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SPECIAL REPORT

Endogenous noradrenaline release from guinea-pig isolated trachea is inhibited by activation of M_2 receptors

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Overflow of endogenous noradrenaline (NA) from guinea-pig isolated tracheae was evoked by electrical field stimulation (3 Hz, 540 pulses). The muscarinic receptor agonist oxotremorine inhibited the evoked overflow of NA in a concentration-dependent manner (EC_{50} 84 nM). Methoctramine, pirenzepine and *p*-fluoro-hexahydrosiladiphenidol (each 1 μM) shifted the concentration-response curves of oxotremorine to the right with apparent pA_2 values of 7.60, 6.74 and 6.18, respectively. It is concluded that sympathetic nerve terminals in the guinea-pig trachea are endowed with inhibitory muscarinic M_2 receptors.

Keywords: Noradrenaline release; trachea; airway; muscarinic receptor; *p*-fluoro-hexahydrosiladiphenidol (*p*-FHHSiD); methoctramine; pirenzepine

Introduction The airways are innervated by cholinergic and aminergic nerves, and there is morphological (e.g. Jones *et al.*, 1980) and functional (e.g. Pendry & Maclagan, 1991b) evidence that cross talk between the two neuronal systems may occur. For example, the sympathetic neurotransmission can be inhibited via presynaptic muscarinic receptors. Based on indirect evidence (experiments in which the relaxation response of isolated tracheae to sympathetic nerve stimulation was measured), Pendry & Maclagan (1991a) concluded that muscarinic receptors on sympathetic nerves in the guinea-pig trachea belonged to the M_3 subtype.

Recently, the overflow of endogenous noradrenaline (NA) from isolated, *in vitro* incubated rat tracheae was directly measured, and it was shown that activation of muscarinic receptors can suppress the release of NA (Racké *et al.*, 1991). Further pharmacological experiments indicated that, in contrast to the above observations in guinea-pig tracheae, the muscarinic receptors inhibiting NA release in the rat trachea belonged to the M_2 subtype (Racké *et al.*, 1992).This discrepancy could reflect a species difference. On the other hand, conclusions about presynaptic muscarinic modulation of NA release based on the airway smooth muscle response are complicated by the fact that M_3 receptors mediate contraction in this tissue. Therefore, the muscarinic modulation of sympathetic neurotransmission in guinea-pig tracheae was characterized in experiments in which the overflow of endogenous NA was measured directly.

Methods Guinea-pigs of either sex weighing 220–270 g (Charles River Wiga, Sulzfeld, Germany) were used. As described in detail for rats (Racké *et al.*, 1991), the isolated tracheae were fixed between two platinum wire field electrodes and incubated in 1.7 ml Krebs-HEPES solution (composition, mM): NaCl 118.5, KCl 5.7, $CaCl_2$ 1.2, $MgCl_2$ 1.2, sodium EDTA 0.03, (+)-ascorbic acid 0.06, HEPES 20.0 (pH 7.4) and D-glucose 11.1. The medium also contained desipramine (1 μM), tyrosine (10 μM) and yohimbine (1 μM) (see Racké *et al.*, 1991). The bath fluid (37°C and gassed with 100% O_2) was collected every 10 min. At the end of the incubation each trachea was blotted and weighed (133 ± 2 mg, $n = 99$) and catecholamines were extracted in 2 ml 0.1 M $HClO_4$.

Electrical field stimulation was carried out after 60 (S1) and 110 (S2) min of incubation. Biphasic pulses of 1 ms, 250 mA, 3 Hz (Grass S9 stimulator) were applied 3 times for 1 min with 1 min intervals. NA was determined by high performance liquid chromatography with electrochemical detection and the evoked overflow of NA was calculated as described previously (Racké *et al.*, 1991). Antagonism was quantified by calculating apparent pA_2 values of EC_{50} values according to equation (4) of Furchgott (1972). EC_{50} values (concentrations causing 50% inhibition of NA overflow) were calculated with a computer programme (see Racké *et al.*, 1991). Mean values of n observations are given ± s.e.mean.

Drugs used were: desipramine hydrochloride (Serva, Germany); *p*-fluoro-hexahydrosiladiphenidol (*p*-FHHSiD, gift of Prof. Lambrecht, Frankfurt, Germany); oxotremorine sesquifumarate (Sigma, Germany); pirenzepine dihydrochloride monohydrate (Thomae, Biberach, Germany); tetrodotoxin (Sigma); tyrosine (Sigma); yohimbine hydrochloride (Boehringer Ingelheim, Germany).

Results The spontaneous outflow of NA was 1.6 ± 0.1 pmol g^{-1} 10 min^{-1} ($n = 99$). None of the test drugs significantly affected this spontaneous outflow (not shown).

In the absence of test drugs, the overflow of NA evoked by the first electrical stimulation (S1) was 42 ± 4 pmol g^{-1} ($n = 40$), corresponding to 4.53 ± 0.53% of the NA content of the tissue. In control experiments, a second stimulation (S2) caused a similar overflow of NA, resulting in a ratio S2/S1 of 0.90 ± 0.11 ($n = 8$). Tetrodotoxin (1 μM) abolished the evoked overflow of NA, as did omission of calcium (each $n = 3$, not shown).

Oxotremorine inhibited the evoked overflow of NA in a concentration-dependent manner, the block being almost complete at a concentration of 1 μM, EC_{50} 84 nM (Figure 1). Methoctramine, *p*-FHHSiD and pirenzepine (each 1 μM, present from the onset of incubation) shifted the concentration-response curve of oxotremorine to the right. Methoctramine was the most potent and *p*-FHHSiD the weakest antagonist (Figure 1). The following apparent pA_2 values were calculated: methoctramine (7.60), pirenzepine (6.74) and *p*-FHHSiD (6.18). The overflow of NA evoked by S1 was not affected by *p*-FHHSiD (4.06 ± 0.42% of tissue NA, $n = 25$). However, it tended to increase in the presence of methoctramine or pirenzepine (6.63 ± 1.37, $n = 22$, and 6.14 ± 1.14, $n = 12$, % of tissue NA, respectively; each group $0.05 < P < 0.1$ vs controls). Tissue NA at the end of the incubation experiments was 1088 ± 139 pmol g^{-1} ($n = 99$).

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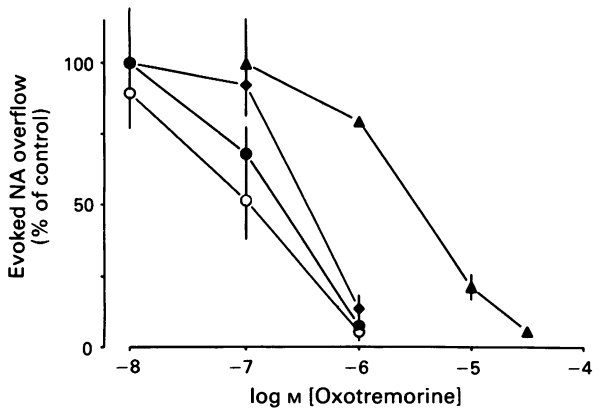

Figure 1 Concentration-dependent effects of oxotremorine on the overflow of noradrenaline (NA) from guinea-pig tracheae evoked by electrical stimulation (3 Hz) in the absence (○) or presence of methoctramine (1 μM, ▲), pirenzipine (1 μM, ◆) or *p*-fluoro-hexahydrosiladiphenidol (1 μM, ●). The antagonists were present from the onset of incubation, oxotremorine was added 10 min before S2. Ordinate scale: evoked NA overflow, expressed as a percentage of the mean overflow (S2/S1) observed in the respective controls (for each antagonist separate control experiments were carried out in which the antagonist was present from the onset of incubation; S2/S1 in all series between 0.89 and 0.96). Means with s.e.mean (vertical bars) of 3–8 experiments.

Discussion Proceeding from previous experiments on rat isolated trachea (Racké *et al.*, 1991), the present study shows that under similar conditions the impulse-induced overflow of endogenous NA from guinea-pig isolated trachea (and presumably the release of NA from the sympathetic axons of the trachea) is also inhibited by activation of muscarinic receptors. The inhibition of NA overflow by oxotremorine was potently antagonized by the M_2 receptor selective antagonist, methoctramine. The apparent pA_2 value of methoctramine determined in the present experiments (7.60), like the pA_2 determined in corresponding experiments in rat tracheae (7.56, Racké *et al.*, 1992), agrees with the reported affinity of methoctramine for M_2 receptors (see Melchiorre, 1988). In addition, the low potency of *p*-FHHSiD in the present experiments (apparent pA_2 value 6.18), like that observed in corresponding experiments on rat tracheae (apparent pA_2 value 5.93, Racké *et al.*, 1992), further indicates that the muscarinic inhibition of NA release in the trachea is mediated via M_2 receptors rather than M_3 receptors (see Lambrecht *et al.*, 1988). Moreover, the potency observed for pirenzepine in the present experiments (apparent pA_2 value 6.74) excludes the involvement of M_1 receptors, but agrees well with the reported affinity of pirenzepine for M_2 receptors (Lambrecht *et al.*, 1988).

In guinea-pig tracheae incubated *in vitro*, Pendry & Maclagan (1991b) observed a vagally-induced inhibition of sympathetic neurotransmission only in about 50% of the experiments. Similarly, under the present incubation conditions an inhibition of the evoked NA release by endogenous acetylcholine also appears to be variable. If there is inhibition, endogenous acetylcholine also seems to act via M_2 receptors, since methoctramine, but not *p*-FHHSiD tended to increase NA release.

In conclusion, the present experiments in which the *in vitro* release of endogenous NA evoked by electrical field stimulation was measured as overflow indicate that sympathetic nerves in the guinea-pig trachea, like those in rat trachea, are endowed with inhibitory muscarinic receptors of the M_2 subtype. The observations stand in contrast to the conclusion which Pendry & Maclagan (1991a) drew from experiments in which the effects of sympathetic nerve stimulation on the smooth muscle response were studied. Presynaptic muscarinic autoreceptors in the guinea-pig trachea have also been characterized as M_2-like (Kilbinger *et al.*, 1991) whereas the postjunctional muscarinic receptors on trachealis muscle belong to the M_3 subtype.

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SPECIAL REPORT

The influence of neuronal 5-hydroxytryptamine receptor antagonists on non-cholinergic ganglionic transmission in the guinea-pig enteric excitatory reflex

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A partitioned bath made it possible to separate the site of recording of the ascending excitatory reflex of the ileal circular muscle (oral compartment) from the site of reflex induction (caudal compartment), evoked by inflating an intraluminal balloon. In the caudal compartment, blockade of cholinergic ganglionic transmission by hexamethonium (100 μM) and hyoscine (0.3 μM) caused an approximately 65% reduction in the amplitude of reflex contractions, suggesting that the remaining response was mediated by non-cholinergic transmission near the distension site. This non-cholinergic component of ganglionic transmission was insensitive to the action of methiothepin (1 μM), ondansetron (1 μM), tropisetron (1.5 μM), DAU 6285 (1 μM) and renzapride (1 μM), agents that antagonize the action of 5-hydroxytryptamine (5-HT) at neural 5-HT_1-like, 5-HT_3, 5-HT_4 and putative 5-HT_{1P} receptors. These findings suggest that the neural pathways subserving non-cholinergic ganglionic transmission in the ascending excitatory reflex in the guinea-pig ileum do not involve 5-HT as neurotransmitter.

Keywords: Isolated small intestine; ascending excitatory reflex; non-cholinergic ganglionic transmission; 5-hydroxytryptamine (5-HT); 5-HT receptor antagonists

Introduction The ascending excitatory reflex is a neurogenic orally-directed motor response of the circular musculature which is triggered by gut wall distension. In the guinea-pig ileum, both cholinergic (nicotinic and muscarinic) and non-cholinergic transmission are involved in this reflex (Holzer, 1989; Tonini & Costa, 1990). Evidence that 5-hydroxytryptamine (5-HT) or substance P might be the transmitter mediating non-cholinergic transmission between myenteric neurones has previously raised considerable debate (see Furness & Costa, 1987). Although substance P is currently considered to be the most likely transmitter candidate (Furness & Costa, 1987), an equivalent role for 5-HT has not yet been ruled out. In the guinea-pig ileum, in fact, 5-HT causes fast and slow synaptic excitation through activation of 5-HT_3 (Surprenant & Crist, 1988; Mawe *et al.*, 1989) and putative 5-HT_{1P} receptors (Mawe *et al.*, 1989), respectively. Recently, other 5-HT receptor subtypes have been characterized in myenteric neurones. These include excitatory 5-HT_4 receptors which cause prejunctional and presynaptic acetylcholine release (for review see Tonini *et al.*, 1991), and inhibitory 5-HT_1-like receptors (Galligan *et al.*, 1988).

This study was designed to assess whether the 5-HT receptor antagonists methiothepin (5-HT_1-like), ondansetron (5-HT_3), tropisetron (5-HT_3/5-HT_4), DAU 6285 (5-HT_4) (Peroutka, 1990; Dumuis *et al.*, 1992) and the prokinetic agent renzapride, which was shown to antagonize putative 5-HT_{1P} receptors (Mawe *et al.*, 1989), affect the non-cholinergic component of ganglionic transmission in the ascending excitatory reflex in the guinea-pig ileum.

Methods The experimental model was similar to that described in detail previously (Tonini & Costa, 1990). Guinea-pigs of either sex (300–350 g) were stunned and bled. Seven cm long segments of ileum were excised about 20 cm from the ileocaecal junction and set up horizontally in a three-compartment bath containing standard Tyrode solution maintained at 37°C and bubbled with a mixture of 95% O_2 and 5% CO_2. In this bath, a small intermediate compartment made it possible to separate the site of recording of the ascending excitatory reflex (oral compartment) from the site of reflex induction (caudal compartment). Reflex contractions of the circular coat were recorded isometrically (tension: 5 mN). Inflation of an intraluminal rubber balloon with 0.3 ml of water was used as a gut distending stimulus to evoke reflex responses at 2 min intervals. Drugs were applied in the caudal compartment only. The non-cholinergic component of ganglionic transmission in this compartment was revealed by concomitant administration of 100 μM hexamethonium and 0.3 μM hyoscine (Tonini & Costa, 1990). 5-HT receptor antagonists were added to the compartment in the presence of cholinergic transmission blockade, and reflex responses were followed for 20 min. Reflex contractions elicited in the presence of hexamethonium and hyoscine with and without 5-HT receptor antagonists were expressed as percentages of the response obtained in the absence of any drug treatment.

All data are expressed as mean ± s.e.mean. The significance of difference between mean values was estimated by one-way analysis of variance with Scheffé F-test for multiple comparisons. P values of 0.05 or less were considered significant.

The following drugs were used: hyoscine hydrochloride (Sigma), hexamethonium bromide (Sigma), tetrodotoxin (Sankyo), dimethyl-phenyl-piperazinium iodide (DMPP) (Fluka), methiothepin maleate (Roche), tropisetron (ICS 205-930) (RBI), ondansetron (GR 38032F) (Glaxo), DAU 6285 (endo-6-methoxy-8-methyl-8-azabicyclo [3.2.1.] oct-3-yl-2,3-dihydro-2-oxo-1H-benzimidazole-1-carboxylate hydrochloride) (Boehringer Ingelheim, Italy) and renzapride (BRL 24924) (Beecham Pharmaceuticals).

Results Distension of the gut wall in the caudal compartment by inflation of an intraluminal balloon with 0.3 ml of water, elicited a reflex monophasic contraction of the circular

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muscle in the oral compartment. As reported previously (Tonini & Costa, 1990), the concomitant addition of hexamethonium (100 μM) and hyoscine (0.3 μM) to the caudal compartment reduced the amplitude of reflex contractions to 37.7 ± 5.5% ($n = 8$, $P < 0.01$) and tetrodotoxin (0.6 μM, $n = 6$) caused their disappearance (Figure 1a). In untreated preparations, 20 μM DMPP applied to the caudal compartment caused a contractile response, the amplitude of which was 84.3 ± 6.5% ($n = 8$) of the reflex responses (Figure 1b). These DMPP-induced contractions were abolished by 100 μM hexamethonium ($n = 8$) (Figure 1c), indicating that the latter drug was able to suppress completely responses to nicotinic receptor activation comparable in amplitude to those evoked by gut distension. In the presence of hexamethonium and hyoscine, blockade of 5-HT_1-like receptors by 1 μM methiothepin ($n = 8$), 5-HT_3 receptors by 1 μM ondansetron ($n = 7$), 5-HT_3 and 5-HT_4 receptors by micromolar concentrations of tropisetron (1.5 μM, $n = 7$), 5-HT_4 by 1 μM DAU 6285 ($n = 8$) and putative 5-HT_{1P} receptors by 1 μM renzapride ($n = 10$), did not affect the amplitude of reflex responses (Figure 1d).

Discussion The ascending excitatory reflex response of the circular muscle in the guinea-pig ileum involves both cholinergic and substance P-mediated non-cholinergic transmission at both the neuro-neuronal and neuromuscular level (Holzer, 1989; Tonini & Costa, 1990). Nevertheless, the possibility that 5-HT might possess a transmitter role at the ganglionic level (Mawe *et al.*, 1989) had not yet been adequately explored in functional motility experiments.

Based on present findings, the failure of a variety of antagonists of neural 5-HT receptors to antagonize the non-cholinergic component of ganglionic transmission in the ascending excitatory reflex, argues against 5-HT subserving this type of transmission. In fact, if 5-HT is released by balloon distension into the caudal compartment (i.e. the site where, under our experimental conditions, non-cholinergic ganglionic transmission occurs), it could act at both excitatory (5-HT_3, 5-HT_4 and putative 5-HT_{1P}) or inhibitory (5-HT_1-like) receptors (Tonini *et al.*, 1991; Mawe *et al.*, 1989; Galligan *et al.*, 1988). The inefficiency of ondansetron, tropisetron and DAU 6285 in modifying reflex contractions, excludes an involvement of 5-HT_3 or 5-HT_4 receptors on distension-induced non-cholinergic transmission in the caudal compartment. In agreement with our results, tropisetron at micromolar concentrations was previously found not to affect the amplitude of ascending contractions of the circular muscle to transmural electrical stimulation in the guinea-pig ileum (Jin *et al.*, 1989). In our experiments, even renzapride at 1 μM concentration did not affect the amplitude of reflex contractions. In myenteric neurones this compound at the same concentration was found to abolish 5-HT-mediated slow depolarizations and slow e.p.s.ps. through antagonism

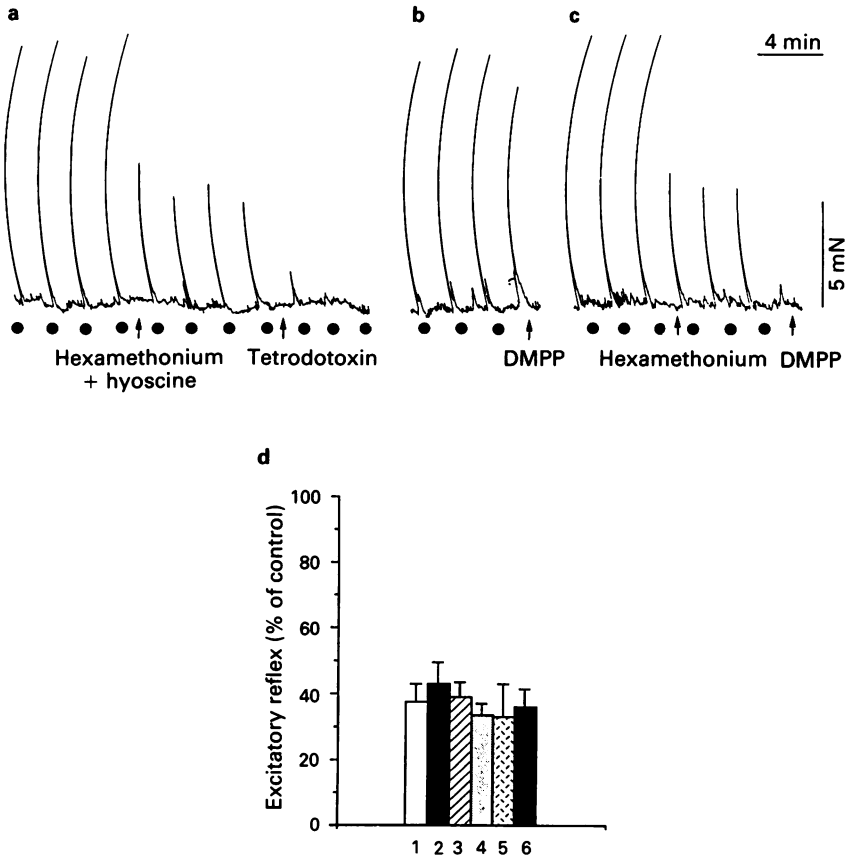

Figure 1 Ascending reflex contractions recorded by a technique which made it possible to separate the site of reflex recording (oral compartment) from the site of reflex induction and drug administration (caudal compartment). In (a) a combination of hexamethonium (100 μM) and hyoscine (0.3 μM) reduced to approximately 35% the amplitude of reflex contractions (dots), which were subsequently abolished by tetrodotoxin (0.6 μM). In (b) dimethylphenylpiperazinium iodide (DMPP, 20 μM) caused the appearance in the oral compartment of contractile responses the amplitude of which was 85% of reflex responses (dots). In (c) administration of hexamethonium (100 μM) depressed by 60% the amplitude of reflex responses (dots), and abolished DMPP-induced contractions. In (d) reflex contractions in the presence of hexamethonium (100 μM) and hyoscine (0.3 μM) before (1) ($n = 8$) and after treatment with (2) methiothepin (1 μM, $n = 8$); (3) ondansetron (1 μM, $n = 7$); (4) tropisetron (1.5 μM, $n = 7$); (5) DAU 6285 (1 μM, $n = 8$); (6) renzapride (1 μM, $n = 10$). Each group of reflex contractions was significantly lower ($P < 0.01$) than those observed in controls. The amplitude of reflex contractions in the presence of hexamethonium and hyoscine plus each of the five 5-HT receptor antagonists was not different from that recorded with hexamethonium and hyoscine alone.

at putative 5-HT_{1P} receptors (Mawe *et al.*, 1989). The latter finding suggests that 5-HT does not mediate non-cholinergic orally-directed slow excitatory ganglionic transmission in the guinea-pig ileum. Inhibitory 5-HT_1-like receptors (probably the 5-HT_{1A} subtype) are widely distributed in the myenteric plexus, in which they reduce neuronal excitability leading to depression of cholinergic and non-cholinergic synaptic transmission (Galligan *et al.*, 1988). The fact that in the presence of methiothepin the amplitude of reflex activity was unaffected further suggests that the nerve pathways subserving the ascending excitatory reflex do not release 5-HT following a physiological stimulus like radial distension of the gut wall.

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Imidazoline antagonists of α_2-adrenoceptors increase insulin release *in vitro* by inhibiting ATP-sensitive K^+ channels in pancreatic β-cells

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1 Islets from normal mice were used to study the mechanisms by which imidazoline antagonists of α_2-adrenoceptors increase insulin release *in vitro*.

2 Alinidine, antazoline, phentolamine and tolazoline inhibited ^{86}Rb efflux from islets perifused with a medium containing 3 mM glucose, i.e. under conditions where many adenosine 5'-triphosphate (ATP)-sensitive K^+ channels are open in the β-cell membrane. They also reduced the acceleration of ^{86}Rb efflux caused by diazoxide, an opener of ATP-sensitive K^+ channels.

3 ATP-sensitive and voltage-sensitive K^+ currents were measured in single β-cells by the whole-cell mode of the patch-clamp technique. Antazoline more markedly inhibited the ATP-sensitive than the voltage-sensitive current, an effect previously observed with phentolamine. Alinidine and tolazoline partially decreased the ATP-sensitive K^+ current.

4 The four imidazolines reversed the inhibition of insulin release caused by diazoxide (through opening of ATP-sensitive K^+ channels) or by clonidine (through activation of α_2-adrenoceptors) in a concentration-dependent manner. Only the former effect correlated with the ability of each drug to increase control insulin release stimulated by 15 mM glucose alone.

5 It is concluded that the ability of imidazoline antagonists of α_2-adrenoceptors to increase insulin release *in vitro* can be ascribed to their blockade of ATP-sensitive K^+ channels in β-cells rather than to their interaction with the adrenoceptor.

Keywords: Imidazolines; K^+ channels; α_2-adrenoceptors; pancreatic β-cells; insulin release; alinidine; antazoline; phentolamine; tolazoline; diazoxide

Introduction

Several *in vivo* studies have shown that phentolamine augments basal and glucose-increased plasma insulin levels in normal subjects and animals (Cerasi *et al.*, 1969; Buse *et al.*, 1970; Misbin *et al.*, 1970; Lundquist, 1972; Ahren & Lundquist, 1985). Since insulin release is inhibited by activation of α_2-adrenoceptors in β-cells (Nakaki *et al.*, 1980), these observations have been interpreted as evidence for a suppression of β-cell function by a constant adrenergic tone. It has been further suggested that this tone could be excessive in non-insulin-dependent diabetic patients and contribute to their impaired β-cell function (Robertson *et al.*, 1976; Broadstone *et al.*, 1987). This concept, therefore, recently led to the development of α_2-adrenoceptor blockers as potential anti-diabetic agents (Kawazu *et al.*, 1987; Ortiz-Alonso *et al.*, 1991; Gautier *et al.*, 1991).

A number of *in vitro* studies have shown that phentolamine increases insulin release even in the absence of any agonist of α_2-adrenoceptors (Efendic *et al.*, 1975; Smith & Furman, 1988; Schulz & Hasselblatt, 1988; Garrino & Henquin, 1990). They suggested that the drug could have effects unrelated to blockade of α-adrenoceptors and possibly linked to its imidazoline structure (Schulz & Hasselblatt, 1989). The insulin-releasing property of phentolamine has recently been attributed to a blockade of ATP-sensitive K^+ channels in pancreatic β-cells (Plant & Henquin, 1990). These channels are indeed a key site of control of insulin release by glucose and several drugs: their closure accounts for the stimulation by tolbutamide, whereas their opening underlies the inhibition by diazoxide (Henquin & Meissner, 1982; Trube *et al.*, 1986). Besides phentolamine, it has also been suggested that two other imidazoline antagonists of α_2-adrenoceptors, efaroxan and midaglizole, increase insulin release by acting on adenosine 5'-triphosphate (ATP)-sensitive K^+ channels (Chan *et al.*, 1991a,b).

In the present study, we evaluated the effects of imidazoline derivatives structurally related to phentolamine on glucose-induced insulin release by pancreatic islets from normal mice. To determine whether their action on release is better explained by a blockade of α_2-adrenoceptors or of ATP-sensitive K^+ channels, we compared the ability of the drugs to relieve the inhibitions produced by clonidine and diazoxide respectively. The effects of these imidazoline derivatives on K^+ channels were also determined by measurements of ^{86}Rb efflux and by patch-clamp techniques.

Methods

Animals

All experiments were carried out with islets obtained by collagenase digestion of the pancreas of fed female NMRI mice.

^{86}Rb efflux experiments

These experiments were carried out at 37°C. After isolation, the islets were loaded with ^{86}Rb (Rb used as tracer for K) for 90 min in a medium containing 15 mM glucose and supplemented with $^{86}RbCl$ (1.5 to 3 MBq ml^{-1}; sp.act. 7.4 to 18.5 TBq mol^{-1}). The Rb concentration never exceeded 0.4 mM (Garrino & Henquin, 1988). ^{86}Rb efflux was then monitored in a dynamic perifusion system (Henquin, 1978). The

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radioactivity lost by the islets was measured immediately by the Cerenkov radiation (Henquin, 1978) in the effluent fractions collected at 2 min intervals. From the sum of the radioactivity remaining in the islets at the end of the experiments and the accumulated effluent radioactivity, the fractional efflux rate was calculated for each period (radioactivity lost by tissue during the time interval/radioactivity present in the tissue during that time interval) and expressed as percent per min. The solutions used had the following ionic composition (in mM): NaCl 120, KCl 4.8, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 24 and were gassed with 94% O_2/6% CO_2 to maintain a pH of 7.4. They were supplemented with 1 mg ml^{-1} bovine serum albumin fraction V (Boehringer, Mannheim, Germany).

Insulin release measurements

These experiments were also performed at 37°C, with solutions similar to those used for ^{86}Rb efflux experiments. After isolation, the islets were first preincubated for 60 min in a medium containing 15 mM glucose. They were then incubated for 60 min, in batches of 3, in 1 ml of medium containing 15 mM glucose and appropriate concentrations of test substances. At the end of the incubation, a portion of the medium was withdrawn and diluted before insulin assay. Insulin was measured by a double-antibody radioimmunoassay with rat insulin as standard (Novo Research Institute, Bagsvaerd, Denmark).

Electrical recordings

After isolation of the islets, islet cells were dispersed and cultured for 1–2 days as previously described (Plant, 1988). Patch-clamp experiments were performed at room temperature (20–24°C) on single β-cells. For measurements of ATP-sensitive and voltage-dependent K^+ currents the bath solution contained (in mM): NaCl 135, KCl 5.6, $CaCl_2$ 2.6, $MgCl_2$ 1.2, HEPES 10, and was titrated to pH 7.4 with NaOH. Pipettes were filled with a solution containing (in mM): KCl 135, $MgCl_2$ 4, $CaCl_2$ 2, EGTA 10, Na_2ATP 0.65, HEPES 20, and titrated to pH 7.15 with KOH. Details of the recording technique and the separation of ATP-sensitive and voltage-dependent currents from other membrane currents have been described previously (Garrino *et al.*, 1989; Plant & Henquin, 1990). In brief, ATP-sensitive K^+ currents were measured by recording the currents at 15 s intervals at the holding potential (− 70 mV) and during 100 ms pulses to − 60 and − 80 mV which were separated by an interval of 100 ms. Under the conditions used, the current which develops with time during dialysis is almost entirely ATP-sensitive K^+ current. To measure voltage-dependent K^+ currents, cells were held at − 70 mV and depolarized at 15 s intervals to 0 mV to activate K^+ currents in a bathing solution which was supplemented with 100 μM tolbutamide to block ATP-sensitive K^+ currents. A short (50 ms) hyperpolarization to − 100 mV, applied 100 ms before the test pulse, was used to estimate the leakage current.

Drugs

The following drugs were used: alinidine hydrobromide, ST 91 (2,6-diethyl-n-2 imidazolidinylidenebenzenamine) hydrochloride and clonidine hydrochloride (Boehringer-Ingelheim, Germany); phentolamine mesylate (Ciba-Geigy, Basel, Switzerland), tolazoline hydrochloride and antazoline hydrochloride (Sigma Chemical Co, St Louis, MO, U.S.A.); tramazoline hydrochloride (Thomae GmbH, Biberach, Germany); yohimbine hydrochloride (Aldrich-Chemie, Steinheim, Germany); diazoxide (Schering-Plough Avondale, Rathdrum, Ireland); tolbutamide-sodium salt (Hoechst AG, Frankfurt, Germany); tetraethylammonium chloride (Buchs, Switzerland). Stock solutions of diazoxide (50 mM) were prepared in 0.1 M NaOH. Other substances were dissolved in water before being added to the appropriate solutions. $^{86}RbCl$ was purchased from the Radiochemical Centre (Amersham, Bucks, U.K.).

Statistical analysis of results

Results are usually presented as means with s.e.mean for the indicated number of experiments. The statistical significance of differences between means was assessed by analysis of variance of the data, followed by a Dunnett's test.

Results

Effects on ^{86}Rb efflux

In the presence of 3 mM glucose, many ATP-sensitive K^+ channels in the β-cell membrane are open and the potassium permeability is high (Rorsman & Trube, 1985; Misler *et al.*, 1986; Ashcroft *et al.*, 1988). Under these conditions, the rate of ^{86}Rb efflux is high and declines slightly with time (Figure 1a). It was rapidly and reversibly decreased by tolbutamide, a selective blocker of ATP-sensitive K^+ channels (Trube *et al.*, 1986). Addition of 10 μM antazoline to the medium also inhibited ^{86}Rb efflux. In the steady state this inhibition was similar to that produced by 10 μM tolbutamide, but its onset was slower and it was not reversible (Figure 1a).

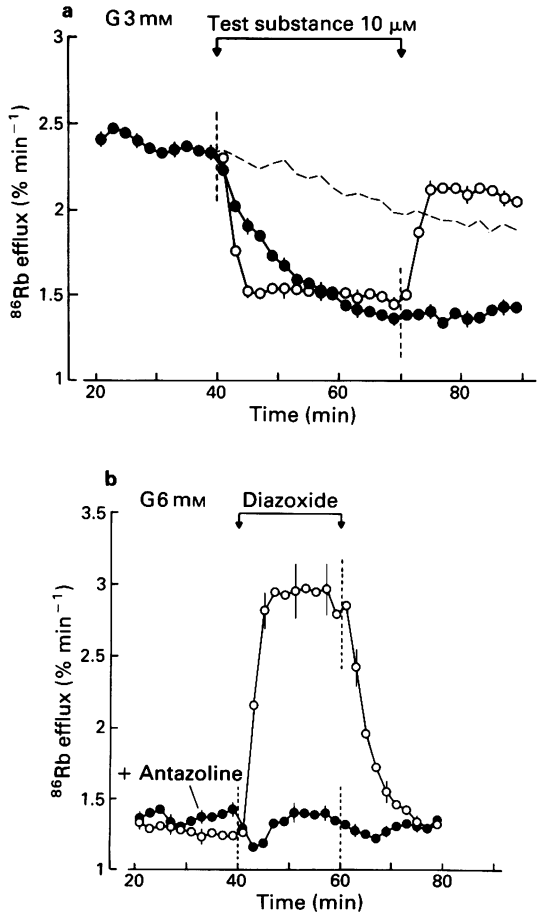

Figure 1 Effects of antazoline, tolbutamide and diazoxide on ^{86}Rb efflux from perifused mouse islets. (a) Antazoline (●) or tolbutamide (○) was added at a concentration of 10 μM to a medium containing 3 mM glucose (G). Controls without test substance are shown by the broken line. (b) Diazoxide (100 μM) was added to a medium containing 6 mM glucose (G) alone (○) or 6 mM glucose plus 10 μM antazoline (●). Values are means for 4–5 experiments with s.e.mean shown by vertical lines.

In similar experiments, five other imidazolines (alinidine, phentolamine, ST 91, tramazoline and tolazoline) produced a slow and irreversible inhibition of ^{86}Rb efflux. Yohimbine also produced a similar effect, which, however, was reversible. A quantitative assessment of these data is presented in Table 1. When the drugs were used at a concentration of 10 μM the order of potency was tolbutamide ⩾ antazoline ⩾ alinidine ⩾ phentolamine > ST 91 ⩾ tramazoline > tolazoline = yohimbine. The two weakest substances were also tested at 100 μM and yohimbine proved to be slightly more potent than tolazoline (Table 1).

Opening of ATP-sensitive K^+ channels by diazoxide (Trube *et al.*, 1986) in the presence of 6 mM glucose causes a marked acceleration of ^{86}Rb efflux that can be inhibited by phentolamine (Plant & Henquin, 1990). This effect of diazoxide was also antagonized by antazoline (Figure 1b) and by other imidazolines tested here (data not shown).

Effects on K^+ currents

As shown previously (Trube *et al.*, 1986; Garrino *et al.*, 1989), ATP-sensitive K^+ currents activate with time during dialysis of the cell interior with a low ATP concentration (Figure 2b). Addition of antazoline to the bathing solution inhibited the ATP-sensitive K^+ current. Effects of antazoline were visible at a concentration of 10 μM, but were difficult to differentiate from run-down of the current because the rate of block was slow. At a concentration of 100 μM, antazoline rapidly blocked the current, by 92 ± 1% (mean ± s.e.mean; $n = 9$). This effect of antazoline, in contrast to that of tolbutamide, was only poorly reversible (Figure 2). Little recovery of the current was observed after applications of antazoline for more than 3–4 min.

In similar experiments, 100 μM alinidine inhibited ATP-sensitive K^+ currents by 58 ± 5% ($n = 4$). Tolazoline had no detectable effect at 50 μM, but produced a small inhibition (by about 20%; $n = 3$) at 500 μM.

The effects of antazoline were also tested on the voltage-dependent K^+ current. In most experiments, currents were recorded in the presence of tolbutamide (100 μM) but without inhibition of the voltage-dependent Ca^{2+} current. With the intracellular Ca^{2+} buffering used in these experiments, a small rapidly-inactivating (100–200 ms) component of the current was probably Ca^{2+}-activated K^+ current activated by Ca^{2+}-entry (Smith *et al.*, 1990). This component is lost as Ca^{2+} currents run down during an experiment and accounts for the disappearance of the inactivating component (compare records A and C in Figure 3a). Antazoline (100 μM) caused a slow block of the current that reached a maximum after 3.5–4 min, but was almost completely reversible even after application of the drug for more than 10 min (Figure 3). This blockade was 69 ± 2% for the peak current and 68 ± 2% for the current at the end of the pulse, when the contamination by the Ca^{2+}-activated current is minimal. The inhibition by 100 μM antazoline was similar to that by tetraethylammonium ions (TEA^+) at a concentration of 4 mM (Figure 3d). The latter inhibition was, however, faster (within 1 min) than that produced by antazoline.

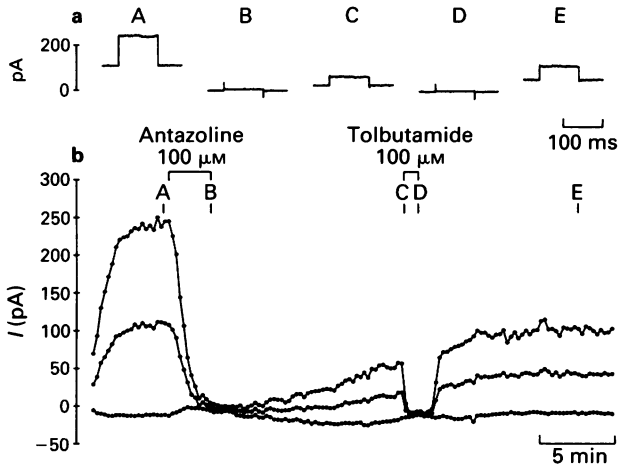

Figure 2 Inhibition of whole-cell ATP-sensitive K^+ currents by antazoline. (a) Currents recorded during 100 ms pulses from − 70 to − 60 mV at the times indicated by the letters in the experiment illustrated in (b). Outward currents are positive and shown as upward deflections. (b) Current values recorded at 15 s intervals at − 60, − 70 and − 80 mV (upper, middle and lower traces respectively). Antazoline (100 μM) was applied for 3 min as indicated by the bar. During recovery from the effects of antazoline, tolbutamide (100 μM) was applied for 1 min.

Table 1 Effects of various imidazolines, of yohimbine and of tolbutamide on ^{86}Rb efflux from mouse islets perifused with 3 mM glucose

Test substance (μM)	*Decrease in ^{86}Rb efflux*
—	5.1 ± 0.4
Antazoline 10	20.5 ± 1.5[b]
Alinidine 10	19.6 ± 2.6[b]
Phentolamine 10	17.9 ± 1.6[b]
ST91 10	14.9 ± 2.1[b]
Tramazoline 10	12.7 ± 1.3[b]
Tolazoline 10	8.1 ± 0.2[a]
Tolazoline 100	13.7 ± 0.7[b]
Yohimbine 10	8.0 ± 0.4[a]
Yohimbine 100	18.7 ± 1.0[b]
Tolbutamide 10	22.7 ± 1.2[b]

All substances were tested in experiments similar to those shown in Figure 1(a). The inhibition of ^{86}Rb efflux was calculated by integrating the area between a hypothetical horizontal line at the level of the average rate of efflux between 36 and 40 min (i.e. before drug addition) and the curve corresponding to the actual rate of efflux between 40 and 70 min (i.e. in the presence of the drug). This integration, which gives a dimensionless value, was calculated for each individual experiment. The value obtained in the absence of test substance reflects the small decrease of the control efflux rate between 40 and 70 min. Values are means ± s.e.mean for 4–5 experiments with each substance. [a]$P < 0.05$; [b]$P < 0.01$ versus controls in 3 mM glucose alone.

Effects on insulin release

Control insulin release by islets incubated in a medium containing 15 mM glucose alone amounted to 6.1 ± 0.1 ng per islet 60 min^{-1} ($n = 271$).

At a concentration of 0.1 μM, tramazoline and compound ST 91 inhibited glucose-induced insulin release by 71 ± 4% ($n = 16$) and 69 ± 3% ($n = 18$) respectively. These inhibitions were antagonized by yohimbine. Tramazoline and ST 91, therefore, behave like α_2-adrenoceptor agonists in pancreatic β-cells.

Figure 4a illustrates the concentration-dependent increase in insulin release brought about by 4 other imidazolines and by tolbutamide and yohimbine in the presence of 15 mM glucose alone. The relative potencies of the drugs were evaluated by determining the concentration at which they doubled insulin release (Table 2). The order of potency was tolbutamide > antazoline > phentolamine > alinidine >> yohimbine >> tolazoline. Interestingly, larger maximal increases in insulin release were produced by antazoline ($P < 0.05$) and phentolamine ($P < 0.01$) than by tolbutamide (Figure 4a).

Diazoxide inhibits glucose-induced insulin release by hyperpolarizing the β-cell membrane through a selective opening of ATP-sensitive K^+ channels (Henquin & Meissner, 1982; Trube *et al.*, 1986). The concentration of 40 μM diazoxide was chosen to achieve 85% inhibition of insulin release induced by 15 mM glucose (1.0 ± 0.1 ng per islet 60 min^{-1} vs

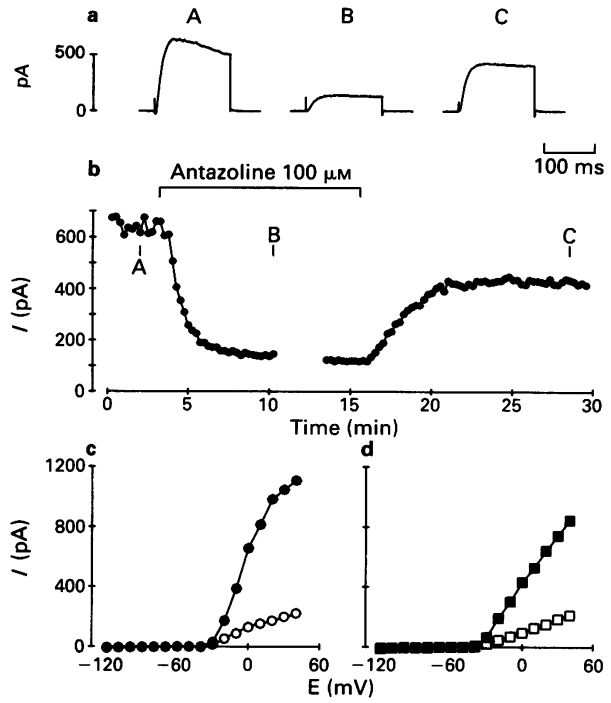

Figure 3 Effects of antazoline on voltage-dependent K^+ currents. (a) Mean currents determined from six consecutive records at the times indicated by the letters in (b). (b) Time course of the effects of antazoline (100 μM) on the maximum value of the voltage-dependent K^+ current during pulses from − 70 mV to 0 mV applied at intervals of 15 s. During the break in the measurements the current-voltage relation was recorded. (c) Current-voltage relations under control conditions (●) and about 8 min after the addition of antazoline (○). (d) Current-voltage relations after wash-out of antazoline (■), and following the subsequent addition of tetraethylammonium chloride (4 mM; □). During all experiments illustrated in this figure the bath solution contained tolbutamide (100 μM) to block ATP-sensitive K^+ currents.

6.5 ± 0.3 ng per islet 60 min^{-1} in controls; $n = 73$). Figure 4b shows that the inhibitory effect of diazoxide was antagonized in a concentration-dependent manner by tolbutamide > antazoline > phentolamine > alinidine. This order of potency was established by determining the concentration of each drug that was necessary to reverse the effect of diazoxide completely (Table 2). On the other hand, the inhibitory effect of diazoxide (85%) was only reduced to 63 ± 4% by 250 μM yohimbine ($P < 0.01$) and to 72 ± 2% by 250 μM tolazoline ($P < 0.05$).

Clonidine inhibits glucose-induced insulin release by activating α_2-adrenoceptors. The concentration of 100 nM clonidine was chosen to achieve 85% inhibition of insulin release induced by 15 mM glucose (0.85 ± 0.04 ng per islet 60 min^{-1} vs 5.8 ± 0.2 ng per islet 60 min^{-1} in controls; $n = 96$). This concentration is at least 50-fold lower than those at which clonidine directly affects ATP-sensitive K^+ channels in β-cells (Plant *et al.*, 1991).

