that ATP and analogues produced spasm of guinea-pig isolated ileum with a potency order compatible with an action at P_{2Y}-purinoceptors but the compounds were not antagonized by reactive blue 2. More recently, Bailey & Hourani (1990) have suggested that the rat colon muscalaris mucosae contains P_{2Y}-purinoceptors which mediate spasm. In the rat gastric fundus, ATP produced a biphasic effect, relaxation followed by spasm (Burnstock *et al.*, 1970; Lefebvre & Burnstock, 1990). Moreover, 2-MeSATP also produced a biphasic effect but α,β-MeATP and adenosine only induced relaxation. The rank order of potency for relaxation was 2-MeSATP > α,β-MeATP > ATP > adenosine which does not correspond with either P_{2X}- or P_{2Y}-purinoceptors. Also, ATP, as a relaxant, was antagonized by reactive blue 2, but not by suramin and the effect of ATP was reduced by desensitization with α,β-MeATP. Thus, Lefebvre & Burnstock (1990) concluded that the subtype mediating relaxation may differ from either the P_{2X}- and or the P_{2Y}-purinoceptor. They did not conduct a full analysis of the purinoceptor mediating spasm to ATP but suggested that it was rebound following the relaxation and involved prostaglandin generation as the spasm was reduced by indomethacin.

The purpose of this study was to clarify the nature of the purinoceptors in the isolated rat gastric fundus where the current classification is problematic. The approach was functional by using the rank order of potency of agonists. Experiments were performed both with agonists alone and in circumstances where confounding factors should be reduced (Kenakin, 1987; O'Connor *et al.*, 1990), that is, in the presence of an antagonist at P_1-purinoceptors (8-sulphophenyltheophylline, 8-SPT; Gustafsson, 1984), an inhibitor of adenosine uptake (nitrobenzylthioinosine, NBTI; Clanachan *et al.*, 1987; Jarvis, 1987), a cyclo-oxygenase inhibitor (indomethacin; Vane, 1971) and/or a desensitizing agent at P_{2X}-purinoceptors (α,β-MeATP; Burnstock & Kennedy, 1985). 5'-N-ethylcarboxaminoadenosine (NECA) was used as a stable and selective agonist at P_1-purinoceptors (Van Calker *et al.*, 1979). In addition, studies were conducted using the purported selective antagonists reactive blue 2 and suramin.

Methods

Rat gastric fundus

Male Sprague Dawley or Allen and Hanburys rats (150–600 g) were killed by a blow to the head and exsanguination. The stomach was removed and two strips of longitudinal fundal smooth muscle were prepared as described by Vane (1957). The tissues were suspended under isometric conditions in 20 ml organ baths containing a physiological salt solution (PSS) of the following composition (mM): Na^+ 143.0, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.55, $H_2PO_4^-$ 1.2, SO_4^{2-} 1.2, Cl^- 128.0, HCO_3^- 25.0 and glucose 11.0. The PSS was maintained at 37°C and gassed with 95% O_2/5% CO_2. Tissues were placed under an initial tension of 1 g and allowed to equilibrate for 1 h.

In most experiments tissue tone was raised by carbachol (0.1 μM; Lefebrve & Burnstock, 1990). The carbachol contact time was 14 min. After the tissues had been exposed to carbachol (0.1 μM) for 10 min, the agonist (for example, ATP) was added and left in contact for 4 min. The tissues were washed to remove carbachol and agonist and left for 6 min before re-exposure to carbachol (0.1 μM) and agonist. In this manner non-cumulative concentration-response curves for agonists were obtained by stepwise increments of 0.5 or 1.0 $\log_{10}$ M concentrations.

Preliminary studies

Some preliminary experiments were conducted to establish the conditions to be used in the main study.

Tone induced by carbachol Two cumulative concentration-response curves for carbachol were constructed to determine a concentration that would produce a submaximal spasm of about an EC_{60}. The two curves were constructed 1 h apart. The amplitude of the spasms were expressed as a percentage of the maximum spasm obtained in each curve.

Effect of ATP at different tone levels Each tissue was initially challenged with carbachol (1 μM; an EC_{100}). Non-cumulative concentration-response curves were obtained to ATP at resting tension and, 1 h later, when tone was raised by carbachol (0.1 μM). The responses to ATP in this set of experiments were expressed as a % of the spasm to carbachol (1 μM) (but see later).

Influence of indomethacin on the responses to ATP A study was conducted to assess the involvement of the products of cyclo-oxygenase in the actions of ATP. Concentration-effect curves were constructed to ATP, with tone raised by carbachol (0.1 μM), before and after incubation with indomethacin (10 μM) for 45 min. Time-matched control tissues were exposed to the vehicle for indomethacin.

P_1-purinoceptors, adenosine uptake and the relaxation to adenosine and ATP

8-SPT (30 μM) was included in the PSS in most experiments to eliminate agonist-induced relaxations mediated directly or indirectly via P_1-purinoceptors. Concentration-response curves were constructed to adenosine, NECA or ATP in the absence and in the presence, after 45 min incubation, of 8-SPT (30 μM). To determine the importance of adenosine uptake on the potencies of adenosine or ATP, non-cumulative concentration-response curves were constructed to the compounds in the absence and presence of NBTI (0.3 and 30 μM; 45 min incubation). Indomethacin (10 μM) was included in the PSS throughout. Time-matched control tissues were exposed to the vehicle for 8-SPT or NBTI as appropriate.

All subsequent experiments were conducted on tissues with spasm induced by carbachol (0.1 μM) and with the PSS containing indomethacin (10 μM) and 8-SPT (30 μM) throughout. 8-SPT was not present for the experiments with suramin.

Agonist potencies

A non-cumulative concentration-response curve was constructed to ATP with spasm induced by carbachol (0.1 μM). After 1 h, a concentration-response curve was constructed to ATP, 2-MeSATP, α,β-MeATP or adenosine, one agonist per tissue.

Influence of desensitization to α,β-methylene ATP

Non-cumulative concentration-response curves were constructed to ATP, 2-MeSATP, adenosine or potassium chloride (KCl) with spasm induced by carbachol (0.1 μM). After 15 min, tissues were exposed to carbachol (0.1 μM) and 10 min later α,β-MeATP (100 μM) added and left in contact with the tissue for 1 h. Initially the α,β-MeATP produced a pronounced relaxation but carbachol-induced tone partially or fully recovered. After 1 h, when necessary, further carba-

chol (0.05–0.1 μM) was added to return spasm to the original level. A second concentration-response curve was constructed to ATP, 2-MeSATP, adenosine or KCl in the continued presence of α,β-MeATP (100 μM). Time-matched control tissues were exposed to the vehicle for α,β-MeATP.

Influence of reactive blue 2 or suramin

Non-cumulative concentration-response curves were constructed to ATP, 2-MeSATP, α,β-MeATP, adenosine, KCl or isoprenaline (reactive blue 2 experiments) or to ATP, adenosine or KCl (suramin experiments). Tissues were subsequently exposed to reactive blue 2 (10 μM; 45 min) or to suramin (100 μM, 90 min; Leff *et al.*, 1990) before a second concentration-response curve was constructed to the appropriate agonist. Time-matched control tissues were exposed to the vehicles for reactive blue 2 or suramin.

Statistical analysis

The response (relaxation or spasm) to each concentration of agonist was expressed as a % of the tone induced by carbachol (0.1 μM). For each tissue strip linear regression of % response versus $-\log_{10}$ M concentration of agonist was conducted, only with data from responses which lay between 20 and 80% of the maximum. From these regressions, the concentrations producing 50% of maximum response to the agonist were calculated and hence pD_2 derived for each agonist on each tissue strip. Differences between the first and second concentration-response curves were expressed as the $\log_{10}$ concentration ratio (log CR). Data were calculated as means ± s.e.mean. The significance of differences between group pD_2 or log CR were determined by paired or unpaired Student's t test, with $P<0.05$ considered to be significant. For illustrative purposes the responses to an agonist at specific drug concentrations from all tissues in the same experimental group were calculated as means ± s.e.mean.

Drugs and solutions

Stock solutions (all of 10 mM) of carbamylcholine chloride (carbachol, Sigma), ATP (disodium salt, Sigma), adenosine (hemisulphate salt, Sigma), 2-MeSATP (Research Biochemicals Inc.), α,β-MeATP (dilithium salt, Sigma), 8-SPT (Research Biochemicals Inc.), reactive blue 2 (cibacron blue 3GA, Sigma) and suramin (Bayer) were made in distilled water. Agonist solutions or solvent in controls were added to the organ baths in volumes usually not exceeding 1 ml. Indomethacin (Sigma) was dissolved initially in 10% sodium bicarbonate solution and subsequently added to the PSS. NECA (Glaxo Group Research) and isoprenaline (Sigma) were dissolved in 0.1 N hydrochloric acid with further dilutions in distilled water. NBTI (Sigma) was dissolved in 0.1 M sodium hydroxide solution with further dilutions in distilled water.

Results

Preliminary studies

Tone induced by carbachol Carbachol, added cumulatively, caused a concentration-dependent spasm. The initial and second concentration-response curves did not differ significantly from each other in terms of position, slope or maximum. Carbachol (0.1 μM) produced 64.3 ± 4.9% (first curve) and 59.3 ± 3.6% (second curves, $n=8$) of the maximum spasm to carbachol.

Effects of ATP at different tone levels Under both resting tone and with tone raised by carbachol (0.1 μM), ATP (0.1 μM–1 mM) induced an initial phasic, concentration-dependent relaxation followed by a sustained, concentration-dependent spasm. At resting tension the relaxation was small and the spasm large, while the converse was seen in the presence of raised tone but the potencies of ATP in these situations did not differ. The pD_2 values of ATP, as a relaxant, at resting and raised tone were 5.31 ± 0.15 and 5.26 ± 0.18 ($n=6$) respectively. The pD_2 values of ATP, as a spasmogen, at resting and raised tone were 5.19 ± 0.23 and 5.09 ± 0.30 ($n=6$) respectively.

Influence of indomethacin on the responses to ATP ATP, as a relaxant, was slightly but significantly ($P<0.05$) potentiated (log CR = 0.30 ± 0.04, $n=6$) by indomethacin (10 μM). The spasm to ATP was nearly abolished by indomethacin (10 μM). The vehicle for indomethacin did not modify responses to ATP. Indomethacin (10 μM) did not modify the spasm to carbachol (0.1 μM).

P_1-purinoceptors, adenosine uptake and relaxation to adenosine and ATP

Adenosine (1 μM–3 mM) caused a maintained and concentration-dependent relaxation. 8-SPT (30 μM), with indomethacin (10 μM) present throughout, antagonized adenosine (log CR = 0.67 ± 0.11, $P<0.05$, $n=6$), although the shift in the concentration-response curve was greater at the foot then the top of the curve. The pD_2 values for adenosine in the absence and presence of 8-SPT (30 μM) were 4.19 ± 0.05 and 3.51 ± 0.19 ($n=6$) respectively.

NBTI (0.3 and 30 μM) potentiated adenosine (log CR = 0.36 ± 0.12 and 0.71 ± 0.08 respectively, $n=6$, $P<0.05$). In the presence throughout of NBTI (30 μM) and indomethacin (10 μM), 8-SPT (30 μM) produced a parallel rightward displacement of the concentration-effect curve to adenosine without reduction in the maximum response. Accounting for a small rightward shift in time-matched controls, the log CR of adenosine due to 8-SPT was 1.26 ± 0.18 ($n=6$).

NECA (0.1–100 μM) caused a maintained and concentration-dependent relaxation of similar time course to that of adenosine. 8-SPT (30 μM), in the presence of indomethacin (10 μM) throughout, caused a parallel, rightward displacement of the concentration-effect curve to NECA without reduction in the maximum response (pD_2 in absence of 8-SPT = 6.15 ± 0.07, pD_2 in presence of 8-SPT = 4.12 ± 0.13, log CR = 1.99 ± 0.13, $P<0.01$, $n=6$).

NBTI (30 μM) did not modify the potency of ATP as a relaxant (pD_2 in absence of NBTI = 5.84 ± 0.28, pD_2 in the presence of NBTI = 5.90 ± 0.15, $P>0.05$, $n=6$). Relaxations produced by ATP were not modified by 8-SPT (30 μM; pD_2 in the absence of 8-SPT = 5.99 ± 0.12, in the presence of 8-SPT = 6.04 ± 0.11, $P>0.05$, $n=6$).

Neither NBTI nor 8-SPT modified the spasm to carbachol (0.1 μM).

Agonist potencies

In the presence of indomethacin (10 μM) and 8-SPT (30 μM), ATP (0.1 μM–1 mM) and 2-MeSATP (1 nM–30 μM) induced concentration-dependent relaxations, followed by small, concentration-dependent spasms while α,β-MeATP (10 nM–0.1 μM) and adenosine (1 μM–3 mM) induced concentration-dependent relaxations only. All compounds produced relaxation log concentration-effect curves which were sigmoidal in nature and had similar maxima (84–87% of the carbachol spasm; Figure 1a). The rank order of potencies for relaxation, with pD_2 values and mean potencies relative to ATP, was 2-MeSATP (7.76 ± 0.13, 59, $n=5$) ≫ α,β-MeATP (6.53 ± 0.03, 3.5, $n=5$) > ATP (5.99 ± 0.14, 1, $n=6$) ≫ adenosine (3.78 ± 0.06, 0.006, $n=6$).

2-MeSATP was more potent (mean 55 fold) than ATP as a spasmogen (pD_2 = 6.99 ± 0.28 and 5.25 ± 0.3, $n=5$ and 6 respectively) but both compounds exhibited similar maxima, about 40% of the carbachol spasm (Figure 1b).

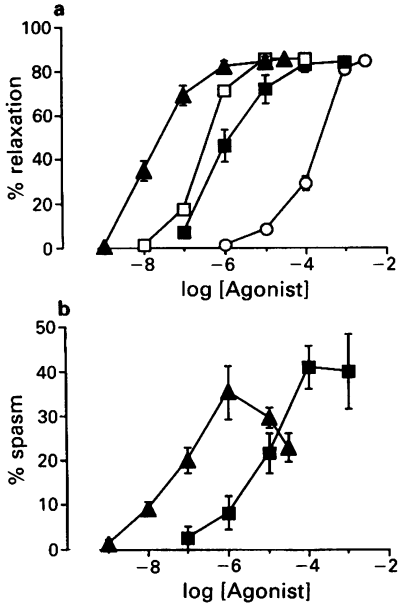


Figure 1 Non-cumulative log agonist concentration-effect curves for (a) relaxations to 2-methylthio ATP (2-MeSATP, ▲), α,β-methylene ATP (α,β-MeATP, □), ATP (■) and adenosine (○) and (b) spasms to 2-MeSATP (▲) and ATP (■) of the rat gastric fundus. Tone was induced with carbachol (0.1 μM) and tissues were incubated in indomethacin (10 μM) and 8-sulphophenyltheophylline (30 μM). The ordinate scale is the relaxation (a) or spasm (b) as a % of the tone to carbachol (0.1 μM). The abcissae are the $\log_{10}$ M concentrations of the agonist. Points represent the means and vertical lines the s.e.mean, $n = 5$–6.

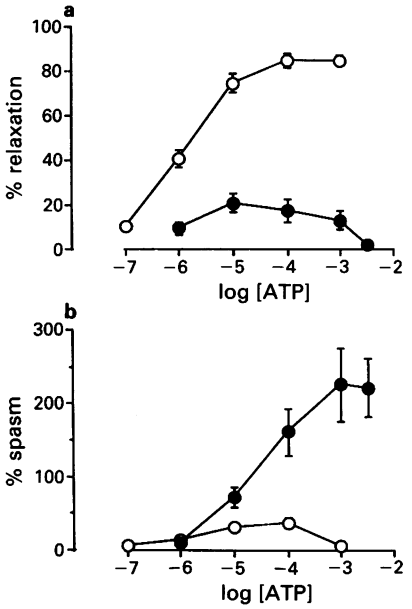


Figure 2 Non-cumulative log agonist concentration-effect curves to ATP for (a) relaxation and (b) spasm of the rat gastric fundus in the absence (○) and presence (●) of α,β-methylene ATP (α,β-MeATP, 100 μM). Tone was induced with carbachol (0.1 μM) and tissues were incubated in indomethacin (10 μM) and 8-sulphophenyltheophylline (30 μM). The ordinate scale is the relaxation (a) or spasm (b) as a % of the tone to carbachol (0.1 μM). The abcissae are the $\log_{10}$ M concentrations of ATP. Points represent the means and vertical lines the s.e.mean, $n = 5$.

Influence of desensitization to α,β-methylene ATP

Continuous exposure to a desensitizing concentration of α,β-MeATP (100 μM) in the presence of 8-SPT (30 μM) and indomethacin (10 μM) markedly reduced the relaxant response to ATP, which was now no longer concentration-dependent (Figure 2a). By contrast, the spasmogenic response to ATP was considerably enhanced during exposure to α,β-MeATP (100 μM, Figure 2b). It appeared that the potency of ATP as a spasmogen was decreased by α,β-MeATP (pD_2 in the absence of α,β-MeATP = 5.77 ± 0.20, pD_2 in the presence of α,β-MeATP = 4.58 ± 0.18, $P < 0.05$, $n = 5$), but the marked increase in maximum amplitude to ATP (from 37.8 ± 6.6% to 225.0 ± 49.6% of the carbachol-induced spasm) make such comparisons problematic. Responses to ATP were unchanged by the solvent for α,β-MeATP (distilled water).

The maximum relaxation to 2-MeSATP was reduced slightly but significantly (80.0 ± 1.9% to 59.8 ± 4.9% of the carbachol-induced spasm, $P < 0.05$, $n = 5$) during exposure to α,β-MeATP (100 μM) but the potency of 2-MeSATP was unaffected (pD_2 in the absence of α,β-MeATP = 7.71 ± 0.19, pD_2 in the presence of α,β-MeATP = 7.64 ± 0.11, $P > 0.05$, $n = 5$; Figure 3a). Like ATP, it appeared that the potency of 2-MeSATP as a spasmogen was decreased by α,β-MeATP (pD_2 in the absence of α,β-MeATP = 7.84 ± 0.15, pD_2 in the presence of α,β-MeATP = 7.28 ± 0.16, $P < 0.05$, $n = 5$), but there was an increase in the maximum amplitude to 2-MeSATP after α,β-MeATP (from 32.6 ± 4.3% to 63.0 ± 10.2% of the carbachol-induced spasm; Figure 3b). Responses to 2-MeSATP were unaffected by the solvent for α,β-MeATP (distilled water).

The potency of adenosine as a relaxant was unaffected by exposure to α,β-MeATP (100 μM; pD_2 in the absence of α,β-MeATP = 3.72 ± 0.11, pD_2 in the presence of α,β-MeATP = 3.76 ± 0.15, $n = 5$, $P > 0.05$) (Figure 4a) or the solvent for α,β-MeATP (distilled water).

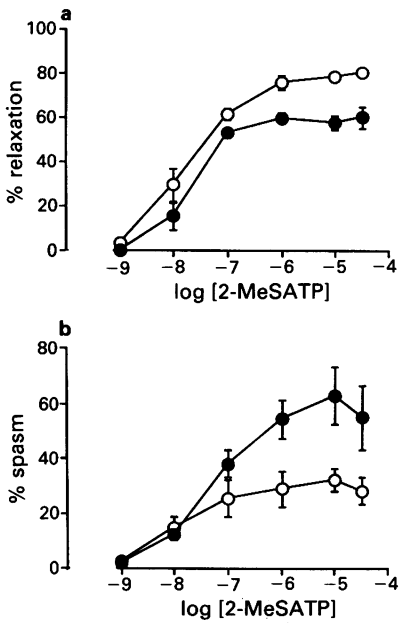


Figure 3 Non-cumulative log agonist concentration-effect curves to 2-methylthio ATP (2-MeSATP) for (a) relaxation and (b) spasm of the rat gastric fundus in the absence (○) and presence (●) of α,β-methylene ATP (α,β-MeATP, 100 μM). Tone was induced with carbachol (0.1 μM) and tissues were incubated in indomethacin (10 μM) and 8-sulphophenyltheophylline (30 μM). The ordinate scale is the relaxation (a) or spasm (b) as a % of the tone to carbachol (0.1 μM). The abcissae are the $\log_{10}$ M concentrations of 2-MeSATP. Points represent the means and vertical lines the s.e.mean, $n = 5$.

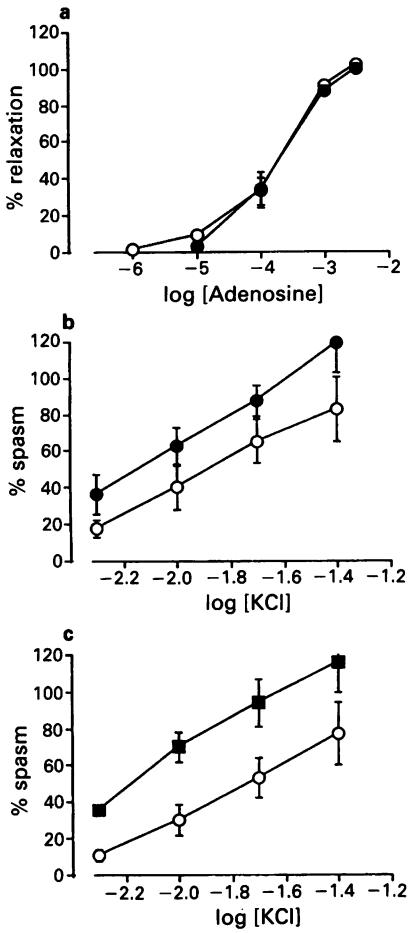


Figure 4 Non-cumulative log agonist concentration-effect curves in rat gastric fundus for (a) relaxation to adenosine and (b) spasm to KCl in the absence (○) and presence (●) of α,β-methylene ATP (α,β-MeATP, 100 μM) and (c) spasm to KCl in the absence (○) and presence (■) of solvent for α,β-MeATP. Tone was induced with carbachol (0.1 μM) and tissues were incubated in indomethacin (10 μM) and 8-sulphophenyltheophylline (30 μM). The ordinate scale is the relaxation (a) or spasm (b,c) as a % of the tone to carbachol (0.1 μM). The abcissae are the $\log_{10}$ M concentrations of adenosine or KCl. Points represent the means and vertical lines the s.e.mean, $n = 5$.

KCl (5–40 mM) produced a concentration-dependent spasm. A small increase in response to KCl occurred in the presence of α,β-MeATP compared to the initial curve (Figure 4b). In time-matched control tissues the responses in the second curve, in the presence of the solvent for α,β-MeATP (distilled water), were similarly greater in magnitude than in the first curve (Figure 4c). Therefore, α,β-MeATP had no effect on responses to KCl.

Influence of reactive blue 2

Reactive blue 2 (10 μM), in the presence of indomethacin (10 μM) and 8-SPT (30 μM) throughout, produced antagonism of ATP (14.5 fold), 2-MeSATP (13.8 fold), α,β-MeATP (3.9 fold), adenosine (3.6 fold) and isoprenaline (3.8 fold) as relaxants (Figure 5 and Table 1). For each of the agonists the log concentration-effect curves were displaced to the right in an approximately parallel manner with no reduction in the maximum response (except for a small reduction in the maximum response to ATP). The potencies of the relaxants were unchanged in solvent (distilled water), time-matched control tissues.

Reactive blue 2 (10 μM) virtually abolished the spasm to ATP and to 2-MeSATP (Figure 6a,b and Table 1). A small increase in response occurred to KCl in the presence of reactive blue 2 (10 μM), but a similar change was seen in time-matched controls (Figure 6c,d).

Neither reactive blue 2 nor its solvent (distilled water) affected the spasm to carbachol (0.1 μM).

Influence of suramin

Suramin (100 μM), in the presence of indomethacin (10 μM) throughout, produced a parallel rightward displacement of the concentration-effect curve to ATP (5.8 fold) as a relaxant with no change in the maximum response (Figure 7a and Table 2). The relaxant adenosine was not antagonized by suramin.

Suramin (100 μM) caused a rightward displacement of the log concentration-effect curve to ATP (4.3 fold) as a spasmogen with a slight reduction in the maximum response (pD_2 in absence of suramin = 5.59 ± 0.11, pD_2 in the presence of suramin = 4.96 ± 0.15, $P < 0.05$, Figure 7b and Table 2). A small increase in response occurred to KCl in the presence of suramin (100 μM), but a similar change was seen in time-matched controls, identical to that shown in Figures 4 and 6.

Neither suramin (100 μM) nor its solvent (distilled water) affected the spasm to carbachol (0.1 μM).

Discussion

This study has attempted to clarify the purinoceptors in the rat gastric fundus using functional criteria under conditions where some of the confounding problems have been reduced. The data suggest the presence of P_1-purinoceptors mediating relaxation and possibly the presence of P_{2X}- and P_{2Y}-purinoceptors mediating relaxation and spasm respectively.

Adenosine uptake and P_1-purinoceptors

NBTI is reported to be a selective inhibitor of adenosine uptake (Clanachan *et al.*, 1987; Jarvis, 1987). In the present study NBTI selectively potentiated adenosine without affecting responses to ATP. These data suggest that uptake of adenosine is a significant factor in terminating the action of adenosine which results in an underestimation of the true potency of adenosine. It is likely that NBTI (30 μM) had produced a complete block of adenosine uptake as this concentration only induced a 2.2 fold greater potentiation of adenosine than NBTI (0.3 μM).

8-SPT produced marked and selective antagonism of adenosine and NECA, as relaxants, without any antagonism of ATP. As NECA is purported to be a selective agonist at P_1-purinoceptors (van Calker *et al.*, 1979) and 8-SPT a selective antagonist at P_1-purinoceptors (Gustafsson, 1984), these results provide strong support for the idea that relaxation to adenosine and NECA in this tissue is mediated mainly via P_1-purinoceptors. It was interesting to note that 8-SPT (100 μM) produced only a 4.7 fold antagonism of adenosine in PSS without NBTI, with a greater shift at the foot rather than the top of the concentration-effect curve. However, in the presence of NBTI where adenosine was more potent, 8-SPT (100 μM) produced an 18.2 fold antagonism of adenosine, with a parallel displacement of the concentration-effect curve. This antagonism was still less than the 97.7 fold antagonism of the more potent NECA. It is likely that relaxation to high concentrations of adenosine is mediated by another mechanism in addition to P_1-purinoceptors. This additional mechanism is unlikely to involve P_2-purinoceptors as adenosine was not antagonized by suramin (see below). Identification of the subtype of P_1-purinoceptors with which adenosine and NECA interact, whether A_1 or A_2 (Kennedy, 1990), requires further studies with selective agonists and antagonists.

It is clear that the relaxant action of ATP does not signi-

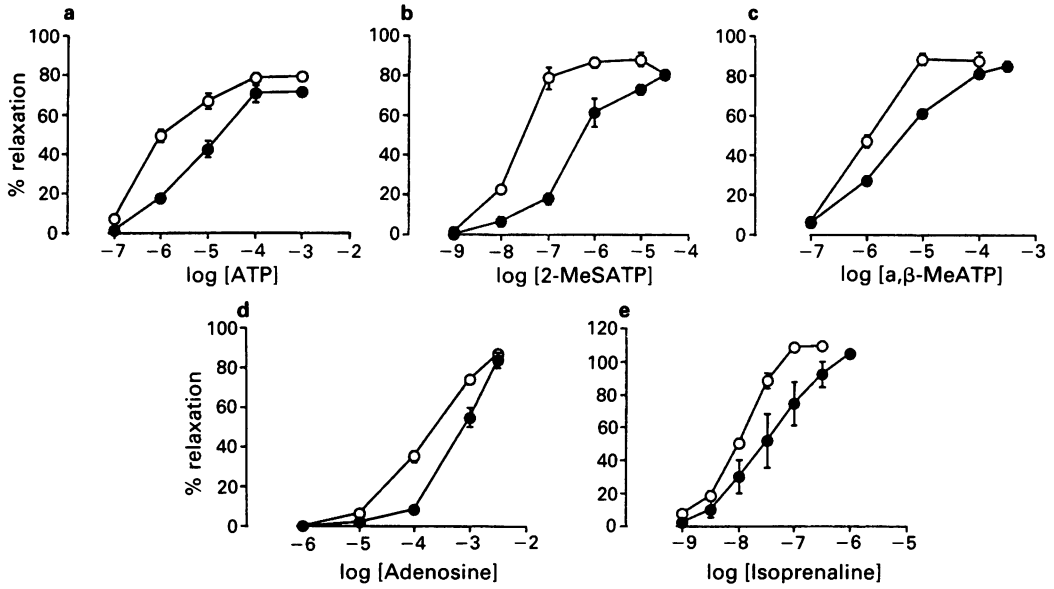


Figure 5 Non-cumulative log agonist concentration-effect curves for relaxations of the rat gastric fundus to (a) ATP, (b) 2-methylthio ATP (2-MeSATP), (c) α,β-methylene ATP (α,β-MeATP), (d) adenosine and (e) isoprenaline in the absence (open symbols) and presence (closed symbols) of reactive blue 2 (10 μM). Tone was induced with carbachol (0.1 μM) and tissues were incubated in indomethacin (10 μM) and 8-sulphophenyltheophylline (30 μM). The ordinate scale is the relaxation as a % of the tone to carbachol (0.1 μM). The abcissae are the $\log_{10}$ M concentrations of the agonist. Points represent the means and vertical lines the s.e.mean, $n = 5-6$.

Table 1 Effect of reactive blue 2 (10 μM), or its solvent (distilled water) in time-matched controls, on parameters of relaxation and spasm concentration-effect curves to several purine analogues

		Test		*Time-matched controls*	
Compound		*Initial*	*Reactive blue 2*	*Initial*	*Solvent*
Relaxation					
ATP	pD_2	6.19 ± 0.07	5.04 ± 0.13*	6.02 ± 0.05	5.99 ± 0.14
	α (%)	78.8 ± 2.0	71.0 ± 1.1*	82.3 ± 1.5	84.0 ± 1.4
2-MeSATP	pD_2	7.59 ± 0.03	6.45 ± 0.10*	7.55 ± 0.18	7.39 ± 0.13
	α (%)	80.8 ± 0.4	80.0 ± 2.0	83.4 ± 3.0	88.8 ± 2.8
α,β-MeATP	pD_2	6.07 ± 0.08	5.48 ± 0.05*	6.01 ± 0.05	5.85 ± 0.12
	α (%)	87.6 ± 4.4	85.2 ± 2.5	93.2 ± 1.8	92.2 ± 2.0
Adenosine	pD_2	3.79 ± 0.10	3.23 ± 0.09*	3.62 ± 0.13	3.77 ± 0.19
	α (%)	86.8 ± 2.0	84.0 ± 3.7	93.6 ± 4.0	96.6 ± 3.80
Isoprenaline	pD_2	7.97 ± 0.03	7.39 ± 0.26*	8.13 ± 0.04	8.03 ± 0.08
	α (%)	109.3 ± 1.9	104.2 ± 2.6	108.0 ± 1.9	106.4 ± 1.2
Spasm					
ATP	pD_2	5.33 ± 0.11	NM	5.43 ± 0.05	5.25 ± 0.30
	α (%)	44.8 ± 4.9	8.0 ± 5.1*	32.8 ± 3.9	41.0 ± 4.9*
2-MeSATP	pD_2	7.42 ± 0.15	6.49 ± 0.42*	7.62 ± 0.18	7.49 ± 0.24
	α (%)	52.0 ± 2.6	16.2 ± 3.0*	34.0 ± 5.5	31.4 ± 4.0

Data are means ± s.e.mean; $n = 5-6$. *indicates significantly different from data in initial concentration-effect curve ($P < 0.05$). α(%) = maximum response as a % of spasm to carbachol (0.1 μM). NM indicates not measurable. The PSS contained indomethacin (10 μM) and 8-sulphophenyltheophylline (30 μM) throughout. 2-MeSATP = 2-methylthio ATP and α,β-MeATP = α,β-methylene ATP.

ficantly involve P_1-purinoceptors either indirectly, via the metabolism to adenosine, or directly as the potency of ATP was unaffected by NBTI or 8-SPT.

Interaction between the two responses to ATP

ATP produced an initial phasic relaxation followed by a maintained spasm. The degree of tone modified the magnitude of the two responses, as reported previously by Lefebvre & Burnstock (1990), but we have found that the potencies for these two effects were unaffected by the extent of tone. Lefebrve & Burnstock (1990) suggested that the spasm was rebound, that is secondary to the relaxation. However, we consider that the two phases are separate but functionally interact. The spasm cannot be significantly rebound in nature as indomethacin increased the potency of ATP as a relaxant but reduced spasmogenic responses to ATP. Additionally, desensitization with α,β-MeATP markedly reduced the relaxant response to ATP but induced a considerable augmentation of the spasmogenic response to ATP. Lefebvre & Burnstock (1990) suggested that the spasmogenic response to ATP involved stimulation of prostaglandin biosynthesis. We concur that indomethacin markedly reduced the spasmogenic response to ATP. However, there was a large spasmogenic response to ATP, after desensitization to α,β-MeATP, in the presence of indomethacin (10 μM, Figure 2b), a concentration sufficient to abolish prostaglandin biosynthesis (Vane, 1971). It is proposed that ATP interacts with different purinoceptors

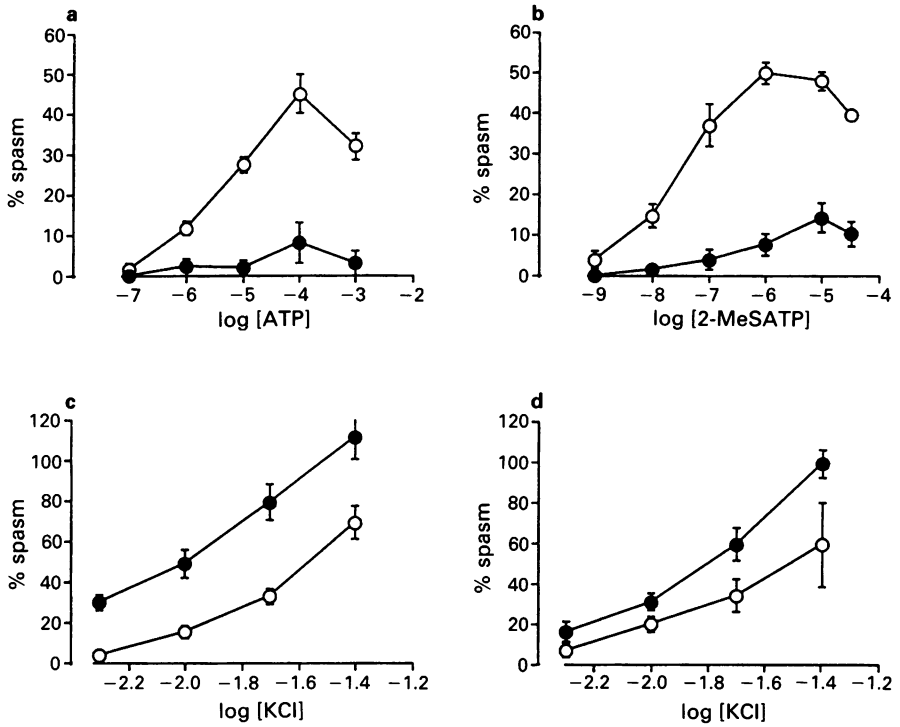


Figure 6 Non-cumulative log agonist concentration-effect curves for the rat gastric fundus for spasm to (a) ATP, (b) 2-methylthio ATP (2-MeSATP), (c) KCl in the absence (open symbols) and presence (closed symbols) of reactive blue 2 (10 μM) and (d) to KCl in the absence (○) and presence (●) of the solvent for reactive blue 2 in time-matched controls. Tone was induced with carbachol (0.1 μM) and tissues were incubated in indomethacin (10 μM) and 8-sulphophenyltheophylline (30 μM). The ordinate scale is the spasm as a % of the tone to carbachol (0.1 μM). The abcissae are the $\log_{10}$ M concentrations of the agonist. Points represent the means and vertical lines the s.e.mean, $n = 5$–6.

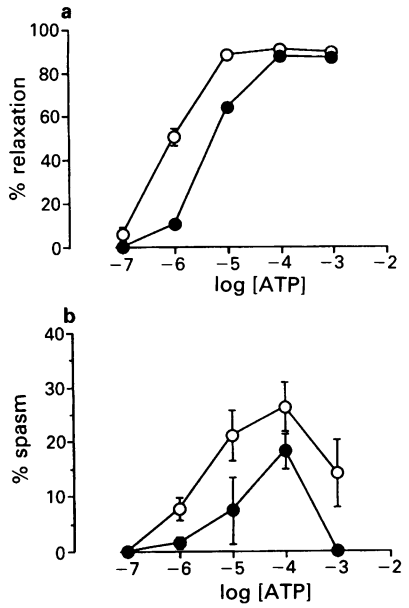


Figure 7 Non-cumulative log agonist concentration-effect curves in rat gastric fundus for (a) relaxation and (b) spasm to ATP in the absence (○) and presence (●) of suramin (100 μM). Tone was induced with carbachol (0.1 μM) and tissues were incubated in indomethacin (10 μM). The ordinate scale is the relaxation as a % of the tone to carbachol (0.1 μM). The abcissae are the $\log_{10}$ M concentrations of ATP. Points represent the means and vertical lines the s.e.mean, $n = 5$.

mediating relaxation and spasm and that the magnitude of each effect is functionally affected by the size of the other response. In addition, prostaglandin biosynthesis plays only a minor role in mediating the spasm.

Relaxant responses to ATP, 2-methylthio ATP and α,β-methylene ATP

Usually P_{2Y}-purinoceptors mediate relaxation of smooth muscle (Burnstock & Kennedy, 1985). The rank order of agonist potency in rat gastric fundus for relaxation was 2-MeSATP ≫ α,β-MeATP > ATP which does not accord with the commonly accepted rank order for the P_{2Y}-purinoceptor which is 2-MeSATP ≫ ATP > α,β-MeATP (Burnstock & Kennedy, 1985). The rank order found in the present study, where tissues were exposed to 8-SPT and indomethacin, is the same as that reported by Lefebvre & Burnstock (1990), where these modifying agents were absent. In the present study the three agonists exhibited similar slopes and maxima for their concentration-effect curves in contrast to the previous study where the relaxants had different slopes and maxima.

The results with the antagonists help to confirm the role of P_2-purinoceptors mediating relaxation to these three agonists but not the sub-type of receptor. Reactive blue 2 is purported to be a slightly selective antagonist at P_{2Y}-purinoceptors (Burnstock & Warland, 1987; Hopwood & Burnstock, 1987; Taylor *et al.*, 1989). However, in the present study reactive blue 2, at a relatively low concentration of 10 μM (Burnstock & Warland, 1987; Lefebvre & Burnstock, 1990), produced marked antagonism of ATP and 2-MeSATP as relaxants as well as an approximately 4 fold antagonism of other relaxants (α,β-MeATP, adenosine and isoprenaline). The latter two compounds do not act via P_2-purinoceptors. Reactive blue 2 may be, at least partially, non-selective preventing relaxation at a post-receptor site.

Recent reports have suggested that suramin is a selective antagonist at P_2-purinoceptors relative to other receptors, but it does not distinguish between P_{2X}- and P_{2Y}-purinoceptors (Dunn & Blakeley, 1988; Den Hertog *et al.*, 1989; Hoyle *et al.*, 1990; Von Kugelgen *et al.*, 1990). Lefebvre & Burnstock

Table 2 Effect of suramin (100 μM), or its solvent (distilled water) in time-matched controls, on parameters of relaxation and spasm concentration-effect curves to purine analogues

		Test		*Time-matched controls*	
Purine		*Initial*	*Suramin*	*Initial*	*Solvent*
Relaxation					
ATP	pD_2	6.11 ± 0.08	5.26 ± 0.04*	6.02 ± 0.05	5.99 ± 0.14
	α(%)	91.8 ± 1.9	89.4 ± 1.2	82.3 ± 1.5	84.0 ± 1.4
Adenosine	pD_2	4.73 ± 0.05	4.68 ± 0.06	4.79 ± 0.19	4.72 ± 0.17
	α(%)	89.0 ± 1.3	88.8 ± 1.4	90.2 ± 2.2	95.6 ± 3.2
Spasm					
ATP	pD_2	5.59 ± 0.11	4.96 ± 0.15*	5.43 ± 0.05	5.25 ± 0.30
	α(%)	26.4 ± 4.5	18.2 ± 3.3*	32.8 ± 3.9	41.0 ± 4.9*

Data are means ± s.e.mean; $n = 6$. *indicates significantly different from data in initial concentration-effect curve ($P < 0.05$). α(%) = maximum response as a % of spasm to carbachol (0.1 μM). The PSS contained indomethacin (10 μM) throughout.

(1990) failed to see any antagonism of ATP, as a relaxant, in rat gastric fundus by suramin (100 μM). It is likely that their period of incubation (10 or 20 min) was too short. Recently Leff *et al.* (1990) have shown that suramin is a slowly equilibrating but competitive antagonist at P_{2X}-purinoceptors in rabbit isolated ear artery. They estimated the times necessary for different concentrations of suramin to achieve at least 95% occupancy at the P_{2X}-purinoceptors in rabbit ear artery and found this to be 86 min for suramin (100 μM). Thus, in the present study the rat gastric fundus was incubated with suramin (100 μM) for 90 min. Suramin was found to produce a parallel shift in the concentration-effect curve to ATP, as a relaxant, with no antagonism of adenosine, demonstrating its selectivity as an antagonist.

Desensitization was readily produced to α,β-MeATP with marked cross-desensitization to ATP but only limited cross-desensitization to 2-MeSATP. The cross-desensitization was selective as none was seen to adenosine. Also the high potency of 2-MeSATP as a relaxant must be considered. One explanation of the data is that the relaxation to α,β-MeATP and to ATP is mediated via a common mechanism (perhaps P_{2X}-purinoceptors) while that to 2-MeSATP is mediated partly via a mechanism which exhibits desensitization with α,β-MeATP, possibly the P_{2X}-purinoceptors, and also partly via an additional mechanism, which does not exhibit desensitization with α,β-MeATP. 2-MeSATP showed a larger maximal response in the absence compared with the presence of α,β-MeATP, perhaps due to the addition of the two actions proposed. Dainty *et al.* (1990) have reported in the rat thoracic aorta that 2-MeSATP is a relaxant and acts as a partial agonist via a mechanism not common to ATP as 2-MeSATP failed to antagonize ATP. A second explanation is that the 3 agonists interact at a common novel purinoceptor which exhibits a rank order of agonist potency of 2-MeSATP ≫ α,β-MeATP > ATP, shows desensitization with α,β-MeATP and at which suramin is an antagonist. The differential cross-desensitization (ATP ≫ 2-MeSATP) could be due to possible differences in intrinsic efficacies of the two agonists at this novel receptor. Responses to an agonist of lower intrinsic efficacy are more subject to a decrease in tissue response capability in the presence of a desensitizing agent and, therefore, not all agonists will be equally affected by receptor desensitization (Kenakin, 1987). If ATP has a lower intrinsic efficacy than 2-MeSATP, at this novel receptor, then the responses to ATP would be preferentially depressed over those to 2-MeSATP. A third explanation is that 2-MeSATP interacts with a P_{2X}-purinoceptor while ATP interacts with this receptor plus another receptor. O'Connor *et al.* (1991) have suggested the presence of a novel 'nucleotide' receptor in certain smooth muscles and other tissues at which ATP and uridine 5'-triphosphate are agonists and with which 2-MeSATP does not interact. In these tissues 2-MeSATP produces a smaller maximum response than ATP. However, in rat fundus the maximum reponses to ATP and 2-MeSATP were essentially the same. Also, there was greater cross-desensitization between α,β-MeATP and ATP than between α,β-MeATP and 2-MeSATP. The first of the three explanations is currently more likely as there is more evidence in support and fewer assumptions have to be made.

Spasm to 2-methylthio ATP and ATP

2-MeSATP and ATP produced spasm of the rat gastric fundus, with 2-MeSATP being 55 fold as potent as ATP, in the presence of 8-SPT and indomethacin. If these two compounds are acting at a common receptor, the relative potency accords with a P_{2Y}-purinoceptor (Burnstock & Kennedy, 1985; Berrie *et al.*, 1989; Kennedy, 1990). α,β-MeATP failed to produce spasm and desensitization with α,β-MeATP enhanced the magnitude of the spasm to ATP and 2-MeSATP. These latter results are in accord with the idea that spasm was mediated via P_{2Y}-purinoceptors. Bailey & Hourani (1990) have provided the first evidence for a P_{2Y}-purinoceptor mediating spasm, in the rat isolated colon.

Reactive blue 2 (10 μM) abolished spasms to ATP and 2-MeSATP with no effect on spasms to KCl or carbachol. Therefore, reactive blue 2 appears to be a selective antagonist against spasm to purinoceptor agonists but not to their relaxation in rat gastric fundus. Burnstock & Warland (1987) suggested that reactive blue 2 is a selective antagonist at P_{2Y}-purinoceptors in rabbit ear artery where purine analogues produced relaxation. By analogy, present data with reactive blue 2 provides further evidence that ATP and 2-MeSATP cause spasm via P_{2Y}-purinoceptors. Suramin selectively antagonized ATP as a spasmogen without effect on responses to KCl or carbachol. The results with the two antagonists support the role of P_2-purinoceptors in the spasmogenic actions of ATP and 2-MeSATP but only aid the classification of the sub-type of receptor involved to a small extent.

In conclusion, rat gastric fundus exhibits relaxation and spasm to a number of purine analogues. It is proposed that adenosine produces relaxation partially via a P_1-purinoceptor and exhibits nucleoside-specific uptake. ATP- and α,β-MeATP-induced relaxation most probably occurs via a P_{2X}-purinoceptor, which is the first report of such a receptor mediating relaxation. 2-MeSATP-induced relaxation may occur via both a P_{2X}-purinoceptor and an additional mechanism. ATP and 2-MeSATP also cause spasm, probably via a P_{2Y}-purinoceptor. Further studies of the complex mixture of purinoceptors in this tissue will require the development of more selective antagonists.

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Inhibitory effect of methysergide on calcitonin gene-related peptide-induced vasodilatation and ocular irritative changes in the rabbit

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1 Calcitonin gene-related peptide (CGRP) is involved in ocular neurogenic inflammation in the rabbit, causing vasodilatation in the anterior uvea, breakdown of the blood-aqueous barrier, increase in the intraocular pressure (IOP) and rise in the adenosine 3':5'-cyclic monophosphate (cyclic AMP) content in the aqueous humour. So far there is no means of preventing these CGRP-induced ocular effects.

2 In the present study, the effect of intravenous methysergide (1–10 mg kg^{-1}, b.w.) on CGRP-induced changes in the IOP, blood-aqueous barrier and cyclic AMP content in the aqueous humour was studied *in vivo*. The effect of methysergide on CGRP-induced vasodilatation both *in vivo* and *in vitro* was also investigated.

3 Methysergide decreased intraocular pressure but had only a transient effect on blood pressure. Methysergide decreased the regional blood flow in ocular tissues by 53–65%, but did not have such a vasoconstrictor effect in most extra-ocular tissues studied.

4 Methysergide inhibited CGRP-induced vasodilatation, increase in the IOP, breakdown of the blood-aqueous barrier and increase in the cyclic AMP content in the aqueous humour *in vivo*.

5 *In vitro*, methysergide alone did not have effects on the vascular tone in isolated ophthalmic artery of rabbit. However, it potentiated noradrenaline (NA)-induced contraction. There were no differences in the IC_{50} values for CGRP on the NA-induced contraction in the presence and absence of methysergide, indicating that methysergide has no direct effect on the vasorelaxant effect of CGRP *in vitro*.

6 The present study demonstrates that in the rabbit eye methysergide inhibits CGRP-induced changes. One inhibitory mechanism of methysergide may be to enhance the effect of a vasoconstrictor (NA) to antagonize the vasodilator effect of CGRP. The present findings suggest that a methysergide-sensitive mechanism may be used to limit some pathophysiological conditions in the eye that involve neurogenic inflammation and the release of CGRP.

Keywords: Calcitonin gene-related peptide (CGRP); vasodilatation; intraocular pressure; blood-aqueous barrier; cyclic AMP; methysergide; regional blood flow

Introduction

Neurogenic inflammation occurs in peripheral target tissues in response to sensory nerve stimulation (Jancso *et al.*, 1967) and is associated with inflammatory and protective states in many tissues, such as in airways, skin and eye (Lundberg & Saria, 1983; Caprioli *et al.*, 1985; Lundblad *et al.*, 1987). In the eye, neurogenic inflammation occurs in response to traumatic stimuli, such as laser therapy of the eye and ocular surgery (Caprioli *et al.*, 1985) and it consists of vasodilatation in the anterior uvea, breakdown of the blood-aqueous barrier, increase in the intraocular pressure (IOP) and miosis (Perkins, 1957).

In ocular neurogenic inflammation, a rapid vasodilatation in the anterior uvea disrupts the epithelial blood-aqueous barrier allowing plasma proteins to leak into the aqueous humour and at the same time it increases the intraocular pressure (Stjernschantz *et al.*, 1982; Unger *et al.*, 1985). Simultaneously the adenosine 3':5'-cyclic monophosphate (cyclic AMP) content in the aqueous humour is increased (Krootila, 1988). Neuropeptides localized in the sensory nerves are thought to be responsible for the changes in the neurogenic inflammation in the rabbit eye. Calcitonin gene-related peptide (CGRP) and substance P (SP) are both localized in the sensory nerves of the eye (Miller *et al.*, 1981; Tervo *et al.*, 1981; Terenghi *et al.*, 1985). They are released into the aqueous humour by antidromic electrical stimulation of the ocular sensory nerves (Bill *et al.*, 1979; Wahlestedt *et al.*, 1986) and their effects mimic the effects of sensory nerve stimulation in the rabbit eye. CGRP causes vasodilatation in the anterior uvea, disruption of the blood-aqueous barrier, rise in the IOP and increase in the cyclic AMP content in the aqueous humour, while SP causes miosis (Stjernschantz *et al.*, 1982; Unger *et al.*, 1985; Krootila, 1988; Krootila *et al.*, 1988).

In a previous study we have demonstrated the inhibitory effect of methysergide on the IOP response in neurogenic inflammation in the rabbit eye (Krootila *et al.*, 1987). Because CGRP is thought to be responsible for the rise in IOP in neurogenic inflammation, the present study was carried out to investigate the effect of methysergide on CGRP-induced microvascular changes in the rabbit eye *in vivo* and on CGRP-induced vascular responses *in vitro*.

Methods

Animals and anaesthesia

Albino rabbits of either sex weighing 2.0–3.5 kg were used. For the experiments the animals were anaesthetized with intravenous urethane (1.5 g kg^{-1}, b.w., supplemented during the experiments). In order to prevent the production of prostaglandins all rabbits were pretreated with 50 mg of intravenous indomethacin 60 min before experiments.

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Experimental procedures

Methysergide (dissolved in physiological saline) was given intravenously at doses 1, 5 or 10 mg kg^{-1}, b.w. Human α-CGRP (0.5 or 2.0 μg) was injected intracamerally with a Hamilton syringe, in a volume of 10 μl, slowly, avoiding a rise in the IOP, 20–30 min after methysergide or saline. The control eyes received intracamerally saline (NaCl) correspondingly. The aqueous humour samples were taken 30 min after injection of CGRP or saline for the measurement of protein and cyclic AMP contents.

Recording of intraocular pressure (IOP) and blood pressure

For the continuous electromanometrical recording of blood pressure, the femoral artery was cannulated with a polyethylene tube and connected to a pressure transducer. The IOP of both eyes was continuously measured electromanometrically with special intracameral needles connected to pressure transducers and to a two-channel recorder. Following cannulations, the IOP was left to equilibrate for 30–60 min before further experiments.

Determination of regional blood flow

The regional blood flows were measured by use of radioactively labelled microspheres described in detail earlier (Krootila *et al.*, 1988; Oksala, 1988). After killing the animals the tissues were carefully dissected under the microscope, the ocular tissues were taken as a whole and the samples of the extra-ocular tissues were weighed after dissection. The results for ocular tissues are given as blood flow in mg min^{-1}/whole tissue and for extra-ocular tissues as blood flow in mg min^{-1} g^{-1} of tissue.

Measurement of protein and cyclic AMP contents in the aqueous humour

The protein content was measured according to the method of Lowry *et al.* (1951). For the measurement of cyclic AMP, the samples were mixed in 1:4 volume for 4 mM EDTA and measured with a ^{125}I-radioimmunoassay kit (Amersham RPA, 509, U.K.).

Contraction measurements on the isolated ophthalmic artery

New Zealand albino rabbits were killed by an overdose of sodium pentobarbitone and decapitated. The ophthalmic artery was isolated, cleaned of excess connective tissue and cut into 2 mm long pieces. The contraction experiments were performed isometrically at 37°C. The drugs were added to the incubation medium in desired concentrations. The composition of the incubation medium was (in mM): NaCl 138, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10 and HEPES 10, pH 7.4.

Drugs

The following drugs were used: Urethane (Fluka Chemika, Switzerland), sodium pentobarbitone (Mebunat, Orion, Finland), methysergide hydrogenmaleate (Sandoz), human α-CGRP (Peninsula, Calif, U.S.A.), indomethacin (Confortid, Dumex, Denmark), noradrenaline HCl (Fluka Chemika).

Statistical analysis

Statistical comparison was made with Student's *t* test or matched-pair *t* test. Results are given as mean ± s.e.mean.

Results

Effect of methysergide on blood pressure, intraocular pressure and regional blood flow

Intravenous methysergide (1–10 mg kg^{-1}, b.w.) caused at 2–3 min a rapid and transient increase in the blood pressure, which recovered to control levels within 30 min (Table 1). In the IOP, a decrease occurred in 30 min after methysergide (i.v., 1–10 mg kg^{-1}, b.w., Table 2). In ocular tissues, decreases of 53–65% in regional blood flows were seen 20–30 min after 5 mg kg^{-1} b.w., i.v. methysergide (Figure 1). In most other tissues studied, kidney, spleen, heart, brain and lung, vasoconstriction did not occur (Figure 2). Only in liver a modest decrease and in pancreas a modest increase in regional blood flow was seen (Figure 2).

Effect of methysergide on CGRP-induced inflammatory changes

Intracameral CGRP (2.0 μg) caused typical inflammatory changes in the rabbit eye consisting of increase in the IOP, increased protein and cyclic AMP contents in the aqueous humour and vasodilatation. The doses 5 and 10 mg kg^{-1} b.w. of methysergide (i.v.) inhibited the CGRP-induced changes in the IOP and protein and cyclic AMP contents in the aqueous humour, while the dose of 1 mg kg^{-1} b.w. (i.v.) was ineffective (Figure 3, Table 3). Intracamerally administered 0.5 μg CGRP increased the regional blood flow in the anterior uvea (iris and ciliary body) by 244.8 ± 80.2 mg min^{-1}/whole tissue. After methysergide (5 mg kg^{-1}, i.v.), the same dose of CGRP increased the regional blood flow only by 54.1 ± 23.3 mg min^{-1}/whole tissue ($P<0.025$, Figure 4). This increase was at the same level as the increase after intracameral injection of corresponding volume of NaCl (33.8 ± 16.2 mg min^{-1}/whole tissue, Figure 4).

Contraction studies on isolated ophthalmic artery

Dose-response curves (DRC) for noradrenaline (NA) in the presence and absence of methysergide (10 nM) were performed (Figure 5). The contraction induced by NA 5 μM in the absence of methysergide was 266 ± 15 mg and was regarded as 100% for further comparison. In the presence of methysergide, there was a shift of the DRC to the left and an increase in the maximal response to NA (Figure 5). Contrac-

Table 1 Effect of methysergide on blood pressure (BP) in the rabbit

		BP (mmHg)		
Dose (i.v.)	n	*Before*	*At 2–3 min*	*At 30 min*
1 mg kg^{-1}	5	101 ± 4	108 ± 5*	98 ± 5
5 mg kg^{-1}	5	95 ± 2	110 ± 4*	96 ± 3
10 mg kg^{-1}	13	102 ± 4	113 ± 5***	101 ± 4

Mean ± s.e.mean.
n = number of animals.
*$P<0.05$; ***$P<0.001$. Matched-pair *t* test.

Table 2 Effect of methysergide on intraocular pressure (IOP) in the rabbit

		IOP (mmHg)	
Dose (i.v.)	n	*Before*	*At 30 min*
1 mg kg^{-1}	5	16.3 ± 0.6	15.0 ± 1.0*
5 mg kg^{-1}	5	20.2 ± 1.5	16.5 ± 0.8*
10 mg kg^{-1}	13	18.4 ± 0.6	14.7 ± 0.8***

Mean ± s.e.mean.
n = number of animals
*$P<0.05$; ***$P<0.001$. Matched-pair *t* test.

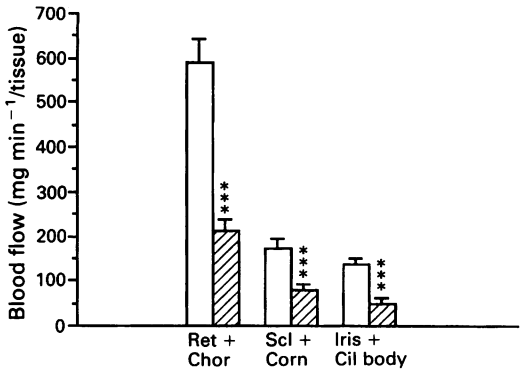


Figure 1 Effect of methysergide (5 mg kg^{-1} b.w., i.v.) pretreatment on regional blood flow in ocular tissues. $n = 9$. The statistical analysis was made between values before (open columns) and 20–30 min after methysergide (hatched columns) using matched-pair t test. ***$P < 0.001$. Ret + Chor = retina and choroid; Scl + Corn = sclera and cornea; Iris + Cil body = iris and ciliary body. Mean values with s.e.mean shown by vertical bars.

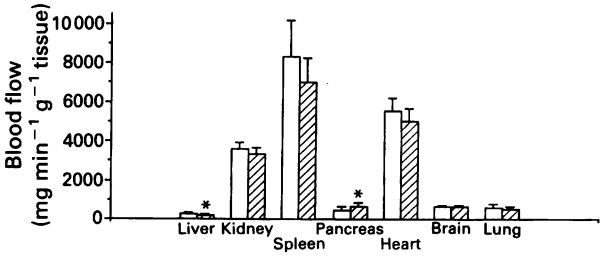


Figure 2 Effect of methysergide (5 mg kg^{-1} b.w., i.v.) pretreatment on regional blood flow in extraocular tissues. $n = 9$. The statistical analysis was made between values before (open columns) and 20–30 min after methysergide (hatched columns) using matched-pair t test. *$P < 0.05$. Mean values with s.e.mean shown by vertical bars.

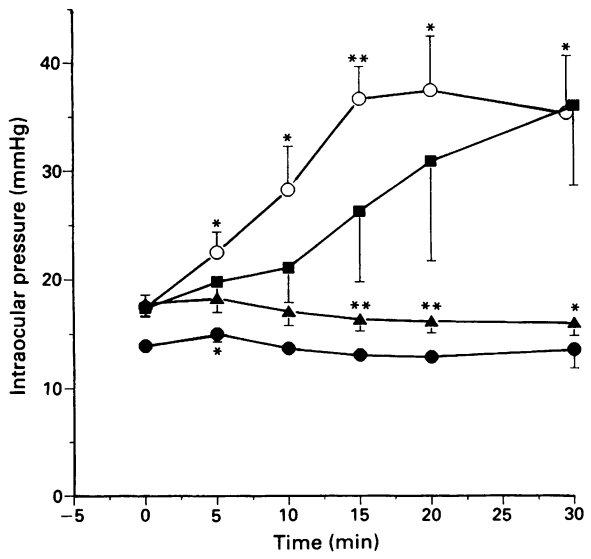


Figure 3 Effect of methysergide (1 mg kg^{-1}, ■, $n = 3$; 5 mg kg^{-1}, ▲, $n = 5$ or 10 mg kg^{-1} b.w., ●, $n = 6$) on the calcitonin gene-related peptide (CGRP)-induced (2.0 μg) increase in the intraocular pressure (IOP). The control animals (○, $n = 6$) received saline correspondingly before CGRP. n = Number of eyes. *$P < 0.05$; **$P < 0.01$. Statistical comparison was made against the values at time 0 using matched-pair t test. Mean values with s.e.mean shown by vertical bars.

Table 3 Effect of methysergide on calcitonin gene-related peptide (CGRP)-induced (2.0 μg) increase in the protein and cyclic AMP content in the aqueous humour in the rabbit

Dose (i.v.)	n	*Protein* (mg ml^{-1})	*Cyclic AMP* (pmol ml^{-1})
Control (CGRP)	6	23.7 ± 2.3	243.2 ± 37.9
1 mg kg^{-1}	3	13.3 ± 6.2	147.0 ± 7.4
5 mg kg^{-1}	5	0.6 ± 0.04***	34.6 ± 3.1***
10 mg kg^{-1}	6	0.6 ± 0.1***	65.3 ± 14.7**

Mean ± s.e.mean.
n = Number of animals.
**$P < 0.01$; ***$P < 0.001$. Student's t test.

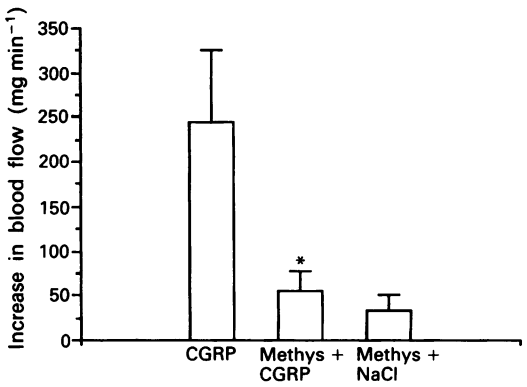


Figure 4 Effect of methysergide (5 mg kg^{-1} b.w.) on calcitonin gene-related peptide (CGRP) – induced (0.5 μg) vasodilatation in the anterior uvea. Number of animals is 7 in group receiving CGRP only and 9 in methysergide (methys) treated group. The results are given as blood flow in mg min^{-1} the whole tissue. *$P < 0.05$. Student's t test. Mean values with s.e.mean shown by vertical bars.

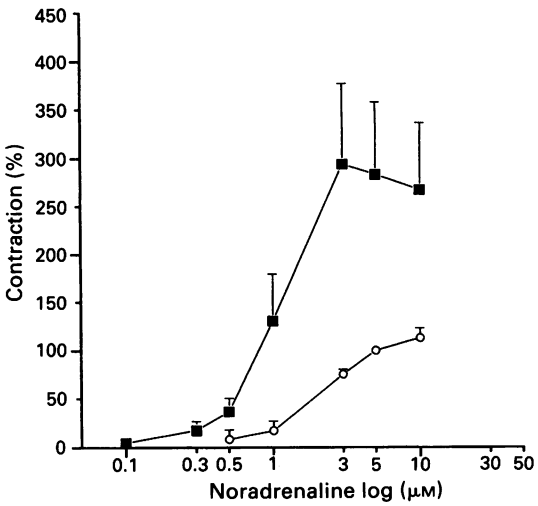


Figure 5 Dose-response curves for noradrenaline (NA) induced contractions in the rabbit isolated ophthalmic artery in the absence (○, $n = 8$) and presence (■, $n = 3$) of methysergide (10 nM). Contraction induced by 5 μM NA was regarded as 100%. Mean values with s.e.mean shown by vertical bars.

tions induced by 10 μM NA were 300 ± 25 mg and 710 ± 150 mg in the absence and presence of 10 nM methysergide, respectively. Methysergide (10 nM) alone did not cause any changes in the tone of isolated opthalmic artery.

The inhibitory effect of CGRP on 5 μM of NA-induced contraction in the absence and presence of methysergide (10 nM) and on 1 μM of NA-induced contraction in the presence of methysergide (10 nM) were measured. Figure 6a shows an original tracing of an experiment where the inhibitory effect of increasing concentrations of CGRP on NA (5 μM) -induced contraction was studied. NA (1 or 5 μM) was added to the incubation solution and after the increase in tension was balanced, the NA was washed out. This was repeated until at least three contractions of the same size were obtained, whereafter the experiment was continued in the presence of increasing concentrations (0.1–3.0 nM) of CGRP. Figure 6b demonstrates the DRCs for increasing concentrations of CGRP on 5 μM NA-induced contraction in the absence and presence of methysergide (10 nM) and on 1 μM NA-induced contraction in the presence of methysergide (10 nM). There were no statistically significant differences in IC_{50} values for CGRP in these three DRCs.

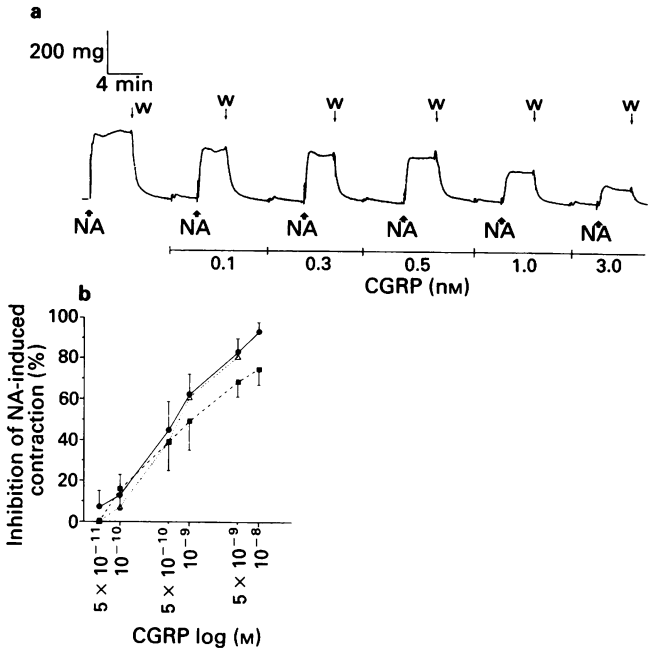


Figure 6 (a) Original tracing demonstrating the effect of increasing concentrations of calcitonin gene-related peptide (CGRP, 0.1–3.0 nM) on the noradrenaline (NA)-induced (5 μM) contraction in the rabbit isolated ophthalmic artery. NA = addition of NA; W = wash out. (b) Dose-response curves (DRC) for CGRP on 5 μM NA-induced contraction in the absence (●—●) and presence (■- - -■) of methysergide (10 nM) and on 1 μM NA-induced contraction in the presence (Δ·····Δ) of methysergide (10 nM). For each DRC for CGRP, the contraction before addition of CGRP was regarded as 100%. $n = 3$ in each. Mean with s.e.mean shown by vertical bars, except in (Δ), where the standard error bars (<15% in all points) have been omitted for clarity.

Discussion

Methysergide is a nonselective 5-hydroxytryptamine (5-HT) antagonist at 5-HT_1 and 5-HT_2 receptors (Peroutka, 1988). It has been shown to contract the intracranial rabbit central ear artery *in vivo* (Apperley *et al.*, 1976), but not the intracranial artery in the cat *in vitro* (Hardebo *et al.*, 1978). Methysergide can also sensitize extracranial vessels to NA both *in vivo* and *in vitro* (de la Lande *et al.*, 1966; Schönbaum *et al.*, 1976). 5-HT receptors have been demonstrated in the rabbit eye (Mallorga & Sugrue, 1987). 5-HT was bound to the iris ciliary body membrane suspension and based on the ability of different antagonists to displace specific 5-HT binding, the binding sites were shown to be of the 5-HT_1 type. Methysergide had strong affinity for the 5-HT binding sites (Mallorga & Sugrue, 1987). In the present study, methysergide decreased the regional blood flow in ocular tissues *in vivo* but methysergide alone did not cause any contraction in the rabbit isolated ophthalmic artery *in vitro*. However, in the presence of methysergide, NA-induced contraction in the ophthalmic artery was potentiated *in vitro*. In this respect, the present findings are in agreement with the previous studies.

In tissues other than the eye, i.v. methysergide had very modest, if any, effects on regional blood flow. This demonstrates a tissue-specific mechanism of action of methysergide. It is possible that the eye is more sensitive to methysergide or there is perhaps local release of NA in the eye from the sympathetic nerves to interact with methysergide. Although in quiet, resting rabbits the sympathetic vasomotor tone to the eye is minimal, under urethane anaesthesia the small sympathetic tone might provide a source of NA for the eye (Bill & Nilsson, 1985).

The relatively intense vasoconstriction in the anterior uvea might explain the IOP lowering effect of methysergide. Although there was not a constant decrease in the regional blood flows in extra-ocular tissues, the transient increase in the blood pressure 2–3 min after the i.v. administration of methysergide might also reflect a transient systemic vasoconstriction.

In rabbit isolated ophthalmic artery, methysergide potentiated the contractile effect of NA. Methysergide has also 5-HT agonistic properties, as demonstrated earlier in other vascular tissues (Apperley *et al.*, 1980) and in rabbit ophthalmic artery (Zschauer *et al.*, 1991). It has also been shown that in the presence of 5-HT the contractile response to different agonists is enhanced (Seabrook & Nolan, 1983). Thus, potentiation of NA-induced contraction in the presence of methysergide in the rabbit ophthalmic artery might be due to 5-HT agonistic properties of methysergide. There were no differences in the IC_{50}-values for CGRP on the NA-induced contraction in the presence and absence of methysergide, indicating that methysergide has no direct effect on CGRP-induced vasodilatation *in vitro*. Thus, the antagonistic effect of methysergide on CGRP-induced inflammatory changes in the rabbit eye is not direct. One of the mechanisms may be to enhance the effect of a vasoconstrictor (NA) to antagonize the vasodilator effect of CGRP.

It is also known that increased sympathetic vascular tone in the eye protects the eye from exogenous insult (Davson & Matchett, 1951) and that sympathectomized eyes show an enhanced responsiveness to noxious stimuli (Butler *et al.*, 1979).

CGRP is localized in the sensory nerves and is one of the most potent vasodilators yet discovered (Rosenfeld *et al.*, 1983; Brain *et al.*, 1985; Struthers *et al.*, 1986). In the eye, CGRP is localized in the trigeminal sensory nerves and CGRP-immunoreactive nerve fibres have been demonstrated in several species including man (Terenghi *et al.*, 1985; Stone & McGlinn, 1988; Uusitalo *et al.*, 1989). CGRP has been shown to be released into the aqueous humour in the rabbit by trigeminal nerve stimulation (Wahlestedt *et al.*, 1986) and CGRP is a vasodilatator in the eye (Krootila *et al.*, 1988; Oksala, 1988). This CGRP-induced rapid vasodilatation in the anterior uvea is thought to cause disruption of the blood-aqueous barrier, subsequent leakage of plasma proteins into the aqueous humour and rise in the IOP in neurogenic inflammation (Stjernschantz *et al.*, 1982; Unger *et al.*, 1985). Thus, CGRP has been thought to act as a mediator in harmful ocular irritative responses (Unger *et al.*, 1985). In a previous study, we have demonstrated the inhibitory effect of methysergide on IOP response in neurogenic inflammation in the rabbit eye (Krootila *et al.*, 1987).

In the present study, the ocular effects of CGRP were inhibited by methysergide *in vivo*. Methysergide inhibited the CGRP-induced vasodilatation in the anterior uvea, increases in the protein and cyclic AMP contents in the aqueous

humour and rise in the IOP. In the rabbit eye, methysergide is a vasoconstrictor *in vivo* and this vasoconstriction seems to protect the eye from the vasodilator effect of CGRP. *In vivo*, methysergide decreased the CGRP-induced increase in regional blood flow in the anterior uvea, while *in vitro*, the magnitude of the CGRP-induced relaxation in the ophthalmic artery was the same in the absence and presence of methysergide. This suggests that *in vivo*, there might be also some other indirect mechanisms to explain how methysergide inhibits the CGRP-induced vasodilatation other than by enhancing the effect of a vasoconstrictor.

The present study addresses the pathophysiological effects of CGRP in the rabbit eye and our results provide the first evidence that the ocular effects of CGRP can be inhibited with methysergide. The effect of methysergide has been shown to be tissue-specific and one of the mechanisms involved may be the potentiation of NA-induced vascular contraction. The present findings suggest that it is pharmacologically possible to limit some pathophysiological conditions in the eye that involve neurogenic inflammation and the release of CGRP.