Figure 4c shows that, with the exception of tolbutamide, all tested drugs antagonized the inhibition by clonidine in a concentration-dependent manner. Based on the drug concentration required to antagonize the effect of clonidine completely (Table 2), the order of potency was phentolamine > yohimbine > antazoline > tolazoline >> alinidine.

As shown in Figure 5, a strong correlation was found between the ability of tolbutamide, antazoline, phentolamine and alinidine to double control insulin release (in 15 mM glucose alone) and to reverse the inhibition by diazoxide ($r^2 = 0.98$ or 0.95 with and without tolbutamide). On the other hand, there was no correlation between the ability of the tested imidazolines to increase control insulin release and to reverse the inhibitory effect of clonidine.

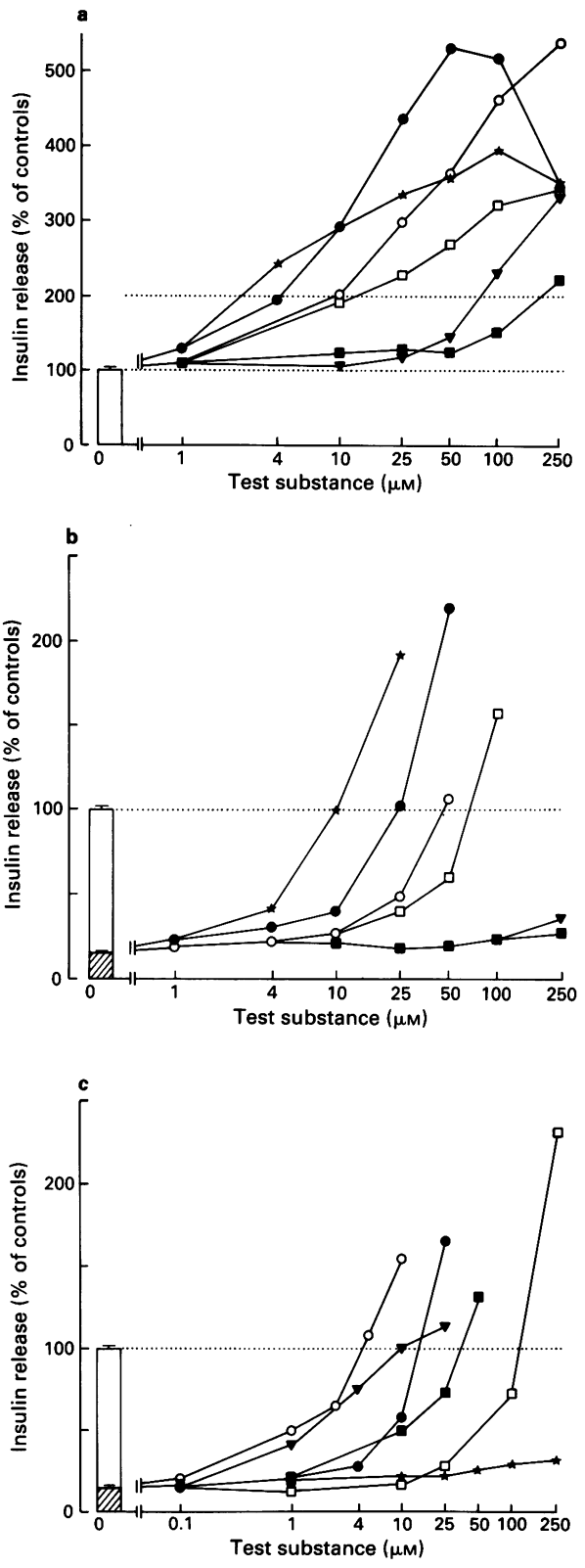

Figure 4 Concentration-dependency of the effects of four imidazolines (antazoline ●; phentolamine ○; tolazoline ■; alinidine □), of tolbutamide (*) and of yohimbine (▼) on insulin release by incubated mouse islets. The incubation medium contained (a) 15 mM glucose alone; (b) 15 mM glucose and 40 μM diazoxide; (c) 15 mM glucose and 100 nM clonidine. Results are expressed as a percentage of insulin release in 15 mM glucose alone (open columns). In (b) and (c), hatched columns show insulin release in the presence of 15 mM glucose and either diazoxide or clonidine. Dotted lines are drawn at the levels of 100 and 200% of control insulin release measured in the presence of 15 mM glucose alone. Values are means for 15–20 batches of islets from 3–4 experiments. S.e.means were omitted for the sake of clarity.

Table 2 Comparison of the efficacy of various imidazolines, of yohimbine and of tolbutamide on insulin release by mouse islets incubated under different conditions

Test substance	*Glucose* 15 mM *alone*	*Experimental conditions* + *Diazoxide* 40 μM	+ *Clonidine* 100 nM
Antazoline	4.3 ± 1.2	23.5 ± 2.9	14.4 ± 2.8
Phentolamine	9.1 ± 5.3	46.0 ± 3.3	4.4 ± 0.3
Tolazoline	190 ± 70	>>250	31.8 ± 8.1
Alinidine	11.3 ± 5.0	68.7 ± 10.3	117 ± 23
Tolbutamide	2.9 ± 1.1	9.8 ± 3.2	>>250
Yohimbine	76 ± 21	>>250	10.0 ± 4.6

The table gives the concentration of test substance (in μM) that doubles control insulin release in the presence of 15 mM glucose alone, or that reverses the inhibition produced by the indicated concentrations of diazoxide or clonidine. This concentration was estimated for each experiment. Values are means ± s.e.mean for 3–4 experiments.

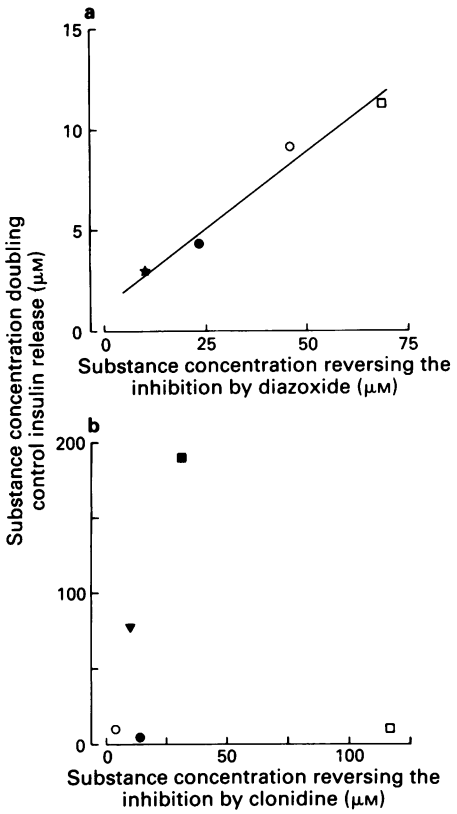

Figure 5 Correlation between the concentrations of test substance doubling control insulin release (in the presence of 15 mM glucose alone) and the concentration reversing the inhibition by (a) 40 μM diazoxide or (b) 100 nM clonidine. The drugs tested here are: antazoline (●); phentolamine (○); tolazoline (■); alinidine (□); tolbutamide (*); yohimbine (▼). In (a) the regression analysis gave a coefficient of correlation of 0.98.

Discussion

All six imidazolines tested in this study were found to affect control insulin release stimulated by glucose alone. Two of them, tramazoline and compound ST 91, inhibited release, whereas the other four increased it in a concentration-dependent manner. This effect of phentolamine has long been established (see Introduction), and those of antazoline and tolazoline have been reported for a single concentration of the drugs (Schulz & Hasselblatt, 1989).

The six imidazolines were also found to act on α_2-adrenoceptors in β-cells. Since the inhibition of insulin release caused by tramazoline and ST 91 was prevented by yohimbine, one may conclude that they behaved as α_2-agonists, as in other tissues (Malta *et al.*, 1981). On the other hand, the other four imidazolines behaved as α_2-antagonists since they relieved the inhibition of insulin release caused by clonidine. This was expected for phentolamine and tolazoline (Schulz & Hasselblatt, 1989; Reynolds, 1989), but less so for antazoline which is usually classified as a histamine receptor antagonist (anti-H_1) (Reynolds, 1989). In another study with mouse islets (Schulz & Hasselblatt, 1989) antazoline was claimed not to antagonize the effects of clonidine, probably because clonidine was used at a 10 fold higher concentration when the effects of antazoline were tested than when the effects of phentolamine were tested. Alinidine is an antiarrythmic agent with weak α_2-antagonist activity (Heinzow *et al.*, 1982). It was clearly the weakest of the antagonists tested here.

The six imidazolines also inhibited ATP-sensitive K^+ channels in β-cells, as demonstrated by the decrease in ^{86}Rb efflux that they produced in islets perifused with a medium containing a low glucose concentration, by their ability to antagonize the acceleration of ^{86}Rb efflux brought about by diazoxide and, for some of them, by the decrease in ATP-sensitive K^+ current. The observation that qualitatively similar effects were produced by agonists and antagonists of α_2-adrenoceptors reinforces our previous conclusion that the action of these substances on ATP-sensitive K^+ channels is independent of the type of action they exert on the α_2-adrenoceptor (Plant & Henquin, 1990; Plant *et al.*, 1991). Quantitative comparisons of the potency of the different drugs on ATP-sensitive K^+ channels are not possible on the basis of the present data. They would not be easily obtained because the slow action of these substances makes it difficult to differentiate small effects from run-down of the current. It is, however, clear that tolazoline was much less effective than phentolamine, antazoline and alinidine. Tolazoline was also found to be less potent than phentolamine and antazoline in inhibiting ATP-sensitive K^+ channels in RINm5F, an insulin-secreting cell line derived from a tumour (Dunne, 1991). In this preparation, the channels were more rapidly, more strongly and more reversibly blocked by phentolamine or antazoline than in the normal β-cells that we used. This difference could be due to the use of distinct experimental models and configurations of the patch-clamp technique. It might also suggest that the site of action of these imidazolines is located at the inner face of the plasma membrane, where it is much more easily reached when open-cell patches are used (Dunne, 1991) than when intact membranes are studied with the whole cell configuration employed here.

Four imidazolines tested here were thus found to inhibit both ATP-sensitive K^+ channels and α_2-adrenoceptors in β-cells, and to potentiate glucose-induced insulin release. The latter property correlated strongly with the ability of the drugs to relieve the inhibition by diazoxide, and not at all with their ability to reverse the inhibition by clonidine. We, therefore, conclude that the increase in insulin release brought about by these agents under control conditions is due to blockade of ATP-sensitive K^+ channels. This conclusion can probably be extended to efaroxan, another imidazoline derivative, which increased control insulin release

and reversed the inhibitory effect of diazoxide within a similar range of concentrations, higher than that required to antagonize the inhibitory effect of noradrenaline (Chan & Morgan, 1990).

The observation that the maximal increase in insulin release produced by antazoline and phentolamine is larger than that produced by tolbutamide suggests, however, that the effect of the two imidazolines is not solely due to blockade of ATP-sensitive K^+ channels. One can exclude the possibility that this difference is due to the simultaneous blockade of α_2-adrenoceptors because yohimbine did not increase insulin release induced by tolbutamide or glibenclamide in the presence of 10–15 mM glucose (Garrino & Henquin, 1990; unpublished observations). Unlike tolbutamide (Ashford, 1990), phentolamine (Plant & Henquin, 1990) and antazoline (this study) also inhibit voltage-dependent K^+ channels in β-cells. This might contribute to their larger maximal effect on insulin release.

The number of tested compounds is too low to draw meaningful conclusions as to structure-activity relationships. We simply note that the three imidazolines (antazoline, phentolamine and tolazoline) were more potent on α_2-adrenoceptors than on ATP-sensitive K^+ channels, whereas the reverse was true for alinidine, which is an imidazolidine. From the present and previous studies (Plant & Henquin, 1990; Plant *et al.*, 1991; Dunne, 1991; Chan *et al.*, 1991a,b), it is clear that imidazolines can be added to the already long list of pharmacological agents able to inhibit ATP-sensitive K^+ channels in β-cells (Ashford, 1990; Henquin, 1990). We previously suggested that an imidazoline-binding site might be involved in the control of ATP-sensitive K^+ channels in B-cells (Plant *et al.*, 1991). It appears, however, that such a site is clearly distinct from the known brain or kidney imidazoline receptors which have a high affinity for idazoxan and tolazoline (Michel & Insel, 1989; Atlas, 1991), two agents that are weakly active on K^+ channels (this study, Chan *et al.*, 1991a).

In conclusion, the ability of several imidazoline antagonists of α_2-adrenoceptors to increase insulin release *in vitro* can be ascribed to their blockade of ATP-sensitive K^+ channels in β-cells rather than to their interaction with the adrenoceptor. A similar mechanism might also account for, or at least contribute to, the changes in plasma insulin levels that this class of compounds may cause. It is, however, premature to exclude formally the possibility that their *in vivo* effects also involve attenuation of inhibitory sympathetic signals in β-cells.

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Characterization of 8-OH-DPAT-induced hypothermia in mice as a 5-HT_{1A} autoreceptor response and its evaluation as a model to selectively identify antidepressants

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1 8-Hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) dose-dependently induced hypothermia in mice.

2 The 5-HT_{1A} receptor partial agonists, buspirone, gepirone and ipsapirone, also dose-dependently induced hypothermia.

3 The 8-OH-DPAT temperature response was antagonized by the 5-HT_1 receptor antagonists quipazine (2 mg kg^{-1}, i.p.), (±)-propranolol (10 mg kg^{-1}, i.p.). (±)-pindolol (5 mg kg^{-1}, i.p.), spiroxatrine (0.5 mg kg^{-1}, i.p.) and metitepine (0.05 mg kg^{-1}, i.p.), but not by 5-HT_2 (ketanserin) or 5-HT_3 (MDL 72222, GR 38032F) receptor antagonists.

4 The response was also antagonized by the dopamine D_2 receptor antagonists, haloperidol and BRL 34778. No other catecholamine or muscarinic receptors were involved in mediating the response.

5 Destruction of 5-hydroxytryptamine (5-HT)-containing neurones with the neurotoxin, 5,7-dihydroxytryptamine (75 μg, i.c.v.), abolished the response to 8-OH-DPAT indicating that the 5-HT_{1A} receptors involved were located on 5-HT neurones.

6 Chronic antidepressant treatment down-regulated this 8-OH-DPAT response. In addition, chronic administration of anxiolytics and neuroleptics was also effective in this respect. Down-regulation was also observed following repeated administration of 8-OH-DPAT (0.5 mg kg^{-1}, s.c.), (±)-pindolol (10 mg kg^{-1}, i.p.) and ketanserin (0.5 mg kg^{-1}, i.p.).

7 In conclusion, these data confirm that 8-OH-DPAT-induced hypothermia is mediated by 5-HT_{1A} autoreceptors. They also indicate that the response involves D_2 receptors. The present study also shows that a wide range of antidepressant drugs down-regulate this response although this property is not restricted to antidepressant treatments. Therefore, care should be exercised when interpreting data from this paradigm.

Keywords: 8-Hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT); hypothermia; mice; antidepressants; 5-HT_{1A} receptors

Introduction

In 1983, Middlemiss & Fozard showed that 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) was a selective agonist at 5-HT_{1A} receptors. Subsequently, it was found that peripheral administration of 8-OH-DPAT induced hypothermia in mice and rats (Hjorth, 1985; Goodwin & Green, 1985; Goodwin *et al.*, 1985a; Gudelsky *et al.*, 1986; Higgins *et al.*, 1988; Wozniack *et al.*, 1988). The studies in mice have shown that this response is dose-dependent and sigmoidal (e.g. Goodwin *et al.*, 1985a). Pharmacological evaluation of the hypothermic response to 8-OH-DPAT led these authors to suggest that it was specifically mediated by 5-HT_{1A} receptors located on 5-hydroxytryptaminergic neurones. Goodwin and his coworkers based this hypothesis on inhibition of the response by ipsapirone (Goodwin *et al.*, 1986), a partial agonist at 5-HT_{1A} receptors (Martin & Mason, 1986), low doses of quipazine (Goodwin *et al.*, 1985a) and abolition of the response by destruction of 5-HT neurones (Goodwin *et al.*, 1985a). However, these authors were unable to prevent the effects of 8-OH-DPAT with a range of other 5-HT_1-like receptor antagonists. Furthermore, haloperidol, a potent D_2 dopamine receptor antagonist, was also found to inhibit this response (Goodwin *et al.*, 1985a). The data suggest, therefore, that the pharmacology of 8-OH-DPAT-induced hypothermia in mice may not be as straight-forward as first thought.

Goodwin *et al.* (1985b) have reported that 8-OH-DPAT-induced hypothermia in mice was selectively attenuated after repeated administration of antidepressant drugs and electroconvulsive shock (ECS). However, as the types of antidepressant tested were limited and the authors failed to investigate the effects of other psychotropic agents, their conclusion that this adaptation was specific to antidepressants required further confirmation.

The aims of the work described here were two fold: first, to extend the pharmacological characterization of the 8-OH-DPAT-induced hypothermia in mice and second, to determine whether the adaptive down-regulation of this response was specific to antidepressant treatment.

Methods

Animals

Adult male C57/Bl/6Ola mice (Olac, Bicester) weighing 25–30 g were used throughout this study. They were housed in groups of 10 on a 12:12 h light:dark cycle (lights on 07 h 00 min) and had free access to food and water. The ambient temperature was maintained at 20 ± 1°C and humidity was approximately 50%.

Agonist studies

Core temperature was measured by inserting the probe of a digital thermometer approximately 2.5 cm into the rectum

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while lightly restraining the animal 10 min prior to (t_{-10}), immediately before (t_0) and 20 min after (t_{20}) an s.c. injection of saline or 5-HT_{1A} receptor agonist and the difference in temperature between t_0 and t_{20} was calculated.

Antagonist studies

Animals received either saline (10 mg kg^{-1}, s.c.) or 8-OH-DPAT (0.5 mg kg^{-1}, s.c.) 30 min after an i.p. injection of saline or antagonist. Rectal temperature was measured immediately before the first injection (t_{-30}) and prior to the second injection of saline or 8-OH-DPAT (t_0). A further measurement was taken 20 min after the second injection (t_{20}). The difference in temperature between t_0 and t_{20} was calculated.

Effect of chonic drug treatments

Groups of mice received either 14 daily injections (i.p.) of drugs or saline. The temperature decrease following an injection of 8-OH-DPAT (0.5 mg kg^{-1}, s.c.) was determined 24 h after the first and last treatments.

5-Hydroxytryptamine-containing neurone lesioning studies

The 5-HT neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT; 75 μg) or saline-ascorbate (4 μl) was injected i.c.v. into halothane anaesthetized mice, pretreated with desipramine (5 mg kg^{-1}, i.p.) to protect noradrenergic neurones, by the method of Heal (1984). The temperature response to 8-OH-DPAT (0.5 mg kg^{-1}, i.p.) was measured seven days later as described above.

Noradrenaline-containing neurone lesioning studies

Mice were given an i.p. injection of N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4, 100 mg kg^{-1}) 30 min after zimeldine (5 mg kg^{-1}, i.p.). Seven days later this procedure was repeated. The temperature response to 8-OH-DPAT (0.5 mg kg^{-1}, i.p.) was measured 12 days after the first DSP-4 injection as described above.

Statistics

Data were analysed by Student's unpaired t test and the null hypothesis was rejected when $P < 0.05$. However, when two treatment groups were compared with a single control group, the level of significance for rejection of the null hypothesis was set at $P < 0.01$.

Drugs

The following drugs were used (abbreviation if any and source shown in parentheses): N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4), 8-hydroxy-2(di-*n*-propylamino)tetralin HBr (8-OH-DPAT); ondansetron (GR 38032F), 1αH,3α,5αH-tropan-3-yl-3,5-dichlorobenzoate (MDL 72222), (+)-7-chloro-8-hydroxy-3-methyl-l-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine maleate (SCH 23390), spiroxatrine (Research Biochemicals Inc.), dothiepin HCl_2, gepirone $(HCl)_2$, ipsapirone $(HCl)_2$, sibutramine HCl (Boots Pharmaceuticals), zimeldine $(HCl)_2$ (Astra), *exo-N*-(9-[(4-fluorophenyl)methyl)-9-azabicyclo [3.3.1]non-3-yl)-4-amino-5-chloro-2-methoxybenzamide (BRL 34778) (Beecham), maprotiline (Ciba-Geigy), metitepine, metergoline (Farmitalia), *erythro*-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol (ICI 118,551) (I.C.I.) ketanserin tartrate (Janssen Pharmaceutica), citalopram (Lundbeck), quipazine (Miles Labs.), mianserin HCl (Organon), prazosin (Pfizer), idazoxan (Reckitt & Colman), chlordiazepoxide, diazepam (Roche), haloperidol (Searle), amitriptyline HCl, buspirone HCl, metoprolol, desipramine HCl, (±)-pindolol, (±)-propranolol (Sigma), tranylcypromine sulphate (Smith Kline & French).

All drugs were dissolved in 0.9% saline and injected intraperitoneally (i.p.) with the exception of 8-OH-DPAT, buspirone, gepirone and ipsapirone which were given subcutaneously (s.c.).

Results

Effects of 5-HT_{1A} receptor agonists on rectal temperature

When mice were injected with increasing doses of 8-OH-DPAT (0.05 to 5.0 mg kg^{-1}, s.c.), this 5-HT_{1A} receptor agonist produced a dose-dependent decrease in temperature (Figure 1). Time-course studies showed that the maximum hypothermic effect was observed 20 min after 8-OH-DPAT (data not shown). Similarly, the 5-HT_{1A} receptor partial agonists, buspirone, gepirone and ipsapirone, also dose-dependently induced hypothermia in mice (Figure 1).

Effects of 5-HT receptor antagonists

The putative 5-HT_1/5-HT_{1A} receptor antagonists quipazine (2 mg kg^{-1}), (±)-propranolol (10 and 20 mg kg^{-1}), (±)-pindolol (5 and 10 mg kg^{-1}), spiroxatrine (0.5 and 5 mg kg^{-1}) and metitepine (0.05 mg kg^{-1}) significantly attenuated the hypothermic response to 8-OH-DPAT (Table 1). Only quipazine had any significant effect (a small increase cf. control) when given alone (Table 1). Metitepine antagonized the response at a dose of 0.05 mg kg^{-1}, which itself had no effect on core temperature. However at a dose of 0.5 mg kg^{-1}, metitepine caused a marked reduction in temperature (3.85 ± 0.79°C; $P < 0.001$). There was no further fall in temperature induced by 8-OH-DPAT. The non-selective 5-HT receptor antagonist, metergoline had similar effects (Table 1).

Ketanserin (0.2 mg kg^{-1}), a 5-HT_2/5-HT_{1C} receptor antagonist, did not significantly alter body temperature itself, nor did it affect the hypothermia induced by 8-OH-DPAT (Table 1).

At moderate doses, the 5-HT_3 receptor antagonists, MDL 72222 and GR 38032F, did not alter the temperature response to 8-OH-DPAT (Table 1).

Effects of catecholamine and muscarinic receptor antagonists

The α_1-adrenoceptor antagonist, prazosin (0.1 mg kg^{-1}) did not attenuate the response to 8-OH-DPAT (0.5 mg kg^{-1}) (Table 2). However, at high dose (1 mg kg^{-1}), it induced a marked fall in core temperature itself; this was not altered by 8-OH-DPAT (Table 2). The α_2-adrenoceptor antagonist,

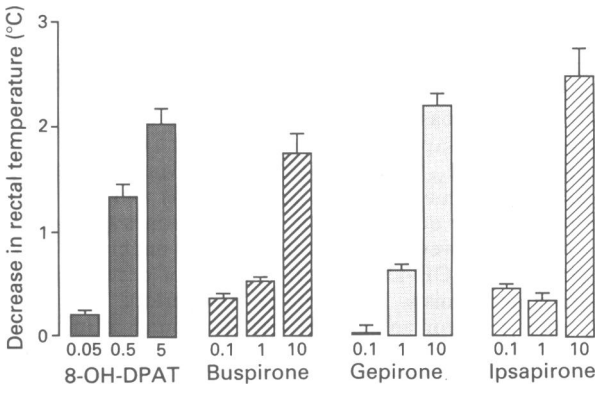

Figure 1 The effect of 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT), buspirone, gepirone and ipsapirone on the rectal temperature of C57/Bl/6O1a mice. Measurements were made immediately before and 20 min after s.c. drug injection. The doses used, in mg kg^{-1}, are shown under each column. Columns represent mean temperature decrease with s.e.mean shown by vertical bars for $n = 10$ in each group.

Table 1 The effects of 5-hydroxytryptamine (5-HT) receptor antagonists on the hypothermic response to 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, 0.5 mg kg^{-1}, s.c.)

Antagonist (mg kg^{-1}, i.p.)	*Change in temperature 20 min after* *Saline*	*8-OH-DPAT*
Saline	− 0.37 ± 0.01 (78)	− 1.63 ± 0.02 (78)
Quipazine (2)	+ 0.07 ± 0.11 (6)*	+ 0.53 ± 0.25 (6)***
(±)-Propranolol (10)	− 0.38 ± 0.08 (6)	− 1.16 ± 0.07 (5)*
(±)-Propranolol (20)	− 0.42 ± 0.07 (6)	− 0.28 ± 0.29 (6)**
(±)-Pindolol (5)	− 0.27 ± 0.04 (6)	− 1.07 ± 0.24 (6)**
(±)-Pindolol (10)	− 0.65 ± 0.18 (6)	− 0.78 ± 0.14 (6)**
Spiroxatrine (0.5)	− 0.5 ± 0.11 (6)	− 0.35 ± 0.21 (6)**
Spiroxatrine (5)	− 0.12 ± 0.09 (5)*	+ 0.66 ± 0.10 (5)**
Metitepine (0.05)	− 0.38 ± 0.13 (6)	− 0.62 ± 0.21 (6)**
Metitepine (0.5)	− 3.85 ± 0.79 (6)***	− 3.8 ± 1.01 (6)*
Metergoline (0.5)	− 1.33 ± 0.20 (6)**	− 2.73 ± 0.44 (6)*
Ketanserin (0.2)	− 0.43 ± 0.07 (6)	− 2.15 ± 0.41 (6)
MDL 72222 (1)	− 0.66 ± 0.13 (5)	− 1.98 ± 0.13 (5)
GR 38032F (0.1)	− 0.48 ± 0.07 (6)	− 2.08 ± 0.17 (6)

Mice received i.p. injections of the antagonists and were injected 30 min later with 8-OH-DPAT (0.5 mg kg^{-1}, s.c.) or saline (10 ml kg^{-1}, s.c.). The temperature change 20 min after the 8-OH-DPAT/saline injection was determined. Data are presented as mean ± s.e.mean (*n*). Each treated group was compared with its own control but these have been pooled for the purpose of clarity. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus saline pretreatment group (Student's *t* test).

Table 2 Effects of catecholamine and muscarinic receptor antagonists on the hypothermic response to 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, 0.5 mg kg^{-1}, s.c.)

Antagonist (mg kg^{-1}, i.p.)	*Change in temperature 20 min after* *Saline*	*8-OH-DPAT*
Saline	− 0.35 ± 0.02 (60)	− 1.60 ± 0.03 (60)
Prazosin (0.1)	− 0.46 ± 0.08 (5)	− 2.90 ± 0.29 (5)**
Prazosin (1)	− 2.35 ± 0.14 (6)***	− 2.57 ± 0.28 (6)**
Idazoxan (0.1)	− 0.62 ± 0.03 (6)*	− 2.18 ± 0.36 (6)
Idazoxan (1)	− 0.58 ± 0.07 (6)	− 2.22 ± 0.20 (6)
(±)-Propranolol (2)	− 0.16 ± 0.03 (5)	− 1.48 ± 0.09 (5)
ICI 118,551 (3)	− 0.26 ± 0.10 (5)	− 1.53 ± 0.09 (5)
Metoprolol (3)	− 0.22 ± 0.11 (5)	− 1.43 ± 0.06 (5)
ICI 118,551 (3) + Metoprolol (3)	− 0.25 ± 0.14 (5)	− 1.63 ± 0.06 (5)
Haloperidol (0.1)	+ 0.22 ± 0.17 (6)**	+ 0.08 ± 0.22 (5)***
Haloperidol (1)	− 0.65 ± 0.14 (6)	− 0.72 ± 0.12 (6)**
BRL 34778 (0.05)	− 0.67 ± 0.11 (6)*	− 0.84 ± 0.10 (5)*
SCH 23390 (0.1)	− 0.68 ± 0.12 (6)	− 1.55 ± 0.13 (6)
Atropine (0.1)	− 0.24 ± 0.06 (5)	− 2.26 ± 0.10 (5)
Atropine (1)	− 0.28 ± 0.02 (5)	− 2.38 ± 0.03 (5)

Mice received i.p. injections of the antagonists and were injected 30 min later with 8-OH-DPAT (0.5 mg kg^{-1}, s.c.) or saline (10 ml kg^{-1}, s.c.). The temperature change 20 min after the 8-OH-DPAT/saline injection was determined. Data are presented as mean ± s.e.mean (*n*). Each treated group was compared with its own control but these have been pooled for the purpose of clarity. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus saline pretreatment group (Student's *t* test).

idazoxan (0.1 and 1 mg kg^{-1}) had no effect on basal temperature and did not alter the hypothermia induced by 8-OH-DPAT (Table 2). When (±)-propranolol was given at the low dose of 2 mg kg^{-1}, the response to 8-OH-DPAT was not altered (Table 2). In addition, the selective β_1- and β_2-adrenoceptor antagonists, ICI 118,551 (3 mg kg^{-1}) and metoprolol (3 mg kg^{-1}), did not modify the response to 8-OH-DPAT (Table 2). These two β-adrenoceptor sub-type antagonists were also without effect on the response to 8-OH-DPAT when used in combination (Table 2). Haloperidol (0.1 and 1 mg kg^{-1}) and BRL 34778 (0.05 mg kg^{-1}), both potent D_2-receptor antagonists, significantly inhibited the effects of 8-OH-DPAT (Table 2). By contrast, SCH 23390 (0.1 mg kg^{-1}) was without effect (Table 2).

Atropine (1 mg kg^{-1}), a muscarinic receptor antagonist, did not alter the effects of 8-OH-DPAT on body temperature (Table 2).

Effects of 5,7-DHT and DSP-4 lesioning

The temperature response to 8-OH-DPAT (0.5 mg kg^{-1}, i.p.) was markedly attenuated following an i.c.v. injection of 5,7-DHT (Figure 2). Furthermore, basal temperatures were not significantly altered (Figure 2). Table 3 shows that the depletions of whole brain 5-HT, noradrenaline and dopamine were respectively 71% ($P < 0.001$), 37% ($P < 0.001$) and + 1% (not significant).

Lesioning of the noradrenergic system with DSP-4 had no effect on the temperature response to 8-OH-DPAT (0.5 mg kg^{-1}, i.p., Figure 2) although basal temperatures were significantly lower in the lesioned animals (Figure 2). DSP-4 lesioning decreased whole brain noradrenaline concentrations by 75% ($P < 0.001$, Table 3). This treatment also resulted in a minor reduction in dopamine (16%, $P < 0.001$) content but had no effect on 5-HT content.

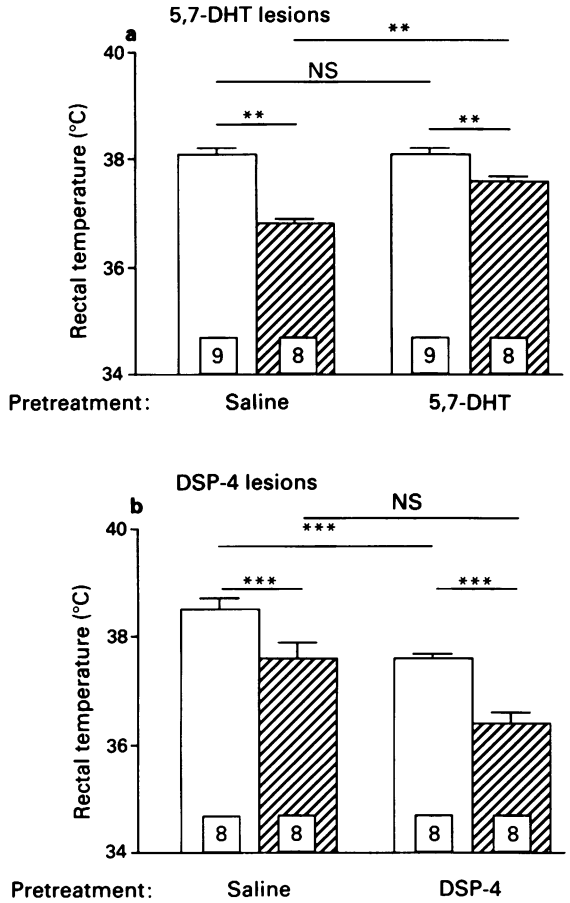

Figure 2 The effects of 5,7-dihydroxytryptamine (5,7-DHT, a) and N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4, b) treatment on the hypothermic response to 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, 0.5 mg kg^{-1}, i.p.). (a) Mice were given desipramine (5 mg kg^{-1}, i.p.) 30 min before 5,7-DHT (75 μg, i.c.v.) or saline-ascorbate (4 μl, i.c.v.) under halothane anaesthesia. Seven days later the temperature change in the 20 min following 8-OH-DPAT (0.5 mg kg^{-1}, i.p.) was measured. (b) Mice were given zimeldine (5 mg kg^{-1}, i.p.) 30 min before DSP-4 (100 mg kg^{-1}, i.p.) or saline (0.25 ml, i.p.). This was repeated seven days later and 12 days after the first dose of DSP-4 the temperature response to 8-OH-DPAT (0.5 mg kg^{-1}, i.p.) was measured. Each column represents the mean rectal temperature with s.e.mean shown by vertical bars. The number of animals is shown at the base of each column. Data were analysed by Student's paired (within subjects) or unpaired (between subjects) *t* test.
NS = not significant; $^{**}P < 0.01$; $^{***}P < 0.001$.

Effects of acute and chronic treatments

Antidepressants A single dose of maprotiline (20 mg kg^{-1}) or citalopram (20 mg kg^{-1}) attenuated the response to 8-OH-DPAT given 24 h later (temperature decrease: -1.88 ± 0.08°C, $n = 10$, saline; -0.92 ± 0.04°C, $n = 10$, maprotiline; -0.69 ± 0.03°C, $n = 10$, citalopram). None of the other drugs tested had any effect after a single dose (data not shown). By contrast, after 14 days of treatment, all of the drugs reduced the magnitude of the hypothermia induced by 8-OH-DPAT (0.5 mg kg^{-1}) (Table 4). In some cases (sibutramine HCl, desipramine, maprotiline, citalopram and zimeldine) this effect was only apparent with relatively large doses.

In common with the established antidepressant drugs, those with more novel mechanisms of action, such as idazoxan, buspirone and gepirone, were also effective in this paradigm (Table 4).

Anxiolytics Repeated administration of diazepam (1 mg kg^{-1}) and chlordiazepoxide (3 mg kg^{-1}) reduced the size of the response to 8-OH-DPAT (0.5 mg kg^{-1}) (Table 5). By contrast, a single dose of these drugs was without effect (data not shown).

Neuroleptic and 5-HT receptor antagonists

Four other psychotropic agents were tested: haloperidol (1 mg kg^{-1}), 8-OH-DPAT (0.5 mg kg^{-1}), (±)-pindolol (10 mg kg^{-1}) and ketanserin (0.5 mg kg^{-1}). Repeated haloperidol and ketanserin weakly attenuated the response, whereas 8-OH-DPAT and (±)-pindolol markedly reduced it (Table 5). None of the drugs had any effect after a single dose (data not shown).

Discussion

In this study, 8-OH-DPAT produced dose-dependent hypothermia in mice in agreement with previously reported findings with this species (Goodwin *et al.*, 1985a) or rats (Goodwin *et al.*, 1986; Hutson *et al.*, 1987). Similarly, the ED_{50} value of approximately 0.5 mg kg^{-1} agrees well with that found by Goodwin *et al.* (1985a). We have also extended these findings by showing that the 5-HT_{1A} receptor partial agonists, buspirone, gepirone and ipsapirone, also dose-dependently induce hypothermia.

Mediation of the 8-OH-DPAT-induced hypothermia by 5-HT_{1A} receptors was confirmed with various 5-hydroxytryptamine and other neurotransmitter receptor antagonists. The 5-HT_1 receptor antagonists, (±)-propranolol (Middle-

Table 3 The effects of 5,7-dihydroxytryptamine (5,7-DHT) and N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) treatment on whole mouse brain noradrenaline (NA), dopamine (DA) and 5-hydroxytryptamine (5-HT) concentrations

Treatment	*Brain monoamine concentration* (ng g^{-1} tissue wet wt.)			
	n	*NA*	*DA*	*5-HT*
5,7-DHT				
Control	9	506 ± 7	1014 ± 14	677 ± 17
5,7-DHT	8	316 ± 20***	1020 ± 18	196 ± 39***
% change		− 37	+ 1	− 71
DSP-4				
Control	8	463 ± 10	1146 ± 14	882 ± 13
DSP-4	7	117 ± 6***	969 ± 13***	859 ± 18
% change		− 75	− 16	− 3

5,7-DHT lesioning Mice were given an i.p. injection of desipramine (5 mg kg^{-1}) 30 min before an i.c.v. injection of 5,7-DHT (75 μg) or saline-ascorbate (4 μl) under halothane anaesthesia. Seven days later, the mice were given a s.c. injection of 8-OH-DPAT (0.5 mg kg^{-1}) and the temperature change in the following 20 min determined.
DSP-4 lesioning Mice were given an i.p. injection of zimeldine (5 mg kg^{-1}) 30 min before DSP-4 (100 mg kg^{-1}, i.p.) or saline (0.25 ml). This procedure was repeated seven days later. Twelve days after the first dose of DSP-4 the temperature changes of the mice in the 20 min following 8-OH-DPAT (0.5 mg kg^{-1}, s.c.) were determined. Twenty-four hours later, the mice were killed and brain NA, DA and 5-HT concentrations (ng g^{-1} tissue wet wt.) were determined by h.p.l.c.-e.c.d. Results are expressed as mean ± s.e.mean and were analysed by Student's unpaired *t* test.
$^{***}P < 0.001$ compared with control.

Table 4 The effects of 14 day treatment with antidepressant drugs on the hypothermic response to 8-hydroxy-2 (di-n-propylamino)tetralin (8-OH-DPAT, 0.5 mg kg^{-1}, s.c.)

Treatment (mg kg^{-1}, i.p.)	*Principle pharmacological action*	*Decrease in temp. 20 min after 8-OH-DPAT*
Control (0.25 ml, i.p.)	–	1.23 ± 0.02 (127)
Amitriptyline (10)	NA + 5-HT uptake	0.87 ± 0.07 (10)***
Sibutramine HCl (10)	NA + 5-HT uptake	1.13 ± 0.07 (10)*
Dothiepin (30)	NA + 5-HT uptake	0.77 ± 0.09 (10)***
Desipramine (10)	NA uptake	1.24 ± 0.09 (10)
Desipramine (20)	NA uptake	0.86 ± 0.10 (7)***
Maprotiline (10)	NA uptake	1.26 ± 0.11 (8)
Maprotiline (20)	NA uptake	0.50 ± 0.05 (10)***
Citalopram (10)	5-HT uptake	1.33 ± 0.10 (9)
Citalopram (20)	5-HT uptake	0.70 ± 0.10 (10)***
Zimeldine (10)	5-HT uptake	1.26 ± 0.10 (9)
Zimeldine (20)	5-HT uptake	0.82 ± 0.06 (9)***
Tranylcypromine (5)	MAO inhibition	0.64 ± 0.04 (10)***
Mianserin (5)	α_2/5-HT$_2$ antagonist	0.83 ± 0.10 (10)***
Idazoxan (0.1)	α_2-antagonist	1.01 ± 0.04 (10)*
Idazoxan (1)	α_2-antagonist	0.99 ± 0.04 (10)***
Buspirone (10)	5-HT$_{1A}$ agonist	1.0 ± 0.05 (10)**
Buspirone (30)	5-HT$_{1A}$ agonist	0.82 ± 0.07 (10)***
Gepirone (10)	5-HT$_{1A}$ agonist	0.69 ± 0.06 (8)***
Gepirone (30)	5-HT$_{1A}$ agonist	0.63 ± 0.07 (6)**

Mice received i.p. injections of the drugs indicated above. Twenty-four hours after the final dose, 8-OH-DPAT (0.5 mg kg^{-1}, s.c.) was administered and the temperature change in the following 20 min determined. Data are mean ± s.e.mean (*n*). Each treated group was compared with its own control but these have been pooled for the purposes of clarity.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus saline pretreatment group (Student's *t* test).

Table 5 The effects of 14 days treatment with psychotropic drugs on the hypothermia induced by 8-hydroxy-2(di-n-propylamino)tetralin (8-OH-DPAT, 0.5 mg kg^{-1}, s.c.)

Treatment (mg kg^{-1}, i.p.)	*Principle pharmacological action*	*Decrease in temp. 20 min after 8-OH-DPAT*
Control	–	1.36 ± 0.03 (54)
Diazepam (1)	BDZ agonist	0.84 ± 0.04 (9)**
Diazepam (2.5)	BDZ agonist	0.83 ± 0.07 (9)**
Chlordiazepoxide (1)	BDZ agonist	1.15 ± 0.08 (10)
Chlordiazepoxide (3)	BDZ agonist	0.97 ± 0.05 (10)**
Haloperidol (1)	D_2/α_1 antagonist	1.14 ± 0.07 (10)*
8-OH-DPAT (0.5 s.c.)	5-HT$_{1A}$ agonist	0.74 ± 0.06 (8)**
(±)-Pindolol (10)	β/5-HT$_1$ antagonist	0.78 ± 0.06 (8)**
Ketanserin (0.5)	5-HT$_2$/5-HT$_{1C}$ antagonist	1.06 ± 0.09 (8)*

Mice received i.p. injections of the drugs indicated above. Twenty-four hours after the final dose, 8-OH-DPAT (0.5 mg kg^{-1}, s.c.) was administered and the temperature change in the following 20 min determined. Data are mean ± s.e.mean (*n*). Each treated group was compared with its own control but these have been pooled for the purposes of clarity. BDZ = benzodiazepine receptor.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus saline-treatment group (Student's *t* test).

miss, 1984), (±)-pindolol (Hamon *et al.*, 1986), spiroxatrine (Nelson & Taylor, 1986) and quipazine (Moret, 1985), inhibited 8-OH-DPAT-induced hypothermia. In addition, metitepine, which is reported to show some 5-HT$_1$ selectivity (Hibert & Middlemiss, 1986), also inhibited the response at low doses. Goodwin *et al.* (1985a) previously reported that 8-OH-DPAT hypothermia was antagonized only by quipazine and that propranolol, pindolol and metitepine were without effect. Our data confirm that quipazine is an antagonist, but disagree with respect to the latter antagonists. In the case of metitepine, the failure to observe inhibition of 8-OH-DPAT hypothermia was probably because at the dose used by Goodwin *et al.* (1985a), metitepine itself produced a marked reduction in core temperature; a finding confirmed by the present study. However, this argument does not explain the differences in respect of propranolol and pindolol and methodological variations are unlikely to be responsible because the identical strain and supplier of mice were used and a similar experimental protocol was employed. Further evidence for the 5-HT$_{1A}$ receptor-mediation of 8-OH-DPAT hypothermia is provided by the observations that ketanserin (5-HT$_2$/5-HT$_{1C}$ antagonist) and MDL 72222 and GR 38032F (5-HT$_3$ receptor antagonists) had no effect.

In addition to demonstrating that 8-OH-DPAT hypothermia was a 5-HT$_{1A}$ receptor-mediated response, the involvement of other neurotransmitters in its expression was also determined. Our findings clearly implicate dopamine receptors because the hypothermia was inhibited by the D_2 receptor antagonists, haloperidol and BRL 34778. However, D_1 receptor involvement can be discounted because the highly selective antagonist, SCH 23390, had no effect on this response.

The hypothermia induced by 8-OH-DPAT was not modified by administration of α_1-, α_2- or selective β-adrenoceptor antagonists and these data argue that the response is unlikely to be expressed via any of the above receptor subtypes. These findings are in agreement with those of Goodwin *et al.* (1985a), but are at variance with the reported potentiation of 8-OH-DPAT hypothermia by the β-adrenoceptor agonist, clenbuterol (Green *et al.*, 1986). Since it has been suggested that 8-OH-DPAT produces hypothermia by activating somatodendritic autoreceptors in the median raphe (Higgins *et al.*, 1988), these observations are also incompatible with the involvement of α_2-adrenoceptors in the 8-OH-DPAT-induced inhibition of 5-HT release (Marsden & Martin, 1986).

Destruction of 5-HT neurones with the neurotoxin, 5,7-DHT, abolished the hypothermia induced by 8-OH-DPAT. This finding confirms the observations of Goodwin *et al.* (1985a), who reported that inhibition of 5-HT synthesis by chronic administration of *p*-chlorophenylalanine also prevented the decrease in temperature induced by 8-OH-DPAT. By contrast, lesioning of the noradrenergic system with DSP-4 had no effect on the temperature response to 8-OH-DPAT. This finding confirms that noradrenaline is not involved in the mediation of this response.

Viewed overall, therefore, the data indicate that 8-OH-DPAT induces hypothermia via an action on 5-HT_{1A} receptors located on 5-HT neurones. These receptors are probably located on 5-HT cell bodies in the midbrain raphe complex (Verge *et al.*, 1985; Higgins *et al.*, 1988).

In the second part of this study, we have re-evaluated the hypothesis put forward by Goodwin *et al.* (1985b) that down-regulation of 5-HT_{1A} receptors initiating 8-OH-DPAT hypothermia is an important adaptive response to antidepressant treatment.

We have now confirmed that repeated administration of desipramine, zimeldine, tranylcypromine and mianserin down-regulated this response. In addition, we have extended the findings by demonstrating identical effects with maprotiline, citalopram, amitriptyline, dothiepin, sibutramine, idazoxan, buspirone and gepirone. Overall, therefore, it is clear that antidepressant treatments with diverse modes of pharmacological effect all attenuate this 5-HT_{1A} receptor-mediated response. However, the present findings also demonstrate that this adaptation is produced by repeated treatment with psychotropic drugs which are not antidepressants. Thus the anxiolytic agents diazepam and chlordiazepoxide, attenuated 8-OH-DPAT hypothermia. While they are not claimed to be antidepressants, they have been shown to reduce significantly HAM-D scores in some clinical trials (Goldberg & Finnerty, 1979). This does not argue that these compounds are antidepressants, rather it indicates that drugs which are used to treat disorders with an affective component may be efficacious in this paradigm. In addition to the anxiolytics, haloperidol, an antipsychotic drug, significantly attenuated the response to 8-OH-DPAT, although this effect was weak in comparison to that observed after antidepressant treatment. We have clearly shown that D_2-receptors are involved in the expression of this hypothermic response and therefore, the apparent attenuation by halperidol may be due to antagonism of D_2-receptors by the haloperidol still present 24 h after the final dose. Alternatively, a direct effect of haloperidol on 5-HT_{1A} receptors cannot be ruled out, because this drug and other butyrophenones have weak affinity for 5-HT_{1A} sites (Pedigo *et al.*, 1981; Martin, unpublished data).

The temperature response to 8-OH-DPAT was markedly attenuated following its repeated administration. This observation indicates that chronic stimulation of the 5-HT_{1A} receptors involved in the response results in tachyphylaxis and is entirely in keeping with pharmacological principles. However, the effects of (±)-pindolol were surprising as this drug is generally considered to be an antagonist at 5-HT_{1A} receptors and chronic administration would be expected to produce up-regulation. Similar effects of ritanserin on 5-HT_2 receptor binding have been reported (Leysen *et al.*, 1986) and this phenomenon may represent an idiosyncracy of 5-HT receptor regulation. Alternatively, Hjorth & Carlsson (1986) have reported that pindolol is a partial agonist at 5-HT_{1A} receptors and if this is correct, it would satisfactorily explain the down-regulation of 5-HT_{1A} receptors following repeated administration of pindolol.

The findings of the second part of the present study are consistent with previous studies using this mouse model (Goodwin *et al.*, 1985b). Furthermore, they are also in agreement with the results of electrophysiological studies in rats. Thus, Blier and co-workers (1984, 1987) have consistently shown that antidepressant treatments attenuate the responses of 5-HT neurones to stimulation of somatodendritic 5-HT_{1A} receptors in the midbrain raphe complex. This effect has been shown to have the overall effect of increasing 5-hydroxytryptaminergic neurotransmission (Blier & de Montigny, 1987).

In conclusion, we have confirmed that the hypothermic response of mice to 8-OH-DPAT is mediated by 5-HT_{1A} receptors, the anatomical location of these sites being on the 5-HT-containing neurones. In addition, we have shown that a wide range of antidepressants attenuate this response. However, the apparent down-regulation of these receptors by other classes of drugs indicates that care should be exercised when interpreting data from this model.

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Different patterns of protein kinase C redistribution mediated by α_1-adrenoceptor stimulation and phorbol ester in rat isolated left ventricular papillary muscle

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1 In rat left ventricular papillary muscle, phenylephrine, an α_1-adrenoceptor agonist, had a staurosporine-sensitive positive inotropic effect and increased the particulate-associated protein kinase C (PKC) activity without significant changes in total PKC activity or in cytosolic Ca^{2+}/phospholipid-independent kinase (PKI) activity.

2 A PKC stimulant, phorbol 12,13-dibutyrate (PDBu), decreased contractility and slightly increased PKC activity in the particulate fractions, with a marked decrease and increase in total PKC and PKI activities, respectively.

3 The PDBu-induced negative inotropic response was attenuated by two protease inhibitors, leupeptine and a microbial peptide isolated from *Aspergillus japonicus* (E-64), which are known to inhibit the conversion of particulate-associated PKC to PKI.

4 Such differences in the patterns of PKC redistribution, i.e. marked increases in particulate PKC and cytosolic PKI activities caused by phenylephrine and PDBu, respectively, may account for the opposite inotropic effects of PKC stimulation by an α_1-agonist and a phorbol ester.

Keywords: Protein kinase C (PKC) redistribution; inotropic effect; α_1-adrenoceptor stimulation; phorbol ester; rat papillary muscle

Introduction

The breakdown of phosphatidylinositol 4,5-bisphosphate (PI-4, 5-P_2), and the resulting generation of inositol trisphosphate (IP_3) and diacylglycerol (DAG) occur during signal transduction induced by a number of neuronal and humoral stimuli (Downes & Michell, 1982; Berridge, 1984). A growing body of evidence indicates that IP_3 mobilizes Ca^{2+} from intracellular Ca^{2+} stores, whereas DAG activates Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C; PKC) (Kikkawa *et al.*, 1983; Somlyo *et al.*, 1985; Nosek *et al.*, 1986). In cardiac muscle, an α_1-adrenoceptor-mediated positive inotropic effect is associated with the receptor-linked degradation of PI-4, 5-P_2 (Otani *et al.*, 1988; Scholz *et al.*, 1988). For example, α_1-adrenoceptor stimulation with phenylephrine in the rat isolated left ventricular papillary muscle preparation results in a triphasic inotropic response: a transient positive inotropic effect followed by a transient negative inotropic effect and then a sustained positive inotropic effect (Otani *et al.*, 1988). Our recent studies suggested that the transient response are mediated by IP_3-induced Ca^{2+} mobilization and that the sustained positive inotropic effect is produced via PKC activation (Otani *et al.*, 1988; 1990).

In contrast, a tumour-promoting phorbol ester, which also activates PKC by substituting for DAG, was shown to decrease the contractility of cardiac muscles (Teutsch *et al.*, 1987; Gwathmey & Hajjar, 1990). Thus, the role of PKC in cardiac contractility is still unclear. Recently, proteolytic degradation of PKC has been proposed as a possible mechanism for the phorbol ester-induced cellular responses observed in a variety of cell types (Tapley & Murray, 1985; Pontremoli *et al.*, 1986; Chida *et al.*, 1986). The cleavage of the membrane-bound PKC by a Ca^{2+}-requiring proteinase such as calpain produces a catalytically active fragment which is then released into the cytosol fraction as a Ca^{2+}/phospholipid-independent kinase (Kishimoto *et al.*, 1983; Melloni *et al.*, 1986; Kishimoto, 1990). Based on these findings, we compared the mode of activation of PKC and the contractility induced by an α_1-adrenoceptor agonist, phenylephrine, with those induced by a phorbol 12,13-dibutyrate (PDBu) in rat isolated left ventricular papillary muscles. Furthermore, the possibility that proteolytic degradation of PKC has a role in the PDBu-induced negative inotropic effect was examined by use of two calpain inhibitors, leupeptin and a microbial peptide isolated from *Aspergillus japonicus* (E-64).

Methods

Preparation and stimulation of rat papillary muscles

Male Sprague Dawley rats weighing 250–300 g were anaesthetized with an intraperitoneal injection of sodium pentobarbitone, and the hearts were quickly removed. Left ventricular papillary muscles, 0.8–1.0 mm in diameter, were suspended in an organ bath containing 20 ml Tyrode solution of the following composition (mM): NaCl 122.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.1, $NaHCO_3$ 24 and glucose 10 (pH 7.4), aerated with 95% O_2 and 5% CO_2 at 32°C. The muscle was initially loaded with 500 mg and driven electrically by rectangular pulses with a frequency of 1 Hz, a duration of 10 ms and a voltage ranging from 2–4 V which was twice threshold. The isometric tension was measured by a force displacement transducer (Shinko-Tsusin, Japan. UL-2) and recorded on a pen-writing recorder (Seconic, Japan. SS-250F) through an amplifier (Shinko-Tsusin, DS-601B). The preparation was allowed to equilibrate for 50 min and was then treated with 10 μM phenylephrine or 0.1 μM phorbol 12,13-dibutyrate (PDBu) for the indicated periods. When phenylephrine was used, 0.3 μM propranolol was always present to inhibit β-adrenoceptor stimulation at this phenylephrine concentration. We used PDBu because of its lower hydrophobicity (compared to other potent phorbol esters) that could facilitate association of the phorbol ester with the inner leaflet of the sarcolemmal membrane of intact tissues (Kikkawa *et al.*, 1983). A calpain inhibitor (1 mM), either

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leupeptin or E-64 (microbial peptide isolated from *Aspergillus japonicus*), was added to the buffer 60 min before the addition of PDBu. Changes in the contractility after the administration of PDBu were measured at the indicated times and were expressed as a % of the contractility just before the addition of PDBu.

Determination of protein kinase C

At the end of each experiment, the single papillary muscle was quickly blotted and frozen in liquid nitrogen. The frozen muscles were weighed and homogenized in a glass homogenizer to which 1.0 ml of ice-cold buffer A (20 mM Tri/HCl, pH 7.4, 250 mM sucrose, 5 mM diNa-EDTA 1, 1 mM EGTA) containing 0.005% leupeptin, 100 units ml^{-1} aprotinin and 0.4 mM phenylmethylsulphonylfluoride (PMSF) was added. The homogenates were centrifuged at 3,000 *g* for 20 min. The supernatants were centrifuged again at 105,000 *g* for 90 min. The resulting supernatants were saved as cytosolic fractions. The pellets resulting from the 105,000 *g* centrifugation were suspended in 0.5 ml of buffer A containing 0.1% Triton X-100. After 15 min of incubation, this suspension was centrifuged again at 105,000 *g* for 60 min, and the resulting supernatant was saved as a solubilized particulate fraction. Both fractions were applied to a 0.2 ml column of DE-52 (Whatman Inc., NJ, U.S.A.) equilibrated with buffer B (buffer A minus sucrose). After being washed with 10 ml of buffer B, PKC was eluted by the addition of buffer B containing 0.1 M NaCl (PKC fraction). In the case of the cytosolic fraction, the column was further eluted with buffer B containing 0.4 M NaCl to determine Ca^{2+}/phospholipid-independent protein kinase activity (PKI fraction) (Chambers & Eilon, 1988).

PKC activity was determined by measuring the incorporation of ^{32}P from [γ-^{32}P]-ATP into histone H1 as described by Kishimoto *et al.* (1983), with a slight modification. The standard reaction mixture of 0.25 ml contained 20 mM Tris-HCl (pH 7.4), 10 mM $Mg(SO_4)_2$, 0.2 mg ml^{-1} histone H1, 50 μM [γ-^{32}P]-ATP (40–60 c.p.m. $pmol^{-1}$), 3 mM $CaCl_2$, 20 μg ml^{-1} phosphatidylserine (PS), 2 μg ml^{-1} diolein and 20–80 μl of PKC fraction. After 20 min of incubation at 30°C, the reaction was terminated by the addition of 3 ml of 25% trichloroacetic acid (TCA). Acid-precipitable materials were collected on a cellulose nitrate membrane filter with 0.45 μm pores (Toyo Roshi, Tokyo, Japan), washed with 25% TCA and transferred to a vial to count the radioactivity. PKC activity was defined as the difference between ^{32}P incorporation into histone in the presence and absence of calcium and phospholipid. Ca^{2+}/phospholipid-independent kinase activity was measured in the PKI fraction and was defined as the ^{32}P incorporation in the presence of EGTA instead of Ca^{2+} and phospholipid. Under these conditions, the ^{32}P incorporation was linear with time up to 30 min. Protein concentration was determined by Lowry's method (Lowry *et al.*, 1951), with bovine albumin used as standard.

Statistical analysis

The data are expressed as mean ± s.e.mean. Student's *t* test was used for statistical analysis. The differences between two groups with *P* values less than 0.05 were considered to be significant.

Results

In rat left ventricular papillary muscles treated with 0.3 μM propranolol, 10 μM phenylephrine produced a triphasic inotropic response: a transient positive inotropic effect followed by a transient negative inotropic effect and then a sustained positive inotropic effect (Figure 1a, left). Changes in the transient positive, the transient negative or the sustained positive inotropic phase following the administration of phenylephrine were 106 ± 2, 85 ± 2, or 151 ± 6% (mean ± s.e.mean; $n = 6$) of that observed before the treatment, respectively, as has been shown in a previous study (Otani *et al.*, 1990). The sustained positive inotropic effect was reduced by staurosporine, a PKC inhibitor, to 118 ± 7% ($n = 6$, $P < 0.01$ vs phenylephrine only) of the basal contractility, without changes in the transient positive and the transient negative inotropic effects (Figure 1a, right). In contrast, 0.1 μM PDBu gradually decreased the contractile force, reaching 43 ± 7% ($n = 6$) of that observed before the treatment with PDBu, as shown in Figure 1b, left. A biologically inactive phorbol ester, 4α-phorbol, had no effect at the same concentration (Figure 1b, right). Staurosporine had no effect

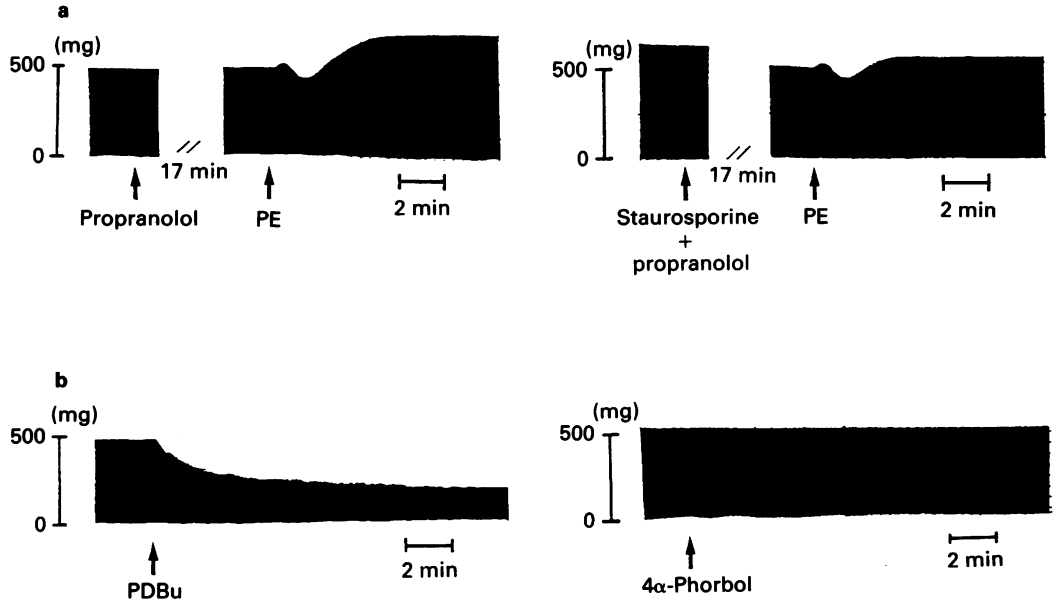

Figure 1 Representative traces of the effects of phenylephrine and phorbol esters on contractile force. Rat left ventricular papillary muscles pretreated with vehicle (0.01% dimethylsulphoxide, a, left) or 100 nM staurosporine (a, right) for 20 min were exposed to 10 μM phenylephrine (PE) in the presence of 0.03 μM propranolol. (b) The preparations were treated with 0.1 μM phorbol 12,13-dibutyrate PDBu (left), or 0.1 μM 4α-phorbol (right) for 10 min.

on this PDBu-induced negative inotropic effect (data not shown).

PKC activities in cytosolic and particulate fractions of rat isolated left ventricular papillary muscles were measured before and after the addition of phenylephrine or phorbol esters (Figure 2). Phenylephrine (10 μM) increased the PKC activity in the particulate fractions in a time-dependent manner, and the maximum level (14 ± 4 pmol min^{-1} mg^{-1} protein, $n = 6$) was reached after 5 min (Figure 2a). Thereafter, the maximum activity in the particulate fraction remained unchanged or declined only slightly. The change in PKC activity in the cytosol fraction showed an inverse relationship to that in the membrane fraction. The minimum activity in the cytosolic fraction was 23 ± 6 pmol min^{-1} mg^{-1} protein ($n = 6$) at 5 min and remained unchanged thereafter.

We next examined the effect of the phorbol ester on the distribution of PKC (Figure 2b). PDBu at 0.1 μM increased the PKC activity in the particulate fraction to 6.3 ± 0.2 pmol min^{-1} mg^{-1} protein ($n = 6$), which was almost half of the maximum activity induced by phenylephrine. This was achieved within 1 min and remained unchanged thereafter. In contrast with the action of phenylephrine, PKC activity in the cytosolic fraction continued to decrease significantly for 20 min. 4α-Phorbol (0.1 μM) had no effect on the PKC activities in either fraction (Figure 2b).

Next, we compared the effects of phenylephrine and PDBu on the total PKC activity (particulate + cytosol) and Ca^{2+}/phospholipid-independent kinase activities (PKI) in the cytosolic fraction. Phenylephrine did not significantly affect either of the kinase activities (Figure 3a), while PDBu decreased the total PKC activity and increased the PKI activity in the cytosolic fraction (Figure 3b). These findings suggest that a decrease in total PKC activity during PDBu treatment is partly due to conversion of PKC into a Ca^{2+}/phospholipid-independent form of the enzyme, probably by Ca^{2+}-dependent proteinase (calpain).

If the phorbol ester-induced negative inotropic effect is mediated by the loss of PKC and the formation of PKI, pretreatment with a calpain inhibitor could attenuate this negative inotropic effect by reducing the proteolytic degradation of PKC. The following study was undertaken to test this possibility. The papillary muscles were first treated with 1 mM of one of the following two proteinase inhibitors (Melloni *et al.*, 1986; Wang, 1990), leupeptin or E-64, for 60 min and exposed to 0.1 μM PDBu (Figure 4). These inhibitors attenuated the PDBu-induced negative inotropic effect, indicating that this negative inotropic effect occurred, at least in part, via stimulated formation of PKI.

Discussion

There is very little information regarding the role of PKC in the regulation of the cardiac contractility. α_1-Agonists and phorbol esters are known to have different PKC-mediated effects on cardiac contractility: the former have positive inotropic effects (Otani *et al.*, 1990; Scholz *et al.*, 1988), while the latter have negative inotropic effects (Teutsch *et al.*, 1987; Gwathmey & Hajjar, 1990). This may be due to a difference in the way that these agents stimulate PKC. Indeed, the

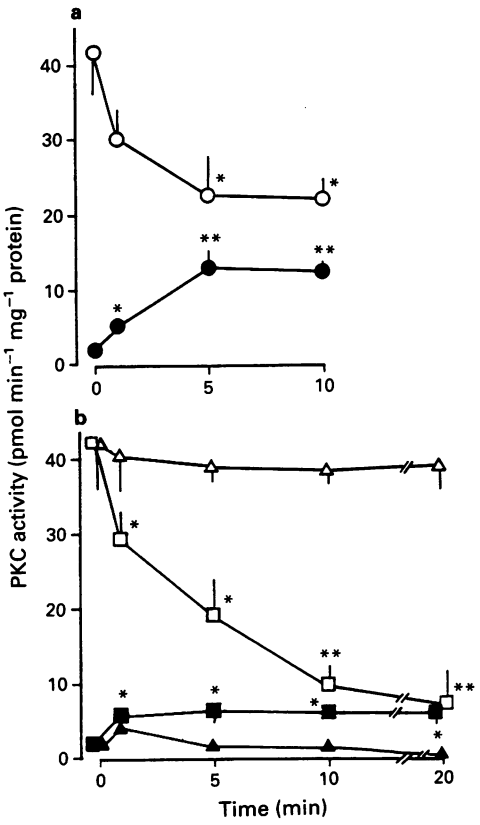

Figure 2 Effects of phenylephrine and phorbol esters on protein kinase C (PKC) activity. Rat left ventricular papillary muscles were treated with 10 μM phenylephrine (a) or 0.1 μM phorbol esters (b) for the indicated periods. PKC activity was determined in the cytosolic (open symbols) and particulate (closed symbols) fractions, which were prepared as described in Methods. ○ ●, Phenylephrine (a); □ ■, PDBu (b); △ ▲, 4α-phorbol (b). Each point represents the mean of 6 preparations; s.e.mean shown by vertical lines. $*P<0.05$, $**P<0.01$ compared with the basal value obtained before the addition of the agents.

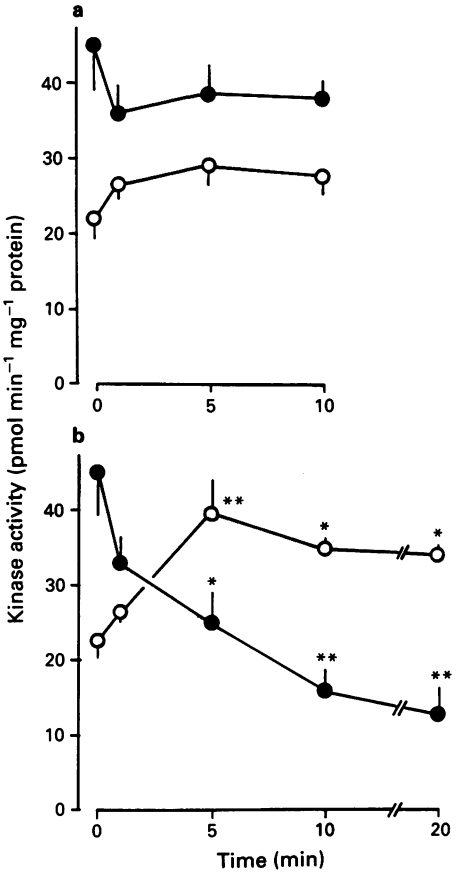

Figure 3 Effects of phenylephrine and phorbol 12,13-dibutyrate (PDBu) on the total protein kinase C (PKC) activity and Ca^{2+}/phospholid-independent kinase (PKI) activity. Rat left ventricular papillary muscles were treated with 10 μM phenylephrine (a) or 0.1 μM PDBu (b) for the indicated periods. Total PKC activity (cytosol plus particulate PKC activity; ●) and PKI activity in cytosol (○) were measured as described in Methods. Each point represents the mean of 6 preparations; vertical lines show s.e.mean; $*P<0.05$; $**P<0.01$ compared with the basal value.

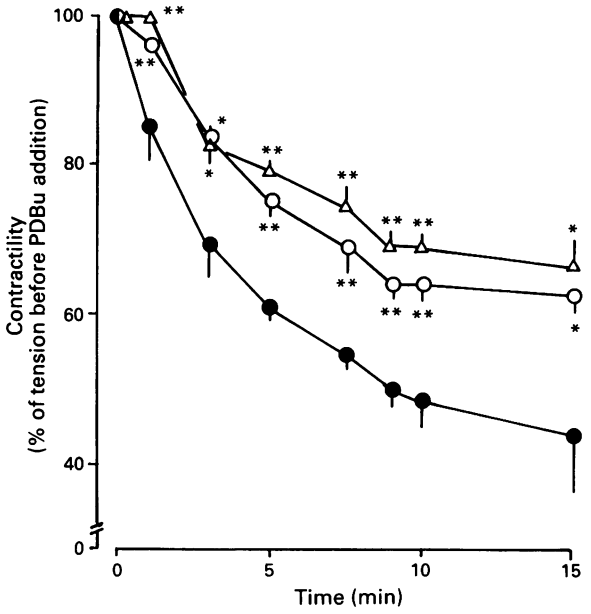

Figure 4 Effects of leupeptin and E-64 on the phorbol 12,13-dibutyrate (PDBu)-induced negative inotropic response. Rat left ventricular papillary muscles were exposed to 0.1 μM PDBu with or without (control; ●) pretreatment with 1 mM of either leupeptin (○) or E-64 (△) for 60 min. Contractile force at the indicated time is expressed as a percentage of that obtained just before the addition of PDBu. Each point represents the mean of 6 preparations; vertical lines show s.e.mean. $*P<0.05$; $**P<0.01$ compared with the corresponding control value at each time.

present study shows that these agents cause different redistribution patterns of PKC with opposite inotropic effects.

It has been shown that α_1-adrenoceptor-stimulation of cardiac muscle causes receptor-linked degradation of PI-4, 5-P_2 and the resulting formation of IP_3 and DAG (Otani *et al.*, 1988; Scholz *et al.*, 1988). In the present study, α_1-adrenoceptor stimulation with phenylephrine had a positive inotropic effect accompanied by an increase in the particulate PKC activity and a decrease in the cytosolic PKC activity, but PKI activity was unaffected by this treatment. A similar α_1-adrenoceptor-mediated translocation of PKC after exposure to noradrenaline has been reported to occur in cultured cardiac myocytes and in brain neurones (Henrich & Simpson, 1988; Kalberg & Sumners, 1990). The present study and others suggest that an increase in the membrane-bound PKC activity via translocation from the cytosol in the presence of a PKC activator, DAG, is responsible for the α_1-adrenoceptor-mediated cellular responses. This hypothesis is also supported by the finding that staurosporine inhibits the phenylephrine-induced positive inotropic effect (Figure 1), since staurosporine is reported to inhibit membrane-bound PKC activity at the concentration used in the present study (0.1 μM) by interacting with the catalytic subunit of PKC (Nakadate *et al.*, 1988). It has been suggested that an increase in the membrane-bound PKC in cardiac myocytes elicits sustained positive inotropic effect via Ca^{2+}-channel activation (Dösemeci *et al.*, 1988) and/or Na^+/H^+ exchange activation (Ikeda *et al.*, 1988). The latter possibility is strongly supported by our recent paper which showed that the α_1-adrenoceptor-mediated positive inotropic effect can be reduced by inhibitors of Na^+/H^+ exchange as well as by PKC inhibitors (Otani *et al.*, 1990).

On the other hand, PDBu increased only slightly particulate-associated PKC activity. In marked contrast to the change in particulate-associated PKC, PDBu decreased the total PKC activity and increased the PKI activity. These data are in good agreement with those obtained from cultured cardiac myocytes and neutrophils treated with phorbol myristate acetate (PMA) (Henrich & Simpson, 1988; Melloni *et al.*, 1986). The biologically inactive phorbol ester, 4α-phorbol, did not cause such a redistribution of PKC, excluding the possibility that there was a nonspecific interaction of PKC with PDBu. When PKC is stimulated by a phorbol ester, membrane-bound PKC is cleaved by a Ca^{2+}-dependant neutral protease such as calpain, to produce a catalytically active fragment, which does not require Ca^{2+} and phospholipid, and which acts as an irreversibly activated cytosolic kinase, i.e. PKI (Kishimoto *et al.*, 1983; Kishimoto, 1990). Such a protease-mediated down-regulation of PKC and the occurrence of PKI, has been implicated as an alternate mechanism of PKC activation and of the resulting cellular response by phorbol ester (Pontremoli *et al.*, 1986; Chida *et al.*, 1986; Melloni *et al.*, 1986). The present study shows that leupeptin and E-64, which are reported to be specific inhibitors of thiol proteinase (calpain) (Wang, 1990), attenuated the PDBu-induced negative inotropic effect, which was relatively insensitive to staurosporine. These two calpain inhibitors have been shown to prevent the loss of total PKC activity and the conversion of membrane-bound PKC to PKI at concentrations similar to or lower than those used in this study (Tapley & Murray, 1985; Pontremoli *et al.*, 1986; Melloni *et al.*, 1986). Furthermore, in neutrophils treated with phorbol 12-myristate 13-acetate (PMA), exocytosis of granule contents such as lysosomal enzymes and vitamin B_{12} binding protein can be inhibited by leupeptin and E-64 (Pontremoli *et al.*, 1986), suggesting that these PMA-induced events occur via the conversion of membrane-bound PKC to cytosolic PKI. When these data are taken together, it is reasonable to conclude that increased PKI activity may be involved in the PDBu-induced negative inotropic effect. There is, however, controversial evidence that leupeptin does not affect the PKC-mediated responses in platelets treated with PMA (Alonso *et al.*, 1989). This may indicate that the roles of PKI may differ, depending on the cell and tissue type. Furthermore, although the mechanism by which PKI caused the negative inotropic effect was not addressed in this study, there are at least the following possibilities: (1) inhibition of Ca^{2+} accumulation in sarcoplasmic reticulum (Rogers *et al.*, 1990) and (2) decrease in myofilament Ca^{2+} sensitivity (Gwathmey & Hajjar, 1990). Either mechanism could result in a decrease in the force of contraction.

In conclusion, α_1-adrenoceptor stimulation with phenylephrine and direct stimulation of PKC by PDBu accelerated the translocation of PKC from the cytosolic to the particulate fractions. Furthermore, the modes of redistribution were different; phenylephrine changed neither the total PKC activity nor PKI activity, while PDBu decreased the total PKC activity and increased PKI in the cytosol. Such a difference in the patterns of PKC distribution may account for the opposite inotropic effects after PKC stimulation.

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Tachykinin receptors in the guinea-pig renal pelvis: activation by exogenous and endogenous tachykinins

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1 The contractile response to substance P, neurokinin A, selective agonists for the NK_1, NK_2 and NK_3 tachykinin receptors and the activity of receptor-selective antagonists has been investigated in circular muscle strips of the guinea-pig isolated renal pelvis in the presence of indomethacin (3 μM).

2 Neurokinin A was the most potent agonist tested, being about 32 times more potent than substance P. The action of both substance P and neurokinin A was enhanced by peptidase inhibitors (bestatin, captopril and thiorphan, 1 μM each). The selective NK_2 receptor agonist [βAla^8] neurokinin A (4-10), was slightly less potent and effective than neurokinin A itself. The selective NK_1 receptor agonist [Sar^9] substance P sulphone was effective at low (nM) concentrations but its maximal effect did not exceed 30% of maximal response to substance P or neurokinin A. The NK_3-selective agonist [MePhe^7] neurokinin B was effective only at high (μM) concentrations.

3 The pseudopeptide derivative of neurokinin A(4-10), MDL 28,564, displayed a clear-cut agonist character, although it was less potent than neurokinin A.

4 The responses to roughly equieffective (25–35% of maximal response) concentrations of [βAla^8] neurokinin A (4-10), MDL 28,564 and [MePhe^7] neurokinin B were antagonized to a similar extent by MEN 10,376 (3 μM), a selective NK_2 tachykinin receptor antagonist, while the response to [Sar^9] substance P sulphone was unchanged.

5 The response to [Sar^9] substance P sulphone was inhibited by the NK_1 receptor-selective antagonist, GR 82,334 (3 μM) while the response to [βAla^8] neurokinin A (4-10) was unchanged.

6 The selective NK_2 receptor antagonists MEN 10,376, L 659,877 and R 396 antagonized competitively the response to [βAla^8] neurokinin A (4-10) with the following rank order of potency (pA_2 values in parentheses): MEN 10,376 (7.41) > L 659,877 (7.15) > R 396 (6.43). MEN 10,376 and L 659,877 also competitively antagonized the response to neurokinin A, although with lower potency as compared to the selective NK_2 receptor agonist.

7 MEN 10,376, L 659,877 and R 396 reduced in a concentration-dependent manner the contractile response produced by electrical field stimulation (1 Hz, 100 V, 0.25 ms pulse width, trains of 10 s). The rank order of potency of NK_2 receptor antagonists in blocking the response to electrical stimulation (MEN 10,376 > L 659,877 > R 396) closely mimicked their potency in antagonizing exogenous tachykinins.

8 The inhibitory effect of MEN 10,376 toward responses produced by electrical field stimulation was significantly reduced when tested in the presence of peptidase inhibitors, which increased significantly the response to nerve stimulation.

9 GR 82,334 (3 μM) did not significantly affect the response to nerve stimulation in untreated preparations and slightly reduced it in the presence of peptidase inhibitors.

10 We conclude that both NK_1 and NK_2 receptors mediate the contractile effect of tachykinins in the circular muscle of the guinea-pig renal pelvis and that the response ascribable to NK_2 receptor stimulation is larger than that ascribed to NK_1 receptor stimulation. The NK_2 receptor in the guinea-pig renal pelvis belongs to the same subtype previously identified in the rabbit pulmonary artery. NK_2 receptors play a dominant role in the physiological response determined by the release of endogenous tachykinins and a contribution of NK_1 receptors becomes evident after inhibition of peptide degradation.

Keywords: Guinea-pig renal pelvis; tachykinins; tachykinin receptors; NK_2 receptor subtypes; tachykinin antagonists

Introduction

In mammals, the renal pelvis and ureter receive a dense afferent peptidergic innervation, which signals ureteral pain. The major part (91%) of afferents in the guinea-pig ureter belong to that subpopulation of sensory neurones which are capsaicin-sensitive (Cervero & Sann, 1989). In both the renal pelvis (Maggi *et al.*, 1992a) and ureter (Maggi & Giuliani, 1991), the capsaicin-sensitive primary afferents participate in the local modulation of ureteral motility through neuropeptide release from their peripheral nerve endings. In the guinea-pig isolated renal pelvis, the local activation of neuropeptide release from capsaicin-sensitive primary afferents mediates a contractile response which reinforces the spontaneous activity sustained by natural pacemakers (Maggi *et al.*, 1992a; Golenhofen & Hannappel, 1973).

This contractile effect is ascribable to the release of endogenous tachykinins, such as substance P and neurokinin A (Maggi *et al.*, 1992a). Both substance P- and neurokinin A-like immunoreactivity have been shown to be present in the guinea-pig renal pelvis and are simultaneously released following application of capsaicin. Neurokinin A is more

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potent than substance P in producing contraction of the isolated renal pelvis, suggesting the presence of tachykinin NK_2 receptors at this level. However, the receptors involved in tachykininergic control of renal pelvis motility have not been fully characterized. Furthermore, recent studies have provided evidence for the heterogeneity of tachykinin NK_2 receptors, possibly reflecting the existence of NK_2 receptor subtypes (Maggi *et al.*, 1990; 1991a; Buck *et al.*, 1990; Van Giersbergen *et al.*, 1991; Patacchini *et al.*, 1991). The aim of this study was twofold: (a) to assess the type of tachykinin receptors mediating contraction of the guinea-pig isolated renal pelvis and (b) to assess the relative contribution of different tachykinin receptors to the response produced by endogenous tachykinins released during nerve stimulation.

Methods

Male albino guinea-pigs (250–300 g b.wt.) were killed by a blow on the back of the head and exsanguination. The whole kidney and attached ureter were removed and placed in oxygenated (96% O_2 and 4% CO_2) Krebs solution, as described previously (Maggi *et al.*, 1992a). The renal pelvis was carefully dissected from the renal parenchyma, separated from the ureter, cut and connected to threads to record motility along the circular axis. The preparation was suspended in a 5 ml organ bath and mechanical activity recorded by means of an isotonic transducer (load 0.15 mN). Transmural electrical field stimulation was applied by means of platinum wire electrodes placed at the top and bottom of the organ bath and connected to a GRASS S 11 stimulator. Square wave pulses (pulse width 0.25 ms, 100 V) were delivered in trains of 10 s duration at a frequency of 1 Hz.

Indomethacin (3 μM) was added to the Krebs solution in order to reduce the amplitude of spontaneous activity and obtain a better quantitative evaluation of the contractile response to stimulants. All experiments started after a 60–90 min equilibration period when the amplitude and frequency of spontaneous activity had reached a steady state (about 15% of maximal contractile responses).

Concentration-response curves to the agonists were constructed in a cumulative manner, the next concentration being added when the effects of the preceding one had reached a steady state.

Preliminary experiments had shown that the contractile response to natural tachykinins and receptor selective synthetic agonists in this preparation do not exhibit significant desensitization. The only exception was the selective NK_1 receptor agonist, [Sar^9] substance P sulphone (Dion *et al.*, 1987) for which non-cumulative concentration-response curves were constructed, because of desensitization. For this agonist, non-cumulative concentration-response curves were constructed by addition of increasing concentrations of the agonist at 15–20 min intervals with washouts intervening between doses.

The effect of antagonists toward contractions produced by agonists were investigated after a contact time of 15 min which, in preliminary experiments, was shown to allow expression of maximal inhibitory effect.

Data evaluation

Previous experiments (Maggi *et al.*, 1992a) had shown that tachykinins produce complex motor response in the spontaneously contracting guinea-pig renal pelvis. The first effect, observed with low concentrations of the agonist is a pure enhancement of amplitude of contractions, accompanied, at higher concentrations, by an increased frequency of contractions. Finally, high concentrations of tachykinins which produce maximal increase in contraction amplitude, also produce an increase in tone. In the text and figures all the effect of agonist and antagonists describe changes in amplitude of contractions produced by test substances as compared to the original baseline.

All changes in mechanical activity were expressed as % of maximal contracture produced by application of barium chloride (10 mM).

Statistical analysis

All values in the text and figures are mean ± s.e. mean. Statistical analysis was performed by means of Student's *t* test for paired or unpaired data or by means of analysis of variance, when applicable. The effect of tachykinin antagonists on the contractile response produced by electrical field stimulation was evaluated by one way analysis of variance and Dunnett's test.

To estimate affinities of NK_2 receptor antagonists toward the responses produced by agonists, concentration-response curves were constructed in the absence and the presence of antagonists and Schild plots constructed accordingly. For slopes of Schild plots not significantly different from unity, the constrained plot method was used to calculate pA_2 values.

Drugs

Drugs used were: indomethacin, captopril, bestatin and thiorphan (Sigma), GR 82,334 (Bachem). Neurokinin A, MDL 28,564, [βAla^8] neurokinin A (4-10), [MePhe^7] neurokinin B, L 659,877 and MEN 10,376 were synthesized by conventional solid phase methods. [Sar^9] substance P sulphone was from Peninsula. R 396 was a kind gift of Prof. D. Regoli, Department of Pharmacology University of Sherbrooke, Canada. The amino acid sequence of peptides investigated is shown in Table 1.

Table 1 Amino acid sequence of peptides used in this study

Agonists	
Substance P	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
[Sar^9]SP sulphone	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Sar-Leu-Met(O_2)-NH_2
NKA	H-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
[βAla^8]NKA (4-10)	H-Asp-Ser-Phe-Val-βAla-Leu-Met-NH_2
NKB	H-Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2
[MePhe^7]NKB	H-Asp-Met-His-Asp-Phe-Phe-MePhe-Gly-Leu-Met-NH_2
MDL 28,564	H-Asp-Ser-Phe-Val-Gly-Leuψ[CH_2NH]Leu-NH_2
Antagonists	
MEN 10,376	H-Asp-Tyr-DTrp-Val-DTrp-DTrp-Lys-NH_2
L 659,877	c(Leu-Met-Gln-Trp-Phe-Gly)
R 396	Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2
GR 82,334	Pyr-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-DPro(spiro-γ-lactam)Leu-Trp-NH_2

NKA = neurokinin A; NKB = neurokinin B.

Results

Effect of agonists

Addition of tachykinins increased amplitude and frequency of spontaneous contactions of the guinea-pig isolated renal pelvis. For low concentrations of tachykinins the effect on contraction amplitude dominated and a clear-cut increase in frequency became evident when the positive inotropic effect had reached its maximum. At this stage, the increased frequency of contractions produced by added tachykinins was also accompanied by an increase in tone (Figure 1). For quantitative evaluation of drug effects the increased amplitude of contractions as compared to the original baseline was determined.

Either substance P or neurokinin A produced a concentration-dependent contraction (Figures 1 and 2) which reached the same maximum produced by addition of barium chloride (10 mM). Neurokinin A was about 32 times more potent than substance P (Table 2).

The response to both substance P and neurokinin A was significantly enhanced by peptidase inhibitors (captopril, bestatin and thiorphan, 1 μM each, 15 min before): in the presence of peptidase inhibitors substance P and neurokinin A were 8.5 and 3.7 times more potent than in control experiments, respectively (Table 2). The selective NK_1 receptor agonist, [Sar^9] substance P sulphone had a low threshold (10 nM) and its maximal effect (at 3 μM) did not exceed 30% of maximal response of the preparations. It was noted that the effect of [Sar^9] substance P sulphone exhibits marked desensitization and the curve shown in Figure 2 has been obtained following non-cumulative addition of the peptide. The action of [Sar^9] substance P sulphone was not affected by peptidase inhibitors (Figure 2).

The selective NK_2 receptor agonist, [βAla^8] neurokinin A (4-10) was less potent and less effective than neurokinin A (Figure 2). The maximal response to [βAla^8] neurokinin A (4-10) did not exceed 85% of maximal barium response with an EC_{50} of 95 nM (49–236 nM are 95% c.l.). The action of [βAla^8] was not significantly affected by peptidase inhibitors (Figure 2). The pseudopeptide, MDL 28,564 displayed a clearcut agonist character, although it was less potent than neurokinin A (threshold concentration 3 μM). At the highest concentration tested (100 μM) the effect of MDL 28,564 approached 75% of maximal response (Figure 2).

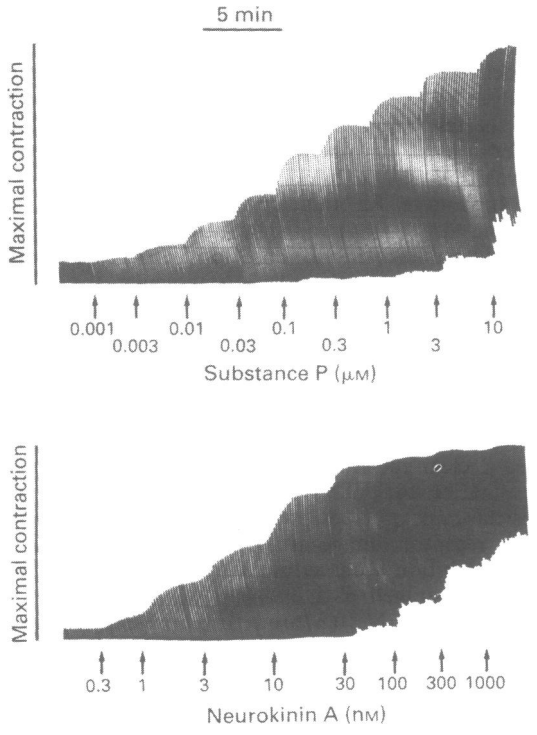

Figure 1 Typical tracing illustrating the contractile effect produced by cumulative addition of substance P or neurokinin A on the spontaneous activity of the guinea-pig isolated renal pelvis.

Table 2 EC_{50}s of substance P and neurokinin A in producing positive inotropic response of the guinea-pig isolated renal pelvis in the absence and presence of peptidase inhibitors

	EC_{50} (nM, 95% c.l. in parentheses)	
	Control	*Peptidase inhibitors*
Substance P	239 (92-602)	28 (16-60)
Neurokinin A	7.4 (6.0–9.6)	2 (1.5–2.4)

Concentration-response curves to substance P or neurokinin A were constructed in the absence (control) or presence of peptidase inhibitors (bestatin, captopril and thiorphan, 1 μM each).