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CP-93,129, sumatriptan, dihydroergotamine block c-fos expression within rat trigeminal nucleus caudalis caused by chemical stimulation of the meninges

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1 The effects of intravenously administered 5-HT_{1B} receptor agonists were examined on c-fos-like immunoreactivity, an indicator of neuronal activation, within the brain stem. C-fos was induced by injecting an algesic, vasoconstrictor substance (0.3 ml of autologous blood) or a pro-inflammatory molecule, carrageenin (1 mg in 0.1 ml saline) into the cisterna magna of pentobarbitone-anaesthetized Sprague-Dawley rats and was visualized in serial sections (50 μm) by use of a polyclonal antiserum.

2 As previously reported, the injection of blood caused significant labelling within laminae I, II_o of the trigeminal nucleus caudalis, a major nociceptive brain stem nucleus, as well as within nucleus of the solitary tract and area postrema. A similar pattern of expression with fewer cells per section was detected after carrageenin instillation. The number of expressing cells was reduced by 54% in trigeminal nucleus caudalis but not within the nucleus of the solitary tract or area postrema when blood was injected in adult rats after neonatal capsaicin treatment.

3 Pretreatment with 5-HT_1 agonists with some selectivity for the 5-HT_{1B} receptor, CP-93,129 (460 nmol kg^{-1} × 2, i.v.), sumatriptan (720 nmol kg^{-1} × 2, i.v.) or dihydroergotamine (86 nmol kg^{-1} × 2, i.v.) reduced positive cells by 39%, 31%, and 33% respectively in trigeminal nucleus caudalis but not in nucleus of the solitary tract or area postrema after blood instillation. Pretreatment with the analgesic morphine (15 μmol kg^{-1}, s.c.) also decreased the number of positive cells by 63% in trigeminal nucleus caudalis.

4 CP-93,129 (460 nmol kg^{-1} × 2, i.v.) reduced the number of c-fos labelled cells by 47% within lamina I, II_o after carrageenin instillation.

5 Drug-induced blockade appeared to be tissue-dependent. Pretreatment with sumatriptan (720 nmol kg^{-1} × 2, i.v.) did not block c-fos expression in trigeminal nucleus caudalis following formalin application to the nasal mucosa.

6 Drug-induced blockade may be mediated by an action on primary afferent (trigeminovascular) fibres in as much as CP-93,129 (460 nmol kg^{-1} × 2, i.v.) did not reduce the number of expressing cells within the trigeminal nucleus caudalis following blood instillation in rats treated as neonates with capsaicin.

7 We infer from these results that the analgesic actions of agonists at 5-HT_{1B} receptors (the receptor subtype analogous to 5-HT_{1D} in man) need not depend upon the presence of vasodilatation and, that 5-HT_{1D} receptor-mediated blockade of neurotransmission contributes significantly to the analgesic effects of these drugs in headache.

8 Based on the demonstrated effects of 5-$HT_{1B/D}$ agonists against the actions of two chemically-unrelated meningeal stimulants, we suggest that treatment with 5-HT_{1D} agonists may be useful for the alleviation of pain in other headache conditions associated with meningeal irritation. Bacterial, viral (including AIDS meningovascular inflammation) and other forms of chemical meningitis merit further investigation.

Keywords: Sumatriptan; CP-93,129; dihydroergotamine; trigeminovascular system; subarachnoid haemorrhage; c-fos expression; 5-$HT_{1B/D}$ receptor agonists; headache

Introduction

The mechanism by which sumatriptan and dihydroergotamine abort or ameliorate acute vascular head pain is not well understood. Both drugs are 5-HT_{1D} receptor agonists (Peroutka, 1990) and exhibit nanomolar binding affinities to 5-HT_{1D} recognition sites as well as to binding sites in rat brain membranes (5-HT_{1B}) which are analogous to the 5-HT_{1D} binding sites in man (Middlemiss & Hutson, 1990).

Explanations for the analgesia have been proposed based on prejunctional (Saito *et al.*, 1988; Buzzi & Moskowitz, 1990; Buzzi *et al.*, 1991a) and postjunctional mechanisms (Saxena & Verdouw, 1985; Humphrey *et al.*, 1990) in cephalic blood vessels. At prejunctional receptors, 5-HT_{1D} and probably 5-HT_{1B} agonists inhibit neuropeptide release from perivascular axons and block the development of neuroinflammation within the meninges and, by so doing, inhibit sensitization of innervating primary afferent fibres. Neuroinflammation in the meninges is characterized by mast cell degranulation, platelet aggregation, and endothelial activation (Buzzi *et al.*, 1992; Dimitriadou *et al.*, 1992). At postjunctional receptors, 5-$HT_{1B/D}$ receptor agonists constrict intracranial vascular smooth muscle (Connor *et al.*, 1989; Feniuk *et al.*, 1989). Pain may be alleviated by constricting the dilatation, the proposed mechanism for pain in this model. The triggering events for vascular headaches are unknown, although there is general agreement that meningeal blood vessels and their associated small unmyelinated nerve

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fibres generate the pain of most headaches (Moskowitz, 1984).

We have recently adapted an animal model to address the possibility that drugs with some selectivity for the 5-$HT_{1B/D}$ subtype exhibit important neurogenic mechanisms as a basis for their actions. The model is based on injecting autologous blood (Delgado *et al.*, 1985; Jackowski *et al.*, 1990) or the sulphated mucopolysaccharide, carrageenin into the cisterna magna and subarachnoid space of Sprague-Dawley rats. Blood in the subarachnoid space in man is quite noxious and causes significant pain, vasoconstriction and reductions in cerebral blood flow. Carrageenin is a potent inflammatory agent when injected into the central nervous system. As in other models of pain and inflammation, injection of a noxious substance promotes c-fos protein expression within cells of lamina I, II_o of the trigeminal nucleus caudalis (TNC) or dorsal horn of the spinal cord (Hunt *et al.*, 1987). TNC (laminae I, II_o) contains the synapses of primary afferent fibres concerned with the transmission of nociceptive information from trigeminal receptive fields and second and higher order neuorones which transmit nociceptive information to rostral centres (Willis, 1985; Menétrey & Basbaum, 1987). In a previous study, the number of positive cells in this nucleus correlated with the amount of injected blood (Nozaki *et al.*, 1992). Similarly, the number of positive cells in the dorsal horn correlated with a nociceptive behavioural response after formalin injection into the hind paw of a rat. In the formalin experiments, the number of expressing cells decreased after morphine administration (Presley *et al.*, 1990; Gogas *et al.*, 1991) in a dose-dependent, naloxone-sensitive manner. Chronic sectioning of the trigeminal nerve innervating the meninges, or destroying small unmyelinated C-fibres by neonatal capsaicin treatment significantly reduced the number of cells expressing c-fos within TNC after blood injection into the cisterna magna (Nozaki *et al.*, 1992).

We chose to address the possible importance of neurogenic mechanisms to the actions of 5-$HT_{1B/D}$ receptors by determining whether CP-93,129, sumatriptan or dihydroergotamine block expression of a marker of sensory activation when given prior to noxious chemical stimulation of the meninges. Our data show that 5-HT_{1B} receptor agonists block selectively the expression of a marker of activation within TNC after noxious trigeminovascular stimulation. These findings suggest a mechanism independent of vasoconstriction for the therapeutic effects of the drugs in alleviating headaches.

Methods

Blood and carrageenin instillation into the subarachnoid space

We chose to study the rat instead of the guinea-pig, a species possessing the 5-HT_{1D} receptor, because of the expected high mortality in the latter and because of the opportunity to monitor physiological parameters more easily. Male Sprague-Dawley rats (250–300 g, Charles River Laboratories, Wilmington, MA, U.S.A.) were anaesthetized with pentobarbitone (45 mg kg^{-1}, i.p.). Polyethylene catheters (22 Ga, 0.7 mm internal diameter; Intracath, Deseret, Sandy, Utah, U.S.A.) were inserted into the femoral artery and vein for blood withdrawal and drug administration. A midline skin incision was made from the occipital protuberance to $cervical_7$ area in order to introduce a soft catheter (PE-10, internal diameter 0.28; Intramedic, Clay Adams, Parsippany, NJ, U.S.A.) into the cisterna magna. Anaesthesia was maintained by periodic injections (i.p. or i.v.) of pentobarbitone (10 mg kg^{-1}) approximately every 2 h. Animals were kept in a prone position. Six hours after catheter placement, arterial blood (0.4 ml) was removed from the femoral catheter and an equivalent volume of saline was injected intra-arterially. Within 1 or 2 min following withdrawal, 0.3 ml of autologous non-heparinized arterial blood or artificial CSF (Na 150 mEq l^{-1}, K 3 mEq l^{-1}, Ca 2.5 mEq l^{-1}, Mg 1.2 mEq l^{-1}, Cl 132 mEq l^{-1}, glucose 3.7 mM, urea 6 mM, HCO_3 25 mEq l^{-1}, pH adjusted to 7.4) was injected into the cisterna magna over about 1 min via a tuberculin syringe (1 ml). Sham animals were treated identically but received no intracisternal injection.

In the carrageenin experiments, animals were treated as described above except that 0.1 ml of carrageenin solution (10 mg ml^{-1} in saline) was injected into the cisterna magna via a 27 gauge needle and tuberculin syringe.

Animals were then kept at 30° head-down for 30 min in order to facilitate the settling of blood or carrageenin in the basal cisterns after which they remained in the horizontal prone position. Animals received no additional anaesthetics.

In selected (chosen at random) animals, arterial PCO_2, PO_2, pH (Corning 178 Blood Gas Analyzer, Ciba Corning Diag Corp., Medford, MA, U.S.A.), haematocrit, blood glucose, and direct arterial blood pressure (Grass Model 7B Polygraph Grass Instrument Co., Quincy, MA, U.S.A.) were monitored along with heart rate and respiratory rate. Intracranial pressure was recorded in animals (administered blood only) equipped with a closed cranial window via an outlet from the cranial window to a pressure transducer (DTX/Plus™ Disposable Transducer Kit, Viggo-Spectramed, Oxnard, CA, U.S.A.) attached to a chart recorder (Gould model RS 3200, Gould Inc., Cleveland, Ohio, U.S.A.) (Morii *et al.*, 1986). Core body temperature was maintained between 36–37°C with a homeothermic blanket system (Harvard Apparatus, Natick, MA, U.S.A.).

Formalin application to the nasal mucosa

Formalin (5% in saline) was applied to the right nasal mucosa. Animals were lightly anaesthetized with pentobarbitone (20 mg kg^{-1}, i.p.) and 50 µl of 5% formalin solution was then dripped onto the right nasal cavity from a Hamilton syringe.

Capsaicin neonatal treatment

Rat pups within the first 48 h of birth were injected with capsaicin (50 mg kg^{-1}, s.c.) to destroy small unmyelinated sensory fibres as previously described (Markowitz *et al.*, 1987) and raised to adulthood. Before experiments, a small amount of capsaicin (1 µM–1 mM) was applied topically to the cornea to confirm that the animals were desensitized to this stimulus.

Drug treatment

Intracisternal blood-CP-93,129 (46, 140 or 460 nmol kg^{-1}), sumatriptan (240 or 720 nmol kg^{-1}) or dihydroergotamine (86 nmol kg^{-1}) (1 ml kg^{-1}) were injected intravenously 60 and 10 min before blood instillation. Drug dosages were chosen to approximate amounts previously shown to activate prejunctional receptors and block neurogenic extravasation within the meninges (Buzzi *et al.*, 1991b; Matsubara *et al.*, 1991). Morphine sulphate (15 µmol kg^{-1}) (0.67 ml kg^{-1}) was injected subcutaneously 20 min before blood instillation (see Presley *et al.*, 1990).

Carrageenin injection CP-93,129 (460 nmol kg^{-1} × 2) was injected 60 and 10 min before carrageenin instillation.

Formalin injection-Sumatriptan (720 nmol kg^{-1}) was injected intravenously 60 and 10 min before formalin application into the right nasal mucosa.

C-fos immunohistochemistry

All animals survived for 2 h after which they were anaesthetized and killed with an overdose of pentobarbitone (60 mg kg^{-1}, i.p.) and perfused through the ascending aorta

with 150 ml of saline (0.9%), followed by 200 ml of paraformaldehyde (4%) in 0.1 M phosphate buffer (PB, pH 7.3). The perfused brain stems with attached upper cervical spinal cords were kept in the same fixative overnight, and then placed in 20% sucrose and 30% ethylene glycol in 0.1 M PB for 48 h at 4°C before sectioning. The brain stem with upper cervical spinal cords were sectioned coronally (50 μm) on a freezing microtome.

Free-floating sections were processed immunohistochemically with the avidin-biotin procedure using commercially available kits (Vectastain ABC; Vector Labs Burlingame, CA) as described previously (Uemura *et al.*, 1991). Briefly, sections were incubated with 10% normal goat serum and 0.03% hydrogen peroxide in 0.1 M phosphate buffered saline (PBS, pH 7.3) for 30 min at room temperature with gentle agitation. Subsequently, sections were incubated with primary antisera for c-fos protein (1:5000 dilution) in PBS with 0.3% triton X-100 (Sigma Labs, St Louis, MO, U.S.A.) overnight at room temperature under gentle agitation. The rabbit polyclonal antiserum was directed against an *in vitro* translated product of c-fos gene (kindly provided by Dr Dennis Slamon, the Department of Hematology and Oncology at University of California, Los Angeles, U.S.A.), and was preabsorbed against acetone-dried rat liver powder overnight to reduce non-specific background staining. The staining pattern produced by this antibody is comparable to a commercially available monoclonal antibody raised against a synthetic peptide which consists of residue 4–17 of the c-fos protein. In some experiments, antisera were reused (maximum of 5 times) and collected and stored at 4°C after use.

After washes in PBS (15 min × 3), sections were placed in biotinylated anti-rabbit IgG antiserum (1:200 dilution in PBS, Vector Labs, Burlingame, CA, U.S.A.) at room temperature for 2 h with gentle agitation. After PBS washes (15 min × 3), sections were placed in ABC-peroxidase complex (Vector Lab) for 2 h at room temperature with gentle agitation. After further PBS washes (15 min × 3) sections were placed in a solution of 3,3'-diaminobenzidine tetrahydrochloride (40 mg%; Sigma Labs) and 0.003% hydrogen peroxide in 50 mM Tris-HCl buffer (pH 7.6) for about 20 min. After the diaminobenzidine reaction, sections were mounted on gelatin-coated slides, air-dried and coverslipped.

Every second section was processed for immunohistochemistry and approximately 150 sections were examined in each animal. Serial sections were evaluated and quantitated for c-fos expression bilaterally with a Zeiss microscope. Anatomical boundaries were determined by co-ordinates established by the Paxinos & Watson rat brain atlas. In order to quantitate c-fos protein expression, cells showing c-fos like immunoreactivity within lamina I, II$_o$ of TNC, nucleus of the solitary tract (NTS) and area postrema (AP) were counted by a 'blind' observer (P.B.) and these results were confirmed independently by K.N. The extent of TNC examined in this study was from AP to cervical 1 segment (about 50 sections in each animal), and those of NTS and AP were from 0.2 mm caudal–0.8 mm rostral to obex (in which the majority of positive cells were observed; about 10 sections in each animal) and from obex–0.5 mm rostral to obex (about 5 sections in each animal) respectively. Because re-use of primary antisera caused an apparent reduction in the number of c-fos expressing cells following blood instillation, it was especially important to include appropriate controls in each experiment.

Statistics

Data are expressed as the number of cells within each 50 μm section (mean ± s.d.) or in the case of carrageenin, the total number of labelled cells per nucleus. Statistical comparisons were performed by unpaired Student's *t* test. Probabilities of less than 0.05 were considered significant.

Drugs

Sumatriptan (Glaxo Ltd, Hertfordshire, England) and dihydroergotamine (DHE) (Sandoz; supplied as D.H.E.45 ampoules [1 mg ml^{-1}]) were diluted in saline; CP-93,129 {3-(1,2,5,b-tetrahydropyrid-4-yl)pyrrolo [3,2-b]pyrid-5-one} (Pfizer, Inc., Groton, CT, U.S.A.) was dissolved in dimethylsulphoxide:saline 1:19. Capsaicin was solubilized in saline:Tween 80 8:1:1 and purchased from Polysciences Inc., Wilmington, PA, U.S.A. Pentobarbitone was obtained from Anthony Products Co, Aracadia, CA, U.S.A. Morphine sulphate (Elkins-Sinn, Inc., Cherry Hill, NJ, U.S.A., [15 mg ml^{-1}]) was used without dilution. Lambda-carrageenin (Sigma Chemical Co, St. Louis, MO, U.S.A.) was mixed in saline.

Results

The mortality following anaesthesia and blood injection was less than 2%. Extensive blood clots were observed around the brain stem and in the basal cistern when rats were killed 2 h after blood instillation.

Physiological parameters

In both vehicle- and drug-treated [CP-93,129 (460 nmol kg^{-1} × 2, $n = 4$, Figure 1a) or sumatriptan (720 nmol kg^{-1} × 2, $n = 3$, Figure 1b)] animals, similar increases in femoral arterial blood pressure and decreases in pulse rate and respiratory rate were observed 1 min after blood injection, and returned to baseline by 20 min. Heart rate and femoral arterial blood pressure remained at baseline levels for the remaining 2 h, although respiratory rate increased slowly. Intracranial pressure increased up to 30–35 mmHg from baseline (4–12 mmHg) 5 min after blood instillation, and slowly returned towards baseline. At 1 h after blood instillation, intracranial pressure was 10–18 mmHg. There were no significant differences in the above physiological parameters between vehicle- and drug-treated animals. Similarly, arterial Pco_2, Po_2, pH, haematocrit or blood glucose did not differ between groups when measured just before blood instillation and after 30 min and 2 h (data not shown).

C-fos expression

In sham animals, few c-fos positive cells were detected in the brain stem. The number of c-fos immunoreactive cells per section in TNC (lamina I, II$_o$), NTS or AP was 6 ± 3, 4 ± 2 or 6 ± 4 ($n = 5$) respectively. In animals which were injected with 0.3 ml of artificial CSF, c-fos positive cells were sparsely observed in the same brain stem nuclei. The number of positive cells per section in TNC (lamina I, II$_o$), NTS or AP was 13 ± 5, 17 ± 5 or 17 ± 6 ($n = 12$), respectively.

C-fos-like immunoreactivity was found bilaterally and most intensely within cells of TNC (lamina I, II$_o$), NTS and AP in animals which were injected with 0.3 ml of blood. The numbers of c-fos containing cells per section in TNC (lamina I, II$_o$), NTS, AP were 39 ± 5, 35 ± 9, 39 ± 10 ($n = 12$), respectively. In adult animals which were treated as neonates with capsaicin, c-fos positive cells were reduced in number by 54% within TNC (lamina I, II$_o$), but not within NTS or AP (Figure 2).

Treatment with CP-93,129 or sumatriptan prior to blood injection dose-dependently reduced the numbers of c-fos positive cells within TNC. CP-93,129 (460 nmol kg^{-1} × 2) or sumatriptan (720 nmol kg^{-1} × 2) reduced the numbers by 39% [38 ± 4 ($n = 11$) versus 23 ± 3 ($n = 7$) in vehicle versus drug treatment, respectively] or 31% [39 ± 6 ($n = 9$) versus 27 ± 2 ($n = 7$) in vehicle versus drug treatment, respectively] (Figures 3 and 4), but not in NTS or AP (Figure 5a,b). Pretreatment with dihydroergotamine (86 nmol kg^{-1} × 2) also reduced the numbers of positive cells in TNC by 33%

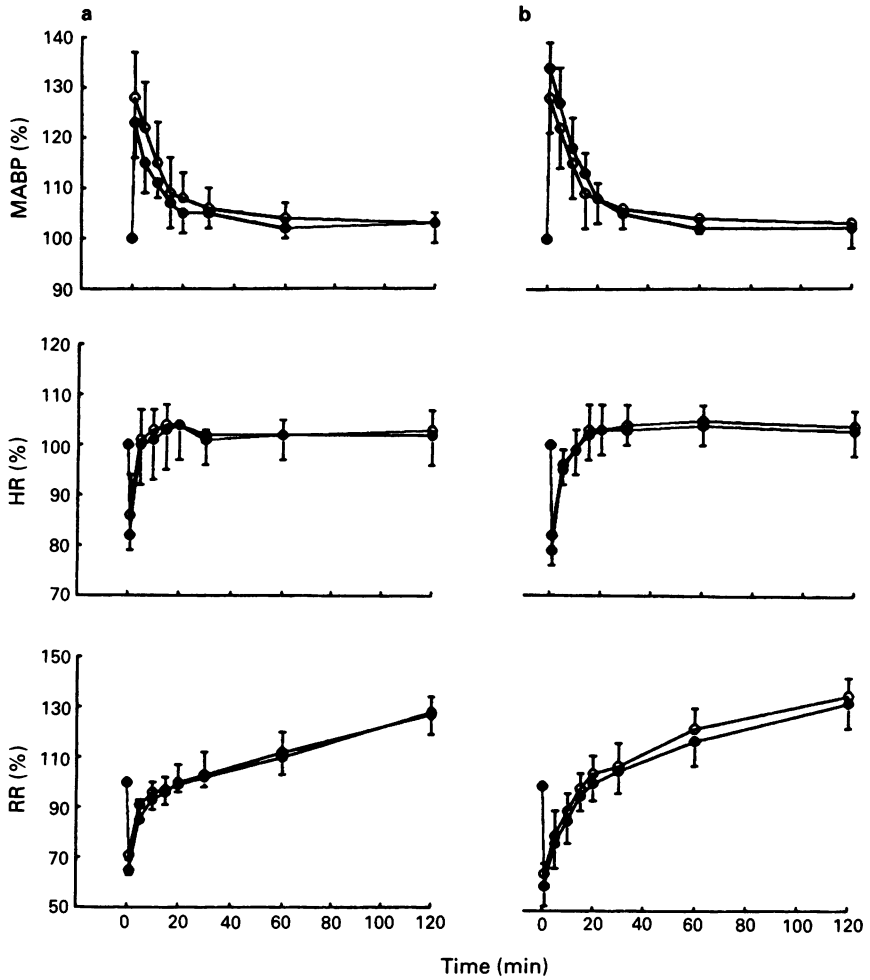


Figure 1 Time-dependent changes in mean arterial blood pressure (MABP), heart rate (HR), and respiratory rate (RR) following intracisternal injection of 0.3 ml of blood in animals which were pretreated with (a) CP-93,129 (460 nmol $kg^{-1} \times 2$, $n = 4$, ●) or vehicle ($n = 4$, ○) and (b) sumatriptan (720 nmol $kg^{-1} \times 2$, $n = 3$, ●) or vehicle ($n = 3$, ○). Data are expressed as percentage of baseline (with s.d. shown by vertical bars). Baseline values of MABP, HR and RR in CP-93-129- or vehicle-treatment were 93 ± 3 or 93 ± 3 mmHg, 383 ± 28 or 403 ± 27 min^{-1} and 68 ± 6 or 71 ± 6 min^{-1}, respectively. Baseline values of MABP, HR and RR in sumatriptan- or vehicle-treatment were 94 ± 5 or 96 ± 6 mmHg, 397 ± 16 or 404 ± 24 min^{-1} and 72 ± 7 or 73 ± 7 min^{-1}, respectively.

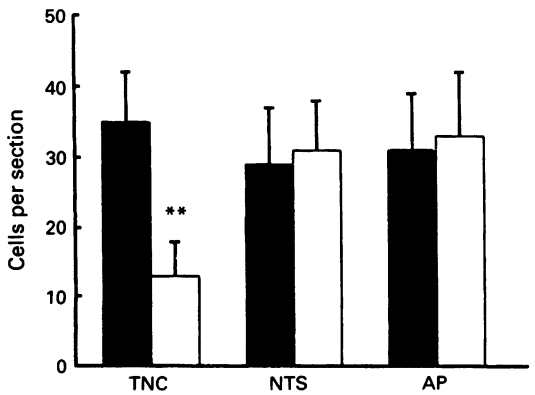


Figure 2 Capsaicin neonatal treatment selectively decreases c-fos expression in trigeminal nucleus caudalis (TNC) after subarachnoid blood injection. Numbers of c-fos immunoreactive cells per 50 μm sections are shown in TNC (lamina I, II_o), nucleus of the solitary tract (NTS) and area postrema (AP) 2 h after intracisternal injection of 0.3 ml of blood to adult animals treated during the neonatal period with vehicle (closed columns, $n = 7$) or capsaicin (open columns, $n = 6$). The numbers of c-fos positive cells per section observed in sham animals (6 ± 3 for TNC, 4 ± 2 for NTS, 6 ± 4 for AP, $n = 5$) are subtracted from each number.
$^{**}P < 0.01$.

[45 ± 3 ($n = 3$) versus 30 ± 5 ($n = 4$) in vehicle versus drug treatment, respectively], but not in AP or NTS (Figure 5c). Pretreatment with morphine (15 μmol kg^{-1}) markedly decreased the number of c-fos positive cells by 63% in TNC [38 ± 10 ($n = 4$) versus 14 ± 4 ($n = 5$) in vehicle versus drug treatment, respectively] and moderately within NTS (Figure 5d).

Pretreatment with CP-93,129 (460 nmol $kg^{-1} \times 2$) did not further reduce blood-induced c-fos expression within TNC in adult animals treated as neonates with capsaicin. The number of c-fos positive cells within TNC in CP-93,129- or vehicle-treated animals was 23 ± 6 ($n = 3$) or 22 ± 8 ($n = 6$), respectively (Figure 6).

Carrageenin instillation was accompanied by c-fos expression within lamina I, II_o of TNC (Figure 7) as well as within AP and NTS (data not shown). The number of labelled cells in each nucleus was significantly reduced as compared to the response following blood injection, which could have been due to the amount of injected carrageenin or to the nature of the stimulus (e.g., carrageenin is viscous and did not distribute as widely as the injected blood). Pretreatment with CP-93,129 (460 nmol $kg^{-1} \times 2$) significantly attenuated the c-fos response in the seven examined animals (531 ± 175 total cells in TNC after vehicle treatment versus 280 ± 171 after CP-93,129 treatment). C-fos labelled cells were not counted within NTS or AP as part of this latter experiment.

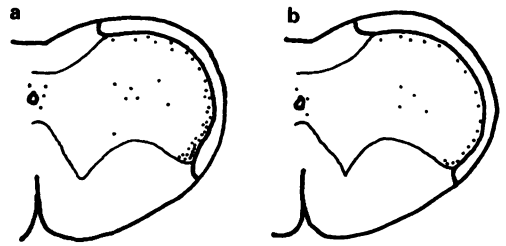


Figure 3 Camera lucida drawings of coronal sections of upper cervical spinal cord showing localization of c-fos immunoreactive cells (dots) within superficial lamina of trigeminal nucleus caudalis (TNC) (3.0 mm below obex) in animals which were pretreated with (a) vehicle or (b) sumatriptan (720 nmol kg^{-1}) 60 and 10 min before intracisternal blood injection. A few positive cells were found within the vicinity of the central canal and diffusely in deeper nuclei.

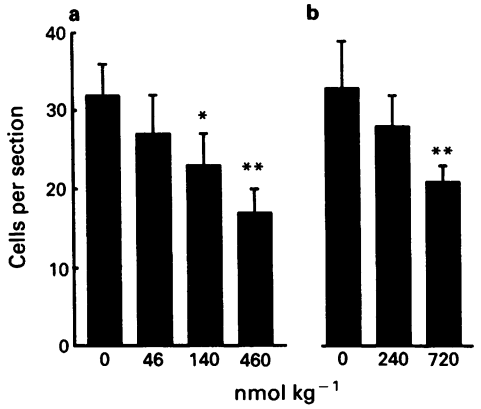


Figure 4 CP-93,129 and sumatriptan decrease the numbers of c-fos immunoreactive cells per 50 μm section within trigeminal nucleus caudalis (TNC) (lamina I, II$_o$) after intracisternal blood injection. (a) CP-93,129: vehicle ($n = 11$), 46 nmol kg^{-1} ($n = 3$), 140 nmol kg^{-1} ($n = 5$), 460 nmol kg^{-1} ($n = 7$); (b) sumatriptan: vehicle ($n = 9$), 240 nmol kg^{-1} ($n = 3$), 720 nmol kg^{-1} ($n = 7$) 60 and 10 min before blood injection. The number of c-fos positive cells per section observed in sham animals (6 ± 3, $n = 5$) is subtracted from each number.
*$P < 0.05$; **$P < 0.01$ compared with vehicle treatment.

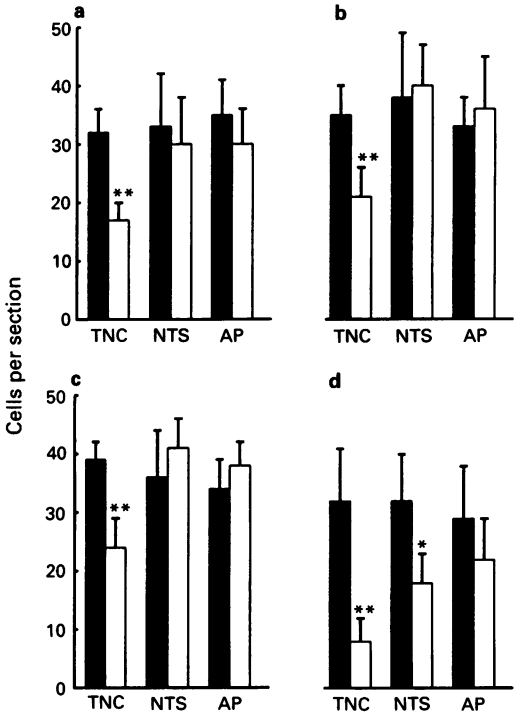


Figure 5 Drug-induced inhibition of c-fos expression within brain stem nuclei. C-fos immunoreactive cells were counted per 50 μm section within trigeminal nucleus caudalis (TNC) (lamina I, II$_o$), nucleus of the solitary tract (NTS) and area postrema (AP) in animals which were pretreated with (a) CP-93,129 (vehicle, closed columns, $n = 8$; 460 nmol kg^{-1} × 2; open columns, $n = 7$), (b) sumatriptan (vehicle, closed columns, $n = 6$; 720 nmol kg^{-1} × 2, open columns, $n = 7$), (c) dihydroergotamine (vehicle, closed columns, $n = 3$; 86 nmol kg^{-1} × 2, open colums, $n = 4$), and (d) morphine (vehicle, closed columns, $n = 4$; 15 μmol kg^{-1}, open columns, $n = 5$) before intracisternal blood injection. The numbers of c-fos positive cells per section observed in sham animals (6 ± 3 for TNC, 4 ± 2 for NTS, 6 ± 4 for AP) are subtracted from each number.
*$P < 0.05$; **$P < 0.01$ compared with vehicle.

Formalin (5%) injected into the right nasal mucosa increased c-fos expression mostly in ipsilateral TNC (lamina I, II$_o$), and in NTS and AP, as well. Treatment with sumatriptan (720 nmol kg^{-1} × 2) before formalin application did not reduce the number of c-fos positive cells in TNC (Figure 8).

Discussion

The expression of c-fos immunoreactivity within brain and spinal cord is useful for mapping the spatial distribution of neurones activated by noxious as well as non-noxious stimulation (Hunt *et al.*, 1987; Presley *et al.*, 1990). The experiments described herein, however, concern noxious stimulation of the meninges because, (a) small calibre, unmyelinated sensory axons which transmit nociceptive information (not larger calibre myelinated afferents) predominate within the pia mater of animals and man, (b) meningeal blood vessels cause pain (and no other sensation) during electrical or mechanical stimulation, (c) blood in the subarachnoid space is noxious in man and causes severe headache and pain, (d) c-fos labelling was most intense in lamina I and II$_o$ of TNC, a region that processes nociceptive information, (e) surgical or chemical sectioning of meningeal afferents reduces the number of labelled neurones in TNC, and (f) the analgesic, morphine, reduced c-fos expression in TNC as did the 5-HT$_1$ agonists.

5-HT$_{1B}$ is probably the receptor subtype mediating inhibition of c-fos expression in this rat subarachnoid haemorrhage model. CP-93,129 is a potent, selective and specific agonist for the 5-HT$_{1B}$ receptor subtype. CP-93,129 exhibits high nanomolar or micromolar affinities for all other 5-HT recognition sites (Macor *et al.*, 1990) and for dopamine, noradrenaline and adenosine binding sites as well. Sumatriptan binds with high affinity to 5-HT$_{1B}$ and 5-HT$_{1D}$ recognition sites, although it seems to be more potent as an agonist at 5-HT$_{1D}$ receptors. Dihydroergotamine exhibits nanomolar affinities at both 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor sites and is the least selective agent among the three. Because 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors are species-specific (Hoyer & Middlemiss, 1989; Matsubara *et al.*, 1991), 5-HT$_{1B}$ receptor-mediated c-fos inhibition in rat may be analogous to the 5-HT$_{1D}$ inhibition of vascular head pain in man.

5-HT$_{1B}$ receptors which inhibit c-fos expression appear closely associated with or directly on perivascular afferent fibres (Buzzi & Moskowitz, 1990; Buzzi *et al.*, 1991a,b; Moskowitz, 1991). The spatial distribution of expressing cells was similar after destruction of primary afferent fibres and after sumatriptan, CP,93-129 or dihydroergotamine pretreatment. Neither blocked cell labelling in NTS or AP after blood injection. A 5-HT receptor on brain stem neurones is less likely to mediate the observed effects because CP-93,129 did not inhibit c-fos expression in capsaicin-treated rats, and because sumatriptan did not block the expression after for-

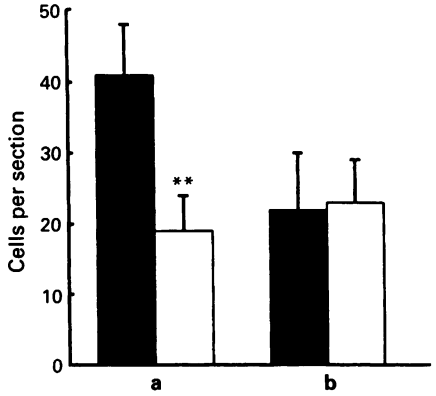


Figure 6 CP-93,129 does not decrease c-fos expression within trigeminal nucleus caudalis (TNC) in adult animals treated as neonates with capsaicin. (a) C-fos immunoreactive cells were counted per 50 μm section within TNC (lamina I, II_o) 2 h after intracisternal blood injection in adult animals after neonatal vehicle or capsaicin pretreatment (closed columns in a, $n = 6$ or open columns in a, $n = 5$, respectively). (b) CP-93,129 (460 nmol $kg^{-1} \times 2$, open columns, $n = 3$) or vehicle (closed columns, $n = 6$) was given 60 and 10 min before blood injection in adult animals after neonatal capsaicin pretreatment.
$^{**}P < 0.01$.

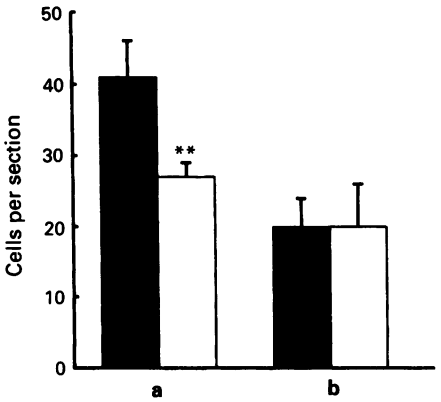


Figure 8 Sumatriptan did not block c-fos expression within trigeminal nucleus caudalis (TNC) (lamina I,II_o) after formalin application to the nasal mucosa. (a) Sumatriptan (720 nmol $kg^{-1} \times 2$, open columns, $n = 7$) or vehicle (closed columns, $n = 6$) was administered 60 and 10 min before intracisternal blood injection. (b) Sumatriptan (720 nmol $kg^{-1} \times 2$, open columns, $n = 3$) or vehicle (closed columns, $n = 3$) was administered 60 and 10 min before formalin application to the right nasal mucosa. The numbers of c-fos immunoreactive cells were counted per 50 μm sections.
$^{**}P < 0.01$.

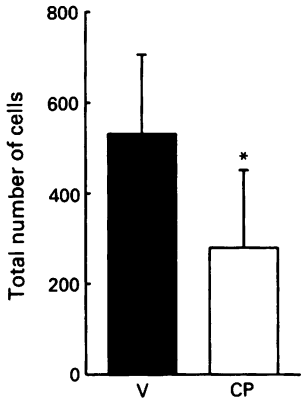


Figure 7 CP-93,129 (460 nmol $kg^{-1} \times 2$) attenuates the expression of c-fos within trigeminal nucleus caudalis (TNC) following the instillation of carrageenin (1 mg in 0.1 ml saline). Vehicle (V) or CP-93,129 (CP) were administered 60 and 10 min before carrageenin administration, and the animals were killed 2 h later ($n = 7$ per group). The data are expressed as the total number of cells (with s.d. shown by vertical bars) within TNC. The brains were processed as described in Methods.
$^{*}P < 0.05$ as compared to vehicle-treated group.

malin application to the nasal mucosa.

Activation of $5\text{-}HT_{1B}$ receptors on vascular smooth muscle does not mediate inhibition of c-fos expression within TNC. In man and animals, blood in the subarachnoid space is accompanied by intense cerebroarterial constriction and reductions in cerebral blood flow (50% lower than normal when tested in our own experiments using laser-Doppler flowmetry). If inhibition of c-fos expression does reflect reduction of a nociceptive signal, then dilated blood vessels are not a requisite for drug-induced blockade of neural transmission in this animal model. If constriction of dilated vessels is a mechanism for analgesia in the clinical situation (which is doubtful; Moskowitz, 1992), then our studies suggest that sumatriptan and dihydroergotamine possess a second and distinct antinociceptive mechanism. Our data show that $5\text{-}HT_{1B/D}$ receptors are coupled to at least two important functions which may diminish pain and sensitization in the meninges (Buzzi & Moskowitz, 1990; Buzzi *et al.*, 1991a,b; Matsubara *et al.*, 1991). The drugs block neural transmission within trigeminovascular fibres (as reflected by inhibition of c-fos protein expression), and also block the neuroinflammatory response by inhibiting neuropeptide release. The threshold doses for both effects are within a similar range and both are related to prejunctional mechanisms. The molecular basis remains to be determined.

The studies reported herein have focused on pain mechanisms related to the irritants blood or carrageenin in the subarachnoid space. The data suggest the possibility that $5\text{-}HT_{1D}$ agonists may be useful in treating other conditions characterized by meningeal pain and inflammation such as viral, bacterial or chemical meningitis.

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Nitric oxide and relaxation of pig lower urinary tract

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1 We studied the non-adrenergic, non-cholinergic (NANC) nerve-mediated relaxation induced by electrical stimulation in pig isolated lower urinary tract smooth muscle, and the possible involvement of the L-arginine (L-ARG)/nitric oxide (NO) pathway in this response.

2 Trigonal strips, precontracted by noradrenaline (NA), carbachol or endothelin-1 (ET-1), relaxed frequency-dependently in response to electrical stimulation. Maximum relaxation was obtained at 6–8 Hz, and amounted to 56 ± 2%, 77 ± 3% and 62 ± 6% of the agonist-induced tension in preparations contracted by NA, carbachol, or ET-1, respectively. Exposure to N^G-nitro-L-arginine (L-NOARG; 10^{-7}–10^{-5} M) concentration-dependently reduced the relaxant response in preparations contracted by NA. L-NOARG (10^{-6} M) reduced the maximal response to 51 ± 8% of control. L-NOARG (10^{-5} M) abolished all relaxation, and unmasked a contractile component; D-NOARG had no effect. Also in trigonal preparations, where the tension had been raised by carbachol or ET-1, L-NOARG (10^{-5} M) markedly reduced relaxations evoked by electrical stimulation.

3 In trigonal preparations contracted by NA, maximal relaxation was increased after pretreatment with L-ARG (10^{-3} M), and the inhibitory effect of L-NOARG (10^{-6} M) was prevented. Incubation of the trigonal strips with methylene blue had no effect on relaxations elicited at frequencies <6 Hz, but a small inhibition was observed at higher frequencies.

4 Administration of NO (present in acidified solution of $NaNO_2$) induced concentration-dependent relaxations in trigonal preparations contracted by NA, carbachol, or ET-1. L-NOARG (10^{-5} M) and L-ARG (10^{-3} M) had no effect on these relaxations. However, methylene blue (10^{-5} M) significantly shifted the concentration-response curve for NO to the right. NANC-relaxation and NO-induced relaxation of trigonal preparations were both inhibited by oxyhaemoglobin (10^{-5} M) and pyrogallol (10^{-4} M).

5 In urethral preparations precontracted by NA, electrical stimulation caused frequency-dependent relaxations. A maximum relaxation of 73 ± 4% was obtained at 10 Hz. Also in the urethra, NANC-relaxation was blocked by L-NOARG (10^{-5} M), and a contractile response generally appeared.

6 Detrusor strips treated with α-β methylene ATP (10^{-5} M) and atropine (10^{-6} M), and then contracted by ET-1, showed relaxations (19 ± 3% of the induced tension) in response to electrical field stimulation (2–20 Hz) only when the tension was high. No response at all, or small contractions, were found in response to electrical stimulation in K^+ (35 mM)-contracted detrusor strips. Detrusor preparations contracted by carbachol were concentration-dependently relaxed by exogenously administered NO, SIN-1 (NO-donor), and isoprenaline, whereas vasoactive intestinal polypeptide had minor effects. NO and SIN-1 induced maximal relaxations of 63 ± 3% and 70 ± 4%, respectively, of the tension induced by carbachol. Isoprenaline produced an almost complete relaxation (96 ± 4%).

7 The results suggest that NANC-nerve mediated relaxation, involving the L-ARG/NO pathway, can be demonstrated consistently in the pig trigonal and urethral, but not in detrusor smooth muscle. The importance of this pathway for lower urinary tract physiology and pathophysiology remains to be established.

Keywords: Non-adrenergic non-cholinergic nerves; nitric oxide; urinary tract; N^G-nitro-L-arginine

Introduction

Previous investigations have shown that isolated, contracted urethral smooth muscles from rabbit, sheep, pig and man respond to transmural stimulation with a relaxant response mediated by a non-adrenergic, non-cholinergic (NANC) mechanism (Klarskov *et al.*, 1983; Andersson *et al.*, 1983; 1991; 1992; Garcia-Pascual *et al.*, 1991). Tetrodotoxin (TTX) blocks the response, suggesting the involvement of transmitter(s) released from nerves. The electrically evoked relaxation could be completely blocked by N^G-nitro-L-arginine (L-NOARG), which inhibits the synthesis of nitric oxide (NO) from L-arginine (L-ARG) (Mülsch & Busse, 1990). The effect was enhanced by L-ARG, whereas no effects were obtained with D-NOARG or D-ARG (Andersson *et al.*, 1991; 1992; Garcia-Pascual *et al.*, 1991). In addition, NO (present in acidified $NaNO_2$ solution) relaxed the preparations, even in the presence of L-NOARG. These results suggest that NO (or a NO-containing compound) is a main mediator of urethral relaxation.

A NANC-mediated relaxation in pig detrusor muscle, preceded by a contraction, and which could be blocked with TTX, was demonstrated by Klarskov (1987). He also claimed to have found an occasional NANC-mediated relaxation in the human detrusor, but the evidence for this was not convincing. Recently, however, James *et al.* (1991) reported that human isolated, atropinized detrusor muscle, depolarized with 20 mM K^+, relaxed on electrical stimulation. The relaxation was TTX-insensitive, but could be blocked with L-NOARG, suggesting the involvement of the L-ARG/NO pathway.

Speakman *et al.* (1988) showed that electrical stimulation of strips of human superficial trigone produced relaxation in 40% of the preparations. This response could not be blocked by propranolol, and it was enhanced by atropine and phentolamine, which suggested that it was NANC-mediated.

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Similar relaxant effects had previously been demonstrated in human and pig trigonal muscle (Klarskov *et al.*, 1983; Hills *et al.*, 1984). The mechanism of this relaxant effect has not been clarified.

The present study was performed, in order to study further NANC-mediated relaxation in lower urinary tract smooth muscle, particularly in the trigone and the detrusor, and the possible involvement of the L-ARG/NO pathway in this response. Pig lower urinary tract was chosen for a comparison between the detrusor, trigone, and urethra, partly because of their similarity to corresponding regions in man, partly because the tissues were readily available.

Methods

Tissue preparations and recording of mechanical activity

The bladder and urethra from female pigs were removed in a slaughterhouse shortly after the animals had been killed. The tissue was transported to the laboratory in cold Krebs solution (for composition, see below). The bladder and urethra were opened longitudinally and smooth muscle strips ($1 \times 2 \times 5$ mm) stripped of mucosa were prepared. Detrusor strips were taken from the anterior wall of the dome, and urethral strips were cut transversally from the proximal (2–3 cm distal to the bladder neck) part of the urethra. The trigonal strips were taken in an oblique direction from the internal urethral orifice toward one of the orifices of the ureter.

Tissue preparations were investigated on the same day as the tissue was obtained, or stored for 24 h at 4°C in Krebs solution before investigation. All experiments with electrical field stimulation were made on the first day. There were no differences in the responses to drugs between preparations investigated on the first day and those studied the day after.

The preparations were transferred to 5 ml organ baths containing Krebs solution maintained at 37°C by a thermoregulated water circuit. The Krebs solution was bubbled with a mixture of 95% O_2 and 5% CO_2, maintaining pH at 7.4. The strips were mounted between two L-shaped hooks by means of silk ligatures. One of the hooks was connected to a Grass Instrument FT03C force-displacement transducer for registration of isometric tension and the other was attached to a movable unit. By varying the distance between the hooks the tension could be adjusted. The transducer output was recorded on a Grass Polygraph model 7D or E. During an equilibration period of 45–60 min, the preparations were stretched until a stable tension of 4–6 mN was obtained.

When subjected to electrical field stimulation, the preparations were mounted between two parallel platinum electrodes (3 mm long and 4 mm apart) in the organ baths. Transmural stimulation of nerves was performed with a Grass S48 or S88 stimulator delivering single square pulses (duration 0.8 ms) at supramaximal voltage. The train duration was 5 s and the stimulation interval 120 s.

Experimental procedure

After the equilibrium period, each experiment was started by exposing the preparations to a K^+ (124 mM) Krebs solution (for composition, see below), until two reproducible contractions (difference $<10\%$) had been obtained. The mean of these contractions amounted to 51 ± 3 mN ($n = 67$, N = 23), 15 ± 1 mN ($n = 127$, N = 29) and 7.8 ± 1.2 mN ($n = 10$, N = 6), in detrusor, trigonal, and urethral preparations, respectively.

Relaxant responses to electrical stimulation and the agonists NO, SIN-1, vasoactive intestinal polypeptide (VIP) and isoprenaline were studied in precontracted preparations. SIN-1 is the active metabolite of molsidomine, and believed to liberate NO non-enzymatically (Reden, 1990). Contractions of detrusor and trigonal preparations were evoked by carbachol 5×10^{-7}–10^{-6} M or endothelin-1 (ET-1) 10^{-8}–10^{-7} M. Noradrenaline (NA) 3×10^{-6}–10^{-5} M was used to induce contractions in the urethra and the trigone. These concentrations gave submaximal contractions of approximately the same relative amplitude (60–90% of the tension induced by K^+ 124 mM).

First, frequency-response curves (electrical stimulation, 1–16 Hz) or concentration-response curves (agonists) were constructed. Next, L-NOARG (10^{-7}–10^{-5} M), D-NOARG (10^{-5} M), L-ARG (10^{-3} M), or methylene blue (10^{-5}–3×10^{-5} M) were given at least 15 min before the preparations were once again subjected to electrical stimulation or exposed to agonists. The relaxant effects of NO (10^{-6}–3×10^{-3} M), SIN-1 (10^{-7}–3×10^{-4} M), VIP (10^{-9}–10^{-6} M) and isoprenaline (10^{-9}–10^{-5} M) were studied by cumulative addition. The concentration was increased only after the response to the previous addition had reached a maximal level.

The effects of oxyhaemoglobin (10^{-5} M) and pyrogallol (10^{-4} M) on trigonal relaxation induced by electrical stimulation (2 and 8 Hz), NO (10^{-4} M) and SIN-1 (10^{-4} M) were subsequently studied on the same NA-induced contraction.

In some experiments, the effects of SIN-1 on spontaneous myogenic activity (in the trigone and urethra) and Bay K 8644-induced myogenic activity (in the detrusor) were studied.

The effect of electrical stimulation on detrusor strips, contracted by K^+ (35 mM) or ET-1, were studied after pretreatment with α-β methylene ATP (10^{-5} M), and in the presence of atropine (10^{-6} M).

Drugs and solution

The following drugs were used: (−)-noradrenaline hydrochloride, carbamylcholine chloride, vasoactive intestinal polypeptide (VIP), isoprenaline, propranolol hydrochloride, tetrodotoxin, atropine sulphate, methylene blue, N^G-nitro-L-arginine (L-NOARG), L-arginine hydrochloride, α-β methylene ATP, pyrogallol, haemoglobin (human) (Sigma, U.S.A.), N^G-nitro-D-arginine (D-NOARG), (Bachem, Germany), endothelin-1 (Peptide Institute, Japan), Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethyl phenyl)-pyridine-5-carboxylate), nifedipine (Bayer, Germany) phentolamine methane sulphonate (Ciba-Geigy, Switzerland). SIN-1 (3-morpholino-sydnonimin hydrochloride) was a gift from Dr Kunstmann, Cassella AG, Germany. Stock solutions were prepared and then stored at − 70°C. Subsequent dilutions of the drugs were made with 0.9% NaCl, and when appropriate, 1 mM ascorbic acid was added as an antioxidant. Bay K 8644, nifedipine and SIN-1 were kept in dark vessels in order to minimize light-induced degradation. ET-1 was diluted with 0.9% NaCl containing 0.05% human serum albumin (Behringwerke, Germany) to counteract the adhesive tendency of the peptide. Acidified solutions (pH 2) of $NaNO_2$ (Sigma, U.S.A.) were prepared immediately before use and protected from atmospheric oxygen by covering the tubes with plastic caps. Separate experiments showed that the vehicle had no relaxant effect *per se*. Oxyhaemoglobin was prepared as described by Martin *et al.* (1985).

The reported concentrations are the calculated final concentrations in the bath solution. The Krebs solution used had the following composition (mM): NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, NaH_2PO_4 1.2, glucose 11. K^+-Krebs solutions (124 mM and 35 mM) were prepared by replacing NaCl with equimolar amounts of KCl.

Analysis of data

The effects of electrical field stimulation and drugs are expressed either as percentage relaxation of the agonist-induced tension, or as percentage of the maximal response obtained in control experiments before drug treatment. Statistical determinations were performed by use of Student's two-tailed *t* test. A probability level <0.05 was accepted as significant.

n denotes the number of preparations and N the number of animals. When the number of preparations and animals is identical, only n is given. Results are given as mean values ± s.e.mean.

Results

In all detrusor preparations, tension decreased during the period of equilibration, and the preparations had to be stretched repeatedly. The majority of trigonal and urethral strips were only stretched initially, since the tension increased spontaneously to a steady level during the recovery period. Trigonal strips (92%) developed spontaneous contractions with large amplitudes (Figure 1) during the period of equilibration. Also urethral strips (80%) displayed spontaneous, myogenic activity, although the amplitudes were smaller than in the trigone. In contrast, spontaneous activity was not regularly present in strips from the detrusor (26%), but could be induced by Bay K 8644. The spontaneous activity persisted after addition of atropine (10^{-6} M), phentolamine (10^{-6}M), and TTX (10^{-6} M), but was abolished by nifedipine (10^{-6} M). SIN-1 reduced or abolished spontaneous contractions in the trigone ($n = 7$, Figure 1) and urethra ($n = 6$), and was also able to reduce Bay K 8644-induced contractile activity in the detrusor ($n = 11$).

The trigone

Trigonal strips, precontracted by NA, carbachol or ET-1, caused TTX-sensitive relaxations in response to electrical stimulation. Maximum relaxation was obtained at 6–8 Hz, and amounted to 56 ± 2% ($n = 46$, N = 15), 77 ± 3% ($n = 9$, N = 7) and 62 ± 6% ($n = 5$) in preparations contracted by NA, carbachol, or ET-1, respectively (Figure 2). Exposure to L-NOARG (10^{-7}–10^{-5} M) concentration-dependently reduced the relaxant response to electrical stimulation in preparations contracted by NA (Figure 3). In untreated control preparations, there was no difference in response between the first and the second period of electrical stimulation. Thus, the second stimulation averaged 104 ± 5% ($n = 5$) of the maximal response during the first period of stimulation. At the lowest concentration of L-NOARG used, 10^{-7} M, no inhibition was obtained (data not shown). L-NOARG 10^{-6} M reduced the maximal response to 51 ± 8% ($n = 5$, $P < 0.01$) of control, and L-NOARG 10^{-5} M totally abolished all relaxation (Figure 3), and a contractile component was generally unmasked. D-NOARG (10^{-5} M) had no effect (Figure 3). Also in trigonal preparations where the tension had been raised by carbachol or ET-1, L-NOARG (10^{-5} M) markedly reduced relaxations evoked by electrical stimulation (data not shown).

The maximal relaxation obtained by electrical stimulation in preparations contracted by NA, was increased to 132 ± 10% ($n = 5$, $P < 0.05$) after pretreatment with 10^{-3} M L-ARG (Figure 4), and the inhibitory effect of L-NOARG (10^{-6} M) was prevented (Figure 4). Incubation of the trigonal strips with methylene blue (10^{-5} and 3×10^{-5} M; $n = 5$ in each case) had no effect on relaxations elicited at frequencies < 6 Hz, but a small inhibition (< 25%) was observed at higher frequencies (data not shown).

L-NOARG did not affect the basal tension of the trigonal strips. However, in about 15% of the preparations L-NOARG produced a small further increase in tension when added to preparations contracted by an agonist.

Administration of NO (present in acidified solution of $NaNO_2$) induced concentration-dependent relaxations in trigonal preparations contracted by NA, carbachol, or ET-1. The maximal relaxation obtained at 3×10^{-3} M amounted to 87 ± 4% ($n = 20$, N = 8), 97 ± 2% ($n = 8$) and 86 ± 7% ($n = 5$) of the agonist-induced contraction, respectively (data not shown). L-NOARG (10^{-5} M) and L-ARG (10^{-3} M) had no effects on the relaxations induced by exogenously applied NO ($n = 5$, in each case; Figure 5). However, methylene blue (10^{-5} M) significantly ($n = 5$, $P < 0.05$) shifted the concentration-response curve for NO to the right (Figure 5).

Figure 6 illustrates that pyrogallol (10^{-4} M) and oxyhaemoglobin (10^{-5} M) significantly inhibited relaxations in response to both nerve-stimulation (2 and 8 Hz) and exogenously applied NO (10^{-4} M). Neither pyrogallol nor oxyhaemoglobin inhibited the relaxation produced by SIN-1 (10^{-4} M).

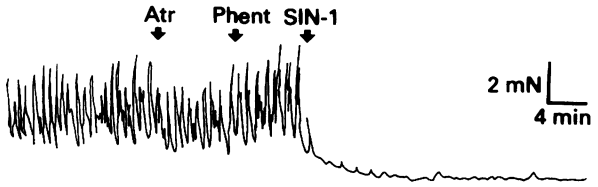


Figure 1 Original tracing from an isolated preparation of the pig trigone showing the effects of atropine (Atr 10^{-6} M), phentolamine (Phent, 10^{-6} M) and SIN-1 (3-morpholino-sydnonimin HCl, 10^{-6} M) on spontaneous myogenic activity.

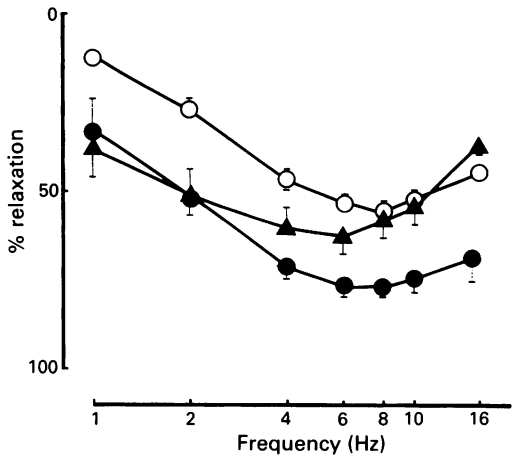


Figure 2 Frequency-response relations for electrically induced relaxations in pig isolated trigonal preparations contracted by (○) noradrenaline (3×10^{-6}–10^{-5} M, $n = 46$, N = 15), (●) carbachol (5×10^{-7}–10^{-6} M, $n = 9$, N = 7) or (▲) endothelin-1 (ET-1, 10^{-8}–10^{-7} M, $n = 5$). Each point is expressed as percentage relaxation of the agonist-induced tension, and represents mean with s.e.mean shown by vertical bars.

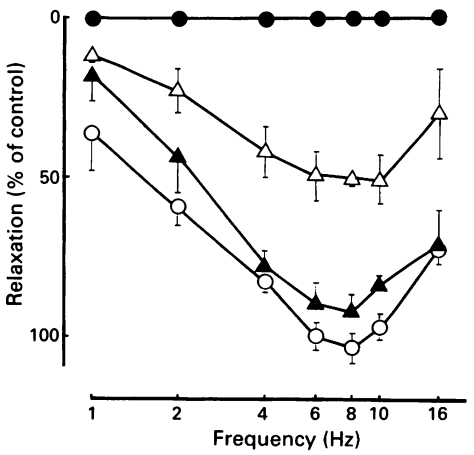


Figure 3 Frequency-response relations for electrically induced relaxations in pig isolated trigonal preparations contracted by noradrenaline (3×10^{-6}–10^{-5} M). Responses were recorded in controls (○) or after pretreatment with N^G-nitro-L-arginine (L-NOARG) 10^{-6} M (△), 10^{-5} M (●) or D-NOARG 10^{-5} M (▲). Each point is expressed as percentage of the maximal response before treatment, and represents mean ($n = 5$) with s.e.mean shown by vertical bars.

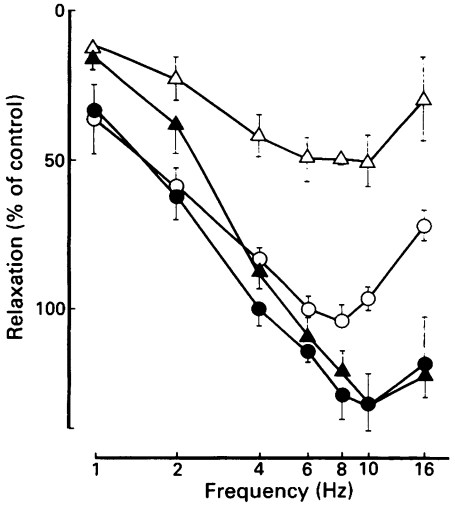


Figure 4 Frequency-response relations for electrically induced relaxations in pig isolated trigonal preparations contracted by noradrenaline (3×10^{-6}–10^{-5} M). Responses were recorded in controls (○) or after pretreatment with N^G-nitro-L-arginine (L-NOARG) 10^{-6} M (△), L-arginine (L-ARG) 10^{-3} M (●) or L-NOARG 10^{-6} M + L-ARG 10^{-3} M (▲). Each point is expressed as percentage of the maximal response before treatment, and represents mean ($n = 5$) with s.e.mean shown by vertical bars.

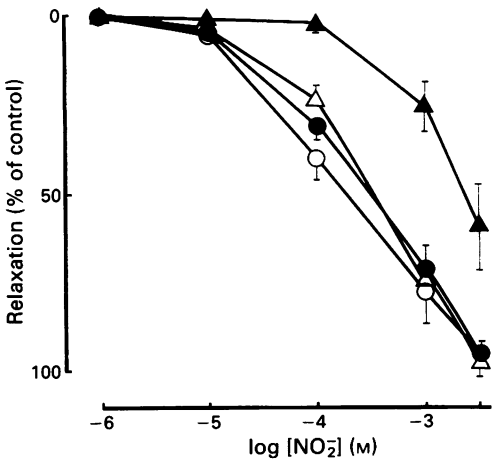


Figure 5 Concentration-response relations for nitric oxide (NO, present in acidified solution of $NaNO_2$) in pig isolated trigonal preparations contracted by noradrenaline (3×10^{-6}–10^{-5} M). Responses were recorded in controls (○) or after pretreatment with L-NOARG 10^{-5} M (△), L-ARG 10^{-3} M (●) or methylene blue 10^{-5} M (▲). Each point is expressed as percentage of the maximal response before treatment, and represents mean ($n = 5$) with s.e.mean shown by vertical bars.

The urethra

In urethral preparations, precontracted by NA, electrical stimulation caused frequency-dependent relaxations that were sensitive to TTX. A maximum relaxation of 73 ± 4% ($n = 10$, N = 6) was obtained at 10 Hz. Also in the urethra, NANC-relaxation was blocked by L-NOARG (10^{-5} M), and a contractile response generally appeared (Figure 7). L-NOARG had no effect on relaxations induced by exogenously applied NO (Figure 7).

The detrusor

In an attempt to suppress contractions induced by potential mediators released by electrical stimulation, detrusor strips



Figure 6 Relaxation induced by electrical stimulation (2 and 8 Hz), nitric oxide (NO, 10^{-4} M, present in acidified solution of $NaNO_2$) and 3-morpholino-sydnonimin HCl (SIN-1, 10^{-4} M) in pig isolated trigonal preparations contracted by noradrenaline (NA, 3×10^{-6}–10^{-5} M). The preparations were pretreated with oxyhaemoglobin (10^{-5} M, hatched columns), pyrogallol (10^{-4} M, solid columns) or remained untreated (open columns). Results are expressed as percentage relaxation of the NA-induced tension, and each column represents mean from 4–5 experiments; s.e.mean shown by vertical bars.

were pretreated with α-β methylene ATP (10^{-5} M) and atropine (10^{-6} M), and then contracted by ET-1 (10^{-8}–3×10^{-8} M) or K^+ (35 mM). ET-1 (10^{-8}–3×10^{-8} M) and K^+ (35 mM) produced contractions amounting to 52 ± 6% ($n = 6$) and 17 ± 3% ($n = 5$) of the K^+ 124 mM-induced response, respectively. α-β methylene ATP *per se* caused transient contractions which were abolished after repeated addition. Electrical field stimulation (2–20 Hz) in no case induced relaxation. In preparations contracted by ET-1 (10^{-8} M–3×10^{-8} M) contractions were observed (Figure 8). However, when the ET-1 concentration was increased to 6×10^{-8}–10^{-7} M (87 ± 4% of the K^+ 124 mM-induced response), 9 out of 11 preparations showed relaxation (19 ± 3% of the induced contraction) in response to electrical stimulation, provided that the preparations were pretreated with atropine (10^{-6} M). L-NOARG abolished the relaxation, but part of the response, particularly that obtained at higher frequencies, persisted after TTX treatment. No response at all, or small contractions, were obtained in K^+ (35 mM)-contracted detrusor strips (Figure 8).

Detrusor preparations contracted by carbachol were concentration-dependently relaxed by NO (10^{-6}–3×10^{-3} M), SIN-1 (10^{-7}–3×10^{-4} M) and isoprenaline (10^{-9}–10^{-5} M), whereas VIP (10^{-9}–10^{-6} M) had only minor effects (Figure 9). NO and SIN-1 produced maximal relaxations of 63 ± 3% ($n = 10$, N = 5) and 70 ± 4% ($n = 10$, N = 5), of the tension induced by carbachol, respectively. Isoprenaline produced an almost complete relaxation (96 ± 4%, $n = 5$, Figure 9). Propranolol (10^{-6} M) did not affect relaxations evoked by NO and SIN-1. Furthermore, NO and SIN-1 also elicited relaxations of detrusor strips precontracted by ET-1 (data not shown). There was no difference in the maximal relaxation produced by NO or SIN-1 in ET-1-contracted compared to carbachol-contracted preparations. Methylene blue (10^{-5} M) inhibited ($P < 0.01$) NO-induced, but not SIN-1-induced relaxations in detrusor strips ($n = 5$, in each case; Figure 10).

Discussion

L-NOARG, an inhibitor of NO synthesis from L-ARG (Mülsch & Busse, 1990), inhibited NANC-relaxation induced by electrical stimulation in smooth muscle preparations of the pig trigone and urethra. The failure of D-NOARG to inhibit the relaxation supports the stereospecific effect of L-NOARG observed on e.g. endothelium-derived relaxing factor (EDRF)-mediated relaxation of vascular smooth mus-

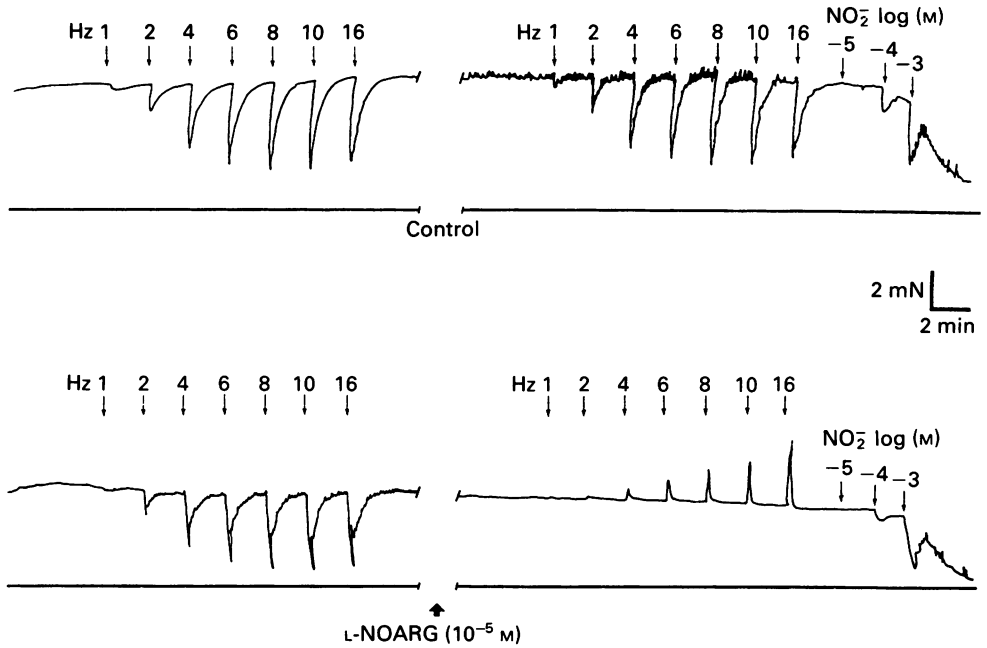


Figure 7 The effect of N^G-nitro-L-arginine (L-NOARG (10^{-5} M) on relaxation induced by electrical stimulation in pig isolated urethra. Precontraction above baseline (shown as a solid line) was induced by noradrenaline (10^{-5} M). The upper panel shows a time-matched control preparation. NO (present in acidified solution of $NaNO_2$) was applied at the end of the experiment.

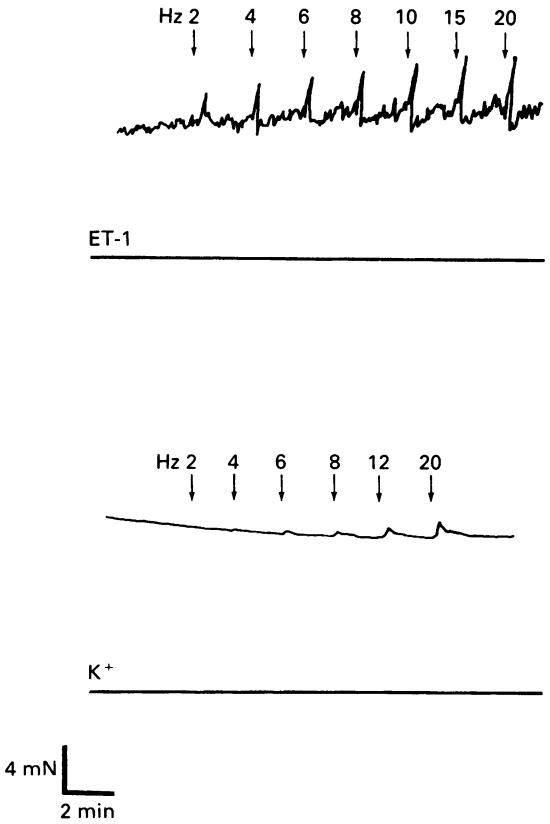


Figure 8 The response to electrical stimulation in pig isolated detrusor preparations contracted above baseline (shown as a solid line) by 10^{-8} M endothelin-1 (ET-1) (upper panel) or K^+ (35 mM) (lower panel). The experiments were performed after pretreatment with α-β methylene ATP (10^{-5} M), and in the presence of atropine (10^{-6} M).

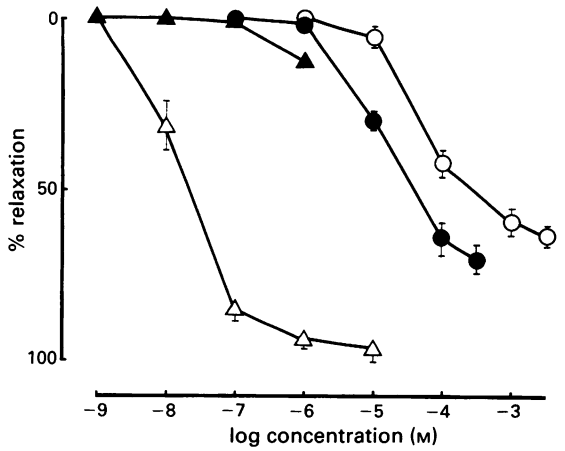


Figure 9 Concentration-response curves obtained by addition of nitric oxide (NO, present in acidified solution of $NaNO_2$) (○, $n = 10$, N = 5), (SIN-1, ●, $n = 10$, N = 5), VIP (▲, $n = 5$) and isoprenaline (△, $n = 5$) to pig isolated detrusor preparations contracted by carbachol (5×10^{-7}–10^{-6} M). Each point is expressed as percentage relaxation of the carbachol-induced tension, and represents mean ± s.e.mean.

cle (Rees *et al.*, 1989). In support of the view that L-ARG is the substrate for the NO-yielding enzyme, it was found that the nerve-induced response increased after pretreatment with L-ARG. L-ARG was also able to reverse the inhibitory effect of L-NOARG, suggesting that L-NOARG is acting competitively. Furthermore, L-NOARG and L-ARG had no effects on the response to exogenously added NO, confirming that their site of action is prejunctional. Taken together, it seems that NO or a NO-containing substance is involved in NANC-relaxation in the pig trigone and urethra. It is not known if NO is released from nerves directly, or whether an unknown nerve-released transmitter causes the generation of NO. However, NO synthase has recently been localised to peripheral neurones (Bredt *et al.*, 1990) and to NANC-nerves

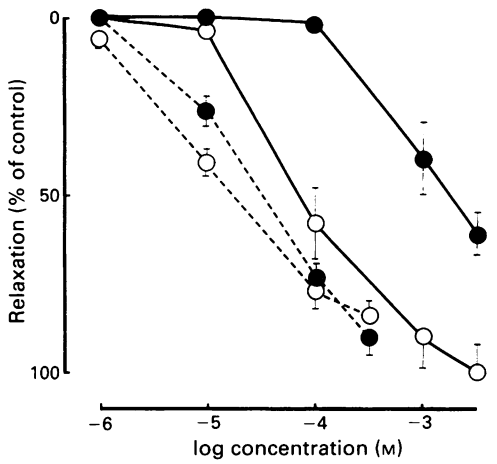


Figure 10 Concentration-response curves obtained by addition of nitric oxide (NO, present in acidified solution of $NaNO_2$) (continuous line) and 3-morpholino-sydnonimin (SIN-1, dashed line) to pig isolated detrusor preparations contracted by carbachol (5×10^{-7}–10^{-6} M) in the absence (○) or presence (●) of methylene blue (10^{-5} M). Each point is expressed as percentage of the maximal response before treatment, and represents mean ($n = 5$) with s.e.mean shown by vertical lines.

(Mitchell *et al.*, 1991) suggesting that NO synthesis by nerves is possible. The fact that exogenously applied NO mimics the response to nerve stimulation gives further support to the view that NO is involved. Thus, the present study extends the previous proposal (Garcia-Pascual *et al.*, 1991; Andersson *et al.*, 1991; 1992) that the L-ARG/NO pathway may have a role in regulation of urethral smooth muscle relaxation, and also suggests a similar mechanism for trigonal relaxation.