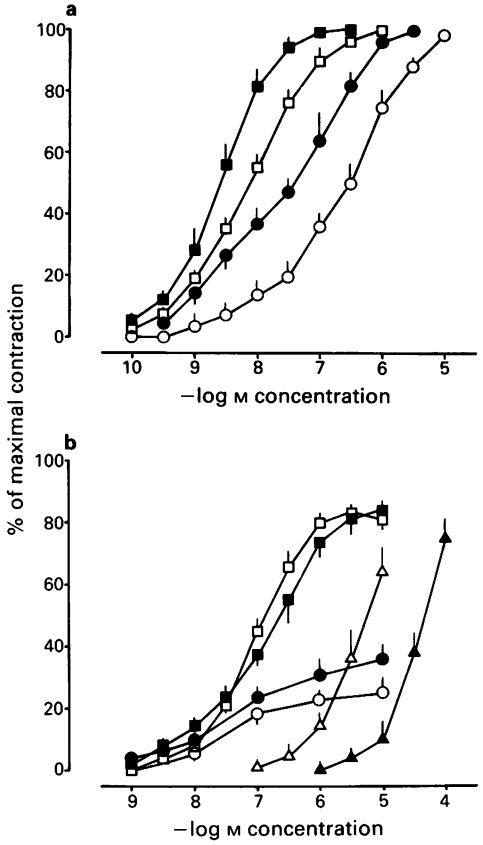

Figure 2 (a) Concentration-dependent contractile effect produced by substance P (SP, circles) or neurokinin A (NKA, squares) in the guinea-pig isolated renal pelvis. Open symbols refer to experiments obtained in the absence of peptidase inhibitors, solid symbols refer to experiments performed in the presence of bestatin, captopril and thiorphan 1 μM each, added 15 min beforehand. Each point is the mean of 12–14 experiments; vertical lines show s.e.mean. (b) Concentration-dependent contractile effect produced by receptor-selective tachykinin agonists in the guinea-pig isolated renal pelvis. For the NK_1 receptor-selective agonist [Sar^9] SP sulphone (circles) and the NK_2 receptor-selective agonist [βAla^8] NKA (4-10) (squares) experiments were performed either in the absence (open symbols) or presence (solid symbols) of peptidase inhibitors (bestatin, captopril and thiorphan 1 μM each, 15 min before). For the NK_2 receptor-selective agonists, MDL 28,564 (▲) and the NK_3 receptor-selective agonists [$MePhe^7$] neurokinin B (△) experiments were performed in the absence of peptidase inhibitors. Each point is mean of 5–8 experiments; vertical lines show s.e.mean.

Also the NK_3 receptor selective ligand, [MePhe^7] neurokinin B displayed agonist activity, although in a concentration-range (0.3–10 μM), much higher than that reported to be effective (low nM) in stimulating NK_3 receptors (Dion *et al.*, 1987).

Effect of antagonists on the response to neurokinin A and [βAla^8] neurokinin A (4-10)

The above experiments indicate that NK_2 receptors prevail in the guinea-pig renal pelvis although the presence of NK_1 and NK_3 receptors may not be ruled out. To address this point further, we studied the effect of MEN 10,376 (3 μM) an NK_2 receptor selective antagonist (Maggi *et al.*, 1991b) toward the response produced by roughly equieffective (25–35% of maximal response) concentrations of [Sar^9] substance P sulphone (3 μM), [βAla^8] neurokinin A (4-10) (30 nM), MDL 28,564 (30 μM) and [MePhe^7] neurokinin B (3 μM). Data in Figure 3 show that MEN 10,376 nearly abolished or greatly reduced the response to [βAla^8] neurokinin A (4-10), MDL 28,564 and [MePhe^7] neurokinin B while leaving the response to [Sar^9] substance P sulphone totally unaffected.

To assess whether the action of [Sar^9] substance P sulphone might involve activation of NK_1 receptors, the NK_1 receptor antagonist, GR 82,334 (3 μM) (Hagan *et al.*, 1991) was studied toward the response produced by [Sar^9] substance P sulphone (3 μM) and [βAla^8] neurokinin A (4-10) (30 nM). Data in Figure 4 show that GR 82,334 significantly inhibited (43%) the response to [Sar^9] substance P sulphone while leaving that to [βAla^8] neurokinin A (4-10) unaffected.

To assess further the nature of the NK_2 receptor involved in the response of the guinea-pig renal pelvis, full concentration-response curves to neurokinin A and [βAla^8] neurokinin A (4-10) were constructed and the effect of different concentrations of MEN 10,376, L 659,877 and R396 was investigated. For each antagonist, at least 4 different concentrations were tested and Schild plots constructed; for each agonist/antagonist combination tested, the slopes of Schild plots were not significantly different from unity (Table 3), and pA_2 values were calculated using the constrained Schild plot method (Tallarida *et al.*, 1979). None of the antagonists tested affected significantly spontaneous contractions of the pelvis. Data shown in Table 3 indicate that both MEN 10,376 and L 659,877 were distinctly more potent (about 10 times for each antagonist) in blocking the action of [βAla^8] neurokinin A (4-10) than in blocking the action of neurokinin A. Antagonist potency toward the effect of the selective NK_2 receptor agonist followed the rank order: MEN 10,376 > L 659,877 > R 396 (Table 3).

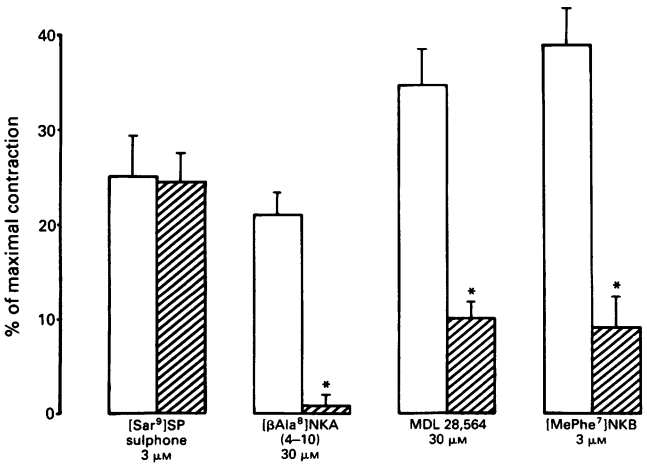

Figure 3 Effect of the NK_2 receptor selective antagonist, MEN 10,376 (3 μM) on the contractile effect produced by roughly equieffective concentrations of receptor-selective synthetic agonists in the guinea-pig isolated renal pelvis. Open columns = control; hatched columns = in the presence of MEN 10,376. Each column is mean of 5–7 experiments; vertical bars show s.e.mean. *Significantly different from the control response, $P<0.05$.

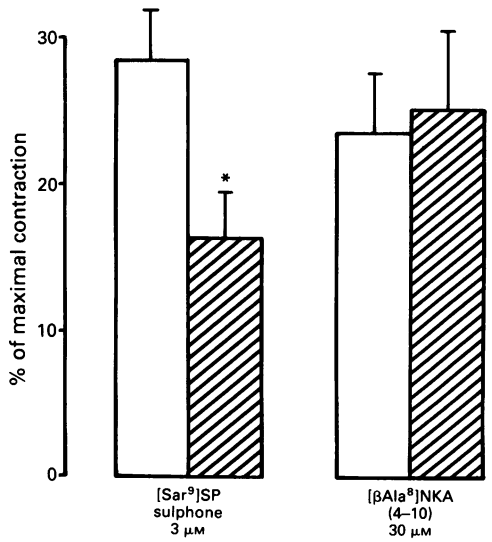

Figure 4 Effect of the NK_1 receptor-selective antagonist, GR 82,334 (3 μM) on the contractile effect produced by an NK_1 and an NK_2 receptor-selective agonist in the guinea-pig isolated renal pelvis. Open columns = control; hatched columns = in the presence of GR 82,334. Each column is mean of 5–6 experiments; vertical bars show s.e.mean. *Significantly different from the control response, $P<0.05$.

Effect of antagonists on the response to electrical field stimulation

The following experiments were designed to assess whether or not the same rank order of potency of NK_2 receptor antagonists observed toward the selective NK_2 receptor agonists [βAla^8] neurokinin A (4-10), occurs for inhibition of the response produced by nerve stimulation. With this aim, the response produced by a train of stimuli (100 V, 0.25 ms pulse width) delivered at a frequency of 1 Hz every 20 min was repeatedly elicited in the presence of increasing concentrations of the test antagonist. Three cycles of stimulation were performed in each strip before addition of the antagonists or of the vehicle.

Data in Figure 5a, indicate that, as compared to the spontaneous decay observed in control strips, MEN 10,376 significantly reduced the response to electrical stimulation at all concentrations tested (0.1–3 μM), whilst the inhibitory effect of L 659,877 and R 396 was statistically significant only at 1–3 μM for L 659,877 and at 3–10 μM for R 396, respectively. In Figure 5b, the effect of antagonists is presented as % inhibition of the evoked responses after the observed values had been corrected for the spontaneous decay observed over repeated stimulation cycles in controls; from this analysis, a clear-cut rank order of potency of antagonists emerges: MEN 10,376 > L 659,877 > R 396 which closely matches the rank order of potency in antagonizing the selective NK_2 receptor antagonist, [βAla^8] neurokinin A (4-10). Maximal inhibition observed with 3 μM MEN 10,376 in these experiments averaged 63 ± 2% ($n = 6$).

The effect of MEN 10,376 on strips pre-exposed to peptidase inhibitors (thiorphan, captopril and bestatin 1 μM each) was also investigated. The amplitude of the response to electrical stimulation (22 ± 4% of barium response, $n = 10$) was significantly enhanced (39 ± 4% of barium response, $n = 10$, $P<0.05$) by peptidase inhibitors. At 0.1–1 μM the inhibitory effect of MEN 10,376 was significantly reduced when tested in the presence of peptidase inhibitors (Figure 6).

Table 3 pA_2 values and slopes of Schild plots of MEN 10,376, L 659,877 and R 396 toward positive inotropic effect produced by neurokinin A or the selective NK_2 tachykinin receptor agonist, [βAla^8] neurokinin A (4-10) on the circular muscle of the guinea-pig isolated renal pelvis

Antagonist	*Neurokinin A*		*[βAla^8] neurokinin A*	
	Slope	*pA_2*	*Slope*	*pA_2*
MEN 10,376	−0.86 (0.67–1.05)	6.56 ± 0.08	−0.75 (0.41–1.19)	7.41 ± 0.09
L 659,877	−1.29 (0.56–2.03)	6.08 ± 0.11	−1.44 (0.80–1.74)	7.15 ± 0.11
R 396	NT	NT	−0.80 (0.59–1.22)	6.43 ± 0.10

Each value was calculated from at least 9 experiments.
pA_2 values were calculated using the constrained Schild plot method
NT = not tested

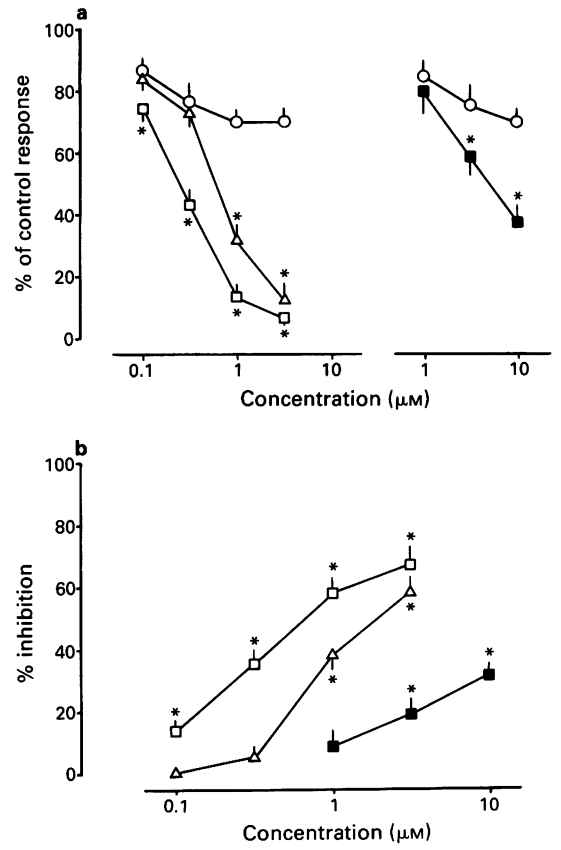

Figure 5 (a) Concentration-dependent inhibitory effect produced by cumulative addition of three NK_2 receptor selective antagonists MEN 10,376 (□), L 659,877 (△) and R 396 (■) on the contractile response to electrical field stimulation in the guinea-pig isolated renal pelvis; (○) indicate the spontaneous decay of the response in control experiments. In (b) the effect of the three antagonists is shown as % inhibition of the response to electrical field stimulation after correction for the spontaneous decay of the response observed in control experiments. Each point is mean of 6 experiments; vertical lines show s.e.mean. *Significantly different from the control response, $P<0.05$.

In a further series of experiments, the effect of MEN 10,376 and GR 82,334 (both at 3 μM) was investigated toward the response produced by electrical field stimulation, in the absence and presence of peptidase inhibitors. MEN 10,376 was effective both in the absence and presence of peptidase inhibitors (65 ± 2 and 49 ± 5% inhibition, respectively). GR 82,334 failed to affect the evoked response in the absence of peptidase inhibitors but slightly reduced it (about 25%) in the presence of bestatin, captopril and thiorphan (Figure 7).

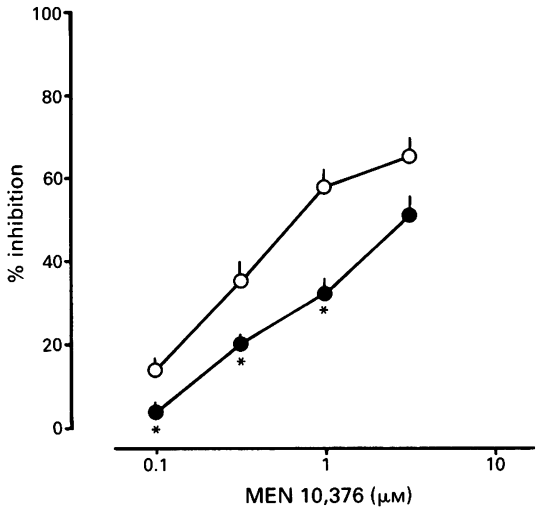

Figure 6 Concentration-dependent inhibitory effect of the NK_2 receptor-selective antagonist, MEN 10,376 on the contractile effect produced by electrical field stimulation in the guinea-pig isolated renal pelvis in the absence (control, ○) and presence of peptidase inhibitors (bestatin, captopril and thiorphan, 1 μM each, ●). Each point is mean of 6–8 experiments; vertical lines show s.e.mean. *Significantly different from the control response, $P<0.05$.

Discussion

Tachykinin receptors in the guinea-pig renal pelvis

The combined results of this study indicate that the NK_2 receptor is the principal mediator of the contractile effect exerted by tachykinins in the guinea-pig isolated renal pelvis. In fact: (a) neurokinin A is more potent than substance P; (b) the NK_2 agonist, [βAla^8] neurokinin A (4-10) was effective at low concentrations; (c) MDL 28,564 which is perhaps the most selective NK_2 receptor ligand available (Harbeson *et al.*, 1990), displayed agonist activity blocked by the NK_2 receptor antagonist MEN 10,376; (d) selective NK_2 receptor antagonists such as MEN 10,376 (Maggi *et al.*, 1991b), L 659,877 (Williams *et al.*, 1988) and R 396 (Dion *et al.*, 1990) were effective against neurokinin A and [βAla^8] neurokinin A (4-10) (see below for discussion about NK_2 receptor subtypes); (e) the response to [βAla^8] neurokinin A (4-10) is unaffected by GR 82,334, a selective NK_1 receptor antagonist (Hagan *et al.*, 1991).

In the past two years, pharmacological evidence has been provided for the heterogeneity of the tachykinin NK_2 receptor (Maggi *et al.*, 1990; 1991a; Buck *et al.*, 1990; Van Giersbergen *et al.*, 1991; Patacchini *et al.*, 1991). Two criteria which allow the distinction between NK_2 receptor subtypes have emerged: (a) rank order of potency of antagonists and

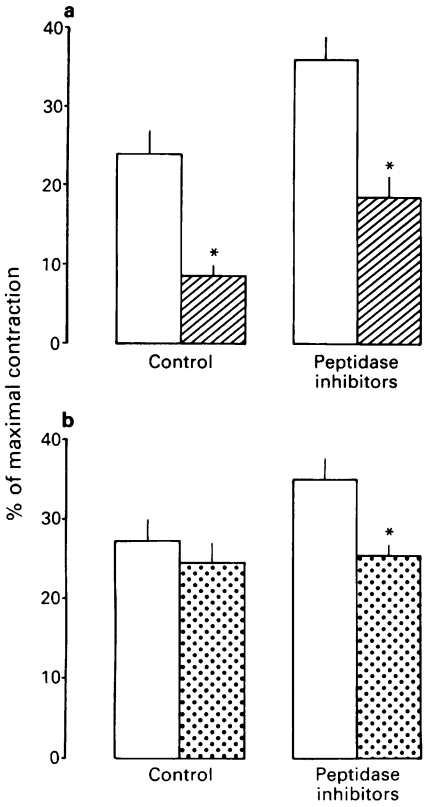

Figure 7 (a) Comparison of the inhibitory effect of the NK_2 receptor-selective antagonist, MEN 10,376 on the contractile response produced by electrical field stimulation in the guinea-pig isolated renal pelvis in the absence or presence of peptidase inhibitors (bestatin, captopril and thiorphan 1 μM each). Open columns = control; hatched columns = in the presence of MEN 10,376. (b) The same as for (a) but with the NK_1 receptor-selective antagonists GR 82,334. Open columns = control; stippled columns = in the presence of GR 82,334. Note that GR 82,334 produced a small inhibitory effect only in the presence of peptidase inhibitors. Each column is mean of 6–8 experiments; vertical lines show s.e.mean. *Significantly different from the control responses, $P<0.05$.

(b) the different agonist/antagonists character of MDL 28,564. On the basis of present findings we conclude that the NK_2 receptor mediating positive inotropic effect in the guinea-pig renal pelvis is of the same subtype occurring in the guinea-pig bronchi (Maggi *et al.*, 1991c) and rabbit pulmonary artery (Maggi *et al.*, 1990).

Our data also consistently indicate the existence of a population of NK_1 receptors mediating contraction of the guinea-pig renal pelvis; this conclusion is based on: (a) the response to [Sar^9] substance P sulphone, which is a highly selective NK_1 receptor agonist (Dion *et al.*, 1987), with negligible or no activity at NK_2 receptors and subtypes (e.g. Maggi *et al.*, 1990); (b) the response to [Sar^9] substance P sulphone was unaffected by MEN 10,376 while it was reduced by GR 82,334.

We can exclude a participation of NK_3 receptors in the contractile response of the pelvis. [MePhe^7] neurokinin B has been shown to stimulate NK_3 receptors in the guinea-pig ileum and rat portal vein at nM concentrations (Dion *et al.*, 1987). This ligand is certainly more selective than neurokinin B itself for NK_3 receptors, but at high concentrations it may act at NK_2 sites (Dion *et al.*, 1987); this probably occurs under our experimental conditions because [MePhe^7] neurokinin B was effective at μM concentrations and its effect was blocked by MEN 10,376 to a similar extent as the response to MDL 28,564 or [βAla^8] neurokinin A (4-10).

Therefore, both NK_1 and NK_2 receptors mediate the response of the guinea-pig isolated renal pelvis to tachykinins. In this study, we concentrated our efforts on the action of test peptides on the amplitude of spontaneous contractions. This parameter is the more sensitive measure of the effect of tachykinins in this preparation. It is evident that, for high concentration of tachykinins, the increased frequency of contractions and the increased tone (possibly related to the failure to relax to baseline because of the increased frequency of contractions) also contribute to the overall increase in amplitude. Although we did not note any major difference in this respect between e.g. neurokinin A and substance P, further studies are needed to assess whether NK_1 and NK_2 receptors might play a differential role in mediating the effect of tachykinins on contraction amplitude and frequency.

While natural tachykinins were capable of producing a maximal response, this was not the case for the NK_1 or NK_2 receptor-selective agonists: their maximal effect approached about 30% (for [Sar^9] substance P sulphone) and 85% (for [βAla^8] neurokinin A(4-10)) of that to neurokinin A or substance P. We interpret these findings as further indication that natural tachykinins have the ability to interact, although at different concentrations, with both NK_1 and NK_2 tachykinin receptors. At the same time, it is evident that the relative contribution of NK_2 receptors to the evoked response of the pelvis is quantitatively greater than that of NK_1 receptors, a conclusion supported by experiments with NK_2 receptor antagonists toward the responses elicited by endogenous tachykinins (see below).

Tachykinin receptors and activation by endogenous ligands

In a previous study (Maggi *et al.*, 1992a) we proposed that endogenous tachykinins are responsible for the contractile effect produced by nerve stimulation of the guinea-pig isolated renal pelvis. Having established that both NK_1 and NK_2 receptors are present at this level, the next logical step was to determine the relative contributions of the two receptors in the response produced by release of endogenous ligands for tachykinins receptors.

Our data indicate that the same subtype of NK_2 receptor which is activated by exogenous tacykinins is also responsible for a large fraction of the response to endogenous tachykinins released by electrical stimulation of intramural nerves. The rank order of potency of NK_2 receptor antagonists in reducing the response to electrical stimulation was the same as that found for their ability to antagonize the response to [βAla^8] neurokinin A (4-10). By contrast GR 82,334 at a concentration which selectively reduces the response to [Sar^9] substance P sulphone did not affect the response to electrical stimulation. Accordingly, a more important role for neurokinin A (or for other ligands with high affinity for the NK_2 receptor) than for substance P in the response produced by activating sensory nerves in the renal pelvis can be postulated. Such an hypothesis is also supported by the observation that the maximal effect of the NK_1 receptor-selective agonist is much lower than that produced by the NK_2-receptor selective agonist.

In the presence of peptidase inhibitors, three major changes occurred: (a) the potency of natural tachykinins was enhanced, particularly for substance P; (b) the response produced by electrical stimulation was increased, suggesting degradation of endogenous tachykinins; (c) the effectiveness of MEN 10,376 in antagonizing the respose to nerve stimulation was decreased while (c) a small but a significant inhibitory effect of GR 82,334 became evident. All together, these findings indicate that breakdown by peptidases limits the expression of activity of endogenous tachykinins acting preferentially at NK_1 receptors (putatively substance P).

Owing to the desensitization of the response to the NK_1 receptor selective agonist, and the spontaneous decay of the response to repeated cycles of electrical stimulation (circles in Figure 5a) the possibility cannot be ruled out, on the basis of present findings, that decay of the response to endogenous

tachykinins involves desensitization of NK_1 receptors by endogenous ligands. Even assuming that such a mechanism may entirely account for the spontaneous decay of the tachykinin response to nerve stimulation it is evident that, in preparations not receiving peptidase inhibitors, the contribution of NK_1 receptors could not exceed 20–25% of the overall response. This estimate originates from the intensity of spontaneous decay during repeated cycles of stimulation in control preparations vs. intensity of inhibition produced by MEN 10,376 in matched preparations (Figure 5a, left panel).

The results of this part of the study could be compared with those obtained in other preparations in which tachykinins released from nerves by electrical stimulation have been shown to produce smooth muscle contraction. In the guinea-pig isolated bronchi (Maggi *et al.*, 1991d) and circular muscle of the human ileum (Maggi *et al.*, 1992b) both NK_1 and NK_2 receptors mediate contraction to tachykinins and endogenous tachykinins mediate atropine-resistant excitatory responses to nerve stimulation. In these preparations, the use of receptor-selective tachykinin antagonists has disclosed a major role of NK_2 receptors in mediating the physiological response i.e., the same finding obtained here for the guinea-pig renal pelvis. Furthermore, in the guinea-pig bronchus the relative contribution of NK_1 receptors becomes more evident after addition of peptidase inhibitors (Maggi *et al.*, 1991d), which is the same pattern found in the renal pelvis. From these studies a major contribution of neurokinin A, or anyway of endogenous ligands with high affinity for NK_2 receptors in physiological responses involving smooth muscle contraction by endogenous peptides of this family can be postulated.

In conclusion, the present findings indicate that both NK_1 and NK_2 receptors mediate the contractile response of the guinea-pig renal pelvis to tachykinins. The NK_2 receptors activated by exogenous and endogenous tachykinins is of the same subtype which has been previously identified in the rabbit pulmonary artery. Activity of endogenous tachykinins acting at NK_1 receptors is limited by degradation by peptidases. The present findings support a more important role for neurokinin A than for substance P as physiological mediators of contraction in the renal pelvis.

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Nucleotide-dependent activation of K_{ATP} channels by diazoxide in CRI-G1 insulin-secreting cells

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1 Patch-clamp recording techniques were used, to examine the effects of diazoxide on K_{ATP} currents in CRI-G1 insulin-secreting cells in the presence of non-hydrolysable nucleotides.

2 In the presence of non- or slowly-hydrolyzed ATP analogues, bathing the intracellular aspect of cell-free membrane patches diazoxide inhibited K_{ATP} channel activity.

3 Under whole-cell recording conditions, with various non-hydrolysable nucleotides present intracellularly (after dialysis), diazoxide induced K_{ATP} current activation. The largest activation occurred with Mg-adenylyl-(β,γ-methylene) diphosphate (Mg-AMP-PCP) present in the dialysing solution. This activation was diazoxide- and nucleotide-concentration-dependent.

4 In the absence of Mg^{2+}, or in the presence of manganese (Mn^{2+}) ions intracellularly, diazoxide did not induce K_{ATP} current activation, regardless of the species of nucleotide present in the pipette.

5 Intracellularly applied trypsin prevented the activation of K_{ATP} currents by diazoxide in the presence of Mg-AMP-PCP, an effect reversed by co-application of intracellular polymethylsulphonyl fluoride with the trypsin.

6 The application, by dialysis, of a CRI-G1 cell lysate, with negligible Mg-ATP, resulted in a substantial activation of the K_{ATP} current by diazoxide.

7 It is concluded that diazoxide can activate K_{ATP} channel currents by two separate pathways, one requiring a phosphorylation process, the other the presence of an intracellular protein coupled with a Mg-purine nucleotide.

Keywords: Diazoxide; K_{ATP} channels; non-hydrolysable nucleotides; CRI-G1 cells; insulin-secretion

Introduction

Adenosine-5′-triphosphate (ATP)-sensitive (K_{ATP}) channels have been described in a variety of tissues including cardiac muscle, skeletal muscle, vascular and arterial smooth muscle, central neurones, pancreatic β-cells and a range of insulin-secreting cell types (see reviews by Ashcroft, 1988; Ashcroft & Ashcroft, 1990; Ashcroft & Rorsman, 1991). Due to the involvement of these channels in the control of cell excitability, their pharmacological modulation has considerable therapeutic potential. For example, the sulphonylureas, tolbutamide and glibenclamide, are useful in the treatment of Type II diabetes since they mimic the effect of glucose by inhibiting K_{ATP} channel opening, albeit by a different mechanism (Sturgess *et al.*, 1985; Ashford, 1990). Closure of these channels induces β-cell depolarization and triggers Ca^{2+} entry through voltage-sensitive Ca^{2+} channels, an effect which ultimately results in insulin secretion (Ashcroft & Rorsman, 1991). In contrast, the sulphonamide diazoxide hyperpolarizes β-cells (Henquin & Meissner, 1982) thereby inhibiting the secretion of insulin and inducing hyperglycemia. This action is believed to be due to the activation of K_{ATP} channels (Trube *et al.*, 1986; Kozlowski *et al.*, 1989).

Initial studies with mouse pancreatic β-cells (Trube *et al.*, 1986) indicated that the presence of ATP was required at the intracellular surface of cell-free patches before diazoxide could activate K_{ATP} channels. Sturgess *et al.* (1988) reported a similar finding with CRI-G1 insulin-secreting cells and Dunne *et al.* (1987), using RINm5F cells, suggested that diazoxide acts by displacing the ATP^{4-} molecule from its binding site thereby reversing the K_{ATP} channel inhibition induced by the nucleotide. However, using isolated inside-out membrane patches from CRI-G1 insulin-secreting cells Kozlowski *et al.* (1989) demonstrated that activation was observed only when Mg-ATP was present in the cytoplasmic bathing solution. There was no activation when ATP, in the absence of Mg^{2+} ions (i.e. ATP^{4-}), or the non-hydrolysable analogue, Mg-adenylylimidodiphosphate (Mg-AMP-PNP) was present to inhibit the K_{ATP} channels. It was therefore concluded that diazoxide-induced activation of K_{ATP} currents involves phosphorylation of the channel or some closely associated membrane protein (Kozlowski *et al.*, 1989). This hypothesis was also found to be applicable to the K_{ATP} channel-activating action of diazoxide observed in RINm5F cells (Dunne, 1989).

However, following additional experiments designed to investigate the nature of the kinase responsible for this putative phosphorylation, it became apparent that under whole-cell recording conditions, diazoxide also activates K_{ATP} channel currents in the presence of non-hydrolysable ATP analogues. Thus we examined further the interactions of diazoxide and Mg-nucleotide complexes on both single channel and whole-cell K_{ATP} currents recorded from CRI-G1 cells.

Methods

Cell culture

Cells of the rat pancreatic islet cell-line (CRI-G1) were cultured and passaged at 3 to 7 day intervals as previously described (Carrington *et al.*, 1986). Cells used for patch-clamp experimentation were plated onto 3.5 cm petri dishes (Sterilin) at a density of approximately 1.5×10^5 cells per dish. Cells were used between 2 and 4 days after plating.

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Electrical recording and analysis

Cell-free and whole-cell recordings of K_{ATP} channel currents were obtained by standard procedures (Hamill *et al.*, 1981). Recording electrodes were pulled from borosilicate glass capillaries and, when filled with electrolyte, had resistances of 8 to 12MΩ for isolated patch experiments and 3 to 5 MΩ for whole-cell recordings. Ionic currents (single channel and whole-cell) were detected with an EPC-7 patch-clamp amplifier (List Electronics) and were recorded onto magnetic tape with a bandwidth (3db down) of 1250 Hz (Racal 4D tape recorder) for off-line analysis at a later date. Records used for illustrative purposes were replaced into a chart recorder (Gould 2200) which filtered the data at ~140 Hz. The potential across the membrane patch is described following the usual sign convention for membrane potential (i.e., inside negative). Outward current (defined as current flowing from the inside to the extracellular side of the membrane) is shown as upward deflections in all traces. Single channel current analysis was determined off-line by use of a programme that incorporates a 50% threshold crossing parameter to detect events (Dempster, 1988) and run on an Apricot XEN-Xi 286/45 microcomputer. Data segments between 30 and 60s were replayed at the recorded speed and filtered at 1.0 kHz (− 3bd; 8-pole Bessel) and digitally sampled at 5.0 kHz with a Data Translation 2801A interface The average channel activity ($N_f.P_o$) where N_f is the number of functional channels in the patch and P_o is the open state probability, was determined by measuring the total time spent at each unitary current level and expressed as a proportion of the total time recorded (Kozlowski *et al.*, 1989; Kozlowski & Ashford, 1990). Changes in $N_f.P_o$ as a result of drug effects are expressed as a percentage of control.

To obtain whole-cell K_{ATP} currents, the cell was voltage-clamped at a holding potential of − 70mV and alternate ± 10mV pulses of 200 ms duration were applied every 2 s (Trube *et al.*, 1986; Kozlowski *et al.*, 1989; Kozlowski & Ashford, 1990). The effects of diazoxide were quantified by measuring the amplitude of the current response during exposure to the drug (I_D) and comparing those recorded under control conditions (I_C) immediately preceding drug application. Because the effect of diazoxide was transient the value for I_D was taken at the peak of the response. Due to the presence of current run-down the inhibitory effects of diazoxide were quantified by taking a value 2.5 min after diazoxide application (Kozlowski *et al.*, 1989).

Solutions

Before use, cells were washed with normal bath saline which contained (in mM): NaCl 135, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, N-2-hydroxyethypiperazine-N'-2-ethane-sulphonic acid (HEPES) 10; pH 7.4 with NaOH (solution A) and this solution was also used to bathe the cells during whole-cell current and outside-out single channel current recordings. For both these configurations the recording pipette contained solution B which consisted of (in mM): KCl 140, $MgCl_2$ 1, $CaCl_2$ 2, K^+-ethyleneglycol-*bis* (β-aminoethyl-ether)-N,N'-tetraacetic acid (KEGTA) 10, HEPES 10; pH 7.2 with KOH resulting in a free calcium and magnesium concentration of ~20 nM and 0.65 mM, respectively. In the presence of nucleotides in the pipette, the concentration of Mg^{2+} was increased to compensate for chelation by the nucleotides, thereby maintaining the free Mg^{2+} concentration at 0.65 mM. Thus for triphosphate nucleotides at concentrations of 0.1, 0.3 and 1.0 mM the concentration of $MgCl_2$ was increased to 1.1, 1.3 and 1.8 mM, respectively. Similarly when 0.1 mM tripolyphosphate or adenosine tetraphosphate was added to solution B the concentration of Mg^{2+} was increased to 1.1 mM, and for 0.1 mM adenosine 5'-diphosphate (ADP), it was raised to 1.05 mM. It was assumed that the affinities of Mg^{2+} for adenylyl-(β,γ-methylene) diphosphate (AMP-PCP), adenosine 5'-o-[3-thioitriphosphate] (ATPγS), guanosine 5'-o-[3-thioitriphosphate] (GTPγS), cytosine 5'-triphosphate (CTP), AMP-PNP and ATP were identical (Yount, 1975). No adjustment of Mg^{2+} concentration was made when adenosine 5'-monophosphate (AMP) or adenosine was added to this solution. When examining the effects of diazoxide in the presence of manganese (Mn^{2+}) ions and AMP-PCP (0.1 mM) intracellularly the 1.1 mM $MgCl_2$ present in solution B was replaced by 1.1 mM $MnCl_2$. In some experiments solution B was replaced with solution C consisting of (mM): KCl 140, $CaCl_2$ 4.6, KEGTA 10 and HEPES 10; pH 7.2 with KOH which resulted in free Ca^{2+} and Mg^{2+} concentration of ~25 nM and < 5.0 nM respectively.

For inside-out patches the extracellular surface was bathed in solution D which contained (in mM): KCl 140, $MgCl_2$ 1, $CaCl_2$ 1 and HEPES 10; pH 7.4 with KOH, whilst the intracellular surface was bathed in solution E, composition (mM): KCl 140, $CaCl_2$ 0.9, $MgCl_2$ 1.0, KEGTA 1.0 and HEPES 10.0; pH 7.4 with KOH which resulted in free Mg^{2+} and Ca^{2+} concentrations of 1.0 mM and < 0.4 μM, respectively. In some experiments triphosphate nucleotides at a concentration of 0.1 mM were present in solution E, and therefore the concentration of Mg^{2+} was increased to 1.1 mM to maintain the free Mg^{2+} concentration of 1.0 mM. The concentration of free divalent cations was determined by use of 'METLIG' a programme for calculating metal ion/ligand binding (England, P. & Denton, R., University of Bristol).

Solutions containing diazoxide were prepared freshly before each experiment (from a 30 to 40 mM stock solution in 0.1 M KOH). Changes in pH due to addition of diazoxide and its vehicle were compensated for in all solutions. All nucleotides were prepared as 20 and 30 mM stock solutions in water. Drugs were applied to cell-free patches or whole-cells by exchanging the bath solution by a gravity feed system, at a rate of approximately 0.5 ml s^{-1}. This allowed complete solution exchange within 45 s (cells were not continuously superfused by the bathing solution). ATP (K^+ salt), AMP-PNP (Li^+ salt), ADP (K^+ salt), AMP (Na^+ salt), adenosine, sodium tripolyphosphate and adenosine tetraphosphate (Na^+ salt), GTPγS (Li^+ salt), CTP (Na^+ salt) and polymethylsulphonylfluoride (PMSF) were obtained from Sigma (Poole, Dorset). ATPγS (Li^+ salt) and AMP-PCP (Li^+ salt) were obtained from Boehringer (Mannheim, Germany). Diazoxide was donated by Glaxo Pharmaceuticals (Greenford, England). All experiments were conducted at room temperature (22–25°C).

All data in text, tables and figures are presented as mean values ± s.e.mean unless otherwise stated. The statistical significance between experimental groups was assessed by Student's *t* test for unpaired data.

Preparation of cell lysate

CRI-G1 cells were grown in 50 ml tissue culture flasks (Nunclon Ltd.), until 80% confluent. Cells were then taken up in 10 ml of solution containing trypsin (1 mg ml^{-1}), NaCl (137 mM), KCl (5 mM), NaH_2PO_4 (1.1 mM), KH_2PO_4 (1.1 mM), glucose (6 mM) and phenol red (0.001%); pH 7.4. Subsequently, 10 ml of culture medium (Dulbecco's modified Eagle's medium; Gibco) containing 10% foetal calf serum (Gibco), glucose (10 mM), penicillin (50 mg ml^{-1}; Gibco) and streptomycin (50 mg ml^{-1}; Gibco) was added. Following centrifugation at 185 *g* for 5 min the supernatant was discarded and the pellet re-suspended in 10 ml of culture medium and the cells counted. This centrifugation procedure was repeated and the pellet washed in 20 ml of solution A and centrifuged for a third time. The resultant pellet was re-suspended in 1 to 1.5 ml of distilled water and allowed to lyse on ice for 15 min. The lysate was centrifuged at 2950 *g* for 5 min, the pellet discarded and an equal volume of solution B (2 × concentrated) added to the supernatant. On occasion 0.2 mM ATP was added to solution B (2 × concentrated) which under these conditions contained 2.2 mM $MgCl_2$. Thus, the free Mg^{2+} concentration in the diluted lysate was main-

tained at 0.65 mM. Prior to filling of electrodes the diluted lysate was passed through a 0.45 μm syringe filter (Millex-GA). This electrode-filling solution contained lysate from a mean of 5.3 ± 0.07 million cells ml^{-1} ($n = 5$) and had a protein concentration of 0.12 to 0.44 mg ml^{-1} determined according to the method of Bradford (1976) using a protein assay kit (Bio-Rad Laboratories GmbH). The ATP content within the cell lysate was assayed with a luciferase bioluminescence assay kit (Boehringer Mannheim Ltd.), based on the method described by Deluca (1969). Luminescence from the samples was recorded with a luminometer (Canberra Packard, Pico-lite). In the experiments where exogenous ATP at a final concentration of 0.1 mM had been added to the lysate (see above), the measured ATP concentration ranged from 0.4 to 15 μM, presumably as a result of its rapid breakdown by endogenous ATPases present within the lysate.

Results

Single K_{ATP} channel studies

In our previous study, using inside-out membrane patches isolated from CRI-G1 cells, we demonstrated that suppression of K_{ATP} single channel current activity with Mg-AMP-PNP or Mg-free ATP resulted in the inability of diazoxide to induce activation (Kozlowski *et al.*, 1989). We have extended these observations to include other nucleotides and have shown that, in agreement with our earlier study, diazoxide is unable to activate K_{ATP} channel currents recorded from isolated membrane patches in the presence of Mg^{2+} complexes of other non-hydrolysable adenine nucleotides. For example, application of diazoxide (0.6 mM) to the extracellular aspect of outside-out patches was unable to elicit activation of K_{ATP} channel currents in the presence of 0.1 mM Mg-AMP-PCP in the electrode solution (Figure 1a). In fact under these conditions, diazoxide induced a $74.7 \pm 16.5\%$ ($n = 4$) reduction in K_{ATP} channel activity relative to control. Upon washout of the drug, little or no reversal was observed. A similar decrease ($75.6 \pm 9.0\%$; $n = 4$) in K_{ATP} channel activity by extracellularly applied diazoxide (0.6 mM) was observed when 0.1 mM Mg-ATPγS was present in the pipette solution. The application of diazoxide (0.6 mM) to the intracellular membrane surface of inside-out patches, where it is more effective in causing K_{ATP} channel activation (Kozlowski *et al.*, 1989), was also unable to reverse near-maximal inhibition or submaximal inhibition induced by 0.1 mM or 0.01 mM Mg-ATPγS, respectively (Figure 1b,c). For example, the application of Mg-ATPγS (0.01 mM) reduced K_{ATP} channel activity by $83.9 \pm 4.5\%$ ($n = 4$) with a slight decrease ($82.6 \pm 9.7\%$; $n = 4$) being observed in the presence of diazoxide.

Whole-cell K_{ATP} currents

On repeating these experiments with non-hydrolysable analogues on whole-cell K_{ATP} currents a completely different result was obtained. The effects of diazoxide (0.6 mM) were examined on whole-cell K_{ATP} currents using a range of nucleotides and related compounds added to solution B at the concentration (0.1 mM) used in cell-free patch experiments. Diazoxide was tested on these currents only after the peak whole-cell K_{ATP} current had been reached (Trube *et al.*, 1986; Kozlowski *et al.*, 1989). In direct contrast to the isolated patch data, application of diazoxide produced a marked activation of whole-cell K_{ATP} currents when Mg-AMP-PNP dialysed the cell interior (Figure 2a). This effect declined rapidly with time in the continued presence of diazoxide and its time course was unaffected by washout of the drug. Subsequent re-application of diazoxide (at least 4 min after washout of the first application of the drug, throughout) resulted in a significant inhibition of the whole-

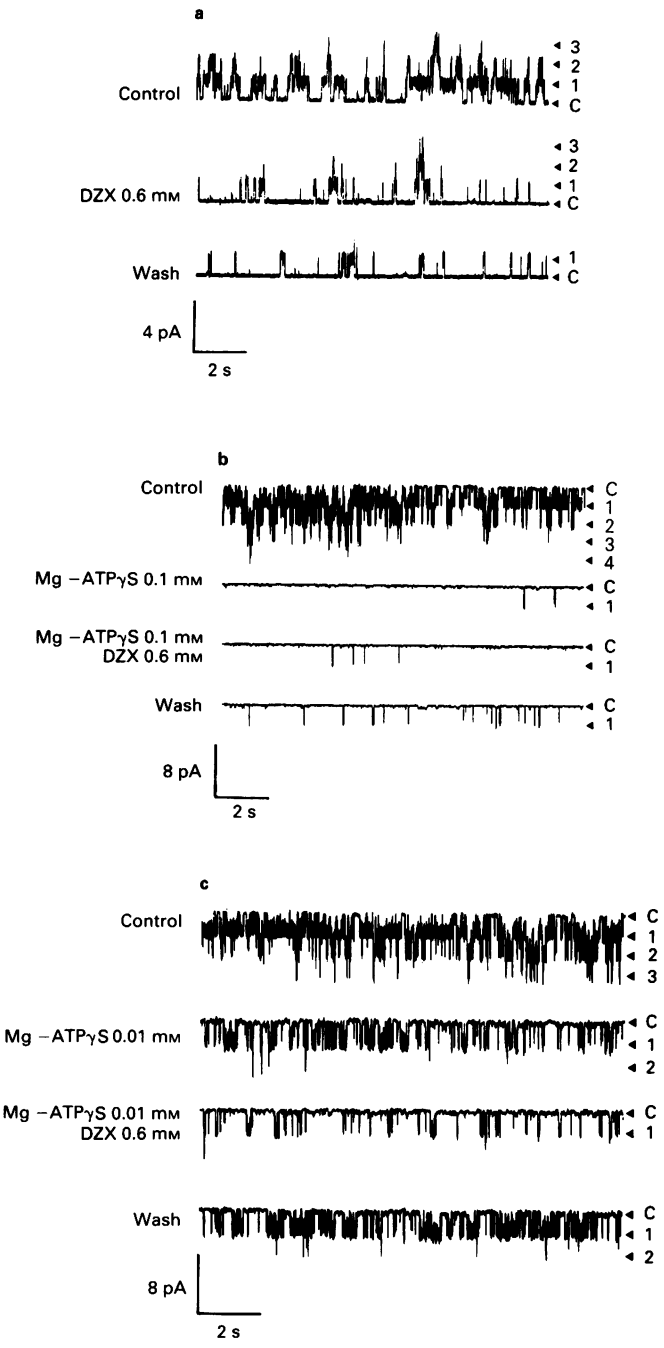

Figure 1 (a) Single channel currents recorded from an outside-out membrane patch exposed to a physiological cation gradient and voltage-clamped at a membrane potential of 0 mV. The bath contained solution A whilst the pipette contained solution B to which 0.1 mM Mg-AMP-PCP had been added (Control). Application of 0.6 mM diazoxide (DZX) to the extracellular surface of the patch inhibited K_{ATP} channel activity an effect not reversed upon wash. The $N_f.P_o$ values were as follows: control 0.947; diazoxide 0.080; wash 0.065. (b) Single channel currents recorded from an inside-out patch exposed to symmetrical 140 mM KCl, held at a membrane potential of − 50 mV. The extracellular surface was in contact with solution D whilst solution E bathed the intracellular surface. Application of 0.1 mM ATPγS virtually abolished K_{ATP} channel opening and concomitant addition of 0.6 mM diazoxide (DZX) had no effect. The inhibition by Mg-ATPγS was poorly reversed on wash. The values for $N_f.P_o$ were as follows: control 0.303; Mg-ATPγS 0.001; Mg-ATPγS and diazoxide 0.001; wash 0.008. (c) A similar experiment to (b) in which 0.01 mM ATPγS was used. At this concentration, where K_{ATP} channel currents were less inhibited, diazoxide (0.6 mM) was still ineffective at increasing channel activity. Only the inhibitory effect of diazoxide was apparent. Washing out of both the Mg-ATPγS and diazoxide resulted in some reversal. The values for $N_f.P_o$ were as follows: control 0.760; Mg-ATPγS 0.216; Mg-ATPγS and diazoxide 0.096; wash 0.154. For abbreviations, see text.

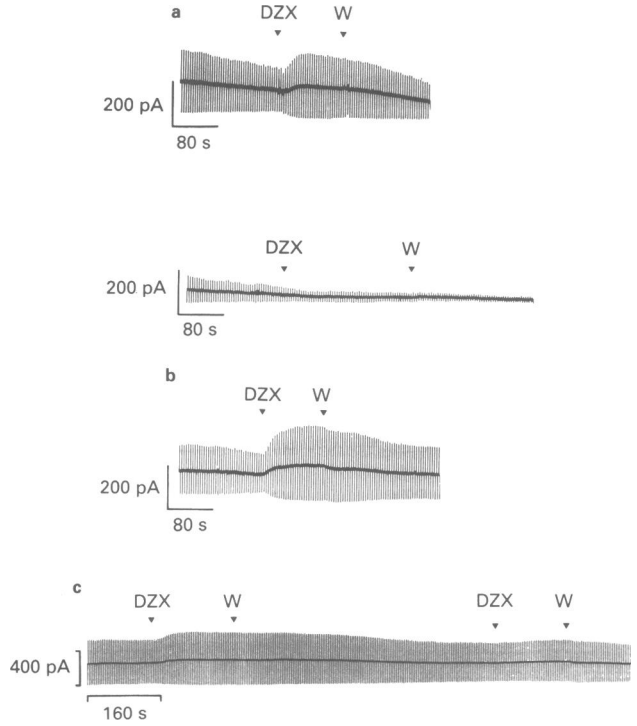

Figure 2 Recordings of whole-cell, voltage-clamped K_{ATP} currents. Cells were voltage clamped at a holding potential of − 70 mV and alternate ± 10 mV pulses of 200 ms duration applied every 2 s. Both records shown in (a) are taken from the same cell bathed in solution A, whilst the pipette contained solution B to which 0.1 mM Mg-AMP-PNP had been added. The upper trace shows the effect of 0.6 mM diazoxide (DZX) soon after the peak whole-cell current had been reached. Note the transient increase in the amplitude of the current pulses (denoted by the vertical lines) and the concomitant change in holding current (indicated by the horizontal line). Washing out of the diazoxide (W) made little difference to the time course for K_{ATP} current run-down. The lower trace is a continuation of the upper with a break of approximately 1 min. A second application of 0.6 mM diazoxide (DZX) was ineffective in eliciting activation of the K_{ATP} current. (b) The effect of diazoxide upon whole-cell K_{ATP} currents recorded under the conditions described above but with 0.1 mM Mg-ATPγS dialysing the cell interior. Diazoxide (0.3 mM) induced an increase in the amplitude of the currents in response to the voltage pulses with a concomitant increase in the holding current. This effect waned gradually with time of exposure and appeared relatively unaffected by the washout of the drug. A second application of the same concentration of diazoxide resulted in a reduced response which also gradually diminished with time. (c) The effect of diazoxide (0.6 mM) on cells dialysed with 0.1 mM Mg-ATP. Note the sustained activating effect of diazoxide under these conditions. For abbreviations, see text.

cell K_{ATP} current (Figure 2a and Table 1). In the presence of Mg-AMP-PCP in the pipette, the activation elicited by diazoxide was greater than in the presence of a similar concentration of Mg-AMP-PNP although a subsequent application of diazoxide, once the response had returned to control, resulted in no significant effect (Table 1). When Mg-ATPγS dialysed the cell interior (Figure 2b) the activation induced by diazoxide was similar to that observed when Mg-AMP-PNP was present intracellularly and a second application also had no significant effect (Table 1). In general it was noted that the diazoxide-induced activation of whole-cell K_{ATP} currents with Mg-ATP present intracellularly was generally longer-lived (Figure 2c) when compared with cells dialysed with the other, poorly hydrolysed, nucleotides. A quantitative assessment of the peak response obtained to diazoxide using various adenine nucleotides and phosphates is given in Table 1.

In order to test whether the diazoxide-induced activation depended upon the K_{ATP} channels being inhibited by the dialysing nucleotide, experiments were performed in which the cells were dialysed with ADP, AMP or adenosine and the effects of diazoxide examined. The results, shown in Table 1, indicate that the potency of diazoxide progressively decreases with a reduction in the length of the phosphate chain (i.e. ATP > ADP > AMP > adenosine). In the presence of adenosine tetraphosphate or sodium tripolyphosphate, diazoxide was poorly effective or ineffective in producing activation. A second application of diazoxide, irrespective of which nucleotide dialysed the cell interior, failed to produce a significant activation of the whole-cell K_{ATP} current (Table 1). This lack of effect may be due to the loss of an unknown cellular component during the course of whole-cell recording.

In cells dialysed with Mg-AMP-PCP, diazoxide induced a concentration-dependent enhancement of whole-cell K_{ATP} currents (Figures 3a,b) which generally declined in the continued presence of the drug with a time course unaffected by washout of the diazoxide. The rate of the current decline appeared to decrease with increasing concentration of diazoxide. The relationship between diazoxide-induced activation and the concentration of the dialysing nucleotide was also examined. Data obtained from cells dialysed with a range of Mg-AMP-PCP concentrations revealed that as the concentration of Mg-AMP-PCP was increased the amplitude of the whole-cell K_{ATP} current diminished (Figure 4a,b) indicating that this analogue is also a potent inhibitor of K_{ATP} channel activity. The half-maximal inhibitory concentration for this nucleotide is approximately 100 μM, a value similar to that obtained for Mg-ATP (Sturgess *et al.*, 1988). As the overall conductance of the cell diminished, application of diazoxide (0.3 mM) resulted in a progressively larger degree of activation relative to pre-drug levels (Figure 4c). Note that mixed results were obtained with 1 mM AMP-PCP dialysing the cell

Table 1 Whole-cell K_{ATP} current activation induced by diazoxide (0.6 mM) in the presence of various dialysing nucleotides and related compounds (0.1 mM)

Dialysing nucleotide or related molecule	*1st Application* (I_D/I_C)	*2nd Application* (I_D/I_C)
AMP-PCP	*1.85 ± 0.15 ($n = 4$)	1.11 ± 0.13 ($n = 4$)
ATPγS	*1.48 ± 0.17 ($n = 5$)	1.10 ± 0.07 ($n = 5$)
AMP-PNP	*1.45 ± 0.10 ($n = 5$)	*0.77 ± 0.06 ($n = 5$)
#ATP	*1.27 ± 0.05 ($n = 7$)	1.18 ± 0.10 ($n = 5$)
ADP	*1.26 ± 0.13 ($n = 5$)	1.06 ± 0.12 ($n = 2$)
AMP	*1.15 ± 0.04 ($n = 3$)	1.19 ± 0.12 ($n = 2$)
Adenosine	*1.08 ± 0.02 ($n = 3$)	ND
Adenosine tetraphosphate	*1.07 ± 0.02 ($n = 2$)	1.01 ± 0.03 ($n = 2$)
Sodium tripolyphosphate	1.04 ± 0.01 ($n = 3$)	ND
Various (saline control)	1.00 ± 0.02 ($n = 8$)	ND

For abbreviations, see text.
Cells were dialysed with solution B, consequently nucleotides are predominantly the Mg^{2+} complexed species.
*$P < 0.05$ relative to control value obtained on perfusion of the bath with normal saline containing no diazoxide. On some occasions the effects of a second application were not determined (ND).
#Taken from Kozlowski *et al.* (1989).

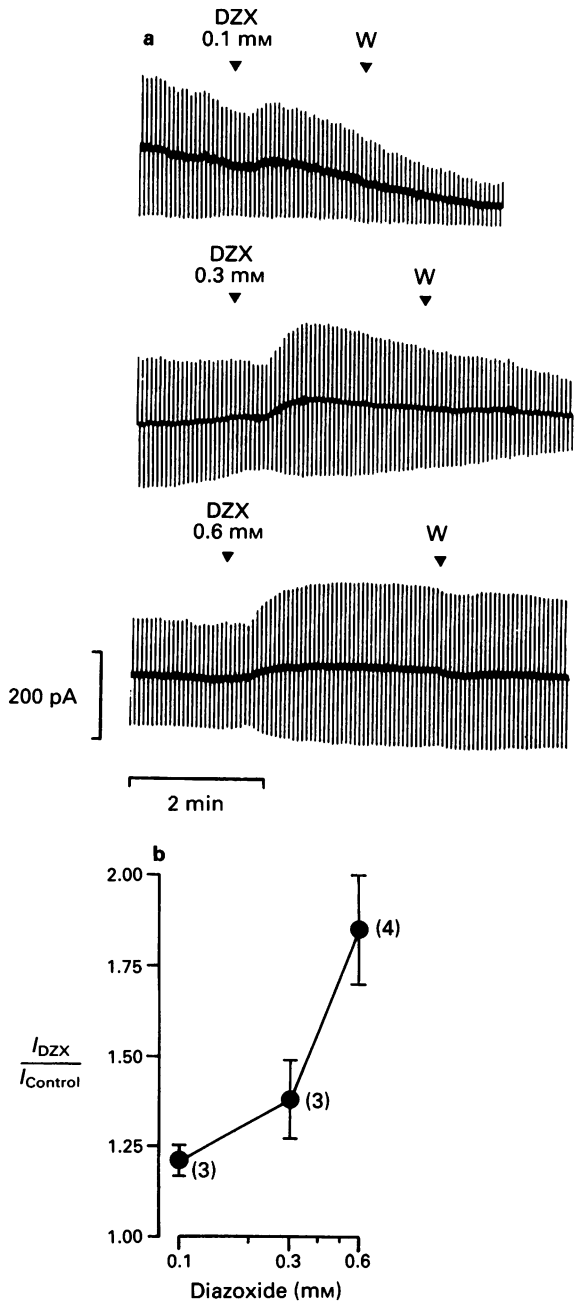

Figure 3 (a) Effects of different concentrations of diazoxide upon whole-cell K_{ATP} currents. In all three records (taken from different cells), solution A bathed the cell exterior, whilst solution B containing 0.1 mM Mg-AMP-PCP dialysed the interior of the cells. Application of diazoxide (DZX) induced a concentration-dependent increase in the whole-cell K_{ATP} current. Note the time course of the decline of whole-cell K_{ATP} currents activated by diazoxide varied between cells and was generally unaffected by washout of the drug (W). (b) Concentration-response relationship for activation of K_{ATP} currents (I) by diazoxide under the conditions described in (a). $I_{DZX}/I_{Control}$ is the ratio of the peak current obtained in the presence of diazoxide (I_{DZX}) and the current amplitude under control conditions ($I_{Control}$) immediately prior to application of the drug. Vertical lines represent the ± s.e.mean values with the number of determinations being indicated in parentheses adjacent to the corresponding data point. Note only one concentration of diazoxide was tested per cell. For abbreviations, see text.

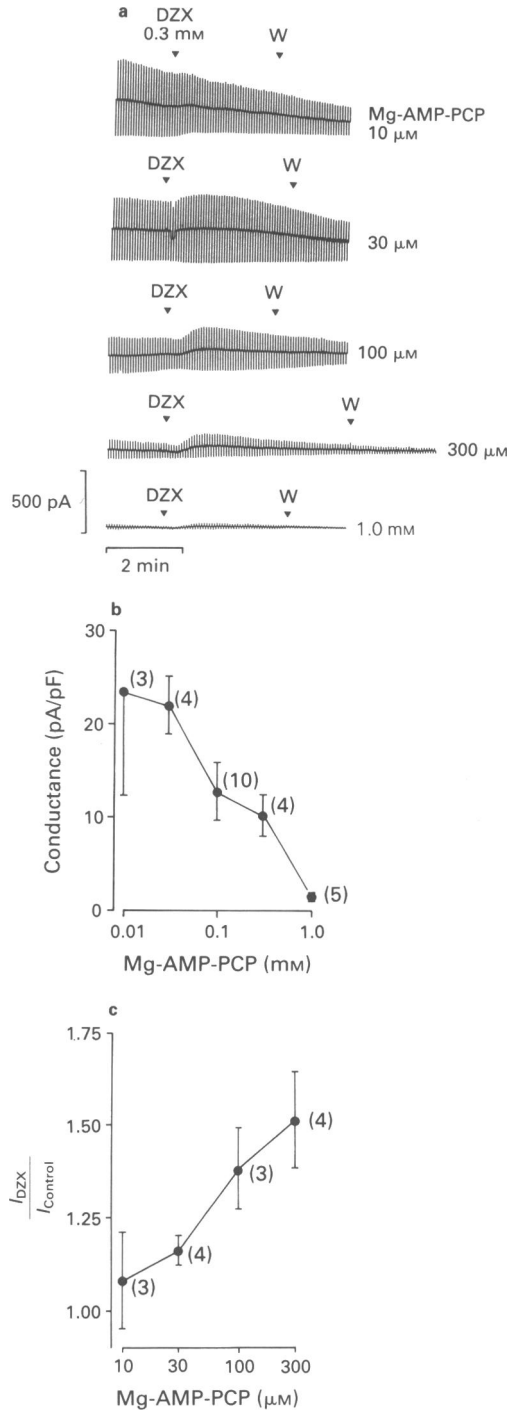

Figure 4 (a) Effect of 0.3 mM diazoxide (DZX) upon whole-cell K_{ATP} currents recorded from cells dialysed with various Mg-AMP-PCP concentrations. The five records shown were recorded from different cells. Each cell was bathed in solution A whilst the recording pipettes contained solution B to which different concentrations of Mg-AMP-PCP were added. The concentration of Mg-AMP-PCP dialysing the cell interior is indicated to the right of each current record. Note that the magnitude of the whole-cell K_{ATP} current became smaller as the concentration of Mg-AMP-PCP was increased, and that as the concentration of the nucleotide was increased the activation induced by diazoxide became larger relative to the amplitude of the current prior to application of the drug. (b) Relationship between the mean peak whole-cell K_{ATP} current (pA/pF) and the concentration of Mg-AMP-PCP dialysing the interior of the cells. The vertical lines indicate ± s.e.mean values whilst the number of determinations is shown adjacent to its corresponding data point. (c) Concentration-response relationship for 0.3 mM diazoxide applied to cells dialysed with a range of Mg-AMP-PCP concentrations under the recording conditions described in (a). Diazoxide has a progressively greater activating effect as the concentration of the nucleotide is increased. Data for 1 mM Mg-AMP-PCP have not been included (see text). For abbreviations, see text.

interior (increases in I_D/I_C of between 0.07 and 7.62 occurred). One explanation for this variability is that, because the whole-cell current was very small under these conditions, it was difficult to determine the current maximum and conse-

quently diazoxide may have been added after the peak and following substantial K_{ATP} current run-down in some experiments. It has been demonstrated that diazoxide does not reverse the rundown process (Sturgess *et al.*, 1989).

In order to determine whether the activation produced by diazoxide was Mg^{2+}-dependent, cells were dialysed with solution C which was nominally Mg^{2+}-free. Application of diazoxide to cells which were dialysed with nucleotides in Mg^{2+}-free conditions resulted in no activating action and a significant inhibition of the whole-cell K_{ATP} current by diazoxide was observed for all nucleotides tested (Table 2 and Figure 5). These inhibitory effects were quantified after 2.5 min exposure to diazoxide since a steady-state inhibition was not attained under these conditions (Kozlowski *et al.*, 1989). There was little variation in the amount of inhibition induced by diazoxide in the absence or in the presence of different dialysing nucleotides. Thus, activation of the whole-cell K_{ATP} current by diazoxide was dependent upon the presence of intracellular Mg^{2+} ions. Substitution of the Mg^{2+} present in solution B by Mn^{2+}, in the presence of 0.1 mM AMP-PCP, prevented K_{ATP} current activation by 0.3 mM diazoxide (I_D/I_C; 1.05 ± 0.05, $n = 6$). This effect was significantly ($P < 0.05$) smaller when compared to the increase observed with 0.1 mM Mg-AMP-PCP intracellularly (I_D/I_C; 1.38 ± 0.11, $n = 3$). These data strongly suggest that the diazoxide-induced activation process has a specific requirement for Mg^{2+} ions.

Base specificity

Application of diazoxide (0.6 mM) to cells dialysed with solution B containing 0.1 mM Mg-GTPγS (Figure 6a) resulted in an increase in the whole-cell K_{ATP} current (I_D/I_C; 1.43 ± 0.13, $n = 4$) similar to that induced in the presence of 0.1 mM Mg-ATPγS (see Table 1). In three of the four cells the effect of a subsequent application of diazoxide after washout was examined. No significant second response being observed (I_D/I_C; 1.16 ± 0.14, $n = 3$). The K_{ATP} current activation induced by diazoxide in the presence of GTPγS was also Mg^{2+}-dependent (Figure 6b). In the absence of Mg^{2+}, diazoxide inhibited the K_{ATP} current to 0.70 ± 0.08 ($n = 3$) of control, a value similar to that obtained in other Mg^{2+}-free conditions (Table 2). In contrast, cells dialysed with a pyrimidine base nucleotide, 0.1 mM Mg-CTP (Figure 6c), were relatively insensitive to 0.6 mM diazoxide (I_D/I_C; 1.11 ± 0.09, $n = 3$).

Protease modulation of diazoxide action

Trube *et al.* (1989) reported that intracellularly-applied trypsin produced an increase in the K_{ATP} current and reduced its sensitivity to nucleotides in pancreatic β-cells. Therefore the effects of trypsin were determined on the diazoxide-induced, Mg^{2+}-nucleotide dependent activation of K_{ATP} currents. In the presence of 0.1 mg ml^{-1} trypsin and 0.1 mM Mg-AMP-PCP, intracellularly, the whole-cell conductance was significantly ($P < 0.05$) higher than the conductance of cells dialysed with 0.1 mM Mg-AMP-PCP alone (Table 3) and was similar to that obtained for these cells in the absence of intracellular ATP under these recording conditions (Sturgess *et al.*, 1988). Furthermore in the presence of trypsin, diazoxide failed to activate the whole-cell K_{ATP} current (Figure 7) which remained at the increased level. Addition of 1 mM PMSF (a serine protease inhibitor which prevents the proteolytic action of trypsin) to the intracellular dialysing solution together with trypsin (0.1 mg ml^{-1}) and Mg-AMP-PCP (0.1 mM) restored the ability of diazoxide to induce activation of the whole-cell K_{ATP} current (Figure 7). In addition, the presence of PMSF opposed the stimulatory effect of trypsin on the whole-cell K_{ATP} conductance in the presence of 0.1 mM Mg-AMP-PCP (Table 3). These latter observations confirm that the effects of trypsin are the result of its proteolytic activity.

Table 2 Inhibitory action of diazoxide (0.6 mM) on whole-cell K_{ATP} currents recorded from cells dialysed with various nucleotides in the absence of Mg^{2+} ions (Solution C)

Nucleotide	I_D/I_C
AMP-PCP 0.1 mM	0.73 ± 0.07 ($n = 4$)
ATPγS 0.01 mM	0.79 ± 0.04 ($n = 3$)
AMP-PNP 0.01 mM	0.79 ± 0.02 ($n = 3$)
*ATP 0.1 mM	0.87 ± 0.03 ($n = 6$)
*ATP 0.01 mM	0.84 ± 0.03 ($n = 4$)
AMP 0.1 mM	0.81 ± 0.03 ($n = 3$)
*No nucleotide (solution C)	0.75 ± 0.05 ($n = 7$)

For abbreviations, see text.
*Taken from Kozlowski *et al.* (1989).

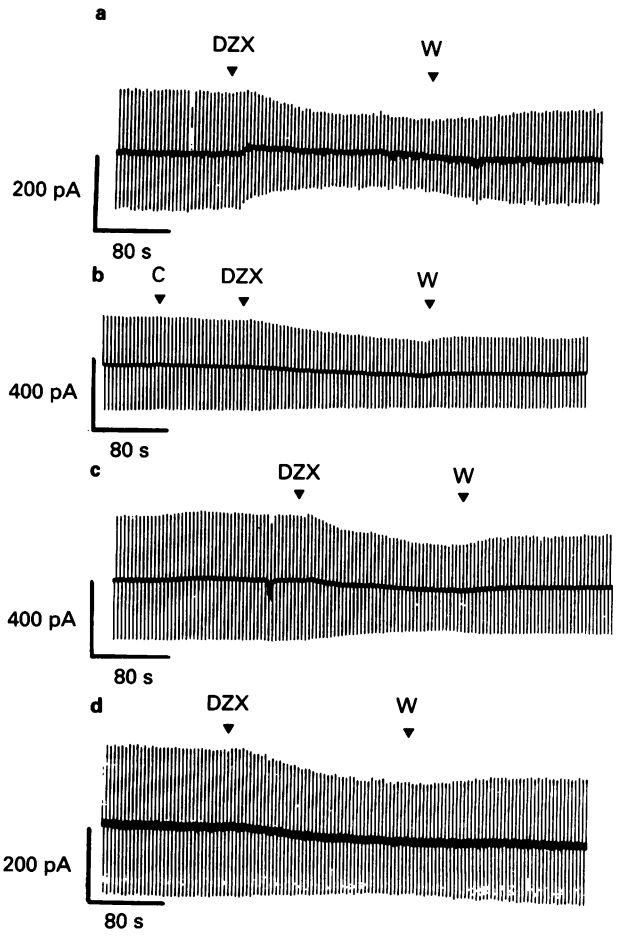

Figure 5 Recordings of whole-cell voltage clamped K_{ATP} currents. Records shown in (a), (b), (c) and (d) are taken from different cells. In all four cases cells were bathed in solution A whilst the pipette contained solution C nominally free of Mg^{2+} ions (< 5 mM) to which nucleotides were added. The records shown were obtained from cells dialysed with (a) 0.1 mM AMP-PCP, (b) 0.01 mM AMP-PNP, (c) 0.01 mM ATPγS and (d) 0.1 mM AMP. In the presence of all the nucleotides application of 0.6 mM diazoxide (DZX) induced an inhibition of the whole-cell K_{ATP} current. Note, in (b) a control perfusion of the bath with bathing solution containing no diazoxide (indicated by the letter C on the current trace) did not alter the magnitude of the current. For abbreviations, see text.

Effects of CRI-G1 cell lysate

In all the aforementioned experiments, diazoxide was added after the peak whole-cell K_{ATP} current had been reached, with the result that the maximal activation did not exceed 100%

of the control levels. However, when diazoxide was added soon after formation of the whole-cell configuration (i.e. before there is a substantial loss, due to dialysis, of intracellular contents as shown by the increase in overall cell conductance (see Kozlowski *et al.*, 1989) a very large activation (>350%, $n = 3$) was observed (Figure 8a). This difference may well be indicative of a process which depends upon a substance lost from the cell during dialysis. To examine this possibility, cells were dialysed with an electrode containing solution B, exogenous ATP and diluted cell lysate prepared according to the protocol described in the Methods. Application of diazoxide to cells dialysed with the lysate solution increased the whole-cell K_{ATP} current (Figure 8b) with I_D/I_C being increased to 1.67 ± 0.34 ($n = 3$) and 1.54 ± 0.15 ($n = 9$) by 0.6 and 0.3 mM diazoxide, respectively. The ATP content of the diluted lysate solution, measured by a luciferase assay, was between 0.4 and 14.8 μM. In control experiments, diazoxide (0.3 mM) was without effect in cells dialysed with 5 μM ATP alone (Figure 8c), with I_D/I_C remaining at approximately control levels (0.98 ± 0.05; $n = 3$). Furthermore, application of diazoxide (0.6 mM) elicited activation (I_D/I_C; 1.66 ± 0.55, $n = 3$) in the presence of lysate but with ATP omitted. Hence, under these whole-cell recording conditions, activation is not solely due to the presence of a nominal concentration of Mg-ATP but also dependent largely upon the presence of some intracellular regulatory factor.

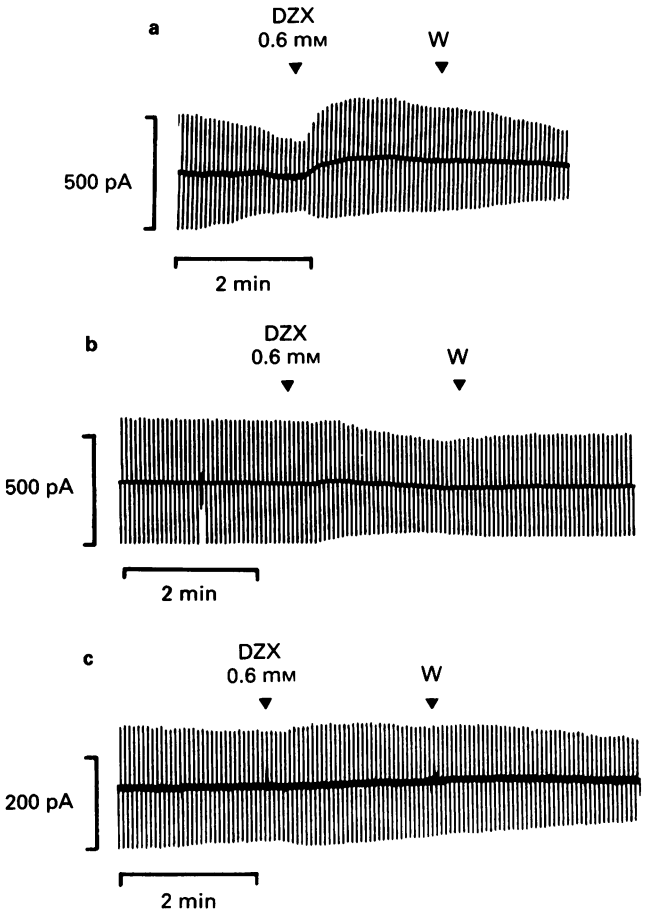

Figure 6 Whole-cell K_{ATP} currents recorded from three different cells with purine and pyrimidine bases. The cells were bathed in solution A. (a) Currents recorded from a cell with solution B containing 0.1 mM Mg-GTPγS dialysing the cell interior. Application of 0.6 mM diazoxide (DZX) induced a marked activation of the K_{ATP} current which waned gradually with time and was unaffected by washout (W) of the drug. (b) Currents recorded from a cell with solution C containing 0.1 mM GTPγS in the absence of Mg^{2+} ions dialysing the cell interior. Application of 0.6 mM diazoxide resulted in an inhibition of whole-cell K_{ATP} currents which was poorly reversed upon washout of the drug (W). (c) Currents recorded from a cell with solution B containing the pyrimidine base Mg-CTP (0.1 mM) as the dialysing solution. Application of 0.6 mM diazoxide resulted in a slight activation of the currents with washout of the drug (W) having little effect. For abbreviations, see text.

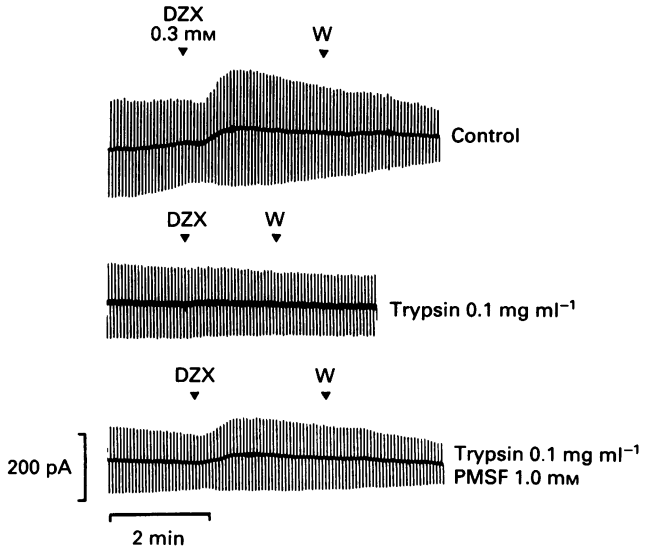

Figure 7 Effects of trypsin upon diazoxide induced activation of whole-cell K_{ATP} currents. Each trace shown was recorded from a different cell. Cells were bathed in solution A with solution B, which also contained 0.1 mM AMP-PCP, as the dialysis solution. Application of 0.3 mM diazoxide (DZX) to the cell under these conditions resulted in a marked activation of the currents (top trace). When 0.1 mg ml^{-1} trypsin was added to the dialysing solution, in the continued presence of AMP-PCP (0.1 mM), diazoxide was unable to elicit an activation (centre trace). In contrast, cells with both 0.1 mg ml^{-1} trypsin and 1.0 mM PMSF in the presence of 0.1 mM Mg-AMP-PCP were responsive to 0.3 mM diazoxide which elicited an activation of the currents (bottom trace). Washout of diazoxide is indicated by (W) on each current record. For abbreviations, see text.

Discussion

Previous studies on isolated patches and whole-cell configurations of insulin-secreting cells have shown that K_{ATP} channel

Table 3 The lack of effect of diazoxide (0.6 mM) on K_{ATP} currents recorded in the presence of intracellular trypsin

Pipette solution	*Whole-cell conductance* (pA/pF)	*Effect of DZX* (I_D/I_C)
Mg-AMP-PCP 0.1 mM	12.7 ± 3.1 ($n = 10$)	1.38 ± 0.11 ($n = 3$)
Mg-AMP-PCP 0.1 mM	27.9 ± 6.6 ($n = 8$)	0.98 ± 0.04 ($n = 9$)
trypsin 0.1 mg ml^{-1}		
Mg-AMP-PCP 0.1 mM	15.4 ± 6.1 ($n = 4$)	1.30 ± 0.12 ($n = 4$)
trypsin 0.1 mg ml^{-1}		
PMSF 1.0 mM		

For abbreviations, see text.
For all experiments the cells were bathed in solution A whilst the cell interior was dialysed with solution B.

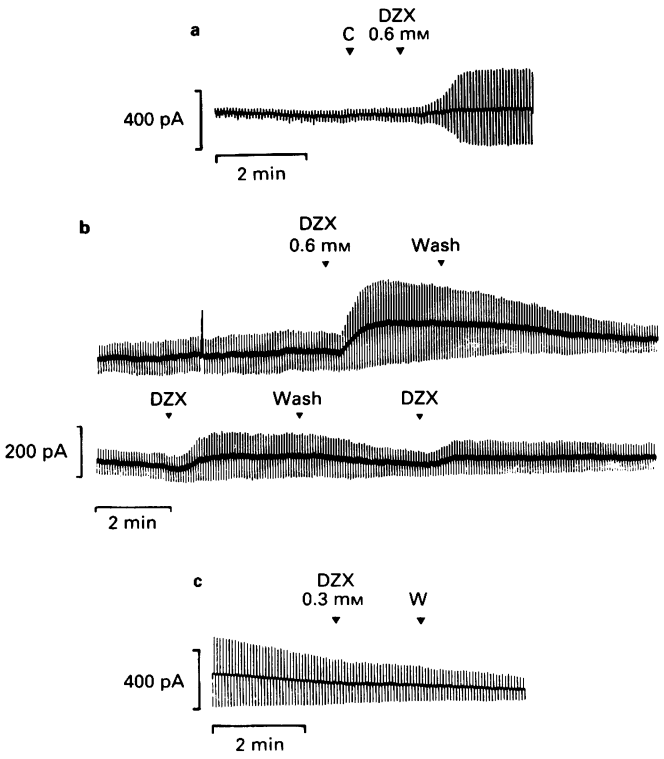

Figure 8 (a) Effect of diazoxide on whole-cell K_{ATP} currents soon after formation of whole-cell mode. The cell was bathed in solution A throughout the experiment, whilst solution C containing 0.1 mM AMP in the absence of Mg^{2+} ions dialysed the cell interior (conditions under which diazoxide does not normally activate K_{ATP} channels; cf. Table 2). Application of 0.6 mM diazoxide (DZX) to the cell soon after achieving a stable voltage-clamp but before the peak whole-cell K_{ATP} current was reached resulted in a large activation of the current. Note, a control perfusion of the bath with bathing solution containing no drug (indicated by the letter C on the current trace) did not alter the magnitude of the current at this stage of the experiment. (b) Effect of diazoxide on whole-cell K_{ATP} currents recorded from a cell bathed in solution A and with solution B, to which the lysate had been added, dialysing the cell interior. Note the large and transient nature of the activation induced by 0.6 mM diazoxide. (c) Effect of diazoxide on whole-cell K_{ATP} currents from a cell bathed in solution A and with solution B containing 5 mM ATP as the dialysis medium. Note that under these conditions, 0.3 mM diazoxide (DZX) is ineffective in eliciting K_{ATP} channel activation.

activation by diazoxide requires the presence of intracellular ATP (Trube *et al.*, 1986; Dunne *et al.*, 1987; Sturgess *et al.*, 1988; Kozlowski *et al.*, 1989). In cell-free patches isolated from insulin-secreting cells, this activation is dependent upon the presence of intracellular Mg-ATP suggesting that phosphorylation is involved (Kozlowski *et al.*, 1989; Dunne, 1989). The findings of the current study are consistent with such a mechanism. Thus, in cell-free patches, diazoxide was unable to activate K_{ATP} channels which were inhibited by the non-hydrolysable ATP analogue, Mg-AMP-PCP. Indeed, only the underlying inhibitory action of the drug was observed (see also Kozlowski *et al.*, 1989). Although Mg-ATPγS has been reported to substitute for Mg-ATP in many processes requiring phosphorylation (Yount, 1975), diazoxide did not activate K_{ATP} channels in cell-free patches in the presence of this nucleotide. Hence the enzyme(s) utilizing ATP, or the protein(s) accepting the phosphate(s) may be specific for ATP. These results parallel the findings of Ohno-Shosaku *et al.* (1987) that in inside-out patches isolated from mouse pancreatic β-cells, K_{ATP} channel refreshment (a transient increase in channel activity relative to control following washout of Mg-ATP) is only mediated by Mg-ATP, whereas Mg-AMP-PNP, Mg-AMP-PCP and Mg-ATPγS are ineffective. The lack of effect observed in the presence of Mg-ATPγS despite the effectiveness of Mg-ATP might be due to differences in the rates of thiophosphorylation, phosphorylation and dephosphorylation (Palvino *et al.*, 1985), suggesting that continuous hydrolysis of ATP is necessary for channel activation.

If phosphorylation is involved in K_{ATP} channel activation then either the channel (or a regulatory protein) has first to be phosphorylated to allow diazoxide to elicit its effect, or diazoxide itself must stimulate a kinase which subsequently mediates phosphorylation. Both protein kinase A and protein kinase C have been reported to increase K_{ATP} channel activity (De Weille *et al.*, 1989; Ribalet *et al.*, 1989), although an interaction of diazoxide with protein kinase C is perhaps unlikely since activation can occur in the absence of intracellular Ca^{2+} (Kozlowski *et al.*, 1989).

The whole-cell, voltage-clamp data clearly indicate that there is also a second mechanism for the activating action of diazoxide on K_{ATP} currents in this cell-line, which is also dependent upon the presence of a purine Mg-nucleotide phosphate in the dialysing solution. However, the activation elicited under these conditions is not phosphorylation-driven as it occurs in cells dialysed with AMP and ADP and the non-hydrolysable ATP analogues Mg-AMP-PNP and Mg-AMP-PCP. This activation is also Mg^{2+}-dependent as in the absence of Mg^{2+} ions, or in the presence of Mn^{2+} ions, a nucleotide-independent inhibitory effect of diazoxide is observed similar to that reported previously in the presence of Mg-free ATP (Kozlowski *et al.*, 1989). These results, using whole-cell and cell-free patch K_{ATP} current recording, imply that diazoxide activation of K_{ATP} channels may be mediated by two distinct processes. In isolated patches, activation by diazoxide appears to depend on phosphorylation whilst under whole-cell recording conditions it depends on the presence of a purine nucleotide and Mg^{2+} ions. It is therefore possible that, for the second mechanism, the Mg^{2+} complexed species of the nucleotide binds to a modulatory site, loosely associated with the channel, which is lost or altered upon patch excision. In support of this hypothesis is the observation that a relatively long-lived activation of the whole-cell K_{ATP} current occurs in cells dialysed with Mg-ATP when compared to cells dialysed with non-hydrolysable ATP analogues, implicating phosphorylation in the sustained effect. Furthermore, in agreement with the single channel data, the activating action of diazoxide in cells dialysed with Mg-ATPγS is similar in duration to that in cells dialysed with Mg-AMP-PCP and Mg-AMP-PNP, which suggests that ATPγS does not substitute for ATP in maintaining a longer-lived activation.

The ability of diazoxide to activate K_{ATP} channels in cells dialysed with AMP or ADP in the presence of Mg^{2+} ions indicates that activation is not due to reversal of K_{ATP} current inhibition since these nucleotides, particularly at the low concentration used in this study (0.1 mM), are poor K_{ATP} channel inhibitors in this cell-line (Sturgess, 1988) and other insulin-secreting cell types (Cook & Hales, 1984; Misler *et al.*, 1986; Ashcroft, 1988). Furthermore, a small but significant activation occurs in the presence of adenosine which is totally ineffective at inhibiting K_{ATP} channel activity in this cell-line (Sturgess, 1988) and RINm5F cells (Ribalet & Ciani, 1987). The small increase in K_{ATP} current observed in the presence of adenosine tetraphosphate also indicates that the triphosphate configuration is optimal. In addition, the relative lack of activation with sodium tripolyphosphate or CTP in the dialysing solution indicates that a purine base in conjunction with a phosphate is also necessary for diazoxide-induced activation. Further experiments using Mg-AMP-PCP, as the most potent nucleotide for diazoxide-induced activation of whole-cell K_{ATP} current, have demonstrated that this activation is diazoxide-concentration dependent (for a constant nucleotide concentration intracellularly) and nucleotide-concentration dependent (at a constant diazoxide concentration).

In the presence of trypsin and Mg-AMP-PCP intracellularly, the whole-cell conductance was increased to a level similar to that observed in the absence of nucleotides or divalent cations (Sturgess *et al.*, 1988; Kozlowski & Ashford, 1990); an effect prevented by the addition of PMSF to the dialysing solution. This observation suggests that proteolysis of some intracellular protein, susceptible to the proteolytic action of trypsin, is responsible for the increase in conductance. Trypsin was also found to prevent the activating action of diazoxide on whole-cell K_{ATP} current, an effect again inhibited by PMSF. In view of these data it is tempting to speculate that trypsin may functionally uncouple the putative nucleotide binding site from its effector, thereby preventing an activating action of diazoxide. A similar hypothesis has been proposed to explain the altered sensitivity of K_{ATP} channels to ATP in the presence of intracellularly applied trypsin in patches isolated from mouse pancreatic β-cells (Trube *et al.*, 1989). Alternatively, it is possible that in the presence of trypsin, the K_{ATP} channels are maximally activated and are therefore not susceptible to further activation by diazoxide.

It was noted that a second application of diazoxide to cells dialysed with a range of nucleotides in the presence of Mg^{2+} ions resulted in a reduced response to the drug, an effect which may be due to the loss or inactivation, through dialysis of the cell contents, of a regulatory protein. To test this hypothesis, cells were dialysed with an electrode solution containing cell extract and the effects of diazoxide examined. The results obtained show that diazoxide is indeed able to activate K_{ATP} currents recorded from whole-cell dialysed with a lysate containing a low concentration (0.4 to 15 μM) of Mg-ATP. At Mg-ATP concentrations of this order of magnitude, diazoxide does not induce K_{ATP} channel activation, suggesting the lysate contains a cellular component lost or altered during cell dialysis which is required for activation. This possibility is supported by the observation that large activating effects of diazoxide are observed soon after formation (i.e., before substantial dialysis can occur) of the whole-cell configuration. It is unlikely that this increased effectiveness of diazoxide is due to the high intracellular ATP content of the cells *per se*, soon after achieving the whole-cell configuration (approximately 5 mM; Ashcroft *et al.*, 1987), since diazoxide is ineffective under conditions of high intracellular ATP in whole-cell recordings (Trube *et al.*, 1986; Sturgess, 1988).

Thus, in conclusion, we suggest that diazoxide can activate K_{ATP} channels in CRI-G1 insulin-secreting cells by two separate pathways. In cell-free membrane patches we propose that diazoxide induces K_{ATP} channel activation by a mechanism involving phosphorylation of the channel or some associated protein by Mg-ATP (see Kozlowski *et al.*, 1989). In intact cells an alternative activatory pathway may exist in which an intracellular regulatory protein, which binds purine Mg^{2+} nucleotides, interacts with the channel and allows diazoxide to induce activation. It is not, at present, clear which of these mechanisms dominates in intact cells where Mg-ATP is the major intracellular purine nucleotide present. It is also not clear as to how diazoxide mediates its inhibitory effects on K_{ATP} channels. However, this secondary inhibitory action is clearly nucleotide-independent and apparent under conditions which do not favour activation (see also Kozlowski *et al.*, 1989). Perhaps under these conditions diazoxide mimics the action of the sulphonylureas. However, in view of the intracellular milieu required for this inhibitory effect it is unlikely to occur in intact pancreatic β-cells. With regard to the potassium channel activating action of diazoxide, it will be interesting to determine whether the dual mechanism proposed above is of general significance and underlies some of the activatory actions of other potassium channel openers in other tissues (Quast & Cook, 1989; Edwards & Weston, 1990).

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The role of complement, platelet-activating factor and leukotriene B_4 in a reversed passive Arthus reaction

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1 The mechanisms underlying oedema formation induced in a reversed passive Arthus (RPA) reaction and, for comparison, in response to zymosan in rabbit skin were investigated.

2 Oedema formation at skin sites was quantified by the accumulation of intravenously-injected ^{125}I-labelled human serum albumin.

3 Recombinant soluble complement receptor type 1 (sCR1), administered locally in rabbit skin, suppressed oedema formation induced in the RPA reaction and by zymosan.

4 The platelet-activating factor (PAF) antagonists, WEB 2086 and PF10040 administered locally, inhibited oedema formation induced in the RPA reaction and by PAF but not by zymosan.

5 A locally administered leukotriene B_4 (LTB_4) antagonist, LY-255283, inhibited oedema formation induced by LTB_4 but did not inhibit oedema responses to PAF, zymosan or the RPA reaction.

6 The results demonstrate a role for complement in oedema formation in both the RPA reaction and in response to zymosan. An important contribution by PAF is indicated in the RPA reaction but not in response to zymosan whereas no evidence was obtained to suggest a role for LTB_4 in either inflammatory response.

Keywords: Reversed passive Arthus reaction; complement; soluble complement receptor type 1 (sCR1); platelet-activating factor (PAF); leukotriene B_4 (LTB_4); oedema formation

Introduction

Maurice Arthus at the turn of this century first described the acute inflammatory and haemorrhagic reaction produced in the skin of rabbits when a local injection of horse serum was administered to previously sensitized rabbits (Arthus, 1903). Although much progress has been made in the elucidation of the mechanisms involved in this complex phenomenon, the mechanisms remain only partially understood (Humphrey, 1955; Cochrane & Janoff, 1974; Williams *et al.*, 1986; Hellewell, 1990). Experimentally it is convenient to investigate the reversed passive Arthus (RPA) reaction. This inflammatory reaction differs from the direct Arthus reaction (described above) in that it is elicited by an intradermal (i.d.) injection of antibody and an intravenous (i.v.) injection of the corresponding antigen. These Arthus reactions, classified as Type III hypersensitivity reactions, are models of vascular injury which are initiated by the deposition of antigen-antibody complexes within the wall of skin microvessels. The ensuing inflammatory reaction is characterized by oedema formation, neutrophil accumulation, platelet accumulation and haemorrhage. In severe reactions the inflammatory response culminates in tissue necrosis.

Early observations showed the depletion of circulating neutrophils with nitrogen mustard or anti-neutrophil antiserum severely depressed oedema in the Arthus reaction suggesting that these cells play a crucial role in this inflammatory reaction (Stetson & Good, 1951; Humphrey, 1955). In addition, systemic depletion of the complement system with cobra venom factor also suppresses the Arthus reaction (Cochrane *et al.*, 1970; Cochrane & Janoff, 1974). A key mediator in the Arthus reaction may be the complement protein fragment C5a which is a potent chemoattractant for neutrophils. C5a was also shown to be potent in inducing oedema formation in the skin (Williams & Jose, 1981). Further, the oedema response although evident very early after injection (5–6 min) was totally inhibited following depletion of circulating neutrophils (Wedmore & Williams, 1981b). In RPA reactions induced in the rabbit peritoneal cavity, C5a has been detected in inflammatory exudate by use of radioimmunoassay (Jose *et al.*, 1983).

We have investigated the role of complement in the RPA reaction and in zymosan-induced oedema formation using the recombinant soluble human complement receptor type I (sCR1) (Weisman *et al.*, 1990; Yeh *et al.*, 1991). The single chain membrane bound glycoprotein, CR1 (C3b/C4b receptor; CD35), exerts a number of inflammatory regulatory functions in the body (Fearon & Wong, 1985; Ross & Medof, 1985; Molines & Lachmann, 1988). In addition, CR1 inactivates C3 and C5 convertases, thereby controlling the activation of the classical and the alternative pathways of the complement cascade (Iida & Nussenzweig, 1981; Weisman *et al.*, 1990; Yeh *et al.*, 1991). Thus, an active soluble form of CR1 (sCR1) may have therapeutic benefits in many inflammatory disease states where activation of the complement cascade is prominent.