NANC-relaxation involving the L-ARG/NO pathway has now been recognized in e.g. the gastrointestinal tract (Bult *et al.*, 1990; Li & Rand, 1990; Boeckxstaens *et al.*, 1991), the oesophageal sphincter (Tøttrup *et al.*, 1991; De Man *et al.*, 1991), tracheal smooth muscle (Tucker *et al.*, 1990; Li & Rand, 1991), the anococcygeus muscle (Gillespie *et al.*, 1989; Hobbs & Gibson, 1990) and in the corpus cavernosum (Ignarro *et al.*, 1990; Holmquist *et al.*, 1991; 1992). Thus, it seems that the L-ARG/NO pathway represents a common pathway mediating NANC-relaxation in a number of tissues.

In this study, the presence of spontaneous contractile activity was clearly dependent on the region from where the strips had been taken. This is in agreement with the results of Sibley (1984), who also found that spontaneous activity in the pig bladder was regularly present in the trigone region, but sparse in the dome. The spontaneous activity in detrusor, trigone and urethral preparations was not affected by TTX, atropine or phentolamine, but depressed by SIN-1. This suggests that the spontaneous activity is of myogenic origin, and that it can be modulated by drugs believed to act as NO-donors.

In trigonal strips, L-NOARG did not affect basal tension. However, when the preparations were contracted, addition of L-NOARG occasionally caused a further increase in tension. This is similar to findings in rabbit urethral preparations where it was found that L-NOARG caused a further increase of the NA-induced tension in about 50% of the strips (Andersson *et al.*, 1992). These results suggest that the L-ARG/NO pathway is not activated during resting conditions, but that NO can be released by contractile activation.

Methylene blue, an agent believed to inhibit the activation of soluble guanylate cyclase (Martin *et al.*, 1985), had only minor effects on the nerve-induced relaxation in the trigone. On the other hand, the relaxation induced by exogenously applied NO was reduced by methylene blue. This result suggests that the mediator of NANC-relaxation is not authentic NO, or alternatively, that the mechanisms for guanylate cyclase activation for exogenous NO and the NANC-mediator may be different. In fact, recent data (Myers *et al.*, 1990) has shown that EDRF is more likely to be a nitrosylated compound such as a nitrosothiol than authentic NO. Oxyhaemoglobin, which binds to NO in the extracellular space (Martin *et al.*, 1985), and pyrogallol, which generates superoxide anion (Moncada *et al.*, 1986), should both be expected to reduce the response to NO. In fact, inhibitory effects of oxyhaemoglobin and pyrogallol were found on relaxations induced by electrical stimulation of NANC-nerves and on relaxations induced by exogenous NO. This further strengthens the view that the NANC-transmitter closely resembles NO. The fact that the effects of SIN-1 were insensitive to methylene blue, pyrogallol, and oxyhaemoglobin may argue against liberation of NO as the predominant mechanism of SIN-1 action in the lower urinary tract. Alternatively, SIN-1 may liberate NO first after cellular metabolism, and in that case be insensitive to extracellular degradation by pyrogallol and oxyhaemoglobin.

Little is known of the mechanisms that keep the detrusor relaxed during urine storage. In the cat, Edvardsen (1968) found an increased sympathetic activity during bladder filling. However, the role of β-adrenoceptor-mediated relaxation during this phase has been questioned. In a previous study (Klarskov, 1987), a NANC-mediated, TTX-sensitive, relaxation in response to electrical stimulation was observed in the pig detrusor after pretreatment with several contractile antagonists. The relaxation was always preceded by a contraction, and was not dependent on the stimulation frequency used. Generally, the NANC-mediated relaxations in the urethra of several species have had a frequency maximum below 10–12 Hz (Klarskov *et al.*, 1983; Andersson *et al.*, 1983; 1991; 1992; Garcia-Pascual *et al.*, 1991). We had difficulties in demonstrating a relaxant effect in our detrusor preparations. Only when the preparations were contracted to a high tension and after pretreatment with α-β methylene ATP and atropine, was a small relaxant response found. The discrepancy in results may be due to differences in experimental approach.

A TTX-insensitive response to electrical stimulation in human detrusor strips, which could be blocked with L-NOARG, has recently been described (James *et al.*, 1991). The preparations were pretreated with atropine, and then contracted by K^+ (20 mM). The relaxation obtained had a maximum at a stimulation frequency of 10 Hz. The authors speculated that NO might be generated by the detrusor muscle itself, and that defects in this system may play a role in the pathophysiology of detrusor instability. We were not able to reproduce the results reported by James *et al.* (1991) in the pig detrusor. This may be due to differences in the experimental approach (we used 35 mM K^+ for contraction) or to species differences. However, the relaxant response we found in some detrusor preparations was abolished by L-NOARG, and a detrusor relaxation involving NO can therefore not be excluded. Considering the difficulties in evoking nerve-induced detrusor relaxations (e.g. blockade of muscarinic receptors was necessary), the physiological relevance of this response might be questioned. In addition, the relaxation of the pig detrusor showed inconsistent responses to TTX, and therefore no conclusion can be drawn about the nature of the relaxation found. Although no endogenous source for NO could be confirmed in the detrusor in this study, it was found that both SIN-1 and exogenous NO had relaxant effects. The possibility that NO generated in other bladder structures (e.g., the urothelium) can exert relaxant effects on the detrusor muscle can therefore not be excluded. The maximal relaxant effect of SIN-1 and NO in the detrusor was, on the other hand, not as pronounced as in the trigone. However, the existence of a functionally important relaxation of detrusor muscle, mediated by the L-ARG/NO pathway, remains uncertain.

Thus, the results show that NANC-nerve mediated relaxation, involving the L-ARG/NO pathway, can be demon-

strated consistently in the trigonal and urethral, but not in the detrusor smooth muscle. The importance of this pathway for lower urinary tract physiology and pathophysiology remains to be established.

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Effects of a potassium channel opener (SDZ PCO 400) on guinea-pig and human pulmonary airways

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1 SDZ PCO 400 evoked dose-related relaxation of isolated airway smooth muscle. For human bronchus precontracted by endogenous tone or addition of carbachol (10^{-5} M), IC_{50} values were 1.74 μM and 1.82 μM respectively. With guinea-pig trachea contracted by endogenous tone, a comparable IC_{50} (1.79 μM) was observed, but no IC_{50} (< 100 μM) could be determined following contraction by carbachol (10^{-6} M).

2 Airway obstruction induced by intravenous bombesin in the anaesthetized ventilated guinea-pig was diminished by intravenous injection of SDZ PCO 400 (ID_{50} 54 μg kg^{-1}) or by introduction into the duodenum (ID_{50} 1.0 mg kg^{-1}). Inhalation of nebulized SDZ PCO 400 (0.1 mg kg^{-1}) diminished airway obstruction due to intravenous injection of histamine (3.2–5.6 μg kg^{-1}) for up to 20 min.

3 Increased bronchoconstrictor responses to bombesin (180–240 ng kg^{-1}) following intravenous infusion of platelet activating factor (PAF) or (±)-isoprenaline, or to histamine (1.0–3.2 μg kg^{-1}) following intravenous injections of immune complexes, were suppressed following concomitant intravenous infusion of SDZ PCO 400 (ID_{50} 0.3 mg kg^{-1} h^{-1}, 1.0 mg kg^{-1} h^{-1} and 0.1 mg kg^{-1} h^{-1} respectively).

4 Intravenous injection of SDZ PCO 400 (0.1 mg kg^{-1}) effected transient (< 10 min) inhibition of histamine-induced bronchospasm, yet diminished, for prolonged periods [up to 40 min] the enhanced bronchoconstrictor responses to histamine that followed intravenous injections of immune complexes. The capacity of SDZ PCO 400 to resolve such established airway hyperreactivity was prevented by prior intraduodenal instillation of a potassium channel antagonist, glibenclamide (30 mg kg^{-1}).

5 In sensitized guinea-pigs, SDZ PCO 400 inhaled as a dry powder (5.7 mg kg^{-1}) suppressed development of allergic airway hyperreactivity to histamine (1.8–3.2 μg kg^{-1}, i.v.), but failed to diminish accumulation of eosinophils or other inflammatory cells within the airway lumen 24 h after inhalation of ovalbumin.

6 Preincubation (30 min) of isolated sensitized trachea of guinea-pig with SDZ PCO 400 (10^{-5}–10^{-4}M) did not influence contractile responses to ovalbumin. However in anaesthetized sensitized guinea-pigs, insufflation of SDZ PCO 400 (1.25 mg) as a powder substantially diminished airway obstruction that followed inhalation of ovalbumin. This effect was prevented by prior vagal section.

7 It is concluded that SDZ PCO 400 reduces airway obstruction not only through direct actions on airway smooth muscle but also by impairing the expression of airway hyperreactivity, without directly influencing inflammatory events in the airways.

Keywords: SDZ PCO 400; potassium channel opener; guinea-pig airways; human airways; hyperreactivity; eosinophils; antigen; vagus nerve; asthma

Introduction

SDZ PCO 400 ((−)-(3S,4R)-3,4-dihydro-3-hydroxy-2,2 dimethyl-4- (3-oxo-cyclopent-1-enyloxy)-2H-1-benzopyran- 6-carbonitrile) opens channels in the sarcolemma of smooth muscle that are selective for potassium ions (Quast *et al.*, 1990). Drugs which open potassium channels may thereby relax smooth muscle, including airway smooth muscle, raising the prospect of potential anti-asthma efficacy. In this context, cromakalim has been shown to diminish histamine-induced airway obstruction in normal subjects (Baird *et al.*, 1988) and to provide symptomatic relief in nocturnal asthma (Owen *et al.*, 1990). In order to ascertain which pharmacological properties of SDZ PCO 400 might contribute to potential anti-asthma efficacy, we have examined the relaxant effects of this compound on isolated airway smooth muscle of guinea-pig and man as well as determining the capacity of SDZ PCO 400 to prevent or reverse established airways obstruction in the anaesthetized guinea-pig. In addition, we have investigated the effects of SDZ PCO 400 upon both the development and expression of airway hyperreactivity and determined whether altered kinetics of inflammatory cell migration might account for inhibition. A preliminary account of part of this work has been presented to the British Pharmacological Society (Chapman *et al.*, 1991a) and at an IUPHAR satellite meeting (Chapman *et al.*, 1991b).

Methods

Animals

Male guinea-pigs (Dunkin-Hartey circa 350–550 g) were used throughout. Food and water were available *ad libitum*.

Sensitization to ovalbumin

Guinea-pigs (350–400 g) were pretreated with cyclophosphamide (100 mg kg^{-1}, i.p.); 24 h later, ovalbumin (10 μg ml^{-1}) was added to aluminium hydroxide (10 mg ml^{-1}) and,

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after mixing with a magnetic stirrer (1 h, room temp), injected intraperitoneally (1.0 ml) coincidentally with *Bordetella pertussis* vaccine (0.25 ml). Injection of ovalbumin, together with adjuvants, was repeated at 21 and 42 days (Andersson, 1980). Hypersensitivity was detected by intracutaneous injection of ovalbumin (1 μg in 0.1 ml) and only animals with positive (> 10 mm diameter) skin reactions were used for experimental studies.

Antiserum to bovine gamma globulin

A solution of bovine gamma globulin in normal saline (1.0 mg ml^{-1}) and an equal volume of Freund's complete adjuvant were homogenized for 3 min in a high speed blender to form an oil-in-water emulsion. This mixture was injected intraperitoneally (1.0 ml per animal) and the procedure repeated two weeks later. After a further week, guinea-pigs were anaesthetized with ether and bled by cardiac puncture. Clotted blood was stored overnight at 4°C; after clot retraction, serum was separated for storage (− 20°C) as aliquots (1 ml).

Relaxation of airway smooth muscle in vitro

Guinea-pigs (350–500 g) were killed by an intraperitoneal injection of sodium pentobarbitone (100 mg kg^{-1}). A section of trachea (2–3 cm) was excised and transected at alternate cartilage plates to give rings of tissue 3–5 mm in depth. The proximal and distal rings were discarded. Individual rings of tissue were mounted on stainless steel supports in a tissue bath (10 ml) under a load of 1 g. Tissue was bathed in Krebs solution at 37°C, aerated with a mixture of oxygen and carbon dioxide (95:5 v/v) and isometric tension (Strain gauge UC3, Gould and Statham, USA) was recorded on a chart recorder (W. & W. Electronic Ltd., Switzerland). SDZ PCO 400 was dissolved in ethanol (100 ml) and polyethylenegylcol (3 ml) and further diluted in saline (0.9%) such that the final concentration of solvent in the organ bath did not exceed 1% at the highest concentration of SDZ PCO 400 used. This concentration of solvent was without effect upon either endogenous tone or tissue precontracted with carbachol (data not shown). Increasing quantities of SDZ PCO 400 were added at intervals (10 min) to define a cumulative concentration-effect curve upon tissue contracted either by endogenous tone (tension generated = 381 ± 50 mg, $n = 10$) or by addition of a spasmogen (carbachol 10^{-6} M; EC_{80}, tension generated = 1088 ± 137 mg, $n = 10$). Tissue from sensitized animals was contracted by addition of ovalbumin (100 μg ml^{-1}) with, or without, prior (30 min) incubation with SDZ PCO 400 (10^{-4} or 10^{-5} M).

Macroscopically normal rings of human bronchus (circa 5 mm diameter), dissected from lung that had been resected for carcinoma, were used immediately, or were immersed in foetal calf serum containing DMSO (1.8 M), frozen (− 70°C) and stored in liquid nitrogen (− 190°C) (Mueller-Schweinitzer, 1988). Freshly collected, or thawed (30 min room temperature, 3 min at 37°C), bronchial rings were mounted in a tissue bath as described for guinea-pig trachea. Such preparations produced tone spontaneously (tension generated = 778 ± 184 mg, $n = 5$) or were precontracted with carbachol (10^{-5}M; EC_{80}, tension generated = 702 ± 78 mg, $n = 10$).

Measurement of lung function

Guinea-pigs (circa 500 g) were anaesthetized by intraperitoneal injection of sodium phenobarbitone (100 mg kg^{-1}) supplemented with sodium pentobarbitone (30 mg kg^{-1}) and were then paralysed by intramuscular injection of gallamine (10 mg kg^{-1}). Animals, maintained at 37°C with a heated pad controlled by a rectal thermistor, were ventilated (8 ml kg^{-1}, 1 Hz) via a tracheal cannula with a mixture of air and oxygen (50:50, v/v). Ventilation was monitored at the trachea by a pneumotachograph (Fleisch 0000, Zabona, Switzerland) connected to a differential pressure transducer (MP 4514871, Validyne, USA). Coincident pressure changes within the thorax were measured via an intrathoracic cannula, using a differential pressure transducer (MP 4524, Validyne, USA); blood pressure and heart rate were recorded from the carotid artery with a pressure transducer (P23Dd, Gould, USA).

From measurements of air-flow and transpulmonary pressure, airway resistance (R_L, $cmH_2O\,l^{-1}\,s^{-1}$) was calculated after each respiratory cycle by use of a digital electronic pulmonary monitoring system (PMS, Mumed, London, U.K.) which displayed blood pressure, intrathoracic pressure and airflow and computed R_L in real time for display on a visual display unit (model AT3, IBM, USA); experimental data were stored electronically and experimental traces or processed data were plotted on a laser printer (LaserJet Series II, Hewlett Packard, USA) as required.

Bronchodilator action

In the anaesthetized ventilated guinea-pig intravenous injection of bombesin (300–600 ng kg^{-1}) caused sustained bronchospasm. When the response was maximal (circa 2 min), SDZ PCO 400 was injected into the jugular vein. Airway resistance was measured at 3 min and reduction has been expressed as a percentile of maximal R_L observed following injection of bombesin.

During an intravenous infusion of bombesin (50–100 ng $kg^{-1}\,min^{-1}$) to induce sustained airway obstruction, SDZ PCO 400 was introduced into the gastric end of the transected duodenum via a 12 gauge metal needle fixed by a purse string ligature. Bronchodilator responses were estimated as the reduction of maximal R_L and expressed as a percentile of maximal R_L prior to introduction of SDZ PCO 400.

SDZ PCO 400 (1 mg ml^{-1}) or vehicle were nebulized for 10 min in a De Villbis nebulizer, (Hospitak, USA) in-line with the inspiratory flow so that aerosols were introduced directly into the trachea of anaesthetized, ventilated animals. Sensitivity of the airways to intravenous injection of histamine (3.2–5.6 μg kg^{-1}) was determined at regular (10 min) intervals and reduction of responses (maximal R_L) have been expressed as a percentile of the pretreatment response to histamine.

Allergic bronchospasm

Either saline or SDZ PCO 400 (1.25 mg powder), were insufflated directly into the trachea of anaesthetized sensitized guinea-pigs (Schiantarelli *et al.*, 1982), with or without prior bilateral vagal section, 15 min prior to inhalation of ovalbumin in saline (1.0 mg ml^{-1} for 5 min) from a De Villbis nebulizer.

Airway hyperreactivity

Intravenous injections of bombesin (180–240 ng kg^{-1}) were used to establish sensitivity of the airways in anaesthetized ventilated guinea-pigs; 20 min after intravenous infusion of PAF (600 ng $kg^{-1}\,h^{-1}$ administered in a graded fashion: 30 ng kg^{-1} for 10 min, followed by 120 ng kg^{-1} for 20 min, followed by 450 ng kg^{-1} for 30 min) or (±)-isoprenaline as free base (100 μg $kg^{-1}\,h^{-1}$), sensitivity to bombesin was redefined. Airway hyperreactivity has been expressed as the paired difference between the maximal value for R_L to bombesin before and after exposure to PAF. Alternatively, an allergic reaction was initiated by intravenous injections of preformed immune complexes (prepared by adding 30 μg of bovine gamma globulin in 0.05 ml of saline to 0.05 ml of guinea-pig anti-bovine gamma globulin anti-serum, with a titre of at least 1 in 1000) at regular (10 min) intervals for 50 min. Intravenous injections of histamine (1.0–3.2 μg kg^{-1} at 10 min intervals) were used to define the sensitivity of the

airways prior to, and following exposure to immune complexes. Airway hyperreactivity has been expressed as the paired difference for the maximal value of R_L in response to histamine before, and after, repeated injection of immune complexes. SDZ PCO 400 was administered as an intravenous infusion (0.1–1.0 $\mu g\ kg^{-1}$) prior to (15 min) and concomitantly with either PAF, (±)-isoprenaline or immune complex injections. In other experiments, SDZ PCO 400 (0.1 $\mu g\ kg^{-1}$) was injected intravenously immediately following induction of airway hyperreactivity to histamine by successive intravenous injections of immune complexes.

Glibenclamide (30 $mg\ kg^{-1}$, bolus) was injected into the duodenum before (10 min) injection of SDZ PCO 400 in normal and hyperreactive animals or in normal animals during intravenous injection of histamine.

In actively sensitized guinea-pigs, SDZ PCO 400 was administered either as a dry powder aerosol by nose only inhalation (RCC Geneva, Switzerland) for 60 min (estimated doses 5.7 $mg\ kg^{-1}$) in conscious animals. Animals were prepared for measurement of pulmonary function 24 h later and sensitivity to histamine (1.8–3.2 $\mu g\ kg^{-1}$, i.v.) was determined.

Eosinophilia

Sensitized guinea-pigs were exposed for 1 h to an aerosol of ovalbumin (10 ml, 0.1% w/v) within 1 h of exposure to an aerosol of SDZ PCO 400 (estimated dose 5.7 $mg\ kg^{-1}$).

Animals were killed with pentobarbitone (100 $mg\ kg^{-1}$, i.p.) and the lungs washed with six aliquots (10 ml) of HBSS (total recovery 57.6 ± 0.4 ml, $n = 10$). Cell suspensions were concentrated by centrifugation (200 *g* for 10 min) and resuspended in HBSS (1 ml). Total cell counts were made in a haemocytometer. Differential cell counts were made from smears fixed in methanol (100%) and stained with Leishman's stain in order to differentiate cell types. A total of at least 500 cells per smear were counted at 1000 fold magnification.

Materials

The following were used: concentrated (10x) Hank's balanced salt solution (HBSS) and phosphate buffered saline (PBS), (Gibco, U.K.), bovine serum albumin (BSA), histamine dichloride, ovalbumin (Fluka, Switzerland), sodium pentobarbitone, sodium phenobarbitone (Siegfried, Switzerland), bombesin (Bachem, Switzerland), bovine gamma globulin (Miles Scientific, USA), gallamine (Davis and Geck, USA), Leishmann's stain, Turk's solution, ethanol, aluminium hydroxide, dimethylsulphoxide (DMSO), (Merck, Germany), cyclophosphamide, (±)-isoprenaline sulphate, carbachol (Sigma, USA), *Bordetella pertussis* vaccine (Swiss serum institute, Switzerland), Freund's complete adjuvant, (Difco, USA), platelet-activating factor (PAF) (Noviobiochem, Switzerland), SDZ PCO 400 (Sandoz, Switzerland).

Krebs solution (composition mM: $NaHCO_3$ 25.0, NaCl 113, KCl 4.7, $MgSO_4 7H_2O$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, glucose 11.7) was prepared freshly from analytical grade reagents. PAF was dissolved in absolute ethanol and stored at − 18°C as a stock solution (1 $mg\ ml^{-1}$). Dilutions of PAF in saline 0.9% containing BSA 0.25% were prepared immediately prior to use.

Statistical comparison

Data are presented as means ± s.e.mean and Student's *t* test was used for comparison between treatments. Estimates of IC_{50} or ID_{50} together with confidence limits were computed by Sigfit (Sandoz Pharma Ltd., Switzerland).

Results

Relaxation of isolated airway smooth muscle

SDZ PCO 400 relaxed guinea-pig isolated trachea, when tension had developed spontaneously, with an IC_{50} value of 1.79 (1.66–2.88) μM ($n = 10$). When tracheal tone was induced by addition of carbachol (10^{-6}M), SDZ PCO 400 was not an effective relaxant ($IC_{50} > 100\ \mu M$, $n = 10$). SDZ PCO 400 relaxed human isolated bronchus, whether tension was generated spontaneously, or by addition of carbachol (10^{-5}M) with IC_{50} values of 1.74 (1.45–2.08) ($n = 5$) and 1.82 (1.3–2.55) μM, respectively ($n = 10$).

Effect upon airway obstruction in vivo

In ventilated anaesthetized guinea-pigs, acute intravenous injection of SDZ PCO 400 (0.01–1.0 $mg\ kg^{-1}$) caused dose-related (ID_{50} 54 $\mu g\ kg^{-1}$, $n = 5$ per dose) reduction of the airway obstruction which resulted from intravenous injection of bombesin (Figure 1). Introduction of SDZ PCO 400 (1.0—3.2 $mg\ kg^{-1}$) into the duodenum of anaesthetized ventilated guinea-pigs also effected a dose-related reduction of bombesin-induced bronchospasm with an ID_{50} value of 1.0 $mg\ kg^{-1}$ ($n = 5$ per dose), 30 min after administration (Figure 1). Prior (10 min) inhalation of SDZ PCO 400 (1.0 $mg\ ml^{-1}$) as an aerosol (estimated dose 0.1 $mg\ kg^{-1}$) impaired the development of airway obstruction in response to intravenous injection of histamine (3.2–5.6 $\mu g\ kg^{-1}$) (Figure 1). Before inhalation of SDZ PCO 400, R_L ($cmH_2O\ l^{-1}\ s^{-1}$) following intravenous injection of histamine was 800 ± 17; immediately after inhalation of SDZ PCO 400, maximal R_L achieved was 61 ± 11 and recovered progressively (R_L 85 ± 14 at 10 min and 600 ± 32 at 20 min, $n = 10$) (Figure 1).

Inhibition of airway hyperreactivity

Intravenous injection of SDZ PCO 400 (0.1–1.0 $mg\ kg^{-1}$) prior to and during intravenous infusion of PAF, (±)-isoprenaline or injections immune complexes caused dose-related suppression of airway hyperreactivity, with ID_{50} values of 0.3 $mg\ kg^{-1}$, 1.0 $mg\ kg^{-1}$ and 0.1 $kg\ kg^{-1}$ respectively ($n = 10$ per dose) (Figure 2). In addition, SDZ PCO 400 (0.1 $mg\ kg^{-1}$) was administered after induction of airway hyperreactivity by successive injections of immune complexes ($n = 10$). SDZ PCO 400 strongly suppressed the enhanced airway obstruction to histamine induced by immune complexes, even though SDZ PCO 400 produced only modest ($< R_L$ before, 310 ± 58, $n = 10$; R_L after 191 ± 86, $n = 10$; $P = 0.05$), transient (< 10 min) inhibition of responses to intravenous histamine in normal animals at this dosage (Table 1). Significant inhibition (R_L before, 654 ± 94, $n = 10$; R_L after 218 ± 62, $n = 5$; $P = <0.05$) of responses to histamine in hyperreactive guinea-pigs was evident 40 min after injection of SDZ PCO 400. The capacity of SDZ PCO 400 (0.1 mg kg^{-1}) to suppress responses to intravenous injection of histamine in hyperreactive animals was prevented by prior (10 min) intraduodenal injection of glibenclamide (30 mg kg^{-1}, $n = 10$) (Figure 3). No effect of glibenclamide on responses to intravenous histamine (3.2 $\mu g\ kg^{-1}$) in hyperreactive animals was observed.

Effect upon eosinophil accumulation

Sensitized guinea-pigs exposed to ovalbumin by inhalation develop a marked inflammatory cell accumulation in the airway lumen 24 h later, that is accompanied by increased airway reactivity to histamine and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Sanjar *et al.*, 1990b). Inhalation of powdered SDZ PCO 400 (5.7 $mg\ kg^{-1}$) before inhalation of ovalbumin (0.1% w/v) precluded the expression of airway hyperreactivity to his-

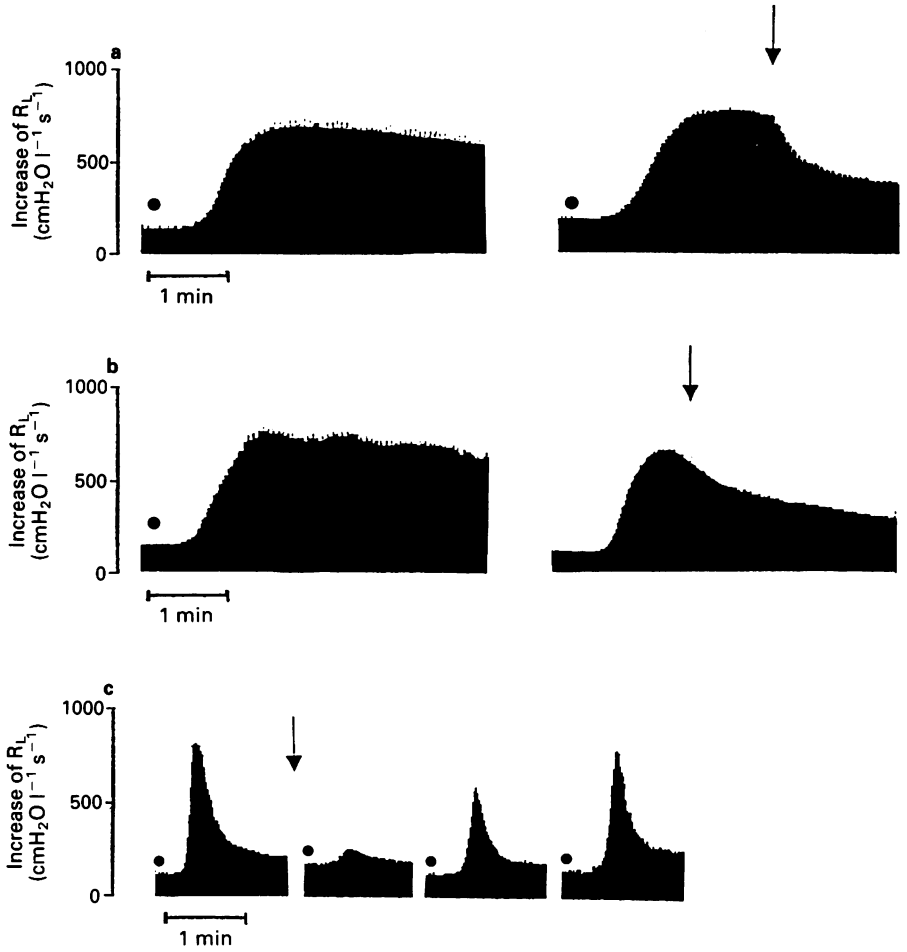


Figure 1 Reduction of airway resistance by SDZ PCO 400 (arrows) following: (a) intravenous injection (0.05 μg kg^{-1}); (b) intraduodenal injection (1.0 mg kg^{-1}) and (c) inhalation of an aerosol (1.0 mg ml^{-1}) in anaesthetized ventilated guinea-pigs in which airway obstruction had been induced by intravenous injection (a) (●), or intravenous infusion of bombesin (200–500 ng kg^{-1}) (b) (●), or histamine (3.2 μg kg^{-1}, ●) at 10 min intervals (c).

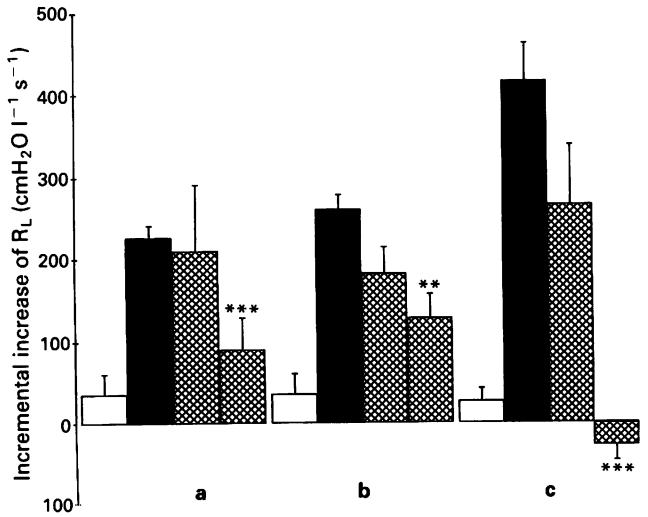


Figure 2 Incremental increased R_L (mean with s.e.mean shown by vertical bars, $n = 10$) following intravenous injection of bombesin (300 ng kg^{-1}, a and b) or histamine (1.8 μg kg^{-1}, c) in animals which have received intravenous infusion of saline (open columns), PAF (a), (±)-isoprenaline (b), and immune complexes (c) (solid columns) and during infusion of SDZ PCO 400 (0.1 and 1.0 mg kg^{-1} h^{-1}) (cross-hatched columns) (**$P = < 0.02$, ***$P = < 0.001$).

tamine, yet no diminution of inflammatory cell accumulation in the airway lumen was observed ($n = 10$) (Figure 4).

Effects upon acute allergic reactions

The acute increased airway resistance that follows inhalation of an aerosol of ovalbumin (1.0 mg ml^{-1} for 5 min) was diminished markedly when powdered SDZ PCO 400 (1.25 mg) was insufflated into the trachea of sensitized anaesthetized animals before (15 min) inhalation of ovalbumin ($n = 10$) (Figure 5). This inhibitory effect of inhaled SDZ PCO 400 was not evident when bilateral vagal section, a procedure which was without detectable effect upon basal airway resistance or responses to inhaled allergen, preceded inhalation of ovalbumin ($n = 10$) (Figure 5). No inhibition of the contractile response was detected when tracheal rings from sensitized animals were exposed to ovalbumin *in vitro*, even in the presence of high concentrations of SDZ PCO 400 (10^{-4}M). Thus, increased tension following addition of ovalbumin in the absence of SDZ PCO 400 (0.43 ± 0.08 g, $n = 10$) was not significantly different from the tension generated in the presence of SDZ PCO 400 (10^{-4} M, 0.53 ± 0.14 g, $n = 10$).

Discussion

SDZ PCO 400 relaxed isolated airway smooth muscle of man and guinea-pig and was bronchodilator *in vivo* in the guinea-pig. In these respects, SDZ PCO 400 resembled cromakalim, which has comparable potency in such preparations (Arch *et*

Table 1 Effect of SDZ PCO 400 upon responses to histamine in normoreactive and hyperreactive guinea-pigs

	Normoreactive				*Hyperreactive*			
Time	0	10	20	30	0	10	20	30
Vehicle	325	309	455	527	675	611	575	547
	(57)	(51)	(63)	(55)	(62)	(52)	(54)	(50)
SDZ PCO 400	310	191	343	526	654	208***	289***	263*
	(58)	(26)	(45)	(37)	(94)	(84)	(92)	(95)

Responses (R_L $cmH_2O\,l^{-1}\,s^{-1}$ ± s.e.mean) to intravenous injections of histamine (3.2 $\mu g\,kg^{-1}$) in guinea-pigs are given immediately before (0) as well as 10, 20, and 30 min after intravenous injection of SDZ PCO 400 (0.1 mg kg^{-1}) or vehicle (0.2 ml) (mean ± s.e.mean in parentheses). Normoreactive animals (vehicle $n = 5$, SDZ PCO 400 $n = 10$) did not receive injections of immune complexes and exhibited responses to histamine that are representative for this strain of guinea-pigs. Hyperreactive animals (vehicle $n = 20$, SDZ PCO 400 $n = 10$) had received three intravenous injections of immune complexes at 10 min intervals prior to histamine exposure. Student's t test was used to assess statistical significance of differences between responses in animals treated with vehicle or SDZ PCO 400. (*$P = <0.05$; ***$P = <0.001$).

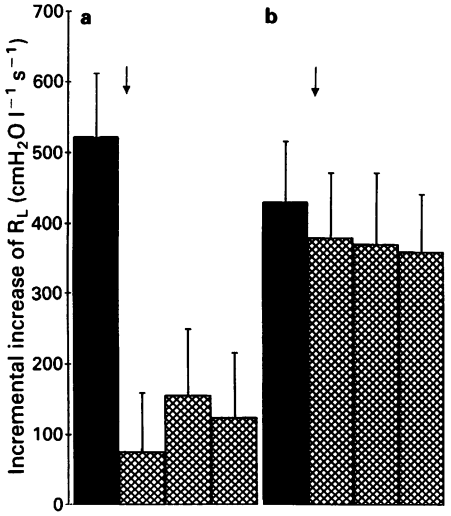


Figure 3 Following intravenous injections of immune complexes, incremental increase of R_L due to repeated (10 min intervals) intravenous injections of histamine (1.8 $\mu g\,kg^{-1}$) was inhibited by SDZ PCO 400 (0.1 $\mu g\,kg^{-1}$) (arrows) following injection of vehicle (ethanol) (a), but not following injection (b) of glibenclamide (30 mg kg^{-1} i.d. bolus, $n = 10$).

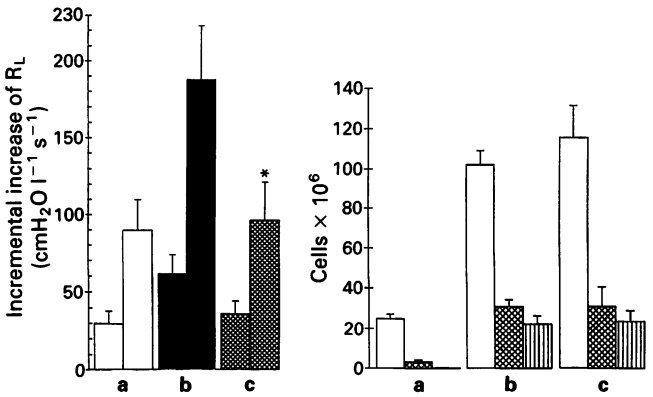


Figure 4 Left panel: incremental increase of R_L due to histamine (1.8 and 3.2 $\mu g\,kg^{-1}$, i.v.) in sensitized guinea-pigs not exposed to ovalbumin (a) and 24 h after inhalation of ovalbumin (0.1%), without (b), or with (c), inhalation of SDZ PCO 400 as a powder (estimated dose 5.7 mg/animal) 1 h before inhalation of ovalbumin. (*$P = <0.05$, $n = 10$ per group). Right panel: total cell numbers (open column), eosinophils (cross-hatched column) and neutrophils (vertically lined column) (mean with s.e.mean shown by vertical bars, $n = 10$ per group) in airway lavage fluid from sensitized animals not exposed to ovalbumin (a) and 24 h after exposure to ovalbumin, without (b), or with (c) inhalation of SDZ PCO 400 as a powder (estimated dose, 5.7 mg/animal), 1 h before inhalation of ovalbumin.

al., 1988; Chapman *et al.*, 1991b). The finding that spasmolytic efficacy of SDZ PCO 400 was independent of the particular contractile agencies used to increase tone of human bronchus is consistent with earlier reports for lemakalim (BRL 38227) (Black *et al.*, 1990). A reduced potency has been reported for RP 49356 when guinea-pig trachea was precontracted with carbachol (Brown *et al.*, 1989) and likewise SDZ PCO 400 was poorly effective when guinea-pig trachea was precontracted by carbachol. We obtained similar results when rabbit trachea and bronchus were used (unpublished data), so that the anomaly cannot be accounted for by differences in airway calibre. Since this difference extends to a phosphodiesterase (PDE) isoenzyme inhibitor, SDZ MKS 492 (Morley *et al.*, 1991) and to other spasmogens (Arch *et al.*, 1988), it may not be directly related to the opening of potassium channels.

In intact animals, SDZ PCO 400 reduced airway obstruction whether administered by the intravenous, oral or inhaled

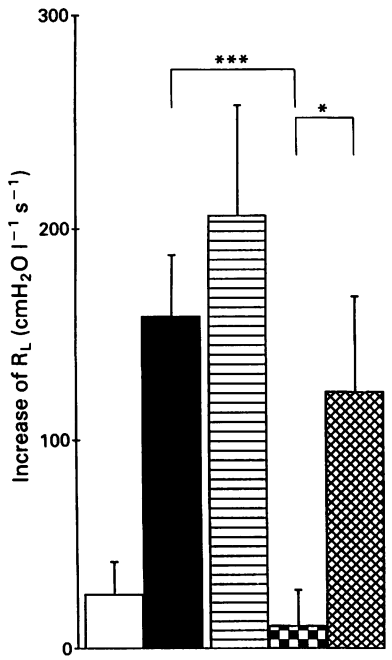


Figure 5 Increased R_L (mean with s.e.mean shown by vertical bars, $n = 10$) in response to inhalation of saline (open column) or an aerosol of ovalbumin (1.0 mg ml^{-1} for 5 min) in sensitized animals without (solid column) or with (horizontally lined column) before bilateral vagotomy and without (checkered column), or with (cross-hatched column) before bilateral vagotomy in animals that had previously (15 min) been administered SDZ PCO 400 powder (1.25 mg) by insufflation. Significant inhibition of the action of SDZ PCO 400 by comparison with the response to ovalbumin in untreated animals (***$P = <0.001$) and by comparison with the response in animals with intact vagi (*$P = 0.05$).

routes. Of particular interest was the observation that intravenous injection of SDZ PCO 400 (0.1 mg kg^{-1}) markedly inhibited the enhanced airway obstruction in response to intravenous injection of histamine in animals rendered hyperreactive by injections of immune complexes, even though this dose of SDZ PCO 400 produced insubstantial and transient bronchodilator responses in control animals. This effect of SDZ PCO 400 on hyperreactive airways was abolished by introduction into the duodenum of glibenclamide, implying that this property of SDZ PCO 400 was dependent upon the opening of ATP-sensitive potassium channels. The capacity of SDZ PCO 400 to influence established airway hyperreactivity may contribute to inhibition of the development of airway hyperreactivity due to immune complexes, PAF or (±)-isoprenaline.

Inhalation of SDZ PCO 400 pre-empted the acute airway obstruction which followed inhalation of ovalbumin. Inhibition was greater than might be anticipated from studies using spasmogens such as histamine or PAF in normal animals (data not shown), raising the possibility that SDZ PCO 400 might impair release or generation of allergic mediators from mast cells. However, preincubation of trachea from actively sensitized animals with high concentrations ($<10^{-4}$M) of SDZ PCO 400 did not inhibit the reaction to allergen. This discrepancy can be accounted for since SDZ PCO 400 failed to suppress allergic bronchospasm in bilaterally vagotomized, actively sensitized animals. This finding implies an effect of SDZ PCO 400 primarily on neural tissue rather than airway smooth muscle, a conclusion which accords with evidence that cromakalim influences airway smooth muscle more effectively when electrical stimuli are used for activation (Ichinose & Barnes, 1990); such effects were attributed to inhibition of excitatory NANC-induced responses a property also evidenced by SDZ PCO 400 (Small *et al.*, unpublished observations). Additionally, or alternatively, SDZ PCO 400 may protect against allergic bronchospasm by modifying neural interaction with mast cells (Leff *et al.*, 1986). The present experiments do not distinguish between these two possibilities. It has been reported that cromakalim, pinacidil and nicorandil do not diminish mediator release in allergic reactions (Nagai *et al.*, 1991), but it cannot be concluded that protective effects of SDZ PCO 400 during allergic reactions depend necessarily upon an interaction with nerves, since SDZ PCO 400 also inhibited airway hyperreactivity induced by infusion of PAF, an effect known to be independent of vagal integrity in this species (Mazzoni *et al.*, 1985).

Prophylactic anti-asthma drugs inhibit the acute change of airway responsiveness to injected or inhaled spasmogens which follows exposure to immune complexes (Boubekeur *et al.*, 1991) or PAF (Sanjar *et al.*, 1989). SDZ PCO 400 inhibited expression of airway hyperreactivity to an equal extent, whether given before or after exposure to immune complexes; hence, it was not possible to ascertain whether prevention contributes to the suppression of airway hyperreactivity observed. In the absence of evidence to the contrary, it may be presumed that the capacity of SDZ PCO 400 to reduce airway hyperreactivity depends upon resolution of airway hyperreactivity, without involvement of a preventative action. Prophylactic anti-asthma drugs impair the influx of eosinophils into the airways in response to inhaled allergen in sensitized animals (Sanjar *et al.*, 1990b), in response to injection of PAF (Sanjar *et al.*, 1990a) or recombinant cytokines (Kings *et al.*, 1991) in normal animals. In the present experiments, no effects of SDZ PCO 400 upon eosinophil accumulation in response to allergen in sensitized animals were observed, nor has an inhibitory effect on eosinophilia been previously described for other potassium channel openers. However, lemakalim (BRL 38227) has been reported to inhibit the peritoneal neutrophil infiltration that follows injection of antigen without affecting other cell types (Buckle & Taylor, 1991) a finding not supported by the present study in which numbers of neutrophils and eosinophils in lavage fluid were unaffected in animals treated with SDZ PCO 400. These observations distinguish SDZ PCO 400 from preventative anti-asthma drugs such as ketotifen which selectively suppresses allergic airway eosinophilia and which pre-empts, but does not suppress airway hyperreactivity in this species (Sanjar *et al.*, 1990b).

The present study shows that SDZ PCO 400 is a modest spasmolytic agent yet is able to reduce airway obstruction most effectively in hyperreactive animals. No mechanism has been identified to account for this property. No inhibition of cellular inflammation in the airways could be detected indicating that modification of airway hyperreactivity by SDZ PCO 400 is not secondary to an anti-inflammatory action. In this context, it should be noted that hyperreactivity due to isoprenaline, which is not associated with inflammatory events (Sanjar *et al.*, 1990c), was more resistant to inhibition, but nonetheless was inhibited effectively. Drugs, with such a profile have been termed anti-hyperreactive agents (Morley, 1991), and may provide an alternative to β_2-adrenoceptor selective sympathomimetics for maintenance symptomatic therapy in asthma. Since there is now evidence that β_2-selective sympathomimetics may not be appropriate for routine use in asthma (Sears *et al.*, 1991), emergence of an alternative pharmacological principle may be timely.

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Effects of acute and chronic electroconvulsive shock on noradrenaline release in the rat hippocampus and frontal cortex

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1 Changes in the extracellular content of endogenous noradrenaline (NA) in frontal cortex and hippocampus were determined by *in vivo* microdialysis following acute and chronic electroconvulsive shock (ECS) in rats anaesthetized with chloral hydrate.

2 Basal release of NA in the frontal cortex (4.9 ± 0.3 pg/sample) did not differ significantly from that in the hippocampus (4.6 ± 0.2 pg/sample).

3 A single ECS resulted in an increase of NA release in the hippocampus (21.1 ± 1.3 pg/sample) and in the frontal cortex (11.6 ± 1.2 pg/sample). In both brain regions extracellular NA had returned to basal values within 30 min.

4 Animals were treated chronically with ECS (once per day for seven days). Twenty-four h later (day 8), basal release of NA into dialysis samples from the frontal cortex was significantly increased (50%) as compared to chronic sham controls. Basal release in the hippocampus was not significantly different from the sham controls. In the chronic ECS animals the increase in NA released in both brain areas following an ECS on day 8 did not differ from either the chronic sham controls or from animals given acute ECS.

5 Animals were challenged 24 h after eight ECS or sham control treatments (once per day) with the α_2-adrenoceptor antagonist, idazoxan (10 mg kg^{-1}, s.c.). Idazoxan increased NA release in the hippocampus in both groups. There was no difference in the magnitude of the response in ECS- and in sham-treated rats. In the frontal cortex, idazoxan increased the extracellular NA content in the chronic sham controls, but the response to idazoxan was significantly attenuated in the chronic ECS animals.

6 Chronic but not acute ECS was found to elicit a sustained (>24 h) increase in the release of NA in the frontal cortex, but not in the hippocampus. The idazoxan data suggest that the increase may be due to a downregulation of presynaptic α_2-adrenoceptors in the frontal cortex. The difference in response of these two brain regions to chronic ECS is discussed in terms of differences in the regulation of extracellular NA content by uptake and autoreceptor activation.

Keywords: Electroconvulsive shock (ECS); noradrenaline; frontal cortex; hippocampus; transmitter release; microdialysis; acute ECS; chronic ECS

Introduction

Since Schildkraut (1965) first proposed that depression may result from an underactivity of cerebral noradrenaline-containing neurones, there have been numerous attempts to explain the effects of antidepressant treatments in terms of changes in cerebral noradrenaline (NA) function (see Sugrue, 1981; Green, 1990). Since antidepressant therapies work only after chronic treatment (Oswald *et al.*, 1972; Fink, 1979; Abrams, 1988), most animals studies have focused on the effects of repeated exposure to antidepressants. It is generally agreed that chronic antidepressant therapies produce consistent decreases in the density of β-adrenoceptors in specific brain regions (Kellar *et al.*, 1981; Stanford *et al.*, 1982). ECS has also been shown to decrease β-receptor number (Kellar *et al.*, 1981; Stanford *et al.*, 1982) and function (Przegalinski *et al.*, 1983). The effects of chronic ECS on α-adrenoceptors are less clearly defined, but point to a downregulation of α_2-receptors (Bergstrom *et al.*, 1979; Stanford *et al.*, 1982). Behavioural data are also consistent with downregulation (Heal *et al.*, 1981; Pilc *et al.*, 1984). The fact that ECS decreases both β- and α_2-adrenoceptor number and function suggests the possibility that brain NA processes are a common site of antidepressant action.

These changes in adrenoceptor number and sensitivity could result from increases in NA release which occur over time to affect the receptors. Yet studies of brain NA content or turnover have singularly failed to find any reliable alterations following chronic drug or ECS treatments (see Sugrue, 1981; Green, 1990). Experiments which measure the turnover and/or content of NA suffer in that these measures of NA neuronal activity are indirect and changes in transmitter release can only be inferred. However, the advent of *in vivo* microdialysis has made it possible to monitor directly changes in the extraneuronal content of NA following its release from nerve terminals in specific brain regions.

Two recent reports have used *in vivo* microdialysis to investigate the effects of acute and chronic ECS on the release of endogenous dopamine from the rat corpus striatum (Glue *et al.*, 1990; Zis *et al.*, 1991). Both groups found evidence for increased striatal dopamine release after chronic ECS. Glue *et al.* (1990) also reported that chronic ECS significantly elevated basal NA release in the frontal cortex; while significantly reducing it in the nucleus accumbens. Both groups suggested that enhanced neurotransmitter release could be due to a downregulation of the appropriate presynaptic autoreceptors for dopamine and NA in the specific brain regions.

We have previously shown that the regulation of the extracellular concentration of NA differs between rat frontal cortex and hippocampus (Thomas & Holman, 1991a,b). In frontal cortex, the primary regulation is by activation of the presynaptic α_2-adrenoceptors; while in hippocampus, NA content in the synaptic cleft is controlled by uptake into the nerve terminal. In the present study the effects of acute and chronic ECS on NA release from rat frontal cortex have been compared with that in the hippocampus. If the effects of ECS

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on NA release are dependent on changes in presynaptic autoreceptor mechanisms then we might expect to see a difference in the response of these two brain regions to chronic ECS. Additionally, to determine whether there are changes in presynaptic α_2-adrenoceptor function, the effects of the α_2-antagonist, idazoxan, on NA release were investigated in chronic ECS-treated animals and in chronic sham-treated controls.

Methods

Male Wistar rats (290–320 g) were anaesthetized with chloral hydrate (400 mg kg^{-1}; 100 mg ml^{-1}, i.p.) and supplemented (90 mg kg^{-1}) as required. Animals were placed in the stereotaxic frame and dialysis probes were implanted into either the frontal cortex (AP: +3.2 mm from bregma, L: −1.2 mm, H: −5.3 mm) or hippocampus (AP: −5.8 mm from bregma, LAT: −5 mm; H: −7 mm) using coordinates according to the atlas of Paxinos & Watson (1986). Body temperature was maintained at 37.5°C by use of a rectal probe and heating blanket. The position of each dialysis probe was verified histologially at the conclusion of each experiment. Concentric dialysis probes were constructed as described by Thomas & Holman (1991a). The dialysis probe membrane (4 mm) was polyacrylonitrile (M.W. cutoff 40,000 and supplied by Hospal Medical, New Jersey, U.S.A.).

Each dialysis probe was dialysed with a physiological medium containing (mM): NaCl 147, $CaCl_2$ 2.3, KCl 4.0 and HEPES 10, final pH 7.4 (Thomas & Holman, 1989) at a flow rate of 2.3 $\mu l\ min^{-1}$ and samples collected every 15 min. At least 1 h passed after implanting the probe before samples were collected. Once sampling commenced, no ECS was administered until basal release was stable and consistent for a minimum of 3 samples. We have previously shown that basal NA release in hippocampus and frontal cortex is neuronal in origin being Ca^{2+}-dependent and tetrodotoxin-sensitive (Thomas & Holman, 1991a). Once steady basal NA release was achieved, electrodes were attached to the rats ears and they received a single ECS of 5 s duration and 100 mA intensity (Constant Current Electroshock Unit, Applegarth Electronics, Oxford). Sham control animals were treated in the same way, however, no current was passed through the electrodes attached to their ears. An effective ECS was assessed by the rat undergoing a full tonic/clonic seizure. Dialysis sampling continued for a further 75 min.

In the chronic treatment experiments, separate groups of rats received either ECS (1 s; 100 mA) or sham (1 s; each clipping) treatment once daily for 7 days in the awake state via electrodes applied to their ears. Care was taken to support the rats during the tonic/clonic phase of the seizure in order to prevent injury. On day 8 these rats were dialysed as described for the acute ECS rats.

Two further groups of rats were also treated chronically with either ECS or sham ear clipping to determine whether any functional changes had occurred in the α_2-adrenoceptors. These were implanted with dialysis probes 24 h after the last treatment and challenged with the α_2-antagonist, idazoxan (10 mg kg^{-1}, s.c.).

Dialysis samples were analyzed for NA content by high performance liquid chromoatography (h.p.l.c.) with electrochemical detection. The mobile phase (sodium acetate 24 mM, citric acid 15 mM, EDTA 3 mM, octane sulphonic acid sodium salt monohydrate 800 μM and 14% (v/v) methanol, pH 5.0) was filtered and degassed before being pumped at a rate of 1 $ml\ min^{-1}$ through a Hichrom column (3 μM o.d., 15 cm × 4.6 mm). Electrochemical detection was performed with a Coulochem (ESA, Boston, MA, U.S.A.) with Det 1: +350 m and Det 2: −350 mV.

Data were analyzed by use of a one-way ANOVA and Dunnett's t test. In situations where the variance of the data could not be assumed to be equal a Kruskal-Wallis one-way analysis of variance with a multiple comparison procedure was used.

For the s.c. injection, idazoxan (Reckitt and Colman) was made up in 0.9% (w/v) saline, the concentration of the solutions was such that the rats received the equivalent of 1 mg kg^{-1}.

Results

Effects of acute electroconvulsive shock on noradrenaline release

In the hippocampus, mean (± s.e.mean) basal release of NA in the acute ECS- and in the sham-treated animals was 4.5 ± 0.2 pg/sample ($n = 5$) and 4.9 ± 0.3 pg/sample ($n = 5$) respectively (Figure 1a). There were no significant differences in basal release between the two groups. ECS administered at the end of the third basal sample resulted in a significant increase in the concentration of NA in the next sample (21.1 ± 1.3 pg/sample, $n = 5$) ($P < 0.01$). The increased release of NA returned to basal values within 30 min of the stimulus. Animals receiving sham treatment did not have a significant increase in the release of the NA as compared with baseline.

In the frontal cortex, mean basal release in the acute ECS- and in the sham-treated animals did not differ, being 4.1 ± 0.3 pg/sample ($n = 4$) and 4.5 ± 0.2 pg/sample ($n = 4$) respectively (Figure 1b). ECS produced a threefold increase in the concentration of NA in the next sample (11.6 ± 1.2 pg/sample, $n = 4$) which was significantly ($P < 0.01$) greater than basal release. As in the hippocampus, NA release in the ECS-treated animals returned to baseline values within 30 min of the stimulus. Sham treatment alone did not elicit a significant change in NA release.

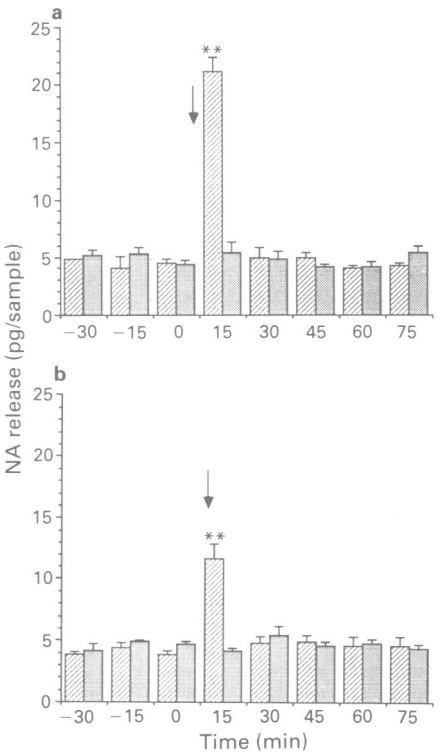


Figure 1 Effects of acute electroconvulsive shock (100 mA, 5 s; hatched columns) and acute sham treatment (5 s, stippled columns) on noradrenaline (NA) release in (a) hippocampus and (b) frontal cortex. Electroconvulsive shock or sham treatment administered at time zero as indicated by arrow. All values are means with s.e.mean shown by vertical bars ($n = 4–5$). Statistical analysis by ANOVA and Dunnett's t test. $^{**}P < 0.01$ versus respective basal release.

Effects of chronic electroconvulsive shock on noradrenaline release

Figure 2a shows that basal NA release in the hippocampus was not significantly different after chronic ECS (4.7 ± 0.4 pg/sample, $n = 6$) as compared with chronic sham treatment (4.3 ± 0.5 pg/sample, $n = 4$). In the chronic ECS group, a further ECS produced a significant fivefold increase in the release of NA above basal levels (20.4 ± 3.8 pg/sample, $n = 5$) ($P < 0.01$). However, this was not significantly different from the ECS-induced release of NA in animals given only a single shock.

In the frontal cortex (Figure 2b), basal release of NA after chronic ECS (6.9 ± 0.9 pg/sample, $n = 4$) was significantly ($P < 0.05$) elevated as compared with the chronic sham (4.6 ± 0.2 pg/sample, $n = 4$) animals. Chronic sham treatment did not alter NA release as compared to the acute sham treatment group. There was a significant ($P < 0.01$) increase in NA release above the basal concentrations immediately after the ECS on day 8 (12.7 ± 2.0 pg/sample, $n = 4$). In order to compare ECS-induced release in acutely treated animals (day 1) and day 8, the respective mean basal release for each experiment was subtracted from the 15 min sample following ECS. A comparison of the NA release in this 15 min sample from animals given either one shock or eight shocks showed there was no significant difference in the response to ECS (day 1: 7.6 ± 1.0 pg/sample, $n = 4$; day 8: 5.8 ± 1.0 pg/sample, $n = 4$).

Effects of chronic electroconvulsive shock on α_2-adrenoceptors function

Basal hippocampus NA release in groups of rats measured 24 h after either eight ECS treatments (3.2 ± 0.3 pg/sample, $n = 5$) or eight sham treatments (3.0 ± 0.2 pg/samples, $n = 5$) was not significantly different with respect to time or treatment group (Figure 3). After a challenge dose of idazoxan, NA release in both the chronic ECS and chronic sham-treated animals was increased, but there was no significant difference between the responses of the two groups. Furthermore, the time course and the magnitude of increased extracellular NA content in the hippocampus following idazoxan in these two groups was similar to that of naive animals challenged acutely with idazoxan (Thomas & Holman, 1991a).

In the frontal cortex (Figure 4), basal release of NA 24 h after the last ECS treatment (5.5 ± 0.1 pg/sample, $n = 5$) was again significantly ($P < 0.01$) elevated in comparison with chronic sham treatment (3.5 ± 0.2 pg/sample, $n = 5$). After a challenge dose of idazoxan to the chronic sham animals (Figure 4a), NA release increased ($P < 0.01$) in a similar manner to acutely challenged animals (Thomas & Holman, 1991b). However, following eight ECS treatments there was no significant increase in NA release in response to idazoxan administration (Figure 4b).

Discussion

We have previously shown that in this anaesthetized rat preparation, the content of endogenous NA present in micro-

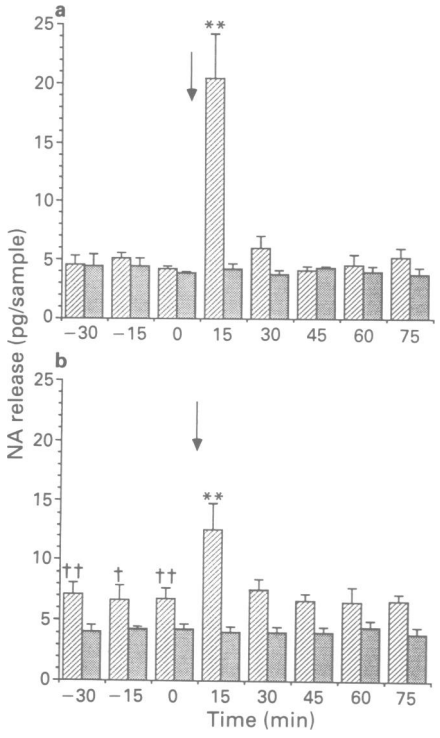


Figure 2 Effects of chronic electroconvulsive shock once daily for 7 days, 100 mA for 1 s (hatched columns) and chronic sham treatment once daily for 7 days, sham treatment for 1 s (stippled columns) on noradrenaline (NA) release in (a) hippocampus and (b) frontal cortex. Electroconvulsive shock or sham administered at time zero indicated by arrow on day 8. All values are means with s.e.mean shown by vertical bars ($n = 4$–6, hippocampus; $n = 4$, frontal cortex). Statistical analysis by two-way ANOVA with Tukey's multiple comparison test, ††$P < 0.01$ and †$P < 0.05$ for comparison of basal release after chronic ECS or sham treatment. **$P < 0.05$ comparing stimulated release with respective basal release by one-way ANOVA with Dunnett's t test.

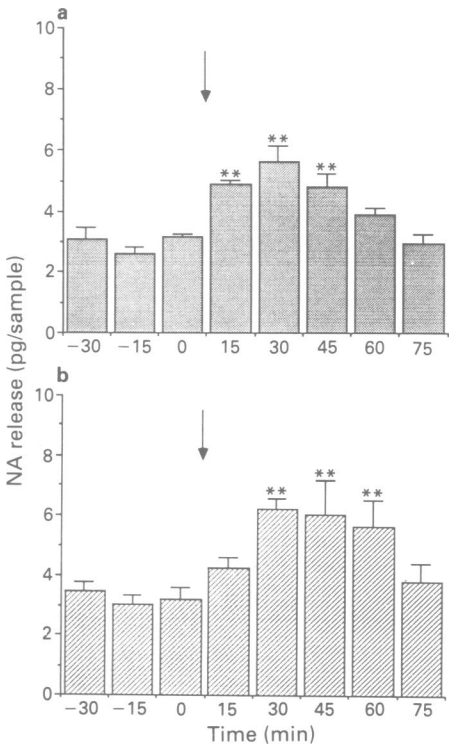


Figure 3 Effects of a challenge dose of diazoxan (10 mg kg^{-1}) on noradrenaline (NA) release in the rat hippocampus 24 h after the last of 8 days of chronic electroconvulsive shock or chronic sham treatment. (a) Chronic sham treatment and (b) chronic electroconvulsive shock treatment. Challenge dose of idazoxan administered at time zero as indicated by arrow. All values are means with s.e.mean shown by vertical bars ($n = 5$). Statistical analysis by two-way ANOVA showed no significant differences between the two treatment groups. One-way ANOVA with Dunnett's t test used for comparison of idazoxan challenge dose with respective basal values, **$P < 0.01$.

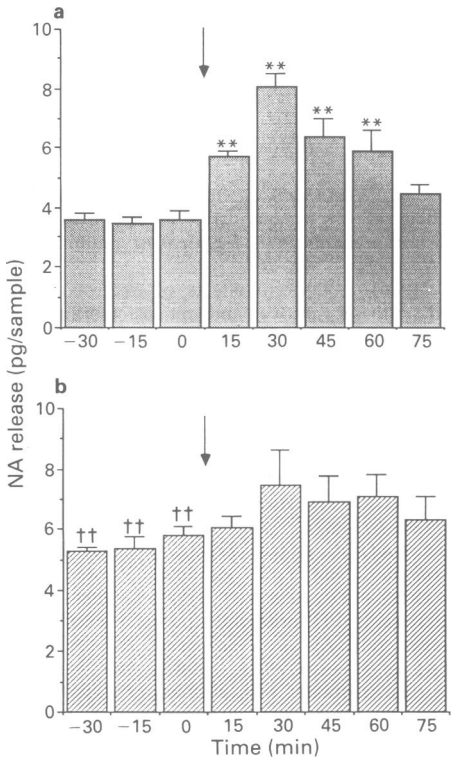


Figure 4 Effects of challenge dose of idazoxan (10 mg kg^{-1}) on noradrenaline (NA) release in the rat frontal cortex 24 h after the last of 8 days of chronic electroconvulsive shock or chronic sham treatment. (a) Chronic sham treatment and (b) chronic electroconvulsive shock treatment. Challenge dose of idazoxan administered at time zero as indicated by arrow. All values are means with s.e.mean indicated by vertical bars ($n = 5$). Statistical analysis by two-way ANOVA showed significant differences between the basal release of the two treatment groups, $\dagger\dagger P < 0.01$. One-way ANOVA with Dunnett's t test used for comparison of idazoxan challenge dose with respective basal values, $^{**}P < 0.01$.

dialysis samples from both the hippocampus and the frontal cortex is Ca^{2+}-dependent and tetrodotoxin-sensitive. Thus the NA appears to be released from neurones in these brain areas in response to normal nervous activity. Further, the basal concentrations of NA and the responses to idazoxan reported here are in agreement with our own and previous reports (L'Heureux *et al.*, 1986; Dennis *et al.*, 1987; Abercombie *et al.*, 1988; Thomas & Holman, 1991a,b).

Despite the similarity of baseline NA release, the NA responses to acute and chronic ECS in the frontal cortex and the hippocampus are very different. A single shock administered to animals that have had no pretreatment elicited short term increases in NA release in both brain regions. Glue *et al.* (1990) reported similar results in frontal cortex. Comparison of the magnitude of the NA release in the two regions shows that the response was greater in the hippocampus (fivefold over basal) versus the frontal cortex (threefold over basal). Since ECS will release transmitters by depolarizing nerve terminals, the difference in the amounts of NA released in the two regions may be affected by: (1) differences in the flow of current, (2) changes in neuronal activity caused by the seizures, (3) the greater tissue concentration of NA in the hippocampus versus the cortex (Holman *et al.*, 1976), or (4) the relative involvement of uptake and presynaptic inhibition in regulating extracellular NA levels. We have previously compared dose-response curves to uptake inhibition (desmethylimipramine, DMI, via the probe), α_2-adrenoceptor inhibition (idazoxan) and to IDX (1.0 mg kg^{-1}) in the presence of increasing concentrations of DMI (via the probe) on extracellular NA content in the hippocampus and in the frontal cortex (Thomas & Holman, 1991b). From these results we have concluded that the synaptic content of NA in the hippocampus is regulated primarily by neuronal uptake; whilst in the frontal cortex, NA activation of presynaptic α_2-adrenoceptors is more important. Thus the release of the transmitter in the frontal cortex would favour activation of the inhibitory feedback mechanism to reduce further release. Whereas in hippocampus released NA is normally taken up into the neurone before it can activate the presynaptic feedback mechanism.

A comparison of the response to the ECS stimulus in acute and chronic animals shows there were no differences in either brain region. This suggests that NA transmission does not adapt or become tolerant to the shocks over the course of the treatment. These results agree with a previous report on the frontal cortex (Glue *et al.*, 1990). Interestingly, increases in striatal dopamine release immediately after the last of a series of chronic ECS are attenuated as compared with a single ECS (Zis *et al.*, 1991). Taken together the data emphasise that different transmitters in different brain regions respond differently to chronic ECS.

The most striking result in the present study was the 50% increase in basal NA release in the frontal cortex following chronic ECS which contrasts with the lack of any change in the hippocampus. Glue *et al.* (1990) reported a comparable increase in NA release in frontal cortex. This change in release was only seen after multiple ECS treatment suggesting a similarity with the requirements for therapeutic effectiveness in man. In addition, the change was present for at least 24 h suggesting some sort of long term adaptation to the chronic treatment.

One explanation for the increase in NA after chronic ECS comes from studies in brain slices in which the synthesis and utilisation of NA has been shown to be increased (Kety, 1967; Modigh *et al.*, 1976). This effect also lasted 24 h beyond the last treatment. However, the result has not been replicated by Nimgaonkar *et al.* (1986). A more plausible explanation is suggested by our results with the idazoxan challenges. These data suggests that after chronic ECS the presynaptic α_2-adrenoceptors in the frontal cortex, but not the hippocampus, are downregulated. Thus the increased basal release of NA would be the result of decreased presynaptic negative feedback due to a downregulation or reduced density of α_2-adrenoceptors. This conclusion is in agreement with previous binding studies (Stanford *et al.*, 1982) and behavioural observations (Heal *et al.*, 1981; Pilc *et al.*, 1984).

Interestingly, chronic ECS has also been shown to increase NA uptake (Hendley *et al.*, 1974; Minchin *et al.*, 1983; Gleiter & Nutt, 1988) which might be expected to counteract the increase in release and the decrease in presynaptic inhibition. In frontal cortex, uptake has only a secondary role in regulating the extracellular content of NA (Thomas & Holman, 1991b) such that any increase may be masked by the changes in the α_2-adrenoceptors. On the other hand, in the hippocampus where uptake plays the major role in the control of extracellular NA, any increase in uptake would serve to prevent changes in presynaptic α_2-receptor function. Further, since the density of α_2-adrenoceptors is much less in the hippocampus than in the cortex (Hudson *et al.*, 1990), any downregulation of these receptors in the hippocampus would be obscured by neuronal uptake and thus would have less of an effect to increase NA release. The similarity of the response to idazoxan in both the chronic and acute ECS and sham groups in this study is consistent with no downregulation of these adrenoceptors in the hippocampus.

In conclusion, our results demonstrate that chronic but not acute ECS increases NA release in the frontal cortex but not in the hippocampus. The increase in basal NA release and the reduced idozaxan sensitivity in the frontal cortex suggests these effects are mediated by downregulation of presynaptic α_2-adrenoceptors. It is tempting to speculate that the increase

in basal NA release in the frontal cortex after chronic ECS may contribute both to the reported decrease in β-adrenoceptors found in this region and to the therapeutic efficacy of ECS. However, such a conclusion must await comparison with the neurochemical effects of other known antidepressant medications.

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Study of mechanisms of glucocorticoid hypertension in rats: endothelial related changes and their amelioration by dietary fish oils

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1 To investigate possible mechanisms of increased systolic blood pressure after 1 weeks treatment with dexamethasone and its amelioration by fish oil feeding, we have examined the reactivity of aortic rings and perfused mesenteric resistance vessels.

2 Thirty six Sprague-Dawley rats were initially divided into two groups and fed a semisynthetic diet containing either (10% by weight) hydrogenated coconut oil and safflower oil mixture (HCO/S) (24 rats) or fish oil (12 rats) for 5 weeks. From the end of the fourth week, dexamethasone (1.25 mg ml^{-1}) in drinking water, was given to half the rats on hydrogenated coconut oil (HCO/S + Dex) and to the fish oil-fed group (fish oil + Dex).

3 One week of dexamethasone treatment raised systolic blood pressure in the HCO/S + Dex rats but not in the fish oil + Dex group.

4 Endothelium-dependent relaxation to acetylcholine (ACh) was decreased in aortic rings taken from HCO/S + Dex rats compared to rats on HCO/S alone. Relaxant responses to ACh of aortic rings from rats given fish oil + Dex were intermediate between the three groups. Aortic endothelium-independent responses to sodium nitroprusside (SNP) were unchanged between the groups, while aortic contractile responses to noradrenaline were similar in all the groups.

5 In the perfused mesenteric resistance artery, sensitivity to noradrenaline was decreased in rats given fish oil and dexamethasone compared to the other two groups. There were no differences in resistance vessel relaxation to ACh or SNP between groups.

6 Serum corticosterone levels, used as a marker of dexamethasone absorption, were substantially suppressed in dexamethasone-treated rats but levels were higher in rats on fish oil than on HCO/S diets.

7 We suggest that the glucocorticoid-induced rise in systolic blood pressure may be due in part to decreased aortic compliance as a consequence of impaired endothelium-dependent relaxation and perhaps reduced nitric oxide synthesis. Fish oil feeding may ameliorate this rise in blood pressure through (i) changes in dexamethasone absorption, (ii) decrease in reactivity to noradrenaline of perfused mesenteric resistance arteries, (iii) an increase in endothelium-dependent relaxation to ACh or a combination of these three factors.

Keywords: Vascular reactivity; dexamethasone-induced hypertension; fish oil

Introduction

Administration of dexamethasone has a hypertensive effect on rats (Handa *et al.*, 1984; Codde & Beilin, 1985; Iijima & Malik, 1985; Tonolo *et al.*, 1988), the mechanism of which is unknown. It has been suggested that this effect may be caused by an increased sensitivity of resistance vessels to vasoconstrictor substances, but variable changes in vascular reactivity to noradrenaline have been reported. In the intact anaesthetized rat, dexamethasone increased pressor responses to noradrenaline (Handa *et al.*, 1984). In isolated perfused mesenteric vessel preparations, Iijima & Malik (1985) found no effect of dexamethasone on noradrenaline responses whereas Luscher & Vanhoutte (1986) reported enhanced reactivity in preparations from rats treated with lower doses of dexamethasone. As noradrenaline responses can be modulated by the endothelium (Cocks & Angus, 1983; Bullock *et al.*, 1986), it is possible that dexamethasone could affect release of vasoactive factors influencing noradrenaline contractile responses in resistant vessels or compliance of conduit arteries.

Endothelium-dependent relaxations to acetylcholine (ACh) have been shown to be impaired in aortae of spontaneously hypertensive rats (SHR) (Konishi & Su, 1983; Vanhoutte & Luscher, 1986), 1-clip, 2-kidney and DOCA-salt hypertensive rats (Van de Voorde & Leusen, 1986) compared to normotensive controls. In SHR, there is also a decrease in relaxations to ACh in isolated mesenteric resistance arterial segments (Watt & Thurston, 1989) with contractions at higher concentrations. Hence, it is plausible that the rise in blood pressure of dexamethasone-induced hypertensive rats may be caused by impaired endothelium-dependent relaxation.

Fish oils rich in eicosapentaenoic acid (EPA) have been claimed to prevent dexamethasone-induced hypertension (Codde & Beilin, 1985) and attenuate blood pressure rise in SHR (Schoene & Fiore, 1981; Yin *et al.*, 1990). The hypotensive effect of fish oil in SHR was associated with decreased constrictor response to noradrenaline, increased dilator responses to sodium nitroprusside (SNP) in perfused mesenteric resistance vessels and an increase in endothelium-dependent relaxation to ACh in aortic rings (Yin *et al.*, 1991).

To investigate the mechanism of dexamethasone-induced hypertension, we have examined the vascular reactivity of aortic rings and perfused mesenteric resistance vessels to vasodilator and vasoconstrictor agents. In addition we examined possible alterations in vascular reactivity in relation to the blood pressure lowering effect of fish oils in this model of hypertension.

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Some of this work was described in an abstract entitled 'Vascular reactivity and effects of fish oil feeding in glucocorticoid hypertensive rats' at the Fifth European Meeting on Hypertension in Milan, Italy, 1991.

Methods

Thirty six male Sprague-Dawley rats (232–370 g) were initially divided into 3 groups matched for blood pressure and body weight. One group was fed a semi-synthetic diet containing 10% (by weight) of 'Max EPA' fish oil while the other two groups were on a semisynthetic diet containing 9.7% hydrogenated coconut oil and 0.3% safflower oil (HCO/S). The major fatty acid composition of these oils was analysed by gas chromatography. 'Max EPA' fish oil contained C16:0 (16%), C18:0 (3%), C18:1 (12%), C20:1 (4%), C20:5 (18%), C22:6 (12%). Safflower oil contained 16:0 (8%), 18:0 (2%), 18:1 (13%), 18:2 (77%). The hydrogenated coconut oil was free of unsaturated fatty acids and safflower oil was added so that the 10% oil mixture contained 3% safflower oil to prevent essential fatty acid deficiency. The dry component of the feed consisted of by weight: casein (7.8%), macrominerals (2.2%), vitamins and trace minerals (0.83%), choline chloride (0.28%), cornflour (50%) and sucrose (16.7%). The feed was mixed in our laboratories before being baked into small biscuits at 120°C for 20 min. Rat feed was kept at 4°C and made up weekly. The rats were placed on this diet for 4 weeks. Systolic blood pressures were taken by tail-cuff sphygmomanometry on rats warmed to a temperature of 39°C for 15 min. Blood pressures were taken as an average of 3 readings before the start of the dietary period and at the end of 4 weeks. After 4 weeks, the rats on fish oil and half the rats on the HCO/S diet were then given dexamethasone (Dex) for 7 days (fish oil + Dex; HCO/S + Dex) while still maintained on their respective diets. The remaining rats received the HCO/S feed alone. The dexamethasone was administered by addition of dexamethasone phosphate to the drinking water (1.25 mg l^{-1}). After one week of dexamethasone administration, blood pressures were measured again.

Vascular reactivity

Isolated perfused mesenteric resistance vessels Rats were anaesthetized with nembutal (0.1 ml 100 g^{-1}) and 2 ml blood was removed for biochemical measurements. Approximately, half the blood was dispensed into a propylene tube for serum thromboxane B_2 (TxB_2), fatty acid and corticosterone analysis. The other half was decanted into a heparin-lined tube for plasma electrolyte analysis. The superior mesenteric artery was cannulated and perfused with Krebs solution at a flow rate of 4.2 ml min^{-1}. Composition of the Krebs solution was (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11.1. The intestine was then carefully cut away from the mesenteric vessels and the preparation mounted in a water jacketed organ bath maintained at 37°C. The methods for obtaining concentration-effect curves have been described elsewhere (Yin *et al.*, 1991) and are briefly described here. Krebs solution was pumped by a 3-channel peristaltic pump (Pharmacia P-3) through polyethylene tubing (SP70) connected to a debubbler (2 ml volume) and then to the cannula (SP40). Perfusion pressure was measured by a pressure transducer (Gould-Statham P231D) via a side arm and connected to a model 7B Grass polygraph. All tubing was immersed in a water bath at 37°C.

All three channels of the peristaltic pump worked simultaneously to deliver solutions at constant flow rate. When drug solutions were to be perfused, tubing running through one channel was simply removed from the Krebs solution and placed in a tube containing the drug. Similarly if two drug solutions were to be perfused simultaneously, then two lines were placed in respective drug solutions. This enabled us to perfuse two drug solutions simultaneously without altering the flow rate. All drug solutions were placed on ice throughout the experiment.

After 30 min equilibration, priming concentrations of phenylephrine (5 μM) were perfused. Concentration-effect curves to relaxants, ACh and SNP, were constructed over a background of phenylephrine precontraction. The level of precontraction was achieved by perfusing phenylephrine (5–12 μM) to a perfusion pressure of 70 mmHg above basal levels. Baseline perfusion pressures were not different between the three groups (fish oil + Dex: 19 ± 0.6 mmHg, $n = 10$; HCO/S + Dex: 18 ± 0.5 mmHg, $n = 9$; HCO/S + 18 ± 0.4 mmHg, $n = 9$). After construction of concentration-effect curves to ACh and SNP, a cumulative concentration-effect curve to noradrenaline was obtained. This was followed by removal of endothelium by perfusion of 0.05% saponin for 60 s before another concentration-effect curve to noradrenaline was constructed. This procedure for removing endothelium had previously been shown to abolish ACh relaxation without altering endothelium-independent relaxations to SNP (data not shown). The removal of endothelium increased the basal perfusion pressure but there was no difference between the three groups (fish oil + Dex: 25 ± 1.7 mmHg, $n = 10$; HCO/S + Dex: 25 ± 1.0 mmHg, $n = 9$; HCO/S: 26 ± 0.9 mmHg, $n = 9$, $P < 0.01$ compared to tissues with endothelium). This protocol was used in all tissues and the order in which the concentration-effect curves were constructed was maintained throughout the experiment.

Aortic rings A length of thoracic aorta was removed. This segment of aorta was cut into 2 rings of equal length (5 mm) with a double-bladed scalpel, one ring had its endothelium removed by gentle rubbing with a cotton probe. The composition of the Krebs was as above except that EDTA (0.026 mM) was added to it. The rings were mounted on platinum wires in Krebs filled organ baths at approximately 2 g for 30 min. One end of the wire was fixed while the other end was attached to a Grass FT03 isometric force transducer. The optimum tension for the aortic rings were obtained by challenging the rings with 30 nM noradrenaline at increasing tensions until this concentration of noradrenaline did not produce an increase in tension. Optimum tensions were not different between any of the three groups or between tissues with and without endothelium. The following were the optimum tensions obtained: Fish oil + Dex: 2.4 ± 0.08 g (with endothelium), 2.3 ± 0.12 g (without endothelium); HCO/S + Dex: 2.5 ± 0.13 g (with endothelium), 2.4 ± 0.08 g (without endothelium); HCO/S: 2.5 ± 0.09 g (with endothelium), 2.5 ± 0.1 g (without endothelium). The aortic rings were then allowed to equilibrate for another 30 min before construction of cumulative concentration-effect curves to ACh, sodium nitroprusside (SNP) and noradrenaline. To obtain concentration-effect curves to ACh and SNP, the rings were precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (1–9 μM) producing a tension of 1.3 g.

Serum thromboxane B_2, fatty acid and corticosterone analysis

Blood was allowed to clot after collection by incubation for 30 min at 37°C. After centrifugation of blood at 1500 r.p.m. for 15 min, 100 μl serum was collected and stored at − 20°C pending assay for serum TxB_2, while 300 μl was kept for fatty acid and corticosterone analysis. Serum TxB_2 was measured by direct radio-immunoassay (RIA). The relative fatty acid composition of serum was determined by gas chromatography of the corresponding methyl esters. Fatty acids were extracted from serum samples (approximately 200 μl) by addition of chloroform:methanol (2:1) and 0.5 ml of saturated NaCl solution with vigorous shaking. After centrifugation at 2500 r.p.m. for 5 min the aqueous phase was aspirated. The organic phase was decanted into glass sealed tubes and evaporated to dryness in a vortex evaporator.

Fatty acid methyl esters were prepared by treatment of serum extracts with 4% H_2SO_4 in methanol at 100°C for 20 min. Methyl esters were extracted into hexane and analysed by gas chromatography (Hewlett-Packard 5890A) using a 10 m × 0.53 m Superox 11 Column (Alltech), temperature programmed from 190°C to 245°C at 5°C min^{-1} with nitrogen carrier gas and a split ratio of 30:1. Peak areas were calculated automatically using a Hewlett-Packard 3393A computing integrator. Serum (60 µl) was used to analyse corticosterone levels by direct radioimmunoassay. This was done by the Anatomy and Human Biology Department of the University of Western Australia.

Aortic thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$

Segments of abdominal aortae (1 cm) were also removed from the rats for measurement of eicosanoid production. The segments of aorta were cleared of fat and connective tissue before incubation in Krebs solution (without EDTA) for 1 h at 37°C. The segments were then removed, blotted dry and weighed. Tissue incubates were stored at − 20°C pending analysis by direct RIA for TxB2 and 6-keto $PGF_{1\alpha}$ (Codde *et al.*, 1984).

Plasma electrolytes

Plasma was placed in heparin-lined tubes and sent for electrolyte analysis at the Biochemistry Department of Royal Perth Hospital.

Drugs and solutions

Acetylcholine chloride, noradrenaline bitartrate, sodium nitroprusside and $PGF_{2\alpha}$ were all purchased from Sigma chemicals. Saponin was from BDH chemicals. A stock solution of 10 mg ml^{-1} of $PGF_{2\alpha}$ was made up in ethanol and stored at − 20°C. On the days of the experiment, $PGF_{2\alpha}$ was freshly prepared by drying down the required amount with nitrogen and reconstituting in Krebs solution. All concentrations mentioned in the text are final bath concentrations.

Statistics

Differences in blood pressures between groups after 4 weeks feeding were analysed by unpaired Student's *t* test. To test the significance of changes in blood pressure after dexamethasone treatment, changes (Δ) before and after dexamethasone treatment were calculated and then compared by Student's unpaired *t* test when examining changes between groups and by Student's paired *t* test when examining changes within the same group.

Areas under individual concentration-effect curves were mathematically derived by methods outlined by Matthews *et al.* (1990). These areas were then compared in a one-way analyses of variance (ANOVA). When significant group effects were found, further comparison between three groups were carried out by Student's unpaired *t* test.

Relaxation responses are expressed as the percentage of the precontractile agent while contractile responses are expressed as increases in tension (g) or perfusion pressure (mmHg) above baseline levels.

pD_2, the log of the mean of 50% of maximum responses was measured by linear regression of the straight part of the concentration-effect curve where pD_2 is a reflection of the sensitivity of the tissue to a particular agonist. Differences between means were assessed by paired or unpaired *t* tests.

Analysis of the serum corticosterone levels was done by the Wilcoxon Rank sum test for non-parametric values.

P values of less than 0.05 were considered significant in all tests.

Results

Body weights

Rats in all groups had similar body weights at the end of the initial 4 week dietary period (Table 1). After a further week, rats fed HCO/S + Dex lost weight (Δ21 ± 4 g, $n = 12$; $P < 0.01$) but rats on fish oil + Dex maintained their weight (Δ0.5 ± 2 g) and those given HCO/S alone gained weight (Δ23 ± 4 g, $n = 12$; $P < 0.01$).

Blood pressure

Systolic blood pressures were not different between the groups after 4 weeks on the different dietary regimes (Table 1). Subsequently, one week of dexamethasone treatment caused a rise in systolic blood pressure in rats fed HCO/S (Δ18 ± 2 mmHg; $n = 12$, $P < 0.01$) but not in fish oil-fed rats (Δ5 ± 4 mmHg; $n = 12$) or in rats fed HCO/S without dexamethasone (Δ6 ± 4 mmHg; $n = 12$).

Vascular reactivity

Aortic rings Aortic rings from rats fed HCO/S + Dex had less endothelium-dependent relaxation to ACh (area: 121.4 ± 7.25, $n = 9$) than rings from rats fed HCO/S alone (area: 166.45 ± 3.57, $n = 10$, $P < 0.01$) between 0.3 and 30 µM (Figure 1). Rats given fish oil + Dex showed greater relaxations (area: 149.14 ± 5.75, $n = 9$) than HCO/S + Dex animals ($P < 0.05$), but still less than those given HCO/S alone between 0.3 and 30 µM ($P < 0.05$) (Figure 1).

Endothelium-independent relaxations to SNP were not different between the 3 groups either with or without endothelium (Figure 2). It is interesting to note that there was a tendency for rings without endothelium to be more sensitive to SNP than rings with endothelium (Table 2).

Neither dexamethasone treatment nor diet had any effect on the contractile responses to noradrenaline (Figure 3a).

Table 1 Summary of body weights and blood pressures in hydrogenated coconut oil and safflower oil (HCO/S) or fish oil-fed rats

	Body weights (g)		
	After 4 wks	*After 5 wks*	*Mean changes*
HCO/S ($n = 12$)	390 ± 12	412 ± 12	23 ± 4†
HCO/S + Dex ($n = 12$)	377 ± 10	356 ± 11	−21 ± 4**†‡
Fish oil + Dex ($n = 12$)	382 ± 8	383 ± 8	−0.5 ± 2†
	Blood pressure (mmHg)		
	4 wks	*5 wks*	*Mean changes*
HCO/S ($n = 12$)	137 ± 2	133 ± 4	−6 ± 4
HCO/S + Dex ($n = 12$)	132 ± 2	151 ± 2	18 ± 2*†‡
Fish oil + Dex ($n = 12$)	136 ± 3	141 ± 3	5 ± 2

*$P < 0.05$ and **$P < 0.01$ when compared to rats in HCO/S group.
† $P < 0.01$ when compared to rats in Fish oil + Dex group.
‡ $P < 0.01$ when compared to blood pressures within the same group measured 1 week previously.
Measurements were taken after 4 weeks feeding of respective diets and after 1 week of dexamethasone (Dex) treatment or continuation of HCO/S diet. Unpaired *t* tests were used to compare changes between groups but paired *t* tests were used when comparing changes within a group.

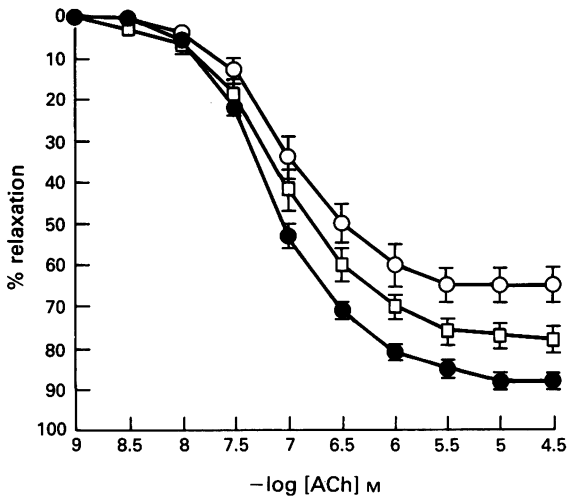


Figure 1 Endothelium-dependent relaxation responses to acetylcholine (ACh) of aortic rings from hydrogenated coconut oil and safflower oil (HCO/S) (●, $n = 10$), HCO/S + dexamethasone (Dex) (○, $n = 9$) and fish oil + Dex (□, $n = 11$) rats. Cumulative concentration-effect curves were constructed after precontraction to a tension of 1.3 g by prostaglandin $F_{2\alpha}$. Values are mean with s.e.mean (shown by vertical bars) of n rats.

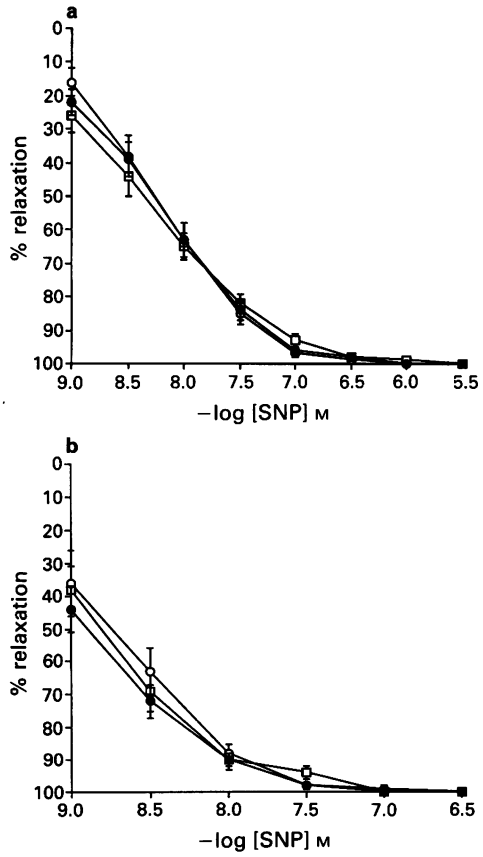


Figure 2 (a) Cumulative concentration-effect curves in response to sodium nitroprusside (SNP) of aortic rings with endothelium. Aortic rings from hydrogenated coconut oil and safflower oil (HCO/S) (●, $n = 8$), HCO/S + dexamethasone (Dex) (○, $n = 8$) and fish oil + Dex(□,$n = 11$) rats were precontracted by prostaglandin $F_{2\alpha}$ to 1.3 g tension before increasing concentrations of SNP were added cumulatively. (b) Concentration-effect curves to SNP were constructed on aortic rings without endothelium from HCO/S (●, $n = 8$), HCO/S + Dex (○, $n = 8$) and fish oil + Dex (□, $n = 9$). Values are mean with s.e.mean (shown by vertical bars) of n rats.

Table 2 pD_2 values of various agonists in aortic rings and perfused mesenteric resistance vessels from hydrogenated coconut oil and safflower oil (HCO/S) or fish oil-fed rats

	HCO/S	*HCO/S + Dex*	*Fish oil + Dex*
Aortic rings			
ACh	7.08 ± 0.07 ($n = 10$)	6.97 ± 0.09 ($n = 9$)	7.01 ± 0.09 ($n = 11$)
SNP(endo[+])	8.10 ± 0.21 ($n = 8$)	8.06 ± 0.18 ($n = 8$)	8.11 ± 0.29 ($n = 11$)
SNP(endo[−])	8.62 ± 0.30 ($n = 8$)	8.49 ± 0.15 ($n = 8$)	8.59 ± 0.17 ($n = 9$)
NA(endo[+])	7.12 ± 0.16 ($n = 11$)	7.21 ± 0.24 ($n = 9$)	7.16 ± 0.15 ($n = 11$)
NA(endo[−])	8.15 ± 0.13** ($n = 10$)	8.19 ± 0.16** ($n = 9$)	8.10 ± 0.21** ($n = 9$)
Perfused resistance vessels			
NA(endo[+])	5.27 ± 0.10† ($n = 9$)	5.30 ± 0.09† ($n = 9$)	5.0 ± 0.08 ($n = 10$)
NA(endo[−])	5.77 ± 0.08†** ($n = 9$)	5.68 ± 0.09†* ($n = 9$)	5.43 ± 0.06** ($n = 10$)

pD_2 values of tissues in response to acetylcholine (ACh), sodium nitroprusside (SNP) and noradrenaline (NA).
*$P < 0.05$ when comparing tissues with endothelium (endo[+]) to tissues without endothelium (endo[−]) in the same dietary group.
**$P < 0.01$ when comparing tissues with endothelium to tissues without endothelium in the same dietary group.
† $P < 0.01$ when comparing tissues of the same endothelium status to tissues from fish oil-fed rats.

Removal of endothelium caused a significant increase in pD_2 (indicating a leftward shift of the concentration-effect curve; Figure 3b and Table 2) but there were no differences between the three groups.

Perfused mesenteric resistance vessels Relaxant responses to ACh and SNP were similar in preparations from all groups (Figure 4a and b). The dexamethasone treatment did not affect contractile responses to noradrenaline (Figure 5a and b). Preparations taken from fish oil + Dex rats had decreased responses to noradrenaline between 1 μM - 100 μM compared to HCO/S-fed rats ($P < 0.01$) as well as HCO/S + Dex rats ($P < 0.01$). The sensitivity of the tissues from fish oil-fed rats to noradrenaline was also decreased compared to the other two groups as judged by their pD_2 values (Table 2). After removal of endothelium, sensitivity of tissues from all groups increased but preparations from the fish oil-fed group remained less sensitive to noradrenaline than the other two groups.

Serum corticosterone

Serum corticosterone was greatly reduced in dexamethasone-treated rats. Levels in rats given fish oil + Dex (65 ± 16 ng-ml^{-1}, $n = 10$) were one-third of serum levels in HCO/S rats (188 ± 21 ng ml^{-1}; $n = 12$, $P < 0.01$) but were higher than HCO/S + Dex-treated rats in which levels were mostly below the limit of sensitivity of the assay which was < 3 ng ml^{-1}.

Eicosanoid production

Levels of aortic TxB_2 from rats fed HCO/S + Dex were less than rats given HCO/S alone, while levels from rats on fish oil + Dex were substantially diminished compared to both other groups (Table 3). A similar trend was observed in the production of serum TxB_2 with levels in rats on HCO/S +

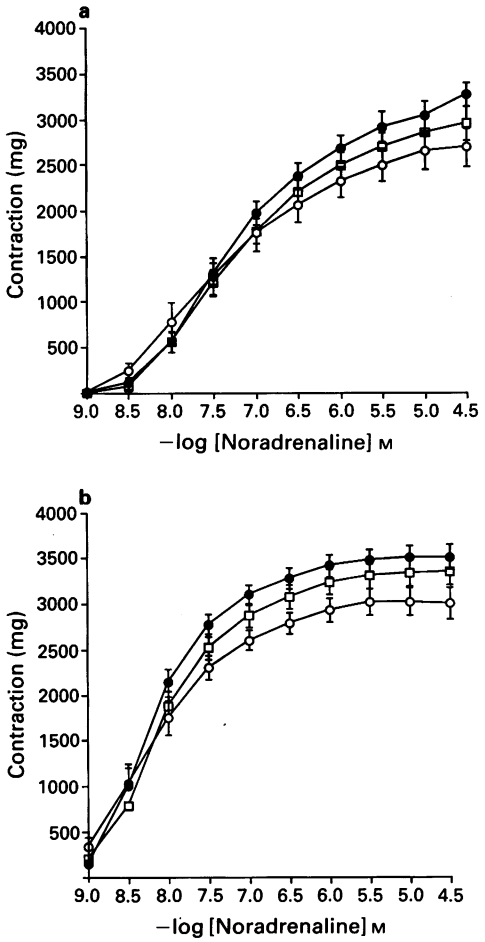


Figure 3 Concentration-effect curves to noradrenaline were constructed in aortic rings with endothelium (a) and without endothelium (b) taken from rats fed hydrogenated coconut oil and safflower oil (HCO/S) (●), HCO/S + dexamethasone (Dex) (○) and fish oil + Dex (□). In rings with endothelium, values are mean with s.e.mean (shown by vertical bars) for $n = 11$ (HCO/S), $n = 9$ (HCO/S + Dex) and $n = 11$ (fish oil + Dex). For rings without endothelium, $n = 10$ (HCO/S), $n = 9$ (HCO/S + Dex) and $n = 10$ (fish oil + Dex).

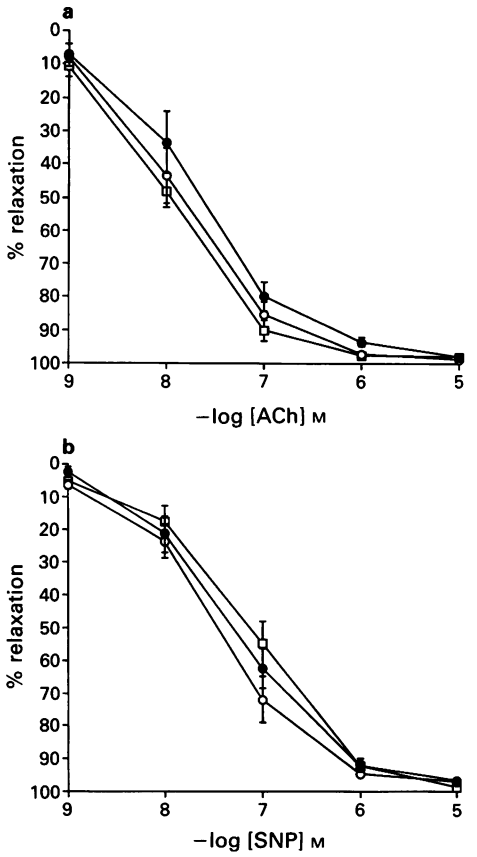


Figure 4 Relaxant response to acetylcholine (ACh) (a) and to sodium nitroprusside (SNP) (b) were assessed in perfused mesenteric resistance arteries. Preparations were preconstricted by phenylephrine to a perfusion pressure of 70 mmHg above basal levels before cumulative concentrations of the vasodilators were perfused. All values are mean with s.e.mean (shown by vertical bars) of % relaxation from preconstricted levels in preparations from rats fed hydrogenated coconut oil and safflower oil (HCO/S) (●, $n = 9$), HCO/S + dexamethasone (Dex) (○, $n = 9$) and fish oil + Dex (□, $n10$).

Dex being less than HCO/S-fed rats and levels in rats on fish oil + Dex being less than other groups. In contrast, dexamethasone treatment did not affect production of 6-keto $PGF_{1\alpha}$ in rats fed HCO/S. On the other hand aortic production of 6-keto $PGF_{1\alpha}$ in rats given fish oil + Dex were diminished compared to the other two groups.

Serum fatty acids

In rats fed fish oils there was an increase in the proportion of EPA (20:5) and a concomitant decrease in arachidonic acid (20:4) (Table 4). There were significantly lower levels of stearic acid (18:0), oleic acid (18:1) and linoleic acid (18:2) in serum of fish oil + Dex rats compared to the other two groups. In addition, serum of HCO/S + Dex rats had greater levels of stearic acid than HCO/S rats but lower levels of oleic acid.

Plasma electrolytes

There was a small difference in plasma potassium levels between rats fed fish oil and dexamethasone and rats fed HCO/S alone (Table 5). Plasma urea levels were higher in rats fed HCO/S + Dex than rats fed fish oil + Dex. These differences are small and do not appear to have a trend.

Discussion

This study confirms that dexamethasone can raise systolic blood pressure and that this increase can be ameliorated by fish oil feeding. Dexamethasone treatment was associated with a decrease in endothelium-dependent relaxation to ACh in aortic rings, offering one potential mechanism for the glucocorticoid-induced blood pressure rise. Fish oil feeding increased the relaxant responses of aortic rings to ACh in dexamethasone-fed rats but the responses were still less than aortic rings from rats fed HCO/S alone. Contractile responses of mesenteric resistance vessels to noradrenaline were decreased in the rats given fish oil as compared to the other two groups.

Dexamethasone caused a rise in systolic blood pressure as seen in other studies (Handa *et al.*, 1984; Codde & Beilin, 1985; Iijima & Malik, 1985; Tonolo *et al.*, 1988). If the associated decrease in endothelium-dependent relaxation in aortic rings occurs *in vivo*, this is likely to cause decreased compliance, which would in turn lead to higher systolic blood pressures.

The lowered endothelium-dependent relaxation of aortic rings taken from rats fed dexamethasone could be caused by a decrease in production or release of endothelium-derived relaxing factor (EDRF), which is now believed to have identical properties to nitric oxide (Palmer *et al.*, 1987). This mechanism is thought to be the cause of the lowered endothelium-dependent relaxant responses to various

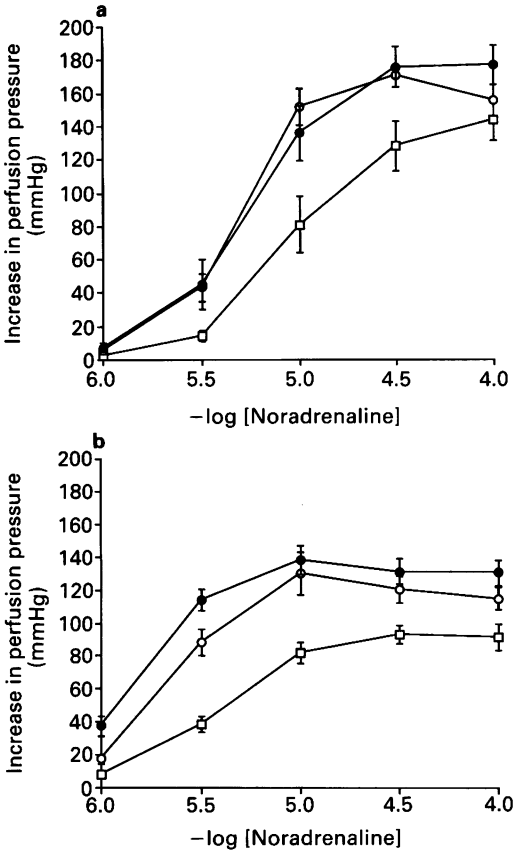


Figure 5 Constrictor responses to noradrenaline were measured in perfused mesenteric resistance arteries with intact endothelium (a) and after endothelium had been removed (b). Endothelium was removed by perfusion of 0.05% saponin for 60 s. All values are mean with s.e.mean (shown by vertical bars) of increase in perfusion pressure above baseline levels of tissues from hydrogenated coconut oil and safflower oil (HCO/S) (●, $n = 9$), HCO/S + dexamethasone (Dex) (○, $n = 9$) and fish oil + Dex (□, $n = 10$).

Table 3 Eicosanoid production of aortae and serum of hydrogenated coconut oil and safflower oil (HCO/S) or fish oil-fed rats

	HCO/S	*HCO/S + Dex*	*Fish oil + Dex*
Aortic TxB_2 ($pg\ mg^{-1}$)	212 ± 26 ($n = 12$)	139 ± 10** ($n = 11$)	54 ± 7† ($n = 11$)
Serum TxB_2 ($ng\ ml^{-1}$)	179 ± 19 ($n = 12$)	110 ± 11** ($n = 11$)	65 ± 8† ($n = 11$)
Aortic 6-keto $PGF_{1\alpha}$ ($ng\ mg^{-1}$)	6.2 ± 0.8 ($n = 12$)	4.8 ± 0.6 ($n = 11$)	2.5 ± 0.4† ($n = 11$)

†$P < 0.01$ when comparing eicosanoid levels in rats on Fish oil + Dex with levels in rats in the other two groups.
**$P < 0.01$ when comparing eicosanoid levels in rats on HCO/S + Dex with levels in rats on HCO/S.

Table 4 Serum fatty acids composition of hydrogenated coconut oil and safflower oil (HCO/S) or fish oil-fed rats

	HCO/S ($n = 8$)	*HCO/S + Dex* ($n = 9$)	*Fish oil + Dex* ($n = 7$)
16:0	23.3 ± 1.8	21.7 ± 1.2	20.3 ± 2.7
16:1	7.0 ± 0.9	5.4 ± 0.4	4.6 ± 0.6
18:0	12.2 ± 0.6*	14.8 ± 0.8†	10.1 ± 0.8
18:1	21.4 ± 1.6†	15.5 ± 0.9**	7.8 ± 0.6
18:2	15.2 ± 1.2	18.2 ± 1.3	5.3 ± 0.5†
20:4	18.2 ± 1.9	20.7 ± 1.1	11.4 ± 0.6†
20:5	trace	trace	27.8 ± 2.4†
22:6	1.6 ± 0.3	1.6 ± 0.6	8.2 ± 1.4†

Results are expressed as % serum fatty acid composition
*$P < 0.05$ as compared to fish oil + Dex.
**$P < 0.01$ as compared to fish oil + Dex.
† $P < 0.01$ as compared to the other two groups.

Table 5 Plasma electrolytes of hydrogenated coconut oil and safflower oil (HCO/S) or fish oil-fed rats

	HCO/S	*HCO/S + Dex*	*Fish oil + Dex*
Na^+(M)	141 ± 0.26 ($n = 10$)	141 ± 0.84 ($n = 10$)	142 ± 0.43 ($n = 12$)
K^+(M)	3.78 ± 0.09 ($n = 10$)	3.88 ± 0.10 ($n = 10$)	4.09 ± 0.09* ($n = 12$)
Urea(M)	5.8 ± 0.4 ($n = 10$)	6.74 ± 0.3 ** ($n = 10$)	5.8 ± 0.23 ($n = 12$)
Creatinine (μM)	47 ± 4 ($n = 10$)	42 ± 3 ($n = 10$)	40 ± 3 ($n = 12$)

*$P < 0.05$ when compared to HCO/S only.
**$P < 0.05$ when compared to the other two groups.

agonists in 1-clip, 2-kidney and DOCA-salt hypertensive rats (Van de Voorde & Leusen, 1986) and in Dahl-salt hypertensive rats (Luscher *et al.*, 1987). However, in some circumstances a decrease in relaxant response to ACh may be caused by an increased production of contractile cyclo-oxygenase product(s) as in SHR (Luscher & Vanhoutte, 1986; Koga *et al.*, 1989; Kato *et al.*, 1990; Yin *et al.*, 1991). In contrast to our study, incubation of aortic rings with dexamethasone for up to 8 h did not affect endothelium-dependent relaxation to ACh (Rees *et al.*, 1990) although in that same study dexamethasone prevented the induction by endotoxin of nitric oxide synthase in neutrophils. The difference between our results and the above mentioned studies is probably a reflection of the difference between *in vivo* and *in vitro* administration of dexamethasone.

The situation with regard to effects of dexamethasone on the synthesis of other local vasodilators or vasoconstrictor substances is unclear. It has been reported that dexamethasone increased glomerular prostaglandin production of PGE_2 and $PGF_{2\alpha}$ via the cylco-oxygenase pathway in rats (Erman *et al.*, 1986). On the other hand there is evidence that in perfused lung and rat peritoneal leukocytes, dexamethasone suppresses prostaglandin formation by promoting the synthesis of the peptide mediator, macrocortin (reviewed by Flower *et al.*, 1984) which exerts its effect by inhibiting the action of phospholipase A_2. In contrast, some *in vivo* studies have reported increased (Codde & Beilin, 1985) urinary excretion of the vasodilator prostanoids PGE_2 and the stable metabolite of prostacyclin, 6-keto $PGF_{1\alpha}$ while Nasjletti *et al.* (1984) claimed that dexamethasone-induced hypertension is not associated with a decline in circulating and renal PGE_2 and prostacyclin. Furthermore, the synthesis of serum TxB_2, the stable metabolite of the vasoconstrictor prostanoid TxA_2, was decreased after dexamethasone treatment without affecting aortic production of 6-keto $PGF_{1\alpha}$ in rats fed HCO/S (Codde & Beilin, 1985). Our results have confirmed the findings of these latter workers. In addition, aortae of rabbits given dexamethasone have been reported to have a decreased PGH_2/TxA_2 receptor number (Sessa *et al.*, 1990) which could explain the accompanying decreased sensitivity specific to PGH_2/TxA_2 receptor agonists and not to phenylephrine, an α_1-adrenoceptor agonist (Sessa & Nasjletti,

1990). Thus it is improbable that changes in prostanoid production could account for the decrease in endothelium dependent relaxation to ACh in HCO/S + Dex treated rats, as a lowered production of TxA_2 or its endoperoxide precursor would tend to increase relaxant responses.

An alternative explanation for the decreased endothelium-dependent responses to ACh after dexamethasone treatment is that this decrease is secondary to the rise in blood pressure. Lockette *et al.* (1986) reported that endothelium-dependent responses could be 'normalized' in aortic rings of hypertensive rats after lowering of blood pressure. This argument is probably not applicable in our study because the blood pressure changes were relatively small. Moreover, there was no significant increase in blood pressure of fish oil + Dex-treated rats which still showed a small but significant decrease in endothelium-dependent relaxation to ACh in aortic rings as compared to rats given HCO/S alone.

In contrast to aortic rings, there was no effect of dexamethasone on endothelium-dependent response to ACh in perfused mesenteric resistance arteries. Endothelium-independent responses to SNP were also unaffected. The finding that dexamethasone does not potentiate responses to noradrenaline in perfused mesenteric resistance vessels agrees with the report of Iijima & Malik (1985) but contrasts with findings of increased noradrenaline reactivity after glucocorticoid treatment in rabbit aortic strips (Kalsner, 1969), in intact rats (Waeber *et al.*, 1983; Handa *et al.*, 1984) and in perfused mesenteric vessels (Russo *et al.*, 1990). With respect to the latter study, differences might be explained by the fact that the increase in noradrenaline reactivity could be biphasic in relation to dosage as the dose of dexamethasone used in that particular study was much lower than in ours (2 μg daily for 14 days compared to approximately 30 μg daily for 7 days). The responses to noradrenaline could also be tissue and species specific. The discrepancy between effects of dexamethasone on the reactivity to noradrenaline and pressor responses in intact whole animals may also be related to central nervous or cardiac effects of glucocorticoids.

The loss in body weight in rats fed HCO/S and dexamethasone has been reported in other studies of this model of glucocorticoid hypertension (Handa *et al.*, 1984; Codde & Beilin, 1985) and reflects the catabolic effect of glucocorticoid hormones. Rats given fish oil before dexamethasone treatment did not lose weight, while rats not treated with dexamethasone continued to gain weight. To test indirectly whether these differences in weight could be due to fish oil affecting dexamethasone absorption we measured serum corticosterone levels. Dexamethasone suppresses ACTH synthesis and thus inhibits production of the main rat adrenal corticosteroid, corticosterone (Williams, 1974). The fact that serum corticosterone levels in rats fed HCO/S before dexamethasone treatment were lower than levels in their fish oil-fed counterparts suggested that the fish oils either affected the absorption of dexamethasone and/or ACTH suppression of corticosterone synthesis. The significant differences in weight loss between the two groups would argue against the latter explanation as lowered corticosterone levels would not in themselves cause significant weight loss.

The effect of fish oils on the endothelium-dependent relaxation responses has been characterized in aortic rings of SHR (Yin *et al.*, 1991) in porcine coronary arteries (Shimokawa *et al.*, 1988; Shimokawa & Vanhoutte, 1989). In SHR, the fish oils affected the endothelium-dependent relaxation responses to ACh by inhibiting a contractile cyclo-oxygenase product (Yin *et al.*, 1991), while in porcine arteries the effect of fish oils was probably via the production/release of endothelium-derived relaxing factor (EDRF) (Shimokawa *et al.*, 1987; Shimokawa & Vanhoutte, 1988).

Fish oil-feeding caused a decrease in production of both TxB_2 and 6-keto $PGF_{1\alpha}$ in dexamethasone-treated rats. This contrasts to the report of Codde & Beilin (1985) who found that fish oils decreased production of serum TxB_2 but not aortic production of 6-keto $PGF_{1\alpha}$. This may be caused by the use of different types of fish oils as we used 'Max EPA' fish oil containing 18% EPA while in the former paper, cod-liver oil, containing 6.7% EPA, was used. This difference in composition could account for the discrepancy in prostanoid production. As already noted above, changes in eicosanoid production do not give an entirely satisfactory explanation for the decrease in endothelium-dependent relaxation in dexamethasone-treated rats. Nevertheless the enhancement of the relaxant response by fish oil feeding in dexamethasone-treated rats might still occur via inhibition of synthesis of contractile endoperoxides, thereby altering the balance of vasoconstrictor and vasodilator substances generated in response to ACh. It is also possible that fish oils rich in EPA might change membrane fluidity (Furchgott *et al.*, 1984) and thus increase EDRF production. In this respect it has recently been claimed that EPA can induce the release of a non-nitric oxide relaxing factor from bovine cultured endothelial cells (Boulanger *et al.*, 1990).

As seen in SHR fed fish oils (Yin *et al.*, 1991), there was a decrease in noradrenaline reactivity of perfused mesenteric resistance vessels taken from fish oil-fed rats given dexamethasone. This attenuation of noradrenaline responses was not endothelium-dependent, indicating that the effect of fish oils was at the level of the smooth muscle in this vascular bed. The mechanism of the decreased responses could be due to change in structure of receptors, in post-receptor mechanisms or in the calcium-mediated contractile process. Recent experiments in the laboratory suggest that fish oil attenuates noradrenaline responses but not responses to vasopressin or KCl in SHR resistance vessels (Chu *et al.*, 1992). While there are specific receptors to vasopressin, this peptide, like noradrenaline, stimulates inositol 1,4,5 trisphosphate production. Hence it is more likely that fish oils affected the contraction at the level of the smooth muscle receptors rather than post-receptor mechanisms of contractions.

In summary, dexamethasone caused a rise in systolic blood pressure which could in part be a consequence of a decrease in endothelium-dependent relaxation of aortae and other conduit arteries. The pressor effect of glucocorticoid may be augmented if as has been suggested, there is an increase in resistance vessel reactivity to pressor stimuli *in vivo*. Fish oil feeding ameliorated this dexamethasone-induced rise in blood pressure. This phenomenon could be caused by fish oil affecting dexamethasone absorption, compounded by an increase in endothelium-dependent relaxant responses in aortic rings and decreased endothelium-independent response to noradrenaline in mesenteric resistance arteries.

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The effects of idazoxan and other α_2-adrenoceptor antagonists on urine output in the rat

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1 In normally-hydrated Wistar rats the α_2-adrenoceptor antagonist, idazoxan (1, 3, 10 mg kg^{-1} i.p.), increased urine output during the 6 h following injection.

2 The more selective and specific α_2-adrenoceptor antagonist, RX811059 (0.3, 1, 3 mg kg^{-1} i.p.), and the peripherally-acting α_2-adrenoceptor antagonist, L-659,066 (1, 3, 10 mg kg^{-1} i.p.), had no effect on urine output in normally-hydrated animals.

3 In rats given a 25 ml kg^{-1} water-load orally, idazoxan (10 mg kg^{-1}, i.p.) produced an initial antidiuretic response which was followed by an increase in urine output which was apparent 4 and 6 h after drug administration.

4 RX811059 (1, 3 mg kg^{-1} i.p.) and L-659,066 (3, 10 mg kg^{-1} i.p.) significantly decreased urine output in water-loaded rats in the 2 h after injection.

5 The antidiuretic effects of L-659,066 were attenuated in Brattleboro rats which are deficient in vasopressin; only the highest dose (10 mg kg^{-1} i.p.) decreased urine output, and this was only a small response in comparison with its virtual abolition of urine output in water-loaded Wistar rats.

6 The results with the selective α_2-adrenoceptor antagonists in Wistar and Brattleboro rats suggest that α_2-adrenoceptors in the periphery may play a physiological role in the control of water balance through a mechanism which involves vasopressin.

7 The paradoxical diuretic effects of idazoxan contrast with the effects of the other α_2-adrenoceptor antagonists and therefore may be attributed to a property of this compound unrelated to α_2-adrenoceptor blockade.

Keywords: Idazoxan; α_2-adrenoceptor antagonists; α_2-adrenoceptors; non-adrenoceptor idazoxan binding sites; urine output

Introduction

We have recently shown that α_2-adrenoceptor antagonists increase water intake in male Wistar rats (Jackson *et al.*, 1991). These effects on water consumption may be secondary to increased urination. However, the effects of α_2-adrenoceptor antagonists on urine output have not been extensively investigated. Thus we have measured urine output in normally-hydrated Wistar rats using three different α_2-adrenoceptor antagonists: idazoxan, a commonly used standard α_2-adrenoceptor antagonist (Chapleo *et al.*, 1983; Doxey *et al.*, 1985) that has been used in a number of studies (Blackburn *et al.*, 1986; Birch & Hayes, 1988; Bianchi, 1991) but which also has high affinity for non-adrenoceptor idazoxan binding sites (NAIBS; reviewed by Michel & Insel, 1989); secondly, RX811059 (2-ethoxy idazoxan) which acts more selectively and specifically than idazoxan at α_2-adrenoceptors (Doxey *et al.*, 1985) and has only low affinity at NAIBS (Mallard *et al.*, 1991) and, thirdly, L-659,066 which does not readily cross the blood-brain barrier (Cline-schmidt *et al.*, 1988) and may therefore be used to determine whether the responses to α_2-adrenoceptor antagonists involve peripheral or central mechanisms.

In addition to these studies in normally-hydrated animals we also examined the effects of α_2-adrenoceptor antagonists in two experimental paradigms in which urine outflow is increased. Firstly, the water-loaded rat was used because yohimbine, a rather non-selective adrenoceptor antagonist with some 5-hydroxytryptamine and dopamine blocking actions (Scatton *et al.*, 1980; Goldberg & Robertson, 1983) has been shown to inhibit diuresis in these animals (Gellai, 1990). Secondly, the Brattleboro rat was used because these animals are deficient in vasopressin (antidiuretic hormone) which controls renal excretion of water and may therefore mediate the action of drugs on urine flow.

Some of the results of this study have previously been presented to the British Pharmacological Society in abstract form (Jackson & Griffin, 1991).

Methods

Animals and environment

All experiments were performed on male Wistar rats (350–450 g) with the exception of the studies in male Brattleboro rats (homozygous for diabetes insipidus; 250–450 g). All animals were bred in the University of Bristol. Rats were housed in groups of 8 at 23 ± 2°C on a 14:10 h light-dark cycle (lights on at 05 h 00 min) with free access to a standard rat and mouse diet (Biosure, Manea, Cambridgeshire) and tap water. Animals were used on one occasion only.

Experimental procedures

On the day of test, animals were transferred to a quiet air-conditioned laboratory (23 ± 2°C). All procedures were carried out between 09 h 00 min and 16 h 00 min. Animals were allocated to one of four treatment groups (each containing 2 animals) and after drug administration placed in simple metaboles. Rats were deprived of both food and water during the experiment. Urine output was measured (to the

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nearest 0.1 ml) after 2 h, 4 h and 6 h in Wistar rats. In some experiments Wistar rats were given a water-load of 25 ml kg^{-1} orally immediately after drug administration. Experiments using Brattleboro rats were performed in a similar manner except that urine output was measured over a 2 h period only, to limit the amount of time that these animals were deprived of water.

Variations in body weight were accounted for by expressing the results as cumulative urine outputs in ml kg^{-1} rat weight (treatment group means ± s.e.mean) and experiments were repeated on at least 3 different days to give a treatment group size of at least 6 animals.

Normally-hydrated control Wistar rats in the weight range used in this study would normally produce up to 2 ml of urine over a 2 h period and water-loaded rats would excrete 6–10 ml. Normally-hydrated Brattleboro rats would normally produce 9–11 ml over 2 h.

Drugs and injections

The following drugs were used: idazoxan (2-[2-(1,4-benzodioxanyl)]-2-imidazoline; 1, 3, 10 mg kg^{-1}), RX811059 (the 2-ethoxy derivative of idazoxan; 0.3, 1, 3 mg kg^{-1}) and L-659,066 ((2**R**,12b**S**)-N-[2-(1,3,4,6,7,12b-hexahydro-2′-oxospiro[2H-benzofuro [2,3-a] quinolizine-2,4′-imidazolidin]-3′-yl) ethyl] methane sulphonamide; 1, 3, 10 mg kg^{-1}). Idazoxan and RX811059 were synthesized at Reckitt & Colman, Hull and L-659,066 was a gift from Merck, Sharp & Dohme, West Point, Pennsylvania, U.S.A. Drug doses are expressed as the hydrochloride salt and all drugs were dissolved in 0.9% saline and administered intraperitoneally in a dose volume of 1 ml kg^{-1}. Doses of drugs were based on those shown by other studies (Clineschmidt *et al.*, 1988; Jackson *et al.*, 1991; unpublished observations) to cover the full pharmacological dose-range.

Statistical analysis

Statistical comparisons between mean group intakes were made using analysis of variance and Dunnett's test (two-tailed).

Results

Effect of idazoxan on urine output

Idazoxan (1, 3 mg kg^{-1}) produced a significant increase in urine output in normally-hydrated Wistar rats in the 2 h following drug administration (Figure 1a). A higher dose (10 mg kg^{-1}) had no effect at 2 h but produced diuresis at the 4 and 6 h readings in these animals.

Low doses of idazoxan (1, 3 mg kg^{-1}) had no effect on urine output in Wistar rats given a 25 ml kg^{-1} water-load (Figure 1b). Idazoxan (10 mg kg^{-1}) produced a significant decrease in urine output in the 2 h following injection. However, at the 4 and 6 h reading the urine output of water-loaded animals treated with this dose of idazoxan was significantly greater than that of the vehicle-treated control animals.

Effect of RX811059 and L-659,066 on urine output

RX811059 (0.3, 1, 3 mg kg^{-1}; Figure 2a) and L-659,066 (1, 3, 10 mg kg^{-1}; Figure 3a) had no effect on urine output in normally-hydrated Wistar rats for up to 6h after drug administration.

On the other hand, RX811059 (1, 3 mg kg^{-1}; Figure 2b) and L-659,066 (3, 10 mg kg^{-1}; Figure 3b) induced significant antidiuretic responses in the 2 h following injection in water-loaded Wistar rats. Urine output was virtually abolished in the animals treated with the highest dose of L-659,066 during this period. The antidiuretic effects of the α_2-adrenoceptor antagonists were relatively short-lasting since cumulative urine outputs of animals treated with RX811059 (0.3, 1, 3 mg kg^{-1}) and L-659,066 (1, 3, 10 mg kg^{-1}) were similar to control levels at the 4 h and 6 h readings.

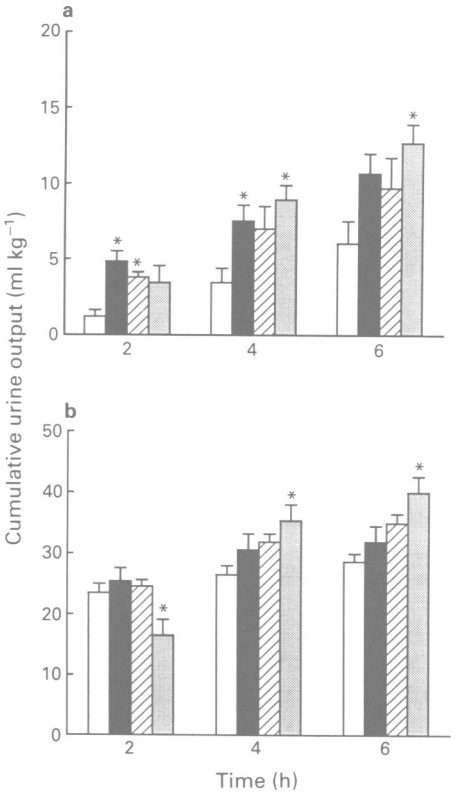


Figure 1 Effect of idazoxan on cumulative urine output in (a) normally-hydrated and (b) water-loaded rats. Groups of 6–8 rats were injected i.p. with either saline (open columns) or doses of idazoxan: 1 mg kg^{-1} (closed columns), 3 mg kg^{-1} (hatched columns) or 10 mg kg^{-1} (stippled columns). Results are expressed as treatment group means; vertical lines represent s.e.mean. Significant differences from control values are indicated by $*P<0.05$.

Effect of L-659,066 on urine output in Brattleboro rats

The urine output of vehicle-treated Brattleboro rats as expected was much higher than that of the normally-hydrated Wistar rat control group and was slightly higher than that of water-loaded Wistar rat controls (Figures 1–4). L-659,066 (1, 3 mg kg^{-1}) had no effect on urine output in Brattleboro rats in the 2 h following drug administration (Figure 4). A higher dose of 10 mg kg^{-1} produced a small but significant antidiuretic response in these animals.

Discussion

Two contrasting findings emerged from the current series of experiments. Idazoxan, but not the other more selective α_2-adrenoceptor antagonists, increased urine flow in normally-hydrated animals. On the other hand, RX811059 and L-659,066 were antidiuretic in water-loaded rats, whereas idazoxan had an initial antidiuretic action which was followed by stimulation of urine output.

The antidiuretic effects of the selective α_2-adrenoceptor antagonists observed in this study confirm and extend previous findings with yohimbine in water-loaded rats (Gellai, 1990). This compound blocks α_2-adrenoceptors but also acts at α_1-adrenoceptors, dopamine and 5-hydroxytryptamine receptors (Scatton *et al.*, 1980; Goldberg & Robertson, 1983). Thus, α_2-adrenoceptors appear to play a physiological role in

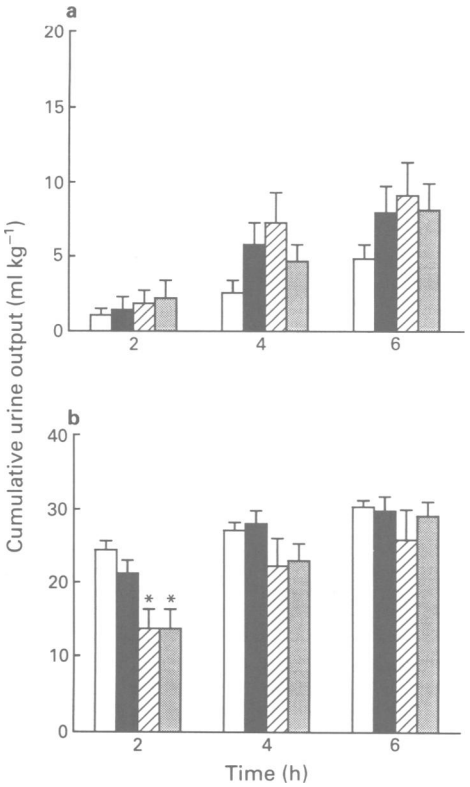


Figure 2 Effect of RX811059 on cumulative urine output in (a) normally-hydrated and (b) water-loaded rats. Groups of 6–8 rats were injected i.p. with either saline (open columns) or doses of RX811059: 0.3 mg kg^{-1} (closed columns), 1 mg kg^{-1} (hatched columns) or 3 mg kg^{-1} (stippled columns). Results are expressed as treatment group means; vertical lines represent s.e.mean. Significant differences from control values are indicated by $^*P<0.05$.

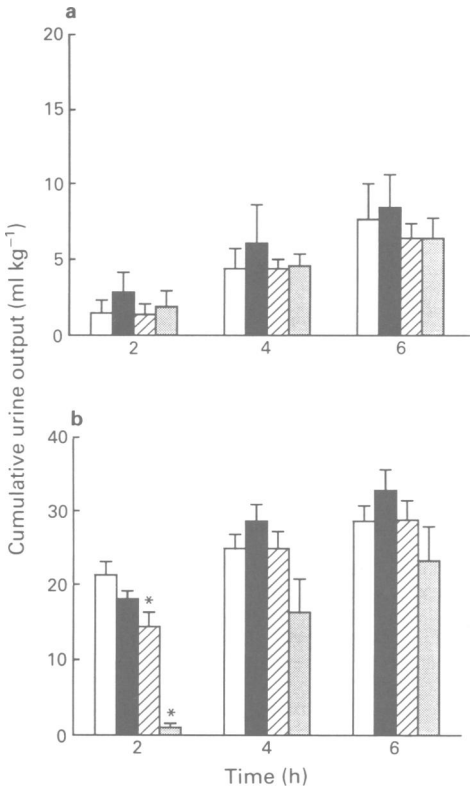


Figure 3 Effect of L-659,066 on cumulative urine output in (a) normally-hydrated and (b) water-loaded rats. Groups of 6 rats were injected i.p. with either saline (open columns) or doses of L-659,066: 1 mg kg^{-1} (closed columns), 3 mg kg^{-1} (hatched columns) or 10 mg kg^{-1} (stippled columns). Results are expressed as treatment group means; vertical lines represent s.e.mean. Significant differences from control values are indicated by $^*P<0.05$.

the control of water balance.

Urine output in water-loaded Wistar rats was almost totally abolished by L-659,066. This antagonist, when administered systemically, acts selectively at α_2-adrenoceptors lying outside the blood-brain barrier (Clineschmidt *et al.*, 1988). Hence, the antidiuretic effects of α_2-adrenoceptor blockade appear to be peripherally-mediated. Recent findings suggest that renal postsynaptic α_2-adrenoceptors inhibit the antidiuretic action of vasopressin in the collecting tubule (reviewed by Gellai, 1990). Blockade of these receptors by α_2-adrenoceptor antagonists presumably removes tonic endogenous inhibition of vasopressin function, thus decreasing urine output. The relatively small antidiuretic effect of L-659,066 in Brattleboro rats, which are deficient in vasopressin, is consistent with this hypothesis although further studies are required to confirm this.

It is interesting that the first effect of idazoxan in water-loaded rats was to inhibit urine output in a fashion similar to but weaker than the other two antagonists. The later diuretic effect was not seen with the other two drugs, a finding which was mirrored in the results from the normally-hydrated rats. Presumably the diuretic actions of idazoxan reflect activity other than α_2-adrenoceptor blockade. One explanation for these results is that idazoxan may act as a partial α_2-adrenoceptor agonist in the periphery as previously suggested by Limberger & Starke (1983) following their experiments in rabbit isolated ear artery. However, there is no evidence that idazoxan acts as a partial α_2-adrenoceptor agonist in peripheral tissues in the rat (Chapleo *et al.*, 1983; unpublished observations).

A more feasible explanation for the paradoxical diuretic effects of idazoxan may be provided by its high affinity for NAIBS. In contrast, RX811059, L-659,066 and yohimbine, have only low affinity at these sites (Brown *et al.*, 1990; Langin *et al.*, 1990; Mallard *et al.*, 1991; A. Hudson & N. Mallard; unpublished observations). NAIBS are present in rat kidney (Michel *et al.*, 1989) and therefore may be involved in regulation of fluid homeostasis. It will be interesting to investigate whether NAIBS-selective ligands increase urine flow, when these compounds become available.

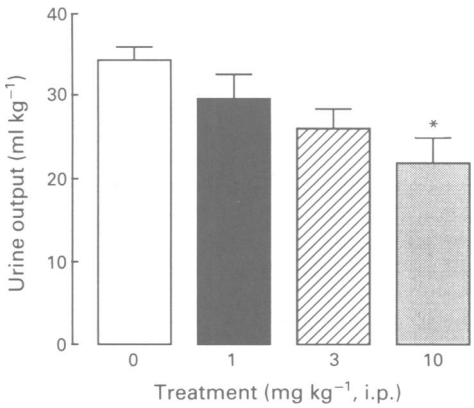


Figure 4 Effect of L-659,066 on urine output in Brattleboro rats. Groups of 6 rats were injected i.p. with either saline (open columns) or doses of L-659,066: 1 mg kg^{-1} (closed columns), 3 mg kg^{-1} (hatched columns) or 10 mg kg^{-1} (stippled columns). Urine was collected during the 2 h following injection. Results are expressed as treatment group means; vertical lines represent s.e.mean. Significant differences from control values are indicated by $^*P<0.05$.

How do the current findings relate to the mechanisms underlying the stimulatory effects of RX811059 and L-659,066 on water intake? Our results show that the increase in drinking induced by α_2-adrenoceptor antagonists (see Jackson *et al.*, 1991) is not simply related to increases in urine flow since these compounds did not produce diuresis in normally-hydrated rats. One possibility is that the effects of α_2-adrenoceptor antagonists on both urine output and water intake represent homeostatic responses to some other action. For instance, both idazoxan and RX811059 decrease blood pressure in conscious normotensive rats, the effects of RX811059 being more pronounced and of longer duration than those of idazoxan (T.L. Berridge, unpublished observations). Hence, RX811059 may inhibit urine output to help maintain blood pressure. Perhaps the small antidiuretic action of L-659,066 in the Brattleboro rat reflects hypotension and it would be interesting to have data on the effects of this drug on blood pressure.

In conclusion, the selective α_2-adrenoceptor antagonist, RX811059 and the peripherally-acting α_2-adrenoceptor antagonist, L-659,066, decreased urine output in water-loaded Wistar rats. The antidiuretic effects of L-659,066 were attenuated in Brattleboro rats, which are deficient in vasopressin. These results suggest that α_2-adrenoceptors in the periphery may play a role in the control of water balance and that this may be mediated, at least in part, by vasopressin. Unlike the other α_2-adrenoceptor antagonist, idazoxan, increased urine output in the rat, paradoxical effects which may presumably be attributed to a property of this compound unrelated to α_2-adrenoceptor blockade.

We would like to thank Merck, Sharp & Dohme for their gift of L-659,066.

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Investigations of the subtype of α_2-adrenoceptor mediating contractions of the human saphenous vein

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1 We have examined the effects of a series of α_2-adrenoceptor antagonists against isometric contractions to noradrenaline in human saphenous vein, and correlated these potencies with affinities for the α_{2A}-ligand binding site of human platelet and the α_{2B}-ligand binding site of rat kidney.

2 The α_{2B}-selective adrenoceptor antagonists, prazosin, ARC 239 and HV 723 showed high, and the α_{2A}-selective antagonist BRL 44408 showed low, potency in human saphenous vein.

3 Potency in human saphenous vein correlated better with affinity for the α_{2B}-ligand binding site ($r = 0.71$, $n = 12$, $P < 0.01$) than with affinity for the α_{2A}-ligand binding site ($r = 0.56$, $n = 12$, non significant).

4 It is concluded that the postjunctional α_2-adenoceptor of human saphenous vein resembles an α_{2B}-ligand binding site more than an α_{2A}-ligand binding site.

Keywords: α_2-Adrenoceptor subtypes; α_{2B}-adrenoceptors; human saphenous vein; prazosin; ARC 239; BRL 44408

Introduction

Molecular cloning techniques have demonstrated that at least 3 genes, on chromosomes 2, 4 and 10, code for α_2-adrenoceptors (Lorenz *et al.*, 1990). However, α_2-adrenoceptors have also been subdivided into 4 subtypes based on selectivities of a series of agonists and antagonists in radioligand binding studies (model tissues for each subtype are also indicated): α_{2A} (human platelet), α_{2B} (neonatal rat lung), α_{2C} (OK cell line), α_{2D} (bovine pineal gland) (Bylund *et al.*, 1988; 1991; Simonneaux *et al.*, 1991). The gene located on chromosome 10 (α_2-C10) codes for the α_{2A}-subtype, whereas the genes on chromosomes 2 and 4 code for previously unrecognised subtypes of the α_{2B}-ligand binding site (Lomasney *et al.*, 1991). The α_{2C}-ligand binding site of the OK cell line (Bylund *et al.*, 1991) may simply be a species difference in the α_{2B}-ligand binding site (Lomasney *et al.*, 1991). The status of the α_{2D}-ligand binding site in relation to cloned receptors has not been established, although it may be identical to the α_2-ligand binding site in rat submaxillary gland (Michel *et al.*, 1989) which may be a species homologue of the human α_{2A}-adrenoceptor (Smith & Docherty, 1992). Little is known of the functional role of subtypes of α_2-adrenoceptor in mediating physiological responses.

The object of the present study was to characterize the functional postjunctional α_2-adrenoceptor mediating contraction of the human saphenous vein by use of a series of antagonists and to correlate these potencies with affinities for the α_{2A}-ligand binding site of human platelet or for the α_{2B}-ligand binding site of rat kidney. The following antagonists were employed in this study: the commonly used but non-selective α-adrenoceptor antagonists yohimbine, phentolamine and idazoxan, and the α_{2B}-subtype selective antagonists, prazosin and ARC 239 (see Bylund, 1988; 1991); chlorpromazine, which is α_{2B}-subtype selective in some (Bylund, 1988) but not all studies (Connaughton & Docherty, 1990a); the relatively non-selective antagonists BDF 893 and SKF 104078 (see Connaughton & Docherty, 1990a), and CH 38083 (see Vizi *et al.*, 1987); the α_{2A}-selective antagonist BRL 44408 (see Young *et al.*, 1989), the 5-HT_{1A}-receptor agonist 8-hydroxy-2-(n-dipropylamino)tetralin (8-OH-DPAT) which has α_2-adrenoceptor antagonist potency (Crist & Surprenant, 1987; Borton *et al.*, 1991), and the potent α_1-adrenoceptor antagonist, HV 723 (see Muramatsu *et al.*, 1990).

Methods

Human saphenous veins were obtained from patients (aged 32–59 years), predominantly female, undergoing surgical removal of varicose veins, and apparently healthy segments of side branches of the main vein were chosen for experimentation. Rings (3–5 mm) were attached to myograph transducers under 1 g tension for isometric tension recording in organ baths at 37°C in Krebs-Henseleit solution of the following composition (mmol l^{-1}): NaCl 119, $NaHCO_3$ 25, (+)-glucose 11.1, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.0, ascorbic acid 0.28. tetrasodium EDTA 0.03, corticosterone 0.03, cocaine 0.003, propranolol 0.001. Rings were pre-exposed to noradrenaline or phenylephrine (10 μmol l^{-1}) for 5 min, and thereafter bathing fluid was changed every 15 min except during agonist administration. After 30 min, a cumulative concentration-response curve was obtained to noradrenaline or phenylephrine (0.5 log unit increments) beginning with 1 nmol l^{-1} and 10 nmol l^{-1}, respectively. Once a maximum response to noradrenaline or phenylephrine had been reached, bathing fluid was changed, and 120 min later, following 60 min exposure to antagonist or vehicle, a second cumulative concentration-response curve to noradrenaline or phenylephrine was obtained in the continuing presence of antagonist or vehicle. Effects of antagonist on the EC_{50} (concentration producing 50% of maximum contraction) and maximum contractile response of noradrenaline or phenylephrine were corrected for changes occurring in paired vehicle experiments. Antagonist potency was expressed as the dissociation constant K_B from the equation $K_B = [B]/(DR-1)$, where [B] is the concentration of antagonist and DR is the agonist dose-ratio produced by the antagonist.

In some experiments, following pre-exposure to noradrenaline, a concentration contractile-response curve was carried

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out to the α-adrenoceptor agonists, xylazine (α_2-selective), phenylephrine (α_1-selective) or oxymetazoline (α_2-selective) or to the α-adrenoceptor antagonists in the absence of agonist, to assess what contractile potency, if any, these agents possessed. Maximum responses to test agents were expressed relative to the maximum response to noradrenaline (100 μmol l^{-1}).

Agonist concentrations producing 10–90% of maximum response were interpolated from each individual concentration-response curve, and graphs were constructed from the mean concentrations obtained. This helps to preserve curve shapes which can be distorted when individual curves are averaged vertically (see Docherty & Starke, 1981). Control curves were corrected for changes occurring in vehicle experiments.

Radioligand binding studies

Male Wistar rats (250–350 g) were obtained from Trinity College Dublin, Ireland.

Preparation of human platelet and rat kidney membranes was carried out exactly as described by Connaughton & Docherty (1990a). The resultant pellets were used immediately or stored at −20°C for later use.

In saturation experiments, aliquots of membrane suspension were incubated with various concentrations of [^{3}H]-yohimbine at 37°C (0.3–20 nmol l^{-1}; human platelet; incubation buffer: Tris-HCl 50 mmol l^{-1}, $MgCl_2$ 8 mmol l^{-1}, EGTA 5 mmol l^{-1}, pH 7.4 at 37°C) or 25°C (0.5–30 nmol l^{-1}; rat kidney; incubation buffer: Tris-HCl 50 mmol l^{-1}, EDTA 5 mmol l^{-1}, pH 7.4 at 25°C) for 30 min. In competition studies, [^{3}H]-yohimbine (5 nmol l^{-1}) was incubated with displacing ligands in concentrations from 0.1 nmol l^{-1} in 0.5 log unit increments for 30 min. Non-specific binding was determined in the presence of phentolamine 10 μmol l^{-1}. Assays were terminated by washing with ice-cold incubation buffer, followed by rapid vacuum filtration through Whatman GF/C filters, using a Brandel Cell Harvester. Radioactivity retained on filters was determined by liquid scintillation spectroscopy.

The inhibition constant (K_i) for displacement of radiolabelled ligand was determined from the formula: $K_i = IC_{50}/(1 + [^3H]/K_D)$ where IC_{50} is the concentration of competing ligand that inhibits radioligand specific binding by 50%, K_D is the dissociation constant for the radioligand (platelet: 5.75 ± 0.78 nmol $^{-1}$, $n = 6$; kidney: 8.80 ± 0.62 nmol l^{-1}, $n = 7$), and ^{3}H is the concentration of tritiated yohimbine employed (5 nmol l^{-1}).

Statistical evaluation

Results are expressed as mean ± s.e.mean or mean and 95% confidence limits. Effects of antagonist on agonist EC_{50} or maximum response were compared with the effects of vehicle, and antagonist K_B or K_i values were compared between tissues; Student's t test was used for unpaired or paired data, where appropriate.

Drugs

The following drugs were used: ARC 239 (2-(2,4-(*o*-methoxyphenyl) -piperazin-1-yl)-ethyl- 4,4 dimethyl-1,3-(2H,4H)-isoquinolindine chloride (gift: Karl Thomae, Biberach, Germany); BDF 8933 (4-fluoro-2-(imidazolin-2-ylamino)-isindoline maleate; gift: Beiersdorf, Hamburg, Germany); BRL 44408 (2-((4,5-dihydro-1H-imidazol-2-yl) methyl)-2,3-dihydro-1-methyl-1H-isoindole; gift: Smith Kline Beecham, Epsom, U.K.); CH 38083 (7,8-(methylenedioxy)-14-α-hydroxyalloberbane hydrochloride; gift: Chinoin, Budapest, Hungary); chlorpromazine hydrochloride (Sigma, Poole, U.K.); cocaine hydrochloride (Sigma); corticosterone (Sigma); HV 723 (α-ethyl-3,4,5-trimethoxy-α-(3- ((2- (2-methoxyphenoxy)ethyl)-amino)-propyl) benzene acetonitrile fumarate; gift: Hokurika, Seiyaku, Katsuyama, Japan); 8-OH-DPAT (8-hydroxy-2- (n-dipropylamino) tetralin; Research Biochemicals, Natick, U.S.A.); idazoxan hydrochloride (Research Biochemicals); noradrenaline bitartrate (Sigma); oxymetazoline hydrochloride (Sigma): phentolamine mesylate (gift: CIBA, Horsham, U.K.); prazosin hydrochloride (gift: Pfizer, Sandwich, U.K.); (±)-propranolol hydrochloride (Sigma); SKF 104078 (6-chloro-9-(3-methyl-2-butenyl) oxyl-3-methyl-1H-2,3,4,5-tetrahydro-3-benzazepine maleate; gift: Smith, Kline & French, King of Prussia, PA, U.S.A.); xylazine hydrochloride (gift: Bayer, Ireland); yohimbine hydrochloride (Sigma).

Drug stocks were dissolved in distilled water and dilutions made up in distilled water, with the exception of corticosterone which was made up in 100% ethanol.

Results

Human saphenous vein

In human saphenous vein, noradrenaline produced isometric contractions with an EC_{50} of 0.60 μmol l^{-1} (95% confidence limits of 0.36–1.02 μmol l^{-1}, $n = 29$ veins) and a maximum contraction of 1.62 ± 0.22 g. Phenylephrine produced contractions with an intrinsic activity of 0.80 relative to noradrenaline, but was approximately 15 times less potent (see Figure 1 and Table 1). Oxymetazoline had an intrinsic activity of 0.70 relative to noradrenaline but was only 1.6 times less potent, whereas xylazine produced virtually no contraction of the tissue (Table 1).

Yohimbine (0.3 μmol l^{-1}), chlorpromazine (1 μmol l^{-1}), BDF 8933 (0.3 μmol l^{-1}), prazosin (1–3 μmol l^{-1}), ARC 239 (0.3–1 μmol l^{-1}), SKF 104078 (3–10 μmol l^{-1}), phentolamine (1 μmol l^{-1}), idazoxan (0.3–1 μmol l^{-1}), CH 38083 (0.1–0.3 μmol l^{-1}), BRL 44408 (3–10 μmol l^{-1}), HV 723 (0.3–

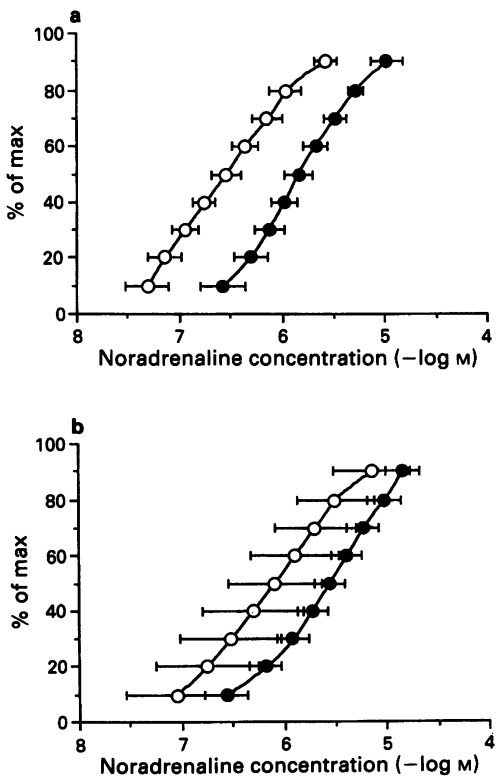


Figure 1 Mean concentration-response curves obtained to noradrenaline before (○) and after (●) 60 min exposure to (a) yohimbine (0.3 μmol l^{-1}) or (b) prazosin (1 μmol l^{-1}). The control curves were corrected for changes occurring in paired vehicle experiments. Horizontal bars represent s.e.mean from 3 (yohimbine) or 4 (prazosin) experiments.