Several membrane-derived lipids have been implicated as important mediators of Arthus reactions (Hellewell & Williams, 1986; Williams *et al.*, 1986). The arachidonic acid metabolite, prostaglandin E_2 (PGE_2) injected i.d. alone into rabbit skin results in little plasma leakage; however, when co-injected with agents that increase microvascular permeability the eicosanoid, by virtue of its vasodilator properties, acts synergistically to augment oedema formation (Williams & Peck, 1977; Wedmore & Williams 1981b). It was subsequently demonstrated that local treatment with the cyclooxygenase inhibitor indomethacin, suppresses the Arthus reaction, an inhibition which can be reversed by local administration of PGE_2 (Williams *et al.*, 1986). The precise contribution made by two other important proinflammatory agents; the ether lipid platelet-activating factor (PAF) and the 5-lipoxygenase product leukotriene B_4 (LTB_4), in the

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Arthus reaction, still remains to be established. In this study we therefore investigated the involvement of complement, PAF and LTB_4 in the RPA reaction and, for comparison, the response to zymosan in the rabbit skin. The agents used were sCR1 (Weisman *et al.*, 1990) the PAF antagonists, WEB 2086 (Casals-Stenzel *et al.*, 1987) and PF10040 (Rossi *et al.*, 1992) and the LTB_4 antagonist, LY-255283 (Jackson *et al.*, 1988; Snyder & Fleisch, 1989).

Methods

Animals

Male New Zealand White rabbits (2–3.5 kg) were purchased from Froxfield Farm, Hampshire.

Generation of antiserum for Arthus reactions

Arthus antiserum, anti-bovine-γ-globulin (anti-BGG) was raised in rabbits as previously described (Hellewell & Williams, 1986). Briefly, subcutaneous injections (4 × 0.25 ml) of BGG (2 mg ml^{-1} in saline) emulsified with an equal volume of Freund's complete adjuvant were administered. This was followed 14 days later by booster subcutaneous injections (4 × 0.25 ml) of the same concentration of BGG in Freund's incomplete adjuvant. At day 28 a subcutaneous injection of alum-precipitated BGG (300 μg/rabbit) was given. Blood was collected by carotid cannulation at day 38, the serum from five rabbits was pooled, heat-inactivated at 56°C for 30 min and stored in aliquots at − 20°C. Heat-inactivated normal rabbit serum was used as the control.

Preparation of zymosan activated plasma (ZAP)

ZAP (a source of C5a des Arg) was prepared by incubating heparinised (10 u ml^{-1}) rabbit plasma with zymosan (5 mg ml^{-1}) for 30 min at 37°C. Zymosan was removed by centrifugation (2 × 10 min, 2500 *g*) and ZAP stored in 1 ml aliquots at − 20°C. The C5a des Arg content of ZAP was approximately 5×10^{-7} M as measured by radioimmunoassay (Collins *et al.*, 1991).

Measurement of local oedema formation in rabbit skin

Rabbits were anaesthetized with i.v. sodium pentobarbitone and the dorsal skin clipped and marked out with 16 treatment skin sites in 6 replicates per animal according to a balanced site plan. Plasma leakage was measured by i.v. injection of ^{125}I-labelled human serum albumin (5 μCi kg^{-1}) together with the visual marker Evans Blue dye (10 mg kg^{-1}) in saline as previously described (Wedmore & Williams, 1981b). After 10 min the agents under investigation, freshly prepared in sterile isotonic saline (unless otherwise stated), were injected i.d. in 0.1 ml volumes. Where indicated, agonists were co-injected with PGE_2 (3×10^{-10} mol/site) to facilitate the measurement of microvascular plasma protein leakage (Wedmore & Williams, 1981b). Cardiac blood samples were collected 4 h after the i.d. injections into heparinised test tubes for the preparation of plasma. The animals were then killed by an overdose of pentobarbitone the dorsal skin removed and the skin sites excised with a 17 mm diameter punch. Radioactivity in the skin sites and in 1 ml plasma samples were counted in an automatic gamma counter. Results are expressed as μl plasma per site by dividing skin sample ^{125}I-counts by ^{125}I-counts in 1 μl of plasma.

For the reversed passive Arthus reaction, 10 min after the i.v. injection of radiolabelled albumin, 0.1 ml volumes of anti-BGG antiserum (undiluted, diluted 1/2, 1/4 and 1/8 with saline) were injected i.d., followed 5 min later by an i.v. injection of the antigen (BGG; 5 mg kg^{-1}). Oedema formation was assessed after 4 h as described above.

Materials

Sagatal (pentobarbitone sodium, 60 mg ml^{-1}) was purchased from May and Baker, Dagenham, Essex. Bovine serum albumin, BGG and zymosan were from Sigma Chemical Co., Poole, Dorset. [^{125}I]-labelled human serum albumin (20 mg albumin per ml of sterile isotonic saline, 50 μCi ml^{-1}) was from Amersham International plc, Amersham, Buckinghamshire. Freund's complete and incomplete adjuvant were from Difco Laboratories, West Molesey, Surrey, Evans blue was from British Drug House, Poole, Dorset. Viaflex (sterile, pyrogen-free isotonic saline solution) was from Baxter Healthcare Ltd, Thetford, Norfolk. Sterile pyrogen-free water was from Phoenix Pharmaceuticals Ltd., Gloucester. Human sCR1 was genetically engineered by site directed mutagenesis and secreted from transfected Chinese hamster ovary cells (Yeh *et al.*, 1991) and was a kind gift from SmithKline & Beecham, Epsom, Surrey. PAF (1-0-hexadecyl-2-acetyl-*sn*-glycero-3-phosphorylcholine) was dissolved in 1% BSA in saline and was purchased from Bachem, Saffron, Walden, Essex. LTB_4 [5(**S**),12(**R**)-dihydroxy-6, 14-*cis*-8,10-trans-eicosatetraenoic acid] was purchased from Cascade Biochem Ltd., Reading, Berkshire, and was a gift from Dr R.M. McMillan, ICI Pharmaceuticals, Alderley Park. PF10040 (1-(3,4-dimethoxyphenylethyl)-6-methyl-3, 4-dihydroisoquinoline hydrochloride) was a gift from Purdue Frederick, Norwark, Connecticut, U.S.A. LY-255283 [1-(5-ethyl-2-hydroxy-4- (6-methyl-6-(1H-tetrazol-5-yl)-heptyloxy) phenyl) ethanone], initially dissolved in 0.01 M NaOH then sequentially diluted in saline, was a gift from Lilly Research Laboratories, Indianapolis, Indiana, U.S.A. WEB 2086 (3-[4-(2-chlorophenyl) -9-methyl-6H-thieno [3,2-f] [1,2,4]-triazolo-[4,3-a] [1,4]-diazepine-2-yl]-1- (4-morpholinyl) -1-propanone) was a gift from Boehringer Ingelheim, Bracknell, Berkshire.

Statistical analysis

The data are presented as the mean ± s.e.mean and have been analyzed by two way analysis of variance. Significant differences ($*P<0.05$, $**P<0.01$) between groups were determined by the Neuman-Kuels procedure.

Results

Effect of sCR1 on plasma leakage

Intradermal injections of zymosan (3–300 μg/site) elicited a dose-dependent oedema formation as measured by the leakage of ^{125}I-human serum albumin at skin sites (Figure 1). In the presence of sCR1 (3 μg/site) these responses were significantly attenuated ($n = 3$ rabbits). Undiluted ZAP, as a source of C5a, mixed with PGE_2 (3×10^{-10} mol/site) induced a response of 126.8 ± 15.1 μl plasma leakage which was unaffected by co-injection of sCR1 (123.1 ± 14.9μl). Injection of sCR1 and PGE_2 alone induced little oedema formation.

In order to determine the role of complement in the RPA reaction we examined the effect of sCR1 on oedema produced by different titres of Arthus antiserum (Figure 2). Arthus antiserum titres (12.5–100%) induced a dose-dependent oedema formation, which in the presence of sCR1 was significantly reduced ($n = 7$ rabbits). Responses to non-immune serum and sCR1 alone were minimal i.e., 2.7 and 1.9 μl above the saline control respectively. The response to zymosan (300 μg/site) in these experiments was 55.6 ± 9.7 μl and with co-injection of sCR1 this was reduced to 38.7 ± 9.7 μl ($P<0.01$; $n = 8$). Note that sCR1, at a concentration of 10 μg/site, also did not affect the response to ZAP plus PGE_2 (data not shown).

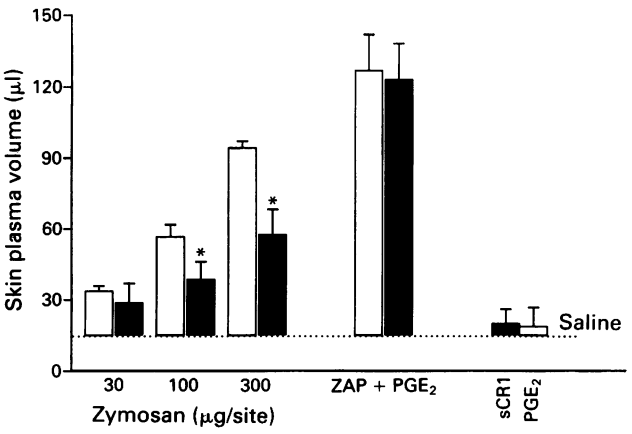

Figure 1 Effect of recombinant soluble complement receptor type I (sCR1) on oedema formation induced by zymosan and zymosan activated plasma (ZAP) plus prostaglandin E_2 (PGE_2) in rabbit skin. Intradermal (i.d.) injections of 30, 100 and 300 μg/site zymosan and undiluted ZAP plus PGE_2 were given alone (open columns) or in the presence of 3 μg/site sCR1 (solid columns). The dotted line shows the control value obtained after i.d. injection of saline. The results are expressed as mean (± s.e.mean, vertical bars) μl plasma volume values from $n = 3$ animals. The significant inhibitory effect of sCR1 is shown as $*P < 0.05$.

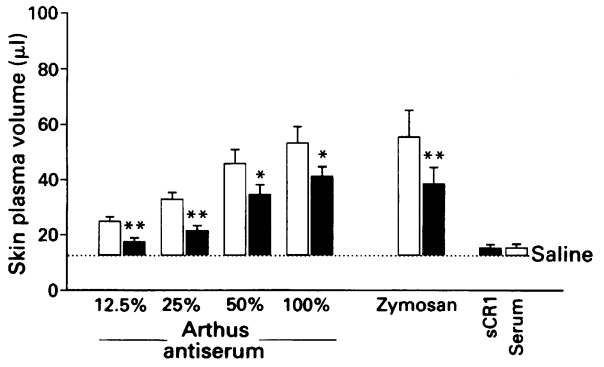

Figure 2 Effect of recombinant soluble complement receptor type I (sCR1) on oedema formation induced by zymosan and produced in the reversed passive Arthus (RPA) reaction in rabbit skin. Intradermal (i.d.) injections of 300 μg/site zymosan and RPA antiserum titres of 12.5%, 25%, 50% and 100% administered alone (open columns) or in the presence of 10 μg/site sCR1 (solid columns). The dotted line shows the control value obtained after i.d. injection of saline. The results are expressed as mean (± s.e.mean, vertical bars) μl plasma volume values from $n = 7$–9 animals. The significant inhibitory effect of sCR1 is shown as $*P < 0.05$ or $**P < 0.01$.

Effect of the PAF antagonists, PF10040 and WEB 2086, on plasma leakage

The next series of experiments was designed to investigate the involvement of PAF in the above inflammatory responses. We therefore examined the effects of the compounds PF-10040 and WEB 2086 on oedema produced in the RPA reaction and, for comparison, oedema induced by PAF plus PGE_2 and zymosan (Figure 3). Plasma leakage induced by PAF (10^{-9} mol/site) plus PGE_2 (3×10^{-10} mol/site) was 45.1 ± 7.6 μl; this response was reduced to 22.5 ± 1.4 μl ($P < 0.01$; $n = 6$ rabbits) by PF10040 (10^{-7} mol/site) and to 17.5 ± 1.9 μl ($P < 0.01$; $n = 6$ rabbits) by WEB 2086 (10^{-7} mol/site). The compounds alone or in the presence of PGE_2 induced little or no oedema formation. In these experiments it was observed that the RPA reaction induced a response of 33.2 ± 6.6 μl; this response was reduced to 22.5 ± 5.5 μl ($P < 0.01$; $n = 5$ rabbits) by PF10040 (10^{-7} mol/site) and to 22.4 ± 3.4 μl ($P < 0.01$; $n = 5$ rabbits) by WEB 2086 (10^{-7} mol/site). For comparison, oedema formation induced by zymosan (300 μg/site) was unaffected ($n = 6$ rabbits) by co-injection of either PAF antagonist.

Effect of the LTB_4 antagonist, LY-255283, on plasma leakage

Figure 4 clearly shows that LY-255283 at 10^{-7} mol/site suppressed the response to LTB_4 (5×10^{-10} mol/site) from 29.9 ± 5.1 μl to 17.6 ± 2.4 μl ($P < 0.01$; $n = 6$ rabbits) and the response to LTB_4 (5×10^{-10} mol/site) plus PGE_2 (3×10^{-10} mol/site) from 54.9 ± 13.7 μl to 19.5 ± 3.1 μl ($P < 0.01$; $n = 6$ rabbits). Oedema formation induced by zymosan (300 μg/

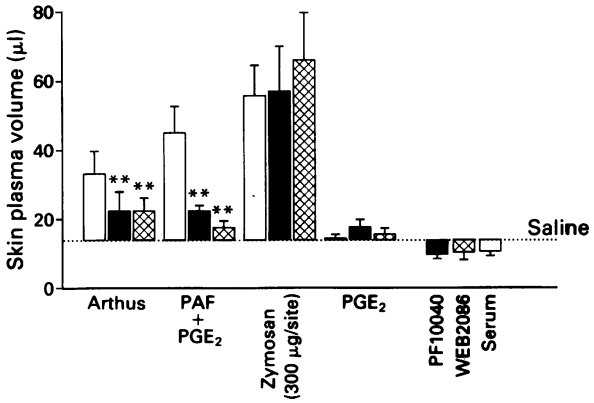

Figure 3 Effect of the platelet-activating factor (PAF) antagonists, PF10040 and WEB 2086 on oedema formation produced in the reversed passive Arthus (RPA) reaction and induced by PAF plus prostaglandin E_2 (PGE_2) and zymosan. RPA antiserum (undiluted), PAF (10^{-9} mol/site) plus PGE_2 (3×10^{-10} mol/site) and zymosan (300 μg/site) injected alone (open columns), with 10^{-7} mol/site PF-10040 (solid columns) or with 10^{-7} mol/site WEB 2086 (hatched columns). The dotted line shows the control value obtained after i.d. injection of saline. The results are expressed as mean (± s.e.mean, vertical bars) μl plasma volume values from $n = 5$–6 animals. The significant inhibitory effect of the PAF antagonists is shown as $**P < 0.01$.

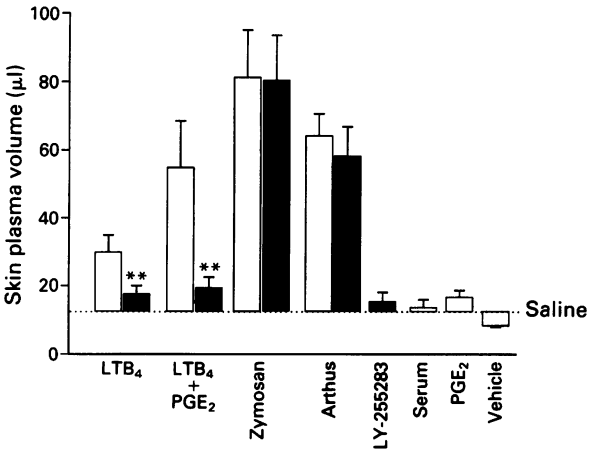

Figure 4 The effect of the leukotriene B_4 (LTB_4) antagonist, LY-255283 on oedema formation produced in the reversed passive Arthus (RPA) reaction and induced by LTB_4, LTB_4 plus prostaglandin E_2 (PGE_2) and zymosan. RPA antiserum (undiluted), LTB_4 (5×10^{-10} mol/site), LTB_4 (5×10^{-10} mol/site) plus PGE_2 (3×10^{-10} mol/site) and zymosan (300 μg/site) were i.d. injected alone (open columns) or with 10^{-7} mol/site LY-255283 (solid columns). The dotted line shows the control value obtained after i.d. injection of saline. The results are expressed as mean (± s.e.mean, vertical bars) μl plasma volume values from $n = 6$ animals. The significant inhibitory effect of LY-255283 is shown as $**P < 0.01$.

site) and the response produced by the RPA reaction were unaffected by co-injection with LY-255283. In a further series of experiments ($n = 7$ rabbits) where LTB_4 plus PGE_2 was similarly inhibited and the Arthus reaction was not affected by the antagonist, the response induced by PAF plus PGE_2 was 57.0 ± 8.1 µl and in the presence of LY-255283, the response was not significantly inhibited and was 53.8 ± 6.4 µl.

Discussion

In this study we investigated the inflammatory mechanisms underlying the Arthus reaction. Specifically, we showed that a truncated and soluble form of CR1 (sCR1) given locally into rabbit skin not only inhibited oedema formation in the RPA reaction, but also suppressed oedema induced by zymosan (Figures 1 and 2). Thus, sCR1 inhibited both the classical pathway of the complement cascade which is activated by the deposition of immune-complexes in the RPA reaction and also inhibited the alternative pathway which is typically activated by yeast cell wall polysaccharides such as zymosan. Previous studies showed that systemic treatment of animals with cobra venom factor or antibodies to complement components suppresses Arthus reactions (Ward & Cochrane, 1965; Cochrane *et al.*, 1970; Cochrane & Janoff, 1974). The present study shows that intradermally-injected complement inhibitor can suppress both the Arthus reaction and the response to zymosan. This emphasizes the importance of local activation of complement in the interstitium in these oedema responses (Williams & Jose, 1981).

Our results are in agreement with a recent paper by Yeh *et al.* (1991). They demonstrated that sCR1 inhibits both the classical and alternative pathways of the complement cascade using an *in vitro* sheep erythrocyte haemolytic assay. Furthermore, using a rat RPA reaction model, they showed that i.d. administration of sCR1 dose-dependently suppresses the inflammatory response as judged by both gross and microscopic examination. These authors also demonstrated by immunological localization of C3 and C5b-9 neoantigen deposition that the immunofluorescence in the RPA reaction in the presence of sCR1 is markedly lower than that produced in the RPA reaction alone. ZAP, as a source of C5a, was included as a control in our experiments described here. Oedema induced by ZAP was unaffected by sCR1 showing that the compound did not interfere with the action of C5a, once formed. These results indicate that complement activation is important in the Arthus reaction and that sCR1 may be a useful inhibitor of the inflammatory response, not only in Type III hypersensitivity reactions but also in other pathological conditions where complement activation is involved. Indeed, Weisman *et al.* (1990) showed that sCR1 had anti-inflammatory activity when administered i.v. in a rat *in vivo* model of reperfusion injury of ischaemic myocardium.

We have previously described experiments indicating that vasodilator prostaglandins are produced in the Arthus reaction and that these mediators have a potentiating role in oedema formation (Williams *et al.*, 1986). Here we have investigated the role of other membrane-derived lipids, namely PAF and LTB_4. Our results show that the PAF antagonists, WEB 2086 and PF10040, specifically inhibit oedema formation produced in the RPA reaction and induced by PAF but not that induced by zymosan (Figure 3). This suggests that PAF plays an important role in the Arthus reaction but not in zymosan-induced plasma leakage. Our results are in accord with a report by Hellewell & Williams (1986) who showed that the PAF antagonist, L-652371, suppresses oedema formation in the RPA reaction. Further evidence supporting a role for PAF in the RPA reaction has been presented by other investigators using different PAF antagonists administered by various routes in a number of animal models (Deacon *et al.*, 1986; Issekutz & Szejda, 1986; Camussi *et al.*, 1987; Warren *et al.*, 1989; Hellewell, 1990).

The role of 5-lipoxygenase products and in particular LTB_4 in the Arthus reaction remains unclear. It has been shown that several 5-lipoxygenase inhibitors given intrapleurally inhibit a reversed passive Arthus pleurisy model in the rat (Berkenkopf & Weichman, 1991) and the 5-lipoxygenase inhibitor, A-63162, suppresses inflammation induced in the RPA reaction in the mouse (Zhang *et al.*, 1991). These observations suggest a role of 5-lipoxygenase products in the Arthus reaction but they do not shed light on the contribution of specific products of 5-lipoxygenase activity (e.g. LTB_4). In the study described here we have shown that local administration of the LTB_4 antagonist, LY-255283, inhibited oedema formation induced by LTB_4 alone and LTB_4 plus PGE_2, but did not affect leakage produced in the RPA reaction or induced by zymosan or PAF (Figure 4). The lack of effect is not due to clearance of LY-255283 from the skin site as LTB_4-induced oedema formation is inhibited when it is administered locally into a site injected 4 h previously with LY-255283 (Von Uexkull *et al.*, unpublished observations). The reported inhibition of the Arthus reaction by various 5-lipoxygenase inhibitors may be due to other bioactive 5-lipoxygenase products such as LTC_4, LTD_4 and LTE_4 or simply non specific effects of the inhibitors. Alternatively, it is possible that LTB_4 is indeed involved in the RPA reaction but that its site of action is not accessible to the antagonist in our model. It is also feasible that the amount of LTB_4 produced in the RPA reaction is so small that its effects are masked by the effects of C5a. Our results with the LTB_4 antagonist, however, suggest that LTB_4 does not play a major role in the induction of oedema in the RPA reaction or in response to zymosan.

A schematic representation of the events that may occur in the RPA reaction is illustrated in Figure 5. Circulating antigen diffuses out of the lumen of the blood vessel and across the microvascular endothelial cells where it meets the antibody, forming immune complexes in the microvessel wall. These complexes activate the classical pathway of the complement cascade resulting in the formation of the chemoattractant C5a. C5a induces neutrophils to adhere to the endothelial cells followed by migration via endothelial junctions. The interaction between neutrophils and endothelial cells triggers increased microvascular permeability by an unknown mechanism (Wedmore & Williams, 1981b). This interaction can explain the effect of the depletion of circulating neutrophils on oedema formation in the Arthus reaction (Stetson & Good, 1951; Humphrey, 1955). The leakage

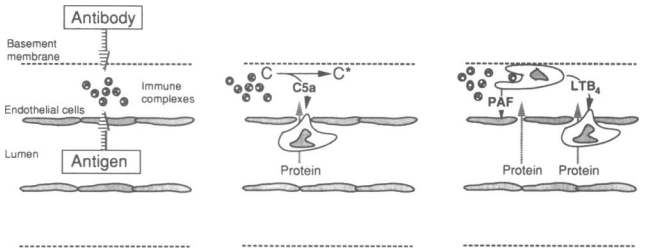

Figure 5 Diagrammatic representation of the possible events occurring in the reversed passive Arthus reaction. Circulating antigen diffuses from the lumen of the micro-blood vessels across the endothelial cell layer where it forms immune complexes with the antibody in the vessel wall. The complexes induce the activation of the classical pathway of the complement cascade leading to the generation of C5a. C5a attracts neutrophils which then adhere to and marginate across the endothelial cell layer resulting in oedema formation. The neutrophils phagocytose the immune complexes and generate PAF. The newly formed PAF then acts directly on the endothelial cells to cause further plasma leakage. The activated neutrophils can also synthesize LTB_4 which may attract other neutrophils to the site of inflammation to produce more oedema formation.

induced provides further antigen to the interstitium thus facilitating further immune complex deposition. Neutrophils that have migrated through the endothelium soon encounter immune complexes under and around the basement membrane and phagocytosis begins. This stimulates the release of PAF from neutrophils which acts directly on endothelial cells to cause further leakage (Wedmore & Williams, 1981a; Braquet *et al.*, 1987). Activated neutrophils may also release LTB_4 which then could attract more neutrophils, although this did not appear to be a major component in this model. Injection of zymosan will also generate C5a, in this case by the alternative pathway of the complement cascade, resulting in plasma leakage. Our results show that sCR1 reduced the plasma leakage produced in both the RPA reaction and in response to zymosan presumably by inhibiting the formation of C5a. In contrast, the PAF antagonists inhibited the oedema formation in the Arthus reaction but did not suppress the response induced by zymosan, suggesting a role for PAF in the former but not in the latter response. An explanation for this observation may be that in the Arthus reaction, phagocytosis takes place when neutrophils are in close contact with endothelial cells. By comparison, when zymosan is injected intradermally subsequent phagocytosis takes place at sites remote from the endothelium. PAF is unstable in tissue fluid and levels in the region of the endothelium may be very low under these circumstances. This argument does not hold for C5a which will also be generated either close to, or remote from the endothelium in the two models. In this case, although there may be some metabolism in tissue fluid (by the action of carboxypeptidase N) the metabolic product C5a des Arg is also highly active as a permeability increasing mediator *in vivo* and is stable in tissue fluid (Jose *et al.*, 1981).

These observations shed some light on the mediators involved in the Arthus reaction. The results illustrate how mediators may be involved in sequence in inflammatory reactions, suggesting that therapeutic agents may be more effective if aimed at the early events in the response. Further, in terms of inflammatory mechanisms, consideration of the exact site of liberation and the stability of an individual mediator is important in determining its relative role in a particular inflammatory reaction.

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Response to atrial natriuretic peptide, endopeptidase 24.11 inhibitor and C-ANP receptor ligand in the rat

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1 The present studies compared the renal and hypotensive response to (a) exogenous atrial natriuretic peptide (ANP) (99–126), (b) an endopeptidase-24.11 inhibitor (candoxatrilat) and (c) an antagonist of ANP clearance receptors (SC 46542) in conscious rats.

2 Infusion of low-dose-ANP (100 ng kg^{-1} min^{-1}) produced a gradual increase in urinary sodium and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) excretion without significant change in glomerular filtration rate (GFR) or fractional lithium clearance (FeLi). There was a significant fall in blood pressure.

3 Infusion of high-dose ANP (300 ng kg^{-1} min^{-1}) produced a brisk, 3 fold increase in urinary sodium and cyclic GMP excretion along with a rise in GFR, but had no significant effect on FeLi compared to the control group. The renal response was accompanied by a pronounced fall in blood pressure.

4 Candoxatrilat or SC 46542, alone, had no significant effect on sodium excretion compared to control animals. Both compounds enhanced the natriuretic and cyclic GMP responses to a low-dose ANP infusion (100 ng kg^{-1} min^{-1}) to levels similar to, or greater than, those observed with the high-dose ANP (300 ng kg^{-1} min^{-1}). However, unlike high-dose ANP, these renal effects were not accompanied by a significant change in GFR and neither compound potentiated the hypotensive effect of the low-dose ANP infusion. Only candoxatrilat when given with ANP produced a marked rise in FeLi.

5 Similarly, combined administration of candoxatrilat and SC 46542 (without exogenous ANP) induced an increase in sodium and cyclic GMP excretion comparable to high-dose ANP but did so without a significant increase in GFR and with a significantly smaller fall in blood pressure. Interestingly, there was no increase in FeLi with the combination of the two compounds, suggesting that the major contribution to sodium excretion came from SC 46542.

6 Both candoxatrilat and SC 46542 increased sodium and cyclic GMP excretion in the rat A-V fistula model of heart failure, a model hyporesponsive to infusions of ANP, without significant change in blood pressure.

7 These data show that candoxatrilat and SC 46542 do not simply reproduce the effects of an ANP infusion but preferentially enhance the natriuretic response to ANP. Inhibition of E-24.11 may potentiate a tubule action of ANP while the renal mechanism of action of the C-ANP receptor ligand needs further study. Both manipulations are of potential value in the management of heart failure.

Keywords: Atrial natriuretic peptide; lithium clearance; cardiac failure

Introduction

The profile of biological activity of atrial natriuretic peptide (ANP 99–126) – natriuresis-diuresis, vasorelaxation and inhibition of the renin-angiotensin system – has led to interest in the therapeutic potential of this peptide in cardiovascular diseases such as heart failure and hypertension (Brenner *et al.*, 1990). Use of the peptide itself as a drug is limited by its short plasma half-life (2–3 min) and its poor oral bioavailability. Instead, attention has focused on enhancing the activity of endogenous ANP, in particular, by inhibiting its clearance.

In addition to filtration at the glomerulus, two mechanisms have been shown to contribute to the clearance of ANP, namely, metabolism by endopeptidase-24.11 (E-24.11) and internalisation via clearance (C-ANP) receptors. E-24.11 is an ectoenzyme with a wide tissue distribution that includes the renal brush border, brain and intestine (Gee *et al.*, 1985; Matsas *et al.*, 1986). The enzyme cleaves ANP at position Cys^{105}-Phe^{106} in the peptide ring to yield an inactive metabolite (Olins *et al.*, 1989). E-24.11 inhibitors prolong the half-life of co-administered ANP *in vivo* and enhance its natriuretic effect (Trapani *et al.*, 1989; Barclay *et al.*, 1991).

ANP receptors can be divided into two major groups; A-ANP and B-ANP receptors are coupled to guanylate cyclase (Chinkers *et al.*, 1989; Koller *et al.*, 1991), while C-ANP receptors are not linked to this enzyme (Porter *et al.*, 1990). A-ANP receptors are thought to mediate the biological actions of ANP. B-ANP receptors may be responsible for the actions of another member of the natriuretic peptide family, known as C-natriuretic peptide (Koller *et al.*, 1991). C-ANP receptors, on the other hand, have been shown to internalise and degrade ANP (Nussenzveig *et al.*, 1990). Widely distributed in a number of tissues (for example, vascular smooth muscle and endothelial cells and renal glomerulus) C-ANP receptors account for the majority of ANP binding sites and are thought to perform a clearance role (Maack *et al.*, 1987). In support of this, specific C-ANP receptor ligands exhibit no intrinsic natriuretic-diuretic activity in the isolated kidney but increase plasma ANP levels and stimulate a natriuresis when infused *in vivo* (Suzuki *et al.*, 1987; Almeida *et al.*, 1989).

If E-24.11 inhibitors and C-ANP receptor ligands act simply to increase plasma ANP levels by reducing the clearance of endogenous ANP, then these compounds should reproduce the effects of an ANP infusion. Moreover, comparison of the effect of C-ANP receptor ligand and E-24.11 inhibitor on the pharmacokinetics of [^{125}I]-ANP suggests that the C-ANP pathway has the greater capacity for clearing circulating ANP (Chiu *et al.*, 1991). It might be predicted

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therefore that C-ANP receptor ligands would be more useful than E-24.11 inhibitors in potentiating the actions of endogenous ANP. However, studies in animal models of heart failure and hypertension show that the renal response to E-24.11 inhibitors does not correlate with their effects on plasma ANP concentration (Wilkins *et al.*, 1990b; Seymour *et al.*, 1991). It has been suggested that the efficacy of E-24.11 inhibitors in heart failure derives, at least in part, from potentiation of the renal tubule actions of ANP (Wilkins *et al.*, 1990b). This concept supported by the observation that the E-24.11 inhibitor, SQ 28603, increases fractional lithium excretion (Margulies *et al.*, 1990), a marker of pre-distal tubule sodium reabsorption. The extent to which this effect is shared by C-ANP receptor ligands is not known.

Accordingly, the present studies compared the renal and hypotensive responses to manipulation of ANP activity with an E-24.11 inhibitor (candoxatrilat) or a C-ANP receptor ligand (SC 46542) with infusion of exogenous ANP in the conscious rat. To assess the therapeutic potential of these manipulations, the effects of candoxatrilat and SC 46542 were also examined in rats with aorto-caval (A-V) fistulae, a model of heart failure with chronically elevated endogenous ANP levels but hyporesponsive to the effects of an ANP infusion (Wilkins *et al.*, 1990b).

Methods

Infusion protocol

Studies were performed on male Wistar rats (250–300 g). Under Hypnorm (fentanyl/fluanisone) anaesthesia, both femoral veins, a femoral artery and the bladder were cannulated with portex tubing (0.58 mm). The animals were allowed to regain consciousness in individual restraining cages. An infusion of sodium chloride 150 mM (0.5 ml h^{-1}) containing lithium choride 25 mM and [^{3}H]-inulin (Amersham) 3 μCi ml^{-1} was given via one femoral vein and continued for the study duration. A second infusion of sodium chloride 150 mM (1 ml h^{-1}) was given via the other femoral vein and used for administration of drugs. Observations were started 2.5 h after beginning these infusions; while still in the acute recovery phase post-surgery, glomerular filtration rate (GFR) and urine output are sufficiently stable to provide a background against which responses can be measured. Urine was collected at 15 min intervals into preweighed tubes for determination of volume, sodium, potassium, lithium, guanosine 3':5'-cyclic monophosphate (cyclic GMP) and [^{3}H]-inulin concentrations. Blood pressure was measured via the femoral artery every 15 min (MacLab instruments). Arterial blood samples (200 μl) were taken into haematocrit tubes 30 and 90 min from the start of observations for lithium and [^{3}H]-inulin measurement.

Rat ANP (99–126) was purchased from Bachem, U.K. Candoxatrilat is a specific E-24.11 inhibitor (Danilewicz *et al.*, 1989) and was a gift from Pfizer UK. SC 46542 is des(Phe^{106}Gly^{107}Ala^{115}Gln^{116})-ANP(5–28) and was synthesized by Monsanto Company, St Louis, U.S.A.; it binds specifically to C-ANP receptors (Koepke *et al.*, 1989).

Studies in normal rats

ANP infusions To examine the response of rats to ANP, animals received exogenous ANP (99–126) 100 ng kg^{-1} min^{-1}, 300 ng kg^{-1} min^{-1} or vehicle (saline) alone as a 60 min infusion ($n = 6$ each group). In a separate set of experiments, animals were set up as per protocol but blood (2 ml) was collected at the end of an infusion of ANP 100 ng kg^{-1} min^{-1} into potassium EDTA (2 mg ml^{-1}) tubes on ice for plasma ANP assay.

Studies with candoxatrilat and SC 46542 To investigate the synergistic effect of inhibition of E-24.11 and co-infusion of ANP, candoxatrilat was given in doses of 1, 3, or 10 mg kg^{-1} ($n = 6$ to 9 each group) by bolus injection over 1 min at the start of a 60 min infusion of ANP 100 ng kg^{-1} min^{-1}. Control animals received candoxatrilat 10 mg kg^{-1} alone ($n = 6$). Doses of candoxatrilat $\geqslant 3$ mg kg^{-1} have been shown to have a maximal natriuretic effect in hydrated rats (Shepperson *et al.*, 1991).

To investigate the effect of a C-ANP receptor ligand on the response to ANP, two doses of SC 46542 (68 μg kg^{-1} bolus; followed by 6.8 μg kg^{-1} min^{-1} for 60 min ($n = 9$); or 680 μg kg^{-1} bolus; then 68 μg kg^{-1} min^{-1} for 60 min ($n = 6$)) were given with a 60 min infusion of ANP (100 ng kg^{-1} min^{-1}). A control group received the higher dose of SC 46542 alone ($n = 6$).

The effect of combined inhibition of both clearance mechanisms in the absence of exogenous ANP was examined by giving candoxatrilat 3 mg kg^{-1} by bolus injection before the higher dose of SC 46542 (680 μg kg^{-1} bolus, then 68 μg kg^{-1} min^{-1} for 60 min ($n = 6$)).

Studies in A-V fistula rats A-V fistula surgery was performed under Hypnorm anaesthesia. The fistula (1–1.5 mm long) was made through a side to side anastomosis between aorta and inferior vena cava approximately 10 mm distal to the renal arteries. Sham-operations were performed by exposing and temporarily clamping (5 min) the aorta and vena cava without cutting or suturing the vessels. After surgery, the animals were returned to their cages and allowed free access to water and a standard rat diet. The rats were studied 10 to 14 days post-surgery. All A-V fistula and sham-operated rats were placed in metabolic cages for 24 h before study for urine collection for cyclic GMP assay. To determine the levels of circulating ANP achieved in this model, A-V fistula and sham-operated animals ($n = 8$ each group) were randomly selected for blood sampling only; carotid artery and femoral vein cannulae were implanted and the animals allowed to recover in restraining cages for 2.5 to 3 h before blood sample collection. Blood (2 ml) was collected into potassium EDTA (2 mg ml^{-1}) tubes on ice. Those animals not used for blood sampling were used for further study. The femoral veins, carotid artery and bladder were cannulated and the infusion protocol described above was used with the exception that lithium was omitted from the infusate. The A-V fistula rats were divided into the following treatment groups: (a) candoxatrilat 3 mg kg^{-1} ($n = 7$) by bolus injection; (b) SC 46542 68 μg kg^{-1} bolus; followed by 6.8 μg kg^{-1} min^{-1} for 60 min, ($n = 6$); (c) SC 46542 680 μg kg^{-1} bolus; followed by 68 μg kg^{-1} min^{-1} for 60 min ($n = 7$); (d) a combination of (a) and (c) ($n = 4$); and (e) vehicle alone ($n = 6$). Sham-operated animals received treatment (a) or (c) with 6 animals in each group. At the end of the experiment the hearts were removed and the weights recorded.

Assays

Urine volume was measured gravimetrically. Sodium concentration was measured by flame photometry (Corning 480) and lithium by atomic absorption spectroscopy. [^{3}H]-inulin levels were determined by liquid scintillation counting in Insta-gel (Packard). Urinary cyclic GMP concentration was measured by radioimmunoassay on appropriately diluted samples as previously described (Wilkins *et al.*, 1990a). Plasma ANP concentrations were measured on unextracted samples with a radioimmunoassay kit (Peninsula Laboratories).

Statistics

Data are presented as mean ± s.e.mean. Blood pressure and renal responses were examined by one way analysis of variance with respect to treatment using time (urine collection) as a covariate. Differences were assessed for statistical significance using least significance difference test. Heart

weight and 24 h urinary cyclic GMP excretion differences were assessed by Students t test, paired or unpaired, as appropriate. All calculations were made with Complete Statistical System (StatSoft) software. Statistical significance was assumed when the P value was <0.05.

Results

ANP infusions in normal rats

Given alone, ANP 100 ng kg^{-1} min^{-1} produced a small increase in urinary sodium and cyclic GMP excretion compared to vehicle treated controls (Figure 1). In the subgroup of rats killed for plasma sampling, the mean plasma ANP level at the end of the ANP infusion was 533 ± 68 pg ml^{-1} compared with 102 ± 33 pg ml^{-1} in the control group ($P<0.05$).

Increasing the dose of ANP to 300 ng kg^{-1} min^{-1} caused a rapid 5 fold rise in sodium output, and a near 8 fold rise in urinary cyclic GMP excretion; both changes were significantly greater than the effect of ANP 100 ng kg^{-1} min^{-1} (Figure 1). The early natriuresis (first 30 min) was accompanied by a significant increase in GFR ($P<0.05$) compared to the vehicle control group, which was not seen with the lower dose of peptide (Table 1). Neither dose of ANP significantly affected fractional lithium excretion (FeLi) when compared to the control group.

Both doses of ANP reduced mean arterial blood pressure compared to vehicle-treated rats (Table 1). The fall in blood pressure with ANP 300 ng kg^{-1} min^{-1} was significantly greater than that produced by 100 ng kg^{-1} min^{-1}.

Studies with candoxatrilat and SC 46542 in normal rats

Candoxatrilat on its own (in doses up to 10 mg kg^{-1}) had no significant effect on sodium excretion compared to vehicle-treated controls, but clearly enhanced the natriuretic response to co-infused ANP (Figure 2). Bolus injection of candoxatrilat 3 or 10 mg kg^{-1} before ANP 100 ng kg^{-1} min^{-1} produced a prompt 5 to 6 fold rise in sodium excretion within 30 min of beginning the ANP infusion. The response to candoxatrilat 1 mg kg^{-1} and ANP was similar in magnitude, but the peak effect was delayed (45 min). Thus 3 mg kg^{-1} was taken as representative of the top of the dose-response curve for the E-24.11 inhibitor. Consistent with potentiation of ANP, the increases in sodium excretion were accompanied by marked rises in urinary cyclic GMP excretion.

Similarly, a small, but non-significant, rise in sodium ex-

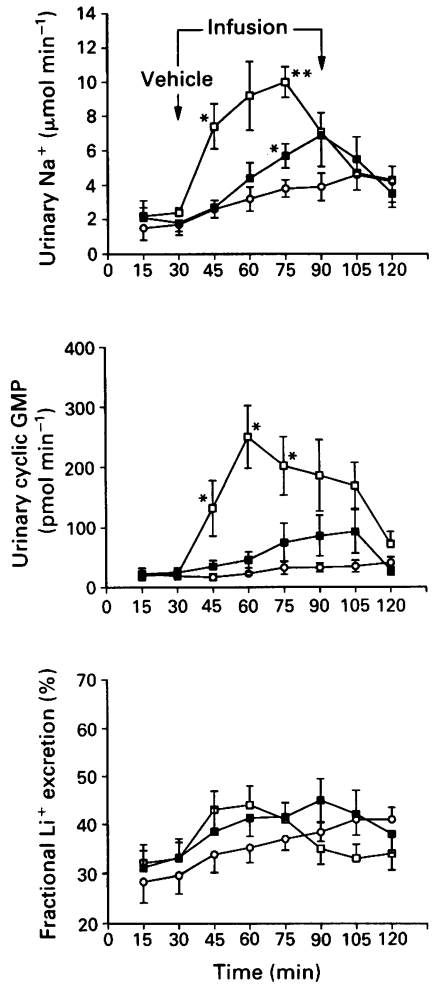

Figure 1 Effect of two doses of atrial natriuretic peptide (ANP, 100 ng kg^{-1} min^{-1}, ■, and 300 ng kg^{-1} min^{-1}, □) and vehicle (○) alone by infusion on urinary sodium (Na^+) excretion, cyclic GMP excretion and fractional lithium (Li^+) excretion in conscious normotensive rats. Saline (100 μl) was given prior to infusion as a control for subsequent experiments. Data are mean with s.e.mean shown by vertical bars. *$P<0.05$ compared to time-controlled vehicle infusion group.

Table 1 Effect of atrial natriuretic peptide (ANP), E-24.11 inhibitor (Can) and C-ANP receptor ligand (SC46542) on mean blood pressure (BP) and glomerular filtration rate (GFR) in conscious normotensive rats

	Time (min)						
	15	30	45	60	75	90	120
BP (mmHg)							
Saline	110 ± 4	109 ± 3	111 ± 4	112 ± 4	109 ± 3	110 ± 4	111 ± 4
ANP 100 ng kg^{-1} min^{-1}	108 ± 4	105 ± 4	103 ± 3	99 ± 3*	97 ± 2*	96 ± 4*	102 ± 5
ANP 300 ng kg^{-1} min^{-1}	109 ± 3	107 ± 4	96 ± 3*	91 ± 3*	85 ± 1*†	84 ± 2*†	87 ± 2*†
ANP + Can 3 mg kg^{-1}	104 ± 5	107 ± 4	105 ± 4	100 ± 3*	96 ± 3*	96 ± 3*	99 ± 3*
ANP + SC 46542	107 ± 3	107 ± 3	102 ± 4	97 ± 3*	93 ± 2*	91 ± 4*	94 ± 4*
Can + SC 46542	107 ± 4	105 ± 4	107 ± 3	104 ± 3*	102 ± 3*	101 ± 4*	101 ± 4*
GFR (ml min^{-1})							
Saline	3.2 ± 0.3	3.5 ± 0.4	3.5 ± 0.3	3.3 ± 0.3	3.6 ± 0.3	3.3 ± 0.3	3.1 ± 0.4
ANP 100 ng kg^{-1} min^{-1}	3.4 ± 0.2	3.4 ± 0.2	3.1 ± 0.3	3.2 ± 0.3	3.3 ± 0.3	3.1 ± 0.3	2.5 ± 0.3
ANP 300 ng kg^{-1} min^{-1}	3.3 ± 0.3	3.1 ± 0.2	4.5 ± 0.4†	4.2 ± 0.5†	3.5 ± 0.4	2.7 ± 0.3	2.9 ± 0.3
ANP + Can 3 mg kg^{-1}	3.0 ± 0.3	3.1 ± 0.3	3.7 ± 0.3	3.2 ± 0.2	3.0 ± 0.2	3.0 ± 0.2	2.8 ± 0.5
ANP + SC 46542	2.7 ± 0.2	3.0 ± 0.3	3.9 ± 0.4	2.9 ± 0.3	2.8 ± 0.5	2.6 ± 0.5	2.5 ± 0.1
Can + SC 46542	3.8 ± 0.4	3.6 ± 0.3	4.2 ± 0.3	3.6 ± 0.4	3.5 ± 0.3	3.4 ± 0.3	3.2 ± 0.2

Data are mean ± s.e.mean. Atrial natriuretic peptide (ANP) was infused from 30 to 90 min. Candoxatrilat (Can 3 mg kg^{-1}) was given prior to ANP 100 ng kg^{-1} min^{-1}. SC 46542 was given as 680 μg kg^{-1} bolus then 68 μg kg^{-1} min^{-1} during ANP 100 ng kg^{-1} min^{-1} or with candoxatrilat.