Table 1 Contractile actions of test agents on the human saphenous vein relative to the maximum contraction to noradrenaline

Agent	*Max contraction* (% of NA max)	EC_{50} (− log M)
Noradrenaline	100%	6.22 ± 0.23
Phenylephrine	80.4 ± 13.8%	5.03 ± 0.42
Oxymetazoline	69.9 ± 43.3%	6.01 ± 0.95
Xylazine	1.5 ± 1.5%	/
SKF 104078	52.2 ± 8.5%	5.20 ± 0.66
8-OH-DPAT	28.9 ± 9.7%	5.06 ± 0.60
BDF 8933	29.4 ± 14.7%	5.49 ± 0.32

Values are mean ± s.e.mean (maximum contraction) or mean and 95% confidence limits (EC_{50}). For full names, see *Drugs* section.

1 μmol l^{-1}) and 8-OH-DPAT (10–30 μmol l^{-1}) all produced approximately parallel shifts in the concentration-response curve for noradrenaline without significantly affecting the maximum contractile response to noradrenaline. The effects of yohimbine (0.3 μmol l^{-1}) and prazosin (1 μmol l^{-1}) against contractions to noradrenaline are shown in Figure 1. None of the antagonists contracted the human saphenous vein in the above concentrations, except for SKF 104078 and 8-OH-DPAT (see Table 1). However, in higher concentrations, BDF 8933 did contract the human saphenous vein in the absence of agonist (Table 1).

Antagonist potencies (K_B) in the human saphenous vein against contractions to noradrenaline are shown in Table 2. Prazosin had similar potencies against noradrenaline and phenylephrine with K_B values of 6.44 ± 0.21 (n = 5) and 6.72 ± 0.30 (n = 5). Yohimbine was 0.7, 2, 9 and 45 times more potent than HV 723, ARC 239, prazosin and BRL 44408, respectively. Hence, the order of potency was yohimbine = HV 723 = ARC 239 > prazosin > BRL 44408.

Radioligand binding studies

K_i values for the displacement by 12 antagonists of [^{3}H]-yohimbine binding to human platelet and rat kidney were obtained (see Table 2). Hill slopes for all antagonists were close to unity, so that it was assumed that a single homogeneous population of ligand binding sites was present in both tissues. The α_{2B}-ligand binding site of rat kidney differed from the α_{2A}-ligand binding site of human platelet in the affinities of prazosin, ARC 239, HV 723 (higher affinity in kidney) and BRL 44408 (lower affinity in kidney).

Yohimbine was 29, 389, 263 and 6 times more potent than HV 723, ARC 239, prazosin and BRL 44408, respectively, in human platelet, and 6, 15, 6 and 66 times more potent, respectively, in rat kidney. Hence, the order of potency was yohimbine > BRL 44408 > HV 723 > prazosin = ARC 239 in platelet, but yohimbine > HV 723 = ARC 239 > prazosin > BRL 44408 in kidney.

Correlation between tissues

The functional postjunctional α_2-adrenoceptor of human saphenous vein correlated better with the ligand binding site of rat kidney (r = 0.71, n = 12, P < 0.01) than with that of human platelet (r = 0.56, n = 12, NS) (Figure 2).

Discussion

The main object of this study was to investigate the subtype of α_2-adrenoceptor mediating contractions of the human saphenous vein, by correlating antagonist potencies with affinities for α_{2A}- and α_{2B}-ligand binding sites. The identification of the α_2-adrenoceptor subtype will be discussed in terms of absolute and relative potencies of antagonists, correlation analysis and in terms of the actions of agonists.

Firstly, it is necessary to consider the validity of the methodologies employed in this study. The K_i values obtained from ligand binding studies give an accurate guide to the affinities of antagonists at the respective subtypes of receptor. It was assumed that the ligand binding sites in human platelet and rat kidney represented homogeneous populations of α_{2A}- and α_{2B}-ligand binding sites, respectively, since there was no evidence from Hill slopes for more than one subtype. However, it has recently been reported that the rat kidney contains both α_{2A}- and α_{2B}-ligand binding sites (Uhlen & Wikberg, 1991), in agreement with Lomasney *et al.*

Table 2 K_i values obtained for α-adrenoceptor antagonists at displacing [^{3}H]-yohimbine binding in human platelet (α_{2A}) and rat kidney (α_{2B}) membranes and K_B values obtained against contractions to noradrenaline in human saphenous vein (hsv)

Antagonist	*Platelet* K_i	*Kidney* K_i	*hsv* K_B
Chlorpromazine	6.48 ± 0.29	6.29 ± 0.08	6.98 ± 0.92
BDF 8933	8.72 ± 0.25	8.91 ± 0.60	7.60 ± 0.17
Prazosin	5.62 ± 0.40	7.12 ± 0.17	6.44 ± 0.21
ARC 239	5.45 ± 0.04	6.76 ± 0.13	7.05 ± 0.61
Yohimbine	8.04 ± 0.25	7.93 ± 0.07	7.37 ± 0.16
BRL 44408	7.28 ± 0.42	6.11 ± 0.32	5.72 ± 0.67
HV 723	6.58 ± 0.42	7.22 ± 0.67	7.52 ± 0.40
Idazoxan	7.30 ± 0.37	7.49 ± 0.23	6.94 ± 0.26
SKF 104078	6.52 ± 0.54	6.25 ± 0.06	6.55 ± 0.19
Phentolamine	7.61 ± 0.28	7.24 ± 0.50	7.04 ± 0.46
CH 38083	8.65 ± 0.41	8.39 ± 0.08	7.52 ± 0.24
8-OH-DPAT	5.44 ± 0.06	6.41 ± 0.13	5.40 ± 0.13

Values are mean and 95% confidence limits (− log M) from at least 3 experiments.
For full names, see *Drugs* section.

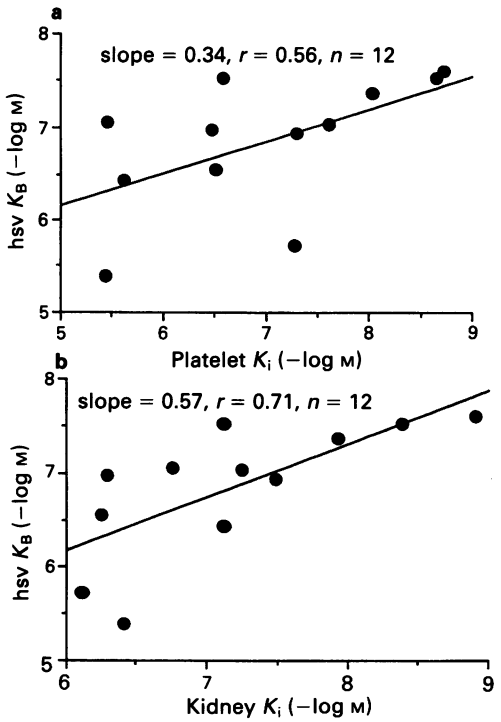


Figure 2 Correlation between antagonist K_i (− log M) obtained at α_{2A}-ligand binding sites of human platelet (a) or at α_{2B}-ligand binding sites of rat kidney (b) and antagonist K_B obtained at postjunctional α_2-adrenoceptors in human saphenous vein (hsv). Values are taken from Table 2.

(1991) who found that rat kidney expresses homologues of both α_2-C10 (α_{2A}) and α_2-C-2 (α_{2B}). However, α_{2A}-sites represented only 15% of the α_2-binding sites labelled in rat kidney by the radioligand [^{3}H]-RX821002, which shows 7 fold selectivity for α_{2A}, and could only be demonstrated by using ARC 239 to remove α_{2B} sites (Uhlen & Wikberg, 1991). In our studies, using [^{3}H]-yohimbine, which shows no selectivity between α_{2A}- and α_{2B}-sites, we were unable to identify α_{2A}-sites. Since a small population of α_{2A}-sites is unlikely to affect K_i values markedly, the K_i values obtained in rat kidney probably represent binding to α_{2B}-ligand binding sites. In their study, Uhlen & Wikberg (1991) also reported two subtypes of α_{2B}-ligand binding site in the rat kidney, based on the selectivity of the compound guanoxabenz, whereas antagonists such as yohimbine, BDF 8933, prazosin, ARC 239 and chlorpromazine showed little selectivity between subtypes of α_{2B}-site. In our study, using the latter compounds, we were unable to find evidence for two subtypes of α_{2B}-ligand binding site in rat kidney.

The model chosen for the α_{2A}-ligand binding site was a human receptor, whereas the model for the α_{2B}-ligand binding site was a rat receptor. If the postjunctional α_2-adrenoceptor in human saphenous vein was an α_{2A}-subtype, an excellent correlation with the human platelet ligand binding site would be expected, but if it was an α_{2B}-site the correlation with the rat kidney ligand binding site might be less than expected due to species differences or because of the complication that there are two α_{2B}-like receptors (see Lomasney et al., 1991). Due to the difficulties inherent in amassing data from the human saphenous vein, it was not possible to obtain pA_2 values from Schild plots for the 12 antagonists, so that the less reliable K_B value was used. Admittedly, we have previously obtained pA_2 values for three of the antagonists employed in this study (yohimbine, prazosin, SKF 104078: Connaughton & Docherty, 1990b), so that these three at least can be regarded as competitive antagonists in the human saphenous vein. The postjunctional α-adrenoceptors of the human saphenous vein appear to be a homogeneous population of α_2-adrenoceptors with no evidence for α_1-adrenoceptors since: yohimbine was approximately 10 times more potent than prazosin in antagonizing contractions to noradrenaline; both yohimbine and prazosin produced parallel shifts in noradrenaline concentration-response curves (see Figure 1); both yohimbine and prazosin behaved competitively against noradrenaline with slopes of Schild plots not significantly different from negative unity (Connaughton & Docherty, 1990a); prazosin inhibited contractions to the α_1-adrenoceptor selective agonist phenylephrine with a K_B similar to that obtained against noradrenaline; the potent α_1-adrenoceptor antagonists HV 723 (see Muramatsu et al., 1990) had relatively low potency against noradrenaline.

In terms of absolute antagonist potencies, it is worth considering separately antagonists which do or do not distinguish between ligand binding sites. The non-selective ligands had lower potency in human saphenous vein than would be expected from ligand binding affinity (with the exception of chlorpromazine), and the potency was more than 1 log unit lower for BDF 8933 and CH 38083. The α_{2B}-subtype selective antagonists ARC 239 and HV 723 showed potency in the human saphenous vein consistent with their high affinity binding site. The α_{2B}-subtype antagonist prazosin showed potency in the human saphenous vein intermediate between its high and low affinities, but this may have been simply due to the fact that many of the antagonists showed lower than expected potency in human saphenous vein, and hence suggests an action of prazosin at its high affinity site.

However, 8-OH-DPAT, which shows selectivity for the α_{2B}-ligand binding site of rat kidney, showed a lower potency in the human saphenous vein which was close to its affinity for α_{2A}-ligand binding sites. The α_{2A}-subtype selective antagonist, BRL 44408, showed potency in the human saphenous vein consistent with its low affinity site. Taken together, these potencies suggest that the α_2-adrenoceptor of the human saphenous vein resembles the α_{2B}-subtype more than the α_{2A}-subtype. Furthermore, the order of antagonist potency in human saphenous vein (yohimbine = HV 723 = ARC 239 > prazosin > BRL 44408) resembles the order of potency at the α_{2B}- (yohimbine > HV 723 = ARC 239 > prazosin > BRL 44408), but not the order of potency at the α_{2A}-ligand binding site (yohimbine > BRL 44408 > HV 723 > prazosin = ARC 239).

In terms of correlation analysis, potency in human saphenous vein correlated better with affinity for the α_{2B}-ligand binding site of rat kidney ($r = 0.71$) than with the α_{2A}-ligand binding site of human platelet ($r = 0.56$). The low correlation with the human platelet sites seems to rule out the possibility that the receptor in the human saphenous vein is α_{2A}. However, the correlation with the α_{2B}-ligand binding site of rat kidney is not strong enough to identify definitely the receptor of human saphenous vein as α_{2B}. Possible explanations for this relatively poor correlation are that the differences between potency and affinity reflect species differences in the peripheral-type α_{2B}-adrenoceptor as found in rat kidney (homologue of human α_2-C-2; see Lorenz et al., 1991) or that the receptor in human saphenous vein represents the α_{2B}-subtype found in brain (α_2-C-4; see Lorenz et al., 1991) and so differs from the rat kidney α_{2B}. Indeed, it has been reported that the human kidney α_2-ligand binding site differs from that of the rat in the affinities of the ligands ARC 239 and prazosin (Neylon & Summers, 1985). The other explanation that the functional α_2-adrenoceptor of human saphenous vein represents a new subtype of receptor is less likely, and is inconsistent with molecular cloning evidence for only 3 genes coding for α_2-adrenoceptors: an α_{2A} and two subtypes of α_{2B} (Lomasney et al., 1991; Flordellis et al., 1991).

Although agonist potencies cannot be directly correlated with affinity in ligand binding studies, some information can also be obtained from our agonist studies. Comparing the phenylethylamines noradrenaline and phenylephrine, which might be expected to have similar receptor occupation/response characteristics, phenylephrine was 15 times less potent than noradrenaline in contracting the human saphenous vein. In ligand binding studies of human α_2-adrenoceptors, phenylephrine was 0.6 times less potent than noradrenaline at α_{2A} but 12 times less potent at α_{2B} (Regan et al., 1988), so that the relative agonist potencies in human saphenous vein suggest an α_{2B}-adrenoceptor. The relatively low potency of oxymetazoline in the human saphenous vein might also indicate an α_{2B}-adrenoceptor, since oxymetazoline has high potency at α_{2A} (Bylund et al., 1988; Michel et al., 1989), although oxymetazoline may have different occupation/response characteristics to phenylephrine and noradrenaline. Of the antagonists which produced contractions of the human saphenous vein, BDF 8933 produced contractions only at concentrations much higher than its antagonist K_B suggesting that they were not due to α_2-adrenoceptor agonism. However, SKF 104078 and 8-OH-DPAT produced contractions at concentrations not much higher than their K_B values, so that the contractions may possibly have been due to α_2-adrenoceptor agonism.

Subtypes of functional α_2-adrenoceptors have been investigated in a variety of other tissues. α_{2A}-Adrenoceptors are reported to be present prejunctionally in the rat vas deferens (Connaughton & Docherty, 1990), rat submandibular gland (Docherty & Connaughton, 1991), and rabbit brain cortex (Limberger et al., 1991). The low potency of prazosin prejunctionally at the cholinergic nerves of guinea-pig ileum (Kaposci et al., 1987), at the noradrenergic and 5-hydroxytryptaminergic nerves of rat brain synaptosomes (Gobbi et al., 1991), and at antagonizing actions of endogenous noradrenaline in rat cerebral cortex (Harsing & Vizi, 1991), may suggest that these receptors are also α_{2A}. However, α_{2A}-adrenoceptors have also been reported on pancreatic islets where they mediate inhibition of insulin secretion (Niddam et al., 1990). α_{2B}-Adrenoceptors have been reported prejunctionally in the rat atrium (Connaughton & Docherty, 1990a), and possibly also in rat

cerebral cortex, mediating the response to exogenous noradrenaline (Harsing & Vizi, 1991). The postjunctional α_2-adrenoceptor mediating contractions of dog saphenous vein has been previously investigated, but results from that tissue are complicated by the presence of α_1-adrenoceptors (Hicks *et al.*, 1991).

In conclusion, the postjunctional α_2-adrenoceptor mediating contractions of the human saphenous vein resembles an α_{2B}-ligand binding site more than an α_{2A}-ligand binding site, but correlation with human α_{2B}-ligand binding sites will be necessary to confirm this identification.

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Differences in evoked dopamine efflux in rat caudate putamen, nucleus accumbens and tuberculum olfactorium in the absence of uptake inhibition: influence of autoreceptors

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1 Dopamine efflux following single pulse or train of pulse stimulations was measured in slices of rat caudate putamen, nucleus accumbens and tuberculum olfactorium, using fast cyclic voltammetry at a carbon fibre microelectrode; 1, 5, 10, 20 or 50 pulses were applied at each location at frequencies varying from 10 Hz to 500 Hz.

2 There are significant differences in the ability of the different regions to increase dopamine efflux following single or repeated electrical stimulation.

3 Highest release in response to a single pulse is observed in the caudate putamen (approximately 250 nM dopamine), but the ratio of the peak dopamine overflow following a train of 20 pulses (50 Hz) when compared to a single pulse is rarely greater than three.

4 Release following single pulse stimulation in the nucleus accumbens (approximately 185 nM dopamine) is often slightly less than in the caudate putamen, but the ratio of peak release when trains of 20 pulses (50 Hz) are compared to single pulse stimulation has been as great as seven fold.

5 The tuberculum olfactorium releases the least dopamine of the three regions following a single pulse stimulation (approximately 40 nM dopamine), but the ratio of peak dopamine release following trains of 20 pulses (50 Hz) when compared to single pulse can result in a value approaching 20.

6 When 20 pulses are applied to the caudate putamen at frequencies ranging from 10 to 500 Hz, the peak efflux is essentially the same (frequency/release profile is flat), with the maximum increase only 140% that of a single pulse.

7 By contrast, in the nucleus accumbens and the tuberculum olfactorium, well defined frequency-release relationships are seen following 20 pulses applied at 10, 20 and 50 Hz; at higher frequencies the dopamine release decreases as frequency is increased.

8 Experiments with sulpiride or metoclopramide (dopamine D_2 receptor antagonists) indicate that dopamine release in the caudate putamen is not regulated by dopamine autoreceptor activation by endogenous dopamine under our experimental conditions (in the absence of reuptake inhibtion). By contrast, in the nucleus accumbens and in the tuberculum olfactorium, we present evidence to show that at frequencies of stimulation up to 20 Hz, endogenous dopamine acts at dopamine autoreceptors to inhibit further release of dopamine but a minimum exposure time of between 500 and 1000 ms is needed for the response to D_2 autoreceptor activation by endogenously released dopamine to be seen.

Keywords: Dopamine release; autoreceptors; fast cyclic voltammetry; striatum; nucleus accumbens; tuberculum olfactorium

Introduction

The dopamine innervation of the forebrain is derived from three clusters of nerve cell bodies located in the region of the retrorubral nucleus, substantia nigra (SN) and ventral tegmental area (VTA), which have been designated as A8, A9 and A10 (Ungerstedt, 1971), respectively. The major target region of areas A8 and A9 in the rat is the caudate putamen (CPu), and from area A10, the mesolimbic structures including the nucleus accumbens (NAc), and the tuberculum olfactorium (TuO).

Studies *in vivo* in anaesthetized or conscious rats (see Chiodo (1988) and White (1991) for reviews), show that the firing rates of nigrostriatal dopaminergic neurones consist of irregular low frequency discharges, interspersed with bursts of higher frequency firing. Grace & Bunney (1984) recorded the non bursting spontaneous firing rate of 597 cells and found a mean rate of 4.5 ± 1.7 Hz, with fewer than 10% of cells discharging at less than 2.5 Hz and fewer than 10% discharging at more than 7 Hz, and Freeman *et al.* (1985) showed that cells firing in bursts could discharge 4 to 10 spikes at rates of up to 25 Hz. The activity of neurones in the VTA has many similarities with those derived from the SN; bursts of high frequency activity are detected and can be linked to external stimuli (Freeman & Bunney, 1987).

Pharmacological evidence suggests that not all dopaminergic neurones respond identically to chemical stimulation. Chiodo & Bunney (1983) and White & Wang (1983a,b) showed that the nerve cell bodies of the VTA respond differentially with respect to the neurones of the SN following long term administration of atypical anti-psychotic drugs such as clozapine, thioridazine, and sulpiride. Whereas conventional anti-psychotic drugs decrease unit activity in the SN and VTA, the atypical anti-psychotics reduce unit activity in the VTA, without affecting the SN. *In vivo* studies in our laboratories using fast cyclic voltammetry (FCV; Millar *et al.*, 1985) to monitor electrically stimulated dopamine release in the CPu or NAc in the anaesthetized rat, indicated differential sensitivity to dopamine receptor agonists and blockers, and inhibitors of dopamine re-uptake in these axon terminal regions (Stamford *et al.*, 1987; 1988; 1991).

Anatomical studies give us information about the projections of dopamine axon terminals from their cells of origin, and pharmacological experiments *in vivo* give qualitative results which indicate that it may be simplistic to regard all dopaminergic neurones as a homogeneous population. None

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of the above approaches allows quantitative characterization of mechanisms which regulate the release of dopamine in the central nervous system. We have recently shown (Palij *et al.*, 1990; Bull *et al.*, 1990; Trout *et al.*, 1991) that dopamine release can be measured by FCV in brain slices following electrical stimulation of the axon terminals, and that this preparation is suitable for both qualitative and quantitative characterization of dopamine autoreceptors which regulate release of dopamine from axon terminals.

While it is important that single electrical pulse stimulation is used in studies where the role of autoreceptors in regulation of dopamine release is under examination (Palij *et al.*, 1990; Bull *et al.*, 1990), the brain slice in conjunction with FCV at small carbon fibre micro-electrodes, offers the opportunity to examine the characteristics of endogenous dopamine release from axon terminals in a time scale not hitherto possible over a wide range of stimulation conditions. This can be done with good anatomical precision, and as the electrophysiological discharge characteristics of neurones in the mesostriatal and mesocorticolimbic systems are different, it was of interest to examine whether this was reflected in differences in dopamine release at the axon terminals. We have compared the effects of single pulse and trains of pulse stimuli applied at different frequencies on dopamine release in three dopaminergic axon terminal regions; in addition, we have examined the role of dopaminergic axon terminal autoreceptors in regulating dopamine release.

Methods

Preparation of brain slices

Male Wistar rats (140–220 g) were killed by cervical dislocation, and the brains gently and rapidly removed into ice cold oxygenated artificial cerebrospinal fluid (ACSF). The brain was placed on a raised plinth covered with an ACSF soaked filter paper, and coronal cuts made at anterior co-ordinates approximately +2.4 and 0.0 mm, relative to the point of Bregma (Paxinos & Watson, 1986) which resulted in a slab of tissue, approximately 2.0 mm thick containing the caudate putamen, nucleus accumbens and tuberculum olfactorium. The slice was kept moist at all times by liberal soaking with oxygenated ACSF. All sectioning was made with razor blades, which had been wiped with 99% ethanol to remove grease. The block was sectioned down the midline, dorsally to bisect the coronal block, and further dorsal cuts were made to remove cortical tissue. The block was then mounted onto the chuck of an Oxford vibratome, using Loctite cyanoacrylate rapid setting glue, orientated such that the coronal cut lay in the horizontal plane. The tissue was irrigated at all times with ice cold, oxygenated ACSF. All cutting with the vibratome was made at a speed of 10 mm per minute, using maximum amplitude, with a Gillette razor blade, set at an angle of 65° to the vertical. A slice was then cut according to the above slicing parameters which removed approximately 400 μm of tissue. The slice was discarded, the stage adjusted and further slices, 350 μm thick, were then cut. A slice approximately +1.0 mm anterior to Bregma, was gently transferred on a small wetted spatula into the brain slice chamber.

Tissue slices were placed on a stainless steel mesh placed in the perfusion well of a thermostatically controlled chamber made to the design of Richards & Tegg (1977). The slice was held in place with a fine nylon mesh, stretched across a framework which was friction held on the walls of the perfusion chamber. Temperature of the incubation chamber was maintained at 32°C by means of an external circulator pump (model HAAKE 1500) and the slice was fully submerged and perfused at a rate of 1 ml per minute by means of one of two Razel model A2 syringe pumps fitted with three way taps such that a uninterrupted flow of ACSF could be maintained during changes of solution.

Fast cyclic voltammetry

Dopamine overflow in brain slices was detected by means of carbon fibre microelectrodes constructed (as described by Armstrong-James & Millar (1979)) of Cortaulds Grafil carbon fibre, embedded in borosilicate glass. The exposed tip of the microelectrode was a carbon cylinder 7 μm in diameter, 30–60 μm long which was located by use of a micromanipulator at a depth of 80 μm below the surface of the brain slice. Fast cyclic voltammetry was performed using a Millar voltameter (PD Systems, Surrey, UK); the stainless steel grid of the perfusion chamber was the auxiliary electrode and a silver chloride pellet in a saline bridge filled with artificial CSF in a plastic pipette tip acted as the reference electrode. A triphasic driving potential (see Figure 1 for voltage waveform) was applied to the working electrode at 480 Vs^{-1}. Each scan lasted approximately 15 ms, and scans were repeated at 2 Hz. Changes at the peak oxidation potential for dopamine (+595 mV) were monitored by a sample and hold circuit and output to a Y-t chart recorder and to a CED 1401 interface; data were stored as files using CED software (SIGAVG and CHART).

Driving potential and current flowing in the working electrode was monitored on a Nicolet 310 digital storage oscilloscope with on board facilities for waveform subtraction; this allowed routine inspection of the faradaic current during the course of any experiment. Following each experiment in a brain slice, the electrode was calibrated in a standard solution of dopamine; electrode sensitivity and voltammetric signals were routinely assessed.

Electrical stimulation

Bipolar tungsten stimulating electrodes (exposed tip length 100 μm, tip separation 200 μm) were placed in a band of grey matter 200 to 400 μm away from the carbon fibre electrode, 80 μm below the surface of the slice. The stimulating electrodes were connected to a DS2 constant voltage stimulator, and timing of single pulses or trains of pulses was achieved by means of Neurolog (Digitimer) signal conditioning apparatus. Single electrical pulses (0.1 ms duration, 20 V) were applied at 2 min intervals; trains of pulses at the frequencies indicated, were applied at 5 min intervals.

Drugs and solutions

ACSF of the following composition was prepared fresh every day (all values mM): NaCl 125, KCl 2, KH_2PO_4 1.25, $MgSO_4$ 2, $NaHCO_3$ 25, D-glucose 11, $CaCl_2$ 2, in distilled water, and equilibrated at 32°C for at least 1 h with 95% O_2:5% CO_2 before use. Nomifensine maleate (Hoechst) and metoclopramide monohydrochloride (Sigma) were dissolved in distilled water, and (−)-sulpiride (Sigma) in tartaric acid (Sigma). Dilutions were made in ACSF to the concentrations indicated.

Statistics

Results are expressed as arthimetic means ± standard error of the mean (s.e.mean). Groups were compared with unpaired Student's *t* test (two-tailed) if analysis of variance tests indicated a significant difference. *n* = number of experiments (= number of brain slices).

Results

Electrical stimulation of brain slices containing CPu, NAc or TuO using single pulse stimulation, or trains of pulses at varying frequencies, resulted in an increase in the oxidation potential monitored at +595 mV on the sample and hold output of the voltammeter. The time course of such a response in each region following a single electrical pulse is

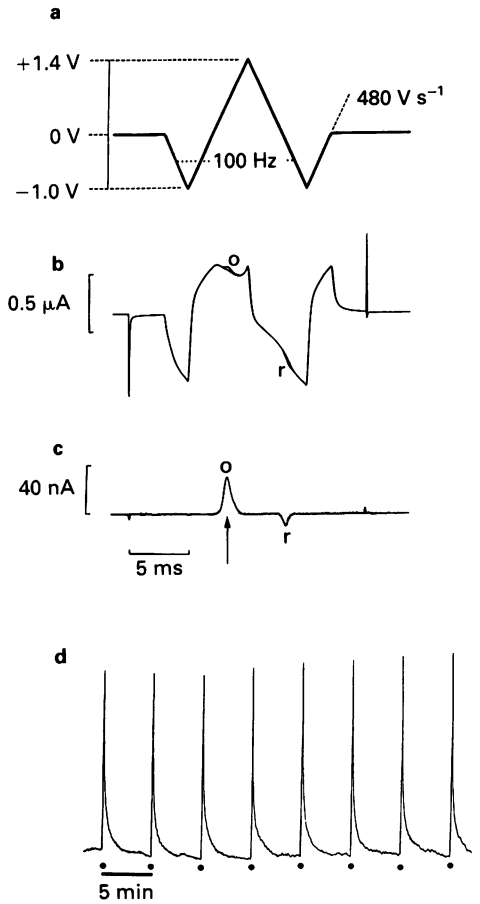


Figure 1 The principles of fast cyclic voltammetry are shown. (a) One and a half cycles of a symmetrical 100 Hz saw-tooth waveform, with a voltage ramp of 480 Vs^{-1}, are applied to a carbon fibre microelectrode twice a second, sweeping from zero volts (relative to a silver/silver chloride reference electrode), to −1.0 V, +1.4 V, −1.0 V and back to 0.0 V. (b) The charging current waveform is shown. When dopamine is present, oxidation (o) and reduction (r) peaks appear on the waveform (charging current plus faradaic current); the oxidation peak appears at a point corresponding to the oxidation potential on the input waveform (approximately +595 mV under these conditions). The reduction peak occurs at approximately −200 mV. (c) The faradaic waveform obtained after the subtraction of the charging current waveform (in the absence of dopamine) from that in the presence of dopamine (shown in b above). The time scale of events in the top three panels is indicated in (c). The Millar voltammeter has a 'sample and hold' facility which, when set at the dopamine oxidation potential (↑), monitors the peak height and produces a record of the peak height with time. The sample and hold signal is fed to a Y-t chart recorder (tracing shown in d), and is digitised (using CED 1401 A-D converter controlled by the CED CHART programme) for subsequent offline analysis (CED SIGNAL AVERAGER programme). The example shown here shows responses to trains of 20 pulses applied at 50 Hz in the nucleus accumbens. The dopamine peaks obtained may be calibrated against those obtained by perfusing known concentrations of dopamine (not shown).

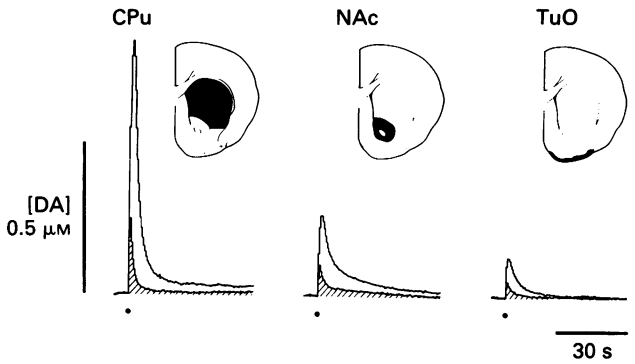


Figure 2 The time course of the dopamine (DA) oxidation signal in three individual experiments, as recorded on a computer from the sample and hold output of the voltammeter. The signals represent a plot of the faradaic dopamine oxidation current with time (method as described in Figure 1d but with an expanded time base). The traces show the changes in dopamine overflow following single electrical pulse stimulation (●) in the brain regions indicated, both in the absence (shaded trace) and presence (open trace) of nomifensine (1.0 μM). The calibration bars indicate extracellular dopamine concentration and time in seconds. CPu = caudate putamen; NAc = nucleus accumbens; TuO = tuberculum olfactorium.

shown in Figure 2. Calibration of electrodes allowed an estimate of the overflow; in response to single pulse stimulation (20 V, 0.1 ms), greatest overflow was consistently achieved in the CPu (0.250 ± 0.028 μM ($n = 24$)), with lower amounts released in the NAc (0.185 ± 0.024 μM ($n = 20$)) and lower still in the TuO (0.042 ± 0.003 μM ($n = 11$)). The response was potentiated in the presence of nomifensine (1.0 μM): increases of 247 ± 26%, 168 ± 18% and 248 ± 51% were seen in CPu ($n = 8$), NAc ($n = 12$) and TuO ($n = 5$), respectively. In the same experiments, the half-times ($t_{\frac{1}{2}}$) of the response decay in these same regions were 1.4 ± 0.1, 1.4 ± 0.1 and 1.5 ± 0.2 s, respectively: significant increases in the $t_{\frac{1}{2}}$ of 106 ± 9, 113 ± 10 and 154 ± 34% were seen in the presence of nomifensine (Figure 2).

The voltammetric signal was routinely monitored on the digital storage oscilloscope; electronic subtraction of the charging current resulted in a faradaic current waveform (a subtractogram) which was indistinguishable from that obtained in solutions of dopamine. At low concentrations, the reduction peak of dopamine is not clearly defined in all cases, but the oxidation peak potential occurred at about +595 mV. A faradaic signal of dopamine at the concentration indicated, and signals obtained in the three brain regions studied following electrical stimulation are shown in Figure 3.

The effects of increasing the frequency of stimulation when 20 pulses were applied to slices containing the regions indicated are shown in Figure 4. These are raw data traces obtained from the sample and hold output on a slower time base than that illustrated for single pulse stimulation in Figure 2. In the CPu, increasing the frequency of a 20 pulse train from 10 to 100 Hz resulted in a small increase in dopamine efflux (maximally 140% that of a single pulse). In the NAc and TuO, peak efflux following 20 pulses when compared to a single pulse was frequency-dependent, with a maximum relative release at 50 Hz, declining at 100 Hz and above.

Increasing the number of pulses in trains of stimuli at 10, 20, 50 and 100 Hz in the three brain regions produced the results illustrated in Figure 5. In this figure (in contrast to Figures 2 and 4), the results are expressed as a ratio of the peak overflow obtained during a train of stimulation pulses divided by that obtained following a single pulse of electrical stimulation. At all frequencies studied, increasing the number of pulses in a train resulted in only a modest (maximally 2.4 fold) increase in peak overflow ratio in the CPu.

Peak overflow ratios in the NAc were significantly greater than in the CPu, at frequencies of 10 Hz and above, and were maximally 7 times greater than that of a single pulse. In the TuO, peak overflow of dopamine at frequencies up to 100 Hz was proportionally related to the number of pulses applied.

Experiments with autoreceptor blockers

In order to study the role of inhibitory dopamine autoreceptors on regulation of dopamine overflow during electrical stimulation by trains of different numbers of pulses applied

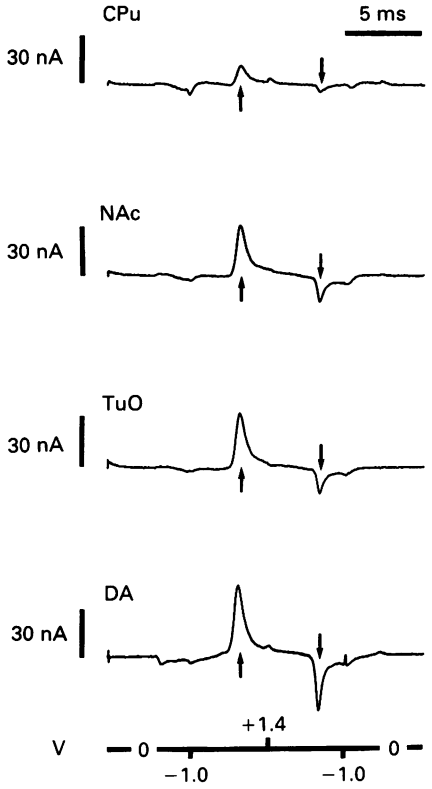


Figure 3 The data shown are faradaic waveform signals derived from scans from which the charging current has been subtracted (see Figures 1b and 1c for explanation and method). The resulting faradaic signals obtained in the brain regions indicated (caudate putamen (CPu), nucleus accumbens (NAc) and tuberculum olfactorium (TuO)) following electrical stimulation with 50 pulses/ 50 Hz are shown. Oxidation and reduction current peaks are visible at +595 (↑ arrow) and −200 mV (↓ arrow), respectively; the bottom signal (DA, dopamine) shows the signal obtained in a 1 μM solution of dopamine in artificial cerebrospinal fluid. The vertical calibration bars indicate the current detected at the carbon fibre microelectrode. The bottom horizontal bar represents the voltage sweeps of the input waveform and the time bar provides a calibration for the duration of the voltammetric scan in milliseconds.

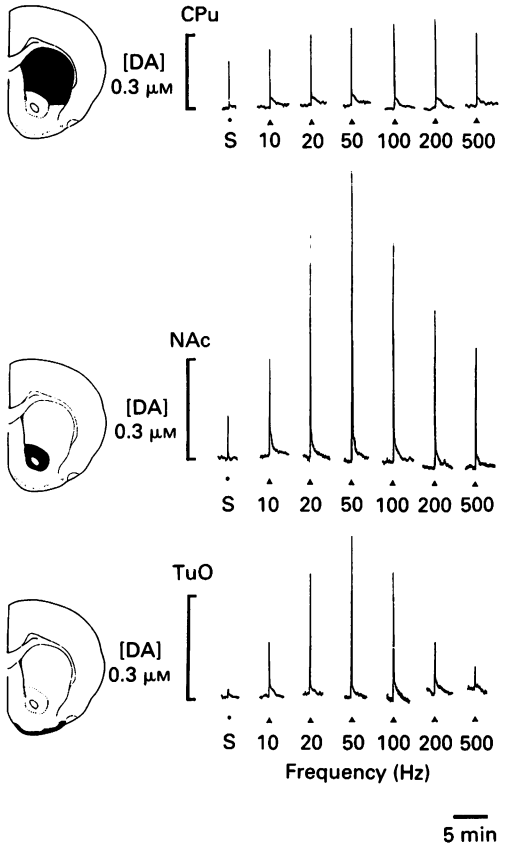


Figure 4 Sample and hold traces representing changes of dopamine (DA) efflux following single pulse (●) or 20 pulse stimulation trains (▲) at the frequencies indicated in caudate putamen (CPu), nucleus accumbens (NAc) and tuberculum olfactorium (TuO). The dopamine calibration bar represents 0.3 μM dopamine, the time bar represents 5 min.

at varying frequencies (which results in stimulations of varying duration), the effects of dopamine D_2 receptor antagonists (sulpiride, 1.0 μM or metoclopramide, 0.3 μM) were tested in the three regions under conditions where the number of pulses was varied from 5 to 50 pulses and the frequency varied from 10 to 100 Hz. Neither antagonist had any significant effect on efflux evoked by single pulse stimulation. The results are shown in Figure 6 for CPu, NAc, and TuO. Under our experimental conditions, the presence of sulpiride (1 μM) or metoclopramide (0.3 μM; Figure 6) did not alter the ratio of each overflow to a train of pulses, relative to overflow following single pulse stimulation in the CPu.

In the NAc, the presence of metoclopramide resulted in an increase in dopamine overflow ratio when 10 or 20 pulses were applied at 10 Hz, and when 20 pulses were applied at 20 Hz (Figure 6). A similar pattern was observed in the TuO; 10 or 20 pulses at 10 Hz or 20 pulses at 20 Hz in the presence of metoclopramide results in a signficantly greater increase of dopamine than in its absence (Figure 6). At higher frequencies (50 and 100 Hz), even when the train duration was increased (by increasing the number of pulses applied to 50), metoclopramide did not increase the dopamine overflow.

Discussion

Single pulse electrical stimulation reproducibly evoked efflux of dopamine in the CPu, NAc and TuO; the peak extracellular dopamine concentration following such stimulation was typically 250 nM (CPu), 185 nM (NAc) and 42 nM (TuO). The dopamine signal declined rapidly following single pulse stimulation. No significant difference was seen between the regions in the decay time of the oxidation peak, nor was there any significant difference in the percentage change of this parameter in the presence of nomifensine (Figure 2). While it cannot be claimed that FCV directly monitors release of dopamine (because the carbon fibre microelectrode does not rest in the synaptic cleft but in the extracellular fluid space), the time course of overflow of dopamine following electrical stimulation is in all likelihood, closely linked to dopamine release into the synaptic cleft. The differences in evoked dopamine overflow do not appear to reflect tissue content of dopamine: radiometric analysis indicates approximately 71, 87 and 95 pg dopamine μg^{-1} protein in CPu, NAc and TuO, respectively (Versteeg *et al.*, 1976).

While dopamine efflux has been monitored in the CPu (Palij *et al.*, 1990) and NAc (Bull & Sheehan, 1991) by fast cyclic voltammetry, we have shown that by using electrochemical, biochemical and pharmacological tests, it is possible to establish that it can also be monitored in the TuO. The TuO is a small structure, and it is only by virtue of the small size of the electrodes used in these studies (a carbon fibre cylinder 30 μm long, 7 μm diameter) that this is possible. Electrochemical and pharmacological data (not all shown) support our conclusion that dopamine is released in slices of

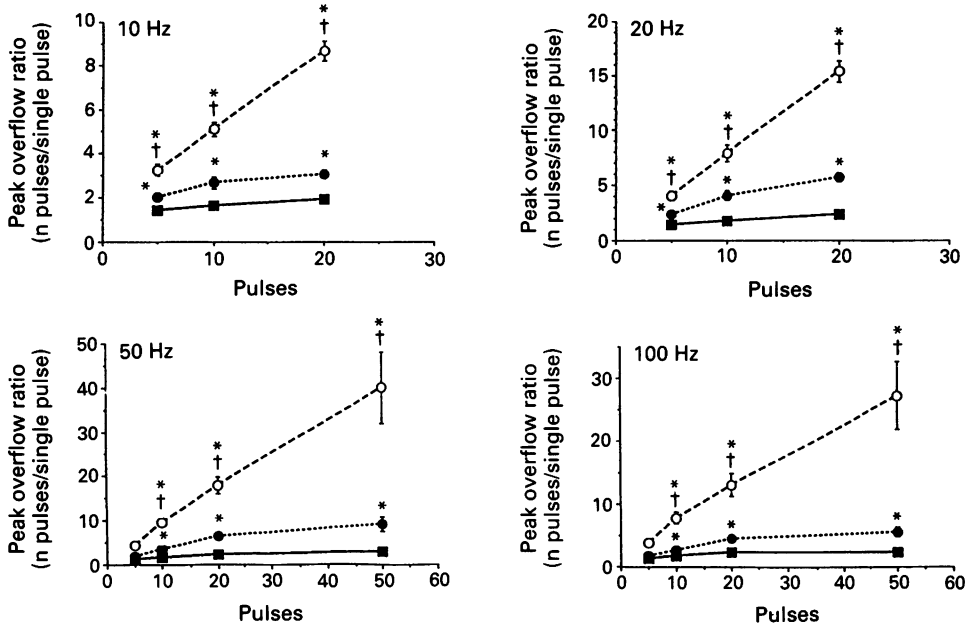


Figure 5 The effects of varying pulse number and pulse frequency on peak dopamine overflow in caudate putamen (CPu; ■), nucleus accumbens (NAc; ●) and tuberculum olfactorium (TuO; ○). Peak overflow is expressed as a ratio of the peak dopamine efflux following the train of 5, 10, 20 or 50 pulses applied at a frequency of 10, 20, 50 or 100 Hz, relative to the overflow evoked following single electrical pulse stimulation in the region (mean with s.e.mean shown by vertical bars; $n = 5$ to 24). $*P<0.05$ significantly different from CPu. $\dagger P<0.05$ significantly different from NAc.

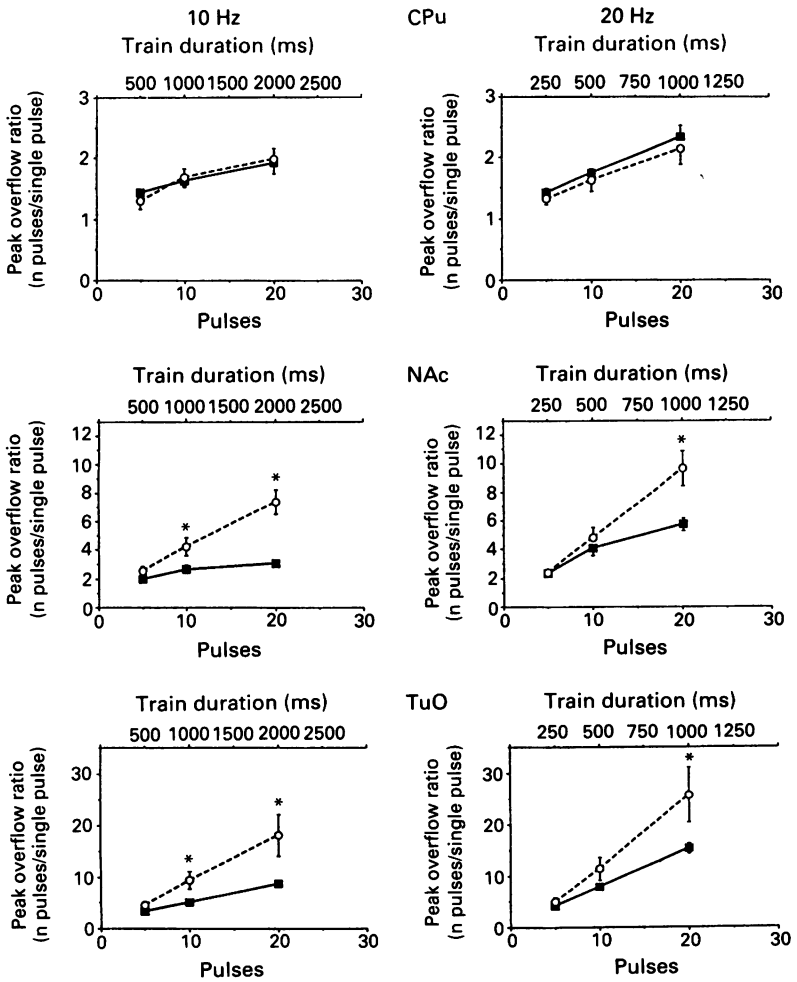


Figure 6 The effects of metoclopramide (0.3 μM) on peak evoked dopamine overflow following trains of pulses, relative to dopamine release following a single pulse stimulation in the caudate putamen (CPu, top row), nucleus accumbens (NAc, middle row) and tuberculum olfactorium (TuO, bottom row). Shown are peak overflow ratios in the three regions for trains of 5, 10 and 20 pulses applied at 10 Hz (left column) and 20 Hz (right column). Control ratios are represented by (■) and those in the presence of the antagonist are shown as (○) (mean with s.e.mean shown by vertical bars; $n = 5$ to 24). The lower abscissa scale shows the number of pulses; the duration of the train of stimulation is shown in the upper abscissa scale. $*P<0.05$; significantly different from control.

TuO upon electrical stimulation. The faradaic current in the TuO after electrical stimulation is indistinguishable from exogenous dopamine using the same electrode (Figure 3). The dopamine signal is clearly different from ascorbic acid, DOPAC (Millar *et al.*, 1985), 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) (O'Connor & Kruk, 1991). The faradaic signal was reversibly abolished by tetrodotoxin (TTX) in the perfusion fluid, and was enhanced by addition of benztropine (2 μM) or nomifensine (1 μM) to the perfusion medium, but unaffected by addition of desmethylimipramine (1 μM) or fluvoxamine (1 μM). Quinpirole, a selective D_2 receptor agonist, inhibited dopamine release (data not shown), while metoclopramide enhanced the dopamine signal in response to 10 and 20 Hz stimulations lasting longer than 500 ms (see Figure 6). For detailed experiments in the CPu and NAc which report such data, see Palij *et al.* (1990), Bull *et al.* (1990) and Bull & Sheehan (1991). A crucial advantage of using such brain slices is that the carbon fibre and stimulating electrodes can be located with confidence in an equivalent site in successive slices under microscopic control. In common with the studies of Palij *et al.* (1990) and Bull & Sheehan (1991), we have studied efflux of dopamine in the absence of precursor loading, uptake of radiolabelled amine or the presence of neuronal uptake inhibitors.

The spontaneous firing rates of the majority of dopaminergic neurones in the nerve cell body regions are generally reported as being between 2.5 and 7 Hz (Grace & Bunney, 1984), but Freeman *et al.* (1985) have shown that the cells may fire at up to 25 Hz in brief bursts. It was of interest to characterize the dopamine release system at the axon terminals more fully, and to compare the ability of axon terminals derived from different groups of nerve cell bodies to release dopamine over a range of frequencies, during trains of stimuli consisting of varying numbers of pulses.

We compared dopamine efflux following single pulse and trains of 20 pulses applied at 10 to 500 Hz (Figure 4). Dopamine efflux from the CPu (where most innervation is derived from the A9 region: the substantia nigra pars compacta) had a frequency-response pattern which contrasted markedly with the NAc and TuO (where most innervation is derived from the A10 region: the ventral tegmental area). In the CPu, release of dopamine from axon terminals following a train of 20 stimuli at up to 500 Hz, is limited maximally to 2 to 3 fold that following a single pulse. In the NAc the 20 pulse/50 Hz to 1 pulse ratio was maximally 7 fold that of a single pulse, while in the TuO this ratio was in the region of 20 fold (comparing single pulse to 20 pulses at 50 Hz). These results show that dopaminergic terminals in the NAc and in the TuO can increase dopamine efflux with increasing number of pulses and increasing frequency (Figure 5); in the TuO there was a near linear relationship between number of pulses, frequency of the train of pulses, and dopamine efflux. This contrasted with the CPu, where a flat frequency: dopamine efflux relationship was observed. The flatness of the frequency: overflow response in the rabbit CPu has been described previously using [^{3}H]-dopamine in the absence of reuptake inhibition (Hoffmann & Cubeddu, 1982).

The decrease in peak overflow ratio with 20 pulses at frequencies above 50 Hz may reflect the inability of the axon terminals to follow such high frequencies; this might arise from the characteristics of the refractory periods of the axons, or a limit on the rate of mobilisation of transmitter from non-releasable to releasable pools in the axonal varicosities. Alternatively, modulatory substances may be released at these high frequencies which act to reduce transmitter release.

It was of interest to establish if these differences could be ascribed to differences in autoreceptor regulated release of dopamine in these three sites. Experiments were done in the presence of either metoclopramide or sulpiride during single pulse stimulation, or trains of 5 to 50 pulses, applied at 10 to 100 Hz. In accord with autoreceptor theory (see Starke *et al.* (1989) for review), single pulse stimulation was not affected by the presence of autoreceptor blocker in any of the three axon terminal regions examined. This can be taken to indicate that under our experimental conditions in the absence of electrical stimulation, there is insufficient dopamine in the synaptic cleft to activate dopamine autoreceptors.

The control of dopamine efflux by autoreceptor activation is not dependent on the frequency of stimulation alone: the duration of the stimulus also plays an important role. In the NAc and TuO, when the number of pulses and frequency of the trains of pulses were varied in the presence of metoclopramide, it was found that only during stimulations lasting at least 500 ms, did blockade of dopamine autoreceptors lead to an increase in the efflux of dopamine evoked by stimulation at 10 and 20 Hz.

These results extend, and to some extent modify the concept of pulse to pulse regulation of monoamine neurotransmitter release (Cejna *et al.*, 1990; Valenta *et al.*, 1989) by indicating that the time for which autoreceptors are exposed to their neurotransmitter may be more significant than the number of pulses applied, and that a minimum exposure time (between 500 and 1000 ms in these experiments) is necessary for the effect of autoreceptor activation by such a transmitter to be seen. This idea has been discussed by Dwoskin & Zahniser (1986), but (unlike the present study), they were unable to estimate the time needed for activation of autoreceptors. The ability to measure changes of dopamine efflux in what approaches real time (a time resolution of 500 ms) is important in allowing us to relate the life time of transmitter in the extracellular space, and activation of mechanisms which regulate its release and efflux.

Examination of curves relating pulse number and frequency to ratio of dopamine efflux following single pulses or trains of stimuli, show that in the NAc or TuO, maximum dopamine efflux conditions occurred with 50 pulses applied at 50 Hz (a stimulus lasting 1000 ms); metoclopramide had no effect on dopamine efflux under these stimulation conditions. If the stimulus did not cause maximal dopamine efflux (10 or 20 pulses at 10 Hz or 20 pulses at 20 Hz in the NAc and TuO), then metoclopramide enhanced dopamine efflux. In the CPu maximum efflux appears to occur under all stimulation conditions used in this study. Using identical techniques however, Limberger *et al.* (1991), showed that 4 pulses applied at 5 Hz did not cause maximal dopamine efflux, and metoclopramide or sulpiride increased dopamine efflux. From these observations a second conclusion might be that autoreceptor block will only influence dopamine overflow if the maximum capacity of the system to release dopamine has not already been reached.

Grace (1991) has suggested that there may be two mechanisms regulating dopamine release in the projections of the dopaminergic systems of the brain. A phasic release might be regulated by depolarization of dopaminergic nerve cell bodies, with close temporal linkage to dopamine release in the axon terminals. A tonic regulation of dopamine release may be present, possibly regulated by excitatory amino acid (glutamate) projections to dopaminergic axon terminals, which may release dopamine independently of electrical activity of dopaminergic neurones. If such systems operate, then it can be suggested that tonic influences may be more significant in the CPu than in the mesolimbic projections (the NAc and TuO in the experiments reported here). This proposal requires further work (and the brain slice preparation in combination with FCV should prove suitable for such studies), as does the suggestion that the distribution of dopamine autoreceptors may be associated with regions where phasic release is a major factor regulating dopamine efflux. It may be significant that the density of the recently described D_3 dopamine receptors, which it has been suggested might act as dopamine autoreceptors (Sokoloff *et al.*, 1990), reflects the frequency dependence of evoked dopamine efflux seen in this study, namely that TuO > NAc > CPu.

The influence of other substances (possibly neuropeptides)

could account for our observations. Peptidergic nerves require higher frequencies to evoke peptide release, and differential releasing effects of frequency have been reported when peptide and classical neurotransmitter release has been measured from the same neurones (Bartfai *et al.*, 1988).

In conclusion, this study shows that the characteristics of dopamine efflux following electrical stimulation show marked regional differences which may reflect the different origins of the innervation. The characteristics of autoreceptor control of dopamine efflux appear to differ in dopaminergic axon terminal regions; demonstration of such heterogeneity of the dopamine efflux mechanisms requires careful selection of stimulation parameters.

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The role of induction of nitric oxide synthesis in the altered responses of jugular veins from endotoxaemic rabbits

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1 Endotoxaemia is characterized by hypotension, peripheral vasodilatation and a reduced response to vasoconstrictors. Clinical studies have indicated that venodilatation contributes to the haemodynamic changes, although there is no direct evidence for abnormal venous reactivity. In the present study, the role of nitric oxide (NO) in modifying the responses of rabbit isolated jugular veins was examined *in vitro*, 4 h after intravenous injection of endotoxin.

2 Treatment with endotoxin reduced the contractile response to the thromboxane-mimetic, 9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U-46619). This affect was endothelium-independent. The response was partially restored by the NO synthase inhibitor, N^G-monomethyl-L-arginine (L-NMMA 300 μM).

3 Jugular veins from control animals did not contract to L-NMMA whereas those from endotoxin-treated animals showed concentration-dependent contractions to L-NMMA. The contractions produced by L-NMMA were reversed by L-arginine but not by D-arginine. Treatment of the animals with dexamethasone (4 mg kg^{-1}) 1 h prior to administration of endotoxin significantly attenuated the response to L-NMMA.

4 The response to sodium nitroprusside did not differ significantly between veins from control and endotoxin-treated animals. Endothelial denudation did not alter the sensitivity of the veins to sodium nitroprusside. Acetylcholine produced endothelium-dependent relaxations which were similar in veins from control and endotoxin-treated animals.

5 The results of this study demonstrate that intravenous administration of endotoxin induces hyporesponsiveness to U-46619 in jugular veins. This effect is mediated, at least in part, by the induction of NO synthesis in smooth muscle. The induction is prevented by prior treatment with dexamethasone.

Keywords: Nitric oxide; arginine; endotoxin; septic shock; veins

Introduction

Endotoxaemia is a major clinical problem; the mortality rate is high and treatment is often ineffective. The syndrome is characterized by vasodilatation, a diminished response to vasoconstrictors, and hypotension (Suffredini *et al.*, 1989; Parillo *et al.*, 1990). Tissue perfusion is eventually compromised by the low blood pressure and this contributes to end organ damage and, ultimately, to death (Ziegler *et al.*, 1991).

Recently, it has been shown that the hypotension (Julou-Schaeffer *et al.*, 1990; Thiemermann & Vane, 1990) and arterial hyporeactivity (Julou-Schaeffer *et al.*, 1990; Rees *et al.*, 1990) produced by endotoxin can be accounted for by increased vascular synthesis of the potent vasodilator nitric oxide (NO). Endotoxin and certain cytokines lead to the induction of a calcium-independent NO synthase in vascular endothelium (Radomski *et al.*, 1990) and smooth muscle (Rees *et al.*, 1990; Busse & Mulsch, 1990; Fleming *et al.*, 1991). This induction can be prevented by dexamethasone or cycloheximide (Rees *et al.*, 1990; Radomski *et al.*, 1990) and, once established, the effects can be reversed with N^G-monomethyl-L-arginine (L-NMMA), a specific inhibitor of NO synthase (Rees *et al.*, 1990; Julou-Schaeffer *et al.*, 1990).

Whether there are changes in the reactivity of veins in sepsis is less clear. Clinical observations suggest that, despite increased levels of vasoconstrictors, the veins are inappropriately dilated in sepsis (Bradley *et al.*, 1945), and initial treatment consists of rapid expansion of intravascular volume to maintain right atrial filling pressure (Parillo *et al.*, 1990; Anon., 1990). In this study we have examined the effects of endotoxaemia on the responses of rabbit jugular veins to the vasoconstrictor thromboxane-mimetic, U-46619 (9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$), and have sought to establish whether NO contributes to the observed changes.

Methods

Studies were performed on New Zealand White rabbits of either sex weighing 2–2.5 kg. Internal jugular veins were removed after induction of terminal anaesthesia with sodium pentobarbitone. Some animals received intravenous (i.v.) endotoxin (500 μg) 4 h before removal of tissues; some received dexamethasone (4 mg kg^{-1}, i.v.) 1 h prior to endotoxin. The veins were placed in ice-cold Krebs solution containing indomethacin (5 μM) and dissected free of surrounding fat and connective tissue. In some experiments the endothelium was removed by inserting a roughened catheter into the lumen of the vessel and gently rotating the vessel between thumb and forefinger. Absence of functional endothelium was confirmed by failure to relax to acetylcholine.

Each vein was cut into 2 rings of 3 mm length. Rings were suspended between 2 hooks connected to a transducer for the measurement of isometric force. The preparations were suspended in a 20 ml organ bath filled with warmed (37°C) oxygenated (95% O_2, 5% CO_2) Krebs solution containing indomethacin (5 μM) and, in some experiments, cycloheximide (100 μM). This concentration of cycloheximide inhibits protein synthesis in the rabbit aorta by 85% (Deblois *et al.*, 1988). Basal tension was initially set at 1.5 g and adjusted to 0.75 g after 1 h equilibration. This tension gives optimal contractile responses in these vessels (Leff *et al.*, 1987).

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Contraction studies

Concentration-response curves were constructed for U-46619 (10^{-10}–10^{-6} M) in the presence or absence of L-NMMA (300 μM added 10 min before addition of U-46619). In all experiments basal tension was again adjusted to 0.75 g before adding U-46619.

In a separate series of experiments the effects of L-NMMA or D-NMMA (0.5×10^{-5}–3.2×10^{-4}M) were tested on vessels pre-contracted with U-46619 (10^{-9} M). Induction of a small amount of tone with a contractile agonist enhances the response of vessels to L-NMMA (Rees *et al.*, 1989).

Relaxation studies

Concentration-response curves were constructed for acetylcholine (0.5×10^{-9}–10^{-6} M), sodium nitroprusside (10^{-9}–10^{-5} M) and L-arginine (10^{-4}–10^{-3} M) in rings precontracted by U-46619 (2×10^{-8} M; EC_{90}).

In a separate series of experiments the effects of L-arginine and D-arginine (10^{-4}–10^{-3} M) were tested on rings pre-contracted with L-NMMA (300 μM).

Drugs

The following drugs were used: acetylcholine bromide (Sigma), L- and D-arginine hydrochloride (Sigma), bacterial endotoxin (*S. Typhosa*; Difco Ltd), cycloheximide (Sigma), dexamethasone sodium phosphate (Decadron Shock-Pak, Merck Sharp & Dohme), indomethacin (Sigma), L- and D-N^G-monomethyl-L-arginine (synthesized by Dr H.F. Hodson), sodium nitroprusside (Sigma), U-44619 (Cayman Chemicals). U-44619 was dissolved in ethanol and diluted in saline. All other drugs were dissolved in saline immediately prior to use.

Statistics

Results are expressed as mean ± s.e.mean and compared by Student's *t* test for paired or unpaired observations as appropriate, where $P<0.05$ is considered significant. For each experiment, values for EC_{30}, EC_{50} and EC_{90} were obtained from sigmoid logistic curves.

Results

After 1–2 h, animals treated with endotoxin became apathetic, respiratory rate increased and diarrhoea was common. Animals treated with dexamethasone appeared healthy after endotoxin. One animal treated with endotoxin died after 2 h and was excluded from analysis.

Contraction studies

The potency of, and maximum response to U-46619 was reduced significantly in veins from endotoxin-treated animals irrespective of endothelial integrity (Figure 1a,b). In veins with functionally intact endothelium the EC_{30} increased approximately fourfold, from $5.8 \pm 1.34 \times 10^{-10}$ M (control animals; $n=8$) to $2.35 \pm 0.58 \times 10^{-9}$ M (endotoxin-treated animals; $n=7$; $P<0.01$). This effect of endotoxin was attenuated by L-NMMA (300 μM): in veins with functionally intact endothelium L-NMMA decreased the EC_{30} for U-46619 from $2.53 \pm 0.44 \times 10^{-9}$ M ($n=11$) to $8.41 \pm 2.48 \times 10^{-10}$ M ($P<0.05$; Figure 2a) and in veins without functionally intact endothelium, L-NMMA decreased the EC_{30} for U-46619 from $2.85 \pm 0.7 \times 10^{-9}$ M ($n=7$) to $7.34 \pm 1.65 \times 10^{-10}$ M ($P<0.05$; Figure 2b). In keeping with previous studies (Martin *et al.*, 1992) inhibition of NO synthase did not increase the potency of U-46619 in veins from control animals ($n=5$ endothelium functionally intact; data not shown).

In pre-contracted (U-46619 10^{-9} M) veins from endotoxin-treated animals, L-NMMA ($EC_{90} = 2.1 \pm 0.4 \times 10^{-5}$ M), but not D-NMMA (data not shown), caused further contractions (Figure 3a) which were endothelium-independent. *Ex vivo* incubation with cycloheximide (100 μM for 2 h before dose-response curves and throughout the remainder of study) did not alter the contractile response to L-NMMA ($n=3$; data not shown). Pretreatment of the animals with dexamethasone (4 mg kg^{-1} i.v. given 1 h before endotoxin) significantly inhibited the contractile response to L-NMMA ($n=4$; Figure 3). L-NMMA (300 μM) also caused slowly-developing endothelium-independent contractions of veins from endotoxin-treated animals which were not pre-contracted with U-46619 ($n=8$). In veins taken from control animals, L-NMMA did not cause significant contractions ($n=7$).

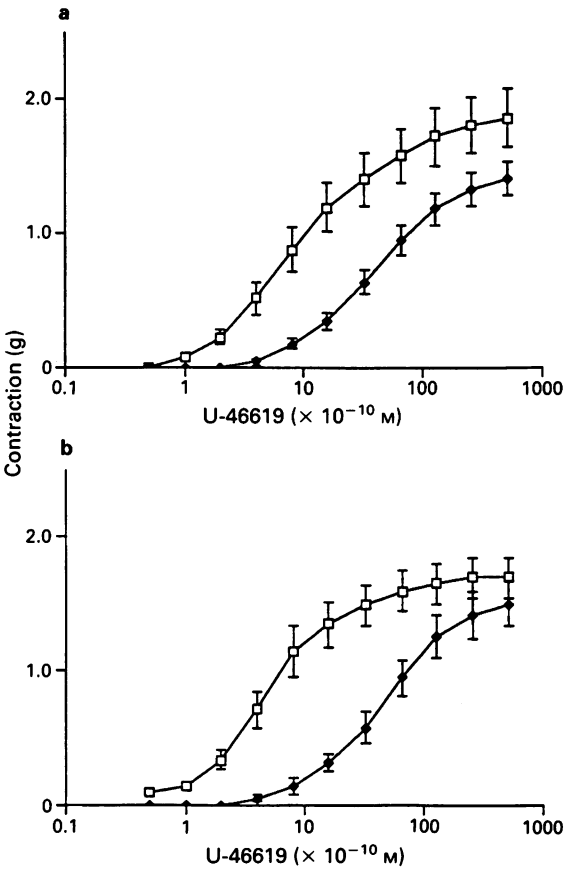


Figure 1 (a) Contraction to U-46619 in endothelium-intact veins taken from endotoxin-treated (◆; $n=7$) and control (□; $n=8$) rabbits; (b) contraction to U-46619 in endothelium-denuded veins taken from endotoxin-treated (◆; $n=7$) and control (□; $n=4$) rabbits.

Relaxation studies

Acetylcholine caused endothelium-dependent relaxation which did not differ significantly between veins taken from endotoxin-treated ($EC_{50} = 6.1 \pm 2.1 \times 10^{-9}$ M) and control animals ($EC_{50} = 4.2 \pm 0.7 \times 10^{-9}$ M; Figure 4).

Sodium nitroprusside caused concentration-dependent relaxations which were not significantly different in endothelium-intact and endothelium-denuded vessels. The potency of sodium nitroprusside did not differ significantly between veins taken from control (EC_{50} intact vein $= 9.11 \pm 1.1 \times 10^{-8}$ M; EC_{50} denuded vein $= 9.87 \pm 4.3 \times 10^{-8}$ M) and endotoxin-treated animals (EC_{50} intact vein $= 9.65 \pm 6.2 \times 10^{-8}$ M; EC_{50} denuded vein $= 2.71 \pm 1.3 \times 10^{-7}$ M; Figure 4).

Veins taken from endotoxin-treated animals which were precontracted with L-NMMA (300 μM) showed dose-depen-

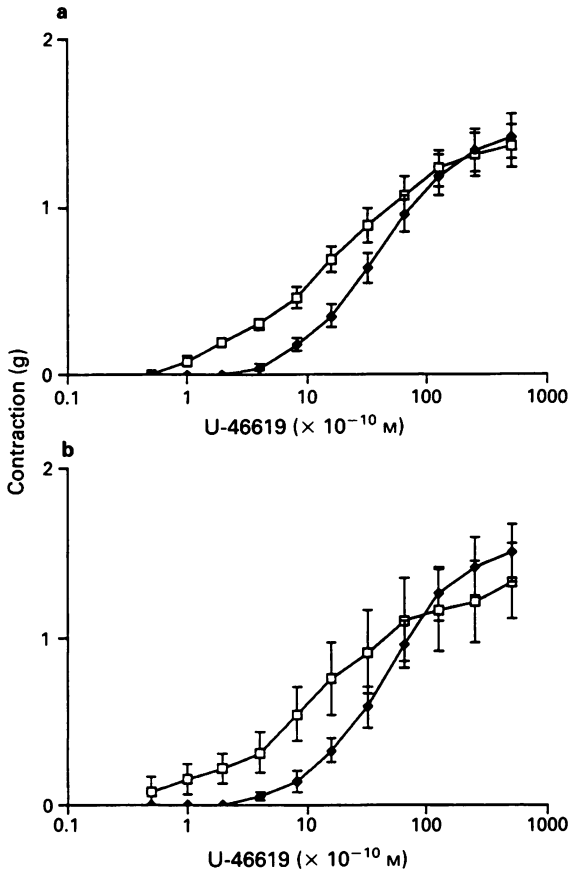


Figure 2 (a) Contraction to U-46619 in endothelium-intact veins from endotoxin-treated animals in the presence (□) and absence (◆) of N^G-monomethyl-L-arginine (L-NMMA, 300 μM); (b) contraction to U-46619 in endothelium-denuded veins from endotoxin-treated animals in the presence (□) and absence (◆) of L-NMMA (300 μM).

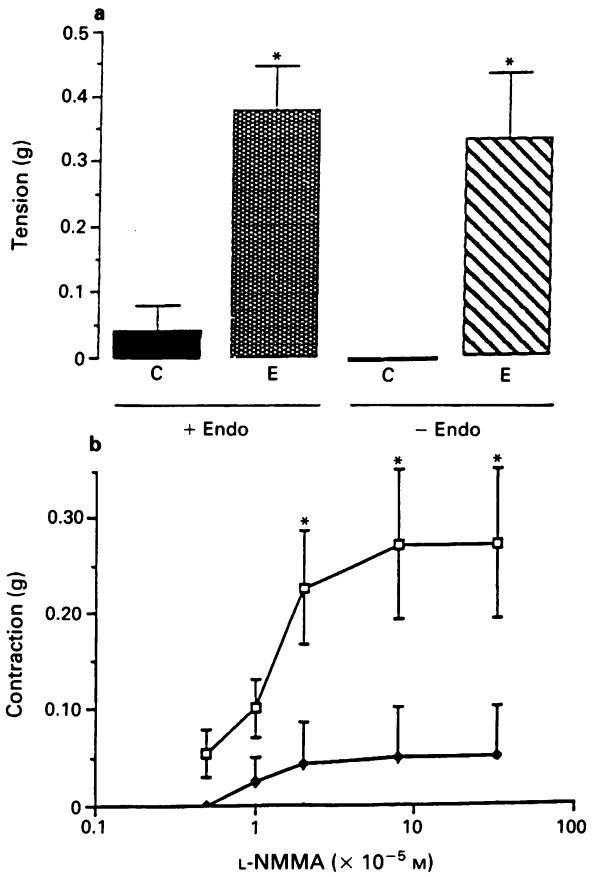


Figure 3 (a) The effects of N^G monomethyl-L-arginine (L-NMMA, 300 μM) on basal tone in endothelium-intact (+ Endo) and endothelium-denuded (− Endo) veins from endotoxic (E; $n = 8$) and control (C; $n = 7$) rabbits. $^*P < 0.01$. Responses shown are maxima. (b) Concentration-response to L-NMMA in endothelium-intact veins taken from endotoxic rabbits (□; $n = 8$), and endotoxic rabbits pretreated with dexamethasone (◆; $n = 4$). The veins were partially contracted with U-46619 (10^{-9} M) prior to addition of L-NMMA. Significant difference from baseline $^*P < 0.05$.

dent relaxations to L-arginine, but not D-arginine ($n = 5$; data not shown). Full relaxation was seen in response to 400 μM L-arginine. In contrast, no significant relaxation to L-arginine was seen when veins from endotoxin-treated animals were precontracted with U-46619 ($n = 3$ endothelium functionally intact; $n = 3$ endothelium functionally absent; data not shown).

Discussion

Bacterial endotoxin, and certain cytokines, diminish arterial contractility (McKenna *et al.*, 1989; Beasley *et al.*, 1990). Recently, it has been demonstrated that this effect is mediated by induction of a calcium-independent NO synthase within vascular endothelial (Radomski *et al.*, 1990) and smooth muscle cells (Rees *et al.*, 1990; Busse & Mulsch, 1990; Joulou-Schaeffer *et al.*, 1990; Fleming *et al.*, 1991). The results of the present study, using rabbit isolated jugular veins, demonstrate that the contractile responses of veins taken from endotoxin-treated rabbits are also impaired and that this impairment is associated with induction of NO synthesis.

Evidence for reduced contractile responses in veins taken from endotoxaemic rabbits is provided by the experiments using U-46619, a thromboxane-mimetic. Veins removed 4 h after intravenous injection of bacterial endotoxin showed diminished contraction to U-46619. The functional abnormality appears to lie in the vascular smooth muscle since the attenuation of the contractile response was the same in endothelium-intact and endothelium-denuded preparations.

The experiments using L-NMMA, a stereospecific inhibitor of NO synthesis (Palmer *et al.*, 1988a,b; Rees *et al.*, 1989) demonstrate that the diminished contraction to U-46619 is associated with induction of continuous NO synthesis. Veins from endotoxin-treated animals showed a dose-dependent contraction to L-NMMA, whereas those from control animals did not. In addition, L-NMMA partially restored the potency of U-46619 in veins from endotoxin-treated animals.

Our finding that L-NMMA, but not D-NMMA, caused dose-dependent contraction of veins from endotoxin-treated animals suggests that under certain pathological conditions, such as endotoxic shock, veins may synthesize NO continuously and that this modifies venous tone. The observation that L-NMMA did not contract veins from control animals is in keeping with previous reports (Leff *et al.*, 1987; Giles *et al.*, 1990; Martin *et al.*, 1992) suggesting that these veins, like many others (Vallance *et al.*, 1989; Ekelund & Mellander, 1990; Yang *et al.*, 1991), do not normally synthesize NO continuously.

The appearance of continuous synthesis of NO in response to endotoxin and the inhibition of this by pretreatment with dexamethasone, suggests induction of NO synthase (Radomski *et al.*, 1990; Rees *et al.*, 1990). The effects of L-NMMA were endothelium-independent, indicating that the induction is occurring in the venous smooth muscle. However, the precise nature of the NO synthase induced in the venous smooth muscle remains to be determined and we do not yet

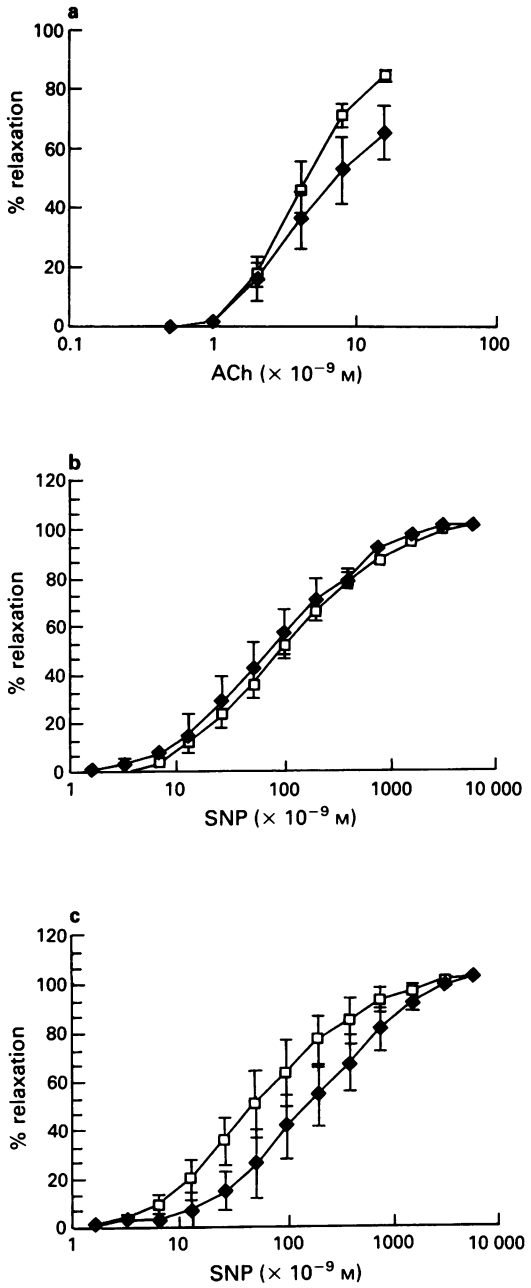


Figure 4 (a) Response to acetylcholine (ACh) in veins from control (□) and endotoxic (◆) rabbits ($n = 4$); (b) response to sodium nitroprusside (SNP) in endothelium-intact (□) and denuded (◆) veins from control rabbits ($n = 4$); (c) response to sodium nitroprusside (SNP) in endothelium-intact (□) and denuded (◆) veins from endotoxin-treated rabbits ($n = 4$).

know whether endotoxin also causes induction of NO synthase in venous endothelium. Incubation of the veins with cycloheximide (100 μM for 2 h *in vitro*), when protein synthesis would be expected to be inhibited by 85% (Deblois *et al.*, 1988), did not alter the responses, indicating that, once induced, the enzyme remains active for several hours without the need for further protein synthesis.

The diminished response to U-46619 seen in veins from endotoxin-treated animals was only partially restored by L-NMMA. This might be due to incomplete inhibition of NO synthesis, and, in support of this, it has been shown that inhibitors of NO synthase do not fully inhibit the NO-mediated relaxant response to acetylcholine in these veins (Martin *et al.*, 1992). Alternatively, additional mechanisms may also contribute to the diminished contractile response. These might include tachyphylaxis to thromboxane A_2 (TXA_2), levels of which rise in endotoxaemia (Cook *et al.*, 1980), induction of other local vasoactive mediators in the wall of the vein, or the onset of irreversible tissue damage. Any contribution by cyclo-oxygenase products to the responses seen was excluded by adding indomethacin to the Krebs solution.

No relaxation was seen when L-arginine was added to vessels from endotoxin-treated rabbits precontracted by U-46619. This is in contrast to studies in arteries which have shown a relaxant effect of L-arginine once the calcium-independent NO synthase has been induced (Rees *et al.*, 1990; Julou-Schaeffer *et al.*, 1990). The reasons for this discrepancy are not yet known but our results suggest that even when NO synthase has been induced, levels of arginine are not rate-limiting for NO synthesis in these veins. It is possible that there are arterio-venous differences in the uptake, storage or utilization of arginine.

In arteries, basal release of NO from the constitutive endothelial NO synthase appears to down-regulate the guanylate cyclase and diminish the sensitivity of smooth muscle to NO and exogenous nitrovasodilators (Moncada *et al.*, 1991). Lack of basal synthesis of NO by venous endothelia may explain the relative venoselectivity of the nitrovasodilators (Collier *et al.*, 1978; Moncada *et al.*, 1991; Benjamin & Vallance, 1991). It might be expected that once continuous NO synthesis has been induced in the veins, their sensitivity to nitrovasodilators would decrease. However, induction of basal synthesis of NO for 4 h did not alter the sensitivity of these vessels to sodium nitroprusside or to acetylcholine, suggesting that longer exposure to NO might be necessary to down-regulate the guanylate cyclase in these vessels.

Circulating levels of a variety of vasoconstrictors, including TXA_2, are increased in endotoxic shock (Cook *et al.*, 1980) and yet there is inappropriately low venous tone (Bradley *et al.*, 1945). Our findings suggest that this may be due to local changes in the blood vessel wall leading to venodilatation and hypo-responsiveness to vasoconstrictors. Induction of NO synthase by endotoxin decreases venous tone and the response to U-46619. If other veins behave similarly to the rabbit jugular veins, significant venous pooling and a postural fall in arterial pressure would follow induction of NO synthase.

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Effects of imipramine on the transient outward current in rabbit atrial single cells

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1. The effects of imipramine on action potential characteristics and transient outward potassium current (I_t) of rabbit isolated atrial myocytes were studied using the whole-cell configuration of the patch-clamp technique.

2 Imipramine, 3 μM, decreased action potential amplitude and lengthened the action potential duration measured at 50% of repolarization, whereas it did not modify the final phase of repolarization or the resting membrane potential. These results are similar to those reported in multicellular rabbit atrial preparations.

3 Imipramine, 0.1–100 μM, induced a concentration-dependent inhibition of the peak amplitude of I_t, a shortening of the time to peak current and an increase in the inactivation rate. The acceleration of the current inactivation is to a major extent responsible for the decrease in the integral of the outward current measured at 50 ms after the start of the pulse.

4 The drug-induced block of I_t was not associated with changes in the voltage-dependence of the steady-state inactivation curve or in the process of recovery from inactivation of the current. Extrapolation to zero block shows that imipramine did not block I_t before its activation at the onset of the depolarization. These results suggested that imipramine does not affect the inactivated or the resting state of I_t channels.

5 It is concluded that in rabbit isolated atrial cells, imipramine inhibits I_t and that this effect is responsible for the lengthening of the action potential duration produced by this drug.

Keywords: Imipramine; rabbit atria; transient outward current; patch-clamp

Introduction

Imipramine (Imip) is a tricyclic antidepressant drug which exhibits cardiotoxic effects (Marshall & Forker, 1982). In multicellular cardiac preparations it has been demonstrated that Imip inhibits the maximum upstroke velocity (V_{max}) of the action potential, an indirect index of the fast inward sodium current, I_{Na} (Rawling & Fozzard, 1979; Rodriguez & Tamargo, 1980). According to the onset and offset kinetics of the frequency-dependent V_{max} block, Imip, like quinidine, has been classified as a class Ia or intermediate kinetics antiarrhythmic drug (Delpón *et al.*, 1990). In fact, very recently it has been demonstrated that it produced a voltage- and frequency-dependent inhibition of the fast inward sodium current (I_{Na}) in guinea-pig isolated ventricular myocytes (Ogata & Narahashi, 1989). Furthermore, Imip decreased cardiac contractility (Marshall & Forker, 1982; Manzanares & Tamargo, 1983) and the amplitude of the slow action potentials elicited by isoprenaline in partially depolarized cardiac fibres (García de Jalón *et al.*, 1978). These results have led to the suggestion that Imip may inhibit the inward calcium current (I_{Ca}). In fact, Imip inhibited, in a concentration-dependent manner the I_{Ca} in bovine (Isenberg & Tamargo, 1985) and guinea-pig (Delpón *et al.*, 1991) isolated ventricular myocytes. It has also been demonstrated that in guinea-pig ventricular myocytes (Delpón *et al.*, 1991) Imip inhibits the potassium delayed outward current (I_K), whereas it does not modify the inward rectifier potassium current (I_{K1}). Despite all these reports, there are puzzling results concerning the effects of Imip on action potential duration (APD). Thus, whereas in guinea-pig and bovine ventricular preparations Imip induced a shortening of the APD (García de Jalón *et al.*, 1978; Rawling & Fozzard, 1979; Isenberg & Tamargo, 1985; Delpón *et al.*, 1990; 1991), a lengthening of the APD has been described in rabbit and rat atria (Matsuo, 1967; Manzanares & Tamargo, 1983). It is well known that ionic currents responsible for the repolarization of the cardiac action potential show important species-dependence. For example, although frog and guinea-pig isolated myocytes exhibit a large, maintained time- and voltage-dependent I_K (Hume & Giles, 1983; Hume & Uehara, 1985), this current is relatively small in atrial and ventricular myocytes isolated from rat, dog, rabbit or human hearts (Hume & Giles, 1983; Josephson *et al.*, 1984; Hume & Uehara, 1985; Escande *et al.*, 1987; Giles & Imaizumi, 1988). In contrast, in these latter four species a larger transient outward current, I_t, is responsible to a major extent for the repolarization of the action potential (Giles & Imaizumi, 1988).

In rabbit atrial cells Clark *et al.* (1988) have found that the threshold for activation of I_t is near − 30 mV and it is fully activated at + 30 mV. Inactivation is fully removed at potentials negative to − 70 mV and it is complete near 0 mV. Moreover, I_t exhibits a very rapid activation and inactivation kinetics, but a very slow time course of recovery from inactivation which can be responsible for the pronounced dependence of the shape of the atrial action potential on stimulus frequency (Giles & Imaizumi, 1988). Furthermore, it has been demonstrated that in rabbit atrial cells I_t is not activated by increases in intracellular sodium or calcium concentration (Giles & Imaizumi, 1988).

Because in rabbit atrial cells, I_t appears to be the major current responsible for repolarization (Giles & Imaizumi, 1988), the present study was undertaken to elucidate the ionic mechanism underlying the lengthening of the APD induced by Imip in rabbit isolated atrial cells and if this effect is mediated by inhibition of the I_t potassium channel.

Methods

Single atrial cells from rabbit hearts were isolated according to the experimental procedure described elsewhere (Isenberg

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& Klöckner, 1982) with minor modifications (Sánchez-Chapula, 1988). Rabbits of either sex (800–900 g weight) were killed by cervical dislocation and their hearts were quickly removed and mounted in a Langendorff perfusion system for retrograde coronary perfusion. Hearts were then initially perfused with Tyrode solution for about 10 min and then for 30 min with nominally calcium-free Tyrode solution containing 0.4 mg ml^{-1} collagenase (Type I, Sigma Chemical Co., St. Louis, MO, U.S.A.). The enzymes were washed out by perfusion with a high K^+, low Cl^- medium for 5 min. Single cells were obtained by mechanical agitation with a pipette. The cells were stored in the high K^+, low Cl^- medium at 4°C for later electrophysiological experiments.

The Tyrode solution contained in mM: NaCl 112, $NaHCO_3$ 24, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, NaH_2PO_4 0.42, glucose 11, taurine 10 (pH = 7.4). The solution was bubbled with 95% O_2: 5% CO_2. Nominally calcium-free Tyrode solution was prepared by simply omitting $CaCl_2$ from the Tyrode solution. The high K^+, low Cl^- medium contained in mM: K-glutamate 50, KCl 50, taurine 20, KH_2PO_4 3, glucose 10, HEPES 10, EGTA 0.2 (pH 7.4 with KOH). A small aliquot of a suspension of dissociated cells was placed in a 0.3 ml chamber mounted on the stage of an inverted microscope (Nikon Diaphot, Nikon Co., Tokyo, Japan). After a brief period during which the cells adhered to the glass at the bottom of the chamber the cells were perfused at a rate of 2–3 ml min^{-1} with bath solution containing (mM): NaCl 130, $CaCl_2$ 1.0, $CoCl_2$ 2, KCl 5.4, $MgCl_2$ 1.0, $NaHCO_3$ 6.0, KH_2PO_4 0.42, tetrodotoxin (TTX) 0.03, HEPES 10, glucose 11; the pH was adjusted to 7.4 with NaOH and the solution was bubbled with 100% O_2. Under these experimental conditions the inward sodium and calcium currents were abolished by TTX and $CoCl_2$, respectively. In the experiments where effects of Imip on action potential characteristics were studied the bath solution was (mM): NaCl 130, $CaCl_2$ 3.6, KCl 5.4, $MgCl_2$ 1.0, $NaHCO_3$ 6.0, KH_2PO_4 0.42, HEPES 10, glucose 11; the pH was adjusted to 7.4 by NaOH. All experiments were performed at room temperature (22–24°C).

Recordings were made in the whole-cell voltage clamp configuration of the patch-clamp technique (Hamill *et al.*, 1981) with a Dagan 8900-1 voltage clamp amplifier (Dagan Co., Minneapolis, MN, U.S.A.). Series resistances (2.6 ± 0.2 MΩ, $n = 18$) were partially compensated (70–80%) by an analog circuit. Since the mean amplitude of I_t at + 50 mV is 1.12 ± 0.2 nA ($n = 18$), the estimated voltage error attributed to uncompensated series resistance was less than 2 mV. Capacitative and linear leak currents were subtracted by an analog circuit or digitally by computer (Delpón *et al.*, 1991). The membrane potential was controlled by computer (IBM PC/AT) via the output of a 12 bit digital-to-analog converter. Membrane currents were filtered at 1 kHz by a 8-pole Bessel filter (Frequency Devices, Haverhill, MA, U.S.A.) before sampling at 2 kHz by a 12 bit analog to digital converter. Membrane potential and current signals were monitored on a storage oscilloscope (Tektronix 5103N, Tektronix Inc., Beaverton, Or, U.S.A.), digitized and stored on the hard disk of the computer using an analog-to-digital converter (Interface TL-1, Axon Instruments Inc., Foster City, CA, U.S.A.) controlled by acquisition and display software (pClamp, versions 5.1, Axon Instruments Inc.). Data were subsequently analyzed with a Clampfit programme. Patch electrodes were made as described by Hamill *et al.* (1981). The recording pipette was filled with an internal solution (mM): K-aspartate 80, KCl 50, KH_2PO_4 10, $MgSO_4$ 1.0, HEPES 5.0, Na_2ATP 3.0, EGTA 0.1 (pH 7.2 with KOH). When filled with the internal solution the electrode resistance was 2–3 MΩ. A liquid junction potential of − 9 mV was corrected for between the pipette solution and the external solution.

Data analysis

When possible, analyzed data are presented as arithmetical means ± s.e.mean. Comparisons between multiple means were made by one-way analysis of variance. To compensate for simultaneous multiple comparisons a method analogous to Bonferroni's method was used (Wallenstein *et al.*, 1980). Differences were considered significant when P values were < 0.05. Curve fitting during data analysis was performed by the nonlinear least-squares Levenberg-Marquardt algorithm (Marquardt, 1963).

Drugs

Imipramine hydrochloride and tetrodotoxin (Sigma Chemical Co, St. Louis, MO, U.S.A.) were dissolved in external solution to obtain final concentrations.

Results

Effects of imipramine on action potential characteristics

It has been shown that Imip increases, in a concentration-dependent manner, the action potential duration in rabbit atria (Matsuo, 1967). Therefore, in the first group of experiments the effects of Imip on action potential characteristics of rabbit atrial myocytes stimulated at a frequency of 0.1 Hz were studied. The result of a typical experiment is shown in Figure 1. It can be observed that at a concentration of 3 μM, Imip decreased the action potential amplitude from 112 ± 3 mV to 88 ± 2 mV and produced a significant lengthening of the APD measured at 50% of the repolarization (from 11 ± 1 ms to 22 ± 2 ms), whereas it did not modify either the final phase of repolarization measured as APD at 90% of repolarization (60 ± 1 ms vs 62 ± 2 ms) nor the resting membrane potential (− 74 ± 1 mV vs − 73 ± 1 mV) ($n = 4$). The effects of Imip became evident within 2 min and reached steady-state values within 5–7 min. All these effects were completely reversible after 15 min of washout with drug-free solution. Therefore, the effects of Imip on rabbit isolated atrial cells were similar to those previously described in multicellular preparations. The lengthening in APD induced by Imip in rabbit atrial cells could have been brought about by an increase in inward currents or a decrease in outward currents. An increase of the inward Na and/or Ca currents flowing during the plateau phase of the action potential is unlikely to be responsible for the lengthening in APD induced by Imip in rabbit atria cells since it has been demonstrated that Imip induced a concentration-dependent inhibition of the I_{Na} and I_{Ca} in single isolated ventricular cells (Isenberg & Tamargo, 1985; Ogata & Narahashi, 1989; Delpón *et al.*, 1991). In guinea-pig ventricular cells Imip also inhibited the delayed outward potassium current (I_K) (Delpón *et al.*, 1991). However, since in the rabbit atrium I_K is relatively small and the largest time- and voltage-dependent current responsible for the control of the action potential

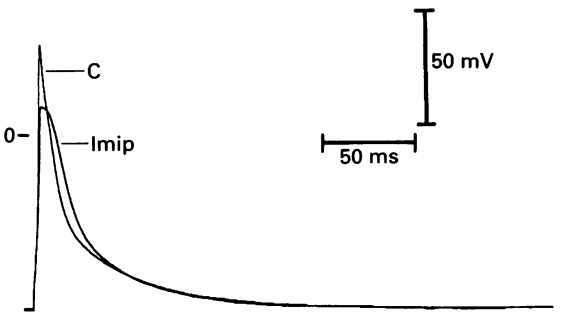


Figure 1 Effects of imipramine (Imip) on the action potential elicited in a rabbit isolated atrial cell stimulated at a frequency of 0.1 Hz. Exposure of a myocyte to 3 μM imipramine decreases the amplitude and lengthens the action potential measured at 50% of repolarization without changing the resting membrane potential. C: control; Imip: imipramine 3 μM.

duration is the transient outward current (I_t) (Giles & Imaizumi, 1988), we decided to study the effects of Imip on the I_t in order to ascertain the ionic mechanism responsible for the lengthening of the APD.

Effects of imipramine on I_t

To study the effect of Imip on potassium currents with minimal interference of calcium and sodium inward currents, experiments were carried out in the presence of 2 mM $CoCl_2$, an inorganic calcium channel blocker, and 30 μM TTX, a specific blocker of the I_{Na}. Single rabbit atrial cells were voltage-clamped to a holding potential of − 70 mV and 1 s duration hyperpolarizing and depolarizing test pulses were applied in 10 mV steps from − 100 to + 50 mV every 30 s. As shown in Figure 2a, under these conditions depolarizing pulses to + 30 mV evoked a transient outward current which activated rapidly and inactivated spontaneously, i.e. I_t. As can be observed, 3 μM Imip significantly decreased the peak amplitude of I_t, but, the most obvious effect of the drug appeared to be an acceleration in the rate of decay of I_t. This effect of Imip was completely reversed after 15 min of washout (Figure 2a). Figure 2b shows the effects of 3 μM Imip on the current-voltage relationship obtained by plotting the peak amplitude of the current as a function of the voltage of the test pulse. As can be observed, Imip reduces the peak amplitude of the current elicited by depolarizing pulses positive to − 30 mV that are mainly carried by the I_t channel (Clark *et al.*, 1988; Giles & Imaizumi, 1988), whereas it did not modify the currents evoked by pulses negative to − 30 mV which are mainly carried by the inward rectifier potassium channel (I_{K1}). The time course of the inactivation of the I_t at potentials in the range between 0 and + 50 mV can be described by the sum of two exponential functions and the kinetic parameters controlling these processes exhibit only very weak voltage-dependence (Clark *et al.*, 1988). In the present experiments we found similar results under control conditions. To quantify the effect of Imip on the rate of inactivation of I_t, the time constants (τ) of inactivation were measured. In five cells following the application of a 1 s depolarizing pulse from − 70 to + 30 mV, the inactivation time course of I_t was generally best fitted by two exponential functions (Figure 2a). Under control conditions, the time constant of the fast (τ_{fast}) and slow components (τ_{slow}) of inactivation were 34 ± 4 ms and 212 ± 14 ms, respectively. Imip at a concentration of 3 μM significantly decreased ($P<0.01$) both time constants to 11 ± 2 ms and 130 ± 9 ms, respectively ($n = 5$). The effect of Imip was concentration-dependent. Thus, 10 μM Imip decreased ($P<0.001$) the τ_{fast} and τ_{slow} values to 4.5 ± 2 ms and 55 ± 9 ms, respectively. At 3 and 10 μM, Imip decreased the amplitude of the slow component of inactivation (A_s) by 34 ± 5% and 69 ± 7%, respectively, whereas the amplitude of the fast component (A_f) was decreased by 13 ± 4% and 40 ± 9%, respectively. Thus, the relative contribution of the A_f to the total time-dependent inactivation of I_t, i.e. the A_f ($A_f + A_s$) ratio, increased from 0.46 under control conditions to 0.53 and 0.66 in the presence of 3 and 10 μM Imip, respectively.

In order to study the concentration-dependent effects of Imip on I_t, concentration-response curves were obtained. Figure 3a shows superimposed traces of the first 50 ms of I_t elicited by 1 s depolarizing pulses to + 50 mV from a holding potential of − 70 mV applied every 30 s in control conditions (C) and in the presence of Imip, 1 and 10 μM. It can be observed that Imip induced a concentration-dependent inhibition of the amplitude of I_t, a shortening of the time to peak

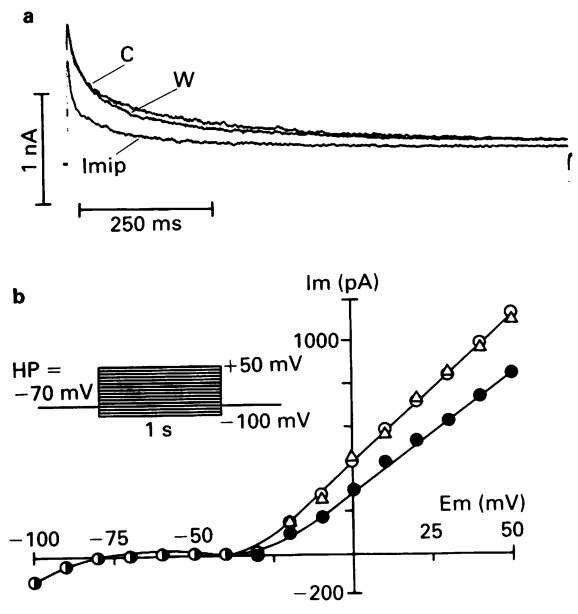


Figure 2 Imipramine (Imip) inhibits I_t but not I_{K1}: (a) shows the membrane current activated by a 1 s depolarizing pulse from a holding potential of − 70 mV to + 30 mV before (C), during (Imip) and after wash-out (W) of 3 μM Imip. In (b) is shown the current-voltage relationship for I_t and I_{K1} observed in the absence of (○), during exposure (●) and after wash-out (△) of 3 μM Imip. I_t was measured as the peak outward current activated by pulses positive to − 25 mV and I_{K1} as the amplitude of the minimum inward current activated in response to pulses more negative than − 30 mV from a holding potential of − 70 mV. At pulses more negative than − 30 mV the values obtained in the absence and presence of Imip were superimposed (◑). Experiments were carried out in the presence of 2 mM $CoCl_2$ and 30 μM tetrodotoxin.

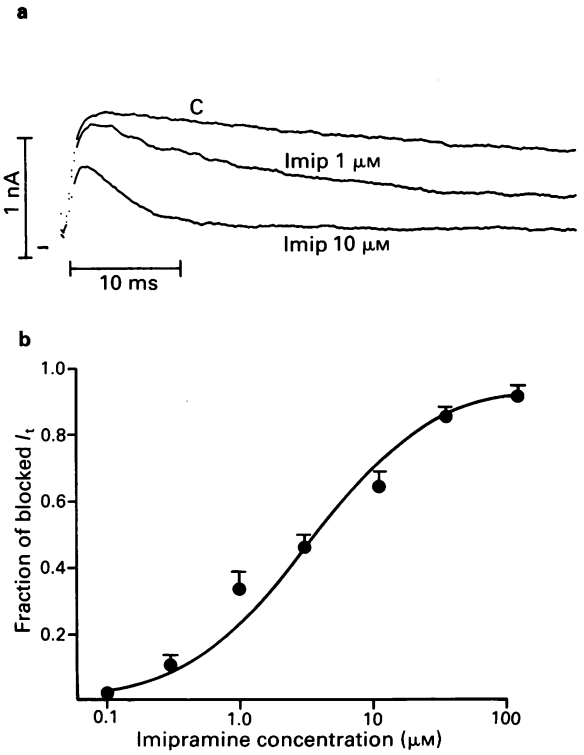


Figure 3 Panel (a) shows superimposed traces of the first 50 ms of I_t in control conditions (C) and in the presence of imipramine (Imip), 1 and 10 μM. The cell was clamped at − 70 mV and 1 s depolarizing pulses to + 50 mV were applied every 30 s. Panel (b) shows the concentration-response curve for the inhibition of the I_t induced by Imip (0.1 − 100 μM) measured as the integral of the first 50 ms of the current elicited by a depolarizing clamp pulse from − 70 mV to + 50 mV applied once every 30 s. The solid curve is best least-squares fit for one-to-one drug receptor interaction: $B = [D]/[D] + K_D$, where B is the fraction of block induced by Imip at the concentration tested [D] and K_D is the dissociation constant. Each point represents the mean value of 8 experiments.

current and an increase in the inactivation rate. Figure 3b shows the concentration-response curve for the inhibition of I_t induced by a wide range of concentrations of Imip (0.1–100 μM), determined as the integral of the current measured from the end of the capacitive current to 50 ms after the start of the pulse. The concentration-dependence of the block is consistent with a first-order reaction with a dissociation constant of 3.7 μM ($n = 8$ cells). This value was calculated by fitting the experimental data by the equation:

$$B = [D]/[D] + K_D \quad (1)$$

where B is the fraction of block induced by Imip at the concentration tested [D] and K_D is the dissociation constant.

In Figure 4 the blockade of I_t produced by Imip during a depolarizing pulse to + 50 mV ($I_{t,Imip}$) is expressed as a proportion of the current observed in the absence of the drug ($I_{t,\ Control}$). The data at two different concentrations of Imip (3 and 10 μM) were well fitted by the following equation:

$$B = B_{max}\ [1\text{-}e(-(k_1[D] + k_2\ t))] \quad (2)$$

where B_{max} is the maximum block produced by the drug concentration [D]; B is the amount of block at time t and k_1 and k_2 are the association and dissociation rate constants for Imip, respectively. Nonlinear least square analysis of the rate of block in the presence of the two concentrations of Imip according to equation (2) provided the following estimates: $k_1 = 51 \times 10^6 \pm 9 \times 10^6\ M^{-1}\ s^{-1}$ and $k_2 = 194 \pm 27\ s^{-1}$ ($n = 4$). The K_D obtained by using these two rate constants ($K_D = k_2/k_1$) for Imip block was 3.8 μM. As can be observed from the analysis of the plots of Figure 4, the blockade of Imip (3 and 10 μM) increased in an exponential fashion during the depolarization. The extrapolation to 'zero block' shows a delay of 0.48 ± 0.18 ms ($n = 4$) between the start of the pulse and the beginning of the block, but this delay was not concentration-dependent. These results suggest that Imip does not block I_t before its activation by depolarization. Furthermore, Figure 4 shows that the maximum block and the rate at which it was attained were increased with increasing Imip concentrations.

Effects of imipramine on inactivation and recovery from inactivation of I_t

To obtain more information about the possible mechanisms of the Imip-induced blockade of I_t, the effect of the drug on the steady-state inactivation and recovery from inactivation of the current were examined. The effects of 3 μM Imip on the voltage-dependence of I_t inactivation were determined by using a standard two pulse protocol. A conditioning step 1 s in duration was applied from a holding potential of − 70 mV; this was followed by an 800 ms 'test' pulse to + 50 mV. The membrane potential during the conditioning step was varied from − 100 to + 10 mV. As is shown in Figure 5 under control conditions, inactivation of I_t was complete at membrane potentials close to 0 mV, and was almost completely removed at membrane potentials more negative than − 70 mV. The continuous line is the best fit obtained with the Boltzmann equation:

$$I_t/I_{max} = 1/[1 + \exp\ (Vm - Vh/s)]$$

where Vh is the voltage at which the one-half inactivation occurs, Vm the conditioning potential and s the slope factor of the curve. The mean control value for Vh and s in 4 cells were − 35.5 ± 2 mV and 5.7 ± 1.3, respectively. It can be observed that a concentration of 3 μM Imip did not modify the inactivation curve of I_t, the values of Vh and s being − 37.0 ± 3 mV and 5.9 ± 1.2 ($n = 4$), respectively.

The effect of 3 μM Imip on the recovery from inactivation time course of I_t was studied by use of a paired-pulse protocol ($P_1 - P_2$) in which P_1 and P_2 (− 70 mV to + 50 mV for 1 s) were applied once per min at a variable coupling interval (T) from 0.2 to 25 s. Figure 6 shows that as the coupling interval becomes progressively longer, I_t reactivates to greater extent. The time course of recovery from inactivation is best described by a monoexponential function with a time constant (τ_{re}) of 6.5 ± 0.1 s. Imip, 3 μM, did not modify the recovery process of I_t, the τ_{re} being 6.4 ± 0.3 s ($n = 5$; $P > 0.05$).

Discussion

In the present paper the effects of Imip, a tricyclic antidepressant drug, on action potential characteristics and transient outward current (I_t) were analyzed in rabbit isolated single atrial myocytes. The results obtained demonstrated that Imip: (a) induced a concentration-dependent reduction in action potential amplitude and a lengthening of the APD without altering the resting membrane potential, and (b) inhibited the I_t in a concentration-dependent manner. All these effects are similar to those previously described in

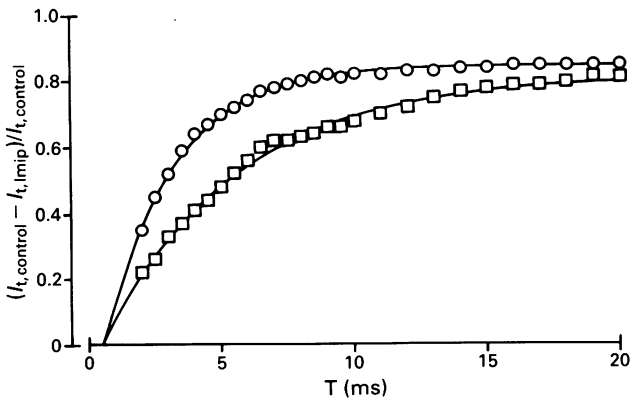


Figure 4 Analysis of the rate of block in the presence of 3 μM (□) and 10 μM (○) imipramine (Imip). The blockade produced by Imip during a depolarizing pulse to + 50 mV expressed as a fraction of the current observed in the absence of the drug is plotted during the first 20 ms of the pulse. $I_{t,control} = I_t$ under control conditions. $I_{t,Imip} = I_t$ in the presence of imipramine. The solid curves show nonlinear least-square fits to the experimental data: $B = B_{max}$ [1-e(-(k_1[D] + k_2t))].

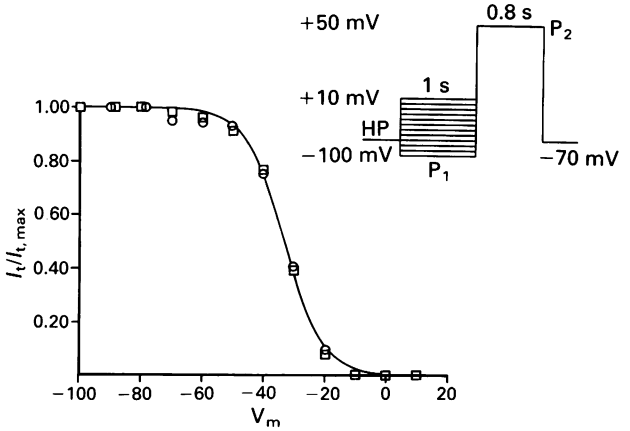


Figure 5 Effect of imipramine (Imip) (3 μM) on the voltage-dependence of steady-state inactivation of I_t. Inset illustrates the double pulse protocol. Pulse 1 (P_1, 1 s in duration) clamped the cell at potentials between − 100 mV and + 10 mV, and pulse 2 (P_2, 0.8 s in duration) depolarized it to + 50 mV. The solid curve through the data points was drawn according to the Boltzmann equation: $I_t/I_{tmax} = 1/[1 + \exp\ (V_m - V_h/s]$, where V_h is the midpotential of the curve and s is the slope of the curve. Under control conditions (○), Vh = − 32.7 mV and s = 5.72. After exposure to 3 μM Imip (□), Vh = − 32.4 mV and s = 5.95.

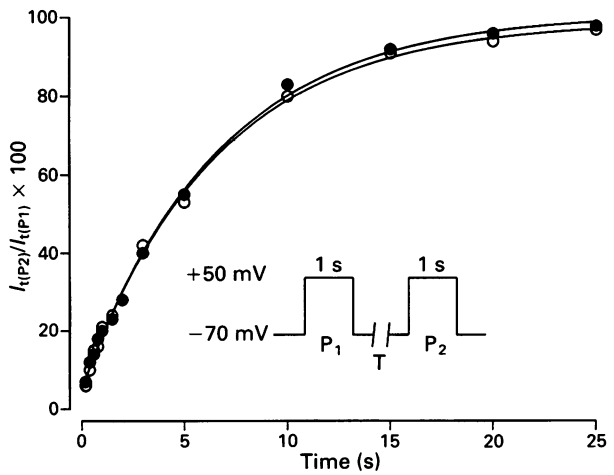


Figure 6 Effects of imipramine (Imip) on the recovery from inactivation of the I_t. Inset shows the experimental protocol. Identical pulses (P_1 and P_2) to + 50 mV from a holding potential of − 70 mV were applied every 60 s and the time interval between them (T) was varied from 0 to 25 s. Both P_1 and P_2 were 1 s in duration. Peak I_t was normalized by dividing the peak amplitude of I_t elicited by P_2 [$I_{t(P2)}$] by the amplitude of I_t elicited by P_1 [$I_{t(P1)}$]. The curves area least-squares fits of a single exponential. (●) Control and (○) 3 μM Imip.

rabbit atria multicellular preparations (Matsuo, 1967).

Imip not only inhibited the peak amplitude of I_t but also significantly accelerated the decay of the current during a sustained depolarization. Thus, Imip enhanced the apparent inactivation kinetics of I_t channels. This effect was reflected by a decrease in the two time constants of the inactivation process. This concentration-dependent acceleration of the current inactivation is to a major extent responsible for the decrease in the integral of the outward current measured at 50 ms after the start of the pulse. This indicator was found to be a sensitive measurement of the drug-induced effect on the I_t channel (Dukes *et al.*, 1990). The acceleration of the rate of decline of I_t observed in the presence of Imip and the decrease in the time to peak current probably reflects a time-dependent block of open channels similar to that proposed to explain the inhibition of I_t produced by quinidine (Imaizumi & Giles, 1987), clofilium (Castle, 1991), tedisamil (Dukes *et al.*, 1990) and bupivacaine (Castle, 1990). On the other hand, Imip had no effect on the voltage-dependence of the steady-state inactivation curve of I_t. However, in the absence of single channel analysis it is impossible to differentiate whether Imip may alter the mean open or closed times of the I_t channel or whether it locks the channel in the drug-occluded inactivated state. In addition, the extrapolation to zero block shows that Imip did not block I_t before its activation at the onset of the depolarization, which suggested that Imip did not interact with the resting state of the I_t channel. Furthermore, the present experiments demonstrated that Imip did not modify the recovery from inactivation process.

The effects of Imip on APD show important species-dependence. Thus, in guinea-pig papillary muscles (García de Jalón *et al.*, 1978; Delpón *et al.*, 1990) and isolated ventricular myocytes (Delpón *et al.*, 1991), bovine ventricular (Isenberg & Tamargo, 1985) and Purkinje fibres (Rawling & Fozzard, 1979), Imip shortened the APD, whereas in rabbit and rat atrial fibres (Matsuo, 1967; Manzanares & Tamargo, 1983) it lengthened the APD. The different effects of Imip on APD may be explained by the important differences in the ionic currents responsible for the repolarization among animal species. Thus, in guinea-pig isolated ventricular myocytes which present relatively little I_t (Giles & Imaizumi, 1988), the APD is controlled by the interaction between inward (I_{Na} and I_{Ca}) and outward currents (I_K and I_{K1}). Imip inhibited the I_{Na} (Ogata & Narahashi, 1989), I_K and I_{Ca} in this order of potency but did not modify the I_{K1} (Isenberg & Tamargo, 1985; Delpón *et al.*, 1991) and thus, it reduced the APD (Delpón *et al.*, 1991). In contrast, in rabbit, rat and human atria (Shibata *et al.*, 1989; Escande *et al.*, 1987; Giles & Imaizumi, 1988) and rat ventricular myocytes (Josephson *et al.*, 1984) the I_K is virtually absent and the I_t appears to be the most important outward K current responsible for action potential repolarization. Therefore, since Imip did not modify the I_{K1}, the inhibitory action on I_t may explain the lengthening of the APD that it induced in rabbit isolated atrial cells.

In the present experiments Imip did not modify the resting membrane potential in rabbit isolated atrial myocytes. This result can be explained by the lack of effect of Imip on the inward rectifier potassium current I_{K1} which in rabbit atrial cells is relatively small (Giles & Imaizumi, 1988) and is responsible for the maintenance of the resting membrane potential).

In this study, the effects of Imip on I_t were studied on a wide range of concentrations (from 0.1–100 μM), which comprised both therapeutic plasma concentrations (0.53–1.07 μM; Marshall & Forker, 1982) and those found after overdosage (>3.57 μM. Glassman & Perel, 1973; Amsterdam *et al.*, 1980). However, it is difficult to relate the *in vivo* plasma concentrations to those of drug-perfusing isolated cardiac myocytes, particularly when the drug is highly bound to plasma and tissue proteins. In fact, it has been found that in experimental animals and man Imip, a highly lipophilic drug, had a selective affinity for cardiac muscle, where it can reach concentrations 20–200 times greater than the concentrations found in plasma (Elonen *et al.*, 1975; Jandhyala *et al.*, 1977; Marshall & Forker, 1982). I_t is the most important outward current not only in rabbit but also in human atrial cells, playing a major role in the regulation of the APD (Escande *et al.*, 1987; Giles & Imaizumi, 1988; Shibata *et al.*, 1989). Therefore, we believe that the Imip-induced prolongation of the APD described in this study may have some clinical relevance. Since the prolongation of the action potential has been recognized as an antiarrhythmic mechanism (i.e. class III; Vaughan Williams, 1984), the inhibition of I_t and the resultant lengthening in atrial APD may be partly responsible for the effectiveness of Imip in supraventricular arrhythmias described in both animal and human models (Marshall & Forker, 1982).

In conclusion, the results obtained in this paper demonstrate that in rabbit isolated atrial cells, Imip inhibits the I_t current and that this effect may be responsible for the lengthening of the APD induced by the drug. These results provide a new insight for the anti/proarrhythmic effects of Imip on those cardiac tissues in which the I_t is important in determining the action potential repolarization process.

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Effect of lignocaine on arginine-vasopressin plasma levels: baseline or induced by frusemide

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1 To assess whether or not lignocaine influences baseline and frusemide-induced (5 mg kg^{-1}) plasma concentrations of arginine-vasopressin (AVP), 2 groups of rabbits received an infusion of lignocaine (130 $\mu g\ min^{-1}\ kg^{-1}$) for 6 h. Lignocaine-induced changes in AVP plasma concentrations were substantiated by measurement of diuresis and natriuresis and hepatic plasma flow, by means of an infusion of indocyanine green (ICG) (249 $\mu g\ min^{-1}\ kg^{-1}$).

2 Baseline plasma AVP levels were 4.9 ± 0.9 pg ml^{-1} ($\pm$ s.e.), and following lignocaine, these values were reduced to 0.7 ± 0.1 pg ml^{-1} ($P<0.01$). Frusemide increased AVP levels to 134.1 ± 73.6 pg ml^{-1} ($P<0.05$) and lignocaine totally prevented this increase, e.g. mean AVP levels of 2.7 pg ml^{-1}.

3 Lignocaine enhanced baseline diuresis secondary to an increase in free water clearance; none of the experimental conditions affected the diuresis and natriuresis induced by frusemide.

4 Frusemide reduced the hepatic plasma flow and this decrease was not reversed by the infusion of lignocaine.

5 It is concluded that in healthy rabbits lignocaine reduces baseline secretion of AVP and its antidiuretic effect; in addition, lignocaine prevents the rise in AVP induced by frusemide.

Keywords: Lignocaine; arginine-vasopressin; renal function; hepatic blood flow; frusemide

Introduction

For more than three decades, lignocaine, a local anaesthetic (Carnegie & Hewer, 1950; Goldberg, 1947), has been used in therapeutics as an antiarrhythmic agent (Zipes, 1984). Although, the electrophysiological properties of lignocaine are well known and acknowledged in the treatment of acute ventricular arrhythmias (Lie *et al.*, 1974), other effects of lignocaine, such as its influence on haemodynamics and on hepatic vascular resistance, are poorly characterized.

Concerning the effect of lignocaine on peripheral haemodynamics, in healthy subjects, low levels ($<3.4\ \mu g\ ml^{-1}$) of lignocaine produced a concentration-dependent elevation in peripheral blood flow resulting from a decrease in peripheral vascular resistance. On the other hand, venous capacitance rose until lignocaine concentrations were around 2 $\mu g\ ml^{-1}$, but decreased at higher concentrations when systolic blood pressure increased (Vyden *et al.*, 1975). The net effect of lignocaine on haemodynamics appears modulated by individual baseline status, since in patients with cardiac failure, an intravenous dose of 50 or 100 mg of lignocaine induced a small decrease in the cardiac index, stroke volume and work indices, as well as in arterial blood pressure (Schumacher *et al.*, 1968).

With respect to the effect of lignocaine on hepatic blood flow, in healthy volunteers, lignocaine appears to produce a concentration-dependent rise in hepatic blood flow, secondary to a fall in splanchnic vascular resistance and an increase in cardiac output (Wiklund, 1977). In anaesthetized dogs, the infusion of lignocaine for 24 h increases hepatic blood flow (Lelorier *et al.*, 1977). These results contrast with those observed in other animals or in patients with heart failure, where lignocaine does not affect hepatic blood flow (Grossman *et al.*, 1969; Burton *et al.*, 1976; Friesen *et al.*, 1986).

Theoretically, in healthy volunteers, the lignocaine-induced decrease in splanchnic vascular resistance could be caused by: (a) a direct effect on smooth muscle vasculature (Aberg & Dhuner, 1972), (b) an effect on the central nervous system (Blair, 1975), or (c) an effect on factors regulating the tonus of the splanchnic vascular bed (Vyden *et al.*, 1975). Since in healthy volunteers, the effect of lignocaine is much more marked on hepatic blood flow than on peripheral resistance or cardiac output (Wiklund *et al.*, 1977), we hypothesized that lignocaine affects the release or effect of factors like arginine-vasopressin (AVP), the activity of which predominantly occurs on the spanchnic vascular bed.

The present study was designed to document in conscious healthy animals whether lignocaine alters (a) baseline plasma AVP levels, and (b) the secretion of AVP induced by a potent stimulus. Frusemide was used to induce AVP secretion, because the depletion of volume produced by frusemide produces a rapid and potent increase in AVP plasma concentrations (Francis *et al.*, 1985). Furthermore, the choice of frusemide was reinforced by the fact that in clinical practice, lignocaine and frusemide are frequently used simultaneously (Pfeifer *et al.*, 1976).

Methods

Animals

Male New Zealand white rabbits weighing 2.2 to 2.7 kg, purchased from Leonard Inc. (Ferme Cunicole, Mirabel, Québec, Canada), were used throughout the study. The rabbits were maintained on Purina pellets and water *ad libitum* in individual, well-ventilated cages. Animals were kept in cages at least 10 days before any experimental work was undertaken.

Each experiment included a group of 6 to 8 rabbits. Before each experiment, a sterile Bardex Foley catheter (No. 8–10) was introduced into the bladder to collect urine specimens. The rabbits were weighed after voiding. Once the animals were in the restraining cage (Plas-Labs, Lansing, MI, U.S.A.), a catheter (Butterfly-21, Abbott Ireland Ltd) was

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placed in the central artery of an ear for blood sampling. A polyethylene tube (PE 50, Becton Dickinson and Co., Rutherford, NJ, U.S.A.) was introduced into the lateral vein of an ear to infuse a solution of 0.9% sodium chloride and 5% dextrose (50:50) at a rate of 14 ml h^{-1}, to replace losses due to ventilation and blood sampling and to inject frusemide or indocyanine green (ICG). To prove that the selected rate of infusion does not affect baseline AVP plasma levels, four rabbits received an intravenous infusion of a solution of 0.9% sodium chloride and 5% dextrose (50:50) at a rate of 14 ml h^{-1} for 6 h; blood samples were withdrawn before the infusion and 90, 180, 270 and 360 min after the beginning of the infusion, to assay plasma AVP. The animals remained conscious throughout the experiment.

In all experiments, renal function and splanchnic circulation were estimated to document the antidiuretic effect of AVP and the vascular resistance of the mesenteric vascular bed, where AVP and other peptides cause vasoconstriction. The splanchnic circulation was assessed to measure hepatic plasma flow, by use of ICG infused at a rate of 249 μg min^{-1} kg^{-1} for various periods of time (Hunton *et al.*, 1960; Caesar *et al.*, 1961; Levy *et al.*, 1962). Preliminary studies demonstrated that ICG serum concentrations remained stable for at least 2 h and that the steady-state was achieved in 8 min. Immediately prior to each infusion of ICG, a new solution was prepared. To verify the hydration status of the rabbits, the animals were weighed again at the end of the experiment.

Experimental protocols

Determination of the effect of lignocaine on baseline levels of arginine-vasopressin Lignocaine was infused into the rabbits at a rate of 130 μg min^{-1} kg^{-1} for 370 min. This rate of infusion was selected because it generates lignocaine steady-state levels between 2 and 3 μg ml^{-1}. Blood samples (2.5 ml) were withdrawn at − 60, 0, 250, 280, 310 and 370 min to assay AVP and measure plasma osmolality and sodium content. To assay serum lignocaine and its metabolites, additional blood samples (1 ml) were withdrawn at 240, 250, 260, 265, 280, 310, 370 min during the infusion and at 20, 30, 40, 50 and 60 min after the infusion. Urine was collected from − 60 to 0 min, from 0 to 250 and from 250 to 370 min. To assess hepatic plasma flow, ICG in 5% dextrose was infused for 12 min, before initiating the infusion of lignocaine, and at 250 min; blood samples were withdrawn after 8 and 10 min of ICG infusion. To facilitate the interpretation of the results, an additional group of 6 rabbits was used to determine the effect of the infusion of lignocaine on mean arterial blood pressure and heart rate; the animals were prepared as described above but the arterial catheter was connected to a pressure transducer (model 1000A, E.M. Instruments, Houston, Tx, U.S.A.) and a recorder (Four A physiograph, E.M. Instruments, Houston, Tx, U.S.A.).

Determination of the effect of lignocaine on frusemide-induced secretion of arginine-vasopressin For this study, the rabbits were subjected to two experiments, in succession, at least 10 days apart. The first one, assessed the effect of frusemide on AVP and the second one, documented the effect of lignocaine on frusemide-induced secretion of AVP.

The infusion of the solution of sodium chloride/glucose was started 238 min before a 75-min infusion of ICG was started (time 0) and at time 12 min, frusemide (5 mg kg^{-1}) was administered intravenously to stimulate AVP secretion. To assay AVP, plasma sodium and osmolality, blood samples (2.5 ml) were withdrawn at 0 and 12 min before, and 5, 10, 15, 30, 45, 60, 75, 90 and 120 min after the administration of frusemide. Other blood samples (0.8 ml) were collected at 0, 2, 4, 6, 8, 10, 12 before and 5, 10, 15, 22, 45 and 60 min after frusemide injection to assay ICG and frusemide plasma concentrations. Urine was collected at the following intervals: − 30–12, 12–30, 30–45, 45–60, 60–90 and 90–120 min, to measure sodium content and osmolality.

In the second experiment, the rabbits received lignocaine at a rate of 130 μg min^{-1} kg^{-1} for 370 min. Frusemide, 5 mg kg^{-1}, was injected intravenously 250 min after the beginning of lignocaine infusion. To assay AVP, blood samples were withdrawn at − 10 and 250 min and thereafter, 30, 60 and 120 min after frusemide administration. Additional blood samples were withdrawn at 5, 10, 15, 22, 45, 80, 90, 100 and 110 min after frusemide administration to measure lignocaine and its metabolites. To assess the effect of frusemide on liver plasma flow in the presence of lignocaine, the infusion of ICG was started 240 min after the beginning of lignocaine infusion and blood samples withdrawn at 250, 255, 260, 265, 272, 295, 330 and 350 min. Urine was collected at 0–250 and 250–310 min.

Analytical methods

Serum concentrations of lignocaine and two of its metabolites (monoethylglycinexylidide (MEGX) and glycinexylidide (GX)) were measured by high performance liquid chromatography (h.p.l.c.) according to the technique described by Marleau *et al.* (1987). Briefly, 0.5 ml of serum was added to 0.35 ml of water, 0.1 ml of 0.5 N NaOH, 0.05 ml of the internal standard solution (ethylmethylglycinexylidide, 50 μg ml^{-1}) and 750 mg of ammonium sulphate. The extraction and h.p.l.c. conditions were as described by Narang *et al.* (1978), except that the mobile phase used in the present study contained 16% acetonitrile.

Serum concentrations of ICG were determined by h.p.l.c. according to the technique described by Rappaport & Thiessen (1982) with few changes. Briefly, to deproteinize the serum, samples were maintained on ice for 10 min; 0.65 ml of acetonitrile and 0.1 ml of the solution containing the internal standard (5 μg ml^{-1} of diazepam in acetonitrile) were then added. The samples were mixed with a Vortex, centrifuged for 5 min at 3000 r.p.m. and a 0.3 ml aliquot evaporated to dryness. The residue was reconstituted in the mobile phase (0.01 M phosphate buffer at pH 3 with 50% acetonitrile). Mobile phase flow rate was 2 ml min^{-1}. ICG was determined with an absorbance detector (Uvicord SD, LKB) at 225 nm. Plasma and urine concentrations of frusemide were assayed by h.p.l.c. as described previously by Lambert *et al.* (1982).

The concentration of sodium in plasma and urine was determined with an automatic flame photometer (Instrumentation Laboratory, Lexington, MA, U.S.A.). Plasma and urine osmolality was measured with an automatic osmometer (Osmette A, Precision Systems Inc., Sudbury, MA, U.S.A.). Plasma levels of AVP were assayed by radioimmunoassay as described elsewhere (Larose *et al.*, 1985); with this method, the within-assay C.V. was 7.6% and the between-assay C.V. was 9.5%.

Drugs used

Lignocaine, monoethylglycinexylidide (MEGX) and glycinexylidide (GX) were donated by Astra Pharmaceuticals Canada Ltd. Frusemide and the methylester of frusemide were provided by Hoechst Pharmaceuticals Canada. AVP was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.), indocyanine green from Aldrich Chemical Company (Milwaukee, Wisconsin, U.S.A.), and diazepam from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Analysis of data

Plasma concentration-time curves for frusemide and lignocaine were well defined by a two-compartment open model with first-order distribution and elimination. The decay of serum concentrations of indocyanine green as a function of time showed only one phase, thus a one compartment open model characterized adequately the decay of ICG concentrations. Pharmacokinetic parameters were estimated by use of

model independent equations as described by Gibaldi & Perrier (1982).

The influence of lignocaine and frusemide on renal function was assessed by measurement of (a) the urinary excretion rate (UV), (b) the rate of sodium excretion in urine ($U_{Na}V$), (c) the renal clearance of sodium (Cl_{Na}) estimated as the ratio of $U_{Na}V$ by sodium plasma concentration, (d) osmolal clearance (Cl_{osm}) calculated from the standard equation: urinary osmolality times UV and divided by plasma osmolality, and (e) free water clearance (Cl_{H_2O}) calculated as UV minus Cl_{osm}.

Statistical tests

The statistical analysis of experimental data was carried out with a one-way analysis of variance for parallel groups and the difference was determined by Dunnett's distribution table (Winer, 1971). The minimal limit of significance was previously fixed at $P<0.05$.

Results

The infusion of a solution of 0.9% sodium chloride and 5% dextrose (50:50) at a rate of 14 ml min^{-1} did not affect baseline plasma AVP levels (Figure 1). This infusion prevented changes in plasma osmolality under all experimental conditions, i.e. plasma osmolality did not differ from baseline values of 285 ± 2 mOsm kg^{-1}. The weight of the animals remained constant throughout the experiments except after frusemide administration, when it decreased by an average of 60 g due to a negative water balance, i.e. losses of water exceeded replacement.

Effects of lignocaine on arginine-vasopressin serum levels

The infusion of lignocaine did not modify mean arterial blood pressure, i.e. 68 ± 6 vs. 70 ± 3 mmHg, but heart rate was slightly increased from 213 ± 22 to 226 ± 8 beats per minute ($P>0.05$). As shown in Figure 1, mean concentrations of 2.4 μg ml^{-1} of lignocaine decreased AVP levels from 4.9 ± 0.9 pg ml^{-1} (mean ± s.e.) to 0.7 ± 0.1 pg ml^{-1} ($P<0.005$). This effect was already apparent 250 min after the beginning of the infusion and was maintained throughout the experiment.

Thirty minutes after its administration, frusemide increased AVP plasma levels from 7.6 ± 2.8 pg ml^{-1} to 134.1 ± 73.6 pg ml^{-1} ($P<0.05$) (Figure 2). AVP plasma concentrations diminished gradually to baseline values in the next 2 h that

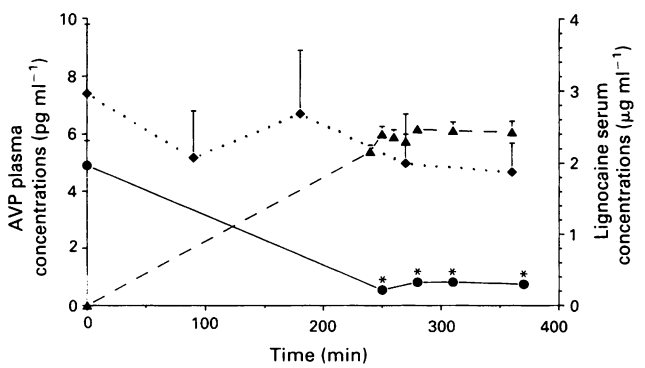


Figure 1 Effect of an infusion of 0.9% sodium chloride and 5% glucose (50:50) at a rate of 14 ml h^{-1} ($n=4$) (◆) or of an infusion of lignocaine (130 μg min^{-1} kg^{-1}) ($n=8$) (●) on average arginine-vasopressin (AVP) plasma levels. The infusion of lignocaine generated mean steady state serum concentrations of 2.44 μg ml^{-1} (▲). Vertical bars are s.e. *$P<0.05$ compared to baseline values.

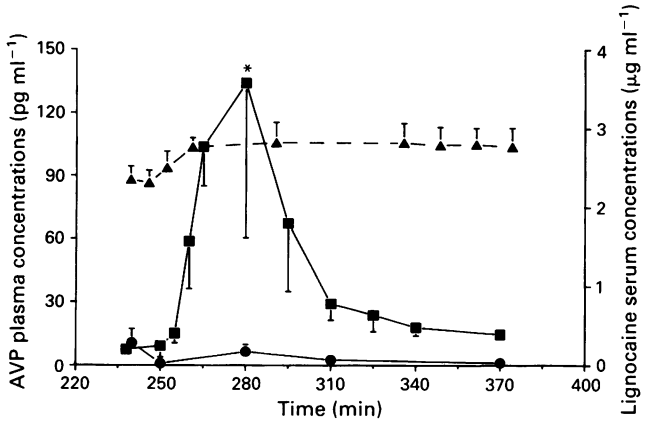


Figure 2 Effect of an infusion of lignocaine (130 μg min^{-1} kg^{-1}) on frusemide-induced arginine-vasopressin (AVP) levels ($n=7$) (●), compared to the influence of frusemide alone ($n=7$) (5 mg kg^{-1}) on average AVP plasma levels (■). The infusion of lignocaine generated mean steady state serum concentrations of 2.83 μg ml^{-1} (▲). Frusemide was injected 250 min after the beginning of the infusion of sodium chloride/glucose (frusemide alone experiment) or of the infusion of lignocaine. Serum AVP and lignocaine levels were estimated 238 min after the beginning of the infusions. Vertical bars are s.e. *$P<0.05$ compared to baseline values.

followed frusemide injection. As shown in the same figure, an infusion of lignocaine, achieving mean steady state serum levels of 2.8 μg ml^{-1}, completely abolished the frusemide-induced increase in AVP plasma concentrations.

Influence of lignocaine on arginine-vasopressin effect

Compared to the control period (from −60 to 0 min), the infusion of lignocaine almost doubled ($P<0.05$) the rate of urinary excretion, although it did not modify sodium renal handling (Table 1). This effect appears to be secondary to the increase in the free water clearance, from -0.203 ± 0.088 to -0.042 ± -0.021 ml min^{-1} ($P<0.05$), possibly associated with the reduction in plasma AVP levels.

By comparison to baseline values, frusemide increased the natriuresis and the diuresis almost tenfold. As shown in Table 1, the inhibition of AVP by the infusion of lignocaine did not change the diuretic and natriuretic response to frusemide. None of the experimental conditions modified frusemide kinetics; aministered alone, frusemide predicted volume of distribution at steady state and systemic clearance were 192 ± 19 ml kg^{-1} and 14.0 ± 0.3 ml min^{-1} kg^{-1}, respectively, and when administered during lignocaine infusion, these values were 224 ± 20 ml kg^{-1} and 14.1 ± 0.9 ml min^{-1} kg^{-1}, respectively.

Effect of lignocaine and frusemide on indocyanine green clearance

ICG systemic clearance was estimated once steady-state was reached. ICG systemic clearance yields a close estimate of hepatic plasma flow, since, once corrected, it coincides with estimates of hepatic blood flow calculated by use of labelled microspheres (Neutze *et al.*, 1968). The value of hepatic plasma flow in the control situation was 39.5 ± 1.2 ml min^{-1} kg^{-1} and decreased by almost 30% ($P<0.05$) following 250 min of infusion of lignocaine (Figure 3). Frusemide caused a 41% decrease ($P<0.05$) in hepatic plasma flow (Figure 3). The infusion of lignocaine did not affect significantly frusemide-induced decrease of hepatic plasma flow.

Table 1 Effect of an infusion of lignocaine (130 μg min^{-1} kg^{-1}) on baseline renal function and on frusemide natriuretic and diuretic response

	*Control period**	*Lignocaine infusion†*	*Control period***	*Frusemide¶*	*Frusemide and lignocaine‡*
UV (ml min^{-1})	0.11 ± 0.02	0.21 ± 0.01§	0.09 ± 0.02	0.98 ± 0.11§	1.18 ± 0.1§
$U_{Na}V$ (μmol min^{-1})	14.7 ± 2.7	10.7 ± 2.0	14.6 ± 1.5	109 ± 16§	142 ± 20§
Cl_{Na} (ml min^{-1})	0.091 ± 0.029	0.090 ± 0.020	0.088 ± 0.016	0.89 ± 0.09§	1.02 ± 0.14§
Cl_{H2O} (ml min^{-1})	− 0.203 ± 0.088	− |†|{ ± 0.021§	− 0.270 ± 0.090	− 0.056 ± 0.082§	0.077 ± 0.026§
Cl_{osm} (ml min^{-1})	0.405 ± 0.088	μψβγγ ± 0.021	0.380 ± 0.030	0.991 ± 0.162§	1.06 ± 0.13§

UV is the urinary excretion rate, $U_{Na}V$ is sodium excretion rate in urine, Cl_{Na} is the renal clearance of sodium; Cl_{H2O} is the free water clearance and Cl_{osm} is osmolar clearance.
*Average values estimated with 60 min urine collections before the infusion of lignocaine ($n = 8$);
†Average values estimated with a 370 min urine collection during lignocaine infusion ($n = 8$);
**Average values estimated with a 42 min urine collection before frusemide administration ($n = 7$);
¶Average values estimated with a 60 min urine collection following frusemide administration ($n = 7$);
‡Average values estimated with a 60 min urine collection following frusemide administration ($n = 7$)
Values are mean ± s.e.
§$P < 0.05$ compared to its respective control period

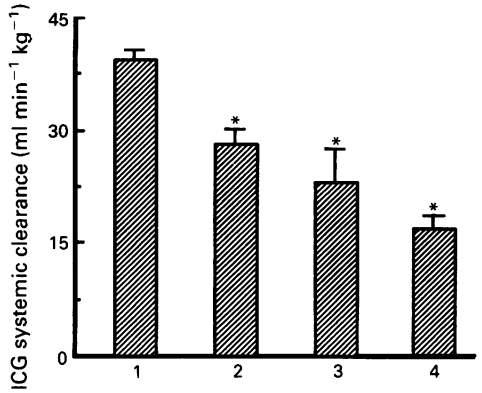


Figure 3 Effect of different experimental conditions on the systemic clearance of indocyanine green (ICG). (1) ICG alone ($n = 6$); (2) when lignocaine was infused (130 μg min^{-1} kg^{-1}) to reach the steady state ($n = 8$); (3) when a dose of 5 mg kg^{-1} of frusemide was administered ($n = 7$), and (4) when frusemide was injected in presence of lignocaine infusion ($n = 7$). *$P < 0.05$ compared to ICG alone. Vertical bars are s.e.

Lignocaine and metabolites kinetics

In the control experiment, the infusion of lignocaine for 4 h generated serum concentrations that remained stable, suggesting that lignocaine steady-state level had been attained. The half-life of lignocaine, estimated during the post infusion phase, was 32.7 ± 5.5 min, supporting that after 4 h of infusion, lignocaine steady state was attained. Compared to control values, frusemide did not affect significantly the systemic clearance of lignocaine (53.2 ± 2.6 vs. 46.1 ± 4.0 ml min^{-1} kg^{-1}).

At steady state of lignocaine, MEGX and GX serum concentrations attained values of around 1.60 and 3.92 μg ml^{-1}, respectively, values that remained quite stable all through the experiment. Frusemide reduced the area under the plasma concentrations (AUC) of MEGX from 193 ± 13 to 137 ± 12 μg min^{-1} ml^{-1} ($P < 0.05$) but did not affect the AUC of GX (380 ± 49 vs. 395 ± 33 μg min^{-1} ml^{-1}).

Discussion

Plasma levels of AVP depend on the rate of secretion of the peptide by the neurohypophysis and on the rate of its degradation. The rate of secretion of AVP is affected by: alterations in plasma osmolality, relayed from central osmoreceptors; non-osmolar stimuli, such as stretch in cardiovascular receptors (baro- and mechanoreceptors) within the thorax; peripheral chemoreceptor-mediated reflexes; sympathomimetic agents; the renin angiotensin system; and drugs (Weitzman & Kleeman, 1980). In animals (Schmitt *et al.*, 1981) or in man (Kimura *et al.*, 1986), the natriuretic and diuretic effect of frusemide produces a decrease in plasma volume similar to the one observed after haemorrhage. As a consequence, AVP secretion is considerably enhanced by baroreceptors, mechanoreceptors (Ledsome *et al.*, 1985), the renin-angiotensin system (Bonjour & Malvin, 1970) and the α-adrenergic system (Kimura *et al.*, 1980; Paller & Linas, 1984; Brunner *et al.*, 1983).

In the current study, lignocaine diminished baseline AVP plasma levels almost sevenfold. This fall in AVP cannot be attributed to changes in plasma osmolality, since the latter remained stable, or to the infusion of sodium chloride-glucose. In the rabbit, exogenous AVP has a very high systemic clearance, about 45 ml min^{-1} kg^{-1} (Lécrivain *et al.*, 1989), and both the splanchnic bed and the liver appear to account for at least half of it (Weitzman & Kleeman, 1980). Therefore, the clearance of AVP should theoretically be considered as a blood-flow-dependent event. Accordingly, increases in splanchnic blood flow should enhance the clearance of AVP. Since lignocaine did not increase hepatic blood flow, we believe that the lignocaine-induced decrease in AVP plasma concentrations is not associated with an increase in AVP clearance. Therefore, we must assume that lignocaine decreases the rate of secretion of AVP. The assumption that lignocaine does not affect AVP levels by changing liver blood flow is reasonable if we bear in mind that frusemide reduced liver blood flow significantly and following the administration of lignocaine, AVP plasma levels were reduced, instead of increased.

Neuronal control of AVP secretion is complex. Baroreceptors and mechanoreceptors, through the vagus nerve to the nucleus tractus solitarius, modulate the stimulation of the neurohypophysis, in such a way that an increase in plasma volume will increase the activity of the baroreceptors, resulting in the inhibition of AVP secretion (Weitzman & Kleeman, 1980). Since lignocaine has very little effect on the autonomic nervous system at low serum concentrations, (Bigger *et al.*, 1985), we speculate that lignocaine decreases AVP baseline plasma concentrations through a direct effect on the hypothalamo-neurohypophysial axis. This hypothesis is supported by the fact that lignocaine affects the excitability of the median preoptic nucleus, which receives afferents from the subfornical organ and sends efferents to the magnocellular region of the hypothalamic paraventricular nucleus,

pathways closely related to AVP secretion (Tanaka *et al.*, 1988).

Lignocaine prevented the increase in AVP plasma concentrations induced by frusemide, probably because of a reduction in AVP secretion by the hypothalamo-neurohypophysial axis rather than an increase in AVP clearance. A 5 mg kg^{-1} dosage of frusemide resulted in a volume depletion of 58 ± 7 ml in 1 h, equivalent to the diuresis produced (Table 1). As mentioned above, this volume depletion will stimulate several systems, all capable of enhancing the release of AVP. The fact that lignocaine was able to inhibit the stimuli responsible for the frusemide-induced secretion of AVP, supports the hypothesis that lignocaine inhibits AVP secretion directly at the hypothalamo-neurohypophysial axis.

The present study does not allow us to conclude whether or not the inhibitory effect on AVP secretion is secondary to lignocaine or its metabolites. Baseline or frusemide-induced AVP plasma concentrations were drastically reduced with lignocaine serum concentrations of about 2.5 $\mu g\ ml^{-1}$. In addition, persistently low levels of AVP were observed three half-lifes after the interruption of lignocaine infusion, when serum concentrations of the parent compound were around 0.3 $\mu g\ ml^{-1}$, but that of the two active metabolites was still showing a plateau. From these results, we speculate that the metabolites may also contribute to the inhibition of AVP secretion.

The inhibition of AVP secretion by lignocaine was reflected by an increase in the diuresis, secondary to an increase in free water clearance. The reduction of AVP plasma levels did not modify significantly the natriuretic and diuretic effect of frusemide; we may speculate that in the presence of frusemide, the inhibition of AVP triggered other homeostatic reactions capable of preventing an increase in frusemide's response. In agreement with another report (Schmitt *et al.*, 1981), frusemide decreased hepatic plasma flow considerably. The inhibition of AVP secretion by lignocaine did not restore hepatic plasma flow to baseline values, suggesting that besides AVP (Kerr *et al.*, 1978; Hock *et al.*, 1984; Schwartz & Reid, 1981) other mechanisms intervene to maintain blood pressure and hepatic plasma flow (Pang, 1983; Burnier *et al.*, 1983; Zerbe *et al.*, 1981). Although we have no evidence on the mechanism underlying the decrease in hepatic blood flow induced by lignocaine, we are tempted to speculate that the inhibition of AVP stimulated compensatory mechanisms that produced a splanchnic vasoconstriction, thus reducing hepatic blood flow. This hypothesis is supported by the fact that during the infusion of lignocaine, despite an inhibition of AVP and a negative balance of water, arterial pressure remained stable.

In summary, in healthy conscious rabbits lignocaine or its metabolites decrease baseline AVP plasma concentrations and inhibit the increase in AVP concentrations induced by frusemide. The reduction in AVP levels by lignocaine entailed an increase in the diuresis, secondary to an increase in free water clearance. We speculate that lignocaine and/or its metabolites directly affect the hypothalamoneurohypophysial axis.

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Effect of the β-adrenoceptor agonist clenbuterol and phytohaemagglutinin on growth, protein synthesis and polyamine metabolism of tissues of the rat

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1 The kidney bean lectin, phytohaemagglutinin (PHA), induced a marked atrophy of skeletal muscle which was evident from the changes in tissue composition (protein, RNA, DNA and polyamine content) and from the reduction in weight and protein synthesis of hind leg muscles of rats fed on kidney bean-diets for four days. The β-adrenoceptor agonist, clenbuterol, induced skeletal muscle hypertrophy by transiently stimulating protein synthesis. As a consequence, the muscle loss caused by a short exposure to PHA was, in part, ameliorated by clenbuterol treatment.

2 Cardiac muscle was affected to a lesser extent than skeletal muscle by both clenbuterol and the lectin. However, there was evidence that protein synthesis in heart was reduced by PHA.

3 PHA had opposite effects on the gut, the lectin-induced hyperplasia of the jejunum was accompanied by a large increase in protein synthesis. Clenbuterol alone had no effect on the jejunum whereas a combination of PHA and clenbuterol appeared to exacerbate the effect of the lectin on gut.

4 Both the lectin-induced gut growth and the hypertrophy of skeletal muscle caused by clenbuterol were preceded by the accumulation of polyamines in the respective tissues. Of particular note was the observation that a significant increase in the proportion of the intraperitoneally injected ^{14}C-labelled spermidine or putrescine taken up by the growing tissues could be detected by the second day. Therefore, the measurement of uptake of labelled polyamines may be used as a sensitive indicator of early alterations in tissue metabolism.

Keywords: Clenbuterol; phytohaemagglutinin (PHA); protein synthesis; small intestine; skeletal muscle; heart; liver; polyamines; growth; atrophy

Introduction

The lectin component of raw kidney bean, the phytohaemagglutinin (PHA) is a potent growth factor for the rat small intestine and induces extensive hyperplastic and hypertrophic growth of the tissue (Oliveira *et al.*, 1988; Pusztai *et al.*, 1988). The basis of this striking physiological activity of PHA is its resistance to proteolytic degradation and consequent binding to the small intestinal epithelium. The lectin survives in an immunologically intact form nearly completely on passage through the entire digestive system (Pusztai *et al.*, 1991a), particularly when bound to the complex carbohydrate moieties of receptors of the luminal membranes of gut epithelial cells (Pusztai *et al.*, 1991b). Binding is followed by endocytosis (King *et al.*, 1986) and partial transcytosis (Pusztai *et al.*, 1991c). Only lectins that are bound and endocytosed by gut cells act as major stimulants of gut growth (Pusztai *et al.*, 1990).

The absorbed PHA induces major shifts in the hormonal balance of the body (Pusztai *et al.*, 1989; 1991a) and interferes with the metabolism of some tissues (Oliveira *et al.*, 1988). One of the most striking effects is the loss of about 30% of skeletal muscle tissue in rats fed with kidney bean diets for a week. Although the mechanism of this atrophy is not fully clear, the reduction of the fractional rate of protein synthesis in the skeletal muscle (Palmer *et al.*, 1987) might be the result of the lowering of the blood insulin levels (Pusztai *et al.*, 1991c).