*$P<0.05$ compared to saline; †$P<0.05$ compared to ANP 100 ng kg^{-1} min^{-1}.

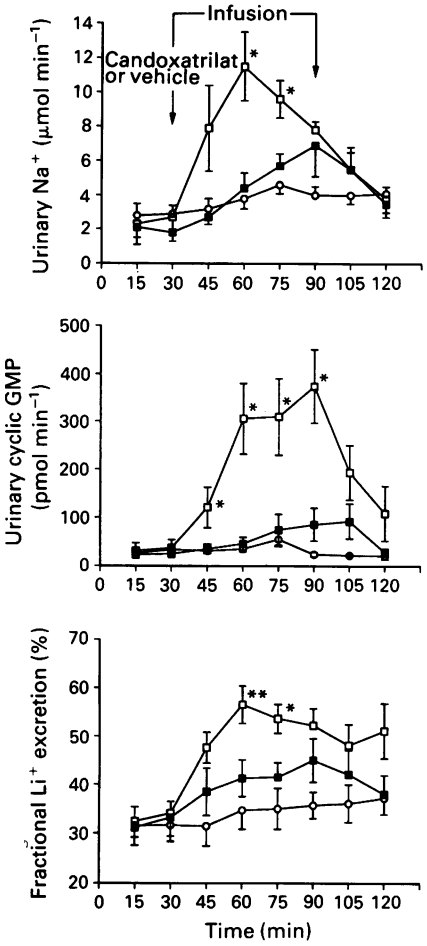

Figure 2 Effect of candoxatrilat (3 mg kg^{-1}, ○) atrial natriuretic peptide (ANP) infusion (100 ng kg^{-1} min^{-1}, ■) and the combination (□) on urinary sodium (Na^+), cyclic GMP, and fractional lithium (Li^+) excretion in conscious normal rats. Data are mean with s.e.mean shown by vertical bars. *$P<0.05$, **$P<0.001$, compared to ANP infusion alone group. (Data on response to co-treatment with candoxatrilat 1 and 10 mg kg^{-1} and ANP are omitted for clarity).

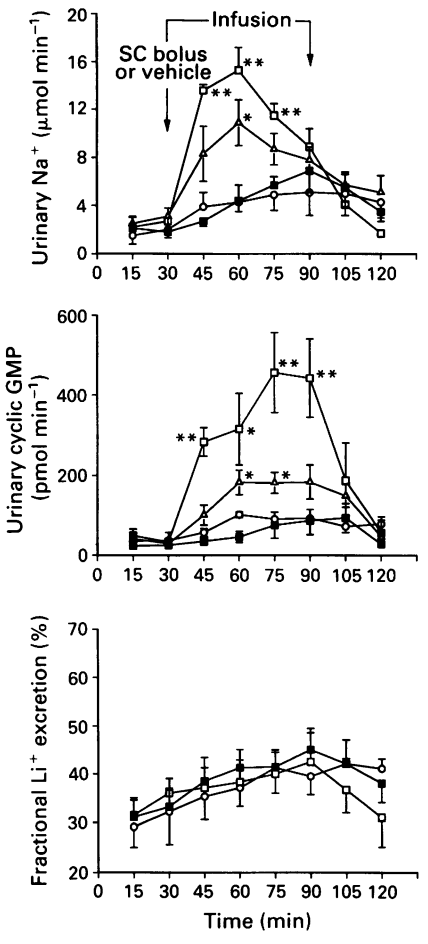

Figure 3 Effect of two doses of SC 46542 (68 μg kg^{-1} bolus, then 6.8 μg kg^{-1}, Δ; or 680 μg kg^{-1} bolus, then 68 μg kg^{-1} min^{-1}, □) in combination with ANP (100 ng kg^{-1} min^{-1}) on urinary sodium (Na^+), cyclic GMP and fractional lithium (Li^+) excretion in conscious rats. Controls received ANP (100 ng kg^{-1} min^{-1}, ■) or SC 46542 (680 μg kg^{-1} bolus, 68 μg kg^{-1} min^{-1}, ○) alone. Data are mean with s.e.mean shown by vertical bars. *$P<0.05$, **$P<0.01$ compared to ANP infusion alone.

cretion was seen with SC 46542 alone, but when co-administered with low-dose ANP, SC 46542 caused a pronounced, dose-dependent natriuresis and rise in urinary cyclic GMP excretion (Figure 3). The effect of combining the higher dose of SC 46542 (680 μg kg^{-1} bolus; 68 μg kg^{-1} min^{-1}) with low-dose ANP exceeded the maximal responses obtained with combined E-24.11 inhibition and ANP, although this did not reach statistical significance ($P = 0.059$).

An important difference between candoxatrilat and SC 46542, however, was observed in their effects of FeLi (Figures 2 and 3). The small, non-significant rise in FeLi seen with ANP alone during the course of the experiment was not significantly affected by the addition of SC 46542. In contrast, a marked and sustained rise in FeLi was obtained when candoxatrilat and ANP were given together.

The combination of candoxatrilat and SC 46542 (without exogenous ANP) produced an increase in urinary sodium and cyclic GMP excretion that was significantly ($P<0.01$) greater than either agent alone (Figure 4); indeed, the peak response matched that produced by combining either of these compounds with a low-dose ANP infusion. Interestingly, the natriuretic response to combined inhibition of both clearance pathways was not associated with a significant rise in FeLi excretion.

The addition of candoxatrilat or SC 46542 to low-dose ANP and the co-administration of the two clearance inhibitors produced only transient rises in GFR in the first period following these manipulations (Table 1). These apparent rises in GFR did not differ significantly from time-matched measurements in the vehicle-treated group, and probably represent a 'wash-out' effect from an increase in urine flow.

The small fall in blood pressure produced by ANP 100 ng kg^{-1} min^{-1} was not significantly changed by the addition of candoxatrilat or SC 46542. The combination of candoxatrilat and SC 46542 had no significant hypotensive effect (Table 1).

Candoxatrilat and SC 46542 in the A-V fistula rat

Typical of this model of heart failure, the A-V fistula rats exhibited cardiac hypertrophy compared to the sham-operated animals (meanheart weight fistulas vs shams; 1.46 ± 0.07 vs 1.08 ± 0.02 g, $P<0.01$). Plasma ANP concentration in the A-V fistula rats sampled was 833 ± 105 pg ml^{-1} compared with 118 ± 33 pg ml^{-1} in the sham-operated animals. Consistent with raised circulating levels of ANP, the A-V fistula rats also had a higher 24 h urinary cyclic GMP excretion (181 ± 23 vs 35 ± 8 nmol, $P<0.01$). Twenty four h urinary cyclic GMP excretion and heart weights did not differ significantly between the A-V fistula treatment groups.

Urinary sodium excretion in the A-V fistula rats receiving saline alone was relatively stable (Figure 5). In contrast to the sham-operated animal, bolus administration of candoxatrilat (3 mg kg^{-1}) to the A-V fistula rat produced a prompt and sustained rise in urinary sodium excretion, accompanied

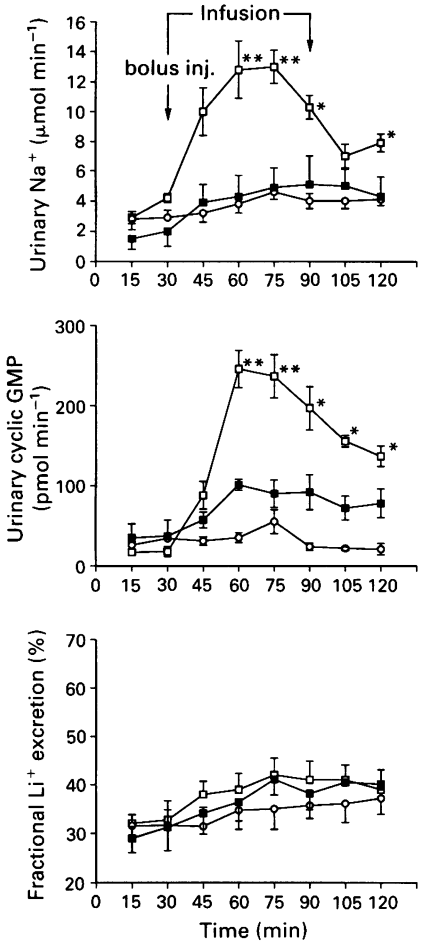

Figure 4 Effect of candoxatrilat (3 mg kg^{-1}, O) and SC 46542 (680 μg kg^{-1}, then 68 μg kg^{-1} min^{-1}, ■) alone and in combination (□) on urinary sodium (Na^+), cyclic GMP and fractional lithium (Li^+) excretion in conscious normal rats. Data are mean with s.e.mean shown by vertical bars. *$P<0.05$, **$P<0.01$ compared to candoxatrilat or SC 46542 alone.

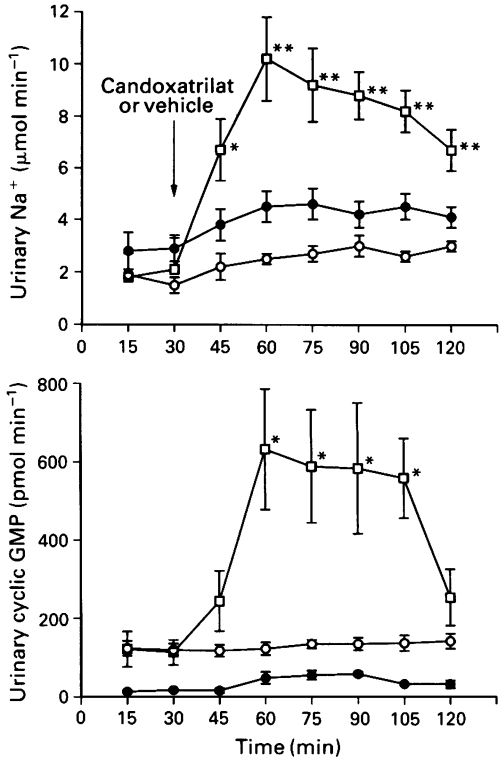

Figure 5 Effect of candoxatrilat (3 mg kg^{-1}) on urinary sodium (Na^+) and cyclic GMP excretion in A-V fistula (□) and sham-operated rats (●). Control A-V fistula rats received saline alone throughout (O). Data are mean with s.e.mean shown by vertical bars. *$P<0.05$, **$P<0.01$ compared to A-V fistula controls.

by a further rise in urinary cyclic GMP excretion (Figure 5).

Similarly, SC 46542 also stimulated a natriuresis and rise in urinary cyclic GMP excretion in the A-V fistula animal but compared with candoxatrilat, there appeared to be some loss of potency with a shift in the dose-response curve to the right (Figure 6), in that the lower dose of SC 46542, which was equipotent with candoxatrilat 3 mg kg^{-1} in the normal rat co-infused with low-dose ANP, had no significant effect on sodium excretion in the A-V fistula model. Increasing the dose 10 fold, however, produced a response closer to that seen with the E-24.11 inhibitor. The natriuretic response to the combination of candoxatrilat and high-dose SC 46542 in the A-V fistula rat was not significantly greater than that produced by either compound alone. Thus, the peak mean sodium excretion with the combined treatment was 12.2 ± 1.8 μmol min^{-1} compared to 10.2 ± 1.5 μmol min^{-1} with candoxatrilat 3 mg kg^{-1} given alone.

Baseline mean arterial blood pressure in the A-V fistula rats receiving vehicle was 103 ± 4 mmHg, and 103 ± 5, 102 ± 4 and 105 ± 5 mmHg in the candoxatrilat and low and high dose SC 46542 treatment groups respectively. This compares with 112 ± 4 mmHg in the sham-operated animals (NS). Blood pressure in the A-V fistula rats receiving active treatment did not differ significantly at any time from the fistula control (vehicle alone) group.

Baseline GFR was 2.8 ± 0.3 ml min^{-1} in the A-V fistula rats receiving saline and 3.0 ± 0.3, 2.8 ± 0.4 and 2.8 ± 0.3 ml min^{-1} in the candoxatrilat and low and high dose SC 46542 groups respectively, compared to 3.2 ± 0.3 ml min^{-1} in the

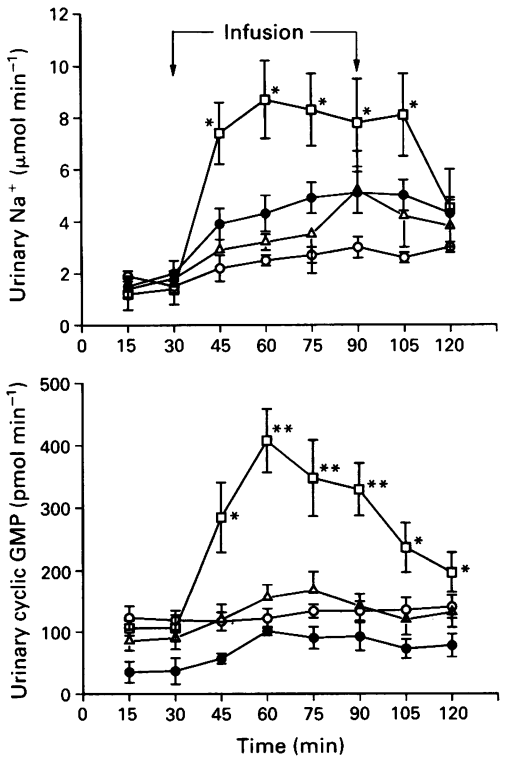

Figure 6 Effect of two doses of SC 46542 (68 μg kg^{-1} bolus then 6.8 μg kg^{-1} min^{-1}, Δ; or 680 μg kg^{-1} then 68 μg kg^{-1} min^{-1}, □) on urinary sodium (Na^+) and cyclic GMP excretion in A-V fistula rats. Sham-operated rats received the higher dose of SC 46542 alone (●). Control A-V fistula rats received vehicle alone throughout (O). Data are mean with s.e.mean shown by vertical bars. *$P<0.05$, **$P<0.01$ compared to A-V fistula controls.

sham-operated rats. None of the treatments significantly altered GFR when compared with the saline controls.

Discussion

Candoxatrilat and SC 46542 inhibit two major mechanisms for clearing ANP – the enzyme E-24.11 and C-ANP receptors respectively. Given alone in the presence of 'physiological' plasma ANP levels, neither candoxatrilat nor SC 46542 significantly affected sodium excretion. It has been suggested that under these conditions, C-ANP receptors are capable of buffering the effects of E-24.11 inhibition on plasma ANP clearance and *vice versa*. In support of this, combined inhibition of both clearance pathways has been reported to produce a greater rise in plasma ANP (Koepke *et al.*, 1989; Seymour *et al.*, 1991) and, as shown in this study, a greater natriuretic response than inhibition of either pathway alone. Similarly, when plasma ANP levels are elevated, either by infusion of exogenous peptide or in pathological states such as heart failure, the capacity of both clearance mechanisms becomes more saturated; the ability of one to compensate for inhibition of the other is limited, and the natriuretic potential of candoxatrilat and SC 46542 is unmasked.

In normal rats with plasma ANP levels elevated 5 fold by infusion of exogenous peptide, SC 46542 showed a trend towards producing a greater natriuresis than candoxatrilat, although this did not reach statistical significance. A greater natriuretic response to SC 46542 might be anticipated from data on the effects of E-24.11 inhibition and C-ANP receptor ligands on the pharmacokinetics of ANP. While both E-24.11 inhibition and the C-ANP receptor ligand, C-ANP(4-23), reduce the clearance of ANP, C-ANP(4-23) in addition reduces the volume of distribution of the peptide leading to a greater increase in plasma ANP levels (Chiu *et al.*, 1991).

However, the present studies suggest that neither candoxatrilat nor SC 46542 act simply through increasing plasma ANP levels. Although the addition of candoxatrilat or SC 46542 to ANP 100 ng kg^{-1} min^{-1}, and the combination of candoxatrilat and SC 46542, enhanced urinary sodium and cyclic GMP excretion to levels similar to, or greater than, those achieved with ANP 300 ng kg^{-1} min^{-1}, these compounds did not reproduce all the effects of the high-dose ANP infusion. First, the increased renal response was produced without matching the fall in blood pressure seen with the higher dose of ANP. The effect of infused ANP on blood pressure is due mainly to a reduction in cardiac output mediated by the action of the circulating peptide on vascular resistance and capillary absorption (Chien *et al.*, 1987; Trippodo & Barbee, 1987). E-24.11 inhibitors (SQ 29072 and SCH 34826) have been reported to increase and prolong the hypotensive response to bolus administration of high doses of ANP in the spontaneously hypertensive rat (SHR) (Seymour *et al.*, 1990) and add to the hypotension produced by C-ANP(4-23) in DOCA-salt hypertensive rats (Seymour *et al.*, 1991; Subbarao *et al.*, 1991). In contrast, other authors have found that co-administration of thiorphan with a low-dose ANP infusion or SC 46542 does not significantly affect blood pressure in normotensive rats (Trapani *et al.*, 1989; Koepke *et al.*, 1989). The explanation for this difference in response between normotensive and hypertensive rats is not known, although it has been suggested that DOCA-salt rats are particularly sensitive to the vascular effects of ANP (Kaneko *et al.*, 1987; Seymour *et al.*, 1991). Nonetheless, the consensus from these data is that in conscious normotensive rats, compared with pharmacological doses of ANP, candoxatrilat and SC 46542 preferentially enhance the renal affects of ANP.

Second, comparison of the renal effects of candoxatrilat or SC 46542 with low-dose ANP and the higher dose ANP infusion suggest that they differ in the renal mechanism by which sodium excretion is increased; that is, each may facilitate a different action of ANP within the kidney. Thus the natriuretic response to the higher dose of ANP was accompanied by a significant rise in GFR but no significant change in FeLi when compared to vehicle-treatment alone. Candoxatrilat and SC 46542 increased sodium excretion without any significant change in GFR and candoxatrilat markedly increased FeLi when given with ANP. The natriuretic response to ANP is thought to result from actions on both the glomerulus and tubule (Cogan, 1990). A rise in GFR is a consistent finding with high doses of ANP (Cogan, 1990) and may be the principal reason for the increased natriuretic response to increasing doses of the peptide. The favoured tubule site of action of ANP is the inner medullary collecting duct, as functional receptors for the peptide have been found there (Zeidel, 1990). However, ANP has also been reported to increase FeLi (Harris *et al.*, 1988), evidence of a pre-distal tubule site of action. The use of FeLi to define the tubule site of action of a drug is not without controversy, not least because doses of lithium that are natriuretic have been reported to inhibit the natriuretic effects of ANP in man (Freestone *et al.*, 1990). The doses of lithium infused in our studies resulted in plasma lithium concentrations <0.2 mmol l^{-1}, i.e. below levels that have been shown to affect sodium excretion (Freestone *et al.*, 1990), and the response to ANP is comparable to our experience in other experiments where lithium was not included in the infusate (Wilkins *et al.*, 1990b). An increase in FeLi with ANP treatment has not been observed by all authors (Cavero *et al.*, 1990) and no clear effect on FeLi was apparent in the studies reported here. This inconsistency may be due to the opposing effects of volume depletion, which increases lithium reabsorption in the distal tubule (Thomsen, 1990), thus reducing overall lithium clearance; no attempt was made to replace volume loss in the present experiments and this may have reduced the effect of ANP on FeLi. Nonetheless, in the same protocol, a marked rise in FeLi was seen with candoxatrilat when given with ANP, suggesting a pronounced pre-distal tubule effect with this drug. A similar rise in FeLi has been observed in a dog model of heart failure with the E-24.11 inhibitor, SQ 28,603 (Cavero *et al.*, 1990). The most likely site of this effect is the proximal tubule. First, E-24.11 is found in great abundance in the renal brush border. Its physiological role at this site is unknown but it is ideally placed to modify the effects of peptides on proximal tubule function. Second, ANP has been shown to antagonize the effect of angiotensin II on sodium reabsorption in the proximal tubule (Garvin, 1989).

The effect of candoxatrilat on FeLi was not shared by SC 46542. The mechanism of the increased natriuretic effect of this ligand is not apparent from this study. It has been proposed that a component of the renal response to ANP may arise from an action on the vasa recta, causing an increase in peritubular capillary hydrostatic pressure and/or an increase medullary blood flow, both of which would favour net sodium excretion (Zeidel, 1990). Given the preponderance of C-ANP receptors in the glomerulus (Rutherford *et al.*, 1991), it is tempting to suggest that inhibition of ANP clearance by these receptors may allow increased delivery of the peptide to the vasa recta, so enhancing these effects, although this remains speculative. An interesting observation is that the combination of candoxatrilat and SC 46542 markedly increased sodium excretion without affecting FeLi, suggesting that the major contribution to the natriuretic effect of this combination came from the C-ANP receptor ligand. In this context it is important to note that SC 46542, like ANP, is a substrate for E-24.11 (A.J. Kenny, personal communication) and would be protected from metabolism by candoxatrilat. Thus, as well as a response to combined inhibition of both clearance pathways, part of the increased response to co-administration of SC 46542 and candoxatrilat may arise from higher plasma levels of SC 46542.

Further evidence that candoxatrilat, SC 46542 and infusions of pharmacological doses of ANP differ in their action

on the kidney comes from the response to these agents in the A-V fistula rat. Consistent with the clinical condition, it has been shown that this model of heart failure is hyporesponsive to infusions of ANP (Wilkins *et al.*, 1990b). In this earlier study, increasing doses of ANP (100 to 1000 ng kg^{-1} min^{-1}) produced a 2 fold increase in sodium excretion in the A-V fistula animal compared to a 4 fold rise in the sham-operated rat. The mechanisms underlying the resistance to this peptide in heart failure are thought to include renal perfusion pressure, receptor down-regulation, increased activity of the renin-aldosterone system and increased renal sympathetic nerve activity. On the other hand, both candoxatrilat and SC 46542 were effective in stimulating a natriuretic response in the A-V fistula rat. Compared to candoxatrilat, there was some resistance to the natriuretic effect of the C-ANP receptor ligand: SC 46542, in a dose that was equipotent with candoxatrilat in producing a natriuresis in the normal rat receiving exogenous ANP, produced only a small, non-significant rise in sodium excretion in the A-V fistula rat. However, increasing the dose 10 fold induced a natriuresis that matched that produced by the E-24.11 inhibitor. The possibility that these compounds enhance other natriuretic factors, in addition to ANP, in A-V fistula animals cannot be excluded but the accompanying rise in urinary cyclic GMP is commensurate with an ANP-mediated response. Interestingly, co-treatment with the two compounds had a less than additive effect in this model. While this would indicate that their renal effects are not completely independent of each other, for example, a component of the response to both compounds is mediated by a rise in plasma ANP levels, a confounding factor in these experiments is the accompanying volume depletion which acts to limit the natriuresis achieved.

In summary, inhibition of E-24.11 with candoxatrilat or C-ANP receptors with SC 46542 enhanced the natriuretic but not the hypotensive effects of low-dose ANP infusion in the rat and induced a natriuresis in the rat A-V fistula model of heart failure. These effects contrast with the response to infusions of pharmacological doses of the peptide and indicate that E-24.11 inhibitors and C-ANP receptor ligands have actions other than those produced by increasing circulating ANP levels. The marked increase in FeLi with candoxatrilat plus ANP and lack of effect of this combination on GFR suggest that candoxatrilat preferentially increases a tubule action of ANP. The mechanism by which SC 46542 increase the natriuretic response to ANP remains to be elucidated.

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Differential effects of endothelin-1 on the vasorelaxant properties of benzopyran and non-benzopyran potassium channel openers

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1. The effects of endothelin-1 (ET-1) on the vasorelaxant properties of structurally different potassium channel openers (PCOs), BRL-38227, Ro 31-6930, SDZ PCO 400, EMD-52692, RP-49356 and pinacidil, were studied.

2. All PCOs evoked concentration-related relaxations of ET-1 (10 nM) or KCl (20 mM) contracted rat isolated aortic rings denuded of endothelium. BRL-38227, EMD 52692, SDZ PCO 400 and Ro 31-6930 were 11–42 times less potent in relaxing contractions to ET-1 than KCl. In contrast, this differential potency was not observed with RP-49356 or pinacidil.

3. BRL-38227 (0.06–3.0 μM), RP-49356 (0.3–3.0 μM) and pinacidil (0.3–3.0 μM) displaced KCl concentration-response curves to the right of controls, without modifying the maximum response. A subcontractile concentration of ET-1 (0.1 nM) prevented the inhibitory effects of low concentrations of BRL-38227 (0.06–0.1 μM) on KCl responses, but failed to modify those to RP-49356, pinacidil or high concentrations of BRL-38227 (0.3–3.0 μM). The inhibitory effects of BRL-38227 (0.1 μM) were also not changed by ET-3 (1.0 nM) or angiotensin II (0.1 nM).

4. In anaesthetized spontaneously hypertensive rats (SHR), cumulative bolus intravenous administrations of BRL-38227 (1–1000 μg kg^{-1}, i.v.), Ro 31-6930 (1–1000 μg kg^{-1}, i.v.), RP-49356 (10–1000 μg kg^{-1}, i.v.) or nitrendipine (0.1–30 μg kg^{-1}, i.v.) produced dose-dependent falls in diastolic blood pressure (DBP). ET-1 (i.v.) evoked a transient fall in DBP (1 μg kg^{-1} = 58 ± 1 mmHg) which returned to pre-administration levels within 4 min.

5. Pretreatment of anaesthetized SHR with ET-1 (1 μg kg^{-1}, i.v.) significantly increased the ED_{15} (dose to evoke a 15% fall in DBP) values for BRL-38227 and Ro 31-6930. However, ET-1 failed to modify the ED_{15} values for RP-49356 or nitrendipine. The ED_{50} values for all of the vasodilators studied were not modified by ET-1.

6. Infusion of BRL-38227 (2 μg kg^{-1} min^{-1}, i.v.) or RP-49356 (4 μg kg^{-1}min^{-1}, i.v.) to anaesthetized SHR evoked dose-related falls in DBP, with a corresponding increase in descending aortic blood flow (DABF) and a decrease in total lower body vascular resistance (TLBVR). Pretreatment with ET-1 (1 μg kg^{-1}, i.v.) significantly attenuated the decreases in DBP and TLBVR observed with low doses of BRL-38227, but not RP-49356 or high doses of BRL-38227. In contrast, ET-3 (3 μg kg^{-1}, i.v.) failed to modify the effects of BRL-38227 on DBP or TLBVR.

7. In conscious SHR, the fall in DBP to BRL-38227 (30 μg kg^{-1}, p.o.) was significantly reduced following ET-1 (1 μg kg^{-1}, i.a.) treatment. ET-1 (1 μg kg^{-1}, i.a.) pretreatment, however, failed to modify the decrease in DBP induced by an equieffective oral dose of RP-49356 (100 μg kg^{-1}).

8. In conclusion, ET-1 selectively attenuated the vasorelaxant effects of the potassium channel opener, BRL-38227 and other substituted benzopyrans. The results are compatible with the hypothesis that benzopyran PCOs and ET-1 have affinity for a site that does not recognise RP-49356 or pinacidil. Thus, ET-1 can differentiate between structurally unrelated potassium channel openers. The cardiovascular effects of some, but not all, PCOs might be radically modified in the clinical setting by elevated endogenous levels of ET-1 associated with certain diseased states.

Keywords: Endothelin-1; potassium channel openers; benzopyran; vasorelaxation; vasodilatation; rat aorta; spontaneously hypertensive rat

Introduction

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide, isolated from the supernatant of cultured vascular endothelial cells (Yanagisawa *et al.*, 1988), which belongs to a family of vasoactive peptides, the endothelins (Yanagisawa & Masaki, 1989). ET-1 has been reported to have a very complex pharmacology on vascular smooth muscle, where contractile effects are reported to involve the activation of ion channels (e.g. Ca^{2+}, Cl^-, K^+, non-selective cation channel) on the plasma membrane and the stimulation of intracellular processes (phosphoinositide hydrolysis, protein kinase C) following interaction with ET receptors. Several electrophysiological studies have reported an interaction of ET-1 with potassium channels in smooth muscle preparations. In rat cultured aortic smooth muscle cells, ET-1 was shown to produce a transient hyperpolarization superseded by a sustained depolarization (Van Renterghem *et al.*, 1988). The former effects were suggested to be a consequence of opening Ca^{2+}-activated potassium channels. Hu *et al.* (1991) demonstrated in smooth muscle cells of porcine coronary artery, that low concentrations of ET-1 (0.1–10 nM) increased the open-state probability of the large Ca^{2+}-activated K^+ channel (BK_{Ca}). Higher concentrations of ET-1 (> 10 nM) irrever-

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sibly inhibited the BK_{Ca} in porcine coronary artery. An interaction of ET-1 with ATP-sensitive potassium channels (K_{ATP}) in *in vitro* (cell culture: Inoue *et al.*, 1990) and *in vivo* studies (Hasunuma *et al.*, 1990; Lippton *et al.*, 1991) has also been demonstrated.

BRL-38227, pinacidil and RP-49356 belong to a novel class of compounds thought to cause smooth muscle relaxation by opening potassium channels in the plasmalemma of smooth muscle cells (see Edwards & Weston, 1990 and references therein).

Therefore, the objective of the present study was to determine if ET-1 could modify the vasorelaxant responses of benzopyran (BRL-38227, Ro 31-6930, EMD 52692, SDZ PCO 400), thioformamide (RP-49356) and pyridyl-cyanoguanidine (pinacidil) openers of potassium channels (PCOs; see Edwards & Weston, 1990) in the rat aorta (*in vitro*) and spontaneously hypertensive rat (*in vivo*) models. Part of this work has been presented in abstract form to the 7th International Symposium on Vascular Neuroeffector Mechanisms (Lawson *et al.*, 1990a), to the IUPHAR, Amsterdam (Lawson *et al.*, 1990b) and to the British Pharmacological Society (Lawson *et al.*, 1991).

Methods

Rat isolated aorta

Ring preparations (4 mm in length) were obtained from the thoracic aorta of male Sprague-Dawley rats (250–300 g) and denuded of functional endothelium by rubbing the intima. The tissues were suspended in isolated organ baths containing Krebs bicarbonate solution (composition in mM: NaCl 118.0, KCl 4.6, $CaCl_2$ 2.5, $MgSO_4.7H_2O$ 1.2, glucose 10.0 and $NaHCO_3$ 25.0) maintained at 37°C and gassed with 95% O_2 and 5% CO_2. An initial resting tension of 2 g was maintained on the preparations during an initial equilibration period of 60 min. The tension of each ring was continuously recorded isometrically with a Grass FT03c force-displacement transducer and displayed on a Gould (BS 274) chart recorder.

Endothelin-1 or KCl-induced contractile responses After a 60 min equilibration period, preparations were contracted with either KCl (20 or 40 mM) or ET-1 (10 or 100 nM); one contractile agent and one concentration was studied per preparation. When the contractile response had stabilized, cumulative concentration-relaxant response curves to BRL-38227 (0.01–30 μM), Ro 31-6930 (0.01–30 μM), EMD 52692 (0.001–3 μM), SDZ PCO 400 (0.01–30 μM), RP-49356 (0.01–10 μM) or pinacidil (0.1–10 μM) were constructed. Only one concentration-response curve was obtained per preparation. The pIC_{50} (log concentration of compound required to relax the aorta by 50%) and E_{max} (maximum relaxation response (%) where 100% = return to precontraction baseline) values were calculated for each individual concentration-response curve.

KCl concentration-response curves Cumulative concentration-response curves to KCl (8–35 mM final bath concentration of K^+ ions) were constructed in separate groups of aortae, before and 30 min after incubation with ET-1 (0.1 nM) or vehicle alone, or with BRL-38227 (0.06–3.0 μM), RP-49356 (0.3–3.0 μM) or pinacidil (0.3–3.0 μM). In a separate series of experiments, concentration-response curves to KCl were constructed before and 30 min after ET-3 (1.0 nM), angiotensin II (0.1 nM) or vehicle alone or with BRL-38227 (0.1 μM). Only one concentration of each potassium channel opener was studied per preparation. The concentrations of ET-1, ET-3 and angiotensin II used were the highest concentration of each peptide that failed to evoke a contractile response in the rat isolated aorta. Responses to KCl after the respective treatments were compared with responses from time-matched control preparations from the same animal. The EC_{50} values (concentration of KCl required to evoke 50% of the control maximal response) and E_{max} values (maximal response to KCl) were calculated for each individual concentration-response curve.

Anaesthetized spontaneously hypertensive rats

Male spontaneously hypertensive rats (SHR: 250–350 g) were anaesthetized with pentobarbitone sodium (50 mg kg^{-1}, i.p.) and maintained with a continuous infusion of 10 mg kg^{-1} h^{-1} pentobarbitone via the femoral vein. The trachea was cannulated to facilitate spontaneous respiration. Arterial blood pressure was recorded from a cannulated carotid artery via a Statham P50 pressure transducer connected to a Gould chart recorder. A femoral vein was cannulated for intravenous administration of compounds.

Cumulative bolus dose-response curves to PCOs After a period of stabilization (30 min), cumulative dose-response curves to BRL-38227 (1–1000 μg kg^{-1}, i.v.), Ro 31-6930 (1–1000 μg kg^{-1}, i.v.), RP-49356 (10–1000 μg kg^{-1}, i.v.) or nitrendipine (0.1–30 μg kg^{-1}, i.v.) were constructed 4 min after intravenous administration of ET-1 (1 or 10 μg kg^{-1}, bolus) or saline (1 ml kg^{-1}, bolus) in separate groups ($n = 5–7$) of SHR. At this time (4 min post ET-1) the acute fall (1 μg kg^{-1} = 58 ± 1 mmHg, $n = 24$; 10 μg kg^{-1} = 77 ± 6 mmHg, $n = 9$) in arterial blood pressure to ET-1 had returned to pre-dose levels. No hypertensive response to the peptide was observed in SHR. The doses of compound required to evoke 15% (ED_{15}) and 50% (ED_{50}) decreases in DBP were calculated from individual dose-response curves.

Continuous intravenous infusion of PCOs For these studies the abdominal aorta below the diaphragm (between the right renal and the mesenteric arteries) of the SHR was cleared and an electromagnetic flow probe (1 mm diameter; Skalar, Delf) placed around the vessel for the measurement of descending aortic blood flow (DABF). Total lower body vascular resistance (TLBVR) was calculated by dividing the mean arterial blood pressure (MBP) by the DABF. The DABF and TLBVR values were corrected for body weight and expressed as ml min^{-1} 100 g^{-1} body weight and mmHg ml^{-1} min^{-1} 100 g^{-1} body weight, respectively.

After a period of stablization (30 min), intravenous infusions of BRL-38227 (2 μg kg^{-1} min^{-1} for 30 min), RP-49356 (4 μg kg^{-1} min^{-1} for 30 min) or vehicle (0.05 ml kg^{-1} min^{-1} for 30 min) were administered 4 min after ET-1 (1 μg kg^{-1}, i.v. bolus) or saline (1 ml kg^{-1}, i.v. bolus) in separate groups ($n = 7–9$) of SHR. In further groups of SHR, an intravenous infusion of BRL-38227 (2 μg kg^{-1} min^{-1} for 30 min) or vehicle (0.05 ml kg^{-1} min^{-1} for 30 min) was administered 4 min after ET-3 (1 μg kg^{-1}, i.v. bolus) or saline (1 ml kg^{-1}, i.v. bolus). Changes in DBP, DABF and TLBVR for each individual animal were calculated from baseline values taken at time zero and are presented as percentage change (%Δ).

Conscious spontaneously hypertensive rats

Male SHR (280–330 g) were anaesthetized with pentobarbitone sodium (50 mg kg^{-1}, i.p.) and a heparinized saline-filled catheter implanted into the descending aorta via a femoral artery, using a modified method of Popovic & Popovic (1960). Animals were housed in individual cages and allowed to recover over a 2 day period. At the end of this period, pulsatile aortic blood pressure was measured directly by means of a Statham P50 pressure transducer connected to the indwelling catheter and recorded on a Gould (S4000) chart recorder.

Separate groups of SHR received a single oral administration of BRL-38227 (30 μg kg^{-1}), RP-49356 (100 μg kg^{-1}) or vehicle (0.5 ml kg^{-1}) 4 min after an intra-arterial (i.a.) injection of either ET-1 (1 μg kg^{-1}, bolus) or saline (0.3 ml kg^{-1},

bolus). Maximum changes in diastolic blood pressure (DBP) were recorded at 5 min intervals for the first 30 min and thereafter at 60 and 120 min. Calculations were made for the percentage changes in DBP for each treatment relative to values after pretreatment. Areas under the time-DBP response curve for the first 60 min ($AUC_{0-60\,min}$) were calculated using the trapezoid rule.

Statistical analysis

Values in the text are expressed as means ± s.e.means or means with 95% confidence limits for n determinations. For studies in anaesthetized SHR receiving a continuous intravenous infusion of PCO, a one-way analysis of variance on DBP and TLBVR at each time point was used to test for differences between ET-1-treated and control groups (Fisher's LSD strategy). A contrast analysis was also performed to account for any haemodynamic effects of ET-1 by testing, for each time point, the nul hypothesis that

$$[(PCO + ET\text{-}1)\text{-}PCO]\text{-}[ET\text{-}1\text{-}control] = 0$$

where PCO + ET-1 is the response to PCO after ET-1 treatment, PCO is the response to the potassium channel opener alone, ET-1 is the response to saline after ET-1 treatment and control is the response to saline after vehicle treatment.

All other data presented as mean ± s.e.means were statistically evaluated by analysis of variance and an unpaired Student's t test for between group comparisons. When P was less than 0.05, two means were considered to be significantly different.

Drugs and solutions

The following compounds were used: endothelin-1 and endothelin-3 were purchased from Novabiochem (Clery-en-Vexin, France). BRL-38227 ((−)-*trans* 3,4-dihydro-3-hydroxy-2,2-dimethyl- 4- (2-oxo-1-pyrrolidinyl)- 2H- 1-benzopyran-6-carbonitrile), Ro 31-6930 (2-(6-cyano-2,2-dimethyl-2H-1-benzopyran-4-yl)-pyridine 1-oxide), EMD 52692 (4-(1,2-dihydro-2-oxo-1-pyridyl)-2, 2-dimethyl- 2H-1-benzopyran-6-carbonitrile), racemic SDZ PCO 400 ((±)-(3S,4R)-3,4-dihydro-3-hydroxy-2,2-dimethyl-4-(3-oxo-cyclopent-1-enyloxy)-2H-1-benzopyran -6-carbonitrile), pinacidil and RP-49356 (N-methyl-2- (3-pyridyl)-tetrahydro-thiopyran-2-carbothioamide-1-oxide) were synthesized by Dr A.W. Lochead, Department of Chemistry, Recherche Syntex France. Angiotensin II and nitrendipine were purchased from Sigma (St Louis, U.S.A.). Stock solutions of BRL-38227, Ro 31-6930, EMD 52692, SDZ PCO 400, pinacidil and nitrendipine were dissolved in absolute ethanol for *in vitro* studies and in PEG-300/ ethanol/glycerol mixture for *in vivo* studies. RP-49356 and endothelin-1 were dissolved in distilled water; concentrated aliquots of peptide were stored at − 80°C, before being thawed and diluted for use. Subsequent dilutions of all compounds used were made with distilled water (*in vitro*) or in 0.9% NaCl solution for *in vivo* studies.

Results

Rat aorta

Endothelin-1 and KCl-induced contractile responses ET-1 (10 and 100 nM) and KCl (20 and 40 mM) evoked contractile responses of the rat aorta that developed a stable plateau for at least 60 min in controls.

EMD 52692, Ro 31-6930, BRL-38227, SDZ PCO 400, RP-49356 and pinacidil all evoked concentration-related relaxations of KCl (20 mM; pIC_{50} values 8.30 ± 0.10, 7.80 ± 0.01, 7.57 ± 0.07, 7.18 ± 0.12, 6.48 ± 0.10 and 6.64 ± 0.06 respectively, $n = 4-8$) or ET-1 (10 nM; pIC_{50} values 7.20 ± 0.05*, 6.18 ± 0.18*, 6.25 ± 0.25*, 6.14 ± 0.16*, 6.27 ± 0.06 and 6.47 ± 0.10 respectively, $n = 4-8$; *indicate a value significantly different ($P<0.05$, unpaired t test) from that on KCl contracted rat aorta (Figure 1). The substituted benzopyran potassium channel openers, BRL-38227, Ro 31-6930, EMD 52692 and SDZ PCO 400 were significantly more potent (11–42 times) in relaxing KCl (20 mM)-contracted than ET-1 (10 nM)-contracted rat aorta (Figure 1). In contrast, the vasorelaxant potencies of RP-49356 or pinacidil on ET-1 (10 nM) or KCl (20 mM) contractions did not differ. Although all PCOs fully relaxed the contraction to KCl (20 mM), BRL-38227, SDZ PCO 400, Ro 31-6930 and EMD-52692 did not completely inhibit the ET-1-induced contractile response (Figure 1).

In a separate series of experiments, when the preparations were contracted with a higher concentration of KCl (40 mM), pinacidil evoked relaxations with a pIC_{50} value of 5.38 ± 0.18 ($n = 4$). In contrast, a 50% relaxant response was not obtained for either BRL-38227 or RP-49356 (up to 30 μM) on contractions to KCl (40 mM). In further groups of aorta, increasing the concentration of ET-1 (to 100 nM) did not significantly modify the relaxant potencies of the PCOs BRL-38227, RP-49356 and pinacidil relative to the potencies calculated with ET-1 at 10 nM.

KCl concentration-response curves KCl (8–35 mM) induced concentration-related contractile responses in the rat aorta. None of the concentrations of ET-1, ET-3, angiotensin II, BRL-38227, RP-49356 or pinacidil employed as pretreatments in this study protocol modified the base-line tension of the aorta.

In separate groups of aorta, BRL-38227 (0.06–3.0 μM), RP-49356 (0.3–3.0 μM) and pinacidil (1.0–3.0 μM) (one concentration per preparation) each progressively displaced the contractile response curves to KCl to the right of controls (Table 1), without modifying the maximal response (control

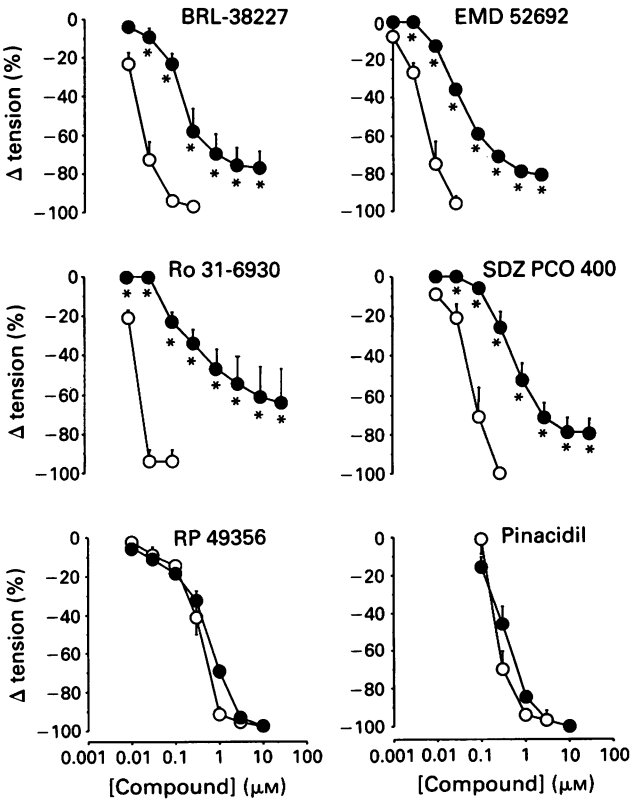

Figure 1 Effects of potassium channel openers on potassium or endothelin-1 contracted rat aorta. Cumulative concentration-response curves to the potassium channel openers were constructed on aortic rings contracted with either KCl (20 mM; ○) or endothelin-1 (ET-1, 10 nM: ●). Data represent mean values with vertical bars indicating s.e.mean ($n = 4-7$ per group). *Indicate responses to PCO that were significantly different ($P<0.05$, unpaired t test) from corresponding responses on KCl (20 mM)-contracted preparations.