The β-adrenoceptor agonist, clenbuterol, increases the mass and protein content of skeletal muscle in many species. It has been proposed that the hypertrophy in rodents was mediated by promoting muscle protein deposition, partly by decreasing protein degradation (Reeds *et al.*, 1986) and partly by a transient rise in protein synthesis rate (Emery *et al.*, 1984; Maltin *et al.*, 1987). The effects of clenbuterol on tissues other than muscle and fat have not been extensively studied although a small reduction in liver weight after feeding rats on clenbuterol for 3 weeks has been reported (Reeds *et al.*, 1986), and another β-agonist, ractopamine, had a similar effect in pigs (Aalhus *et al.*, 1990). Reductions in the protein content of both liver and gut have also been reported in steers (Williams *et al.*, 1987). The mechanism of action of clenbuterol is not known; its effectiveness in diabetic rats suggests that insulin may not be involved (McElligott *et al.*, 1987) but others have shown changes in insulin binding (Webster *et al.*, 1986) and insulin concentrations (Beermann *et al.*, 1986; O'Connor *et al.*, 1988) after feeding clenbuterol. It has also been suggested that clenbuterol may exert some of its effects through interaction with hormones, in the case of cardiac hypertrophy these effects may be mediated by prostaglandins (Palmer *et al.*, 1990). Since clenbuterol might have the opposite effect to PHA on hormones, it may prevent or compensate for the PHA-induced muscle atrophy by reversing some of the changes caused by the lectin.

The intimate involvement of polyamines in growth has long been established (Jänne *et al.*, 1978), although their precise role(s) and mode of action remain unknown. PHA-induced gut growth is preceded by polyamine accumulation in the tissue (Pusztai *et al.*, 1989), not as the result of increased *de novo* synthesis of polyamines *in situ*, but by stimulation of polyamine uptake from the circulation (Bardocz *et al.*, 1990a). Thus, the uptake of putrescine through the basolateral membrane increased within the first hour of lectin exposure. However, the effect wore off quickly, lasting for 24 h only (Bardocz *et al.*, 1990b). In contrast, the stimulation of spermidine uptake from the circulation was not immediate but remained elevated and was stimulated

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further for as long as PHA was present in the bowel (Bardocz *et al.*, 1990b).

The present study was undertaken to establish firstly whether accumulation of polyamines occurs during clenbuterol-induced muscle hypertrophy and whether clenbuterol affects the uptake of polyamines from the circulation by skeletal muscle. Since PHA and clenbuterol have opposite actions on skeletal muscle, a second objective was to investigate the possibility that clenbuterol could protect the muscle against the lectin-induced atrophy by preventing the fall in rates of protein synthesis. The skeletal musculature represents a pool of amino acids which may be mobilised in times of dietary insufficiency (Millward *et al.*, 1976), trauma and sepsis (Clowes *et al.*, 1983; Jepson *et al.*, 1986). Therefore the study was designed also to investigate the possibility that, if the muscle atrophy was a prerequisite for the gut hyperplasia, i.e. substrates for the latter were released by mobilising compounds stored in muscle, then clenbuterol might also reduce gut growth. The feasibility of using measurements of polyamine uptake to detect early changes in the metabolism of the gut and gastrocnemius muscle in rats given both PHA and clenbuterol was also investigated.

Methods

Male Hooded-Lister rats of the Rowett strain (about 80 g) were pre-fed *ad libitum* on a diet containing powdered egg albumin (10% protein) for 3 days, then divided into 12 groups, each containing 8 rats; 6 groups were pair-fed with egg albumin (control) diet with or without 4 mg kg^{-1} clenbuterol (Boehringer-Ingelheim). The other 6 groups were fed on a diet in which raw kidney bean and egg albumin each provided 5% protein (Oliveira *et al.*, 1988), with or without 4 mg kg^{-1} clenbuterol. Phytohaemagglutinin (PHA), which is responsible for most of the antinutritional effects of kidney bean diet (Oliveira *et al.*, 1988), accounted for 15% of the bean protein and therefore for 7.5% of total dietary protein. Food consumption of all groups of rats was restricted to 5.5 g per rat daily, with free access to acidified water. The animals were fed for 2, 4 or 7 days, fasted overnight and then refed with 2 g of the appropriate diet 2 h before being killed by decapitation.

Four animals of each group were injected intraperitoneally with 8.5 nmol (3.0×10^6 d.p.m.) [^{14}C]-putrescine and the other four with 9.9 nmol (3.3×10^6 d.p.m.) [^{14}C]-spermidine precisely 1 h before they were killed. Some of the rats also received 150 μmol L-phenylalanine plus 75 μCi L-[2,6-^{3}H]-phenylalanine per 100 g body weight by injection via the lateral tail vein 10 min before death for the measurement of protein synthesis rates (Garlick *et al.*, 1980). All isotopes used were from Amersham International plc (Amersham, Bucks.). Tissues were dissected as appropriate for measuring polyamine uptake, chemical composition or protein synthesis rate.

The small intestine was removed, washed with ice-cold saline, weighed and its length measured. Six sections of 2 cm were cut out from each small intestine at 3; 9; 21; 41; 63 and 81 cm from the pyloric junction and placed into NE 265 scintillation fluid (NE Technology Limited, Edinburgh). After two days of extraction, the sections were counted for radioactivity as previously described (Bardocz *et al.*, 1990a). Extraction for this time yielded maximum counts. The uptake of polyamines by the rat small intestine was calculated *pro rata* from the amounts of radioactivity incorporated into the 6 sections during the 1 h labelling period. The remaining parts of the small intestine were freeze-dried with the exception of a 2 cm section (7–9 cm from pyloric junction) which was used for the protein synthesis measurement.

Both gastrocnemius muscles were excised. That from the right hind limb was treated with 1 ml of NCS tissue digester (Amersham International plc) at 60°C for 16 h. The solution was neutralized by the addition of 30 μl of concentrated acetic acid and the samples were counted for radioactivity after the addition of 10 ml scintillation liquid, NE 265. The left gastrocnemius was freeze dried.

The plantaris, half of the heart, a 250 mg sample of liver and a 2 cm section of the jejunum were immediately frozen in liquid N after dissection. These samples were used for measurement of protein synthesis (Garlick *et al.*, 1980) as described previously (Reeds *et al.*, 1986).

Chemical analysis was performed on the freeze-dried small intestine and muscle tissues. An 18 cm piece of the upper jejunum and the whole left gastrocnemius muscle from each rat were homogenized in 25 and 20 ml of 2% (w/v) perchloric acid respectively and the homogenates centrifuged for 15 min at 10,000 *g*. The polyamine content of the supernatants was analyzed by high performance liquid chromatography (h.p.l.c.) according to Seiler & Knödgen (1980). Protein (Schacterle & Pollak, 1973), RNA (Sneider, 1957), and DNA (Lovtrup & Roos, 1961) estimations were carried out on the precipitates redissolved in a volume of 0.3 M NaOH which was the same as that of perchloric acid used for homogenization. All chemicals used were the highest grade of purity from Sigma Chemical Co. (Poole, Dorset).

Statistical analysis

All data are expressed as means for 4 rats per group. Statistical analysis was carried out by a multiple way analysis of variance 'ANOVA' from 'Minitab' (Statistical Software) and this programme was used to calculate pooled standard deviations. The residual variance (δ^2) was obtained from the analysis of variance tables. The standard errors of difference (s.e.d.) for comparing two groups of size 4 was calculated from the formula:

$$\text{s.e.d.} = \sqrt{\delta^2\ (1/4 + 1/4)}$$

For the analysis of variance the following factors had been taken into consideration: time (2, 4 and 7 days), clenbuterol and kidney bean treatments. Groups statistically different from control at any time were marked with different superscripts. [a] and [b] refer to significant ($P<0.01$) effects of clenbuterol or kidney bean treatment; [c] mark significant exchanges by time and [d] refers to interaction between clenbuterol and kidney bean treatment.

Results

The lectin from kidney bean was found to be a potent growth factor for the rat small intestine. The dry weight of jejunum increased steadily and by 7 days it was 53% above the control value (Table 1). The jejunal contents of RNA, putrescine and spermidine were also significantly increased by the second day (Table 1 and Figure 1). Similarly, 20% and 46% increases in the rate of gut protein synthesis were observed on days 2 and 4 respectively (Table 2). In contrast, the total protein, spermine and DNA contents of the jejunum were not significantly increased until the fourth day. Jejunal hypertrophy continued until day 7 with further increases of 96% in spermine and spermidine and a 120% increase in putrescine content (Figure 1). Clenbuterol had no effect on the growth of the jejunum in rats given either the control or bean-containing diet (Table 1), but it did inhibit the PHA-induced accumulation of putrescine in the jejunum at day 4 and the increments in jejunal polyamine contents in rats fed kidney beans for 7 days (Figure 1). The amounts of polyamines in the jejunum of clenbuterol-treated rats were 40, 83 and 80%, respectively of those in rats given kidney bean alone (Figure 1). In contrast, the presence of clenbuterol appeared to exacerbate the lectin-induced increase in gut protein synthesis by 20% on day 2 and by 12% on day 4 (Table 2).

Table 1 Effect of kidney beans and clenbuterol on the growth of rat jejenum

		(mg)	*Dry weight* (per 100 g body weight)	*Protein* (mg)	*RNA* (mg)	*DNA* (mg)
Diet:	*Days treatment*					
Control	2	118	0.60	69	7.0	3.1
	4	120	0.60	71	6.0	3.0
	7	121	0.58	71	7.0	3.0
Clenbuterol	2	107^{a}	0.60	70	5.4^{a}	3.0
	4	111^{a}	0.55^{a}	72	6.7^{ac}	3.2
	7	109^{a}	0.55^{a}	66	7.4	3.5
Kidney bean	2	142^{b}	0.70^{b}	73^{b}	9.2^{b}	3.6^{b}
	4	155^{bc}	1.06^{bc}	97^{bc}	16.0^{bc}	4.0^{b}
	7	185^{bc}	1.07^{bc}	112^{bc}	17.0^{bc}	5.2^{bc}
Clenbuterol + kidney bean	2	136^{d}	0.70^{b}	77^{b}	9.3^{b}	3.8^{b}
	4	148^{dc}	1.02^{d}	89^{d}	16.2^{b}	4.7^{d}
	7	189^{bc}	1.10^{dc}	119^{dc}	18.4^{bc}	5.7^{dc}
s.e.d.		3	0.01	2	0.3	0.2

Rats (groups of 8) were fed on egg albumin (control) or kidney bean diets with or without 4 mg kg^{-1} clenbuterol; 20 cm of the upper jejunum was used for analysis. Values are means of 4 and s.e.d. Statistical treatment was by analysis of variance taking out time, clenbuterol and kidney bean effects and their interactions. Data within a column with different superscripts are significantly different from control: $P<0.01$. [a] and [b] represent significant effects of clenbuterol or kidney bean treatments, [c] marks the effect of time on a treatment and [d] represents interaction between clenbuterol and the kidney bean treatments.

Table 2 Fractional rates of protein synthesis (k_s; % per day) in the liver and jejunum of rats fed kidney beans (PHA) and/or clenbuterol for 2 or 4 days

	Jejunum	*Liver*
2 days		
Control	108.9	90.7
Clenbuterol	107.1	93.6
PHA	130.8^{b}	87.5
PHA + Clenbuterol	157.7^{d}	93.2
4 days		
Control	99.2	88.5
Clenbuterol	108.6	89.4
PHA	145.0^{b}	75.1^{b}
PHA + Clenbuterol	162.5^{b}	68.1^{b}
s.e.d.	10.0	8.6

Values are means of 4 and s.e.d. Statistical treatment was by analysis of variance taking out time, clenbuterol and kidney bean effects and their interactions. Data within a column with different superscripts are significantly different from control: $P<0.01$. [a] and [b] represent significant effects of clenbuterol or kidney bean treatments, [c] marks the effect of time on a treatment and [d] represents interaction between clenbuterol and the kidney bean treatments.

The expected effects of clenbuterol on skeletal muscle hypertrophy were evident from the increased weight, protein and DNA contents of the hind leg gastrocnemius tissue by the 4th day of treatment (Table 3). Moreover, RNA (Table 3) and spermidine (Figure 2) contents of the gastrocnemius muscle were significantly increased by the second day. In contrast, in rats fed on kidney beans for 4 or more days, muscle atrophy was observed (Table 3). Clenbuterol partially protected the muscle against the atrophy caused by the kidney bean lectin by ameliorating the loss of weight, protein, and RNA (Table 3) and reversing the kidney bean-induced loss of spermidine and spermine (Figure 2).

Fractional rates of protein synthesis and RNA content were measured in the plantaris muscle and were affected by both clenbuterol and kidney bean lectin (Table 4). By day 2 of treatment clenbuterol had increased the RNA content by 15% and the fractional rate of protein synthesis by over 30%, whilst the lectin had significantly decreased both RNA and protein contents and the fractional rate of protein synthesis. Clenbuterol appeared partially to protect the muscle from the lectin-induced atrophy at this time. Both the RNA content of the muscle and the rate of protein synthesis were significantly higher on the combined treatment than when the

Table 3 Effect of clenbuterol and kidney bean on the growth of rat gastroecnemius

		(mg)	*Dry weight* (per 100 g body weight)	*Protein* (mg)	*RNA* (mg)	*DNA* (mg)
Diet:	*Days treatment*					
Control	2	87	0.38	68	0.97	0.27
	4	93^{c}	0.39	70	1.07^{c}	0.29
	7	98^{c}	0.39	69	1.03^{c}	0.29
Clenbuterol	2	89	0.39	70	1.22^{a}	0.29
	4	104^{a}	0.46^{a}	76^{ac}	1.40^{ac}	0.33^{a}
	7	111^{ac}	0.46^{a}	79^{ac}	1.70^{ac}	0.44^{ac}
Kidney bean	2	80^{b}	0.41^{b}	67	0.76^{b}	0.24^{b}
	4	73^{bc}	0.39	55^{b}	0.67^{bc}	0.24^{b}
	7	71^{bc}	0.35^{b}	43^{bc}	0.43^{bc}	0.22^{b}
Clenbuterol + kidney bean	2	86	0.43^{d}	70	0.85^{d}	0.28
	4	78^{d}	0.42^{d}	63^{d}	0.80^{cd}	0.24^{b}
	7	84^{dc}	0.41^{d}	57^{b}	0.65^{bc}	0.22^{b}
s.e.d.		1	0.01	2	0.2	0.1

Rats (groups of 8) were fed on egg albumin (control) or kidney bean diets with or without 4 mg kg^{-1} clenbuterol. Freeze dried gastrocnemius muscles from both hind limbs were analysed. Values are means of 4 and s.e.d. Statistical treatment was by analysis of variance taking out time, clenbuterol and kidney bean effects and their interactions. Data within a column with different superscripts are significantly different from control: $P<0.01$. [a] and [b] represent significant effects of clenbuterol or kidney bean treatments, [c] marks the effect of time on a treatment and [d] represents interaction between clenbuterol and the kidney bean treatments.

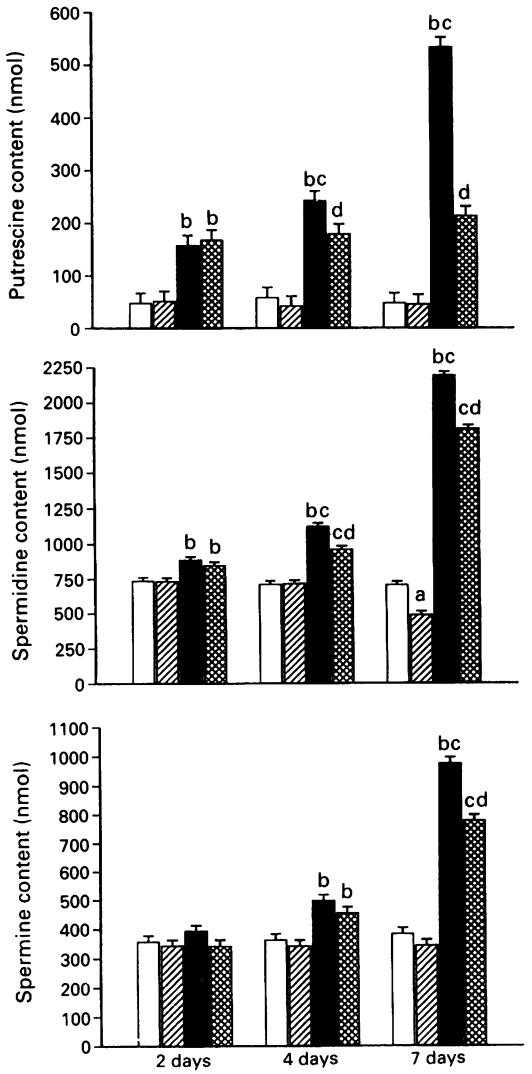


Figure 1 Effect of kidney bean and clenbuterol on the polyamine content of rat jejunum. Rats (groups of 4) were fed on egg albumin (control) with (hatched column) or without (open column) 4 mg kg^{-1} clenbuterol or kidney bean diets with (cross-hatched column) or without (solid column) 4 mg kg^{-1} clenbuterol; 20 cm of their upper jejunum was measured and analysed. Values are means of 4 and s.e.d. (vertical bars). Statistical treatment was by analysis of variance taking out time, clenbuterol and kidney bean effects and their interactions. Data with different superscripts are significantly different from control: $P<0.01$. [a] and [b] represent significant effects of clenbuterol or kidney bean treatments, [c] marks the effect of time on a treatment and [d] represents interaction between clenbuterol and the kidney bean treatments.

lectin was fed alone and were not significantly different from the control group. By 4 days the lectin had induced further losses of RNA and protein from the plantaris muscle and the fractional synthesis rate remained depressed. The effect of clenbuterol on protein synthesis rate appeared to have diminished and was no longer significant. Also, the ability of clenbuterol to protect against the lectin-induced atrophy appeared to have been lost as both the RNA and the fractional rate of protein synthesis were significantly below the control values by this time (Table 4).

Effects of both lectin and clenbuterol on the heart (Table 5) were similar to those on the skeletal muscle. Clenbuterol increased the heart weight and RNA content but did not reduce the rate of protein synthesis. Kidney bean lectin tended to reduce heart weight and protein synthesis rates were lower in the two lectin-fed groups (with or without clenbuterol) at both 2 and 4 days.

Liver protein synthesis rates were unaffected by any treatment on day 2 (Table 2), polyamine content of the liver was also unaffected (data not shown). The only significant effect on the liver was a reduction in the rate of protein synthesis by day 4 on the combined kidney bean plus clenbuterol diet and there were effects on polyamine content at the same time. Spermidine content of the liver was unchanged by clenbuterol (3122 ± 205 nmol; control = 3328 ± 170 nmol) but significantly decreased by the lectin (2434 ± 26 nmol, $P<0.05$) and by the combined diet (2284 ± 52 nmol, $P<0.05$). Spermine content was unaffected by lectin or clenbuterol alone (1905 ± 92 nmol and 1946 ± 98 nmol respectively; control 1922 ± 135 nmol) but, like spermidine, was decreased on the diet containing both kidney beans and clenbuterol (1717 ± 29 nmol, $P<0.05$).

Early changes in metabolic activity of all the tissues studied were detected by measurement of the uptake of ^{14}C-labelled polyamines from the peritoneum. Rats given PHA

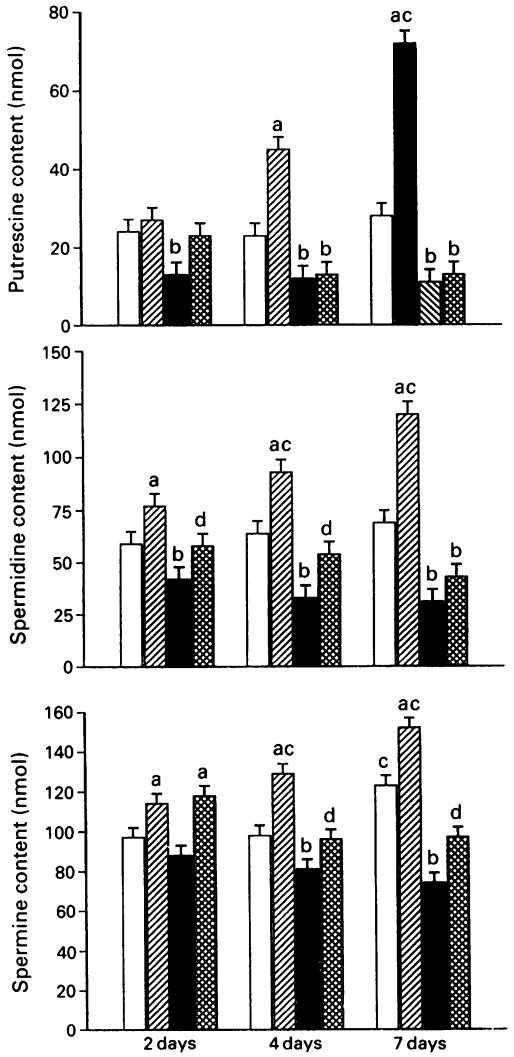


Figure 2 Effect of clenbuterol and kidney bean on the polyamine content of rat gastrocnemius. Rats (groups of 4) were fed on egg albumin (control) with (hatched column) or without (open column) 4 mg kg^{-1} clenbuterol or kidney bean diets with (cross-hatched column) or without (solid column) 4 mg kg^{-1} clenbuterol. Freeze-dried gastrocnemius muscles from both hind limbs were analysed. Values are means of 4 and s.e.d. (vertical bars). Statistical treatment was by analysis of variance taking out time, clenbuterol and kidney bean effects and their interactions. Data with different superscripts are significantly different from control: $P<0.01$. [a] and [b] represent significant effects of clenbuterol or kidney bean treatments, [c] marks the effect of time on a treatment and [d] represents interaction between clenbuterol and the kidney bean treatments.

Table 4 Effect of kidney beans (PHA) and clenbuterol on weight, RNA and protein content and fractional rate of protein synthesis of plantaris muscle

	Wt per 100 g body weight	*RNA* (μg)	*Protein* (mg)	k_s (%per day)
2 days				
Control	91.9	119.4	17.5	15.2
Clenbuterol	98.8[a]	137.6[a]	17.7	20.3[a]
PHA	82.9[b]	91.9[b]	14.0[b]	10.3[b]
PHA + clenbuterol	91.5	107.9[d]	15.5[b]	14.7
4 days				
Control	90.6	111.7	15.1	15.3
Clenbuterol	98.5[a]	138.6[a]	15.6	17.7
PHA	84.4[b]	68.2[bc]	12.1[bc]	10.1[b]
PHA + clenbuterol	91.4	78.2[b]	13.0[bc]	12.5[d]
s.e.d.	2.8	6.0	0.9	1.3

Values are means of 4 and s.e.d. Statistical treatment was by analysis of variance taking out time, clenbuterol and kidney bean effects and their interactions. Data within a column with different superscripts are significantly different from control: $P<0.01$. [a] and [b] represent significant effects of clenbuterol or kidney bean treatments, [c] marks the effect of time on a treatment and [d] represents interaction between clenbuterol and the kidney bean treatments.

Table 5 Effect of kidney beans (PHA) and clenbuterol on weight, RNA and protein content and fractional rate of protein synthesis of heart

	Wt per 100 g body weight	*RNA* (mg)	*Protein* (mg)	k_s (%per day)
2 days				
Control	389	1.42	59.3	14.3
Clenbuterol	472[a]	2.02[a]	59.5	15.2
PHA	392	1.32[b]	56.6[b]	12.4
PHA + clenbuterol	443[d]	1.75[d]	66.6[d]	13.1
4 days				
Control	393	1.60	54.2	13.9
Clenbuterol	430[ac]	1.97[a]	55.4	13.4
PHA	350[b]	1.21[b]	54.2	11.4[b]
PHA + clenbuterol	401[d]	1.33[d]	51.3[d]	11.2[b]
s.e.d.	15	0.04	1.2	1.2

Values are means of 4 and s.e.d. Statistical treatment was by analysis of variance taking out time, clenbuterol and kidney bean effects and their interactions. Data within a column with different superscripts are significantly different from control: $P<0.01$. [a] and [b] represent significant effects of clenbuterol or kidney bean treatments, [c] marks the effect of time on a treatment and [d] represents interaction between clenbuterol and the kidney bean treatments.

showed increased jejunal uptake of both putrescine and spermidine (Figure 3). Clenbuterol did not significantly affect the uptake of either putrescine and spermidine by the gut in rats fed egg albumin but further stimulated spermidine uptake in rats fed the bean diet (Figure 3).

The negative effect of PHA on muscle growth was reflected by a significant decrease in the uptake of labelled spermidine by the gastrocnemius muscle on day 4 (Figure 4). Conversely clenbuterol-induced muscle hypertrophy was accompanied by a stimulation of putrescine uptake (+ 80%) and spermidine uptake (+ 137%) on day 2 (Figure 4). Like the effects of clenbuterol on protein synthesis (Table 4), there was a tendency for the stimulation by clenbuterol of putrescine and spermidine uptake to fall with time.

Discussion

Although the biochemical mechanism whereby clenbuterol promotes muscle protein deposition is unclear, it has been suggested that it involves mobilization of body lipids through interaction with β-adrenoceptors and occurs at the expense of the growth of other tissues of the body (Reeds *et al.*, 1986). The growth of the small intestine induced by PHA also involves mobilization of body lipids (Oliveira *et al.*, 1988; Pusztai *et al.*, 1989). The present study was designed therefore to investigate the combined effects of the two different growth factors on muscle and small intestinal metabolism.

The effects of clenbuterol on muscle mass were small in comparison with previous studies where increases in protein content in excess of 20% have been observed (Reeds *et al.*, 1986; Maltin *et al.*, 1989). The rats in earlier experiments were fed *ad libitum* whilst those in the present study were pair-fed on the amount of diet consumed by the kidney bean-treated groups, receiving approximately half their normal dietary intake. Although the clenbuterol content of the diet was doubled to ensure similar daily intakes of the drug, the dietary restriction may account for the relatively poor hypertrophy of the plantaris. In other respects however the plantaris did respond well to clenbuterol, the significant increase in RNA content and fractional rate of protein synthesis by the second day, and the tendency for synthesis rates to

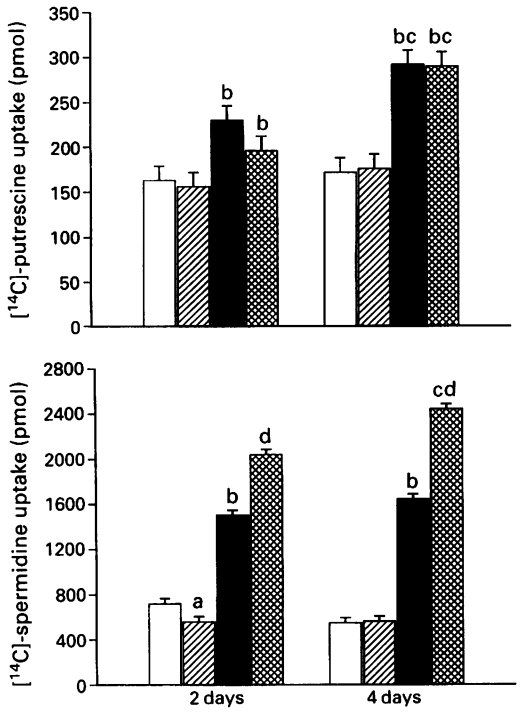


Figure 3 The effects of clenbuterol and kidney bean on the uptake of [^{14}C]-putrescine or [^{14}C]-spermidine from the peritoneum by the rat small intestine. Rats (groups of 4) were pair fed with egg albumin (control) with (hatched column) or without (open column) 4 mg kg^{-1} clenbuterol or kidney bean diets with (cross-hatched columns) or without (solid column) containing 4 mg kg^{-1} clenbuterol (22 μg per rat daily) for 2 or 4 days. Exactly 1 h before killing each group was injected with either [^{14}C]-putrescine or [^{14}C]-spermidine intraperitoneally, and the radioactivity accumulated to their entire small intestine measured. Values are means of 4 and s.e.d. (vertical bars). Statistical treatment was by analysis of variance taking out time, clenbuterol and kidney bean effects and their interactions. Data with different superscripts are significantly different from control: $P<0.01$. [a] and [b] represent significant effects of clenbuterol or kidney bean treatments, [c] marks the effect of time on a treatment and [d] represents interaction between clenbuterol and the kidney bean treatments.

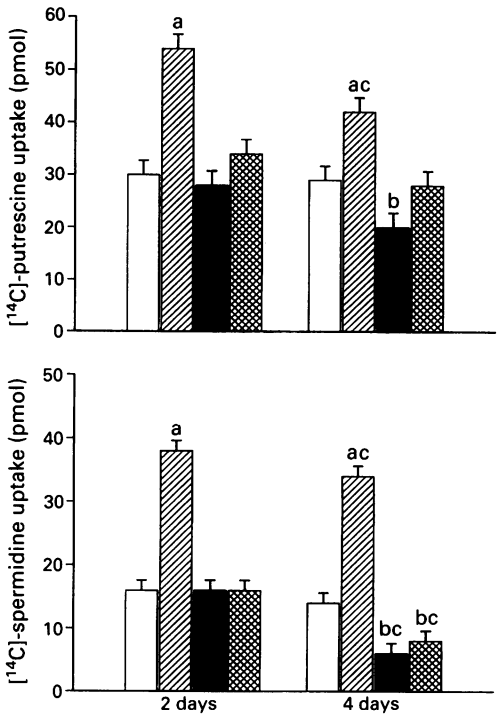


Figure 4 Effect of clenbuterol and kidney bean on the growth of rat gastrocnemius. Rats (groups of 4) were fed on egg albumin (control) with (hatched column) or without (open column) 4 mg kg^{-1} clenbuterol or kidney bean diets with (cross-hatched column) or without (solid column) 4 mg kg^{-1} clenbuterol. Freeze dried gastrocnemius muscles from both hind limbs were analysed. Values are means of 4 and s.e.d. (vertical bars). Statistical treatment was by analysis of variance taking out time, clenbuterol and kidney bean effects and their interactions. Data with different superscripts are significantly different from control: $P<0.01$. [a] and [b] represent significant effects of clenbuterol or kidney bean treatments, [c] marks the effect of time on a treatment and [d] represents interaction between clenbuterol and the kidney bean treatments.

return towards control values by day 4 (Table 4) seem to be typical of the response of rats to the drug. It has been proposed that in rats, clenbuterol both increases synthesis and decreases degradation of muscle protein, the former of these effects being transient in nature and responsible for the early hypertrophic effects of the drug (Table 4 and Maltin *et al.*, 1989). It is interesting therefore that the protection against the lectin-induced muscle atrophy also appears to be transient and occurred only during the period when clenbuterol was acting to stimulate the rate of protein synthesis (Table 3).

As with other growth processes (Jänne *et al.*, 1978), the clenbuterol-induced muscle hypertrophy was accompanied by an increase in the polyamine content of the gastrocnemius muscle (Figure 2). In fact, the accumulation of spermidine was significantly increased within two days of exposure (before the changes in weight, protein or DNA content were observed). Therefore, like the changes in muscle RNA content and in the fractional rate of protein synthesis, which, in clenbuterol-induced hypertrophy usually precede detectable muscle growth (Table 4), changes in polyamine levels (Figure 2) may be a useful early marker for muscle hypertrophy.

In the muscle tissue of PHA-fed rats, treatment with clenbuterol initially maintained the concentration of putrescine at levels close to those in control rats whilst rats fed on the lectin alone showed reduced muscle spermidine levels on day 2 (Figure 2). By 4 days, putrescine content in the gastrocnemius of rats fed lectin plus clenbuterol had declined dramatically and was no longer different from the level in the rat fed the lectin only; by day 7 the spermidine content was also significantly below control values (Figure 2). The reduction in polyamines in response to the lectin suggests that a drop in tissue polyamine levels may provide an indication of incipient tissue loss just as increases in tissue polyamine levels can be used as indicators of gut hypertrophy (Pusztai *et al.*, 1989). Furthermore the transient partial protection by clenbuterol against the effect of the lectin seems to coincide with the partial protection against the lectin-induced muscle hypertrophy discussed above.

Cardiac hypertrophy is a well known effect of clenbuterol in the rat (Reeds *et al.*, 1986) but the data presented here suggest that this effect is not associated with an increase in the fractional rate of protein synthesis (Table 5). Although the lectin did not affect heart weight and clenbuterol did, it was the lectin treatment which was shown to have significant effects on cardiac protein synthesis. These observations suggest that in response to lectin treatment, cardiac atrophy is prevented by proportionately similar decreases in the rates of protein synthesis and degradation. In this respect the heart appears to respond similarly to the tonic soleus muscle which, unlike the phasic plantaris and gastrocnemius, did not atrophy in response to kidney bean lectin in spite of a marked reduction in the rate of protein synthesis (Palmer *et al.*, 1987).

The liver was the tissue least affected by either clenbuterol or the kidney bean lectin. The only significant effect on the rates of liver protein synthesis was a reduction by day 4 in the group of rats fed on kidney beans plus clenbuterol (Table 2). The close temporal correlation between this effect on protein synthesis and the changes in polyamine content, which were only apparent in the combined diet (lectin plus clenbuterol) provide further support for the suggestion that polyamines may be a useful early marker for changes in rates of tissue metabolism that precede growth.

The phytohaemagglutinin, PHA, is a powerful growth stimulant for the gut. Thus, raw kidney bean (Pusztai *et al.*, 1988) and particulary its lectin component (PHA) (Oliveira *et al.*, 1988) induce hypertrophic and hyperplastic growth of the rat small intestine *in vivo* (Pusztai *et al.*, 1991a,b,c). Both the binding of PHA to gut carbohydrate receptors and its endocytosis by epithelial cells appear to be necessary for the stimulation of cellular protein (Table 2) and glycoprotein synthesis (Palmer *et al.*, 1987) and the increase of RNA, DNA and polyamine content of the gut tissue (Table 1, Pusztai *et al.*, 1988).

The increased rate of uptake of ^{14}C-labelled putrescine and spermidine by the jejunum of rats given kidney bean diet (Figure 3) and by the gastrocnemius muscle of rats treated with clenbuterol (Figure 4) indicates that polyamines may play an early role in the growth process. Previous studies have indicated that most of the polyamines required to support the growth of the gut come from external sources via the circulation (Bardocz *et al.*, 1990a,b), suggesting that polyamines may be exchanged between various organs of the body following a growth stimulus. This idea is very attractive. On exposure to PHA, the gastrocnemius (which is representative of the majority of the skeletal musculature) loses more than 20% of its mass and polyamine content in a week (Table 3). Therefore, from the atrophy of the skeletal muscles, which represent about 40% of the total body mass and protein content, relatively large amounts of polyamines might be available for the replacement of those which had been taken up by organs such as the gut, whose growth is stimulated by PHA.

As both the increase in gut polyamine levels and the outflow of polyamines from the muscle occurs very early on PHA stimulation, it seems probable that the inter-organ communication link is not passive and that there is some hormonal signal. The finding that PHA in the gut lumen can immediately reduce circulating insulin levels (Pusztai *et al.*, 1991c) appears to support a direct signalling mechanism. On the basis of the present experimental evidence it appears that there is a parallel between polyamine uptake and protein synthesis rate in the tissues examined. However, it is difficult

to suggest a precise role for polyamines in this signalling process, i.e. whether they act as second messengers or simply follow the changes occurring in muscle. The latter view appears to be more realistic. As the polyamine pool of the body is limited in size, there is competition between the organs for the polyamines available to support the growth induced by a combination of growth factors.

In this paper three different effects of clenbuterol and the kidney bean lectin PHA on tissue growth have been identified. In phasic skeletal muscle clenbuterol imparts a partial and temporary protection against the severe atrophy caused by the lectin. In the heart a previously unidentified action of the lectin appears to be a reduction in protein synthesis; this however does not result in a marked cardiac atrophy, nor is it affected by clenbuterol. Finally, the PHA-induced hyperplasia of the jejunum leading to rapid increase in protein synthesis, is not reversed but accentuated by clenbuterol. It has been shown for the first time that clenbuterol-induced skeletal muscle hypertrophy is accompanied by polyamine accumulation in that tissue. Indeed, this increase is clear before growth and protein accretion could be detected. More importantly, measurements of the uptake of labelled putrescine and spermidine from the circulation by specific organs induced to grow by different stimuli can predict metabolic changes in these tissues at an even earlier stage and can reflect changes in growth and metabolism even when they result from the combined effects of more than one stimulant.

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Coeliac haemodynamic effects of endothelin-1, endothelin-3, proendothelin-1 [1–38] and proendothelin-3 [1–41] in conscious rats

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1 Conscious, chronically-instrumented, Long Evans rats were given bolus doses of endothelin-1, endothelin-3 (both at 0.01 and 0.1 nmol kg^{-1}), proendothelin-1 [1–38] and proendothelin-3 [1–41] (both 0.1 and 1 nmol kg^{-1}) in order to compare their effects on coeliac haemodynamics, because it has been reported that, in conscious dogs, endothelin-1 has paradoxical, prolonged hyperaemic vasodilator effects in this vascular bed. Measurements were made also of mesenteric and hindquarters haemodynamics for comparison. In a separate experiment, endothelin-1 (0.1 nmol kg^{-1}) was given before and 20 min after the onset of an infusion of mecamylamine (50 μmol $kg\ h^{-1}$) to ensure that the responses measured were not confounded by rapid reflex changes in autonomic activity.

2 None of the peptides caused any increases in coeliac flow or any sustained rises in coeliac vascular conductance, although such changes were clear-cut in the hindquarters vascular bed following the higher dose of endothelin-1 and endothelin-3. In animals treated with mecamylamine the regional haemodynamic effects of the higher dose endothelin-1 were not different from those in animals with intact baroreflexes.

3 Although the lower dose of both endothelin-1 and endothelin-3 caused less marked coeliac than mesenteric vasoconstriction, this difference was not apparent with the higher dose of the peptides, or with proendothelin-1 [1–38]. However, proendothelin-3 [1–41] had less marked coeliac and hindquarters vasoconstrictor effects than proendothelin-1 [1–38], in spite of both peptides causing similar changes in mesenteric haemodynamics. These differences could have been due to regional differences in the conversion of proendothelin-1 [1–38] and proendothelin-3 [1–41] to endothelin-1 and endothelin-3, respectively. Our findings contradict the proposition that exogenous proendothelin-3[1–41] is not converted into endothelin-3 *in vivo*.

4 Since, in contrast to endothelin-1 and endothelin-3, proendothelin-1 [1–38] and proendothelin-3 [1–41] caused only small hindquarters vasodilatations, but large vasoconstrictions, it is feasible that the vasodilator responses to exogenous endothelin-1 and endothelin-3 are pharmacological phenomena, and that, *in vivo*, the production of endothelins from proendothelins exerts widespread vasoconstrictor effects.

Keywords: Haemodynamics; endothelin-1; endothelin-3; proendothelin-1 [1–38]; proendothelin-3 [1–41]; conscious rats

Introduction

Although endothelin-1 was originally described as the most potent vasoconstrictor peptide yet discovered (Yanagisawa *et al.*, 1988), it was soon found to cause marked, initial vasodilatations *in vivo*, particularly in the hindquarters and carotid vascular beds (Wright & Fozard, 1988; Gardiner *et al.*, 1989). These effects were observed in anaesthetized, areflexic rats (Wright & Fozard, 1988) and in conscious, intact animals (Gardiner *et al.*, 1989). It has been claimed that endothelin-3 has greater vasodilator effects than endothelin-1 (Warner *et al.*, 1989), although we have not been able to confirm that in conscious rats (Gardiner *et al.*, 1990a,b). Furthermore, although both endothelin-1 and endothelin-3 have been found to elicit mesenteric vasodilator effects *in vitro* (De Nucci *et al.*, 1988; Warner *et al.*, 1989), such responses have not been seen *in vivo* (Wright & Fozard, 1988; Gardiner *et al.*, 1989; 1990a,b). However, recently Bigaud & Vatner (1990) described a substantial, and persistent, coeliac vasodilator effect of endothelin-1, in association with mesenteric and iliac vasoconstrictions in conscious dogs. Therefore, in the present work one objective was to investigate the coeliac haemodynamic effects of endothelin-1 and endothelin-3 in conscious rats. In the same animals we made simultaneous measurements of mesenteric and hindquarters blood flows to ensure that the responses we had described earlier were reproducible, and also to act as comparisons for the changes in coeliac haemodynamics. Since there is a possibility that rapid autonomic reflex changes could influence the regional haemodynamic responses to vasoactive agents *in vivo*, we carried out an additional experiment in a separate group of rats in which we assessed regional haemodynamic responses to endothelin-1 (0.1 nmol kg^{-1}) before and after administration of mecamylamine (Wright & Fozard, 1990).

There is increasing evidence that the *in vivo* haemodynamic effects of proendothelin-1 [1–38] are due to its conversion to endothelin-1 (Kimura *et al.*, 1989; Kashiwabara *et al.*, 1989; D'Orléans-Juste *et al.*, 1990; Matsumura *et al.*, 1990; Fukuroda *et al.*, 1990; Douglas & Hiley, 1991; Gardiner *et al.*, 1991) and that this conversion occurs locally rather than in the systemic circulation (Gardiner *et al.*, 1991). If this is the case, then administration of proendothelin-1 [1–38] might produce haemodynamic effects that more closely resemble the actions of endogenous endothelin-1 than does administration of exogenous endothelin-1. It is not known if a similar argument can be advanced for proendothelin-3 [1–41], since *in vitro* data indicate this peptide is not cleaved by the same

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enzyme that cleaves proendothelin-1 [1–38] (Okada *et al.*, 1990), and there are no data on the *in vivo* regional haemodynamic effects of proendothelin-3 [1–41] in conscious rats. Therefore, another objective was to assess the effects of proendothelin-1 [1–38] and proendothelin-3 [1–41] on coeliac, mesenteric and hindquarters haemodynamics in the same animals that received endothelin-1 and endothelin-3, to allow a comparison of the responses to all these peptides.

Methods

Male, Long Evans rats (350–450 g) were anaesthetized (sodium methohexitone, 60 mg kg^{-1}, i.p., supplemented as required) and had miniaturized pulsed Doppler probes (Haywood *et al.*, 1981) sutured around the coeliac and superior mesenteric arteries (Gardiner *et al.*, 1990c) and the distal abdominal aorta, below the level of the ileocaecal artery, to monitor hindquarters blood flow (Gardiner *et al.*, 1989; 1990a,b). The probe wires ran subcutaneously and emerged at the back of the neck where they were anchored by a suture. Animals were given ampicillin (Penbritin, Beecham: 7 mg kg^{-1}, i.m.) and were then returned to their individual home cages with free access to food (Biosure, GLP grade diet 41B (M)) and water. At least 7 days later, animals were briefly re-anaesthetized (sodium methohexitone, 40 mg kg^{-1}, i.p.) and had intravenous (right jugular vein) and intraarterial (distal abdominal aorta via ventral caudal artery) catheters implanted. Following a recovery period of at least 24 h, experiments were begun and they were completed over the following 3 days.

In the first experiment, all animals ($n = 9$) received 2 doses of endothelin-1 and endothelin-3 (0.01 and 0.1 nmol kg^{-1}), and 2 doses of proendothelin-1 [1–38] and proendothelin-3 [1–41 amide] (0.1 and 1.0 nmol kg^{-1}). The order of administration of the peptides was randomised, but the lower doses were always given before the higher doses because the effects of the latter were so prolonged. We did not give endothelin-1 or endothelin-3 at a dose of 1.0 nmol kg^{-1} because in pilot experiments it caused cardiovascular deterioration. No animal received more than 3 doses of peptides on any day, and at least 2 h elapsed between doses.

In the second experiment, animals ($n = 7$) were challenged with endothelin-1 (0.1 nmol kg^{-1}) in the absence and in the presence of mecamylamine, to block ganglionic transmission. In pilot experiments we gave mecamylamine at the dose used by Wright & Fozard (1990) (0.25 mg kg^{-1} over 15 min), but found this did not abolish reflex heart rate changes in our conscious rats. Therefore, in the full experiment, we gave a 10 fold higher dose (i.e. 50 μmol kg^{-1} h^{-1}) and started the infusion of mecamylamine 20 min before challenging with endothelin-1. The experiments in the absence and presence of mecamylamine were carried out on separate days.

In the first experiment, for 30 min before any peptide was injected, continuous measurements were made of mean systemic arterial blood pressure (MAP), instantaneous heart rate, and mean coeliac, mesenteric and hindquarters Doppler shift signals. Percentage changes in the latter were taken as indices of changes in flow, and % changes in regional vascular conductance were calculated from mean Doppler shift signals and mean systemic arterial blood pressure (Gardiner *et al.*, 1990a,b).

In the second experiment there were significant differences in resting cardiovascular variables in the absence and presence of mecamylamine (see Results). In order not to obscure any possible differences between responses to endothelin-1 in the two conditions, we compared changes in the form of the absolute values rather than percentages.

The results given in the text refer to group means (± s.e.mean) derived from average values obtained over periods of 20 s immediately before any intervention and the 20 s periods around the time points at which measurements were made.

Peptides

Endothelin-1, endothelin-3, proendothelin-1 [1–38] and proendothelin-1 [1–41] amide were obtained from the Peptide Institute (Osaka) through their UK agents (Scientific Marketing Associates). In the text, for brevity, proendothelin-1 [1–38] and proendothelin-3 [1–41 amide] are referred to as proendothelin-1 and proendothelin-3, respectively.

The peptides were dissolved in a small volume of glacial acetic acid, diluted with isotonic saline (157 mmol l^{-1} NaCl) containing 1% bovine serum albumin (Sigma) and 100 μl aliquots were frozen at − 80°C. All injections were given in 100 μl and flushed in with 150 μl saline. Peptide vehicle alone in these volumes had no consistent cardiovascular effects. Mecamylamine hydrochloride (Sigma) was dissolved in isotonic saline and infused at 0.3 ml h^{-1}.

Data analysis

Changes relative to pre-injection baseline values were assessed by Friedman's test (Theordorsson-Norheim, 1987). Comparisons between responses to different peptides were made by Wilcoxon's ranks sums test applied to areas under or over curves (AUC or AOC, respectively) derived from the raw data using a Fortran programme (Gardiner *et al.*, 1990e). Baseline variables in the absence and presence of mecamylamine were compared by Student's t test for paired samples. A P value <0.05 was taken as significant.

Results

Experiment 1

The baseline values for all protocols in experiment 1 are given in Table 1.

Table 1 Average resting cardiovascular variables before administration of endothelin-1, endothelin-3, proendothelin-1 [1–38] or proendothelin-3 [1–41] in the same conscious, Long Evans rats ($n = 9$)

		Endothelin-1	*Endothelin-3*	*Proendothelin-1 [1–38]*	*Proendothelin-3 [1–41]*
Heart rate (beats min^{-1})		337 ± 10	338 ± 7	328 ± 16	336 ± 12
Mean arterial blood pressure (mmHg)		106 ± 1	105 ± 1	108 ± 2	107 ± 2
Doppler shift (kHz)	Coeliac	9.0 ± 1.0	9.2 ± 1.1	8.6 ± 1.0	8.4 ± 1.2
	Mesenteric	6.0 ± 0.5	5.8 ± 0.6	5.9 ± 0.7	5.6 ± 0.7
	Hindquarters	4.2 ± 0.2	4.4 ± 0.2	3.8 ± 0.3	3.8 ± 0.2
Vascular conductance ([kHz $mmHg^{-1}$]10^3)	Coeliac	86 ± 11	87 ± 10	80 ± 8	78 ± 10
	Mesenteric	57 ± 5	55 ± 5	55 ± 7	53 ± 6
	Hindquarters	40 ± 3	42 ± 2	35 ± 3	37 ± 2

Values are mean ± s.e.mean.

Responses to endothelin-1

The lower dose (0.01 nmol kg^{-1}) of endothelin-1 caused a transient tachycardia (AUC, 90 ± 19 units) followed by a slight bradycardia (AOC, 44 ± 17 units) but no change in MAP (Figure 1). However, there was a significant, although slight, reduction in coeliac blood flow (AOC, 46 ± 6 units) and a more marked reduction in mesenteric blood flow (AOC, 107 ± 13 units) (Figure 1). These changes were associated with a slight coeliac (AOC, 39 ± 7 units) and a greater mesenteric (AOC, 97 ± 12 units) vasoconstriction (Figure 1). In contrast, there was a transient rise in blood flow (AUC, 81 ± 11 units) and vascular conductance (AUC, 92 ± 15 units) in the hindquarters (Figure 1).

The higher dose (0.1 nmol kg^{-1}) of endothelin-1 caused tachycardia (AUC, 116 ± 16 units) followed by bradycardia (AOC, 144 ± 25 units) in company with a fall (AOC, 43 ± 4 units) and subsequent rise (AUC, 48 ± 11 units) in MAP (Figure 2). Reductions in coeliac and mesenteric flows (AOC, 83 ± 15 and 117 ± 9 units, respectively) and vascular conductances (AOC, 101 ± 13 and 119 ± 13 units, respectively) occurred, but in contrast to the responses to the low dose of endothelin-1, there were no significant differences between the responses in the coeliac and mesenteric vascular beds, although there was a slight, initial vasodilatation in the former that was absent in the latter (Figure 2). There was a substantial rise in hindquarters flow (AUC, 139 ± 26 units) in association with a large vasodilatation (AUC, 211 ± 31 units) that was succeeded by a slight, and transient vasoconstriction (AOC, 48 ± 10 units) (Figure 2).

Responses to endothelin-3

The lower dose (0.01 nmol kg^{-1}) of endothelin-3 elicited responses that were not different from those following the lower dose of endothelin-1 (heart rate: tachycardia AUC, 63 ± 22 units, bradycardia AOC, 55 ± 16 units; coeliac flow AOC, 49 ± 15 units; mesenteric flow AOC, 66 ± 13 units; hindquarters flow AUC, 69 ± 15 units; coeliac conductance AOC, 44 ± 15 units; mesenteric conductance AOC, 60 ± 11 units; hindquarters conductance AUC, 78 ± 18 units, Figure 1).

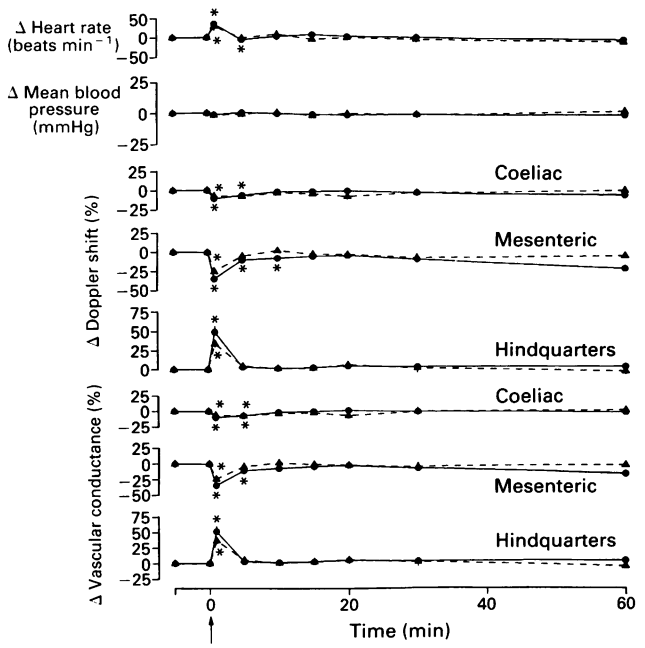


Figure 1 Cardiovascular changes following an i.v. bolus injection (at arrow) of endothelin-1 (0.01 nmol kg^{-1}; ●—●) or endothelin-3 (0.01 nmol kg^{-1}; ▲---▲) in the same conscious, Long Evans rats ($n = 9$). Values are mean, and vertical bars show s.e.mean; where the bars are not visible they lie within the symbols. $*P < 0.05$ versus baseline.

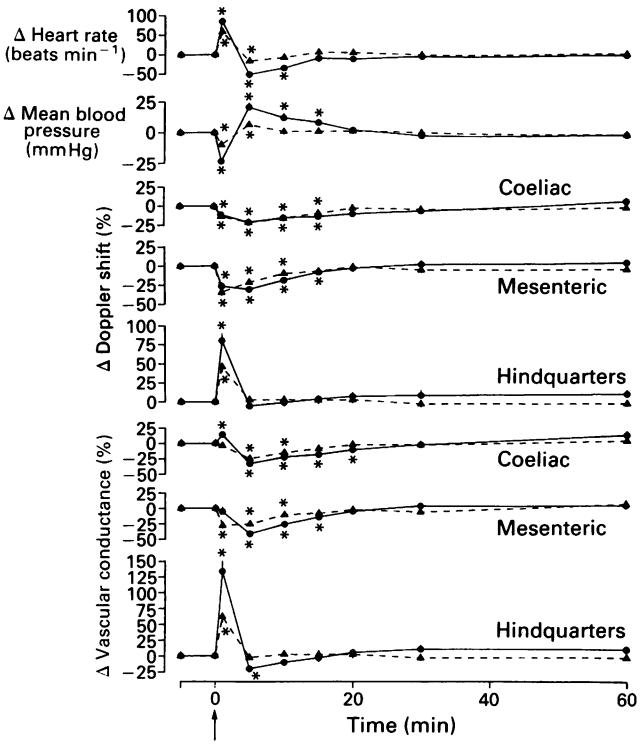


Figure 2 Cardiovascular changes following an i.v. bolus injection (at arrow) of endothelin-1 (0.1 nmol kg^{-1}; ●—●) or endothelin-3 (0.1 nmol kg^{-1}; ▲---▲) in the same conscious, Long Evans rats ($n = 9$). Values are mean, and vertical bars show s.e.mean; where the bars are not visible they lie within the symbols. $*P < 0.05$ versus baseline. The statistics for differences between the integrated responses to endothelin-1 and endothelin-3 are given in the text.

Although the general pattern of cardiovascular response to the higher dose (0.1 nmol kg^{-1}) of endothelin-3 was qualitatively similar to that of endothelin-1 (Figure 2), the later bradycardic effect of endothelin-3 (AOC, 59 ± 17 units) was less than that to endothelin-1, while the initial tachycardic response to the former (AUC, 102 ± 14 units) was not different from that of the latter (Figure 2). However, the early fall (AOC, 27 ± 6 units) and subsequent rise (AUC, 20 ± 5 units) in MAP following endothelin-3 were both less than those following endothelin-1 (Figure 2), although the reductions in coeliac and mesenteric blood flows and vascular conductances were not different following endothelin-3 (coeliac flow AOC, 73 ± 13; mesenteric flow AOC, 110 ± 12 units; coeliac conductance AOC, 75 ± 14 units; mesenteric conductance AOC 108 ± 11 units) and endothelin-1 (Figure 2). But both the increase in hindquarters flow and the rise in vascular conductance were significantly less with endothelin-3 (AUC, 75 ± 19 and 103 ± 23 units, respectively) than with endothelin-1, and there was no subsequent hindquarters vasoconstriction with endothelin-3 (Figure 2).

Responses to proendothelin-1

The lower dose (0.1 nmol kg^{-1}) of proendothelin-1 caused a slight rise (AUC, 47 ± 17 units) followed by a fall (AOC, 114 ± 23 units) in heart rate and a gradual, but small, pressor effect (AUC, 41 ± 13 units) (Figure 3). There were slight, but persistent, reductions in coeliac flow (AOC, 95 ± 13 units) and mesenteric flow (AOC, 73 ± 18 units) and conductances (AOC, 122 ± 15 and 101 ± 17 units, respectively) that were not different (Figure 3). There was an initial increase and a subsequent decrease in hindquarters flow (AUC, 34 ± 14 units; AOC, 76 ± 17 units) and vascular conductance (AUC, 33 ± 14 units; AOC, 96 ± 23 units) (Figure 3).

The higher dose (1.0 nmol kg^{-1}) of proendothelin-1 caused no significant tachycardia, but a marked and sustained reduction in heart rate (AOC, 571 ± 58 units) and increase in MAP (AUC, 204 ± 7 units) (Figure 4). These effects were accompanied by substantial, and similar, falls in coeliac flow (AOC, 240 ± 25 units) and mesenteric flow (AOC, 252 ± 27 units) and vascular conductances (AOC, coeliac 326 ± 18 units; mesenteric 333 ± 21 units) (Figure 4). There were early, slight, and transient increases in hindquarters flow (AUC, 27 ± 7 units) and conductance (AUC, 19 ± 6 units) but, thereafter, both showed prolonged reductions (AOC, flow 238 ± 27 units; conductance 319 ± 20 units) (Figure 4).

Responses to proendothelin-3

The lower dose (0.1 nmol kg^{-1}) of proendothelin-3 had no effect on MAP but there was a slight increase (AUC, 38 ± 12 units) followed by a decrease (AOC, 104 ± 15 units) in heart rate (Figure 3). These effects were not different from those following proendothelin-1. There were modest reductions in coeliac and mesenteric flows (AOC, 119 ± 14 and 70 ± 9 units, respectively) and vascular conductances (AOC, coeliac 124 ± 14 units; mesenteric 73 ± 12 units) following proendothelin-3 that were similar to those after proendothelin-1 (Figure 3). However, proendothelin-3 had no significant effects on hindquarters haemodynamics (Figure 3).

The higher dose (1.0 nmol kg^{-1}) of proendothelin-3 caused a slight, initial tachycardia (AUC, 42 ± 9 units), but subsequently there was a bradycardia (AOC, 292 ± 37 units) in association with a rise in MAP (AUC, 116 ± 18 units (Figure 4). The bradycardic and pressor effects of proendothelin-3 were significantly less than those of proendothelin-1. Proendothelin-3 caused reductions in coeliac and mesenteric blood flows (AOC, coeliac 152 ± 13 units; mesenteric 236 ± 16 units) and vascular conductances (AOC, coeliac 209 ± 8 units; mesenteric 284 ± 21 units). The effects of proendothelin-3 on coeliac haemodynamics were significantly less than those of proendothelin-1 (Figure 4).

There was a tendency towards an increase in hindquarters flow (AUC, 27 ± 13 units) and in vascular conductance (26 ± 17 units) following proendothelin-3, but the changes did not reach significance, although were not significantly different from those following proendothelin-1 (Figure 4). In contrast, the later reduction in hindquarters flow (AOC, 11 ± 21 units) and vascular conductance (AOC, 175 ± 24 units) were significantly less than those following proendothelin-1 (Figure 4).

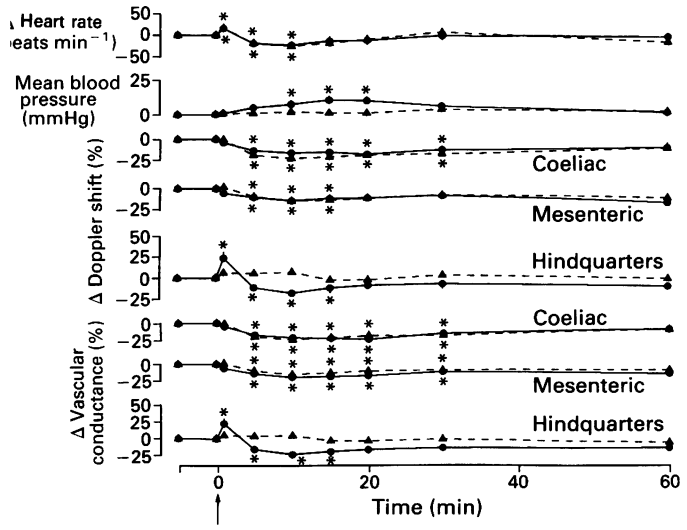


Figure 3 Cardiovascular changes following an i.v. bolus injection (at arrow) of proendothelin-1 [1–38] (0.1 nmol kg^{-1}; ●—●) or proendothelin-3 [1–41 amide] (0.1 nmol kg^{-1}; ▲---▲) in the same conscious, Long Evans rats ($n = 9$). Values are mean, and vertical bars show s.e.mean; where the bars are not visible they lie within the symbols. *$P < 0.05$ versus baseline.

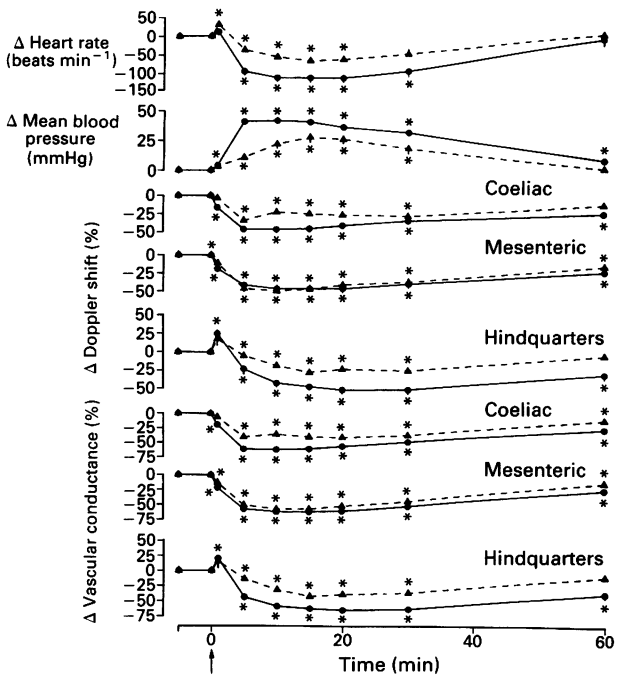


Figure 4 Cardiovascular changes following an i.v. bolus injection (at arrow) of proendothelin-1 [1–38] (1.0 nmol kg^{-1}; ●—●) or proendothelin-3 [1–41 amide] (1.0 nmol kg^{-1}; ▲---▲) in the same conscious, Long Evans rats ($n = 9$). Values are mean, and vertical bars show s.e.mean; where the bars are not visible they lie within the symbols. *$P < 0.05$ versus baseline. The statistics for differences between the integrated responses to proendothelin-1 [1–38] and proendothelin-3 [1–41] amide are given in the text.

Experiment 2

The baseline values in the absence and presence of mecamylamine are shown in Table 2. In the absence of mecamylamine haemodynamic variables were not different from those in experiment 1 (see Table 1), and the cardiovascular responses to endothelin-1 (0.1 nmol kg^{-1}) were similar to those seen in experiment 1 (compare Figures 2 and 5).

In the presence of mecamylamine it was difficult to assess true baseline values for cardiovascular variables, since any motor activity of the animals was accompanied by marked swings in MAP and blood flows.

However, on average, there was resting hypotension and bradycardia and reductions in mesenteric and hindquarters blood flows, together with a mesenteric vasoconstriction (Table 2, Figure 5). Administration of endothelin-1 in the presence of mecamylamine caused an initial reduction in MAP (– 22 ± 1 mmHg) that was not different from that seen in the absence of mecamylamine (– 19 ± 2 mmHg) (Figure 5). Although the subsequent increase in MAP was significantly greater in the former than in the latter condition, the absolute level of MAP reached was not different (Figure 5). The changes in regional vascular conductances elicited by endothelin-1 under the two conditions were not significantly different (Figure 5). There were no significant changes in heart rate following administration of endothelin-1 in the presence of mecamylamine (Figure 5).

Discussion

Elsewhere we have shown that reflex effects are not responsible for the changes in cardiac function (other than heart rate)

Table 2 Average resting cardiovascular variables before and 20 min after onset of mecamylamine infusion (50 μmol kg^{-1} h^{-1}) in the same conscious, Long Evans rats ($n = 7$)

		Before mecamylamine	*During mecamylamine*
Heart rate (beats min^{-1})		334 ± 7	284 ± 7*
Mean arterial blood pressure (mmHg)		106 ± 2	83 ± 1*
Doppler shift (kHz)	Coeliac	8.1 ± 0.8	7.0 ± 0.5
	Mesenteric	6.1 ± 0.5	3.5 ± 0.2*
	Hindquarters	3.8 ± 0.4	2.7 ± 0.4*
Vascular conductance ([kHz $mmHg^{-1}$]10^3)	Coeliac	77 ± 8	85 ± 7
	Mesenteric	57 ± 4	42 ± 3*
	Hindquarters	36 ± 4	33 ± 5

Values are mean ± s.e.mean. *$P < 0.05$ versus before mecamylamine (paired t test).

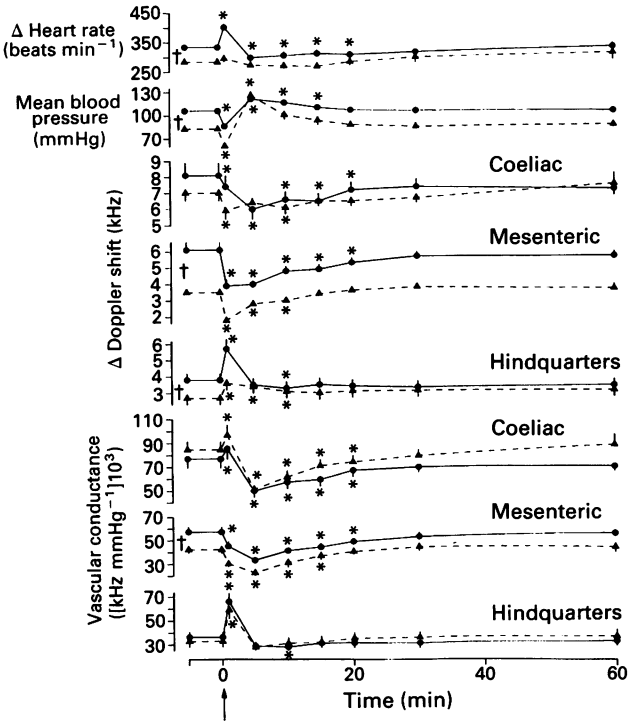


Figure 5 Cardiovascular responses to an i.v. bolus injection (at arrow) of endothelin-1 (0.01 nmol kg^{-1}) in the same conscious, Long Evans rats ($n = 7$) in the absence (●—●) or presence (▲---▲) of mecamylamine (50 μmol kg^{-1} h^{-1} begun 20 min before administration of endothelin-1). Values are mean and vertical bars show s.e.mean; where the bars are not visible they lie within the symbols. *$P < 0.05$ versus baseline; †$P < 0.05$ for resting values in the absence and presence of mecamylamine.

following administration of endothelin-1 (Gardiner *et al.*, 1990d). In addition, the results of the second experiment in the present study indicate that rapid reflex changes in autonomic activity were not confounding the observations by attenuating or potentiating the direct regional haemodynamic effects of endothelin-1. Thus, there is no reason to assume that any of our findings were not due, straightforwardly, to the cardiovascular effects of the peptides studied.

Our main objective was to obtain data on the coeliac haemodynamic effects of endothelin-1 and endothelin-3 in conscious rats, since Bigaud & Vatner (1990) have reported unusual, prolonged vasodilator responses to endothelin-1 in this vascular bed in conscious dogs, and *in vitro* data indicate endothelin-3 is a more potent vasodilator than endothelin-1 (Warner *et al.*, 1989). However, it was clear that under none of our experimental conditions did either peptide elicit increases in coeliac blood flow, although, at the higher dose, endothelin-1 caused a transient increase in coeliac vascular conductance (Figure 2), and the reductions in flow and vascular conductance in this vascular bed following the lower dose of endothelin-1 and endothelin-3 were less than those in the mesenteric vascular bed. Our failure to observe increases in coeliac blood flow was clearly not due to an inability to detect hyperaemia since, in the same animals, we confirmed the occurrence of marked increases in hindquarters flow in response to both endothelin-1 and endothelin-3 (Gardiner *et al.*, 1990a,b). Furthermore, in other studies we have demonstrated clear increases in coeliac blood flow in response to peptides such as neuromedin U-25, rat α-calcitonin gene-related peptide and vasopressin (Gardiner *et al.*, 1990c).

In the present experiments the initial hypotensive and hindquarters hyperaemic vasodilator effects of endothelin-1 were greater than those of endothelin-3. Moreover, only the former peptide caused coeliac vasodilatation. Hence, in conscious rats there is no evidence that endothelin-3 is a more potent vasodilator agent than endothelin-1 (Warner *et al.*, 1989).

The lower dose of exogenous endothelin-1 and endothelin-3 had less marked coeliac, than mesenteric, vasoconstrictor effects, and no hindquarters vasoconstrictor action. However, the higher dose of both peptides caused similar vasoconstrictions in coeliac and mesenteric beds, without reducing hindquarters flow. One possible explanation of these differences is that bolus doses of endothelin-1 or endothelin-3 cause marked stimulation of vasodilator mechanisms that oppose their vasoconstrictor action, as clearly seen in the hindquarters. Thus, the same could be true to a lesser extent in the coeliac and mesenteric vascular beds, even though overt increases in flow did not occur. If this were the case, it is feasible that the more marked vasoconstrictor effects of endothelin-1 and endothelin-3 in the coeliac and mesenteric, than in the hindquarters, vascular beds was due to more effective stimulation of vasodilator mechanisms in the latter. However, another possibility is that rapid enzymatic degradation of exogenous endothelin-1 and endothelin-3 (Sokolovsky *et al.*, 1990; Vijayaraghavan *et al.*, 1990) in the hindquarters vascular bed prevents them exerting more marked effects at that site.

An additional objective was to compare the profiles of effect of proendothelin-1 and proendothelin-3, and to consider them against those of endothelin-1 and endothelin-3. The first point of note is that neither proendothelin-1 nor proendothelin-3 evoked increases in coeliac flow or vascular conductance. But, although in several instances proendothelin-1 had greater vasoconstrictor effects than proendothelin-3, the latter, nevertheless, did exert marked coeliac, mesenteric and hindquarters haemodynamic effects. Hence, the lack of conversion of proendothelin-3 to endothelin-3 *in vitro* by enzymes that act upon proendothelin-1 (Okada *et al.*, 1990; 1991) is likely to be a feature of the isolated system studied in those experiments. Our findings differ from those reported recently by D'Orléans-Juste *et al.* (1991), in anaesthetized guinea-pigs. These workers observed that doses of proendothelin-3 up to 20 nmol kg^{-1} were without effects on

MAP in their experiments, whereas endothelin-1, proendothelin-1 and endothelin-3 at doses of 2 nmol kg^{-1} caused rises in MAP of about 25 mmHg. In recent experiments in animals instrumented for measurement of renal, mesenteric and hindquarters haemodynamics, we have confirmed the pressor and vasoconstrictor effets of proendothelin-3 (Gardiner, S.M. *et al.*, unpublished observations). Furthermore, in those same experiments, we found that proendothelin-2 exerted pressor and vasoconstrictor effects. These observations support our proposition (see above) that *in vitro* assessment of enzyme activity may produce incomplete results, since Okada *et al.* (1991) have reported that the enzyme that converts proendothelin-1 to endothelin-1 is inactive against proendothelin-2.

From the present results it appears that proendothelin-1 can exert similar vasoconstrictor effects on coeliac, mesenteric and hindquarters vascular beds, but proendothelin-3, while having similar mesenteric vasoconstrictor effects to proendothelin-1, has less marked coeliac and hindquarters vasoconstrictor effects. Hence the mesenteric vascular bed may be endowed with enzyme systems that effectively convert both proendothelin-1 and proendothelin-3 to endothelin-1 and endothelin-3, respectively, whereas the local conversion of proendothelin-3 to endothelin-3 might be less in the coeliac and hindquarters vascular beds. In this context, it is notable that the mesenteric vasoconstrictor effets of proendothelin-1 are more resistant to phosphoramidon than are its renal or hindquarters vasoconstrictor actions (Gardiner *et al.*, 1991).

The present results with proendothelin-1 and proendothelin-3 indicate that activation of vasodilator mechanisms (except, perhaps in the hindquarters) may be a feature only of the pharmacology of exogenous endothelin-1 and endothelin-3 and that, *in vivo*, the local genesis of endothelin-1 or endothelin-3 from proendothelin-1 or proendothelin-3, respectively, might exert vasoconstrictor effects in all vascular beds.

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Peripheral analgesic activities of peptides related to α-melanocyte stimulating hormone and interleukin-1β[193–195]

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1 The hyperalgesic effects of interleukin-1β (IL-1β) and prostaglandin E_2 (PGE_2) were measured in rats.

2 Hyperalgesic responses to IL-1β were inhibited in a dose-dependent manner by α-melanocyte stimulating hormone (α-MSH)-related peptides with the following order of potency: [N1[4],D-Phe[7]]α-MSH > α-MSH > Lys-D-Pro-Val > Lys-Pro-Val > Lys-D-Pro-Thr > D-Lys-Pro-Thr.

3 Hyperalgesic responses to PGE_2 were not inhibited by Lys-D-Pro-Thr and D-Lys-Pro-Thr but were inhibited in a dose-dependent manner by the other peptides with the same order of potency as against IL-1β.

4 The potencies of [N1[4], D-Phe[7]]α-MSH and α-MSH were greatly diminished by deletion of their C-terminal tripeptide, Lys[11]-Pro-Val[13].

5 Nor-binaltorphimine (Nor-BNI) largely reversed the analgesic effects of α-MSH, [N1[4], D-Phe[7]]α-MSH, Lys-Pro-Val and Lys-D-Pro-Val indicating that κ-opioid receptors mediated the analgesic activity of these peptides.

6 Nor-BNI did not antagonize the inhibition by Lys-D-Pro-Thr and D-Lys-Pro-Thr of IL-1β evoked hyperalgesia indicating that these peptides were not acting via κ-opioid receptors.

Keywords: Interleukin-1β; hyperalgesia; α-MSH; analgesic peptides

Introduction

The term interleukin-1 (IL-1) describes two pluripotent inflammatory proteins, IL-1α and IL-1β (March *et al.*, 1985), produced by activated macrophages and other cell types (Oppenheim *et al.*, 1986). In rats, recombinant human (rh) IL-1β is a potent hyperalgesic (nociceptive) agent that is inhibited by the tripeptide analogues of IL-1β, Lys[193]-Pro-Thr[195] and Lys-D-Pro-Thr (Ferreira *et al.*, 1988) and by α-melanocyte stimulating hormone (α-MSH) and its stable analogue [N1[4], D-Phe[7]]α-MSH (Follenfant *et al.*, 1989). α-MSH and [N1[4], D-Phe[7]]α-MSH inhibit other responses to IL-1 including fever and the production of acute-phase proteins (Daynes *et al.*, 1987; Robertson *et al.*, 1988). Also, the C-terminal tripeptide of α-MSH, Lys[11]-Pro-Val[13], is antipyretic against leucocyte pyrogen (Richards & Lipton, 1985), of which IL-1 is a major component (Dinarello, 1984), and is reported to have anti-inflammatory activity (Hiltz & Lipton, 1989; 1990).

To obtain information about the structure-activity relations of analgesic peptides we have tested the analgesic activities of a series of peptides related to α-MSH and Lys-D-Pro-Thr against the hyperalgesic agents IL-1β and prostaglandin E_2 (PGE_2, Ferreria, 1972). To investigate the possible involvement of κ-opioid receptors in the analgesic responses, the effects on the responses of the selective κ-opioid receptor antagonist, nor-binaltorphimine (nor-BNI, Portoghese *et al.*, 1987) were measured.

Methods

Hyperalgesia (nociception) in rats (Wistar, male, 135–170 g) was induced by IL-1β (0.1 international units, i.u.) or PGE_2 (100 ng) injected into the paw (intraplantar, i.pl.). Putative analgesic peptides were given intraperitoneally (i.p.) 1 h before injection of IL-1β and 2 h after injection of PGE_2. Nor-BNI was injected i.pl. at 5 μg/paw, 90 min before injection of IL-1β and 90 min after injection of PGE_2. There were five rats per treatment group and injection volumes were 0.1 ml and 0.3 ml for i.pl. and i.p. injections, respectively. Hyperalgesia was measured by a modification of the Randall-Selitto rat paw pressure test (Ferreria *et al.*, 1978; Nakamura & Ferreria, 1987). A constant pressure of 20 mmHg was applied to the hind paws of rats and discontinued when animals presented a characteristic freezing reaction (reaction time). The intensity of hyperalgesia was quantified as the variation of the reaction time (Δ reaction time) obtained by subtracting the value measured 3 h after administration of the hyperalgesic agent (IL-1β or PGE_2) from the pre-injection control reaction time (zero time). At this time interval after injection of the above doses of IL-1β and PGE_2, Δ reaction times were maximal (Ferreira *et al.*, 1988). The experimenter was unaware of the group treatments. Results are presented as means with s.e.mean ($n = 5$). ED_{50} values were calculated from $\log_e$ dose v. probit plots.

Materials

IL-1β produced in *E. coli* with a specific activity of 10^8 i.u. mg^{-1} and an endotoxin content of 0.25 endotoxin units/100,000 i.u. was the NIBSC preparation coded 86/680. PGE_2 was a gift from the Upjohn Co (U.S.A.) and Nor-BNI was a gift from Dr K. Rice, N.I.H. (U.S.A.). α-MSH and [N1[4], D-Phe[7]]α-MSH were purchased from Sigma (Poole, Dorset). α-MSH[1–10] and [N1[4], D-Phe[7]] α-MSH[1–10] were custom synthesized by Multiple Peptide Systems (San Diego, U.S.A). Lys-D-Pro-Thr, D-Lys-Pro-Thr, Lys-Pro-Val and Lys-D-Pro-Val were custom synthesized by Cambridge Research Biochemicals (Cambridge, England). The peptides were characterized by fast atom bombardment mass spectrometry, amino acid analysis and analytical reverse-phase high performance liquid chromatography (h.p.l.c.) by the manufacturers and purified to ⩾95% by preparative h.p.l.c. at NIBSC.

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Results

Interleukin-1β

IL-1β (0.1 i.u.) evoked hyperalgesia in rats: mean Δ reaction times were 21.2 ± 0.4 to 23.9 ± 0.5 s (Figures 1, 3 and 5). The inhibition of IL-1β-evoked hyperalgesia by eight peptides is shown in Figure 1 and ED_{50} values, with 95% confidence intervals, are given in Table 1. [Nl^4, D-Phe^7]α-MSH and α-MSH were the most potent inhibitors of the hyperalgesic effect of IL-1β and their potencies were greatly diminished by deletion of their C-terminal tripeptide, Lys^{11}-Pro-Val^{13} (KPV). Dose-response curves for the tripeptides Lys-D-Pro-Val (K(D)PV), Lys-Pro-Val (KPV), Lys-D-Pro-Thr (K(D)PT) and D-Lys-Pro-Thr ((D)KPT) lay to the right of curves for the larger peptides. Of the tripeptides, those with valine (V) at the C-terminus were 2–3 times more potent than those with threonine (T) in this position.

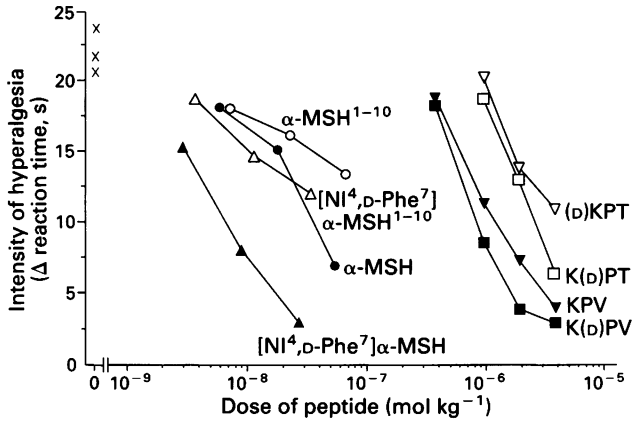


Figure 1 Hyperalgesic responses to interleukin-1β (IL-1β, 0.1 i.u., i.pl.) in rats treated with saline (X) and the inhibitory effects of eight peptides on the responses. The peptides (or saline) were injected (i.p.) 1 h before IL-1β. Symbols represent mean values in 5 rats; s.e.means were within the dimensions of the symbols. For abbreviations, see text.

Prostaglandin E_2

PGE_2 (100 ng) evoked hyperalgesia in rats: mean Δ reaction times were 16.8 ± 0.3 to 19.3 ± 0.5 s after PGE_2 (Figures 2, 4 and 5). The effects of eight peptides on PGE_2-evoked hyperplasia are shown in Figure 2 and Table 1, which gives ED_{50} values with 95% confidence intervals. Except for K(D)PT and (D)KPT the peptides inhibited PGE_2-evoked hyperalgesia. [Nl^4, D-Phe^7]α-MSH and α-MSH were the most potent peptides and their potencies were greatly diminished by deletion of their C-terminal tripeptide, Lys^{11}-Pro-Val^{13}. Dose-response curves for K(D)PV and KPV lay to the right of curves for the larger peptides. Approx 3 times higher doses of K(D)PV and KPV were required to inhibit PGE_2-evoked hyperalgesia than to inhibit IL-1β-evoked hyperalgesia.

Effect of nor-binaltorphimine on the analgesic activity of peptides against interleukin-1β

Inhibition by α-MSH and [Nl^4, D-Phe^7]α-MSH of IL-1β-evoked hyperalgesia was abolished by treatment with the

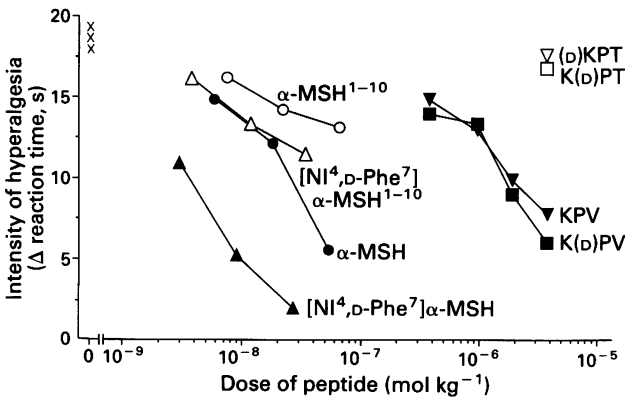


Figure 2 Hyperalgesic responses to prostaglandin E_2 (PGE_2, 100 ng, i.pl.) in rats treated with saline (X) and the effects of eight peptides on the responses. The peptides (or saline) were injected (i.p.) 2 h after PGE_2. Symbols represent mean values in 5 rats; s.e.means were within the dimensions of the symbols. For abbreviations, see text.

Table 1 Comparison of the inhibitory effects of eight peptides on the hyperalgesic effects of interleukin-1β (IL-1β, 0.1 i.u., i.pl.) and prostaglandin E_2 (PGE_2, 100 ng i.pl.)

Agonist	*Peptide inhibitor*	*ED_{50}* (mol kg^{-1})	*95% confidence interval* (mol kg^{-1})
IL-1β	[Nl^4,D-Phe^7]α-MSH	6.3×10^{-9}	3.0×10^{-9}–1.0×10^{-8}
	[Nl^4,D-Phe^7]α-MSH$^{1-10}$	4.5×10^{-8} [1]	—[2]
	α-MSH	3.2×10^{-8}	1.9×10^{-8}–8.0×10^{-8}
	α-MSH$^{1-10}$	2.0×10^{-8} [1]	—[2]
	K(D)PV	7.6×10^{-7}	4.4×10^{-7}–1.1×10^{-6}
	KPV	9.9×10^{-7}	5.9×10^{-7}–1.5×10^{-6}
	K(D)PT	2.1×10^{-6}	1.5×10^{-6}–3.3×10^{-6}
	(D)KPT	3.4×10^{-6}	2.4×10^{-6}–1.1×10^{-5}
PGE_2	[Nl^4,D-Phe^7]α-MSH	4.1×10^{-9}	1.2×10^{-9}–7.2×10^{-9}
	[Nl^4,D-Phe^7]α-MSH$^{1-10}$	7.0×10^{-8} [1]	—[2]
	α-MSH	2.5×10^{-8}	1.6×10^{-8}–7.0×10^{-8}
	α-MSH$^{1-10}$	4.6×10^{-7} [1]	—[2]
	K(D)PV	2.4×10^{-6}	1.5×10^{-6}–6.2×10^{-6}
	KPV	3.2×10^{-6}	1.8×10^{-6}–1.5×10^{-5}
	K(D)PT	—[3]	—
	(D)KPT	—[3]	—

The peptides (or saline) were injected (i.p.), 1 h before IL-1β and 2 h after PGE_2., ED_{50} values and 95% confidence intervals were calculated from $\log_e$ dose v. probit plots with the assumption that the peptides had the capacity to reverse fully the hyperalgesic responses.

[1]Crude estimates based upon relatively flat slopes for dose-response curves suggesting an inability of these compounds to reverse fully hyperalgesic responses.

[2]Flat slopes for dose-response curves precluded calculation of meaningful confidence intervals.

[3]At 1.3 mg kg^{-1} (= 3.8 μmol kg^{-1}) the peptides did not inhibit PGE_2-evoked hyperalgesia.

For abbreviations, see text.

κ-opioid antagonist, Nor-BNI (5 μg, i.pl., Figure 3). In contrast inhibition by α-MSH$^{1-10}$ and [Nl^4, D-Phe^7]α-MSH$^{1-10}$ of IL-1β-evoked hyperalgesia was only partially attenuated by Nor-BNI (Figure 4) and there remained a marginal regression with dose of α-MSH$^{1-10}$ and [Nl^4, D-Phe^7]α-MSH$^{1-10}$ ($0.01 < P < 0.2$) in the presence of Nor-BNI.

Nor-BNI effectively abolished inhibition of IL-1β-evoked hyperalgesia by K(D)PV and KPV but had no effect on inhibition by K(D)PT and (D)KPT (Figure 5).

Effect of nor-binaltorphimine on the analgesic activity of peptides against prostaglandin E_2

Inhibition by α-MSH and [Nl^4, D-Phe^7]α-MSH of PGE_2-evoked hyperalgesia was abolished by treatment with Nor-BNI whereas inhibition by α-MSH$^{1-10}$ and [Nl^4, D-Phe^7]α-MSH$^{1-10}$ was only partially attenuated by Nor-BNI (Figure 4) and there remained marginal regression with dose of α-MSH$^{1-10}$ and [Nl^4, D-Phe^7]α-MSH$^{1-10}$ ($0.01 < P < 0.2$) in the presence of Nor-BNI.

Nor-BNI effectively abolished inhibition of PGE_2-evoked hyperalgesia by K(D)PV and KPV but did not alter the lack of effect of K(D)PT and (D)KPT on responses to PGE_2.

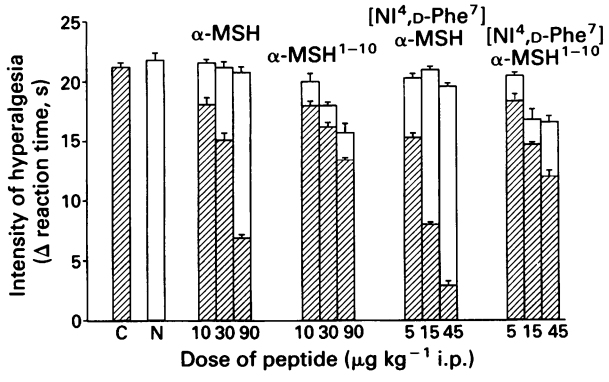


Figure 3 Hyperalgesic responses to interleukin-1β (IL-1β, 0.1 i.u., i.pl.) in rats treated with saline (hatched columns) or nor-binaltorphimine (Nor-BNI, 5 μg, i.pl., open columns) and the inhibitory effects of α-MSH, [Nl^4, D-Phe^7]α-MSH, α-MSH$^{1-10}$ and [Nl^4, D-Phe^7]α-MSH$^{1-10}$ on the responses. Nor-BNI was injected 90 min before IL-1β; peptides were injected (i.p.) 1 h before IL-1β. C = rats injected with saline/IL-1β; N = rats injected with Nor-BNI/IL-1β. The vertical bars are s.e.means of values obtained in groups of 5 rats. For abbreviations, see text.

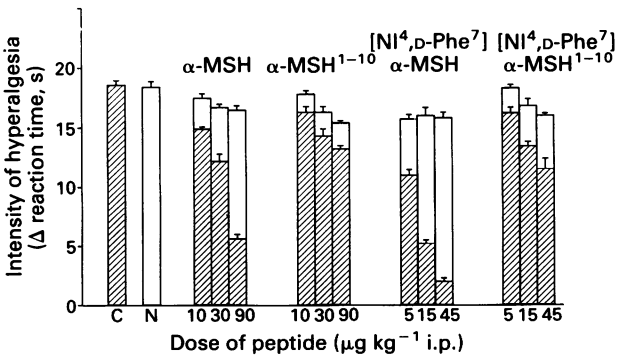


Figure 4 Hyperalgesic responses to prostaglandin E_2 (PGE_2, 100 ng, i.pl.) in rats treated with saline (hatched columns) or nor-binaltorphimine (Nor-BNI, 5 μg, i.pl., open columns) and the inhibitory effects of α-MSH, [Nl^4,D-Phe^7]α-MSH, α-MSH$^{1-10}$ and [Nl^4, D-Phe^7]α-MSH$^{1-10}$ on the responses. Nor-BNI was injected 90 min after PGE_2; peptides were injected (i.p.) 2 h after PGE_2. C = rats injected with saline/IL-1β; N = rats injected with Nor-BNI/IL-1β. The vertical bars are s.e.means of values obtained in groups of 5 rats. For abbreviations, see text.

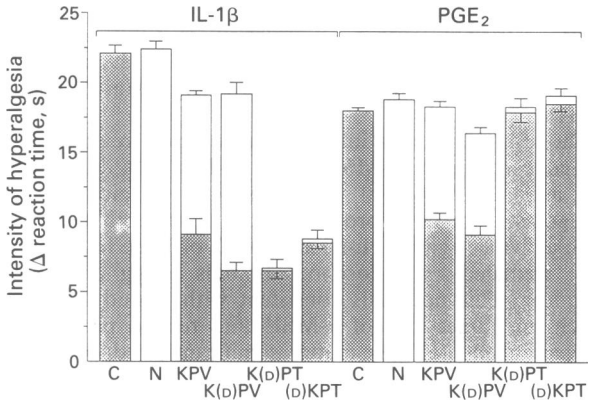


Figure 5 Hyperalgesic response to interleukin-1β (IL-1β, 0.1 i.u., i.pl.) or prostaglandin E_2 (PGE_2 100 ng, i.pl.) in rats treated with saline (hatched columns) or nor-binaltorphimine (Nor-BNI, 5 μg, i.pl., open columns) and the effects of 4 tripeptides (3.8 μmol kg^{-1}, i.p.) on the responses. Nor-BNI was injected 90 min before IL-1β and 90 min after PGE_2; peptides were injected 1 h before IL-1β and 2 h after PGE_2. C = rats injected with saline/IL-1β; N = rats injected with Nor-BNI/IL-1β. The vertical bars are s.e.means of values obtained in groups of 5 rats. For abbreviations, see text.

Discussion

α-MSH and its stable analogue [Nl^4, D-Phe^7]α-MSH (Sawyer *et al.*, 1980) inhibit a number of biological responses to IL-1 including fever, increased plasma concentrations of acute phase proteins and corticosterone, numbers of circulating neutrophils (Daynes *et al.*, 1987) and hyperalgesia (Follenfant *et al.*, 1989). Except for IL-1-evoked proliferation of thymocytes (Cannon *et al.*, 1986), [Nl^4, D-Phe^7]α-MSH has been found to be a more potent inhibitor than the natural hormone. In the present study [Nl^4, D-Phe^7]α-MSH was the most potent of the peptides tested, with an ED_{50} value for inhibition of IL-1β-evoked hyperalgesia of 6.3 nmol kg^{-1}, similar to that, 9.0 nmol kg^{-1}, reported by Follenfant *et al.* (1989). The ED_{50} values of α-MSH (3.2×10^{-8} mol kg^{-1}) and Lys-D-Pro-Thr (2.1×10^{-6} mol kg^{-1}) also were very similar to the values obtained in the earlier study.

The marked loss in analgesic activity that resulted from deletion of the C-terminal peptide, Lys^{11}-Pro-Val^{13}, from α-MSH and [Nl^4, D-Phe^7]α-MSH together with the analgesic activity of the tripeptide show its importance to the analgesic activities of α-MSH and its stable analogue. This finding is consistent with the work of Deeter *et al.* (1989), who tested a series of α-MSH analogues with truncated N-termini for antipyretic activity against IL-1 and found the order of potency to be α-MSH > α-MSH$^{8-13}$ > α-MSH$^{11-13}$ > α-MSH$^{10-13}$. The residual activity in α-MSH$^{1-10}$ and [Nl^4, D-Phe^7]α-MSH$^{1-10}$ suggests an additional site with analgesic activity within these decapeptides. The increases in analgesic activity resulting from the substitution of D-Pro for L-Pro in Lys-Pro-Thr (Ferreira *et al.*, 1988) and Lys-Pro-Val suggest that a similar substitution made in the stable analogue of α-MSH, giving [Nl^4, D-Phe^7,D-Pro^{12}]α-MSH, would yield a potent and long-lasting analgesic agent.

Of the peptides studied only one, Lys-D-Pro-Thr, had been tested previously for inhibitory activity against hyperalgesia evoked by PGE_2, with a negative result (Ferreira *et al.*, 1988; Follenfant *et al.*, 1989). This finding was confirmed and D-Lys-Pro-Thr also was found not to have analgesic activity against PGE_2. In contrast, the six other peptides tested had analgesic activity against PGE_2 with the same order of potency as against IL-1β. The capacity of Lys-Pro-Val and Lys-D-Pro-Val but not Lys-D-Pro-Thr and D-Lys-Pro-Thr to inhibit PGE_2-evoked hyperalgesia suggests that the analgesic effect of the threonine compounds might be specific to IL-1β although there is no evidence that these tripeptides compete

for IL-1 receptors since Lys-D-Pro-Thr was not an antagonist of IL-1β-evoked fever or stimulation of EL-4 mouse thymoma cells (Ferreira *et al.*, 1988) or of IL-1-evoked relaxation of rabbit isolated mesenteric artery (Marceau *et al.*, 1991).

The marked attenuation or near complete reversal of the analgesic effects of α-MSH, [N1[4], D-Phe[7]]α-MSH, Lys-Pro-Val and Lys-D-Pro-Val by the specific κ-opioid receptor antagonist, Nor-BNI, provides good evidence that the analgesic activities against IL-1β and PGE_2 of these four peptides were mediated by endogenous opioids acting on κ-opioid receptors. This notion is consistent with a study in rats in which microinjections of α-MSH and related peptides into the periaqueductal grey matter significantly reduced responsiveness to pain, with α-MSH eliciting a behavioural profile similar to that produced by β-endorphin (Walker *et al.*, 1980). Similarly, in mice, the opioid receptor antagonist, naloxone, (given subcutaneously) prevented the analgesic action of α-MSH (given intracerebroventricularly, Ohkubo *et al.*, 1984). The partial reduction in the analgesic activity of $\alpha\text{-MSH}^{1-10}$ and [N1[4], D-Phe[7]]$\alpha\text{-MSH}^{1-10}$ in the presence of Nor-BNI suggest that the residual analgesic activities of these peptides (present after deletion of Lys^{11}-$Pro\text{-}Val^{13}$) was only in part mediated by κ-opioid receptors. The failure of Nor-BNI to diminish the analgesic effects of Lys-D-Pro-Thr and D-Lys-Pro-Thr indicates that these peptides were not working via κ-opioid receptors. This finding is consistent with the lack of effect of the opioid receptor antagonist, naloxone, on inhibition by Lys-D-Pro-Thr of IL-1β-evoked hyperalgesia (Follenfant *et al.*, 1989). Also consistent with the non-opioid mechanism of action of Lys-D-Pro-Thr is its failure to antagonize PGE_2-evoked hyperalgesia (Ferreira *et al.*, 1988), in contrast to morphine (Lorenzetti & Ferreira, 1985).

Although α-MSH has been shown to have analgesic activity when given centrally (Walker *et al.*, 1980; Ohkubo *et al.*, 1984) it lacked effect on PGE_2-evoked hyperalgesia when given i.v. in a hot-plate test, in contrast to morphine (Follenfant *et al.*, 1989). Similarly Lys-D-Pro-Thr, Lys-Pro-Val and Lys-D-Pro-Val were not effective in a hot-plate test, in contrast to morphine (Ferreira *et al.*, 1988; Ferreira: unpublished data). These data suggest that the peptides tested in the present study had a peripheral rather than a central site of action.

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Synergistic anti-nociceptive effect of L-N^G-nitro arginine methyl ester (L-NAME) and flurbiprofen in the mouse

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1 L-N^G-nitro arginine methyl ester (L-NAME) administered i.p. produces anti-nociception in the mouse assessed by the formalin-induced paw licking and acetic acid-induced abdominal constriction models. The non-steroidal anti-inflammatory drug (NSAID), flurbiprofen, was similarly anti-nociceptive in both models.

2 Combination of a sub-threshold dose of L-NAME (10 mg kg^{-1}) with increasing doses of flurbiprofen (25– 75 mg kg^{-1}) or a sub-threshold dose of flurbiprofen (50 mg kg^{-1}) with increasing doses of L-NAME (10– 100 mg kg^{-1}) resulted in potentiated anti-nociception in the formalin model. Combined therapy with sub-threshold doses of L-NAME (10 mg kg^{-1}) and indomethacin (10 mg kg^{-1}) also resulted in significant anti-nociception. In addition, combining sub-threshold doses of L-NAME (12.5 mg kg^{-1}) and flurbiprofen (2 mg kg^{-1}) significantly reduced acetic acid-induced abdominal constriction.

3 L-NAME (10 mg kg^{-1}) administered i.p. caused a significant (approximately 35%) increase in MAP in the urethane-anaesthetized mouse. Flurbiprofen (50 mg kg^{-1}) was inactive. Combination treatment with L-NAME (10 mg kg^{-1}) and flurbiprofen (50 mg kg^{-1}) failed to elevate MAP above that observed with L-NAME alone. Neither L-NAME (10 mg kg^{-1}) nor flurbiprofen (50 mg kg^{-1}) either alone or in combination significantly altered mouse locomotor activity.

4 These results suggest that L-NAME and flurbiprofen/indomethacin act synergistically in their anti-nociceptive action in the mouse. Combination therapy with L-NAME and flurbiprofen and a similar NSAID may provide an alternative to the clinical control of pain in man.

Keywords: L-N^G-nitro arginine methyl ester (L-NAME); flurbiprofen; indomethacin; anti-nociception in mouse; formalin; nitric oxide

Introduction

The involvement of nitric oxide (NO) in central nervous system function was first suggested by Garthwaite and colleagues who demonstrated the release of NO from immature rat cerebellar neurones challenged with the glutamate receptor agonist, N-methyl-D-aspartate (NMDA) (Garthwaite *et al.*, 1988). NO release has subsequently been reported from cerebellar slices challenged with other glutamate agonists including kainate and quisqualate (Garthwaite *et al.*, 1989). Additionally, NMDA releases NO from immature rat hippocampal slices (East & Garthwaite, 1991).

The NO synthase enzyme responsible has been purified, cloned (Bredt *et al.*, 1991) and its regional distribution determined (Bredt *et al.*, 1990; Forstermann *et al.*, 1990). Enzyme activity is present in varying amounts in all brain areas examined with particular accumulation in the cerebellum, olfactory bulb, superior and inferior colliculus and the superficial layers of the cerebral cortex.

Despite considerable progress in mapping out so-called 'nitrergic' neurones in the brain and in biochemical characterisation of NO synthase the precise biological function(s) of NO in the central nervous system still require extensive investigation. A major role may well be in the control of neuronal development in the immature cerebellum and hippocampus (Garthwaite, 1991).

We have also presented evidence that the brain NO system may play a part in the mechanisms underlying pain perception. This conclusion is based upon the finding that L-N^G-nitro arginine methyl ester (L-NAME), a selective inhibitor of NO synthase, produces a potent, long-lasting and centrally-mediated anti-nociception in the mouse (Moore *et al.*, 1991).

Anti-nociception due to L-NAME after i.p. administration is associated with peripheral side effects including an increase in blood pressure due to inhibition of vascular endothelial NO biosynthesis. For this reason, we have investigated alternative experimental approaches in an attempt to augment the anti-nociceptive effect of L-NAME without similarly augmenting its potentially harmful cardiovascular side effects. To this end we have investigated the anti-nociceptive effect in mice of combination therapy with L-NAME and the non-steroidal anti-inflammatory drugs (NSAID), flurbiprofen and indomethacin. This approach was adopted since not only are NSAID established as therapy for mild to moderate pain in man (Kantor, 1982) but these drugs, unlike L-NAME, are devoid of hypertensive side effects (Rainsford, 1989).

Methods

Full details of the methods involved in this study have been described elsewhere (Moore *et al.*, 1991). Briefly, male mice (LACA, 28–35 g) were allowed food and water *ad libitum* and transported to a quiet laboratory at least 1 h before the experiment. Experiments were carried out in the period between 13 h 00 min and 17 h 00 min in normal room light and temperature (22 ± 2°C).

Assessment of anti-nociceptive effect

The anti-nociceptive effect of L-NAME either alone or in combination with flurbiprofen or indomethacin was assessed by the formalin-induced paw licking procedure essentially as described by Hunskaar & Hole (1987). Briefly, animals were injected sub-plantar in one hindpaw with formalin (5% v/v, 10 µl) and the duration of paw licking (an index of nociception) monitored in the periods 0–5 min (early phase) and

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15–30 min (late phase). L-NAME (10–100 mg kg^{-1}), flurbiprofen (25–100 mg kg^{-1}), indomethacin 10–100 mg kg^{-1}) or combinations thereof were injected i.p. 15 min before formalin injection. Additional animals received 0.1 ml 10 g^{-1} 0.9% w/v NaCl (saline; control for L-NAME-treated animals), 0.1 ml 10 g^{-1} 0.5% w/v Na_2CO_3 (control for flurbiprofen- and indomethacin-treated animals) or a combination of both vehicles as appropriate. In separate experiments, L-NAME (12.5 and 50 mg kg^{-1}), flurbiprofen (2–50 mg kg^{-1}) or a combination of subthreshold doses of each were administered i.p. 15 min before i.p. administration of acetic acid (0.6% v/v, 10 ml kg^{-1}). The resulting abdominal constrictions were counted for 30 min thereafter. Control animals received appropriate volumes of saline, Na_2CO_3 or a combination of the two as above.

Effect of L-NAME and flurbiprofen alone and in combination on mouse blood pressure

Mice were anaesthetized with urethane (10 g kg^{-1}, i.p.) and a cannula inserted into the carotid artery. Blood pressure was monitored continuously with a Bell & Howell pressure transducer connected to a Grass 4-channel pen recorder. L-NAME (10 mg kg^{-1}), flurbiprofen (50 mg kg^{-1}) or a combination of the two at these doses were administered i.p. and MAP determined for up to 45 min thereafter. Control animals received appropriate volumes of saline, Na_2CO_3 or a combination thereof. Results indicate MAP at 45 min post-injection chosen to coincide with the end of the monitoring period in the formalin and acetic acid-induced abdominal constriction assays.

Effect of L-NAME and flurbiprofen alone and in combination on locomotor activity

Locomotor activity was determined after i.p. administration of L-NAME (10 mg kg^{-1}), flurbiprofen (50 mg kg^{-1}) or a combination of the two at these doses in automated activity cages (Ugo Basile, Italy). Animals were placed in the cages in pairs. Results are expressed as counts obtained per pair of animals over a 5 min period from 40–45 min following drug administration. Again control animals received appropriate volumes of saline, Na_2CO_3 or a combination of the two.

Drugs and chemicals

All drugs were purchased from Sigma. L-NAME was dissolved in saline whilst flurbiprofen and indomethacin were dissolved in 0.5% Na_2CO_3. Drug stocks were prepared fresh on the morning of each experiment.

Statistical analysis

Results indicate mean ± s.e.mean except for acetic acid-induced abdominal constriction experiments in which medians with inter-quartile ranges (Q1–Q3) are shown. Statistical significance of differences between groups was determined either by ANOVA followed by Dunnett's t test, unpaired Student's t test or Mann-Whitney U test as appropriate. In each case, a probability (P) value of 0.05 or less was taken to indicate statistical significance.

Results

Anti-nociceptive effect of L-NAME, flurbiprofen and indomethacin alone and in combination

L-NAME, flurbiprofen and indomethacin produced dose-related reductions in paw licking time (anti-nociception) in the late phase of the response. L-NAME and indomethacin were without effect on the early phase whilst flurbiprofen reduced early phase paw licking by approximately 60% only at the highest dose used in this study (100 mg kg^{-1}) (Figures 1–3). At this dose animals appeared immobile and sedated,

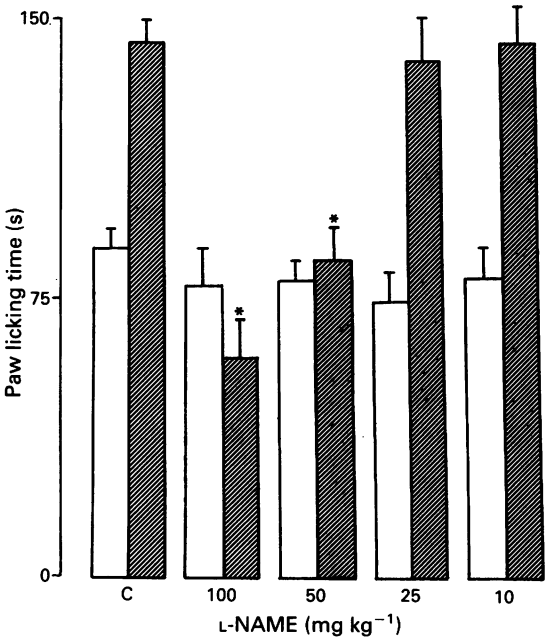


Figure 1 Anti-nociceptive effect of L-N^G-nitro arginine methyl ester (L-NAME) in the formalin-induced paw licking model. Doses (mg kg^{-1}, i.p.) are indicated beneath each pair of columns. Open columns represent paw licking time in the early (0–5 min) phase. Hatched columns represent paw licking time in the late (15–30 min) phase. L-NAME was administered i.p. 15 min prior to subplantar injection of formalin. Control animals (columns labelled C) were injected with an appropriate volume of saline. Results show mean (± se.mean, vertical bars) $n = 5–11$.
*$P < 0.01$ (ANOVA plus Dunnett's t test).

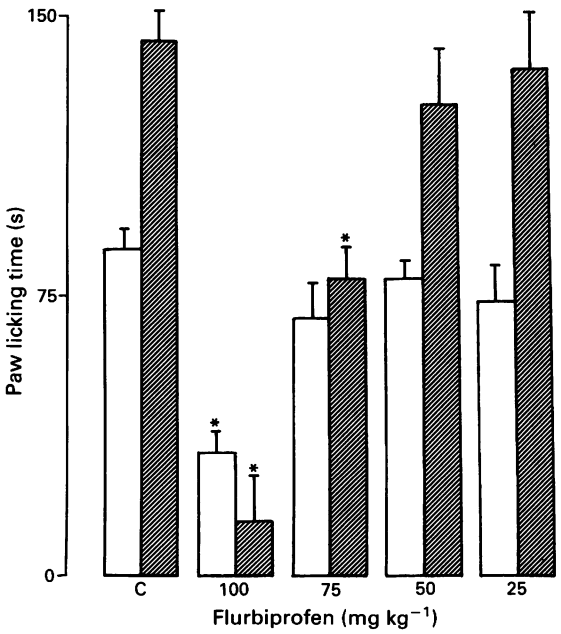


Figure 2 Anti-nociceptive effect of flurbiprofen in the formalin-induced paw licking model. Doses (mg kg^{-1}, i.p.) are indicated beneath each pair of columns. Open columns represent paw licking time in the early (0–5 min) phase. Hatched columns represent paw licking time in the late (15–30 min) phase. Control animals (columns labelled C) were injected with an appropriate volume of 0.5% Na_2CO_3. Results show mean (± s.e.mean, vertical bars) $n = 6–14$.
*$P < 0.01$ (ANOVA plus Dunnett's t test).

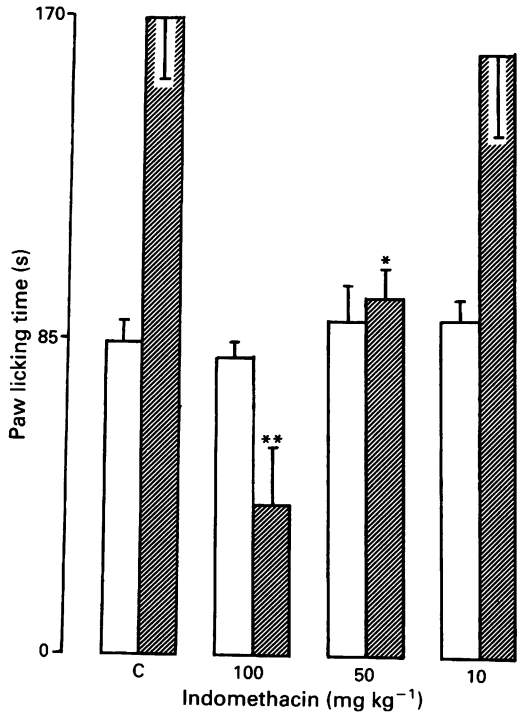


Figure 3 Anti-nociceptive effect of indomethacin in the formalin-induced paw licking model. Doses (mg kg^{-1}, i.p.) are indicated beneath each pair of columns. Open columns represent paw licking time in the early (0–5 min) phase. Hatched columns represent paw licking time in the late (15–30 min) phase. Control animals (columns labelled C) were injected with an appropriate volume of 0.5% Na_2CO_3. Results show mean (± s.e.mean, vertical bars) $n = 6-14$. *$P<0.05$; **$P<0.01$ (ANOVA plus Dunnett's t test).

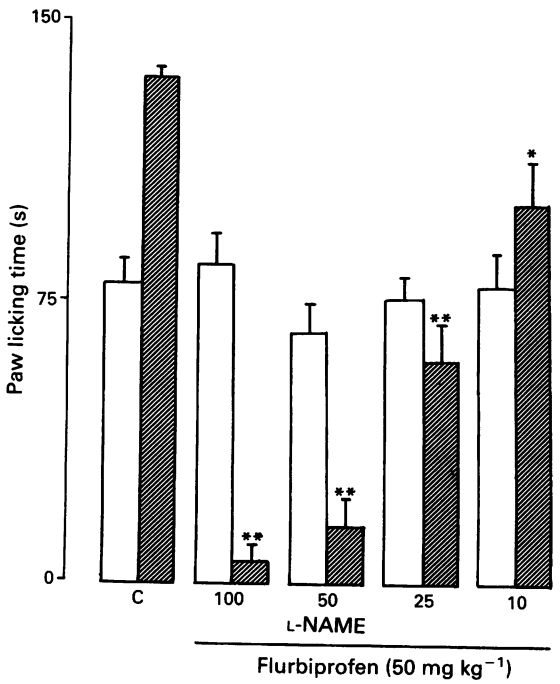


Figure 4 Anti-nociceptive effect of flurbiprofen (50 mg kg^{-1}) combined with increasing doses of L-N^G-nitro arginine methyl ester (L-NAME, mg kg^{-1}, i.p.) indicated beneath each pair of columns. Open columns represent paw licking time in the early (0–5 min) phase. Hatched columns represent paw licking time in the late (15–30 min) phase. Drugs were administered i.p. 15 min prior to subplantar injection of formalin. Control animals (columns labelled C) were injected with an appropriate volume of saline plus 0.5% Na_2CO_3. Results show mean (± s.e.mean, vertical bars) $n = 5-9$. *$P<0.01$; **$P<0.001$ (Student's t test).

suggesting a possible toxic reaction. No discernible gross changes in behaviour were apparent in other animals.

From the dose-response relationships obtained, sub-threshold doses of L-NAME (10 mg kg^{-1}) or flurbiprofen (50 mg kg^{-1}) were combined with increasing doses of flurbiprofen (25–75 mg kg^{-1}) or L-NAME (10–100 mg kg^{-1}) respectively. In separate experiments some animals received sub-threshold doses of L-NAME (10 mg kg^{-1}) and indomethacin (10 mg kg^{-1}). Combination treatment with L-NAME and flurbiprofen acted synergistically to produce significantly greater late phase anti-nociception even at the lowest doses studied (Figures 4,5). For example, combined therapy with L-NAME (10 mg kg^{-1}) and flurbiprofen (50 mg kg^{-1}) resulted in a highly significant reduction in late phase paw licking of 57.4 ± 4.5% ($n = 6$, $P<0.001$). Administered alone, L-NAME at this dose produced a non-significant ($P>0.05$) increase in paw licking time of 5.1 ± 3.4% ($n = 8$) whilst flurbiprofen produced a non-significant ($P>0.05$) decrease in paw licking time of 11.6 ± 7.6% ($n = 14$). In contrast, combination therapy with L-NAME and flurbiprofen failed to result in significant early phase anti-nociception. Administration of sub-threshold doses of a combination of L-NAME (10 mg kg^{-1}) and indomethacin (10 mg kg^{-1}) also significantly reduced the late phase response (85.8 ± 37.0 s, $n = 14$, $P<0.05$ cf. saline-injected controls) without influencing the first phase response (76.9 ± 9.4 s, $n = 14$, $P>0.05$ cf. saline-injected controls).

L-NAME (12.5 and 50 mg kg^{-1}) and flurbiprofen (2–50 mg kg^{-1}) were also assessed for their ability to inhibit acetic acid-induced abdominal constrictions. Only at the highest dose of each drug was a significant anti-nociceptive effect observed. However, combination of sub-threshold doses of L-NAME (12.5 mg kg^{-1}) and flurbiprofen (2 mg kg^{-1}) again synergised to produce potent anti-nociception (Table 1).

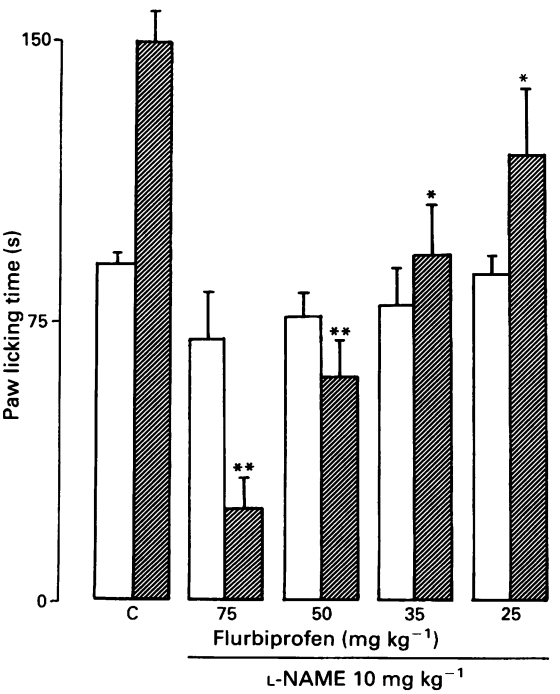


Figure 5 Anti-nociceptive effect of L-N^G-nitro arginine methyl ester (L-NAME, 10 mg kg^{-1}) combined with increasing doses of flurbiprofen (mg kg^{-1}, i.p.) indicated beneath each pair of columns. Open columns represent paw licking time in the early (0–5 min) phase. Hatched columns represent paw licking time in the late (15–30 min) phase. Drugs were administered i.p. 15 min prior to subplantar injection of formalin. Control animals (columns labelled C) were injected with an appropriate volume of saline plus 0.5% Na_2CO_3. Results show mean (± s.e.mean, vertical bars) $n = 5-9$. *$P<0.01$; **$P<0.001$ (Student's t test).

Table 1 Effect of L-N^G-nitro arginine methyl ester (L-NAME) and flurbiprofen alone and in combination on acetic acid-induced abdominal constrictions in the mouse

Drug treatment	*Abdominal constrictions per 30 min* Median	Q1–Q3	*n*
Flurbiprofen			
vehicle (0.5% Na_2CO_3)	11.5	10.5–15.0	15
2 mg kg^{-1}	11.0	9.5–11.0	5
6.25 mg kg^{-1}	10.5	10.0–12.0	8
12.5 mg kg^{-1}	10.0	8.0–11.0	6
50.0 mg kg^{-1}	5.0	5.0–8.0	8**
L-NAME			
vehicle (saline)	12.0	9.5–12.0	6
12.5 mg kg^{-1}	13.5	12.0–15.0	4
50.0 mg kg^{-1}	7.0	6.0–9.0	6*
Combination treatment			
0.5% Na_2CO_3 + saline	13.5	12.0–17.0	6
flurbiprofen (2 mg kg^{-1}) + L-NAME (12.5 mg kg^{-1})	3.0	2.0–5.5	8**

Drug or vehicle were administered i.p. 15 min before i.p. injection of 0.6% acetic acid. Abdominal constrictions were counted for the following 30 min. Results show medians, inter-quartile ranges (Q1–Q3) and number of experiments (*n*).
*$P<0.05$; **$P<0.01$ determined by Mann-Whitney U test.

Effect of L-NAME and flurbiprofen alone and in combination on mouse blood pressure

L-NAME (10 mg kg^{-1}) significantly increased MAP recorded 45 min after injection (87.4 ± 6.5 mmHg, cf. 64.6 ± 1.3 mmHg before L-NAME injection, $n = 5$, $P<0.05$). In contrast, flurbiprofen (50 mg kg^{-1}) did not alter MAP over the same time period (62.0 ± 1.9 mmHg, cf. 55.0 ± 5.1 mmHg, $n = 3$, $P>0.05$). Combination therapy with both drugs failed to result in a significantly enhanced vasopressor effect over that of L-NAME alone (80.0 ± 13.4 mmHg, cf. 58.1 ± 5.0 mmHg, $n = 7$, $P<0.05$).

Effect of L-NAME and flurbiprofen alone and in combination on mouse locomotor activity

Administration of L-NAME (10 mg kg^{-1}) or flurbiprofen (50 mg kg^{-1}) either alone or in combination failed to influence mouse locomotor activity which was recorded for pairs of animals in the period between 40 and 45 min following drug or vehicle injection. Administration of L-NAME resulted in a combined activity count of 195.2 ± 55.9 counts (cf. 187.6 ± 31.2 counts in saline-injected control animals, $n = 6$, $P>0.05$). Flurbiprofen injection caused a locomotor count of 175.6 ± 54.7 counts (cf.166.9 ± 23.8 counts in 0.5% Na_2CO_3-injected control animals, $n = 7$, $P>0.05$). Combination therapy with both drugs resulted in a locomotor activity of 176.0 ± 39.2 counts (cf. 187.6 ± 20.0 counts in control animals injected with appropriate volumes of both saline and 0.5% Na_2CO_3, $n = 6$, $P>0.05$).

Discussion

The results of the present experiments confirm published data indicating an anti-nociceptive effect of L-NAME in two experimental models in the mouse (Moore *et al.*, 1991). In these experiments L-NAME was less potent (at least in the formalin-induced hindpaw licking test) than in results published previously. Variability in experiments of this type may relate to differences between observers, seasonal changes in animal nociception threshold or perhaps to other unidentified environmental factor(s). Flurbiprofen, a clinically used NSAID with potent analgesic activity in man (Busson, 1986), exhibited anti-nociceptive activity against acetic acid-induced abdominal constrictions as well as in the late phase following hindpaw formalin injection in the mouse. Indomethacin was also active in the late phase response of the formalin model. Both flurbiprofen and indomethacin were less potent than L-NAME in the formalin-induced paw licking model but flurbiprofen was approximately equipotent in the acetic acid-induced abdominal constriction assay.

Treatment of mice with a combination of L-NAME and flurbiprofen resulted in a significantly greater anti-nociceptive effect in both the acetic acid-induced writhing procedure and in the late phase of the formalin model than following administration of either drug alone. The degree of potentiation observed is too great to be accounted for a by a simple additive effect thereby suggesting a synergistic interaction between the two drugs. Perhaps the best indication of such synergism is the result of combining sub-threshold doses of L-NAME and flurbiprofen which caused a substantial (> 50%) reduction of late phase paw licking in the formalin-model as well as a significant anti-nociception in the acetic acid-induced abdominal constriction model. That this effect is not selective for flurbiprofen is indicated by the observation that treatment of mice with sub-threshold doses of L-NAME and indomethacin, also resulted in significant late phase (but not early phase) anti-nociception in the formalin model.

The experimental manoeuvre of combining L-NAME with NSAID such as flurbiprofen results in a therapy with pronounced anti-nociceptive potential but lacking significant sedative activity as indicated by the lack of effect on locomotor activity. Similarly, combining L-NAME with flurbiprofen did not result in an enhanced increase in MAP suggesting that NO and prostanoids (particularly prostacyclin) do not act synergistically in their effect on the vasculature. With a view to the possible clinical applications of such combination therapy it should be made clear that additional interactions between NO synthase inhibitors and non-steroidal anti-inflammatory agents may occur. For example, Whittle and colleagues (1990) observed that L-NAME potentiates the ulcerogenic effect of indomethacin in the rat stomach.

The mechanism of the interaction between L-NAME and NSAID is clearly of interest but cannot be established from the present data. We have previously demonstrated that L-NAME exhibits anti-nociceptive activity by a naloxone-independent mechanism within, as yet, unidentified sites in the central nervous system (Moore *et al.*, 1991) whilst NSAID may exert their effect either peripherally (Ferreira, 1972) or centrally (e.g. Brown *et al.*, 1968; Ferreira *et al.*, 1978; Groppetti *et al.*, 1988). At which level (i.e. central or peripheral) and by what mechanism L-NAME and NSAID interact is thus not clear. However, it may be of interest that another NSAID, diclofenac, reportedly produces naloxone-dependent anti-nociception following either i.p. or i.c.v. administration in the rat (Bjorkmann *et al.*, 1990). Thus, the synergistic anti-nociceptive effect of L-NAME and NSAID observed in the present study may conceivably result from an effect of flurbiprofen/indomethacin on central opioid receptors.

In addition, the recent experiments of Coderre and colleagues (1990) may also be relevant in this context. These authors have provided evidence that the first phase nociception response following hindpaw formalin injection in mice invokes long-term adaptive changes in central neuronal function which predispose to the nociceptive response in the late phase. Since NO may play a role in mediating long-term adaptational changes in central neuronal function (Garthwaite, 1991; Southam *et al.*, 1991) it is tempting to speculate that part of the anti-nociceptive effect of L-NAME, at least in the formalin model, may be secondary to an inhibition of this adaptive process.

In conclusion, the results of the present study indicate a synergistic interaction between the anti-nociceptive effects of L-NAME and NSAID such as flurbiprofen and indomethacin in the mouse thereby lending support to our claim that NO plays a part in nociception. Whether combination therapy in this way has potential clinical application in the provision of analgesic cover warrants further investigation.

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Pituitary adenylate cyclase activating polypeptides, PACAP-27 and PACAP-38: stimulators of electrogenic ion secretion in the rat small intestine

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1 The effects of pituitary adenylate cyclase activating polypeptide (PACAP)-27 and PACAP-38 were investigated and compared with vasoactive intestinal polypeptide (VIP) responses in voltage clamped preparations of rat jejunum. Under these conditions electrogenic ion secretion was continuously recorded.

2 PACAP-27 is the most potent secretagogue described thus far, exhibiting a concentration-dependent dual secretory action. At low concentrations it stimulated rapid, transient secretory responses (not seen with either PACAP-38 or VIP) and these were inhibited by tetrodotoxin (TTX). At higher nM concentrations of PACAP-27 more prolonged secretory responses predominated which were insensitive to TTX.

3 In the presence of TTX, the concentration-response curve to PACAP-27 gave an EC_{50} value of 29.4 ± 5.4 nM ($n = 4$) compared with 0.8 ± 0.1 nM ($n = 9$) for PACAP-27 alone and 30.6 ± 5.6 nM ($n = 5$) for PACAP-38. C-terminal fragments of PACAP-38 were not significantly effective.

4 Blockade of muscarinic and nicotinic receptors partially inhibited the low concentration effects of PACAP-27. Substance P densensitization and capsaicin pretreatment were effective at inhibiting the transient secretory PACAP-27 responses. Evidence is presented for selective, high affinity PACAP-27 receptors on submucous neurones innervating the mucosal region of the rat jejunum.

Keywords: PACAP-27; PACAP-38; electrogenic ion transport; vasoactive intestinal polypeptide

Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) was first isolated from ovine hypothalami (Miyata *et al.*, 1989) and subsequently found to exist in two variant forms, either PACAP-27 (Miyata *et al.*, 1990) or the C-terminally extended form, PACAP-38. Both originate from the same precursor (Kimura *et al.*, 1990; Ogi *et al.*, 1990) and both stimulate adenylate cyclase activity in pituitary cells (Miyata *et al.*, 1989) as well as in pancreatic (Buscail *et al.*, 1990; Le Meuth *et al.*, 1991) and neuroblastoma cells (Cauvin *et al.*, 1990). The N-terminal 27 amino-acid residues are required for full biological activity and this sequence exhibits 67% homology with the sequence of vasoactive intestinal polypeptide (VIP, see Table 1). A comparison of the binding affinities and potencies of the two PACAP peptides described thus far shows them to be 300 to 1000 times more potent than VIP in certain systems (Buscail *et al.*, 1990; Tatsuno *et al.*, 1990; Cauvin *et al.*, 1991; Shivers *et al.*, 1991). It is becoming apparent that rather than simply being new variant forms of VIP, PACAP-27 and PACAP-38 can exert their effects via specific receptors for which VIP (and other related peptides such as secretin, peptide histidine isoleucine (PHI) and glucagon) have a low affinity (Cauvin *et al.*, 1990; Shivers *et al.*, 1991). The distribution of PACAP-27 and VIP binding sites do appear to be similar with a few notable exceptions (Gottschall *et al.*, 1990). These are primarily the anterior pituitary and hypothalamus where PACAP-27 specific binding far outstripped that of VIP. In the rat duodenum relatively low, but significant levels were observed for both peptides, but the cellular location of these sites remains to be determined. The recent cloning of the human VIP receptor from various cells including epithelial HT-29 cells (Sreedharan *et al.*, 1991) has confirmed its membership of the seven-transmembrane-spanning, G-protein linked group of receptors.

In the rat small intestine, PACAP-immunoreactive neurones are distinct from those containing VIP and are predominantly found in the myenteric plexus (Sundler *et al.*, 1992). Gastrin-releasing peptide may be colocalized with PACAP in these neurones. Relatively few cell bodies were seen in the submucous ganglia and PACAP containing fibres were rarely observed in the mucosal area of the rat small intestine. This is in marked contrast with PACAP localization in fibres and cell bodies of the mucosal region and in both muscle layers of the small and large intestine in man (Sundler *et al.*, 1992). This pattern of PACAP localization in man more closely matches the immunohistochemical localization of VIP in the rat small intestine (Ekblad *et al.*, 1987). Given the sequence homology of these peptides with VIP and their capacity to stimulate adenylate cyclase, they are prime candidate secretagogues in the intestine. This study set out to characterize the effect(s) of PACAP-27 and PACAP-38 and to compare them with those of VIP and other related peptides.

Methods

Short circuit current measurements

Mucosal sheets of rat jejunum (Sprague-Dawley, 200–300 g, male) were prepared by opening longitudinally and removing overlying smooth muscle layers by blunt dissection. Four stripped preparations were obtained from each length of jejunum and these were lightly clamped between two halves of perspex Ussing chambers, exposing an area of 0.6 cm^2. Krebs-Henseleit solution with the composition (mM): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 24.8, KH_2PO_4 1.2 and glucose 11.1 was used and gassed with 95% O_2/5% CO_2. Tissues were automatically voltage-clamped using WPI DVC 1000 dual voltage clamps as described previously (Cox

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et al., 1988) and although preparations from adjacent jejunal areas exhibited similar basal short circuit current (SCC) and potential differences they were not paired. Under voltage clamp conditions the resultant baseline SCC and any subsequent changes in SCC after peptide exposure were continuously recorded. All additions were made to the basolateral side only. Changes in SCC are quoted as μA 0.6 cm^{-2}, mean ± s.e.mean, throughout. Unpaired Student's *t* tests were used to compare data groups and a *P* value of less than 0.05 was considered statistically significant. Concentration-response curves were performed cumulatively and each curve was analysed by the iterative curve fitting programme, Graphpad, from which EC_{50} values were calculated.

Materials

PACAP-38, PACAP-27, and the fragments of PACAP-38, 16–38, 28–38 and 31–38 plus VIP were purchased from Peninsula Laboratories, Europe Ltd (St Helens, Lancashire). Tetrodotoxin (TTX), capsaicin and atropine were obtained from Sigma (Poole, Dorset) and trimethaphan was a gift from Roche (Welwyn Garden City, Herts). All other standard reagents were of analytical grade.

Results

PACAP-27 produced increases in SCC the profile of which altered in the course of constructing the concentration-response relationship (Figure 1). Initial secretory responses to low concentrations (0.03–10 nM) of PACAP-27 were rapid and transient (see inset, Figure 1) and became prolonged (and VIP-like) at higher concentrations (30–100 nM). At the end of each cumulative concentration-response curve, VIP (100 nM) was added to define the maximum secretory capacity of each preparation. An increase of 59.2 ± 5.8 μA 0.6 cm^{-2} (n = 5) was observed after both PACAP-27 and VIP exposure from a starting baseline prior to peptide additions, of 43.0 ± 6.2 μA 0.6 cm^{-2} (n = 5). Pretreatment of tissues with TTX (100 nM, basolaterally) resulted in loss of the initial transient secretory profile, and much smoother prolonged responses were obtained, with a resultant right shift in the response curve (Figures 1 and 2). Pooled data (Figure 2) show the concentration-response curve for PACAP-27 in the presence of TTX to be superimposable upon that of PACAP-38 alone, and upon the VIP curve obtained in earlier studies (Cox & Cuthbert, 1989). In contrast with PACAP-27, PACAP-38 only produced prolonged secretory responses (Figure 2) that were unaffected by TTX pretreatment. The EC_{50} values for PACAP-27, with and without TTX, were 29.4 ± 5.4 nM (n = 4) and 0.8 ± 0.1 nM (n = 9) respectively, compared with 30.6 ± 5.6 nM (n = 5) for PACAP-38 alone and 23.8 ± 2.4 nM (n = 7) for VIP. Analysis of the residuals obtained from the difference between PACAP-27 curves with and without TTX, produced an EC_{50} value of 1.4 nM. C-terminal fragments of PACAP-38 namely sequences 16–38, 28–38 and 31–38, exhibited only limited, if any, secretory capacity (Figure 2) and had no significant effect upon VIP (100 nM) responses which were 53.5 ± 6.5 μA 0.6 cm^{-2} (n = 3), 43.0 ± 7.0 μA 0.6 cm^{-2} (n = 2) and 48.0 ± 8.0 μA 0.6 cm^{-2} (n = 3) after each fragment respectively, compared with controls of 48.0 ± 6.2 μA 0.6 cm^{-2} (n = 6). These VIP secretory responses were also not significantly different from those observed after exposure of preparations to increasing PACAP-27 concentrations (see above).

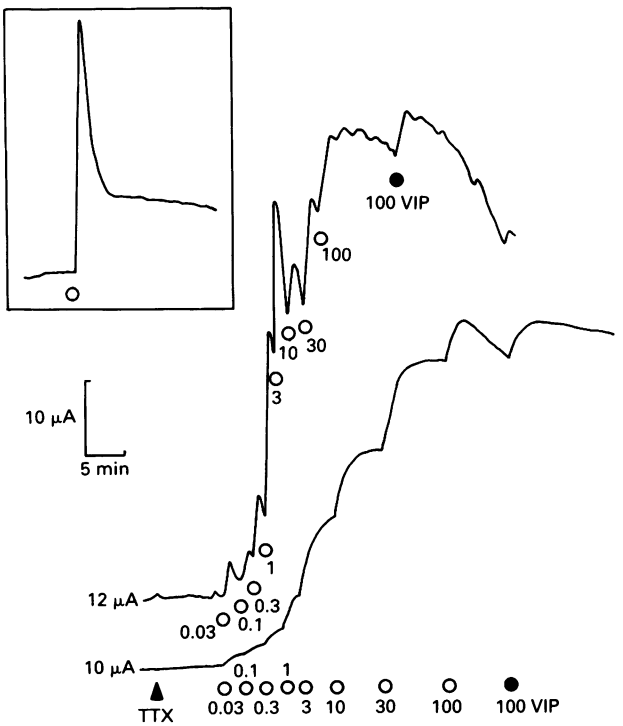


Figure 1 Representative traces of the cumulative concentration-response relationship for PACAP-27 (○) in the absence (top trace) and presence of 100 nM tetrodotoxin (TTX, lower trace) both added basolaterally. Respective baseline currents are given to the left of each recording, and all concentrations are given as nM. Vasoactive intestinal polypeptide (VIP) (100 nM, ●) was added at the end of each response curve to define the maximum secretory response. The inset shows the prolife of a secretory response to a single concentration of PACAP-27 (1 nM).

In attempting to identify the mediator(s) involved in the early phase of the PACAP-27 concentration-response curve, tissues were exposed to either a combination of cholinoceptor blockers, to capsaicin or to a desensitizing concentration of substance P (SP). The resulting responses to increasing concentrations of PACAP-27 were compared with PACAP-27 alone (Figure 3 and Table 2). Pretreatment of tissues with atropine (10 μM) and trimethaphan (100 μM, a concentration that blocks the nicotinic component of field stimulated secretion in this tissue) together resulted in a shift to the right of the PACAP-27 concentration-response curve with an EC_{50} of 11.0 ± 3.6 nM (n = 4). This was compared with an EC_{50} of 4.5 ± 1.7 nM (n = 6) obtained after atropine (10 μM) pretreatment alone although the two values were not significantly

Table I Amino acid sequences of pituitary adenylate cyclase activating polypeptides, PACAP-38, PACAP-27, vasoactive intestinal polypeptide (VIP) and peptide histidine isoleucine (PHI)

	1 5 10 15 20 25 30 35
PACAP-38	H.S.D.G.I.F.T.D.S.Y.S.R.Y.R.K.Q.M.A.V.K.K.Y.L.A.A.V.L.G.K.R.Y.K.Q.R.V.K.N.K.-NH_2.
PACAP-27	H.S.D.G.I.F.T.D.S.Y.S.R.Y.R.K.Q.M.A.V.K.K.Y.L.A.A.V.L.-NH_2
VIP	H.S.D.A.V.F.T.D.N.Y.T.R.L.R.K.Q.M.A.V.K.K.Y.L.N.S.I.L.N.-NH_2
PHI	H.A.D.G.V.F.T.S.D.Y.S.R.L.L.G.Q.L.S.A.K.K.Y.L.E.S.L.I.-NH_2

Amino acid residues that differ from those of the PACAP sequence are underlined. Both PACAP sequences are ovine, while VIP is bovine and porcine and the PHI sequence is bovine.

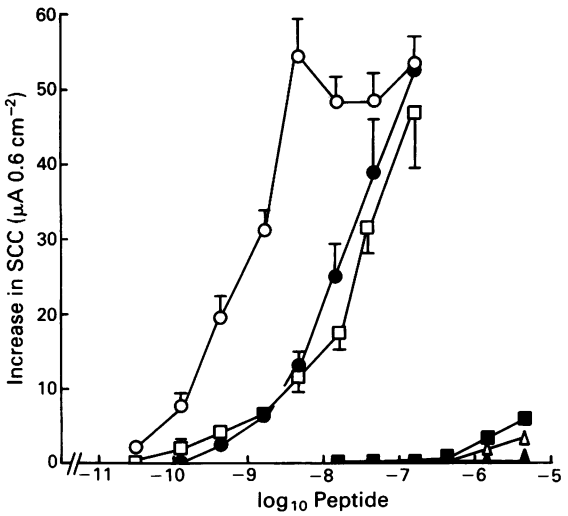


Figure 2 The concentration-response curves for PACAP-27 alone (○, $n = 9$) and following tetrodotoxin TTX (100 nM) pretreatment (●, $n = 4$). Also included are cumulative responses to increasing concentrations of PACAP-38 (□, $n = 5$) and fragments of PACAP-38 namely 16–38 (■, $n = 4$) 28–38 (△, $n = 3$) and 31–38 (▲, $n = 3$). All points represent the mean with vertical lines indicating s.e.mean.

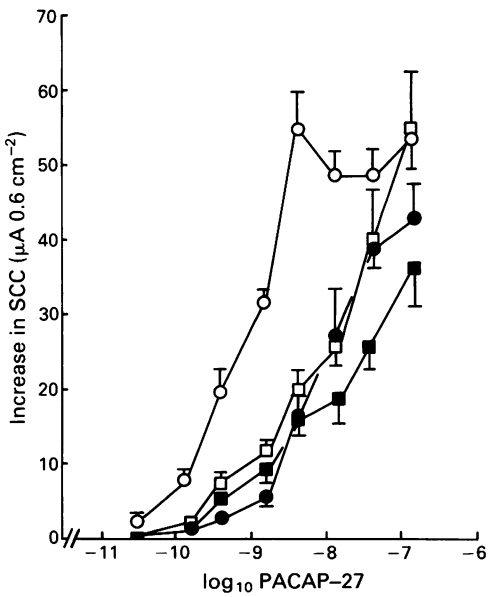


Figure 3 The effects of various pretreatments upon the concentration-response profile to PACAP-27. Nontreated, controls are included for comparison (○, $n = 9$, as shown in Figure 2). Tissues were exposed to either 3 μM capsaicin (□, $n = 4$) two additions of 1 μM substance P (■, $n = 5$) or a combination of atropine (10 μM) and trimethaphan (100 μM, ●, $n = 4$) all added to the basolateral compartment. All points are the mean with vertical lines indicating s.e.mean.

different ($P = 0.07$). Atropine abolished carbachol (10 μM) secretory responses, from 90.2 ± 10.8 μA 0.6 cm^{-2} ($n = 3$) to 0.0 ± 0.0 μA 0.6 cm^{-2} ($n = 3$). Capsaicin pretreatment (3 μM, basolaterally) reduced the basal SCC by −4.6 ± 1.5 μA 0.6 cm^{-2} ($n = 4$) and following this the PACAP-27-response curve was shifted to the right with a resultant EC_{50} of 28.3 ± 5.5 nM ($n = 4$). Preparations of rat jejunum desensitize rapidly to SP, an initial exposure (1 μM) increased SCC transiently by 40.3 ± 5.2 μA 0.6 cm^{-2} ($n = 5$) from a baseline of 21.7 ± 6.2 μA 0.6 cm^{-2} ($n = 5$). A second addition of SP (1 μM) 30 min later when the SCC had stabilized at 36.6 ± 8.5 μA 0.6 cm^{-2} ($n = 5$) produced a transient response of only 2.5 ± 1.1 μA 0.6 cm^{-2} ($n = 5$, $P < 0.001$). Subsequent addition of increasing concentrations of PACAP-27 to SP desensitized preparations resulted in an EC_{50} of 21.8 ± 2.9 nM ($n = 5$) (Figure 3). It is important to note that SP densensitization had no significant effect upon the VIP concentration-response curve in this tissue (EC_{50} of 28.7 ± 2.7 nM, $n = 4$ compared with controls of 23.8 ± 2.4 nM, $n = 7$).

Table 2 Potencies of pituitary adenylate cyclase activating polypeptides, PACAP-27 and PACAP-38, compared with vasoactive intestinal polypeptide (VIP) in control and pretreated preparations of rat jejunum mucosa

Peptide and pretreatment		n	EC_{50} (nM)	P
PACAP-27	Control	(9)	0.8 ± 0.1	—
PACAP-38	Control	(5)	30.6 ± 5.6	<0.001
VIP*		(7)	23.8 ± 2.4	<0.001
PACAP-27	+TTX	(4)	29.4 ± 5.4	<0.001
	+Atr	(6)	4.5 ± 1.7	<0.02
	+Atr/Tri	(4)	11.0 ± 3.6	<0.001
	+SP	(5)	21.8 ± 2.9	<0.001
	+Capsaicin	(4)	28.3 ± 5.5	<0.001

An asterisk denotes reanalysis of previously published data (Cox & Cuthbert, 1989). Atr/Tri is the combination of atropine (10 μM) and trimethaphan (100 μM). Other concentrations are as follows: TTX (100 nM); substance P (SP, 2 × 1 μM) and capsaicin (3 μM), all additions being made to the basolateral surface. *P* values quoted are for each respective EC_{50} value compared with the PACAP-27 controls.

Discussion

A comparison of the response profiles to PACAP-27, PACAP-38 and VIP shows clearly that PACAP-27 is probably the most potent intestinal secretory agent described so far. The TTX sensitivity exhibited by transient secretory responses to low concentrations of PACAP-27 indicates the presence of specific, high affinity receptors for this peptide on submucous neurones in rat jejunum. PACAP-38 and VIP have no neuronal component in their respective secretory responses in this tissue. VIP affects epithelial transport processes directly via specific receptors within the basolateral domain (Dharmsathaphorn *et al.*, 1983) and it is probable that PACAP-38 also affects these receptors. Neither of these peptides therefore appear to have a high enough affinity for the neuronal PACAP-27 receptors. This is similar to the order of agonist potency observed recently for stimulation of pancreatic amylase secretion (Mungan *et al.*, 1991) and for PACAP binding described as 'Type 1' (Shivers *et al.*, 1991) in adrenal medullary chromaffin cells and epididymal cells. These Type 1 binding sites could be similar to those described in astrocytes (Tatsuno *et al.*, 1990) and neuroblastoma cells (Cauvin *et al.*, 1990) for which VIP exhibits an affinity in the μM range. These two studies however, found PACAP-38 to be almost equipotent with PACAP-27, as also seen in pancreatic cell membranes (Robberecht *et al.*, 1991), a situation not observed in Type 1 binding (Shivers *et al.*, 1991). Very little information exists concerning the presence and/or differences between PACAP and VIP receptor binding in the intestine. Preliminary studies have so far simply noted low levels of [^{125}I]-PACAP and [^{125}I]-VIP binding in crude membrane preparations from rat duodenum (Gottschall *et al.*, 1990). What is clear from the functional studies described above is that PACAP-27 has both indirect and direct secretory effects, the latter being similar in potency to both PACAP-38 and VIP.

The primary neurotransmitter candidates for the TTX-sensitive secretory component of PACAP-27 responses are acetylcholine (ACh, though carbachol was used here) and/or SP. Both are secretory neurotransmitters in the intestine (Szerb, 1976; Morita *et al.*, 1980) and both afford transient epithelial anion secretion of similar latency and period to the PACAP-27 responses observed above. Pharmacological manipulations were chosen to inhibit the likely mediators, these being either a combination of cholinoceptor antagonists or SP desensitization. Atropine alone did significantly shift the PACAP-27 concentration-response curve to the right compared with controls ($P<0.02$), and carbachol responses were abolished in these preparations. Pretreatment with atropine plus the nicotinic antagonist, trimethaphan, further shifted the PACAP-27 curve to the right ($P<0.001$, compared with controls) and this was still significantly different ($P<0.01$) from the PACAP-27 curve in the presence of TTX. A significant noncholinergic (and nonadrenergic, as noradrenaline reduces the SCC) component is therefore involved. The primary secretory peptide candidate is SP, not only because of the transient character of its secretory responses, but also because of the significant mucosal innervation by SP positive fibres emanating from cell bodies present in the submucous ganglia of the rat small intestine (Ekblad *et al.*, 1987). Desensitization of preparations to SP resulted in complete loss of the early transient secretory responses to PACAP-27 and a resultant EC_{50} of 21.8 ± 2.9 nM ($n = 5$), which was not different from the EC_{50} obtained after TTX pretreatment. That a portion of the responses to low concentrations of PACAP-27 are attributable to ACh and yet can be abolished by SP densensitization, finds explanation in the respective neurotransmitter receptor localisations. Both carbachol and SP act directly upon epithelial muscarinic and neurokinin$_1$ (NK_1) and NK_2 receptors respectively in the rat small intestine, there being little TTX-sensitivity to either secretory response. Thus the greater inhibitory effect of atropine in conjunction with trimethaphan indicates the presence of PACAP-27 receptors on presynaptic as well as postsynaptic/prejunctional submucous neurones. The former by virtue of ACh release, affect nicotinic receptors and excitation of cholinergic postsynaptic submucous neurones that in turn innervate the mucosa and may also contain SP. Alternatively these selective PACAP-27 receptors may be located on a different neuronal population, possibly sensory (SP-containing) nerves as indicated by the effects of capsaicin. This sensory neurotoxin has an inhibitory effect of the same magnitude as TTX and whilst nonspecific effects of capsaicin upon other nonsensory neurones cannot be ruled out at present (Yarrow *et al.*, 1991), it appears that PACAP-27 may affect sensory nerves in this preparation. By densensitizing epithelial NK receptors, attenuation of PACAP-27 responses could be expected if the final event was indeed SP release from submucous neurones innervating the mucosa. This appears to be the case, SP desensitization caused a shift in the PACAP-27 concentration-response curve of the same magnitude as TTX, and yet had no significant effect upon the VIP concentration-response curve.

In conclusion, PACAP-27 and PACAP-38 are secretory neuropeptides in the rat small intestine and both have the capacity to act directly upon basolateral epithelial receptors. Whether these receptors are the same as those previously characterized for VIP remains to be determined, there being no VIP antagonists with sufficiently high affinity for the epithelial receptor at present (Cox & Cuthbert, 1989). PACAP-27 is notably different from PACAP-38 and VIP, in its capacity to stimulate anion secretion indirectly at pM and low nM concentrations. Thus selective high affinity receptors for PACAP-27 are present on submucous neurones in rat jejunum, the stimulation of which appears to result in SP-mediated epithelial secretion.

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