Table 1 Effects of BRL-38227, RP-49356 and pinacidil on KCl concentration-response curves in rat aortic rings in the absence and presence of endothelin-1 (ET-1)

	EC_{50} (mM)	After ET-1 (0.1 nM) EC_{50} (mM)
Control	10.9 (10.5–11.3)	9.7 (7.8–11.6)
BRL-38227 (μM)		
0.06	18.6 (17.7–19.5)*	15.1 (13.9–16.3)*†
0.10	20.3 (16.5–24.1)*	12.9 (11.1–14.7)†
0.30	24.4 (21.5–27.3)*	25.1 (22.4–27.8)*
3.00	27.3 (22.8–31.8)*	26.2 (22.9–29.5)*
RP-49356(μM)		
0.30	14.5 (13.0–15.9)*	13.8 (11.8–15.8)*
1.00	18.9 (17.9–19.9)*	17.9 (17.1–18.7)*
3.00	25.6 (24.0–27.2)*	25.0 (20.9–29.1)*
Pinacidil (μM)		
0.30	12.7 (10.3–15.1)	15.0 (10.1–19.9)
1.00	23.0 (20.7–25.3)*	23.0 (18.1–28.0)*
3.00	26.2 (23.4–29.0)*	26.6 (21.4–31.8)*

Cumulative concentration-response curves to KCl were constructed 30 min after respective treatments. For each curve to KCl the EC_{50} values are represented as means with 95% confidence limits for each treatment group ($n = 4–38$). *Indicates EC_{50} values that are significantly ($P<0.05$) different from control group; †indicates EC_{50} values that are significantly ($P<0.05$) different from the corresponding results determined in the absence of ET-1.

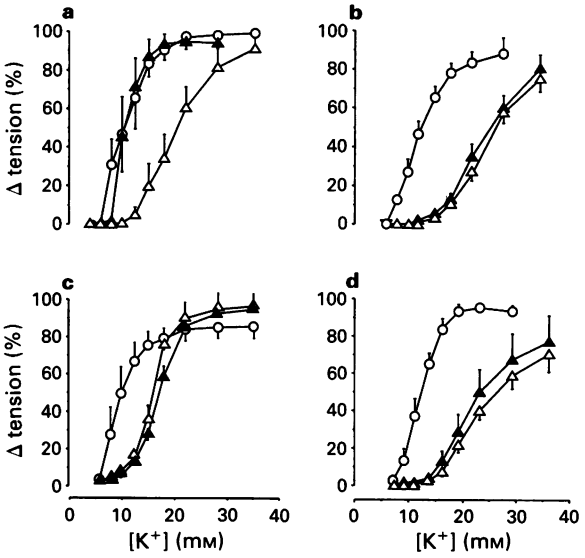

Figure 2 Effects of BRL-38227 (a and b), RP-49356 (c) and pinacidil (d) in the presence or absence of endothelin-1 (ET-1) on concentration-response curves to KCl in rat aortic rings. Cumulative concentration-response curves to KCl were constructed before and 30 min after vehicle (○), a potassium channel opener (Δ: in (a) = BRL-38227 0.1 μM, (b) = BRL-38227 3.0 μM (c) = RP-49356 1.0 μM and (d) = pinacidil 1.0 μM) or a potassium channel opener (as above) with ET-1 (0.1 nM: ▲). Results (means with s.e.mean shown by vertical bars, $n = 5–6$ per group) are expressed as a percentage of the maximum response to the KCl response curve before treatment.

E_{max} 1.84 ± 0.06 g, $n = 38$). ET-1 (0.1 nM), ET-3 (1.0 nM) or angiotensin II (0.1 nM) alone failed to modify the KCl contractile responses. In the presence of ET-1 (0.1 nM), the rightward shifts of the KCl response curves induced by low concentrations of BRL-38227 (0.06 and 0.1 μM) were decreased (Figure 2 and Table 1). In contrast, the inhibitory effects of RP-49356 (0.3–3.0 μM), pinacidil (0.3–3.0 μM) or high concentrations of BRL-38227 (0.3–3.0 μM) on responses induced by KCl were not attenuated by ET-1 (0.1 nM; Figure 2 and Table 1).

ET-3 (1.0 nM) and angiotensin II (0.1 nM) failed to modify the inhibitory effects of BRL-38227 (0.1 μM) on KCl-induced responses (Figure 3).

Anaesthetized SHR

Cumulative bolus dose-response curves to PCOs In anaesthetized spontaneously hypertensive rats (SHR), the group mean baseline DBP value was 139 ± 3 mmHg ($n = 49$); no significant differences were observed between the individual group values or for each individual group value from the collated mean. BRL-38227 (1–1000 μg kg^{-1}, i.v.), Ro 31-6930 (1–1000 μg kg^{-1}, i.v.), RP-49356 (10–1000 μg kg^{-1}, i.v.) or nitrendipine (0.1–30 μg kg^{-1}, i.v.), as cumulative bolus administrations, produced dose-related decreases in diastolic blood pressure (DBP).

ET-1 (1 or 10 μg kg^{-1}, i.v.) pretreatment of the anaesthetized SHR significantly increased the ED_{15} values (dose to lower DBP by 15%) for BRL-38227 (control 8.2 ± 2.2 μg kg^{-1}, $n = 6$; after 1 μg kg^{-1} ET-1 13.2 ± 0.7 μg kg^{-1}, $n = 4$, $P<0.05$; after 10 μg kg^{-1} ET-1 23.7 ± 3.2 μg kg^{-1}, $n = 5$, $P<0.05$) and Ro 31-6930 (control 5.2 ± 1.0 μg kg^{-1}, $n = 6$; after 1 μg kg^{-1} ET-1 11.7 ± 2.5 μg kg^{-1}, $n = 6$, $P<0.05$). However, ET-1 failed to modify the ED_{15} values for RP-49356 (control 53.3 ± 7.0 μg kg^{-1}, $n = 6$; after 1 μg kg^{-1} ET-1 55.0 ± 6.0 μg kg^{-1}, $n = 6$) or nitrendipine (control 1.2 ± 0.3 μg kg^{-1}, $n = 6$; after 10 μg kg^{-1} ET-1 0.7 ± 0.1 μg kg^{-1}, $n = 4$). In addition, the ED_{50} values (50% fall in DBP) for all of the vasodilators studied were not different after ET-1 relative to controls.

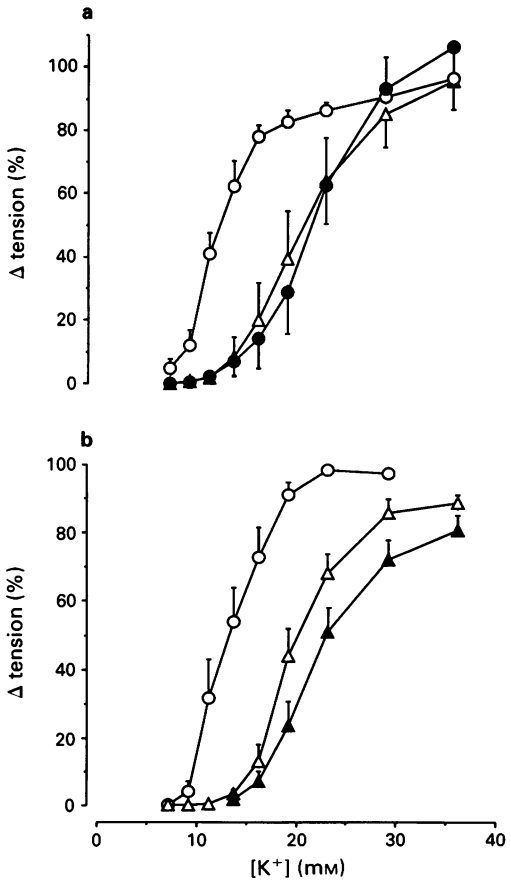

Figure 3 Effects of BRL-38227 in the presence or absence of endothelin-3 (ET-3) (a) or angiotensin II (b) on concentration-response curves to KCl in rat aortic rings. Cumulative concentration-response curves to KCl were constructed before and 30 min after vehicle (○), BRL-38227 (0.1 μM: Δ), BRL-38227 (0.1 μM) plus ET-3 (1.0 nM: ●) or BRL-38227 (0.1 μM) plus angiotensin II (0.1 nM: ▲). Results (means with s.e.mean shown by vertical bars, $n = 5–6$ per group) are expressed as a percentage of the maximum response to the KCl response curve before treatment.

Continuous intravenous infusion of PCOs In separate series of experiments, the effects of a bolus pretreatment with ET-1 (1 μg kg^{-1}, i.v.) or ET-3 (1 μg kg^{-1}, i.v.) on the haemodynamic effects of an intravenous infusion of either BRL-38227 or RP-49356 in anaesthetized SHR were studied. The group mean baseline DBP, DABF and TLBVR values were 139 ± 2 mmHg, 7.8 ± 0.1 ml min^{-1} 100 g^{-1} body weight and 20.0 ± 0.4 mmHg ml^{-1} min^{-1} 100 g^{-1} body weight (n = 80), respectively. No significant differences were observed between the individual group values or for each individual group value from the collated mean. ET-1 and ET-3 decreased DBP by 58 ± 1% (n = 30) and 39 ± 3% (n = 14) respectively; this parameter returned to pre-dose levels by 4 min with no significant overshoot.

BRL-38227 (2 μg kg^{-1} min^{-1}) and RP-49356 (4 μg kg^{-1} min^{-1}) produced dose-dependent falls in DBP and TLBVR (significant from vehicle-treated groups from 10 and 28 μg kg^{-1} doses, respectively; Figures 4 and 5), which were associated with an increase (5–15%) in DABF (not shown). Pretreatment with ET-1 (1 μg kg^{-1}) significantly attenuated the decreases in DBP and TLBVR observed during 7–20 min and 7–15 min, respectively, infusion of BRL-38227 (dose equivalence 14–40 μg kg^{-1}, Figure 4). The responses to BRL-38227 following 25 min of infusion (dose equivalence 50 μg kg^{-1}) were not modified by ET-1 (1 μg kg^{-1}). After treatment with ET-1 the decreases in DBP and TLBVR to RP-49356 were only slightly attenuated (significant at 28–44 μg kg^{-1} dose equivalence; Figure 5) compared with RP-49356 alone, with no modification of the increase in DABF (not shown). However, when the haemodynamic effects of the peptide alone are taken into consideration using contrast analysis of the results (see methods), the DBP and TLBVR responses to RP-49356 in the presence of ET-1 (1 μg kg^{-1}) were not significantly different from those to RP-49356 alone (Figure 5). The DBP and TLBVR responses to BRL-38227 (14–22 μg kg^{-1}) were, however, still significantly attenuated after ET-1, when the haemodynamic effects of the peptide alone are taken into consideration (Figure 4).

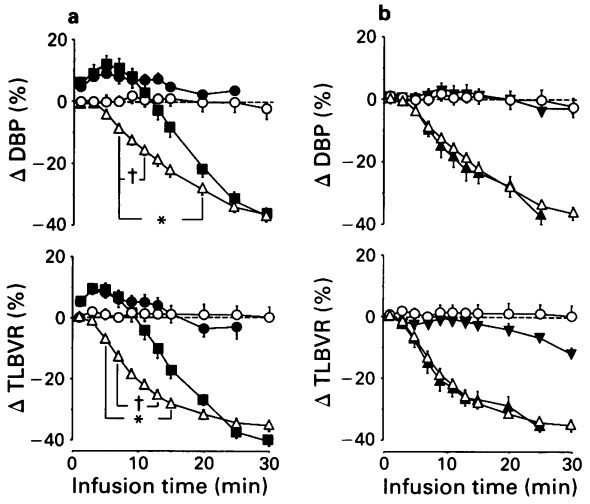

Figure 4 Effects of endothelin-1 (ET-1) (a) and ET-3 (b) on the responses to an infusion of BRL-38227 in anaesthetized spontaneously hypertensive rats (SHR). BRL-38227 was infused (2 μg kg^{-1} min^{-1}, i.v.) 4 min after ET-1 (1 μg kg^{-1} min^{-1}, i.v: ■), ET-3 (1 μg kg^{-1}, i.v: ▲) or saline (1 ml kg^{-1}, i.v.: Δ). In a separate group of SHR, saline was infused (0.05 ml kg^{-1} min^{-1}, i.v.) 4 min after ET-1 (1 μg kg^{-1} min^{-1}, i.v: ●), ET-3 (1 μg kg^{-1}, i.v: ▼) or saline (1 ml kg^{-1}, i.v.: ○). Data, expressed as percentage change in diastolic blood pressure (%Δ DBP) and total lower body vascular resistance (%Δ TLBVR), calculated from the 4 min post ET-1 time-point represent mean ± s.e.mean (vertical bars) (n = 7–18 per group). *Indicates the range of hypotensive responses to BRL-38227 that are significantly different (P < 0.05) after ET-1 from those to BRL-38227 alone on direct comparison. †Indicates the range of hypotensive responses to BRL-38227 that are significantly different (P < 0.05) after ET-1 from those to BRL-38227 when the effects of the peptide alone are taken into consideration.

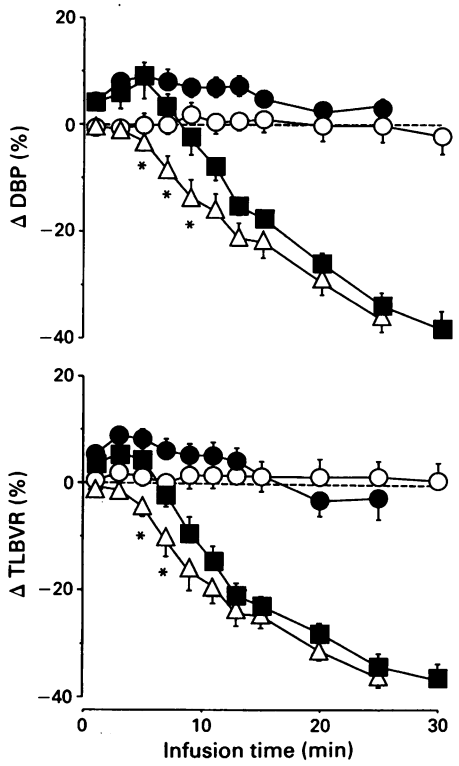

Figure 5 Effects of endothelin-1 (ET-1) on the responses to an infusion of RP-49356 in anaesthetized spontaneously hypertensive rats (SHR). RP-49356 was infused (4 μg kg^{-1} min^{-1}, i.v.) 4 min after a bolus administration of ET-1 (1 μg kg^{-1}, i.v.: ■) or saline (1 ml kg^{-1}, i.v.: Δ). In a separate group of SHR saline was infused (0.05 ml kg^{-1} min^{-1}, i.v.) 4 min after a bolus administration of ET-1 (1 μg kg^{-1}, i.v.: ○) or saline (1 ml kg^{-1}, i.v.: ●). Data, expressed as percentage change in diastolic blood pressure (%Δ DBP) and total lower body vascular resistance (%Δ TLBVR), calculated from the 4 min post ET-1 time-point represent mean ± s.e.mean (vertical bars) (n = 7–12 per group). *Indicates range of hypotensive responses to RP-49356 that are significantly different (P < 0.05) after ET-1 from those to RP-49356 alone. No significant differences were observed between the effects of RP-49356 with or without ET-1 when the effects of ET-1 alone were taken into account.

In contrast, ET-3 (1 μg kg^{-1}) failed to modify the effects of BRL-38227 (2 μg kg^{-1} min^{-1}, i.v.) on DBP or TLBVR (Figure 4); however, the increase in DABF (not shown) over the dose range 22–60 μg kg^{-1} of BRL-38227 was slightly attenuated by ET-3 (1 μg kg^{-1}) compared with non-ET-3-treated controls. The interaction of a higher dose of ET-3 with the PCOs could not be studied due to pronounced hypotensive effects of the peptide.

Conscious SHR

In conscious SHR, the group mean baseline DBP value was 156 ± 4 mmHg (n = 33); no significant differences were observed between the individual group values or for each individual group value from the collated mean. BRL-38227 (30 μg kg^{-1}, p.o.) and RP-49356 (100 μg kg^{-1}, p.o.) produced equieffective decreases in the DBP that were maintained for at least 60 min (Figure 6). ET-1 (1 μg kg^{-1}, i.a.) produced a transient fall in DBP (29 ± 9 mmHg, n = 10) in conscious SHR, which returned to base-line levels within 4 min. No significant hypertension to ET-1 (1 μg kg^{-1}, i.a.) was observed over a 2 h recording period.

The fall in DBP induced by BRL-38227 (30 μg kg^{-1}, p.o.; peak effect − 24.3 ± 2.5 mmHg, $AUC_{0-60\,min}$ 959 ± 100% min,

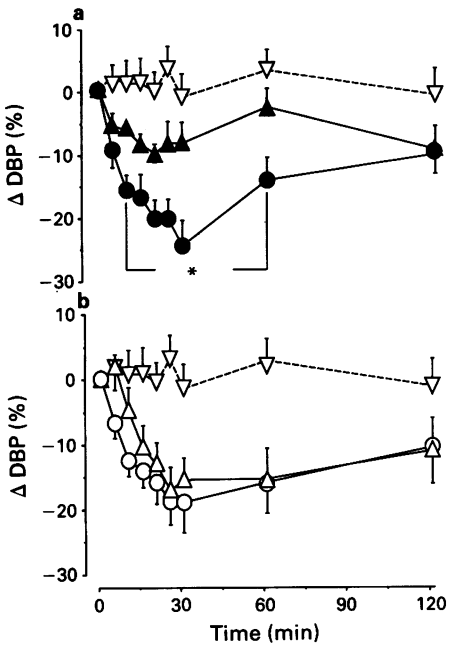

Figure 6 Effects of endothelin-1 (ET-1) on the vasodepressor responses to BRL-38227 (a) and RP-49356 (b) in conscious spontaneously hypertensive rats (SHR). BRL-38227 (30 $\mu g\,kg^{-1}$, p.o.; closed symbols) or RP-49356 (100 $\mu g\,kg^{-1}$, p.o.; open symbols) was administered 4 min after intra-arterial administration of ET-1 (1 $\mu g\,kg^{-1}$: Δ, ▲) or saline (1 $ml\,kg^{-1}$: ○, ●). A separate group of SHR received saline (0.3 $ml\,kg^{-1}$, p.o.), 4 min after ET-1 (1 $\mu g\,kg^{-1}$, i.a.: ▽--▽). Data, expressed as percentage change in diastolic blood pressure (%Δ DBP) relative to time, represent mean ± s.e.mean (vertical bars) ($n = 5-8$ per group). *Indicates range of BRL-38227 responses that are significantly different ($P < 0.05$) from the corresponding results in the BRL-38227 alone group.

$n = 8$; Figure 6) was significantly reduced in conscious SHR pretreated with ET-1 (1 $\mu g\,kg^{-1}$, i.a.; peak effect -9.3 ± 1.6 mmHg, $AUC_{0-60\,min}$ 382 ± 180%.min, $n = 8$, $P < 0.05$). In contrast, ET-1 (1 $\mu g\,kg^{-1}$, i.a.) pretreatment failed to modify the decrease in DBP induced by RP-49356 (100 $\mu g\,kg^{-1}$, p.o., control: peak effect -18.8 ± 4.7 mmHg, $AUC_{0-60\,min}$ 903 ± 214%.min, $n = 5$; after ET-1: peak effect -17.0 ± 3.5 mmHg, $AUC_{0-60\,min}$ 671 ± 212%.min, $n = 5$; Figure 6). In conscious SHR that received saline after ET-1 treatment, the peak effect change in DBP and the $AUC_{0-60\,min}$ value were 2.3 ± 2.9 mmHg and 53 ± 129%.min ($n = 7$), respectively (Figure 6).

Discussion

This present study has demonstrated that the vascular effects of substituted benzopyran potassium channel openers, e.g. BRL-38227, were selectively attenuated by the peptide, ET-1. This was a property of ET-1, but not of the isopeptide ET-3. The vasodilator responses to the structurally different potassium channel openers, RP-49356 (thioformamide) and pinacidil (pyridyl-cyanoguanidine), were insensitive to ET-1.

All potassium channel openers evoked concentration-related relaxant responses of endothelin-1- or low potassium (20 mM)-contracted isolated aortic rings of the rat; however, only pinacidil (amongst the PCOs tested) relaxed contractions to KCl (40 mM). Low K^+ activates a contractile mechanism against which the PCOs demonstrate a rank order of potency for relaxation of: EMD 52692 > Ro 31-6930 > BRL-38227 > SDZ PCO 400 > pinacidil = RP-49356. The benzopyrans (EMD 52692, Ro 31-6930, BRL-38227, SDZ PCO 400) were the more potent vasorelaxants giving IC_{50} values 5–60 times greater than those for the thioformamide, RP-49356, or the pyridyl-cyanoguanidine, pinacidil. In contrast, the three groups of structurally unrelated PCOs (with the exception of EMD 52692) exhibit similar potency in relaxing the contraction to ET-1. Although EMD 52692 was at least 5 times more potent than the other PCOs against contractions to the peptide, EMD 52692 was still significantly less potent (10 times) in relaxing ET-1-contracted than K^+ (20 mM)-contracted rat aorta. Therefore, the greater potency of the benzopyrans over other chemical classes of PCOs is no longer evident in the presence of an ET-1-induced contraction. These results are consistent with the hypothesis that benzopyran PCOs activate a relaxant mechanism in KCl contracted rat aorta, which is not operational in ET-1 contracted tissues. Presumably, it is this mechanism that is responsible for the potency advantage of this class of PCOs. The fact that the potencies of RP-49356 and pinacidil were not modified by ET-1 would suggest that these PCOs do not interact with this benzopyran-sensitive relaxant mechanism.

In a separate series of experiments BRL-38227, pinacidil and RP-49356 when applied to the tissue as pretreatments failed to modify resting tone, but selectively attenuated the contractile responses induced by low concentrations of KCl (5–30 mM), results which are consistent with activation of potassium channels (Lawson & Cavero, 1989). ET-1 (0.1 nM), at a concentration which also failed to modify resting tone in this preparation, selectively decreased the rightward shift in the KCl concentration-response curve in the presence of low concentrations of BRL-38227, but failed to modify the inhibitory effects of RP-49356 or pinacidil. These results support the findings obtained in the ET-1/KCl contraction studies and suggest that ET-1, even at subcontractile concentrations, can discriminate between structurally different PCOs by selectively inhibiting the effects of benzopyran-type PCOs.

These results are consistent with the hypothesis that ET-1 blocks a site (Site 1) on the smooth muscle cell membrane for which the benzopyran BRL-38227 has high affinity. RP-49356 and pinacidil interact with a second site (Site 2), which is apparently insensitive to ET-1, to open the K^+-channel in the rat aorta and cause smooth muscle relaxation. The lack of inhibitory effect of ET-1 against the high concentrations of BRL-38227 on the KCl concentration-response curve could be explained if ET-1 is a competitive antagonist at 'Site 1'. In this case, high concentrations of BRL-38227 would be able to surmount the blockade by ET-1. Binding experiments using [^{125}I]-ET-1, however, have demonstrated that bound ET-1 hardly dissociates from its receptor(s) and thus the peptide can be considered as an irreversible ligand (Hirata *et al.*,1988; Clozel *et al.*, 1989; Kanse *et al.*, 1989). Therefore, 'surmountable antagonism' of the benzopyrans by ET-1 in our study would involve a site distinct from an ET receptor, possibly a direct interaction at the site on the potassium channel that recognises BRL-38227 (and other benzopyrans). An alternative explanation could be that BRL-38227 also has affinity (although low) for 'Site 2' and, thus in the presence of ET-1 (i.e. after removal of 'Site 1'), BRL-38227 can at high concentrations still open K^+-channels. Consequently, it would be the activation of 'Site 1' that is responsible for the potency advantage of benzopyrans over other PCOs as described above. No attempt has thus far been made to study this interaction by use of electrophysiological techniques. The proposed 'two-site' hypothesis would be best resolved by use of radioligand binding techniques; however, to date such techniques are not routinely available. Although Quast & Bray (1992) have described a purported specific PCO binding site in rat aorta, further work is still required to validate this finding. Recently, Waugh *et al.* (1992) suggested that BRL-38227, but not BRL-38226 ((+)-*trans* enantiomer of cromakalim) could displace [^{125}I]-ET-1 from rat cardiac membranes, an initial indication of a direct affinity of a PCO for ET-1 binding sites. However, the stereoselective displacement reported represented only 30–40% of total specific binding. Whether this represents an affinity of BRL-38227 for one of

the ET-receptors in this tissue or an allosteric modulation of the ET-1 binding sites is unknown.

The apparent inhibitory effects of ET-1 on the smooth muscle relaxant responses to benzopyran PCOs may be indirectly mediated, for example through an intracellular interaction on K^+-channel function, or may imply that the benzopyrans activate a different channel (or site on the channel) from that activated by non-benzopyran PCOs. An indirect mechanism for the above effects of ET-1 would suggest that, although all PCOs interact with the same membrane site, the subsequent intracellular processes vary in sensitivity to PCOs of different chemical structure. Whilst this remains tenable at a concentration of the peptide (e.g. 10 nM) which induces tone, it would be less probable at a concentration of ET-1 (e.g. 0.1 nM) sub-threshold for contraction where no obvious functional intracellular cationic exchange occurs. In a preliminary report, Inoue *et al.* (1990) showed that ET-1 evoked a direct (inhibitory) effect on an ATP-sensitive K^+-channel (which BRL-38227, pinacidil and RP-49356 are purported to open) in primary cultured smooth muscle cells from porcine coronary artery. The discrimination by ET-1 between BRL-38227 and pinacidil or RP-49356, under both the *in vitro* experimental protocols, strongly suggests an interaction of the peptide with K^+-channels and that different sites for the PCOs could be present in the rat aorta. Thus, ET-1 can be used as a pharmacological probe to study further the high affinity site, which is only activated by the benzopyran PCOs. Finally, these results do not exclude the possibility that ET-1 and BRL-38227 interact with a K^+-channel that is different from that activated by RP-49356 and pinacidil.

The failure of endothelin-3 and angiotensin II, as pretreatments, to modify the effects of BRL-38227 on the KCl concentration-response curve indicates that the modulation of K^+-channels in rat aorta is a pharmacological property specific to ET-1 and is probably not general for peptides.

In this study we have also attempted to show functional evidence for this proposed interaction using *in vivo* models. In anaesthetized SHR, ET-1 (i.v.) attenuated part of the vasodepressor response to cumulative bolus administration of BRL-38227 and Ro 31-6930, but not that to RP-49356 or the L-type calcium channel antagonist, nitrendipine. Thus, in an *in vivo* model, and with a dose of ET-1 that failed to evoke a hypertensive response, this peptide can also discriminate between different chemical classes of PCOs. The failure of ET-1 to modify the vasodepressor responses to the calcium channel antagonist, nitrendipine, further supports the selectivity observed *in vitro* of the interaction of ET-1 with a mechanism common to benzopyran PCOs. In further studies in the anaesthetized SHR, only the vasodilator responses to low dose infusion of BRL-38227 were attenuated by ET-1, again suggesting that the antagonism induced by the peptide was surmountable or that high doses of BRL-38227 lowered blood pressure by a second ET-1-insensitive mechanism. In these studies, the fall in TLBVR (which comprises renal and all distal hindquarters blood flow) clearly indicates that peripheral vasodilatation occurs to both BRL-38227 and RP-49356. Both compounds increased descending aortic blood flow which is consistent with an arterial vasodilator profile, particularly since heart rate was not modified by either PCO in these studies. The possibility, that due to the design of the experiment, the inhibitory effects of ET-1 faded with time is unlikely since the attenuation, by the peptide, of the BRL-38227 antihypertensive response after oral administration in conscious SHR was apparent over at least a 60 min period. In these experiments, although ET-1 was administered intra-arterially (to obviate immediate cardiac effects), both the peak effect and duration of the blood pressure effect of BRL-38227 were significantly attenuated, in the absence of a hypertensive response to the peptide. An equieffective antihypertensive dose of RP-49356 (p.o.) was clearly not modified by ET-1 in conscious SHR.

ET-3, given intravenously as a pretreatment, induced similar short-lasting hypotensive effects as seen with ET-1, but failed to modify the vasodepressor effects of BRL-38227 in the anaesthetized SHR. These data therefore add additional support to the findings *in vitro* with ET-3 and angiotensin II, that the interaction between the benzopyran PCO, BRL-38227, and ET-1 is an action specific to this peptide. Finally, the acute fall in DBP which was observed with both ET-1 and ET-3 prior to administration of the PCOs is probably not *per se* responsible for the inhibitory effects of ET-1 against BRL-38227.

In conclusion, evidence has been provided which demonstrates functionally that ET-1 exhibits selective inhibitory activity against one chemical class of potassium channel opener, the substituted benzopyrans. The hypothesis that ET-1 and BRL-38227 have affinity for a common site (Site 1), on the potassium channel, that does not recognise RP-49356 or pinacidil is proposed. RP-49356 and pinacidil could therefore interact with a second site (Site 2) to activate the K^+-channel in rat vascular smooth muscle. This discriminatory property of ET-1 could allow sub-classification of this group of vasodilator agents. In addition, the vascular effects of some, but not all, potassium channel openers might be radically modified, when administered as therapeutic agents, by elevated endogenous levels of ET-1 associated with certain diseased states.

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Effect of neuropeptide Y on adrenergic and non-adrenergic, non-cholinergic responses in the rat anococcygeus muscle

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1 The effects of neuropeptide Y (NPY) were examined on adrenergic and non-adrenergic, non-cholinergic (NANC) neurotransmission in the rat anococcygeus muscle.

2 NPY (0.1–0.3 μM) greatly potentiated the contractile responses induced by field stimulation. Prazosin (0.1 μM) completely abolished the stimulation-induced responses either in the absence or presence of NPY.

3 NPY (0.1–0.3 μM) enhanced only the contractile responses to low doses of noradrenaline (NA, 0.003–0.01 μM). Responses to tyramine were unaffected by the same concentrations of NPY.

4 In superfused anococcygeus, previously loaded with [^{3}H]-NA, NPY (0.1–0.3 μM) failed to modify the basal, as well as the stimulation-evoked, release of tritium at 2 and 4 Hz.

5 NANC relaxations induced by electrical stimulation were significantly reduced, in a concentration-related manner, by 0.1–0.3 μM NPY.

6 L-N^G-nitro-arginine (L-NOARG, 30 μM) enhanced the stimulation (0.25–1 Hz)-induced motor responses. In the presence of L-NOARG (30 μM), NPY (0.1 μM) did not modify the motor responses induced by field stimulation (0.25–0.5 Hz). L-Arginine did not reverse the NPY-induced potentiation of stimulation-induced motor responses.

7 The relaxations of anococcygeus muscle induced by sodium nitroprusside (SNP, 0.01–0.3 μM) were diminished by NPY (0.1–0.3 μM).

8 Our study suggests that NPY, at concentrations devoid of contractile effect, potentiates the motor responses of rat anococcygeus muscle as a consequence, at least in part, of the inhibition of NANC relaxing responses by a different mechanism from L-NOARG.

Keywords: NPY, Neuropeptide Y, NANC transmission; rat anococcygeus muscle; L-N^G-nitroarginine

Introduction

Neuropeptide Y (NPY) is a 36 amino acid peptide originally isolated from porcine brain (Tatemoto, 1982). The coexistence and corelease of NPY with noradrenaline (NA) in adrenergic nerve terminals in the sympathetic nervous system has been demonstrated in several tissues (for review see: Lundberg *et al.*, 1990). In peripheral smooth muscle tissues, the effects of NPY that seem to vary with species and tissues include: (i) direct smooth muscle contraction at relatively high concentrations of NPY, in some but not all tissues; (ii) potentiation of contractile responses, at subthreshold concentrations of NPY, induced by other agents including NA and (iii) modulation of its own release as well as the release of NA (Potter, 1988; Lundberg *et al.*, 1990).

The anococcygeus muscle has a motor noradrenergic and an inhibitory non-adrenergic, non-cholinergic (NANC) innervation (Gillespie, 1972). The neuronal release of NA produces contraction mainly via α_1-adrenoceptors (Docherty & Starke, 1981), demonstrated by the fact that motor responses were blocked by prazosin, a selective α_1-adrenoceptor antagonist, but not by rauwolscine, a selective α_2-adrenoceptor antagonist. Although the identity of the NANC neurotransmitter has not yet been clearly elucidated, the results obtained by Gillespie & Sheng (1990) suggest that the NANC neurotransmitter could be nitric oxide (NO) or a substance that can liberate NO.

The aim of this study was to characterize the effects of NPY on both noradrenergic and NANC transmission in the rat anococcygeus muscle.

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Methods

Male Sprague-Dawley rats (300–500 g) were stunned and exsanguinated. The paired anococcygeus muscles were dissected as described by Gillespie (1972) and set up in isolated organ baths containing 20 ml of Krebs bicarbonate solution of the following composition (in mM): NaCl 112.0, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.1, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 11.1. The solution was maintained at 37 ± 0.5°C and continuously gassed with 95% O_2, 5% CO_2. The organ-responses against 0.5 g tension were recorded by means of an isometric transducer (UF-1) on an Omniscribe pen-recorder. The preparations were allowed to equilibrate for at least 30 min during which time the Krebs solution was replaced repeatedly. Following equilibration, the preparation was primed with 30 μM NA.

Effect of neuropeptide Y on stimulation-induced contractile responses

Electrical field stimulation was carried out via platinum ring electrodes, between which the muscle was freely suspended. Square wave pulses of 0.1 ms duration and supramaximal voltage at 0.25 to 5 Hz were applied for 10 s every 3 min with a Harvard stimulator. Frequency-response curves were generated and the tissues washed for 15 min. NPY (0.1–0.3 μM) or bovine serum albumin (BSA, 0.1%) was then added and after 6 min the frequency-response curve was repeated in the same muscle. Responses were calculated as percentage of the control response elicited by the highest frequency of stimulation used (5 Hz). Only one concentration of NPY was applied per muscle. In some experiments desmethylimipramine (DMI, 0.1 μM) was present in the Krebs solution.

Effect of neuropeptide Y on noradrenaline and tyramine contractile responses

Thirty min after the contraction with 30 μM NA, concentration-response curves to NA were constructed in a cumulative manner. After a washout period of 30 min, NPY (0.01–0.3 μM) or BSA (0.1%) was applied 6 min before the cumulative addition of NA was repeated. Each tissue was exposed to only one concentration of NPY. In preliminary experiments we found that, after a second concentration-response curve to NA, responses were always stabilized. Therefore, since the maximum contraction with NA in the presence of the vehicle or NPY was always higher than the maximum contraction obtained in the first curve, the results for NA were calculated as a percentage of the maximum response obtained in the second curve in the presence or absence of NPY. DMI (0.1 μM), normetanephrine (1 μM), propranolol (1 μM) and Na_2EDTA (23 μM) were present in the Krebs solution throughout the NA experiments to block neuronal and extraneuronal uptake, β-adrenoceptors, and to prevent oxidative degradation of NA respectively. When the effect of NPY on tyramine responses was studied, only one concentration-response curve per muscle in the absence or presence of NPY was recorded. The responses were expressed as a percentage of the maximum response to NA (30 μM) applied after 30 min of tyramine washout.

Effect of neuropeptide Y on NANC relaxations

To record NANC relaxations, tone was raised by addition of guanethidine (30 μM). When tone had reached a plateau a frequency-response, or sodium nitroprusside (SNP, 0.01–10 μM) concentration-response, curve was constructed. After a washout of 15 min, NPY (0.1–0.3 μM) or vehicle (BSA 0.1%) was applied during 6 min and a second frequency or SNP response curve was repeated. Field stimulation was applied at supramaximal voltage, 1 ms duration at 0.25 to 5 Hz applied for 10 s every 3 min. SNP (0.01–10 μM) was cumulatively added to the bath. Responses were calculated as the percentage reduction in the degree of tone existing at the moment of nerve stimulation or before applying SNP.

Effect of L-N^G-nitroarginine (L-NOARG) on stimulation-induced contractile responses in presence or absence of NPY

Frequency-response curves were generated and the tissues were washed for 15 min. L-NOARG (30 μM) or L-NOARG (30 μM) plus NPY (0.1 μM) was then added and after 18 min (L-NOARG) and 6 min (NPY) the frequency-response curve was repeated in the same muscle. Responses were calculated as a percentage of the response elicited by the highest frequency of stimulation used (5 Hz).

Comparison of L-N^G-nitroarginine and neuropeptide effects on noradrenergic and NANC responses

Contraction and relaxation to electrical stimulation (0.5 and 2.5 Hz) were studied in preparations from the same anococcygeus muscle. In one of them tone was raised as previously described. After responses to electrical stimulation were stabilized, NPY (0.1–0.3 μM, 6 min) or L-NOARG (3–10 μM, 18 min) was added and a second set of responses was obtained. The degree of potentiation (noradrenergic responses) and inhibition (NANC responses) by NPY and L-NOARG was calculated for each frequency of stimulation in relation to their own control response.

Effect of neuropeptide Y on tritium overflow

The tissues were placed in a nylon net and immersed for 30 min in 10 ml of Krebs bicarbonate solution at 37°C continuously gassed with 95% O_2, 5% CO_2 (stabilization period). Thereafter, they were incubated for 60 min in 1 ml oxygenated Krebs solution at 37°C containing (±)-[^{3}H]-NA (2 μCi ml^{-1}, 0.2 μM, sp. act. 12.8 Ci mmol^{-1}). The anococcygeus muscles were transferred into a superfusion chamber with two parallel platinum electrodes, 0.5 cm apart, connected to a stimulator (Cibertec model CS9, modified to supply the adequate current strength) for field electrical stimulation (200 mA, 0.3 ms, 2–4 Hz, for 1 min). The muscles were superfused at a rate of 2 ml min^{-1} with oxygenated Krebs solution at 37°C for 100 min during which the basal level of tritium efflux reached a steady state. The superfusate was collected in vials (10 in total) at 30 s intervals. These vials were distributed in the following manner: two before stimulation to determine the basal level of tritium efflux, two during and six after the stimulation, covering the period needed to recover the basal levels of tritium efflux. Ready-Protein (Beckman) was added to the vials and the radioactivity measured in a scintillation counter (Beckman LS 2800).

Two or three electrical stimulation periods (S_1, S_2 and S_3) were applied to the tissues at 30 min intervals. To determine the effect of NPY on tritium release, 0.1 μM, and in some experiments 0.3 μM, of the peptide was added to the superfusate 8 min before S_2 and S_3 respectively. Cocaine (10 μM) and normetanephrine (10 μM) were added to the superfusion fluid after the incubation period to block neuronal and extraneuronal uptake of [^{3}H]-NA. To ensure the ability of our system to detect changes in overflow during stimulation, in a few experiments, tetrodotoxin (TTX, 1 μM) was added to the superfusate 15 min before S_2.

The stimulation-evoked tritium release was calculated by subtraction of basal tritium release from the electrical stimulated evoked release. Thereafter, the ratios S_2/S_1 and S_3/S_1 were calculated in order to eliminate differences between tissues. These ratios were not significantly different. The effects of NPY on stimulated efflux were analysed by calculating their effects on these ratios.

Drugs

The following drugs were used: L-arginine (Sigma), cocaine hydrochloride (Depósito de estupefacientes, Ministerio de Sanidad y Consumo), desmethylimipramine hydrochloride (Sigma), noradrenaline hydrochloride (Sigma), prazosin hydrochloride (Fides), L-N^G-nitroarginine (Carl Biochem), normetanephrine hydrochloride (Sigma), neuropeptide Y (Cambridge Research Biochemical), propranolol hydrochloride (Sigma), sodium nitroprusside (Sigma), tetrodotoxin (Sigma), tyramine hydrochloride (Fluka).

All drugs were dissolved in sterile saline with the exception of NPY which was dissolved in BSA (0.1% w/v), aliquoted and stored at −70°C in silicone coated tubes.

Analysis of results

Results are expressed as mean ± s.e. pD_2 values for NA were calculated as the negative logarithm of the EC_{50}, i.e. the concentration that produced 50% of its maximal effect. Data were analysed by a paired or unpaired Student's *t* test.

Results

Effect of neuropeptide Y on motor responses induced by electrical stimulation, noradrenaline (NA) or tyramine

As shown in Figure 1, field stimulation of the rat anococcygeus muscle induced a frequency-dependent contraction. These contractions were markedly potentiated by 0.1 μM (Figure 1b) and 0.3 μM (Figure 1c) NPY. The vehicle used to dissolve the peptide was without effect on the electrically-stimulated contractions (Figure 1a). The potentiation by NPY of electrically-induced contractions was both frequency-

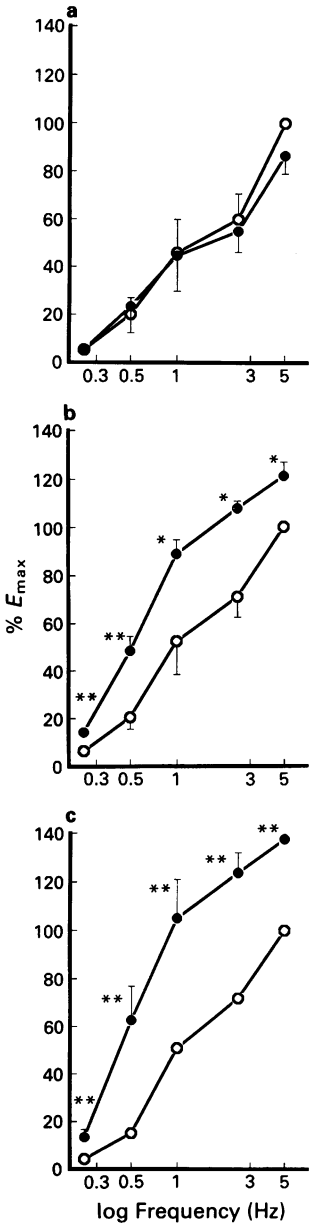

Figure 1 Frequency-response curves for the contractile responses of rat anococcygeus muscles induced by field stimulation (0.25–5 Hz, 0.5 ms, supramax V, 10 s every 3 min) in the absence (○) or presence (●) of (a) 0.1% BSA; (b) 0.1 μM neuropeptide Y (NPY) and (c) 0.3 μM NPY. Results are expressed as a percentage of the contraction induced by 5 Hz before treatment. Each point is the mean of 6–8 experiments. The vertical bars show s.e.mean. $^{**}P<0.01$; $^{*}P<0.05$ by Student's t test for paired observations

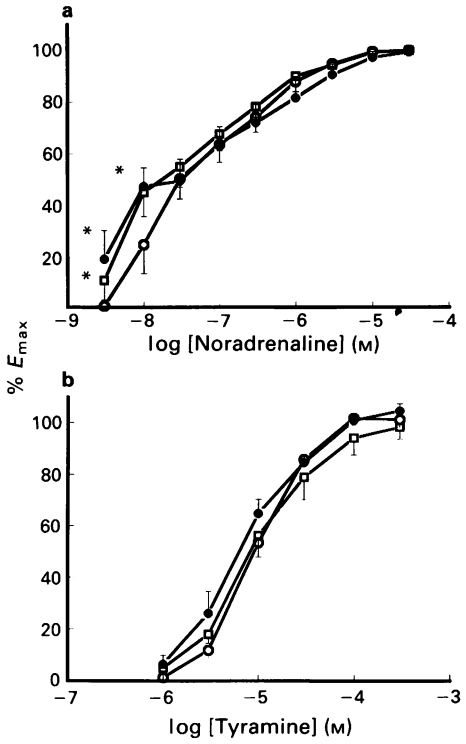

Figure 2 Concentration-response curve for the contractile effect of (a) noradrenaline and (b) tyramine in the absence (○) or presence of 0.1 μM neuropeptide Y (NPY) (●) or 0.3 μM NPY (□). Results are expressed as a percentage of the maximum contraction to NA. Each point is the mean of 6–8 experiments. The vertical bars show s.e.mean. $^{**}P<0.01$, $^{*}P<0.05$ by Student's t test for unpaired observations.

and concentration-dependent being statistically significant at all the frequencies studied. The presence of prazosin (0.1 μM) completely abolished the frequency-response curves in the presence of 0.3 μM NPY (results not shown). In three experiments in which DMI (0.1 μM) was present in the Krebs solution, the potentiation of field stimulation was the same in the presence or absence of NPY (results not shown). In contrast to the effects of NPY on responses to electrical stimulation, the peptide (0.1–0.3 μM) produced a significant potentiation effect on low concentrations of exogenous NA (0.003–0.01 μM) in a non-concentration-dependent manner (Figure 2a) without modifying the pD_2 values (control: 7.86 ± 0.46; 0.1 μM NPY: 7.7 ± 0.34; 0.3 μM NPY: 7.96 ± 0.37). Since lower concentrations of NPY (0.01–0.03 μM) were without effect on NA-induced contractile responses (results not shown), a concentration-range of 0.1–0.3 μM of NPY was selected to elucidate the differences on responses induced by NA exogenously applied or endogenously released. In addition, the responses induced by tyramine (1–300 μM) were not affected by 0.1 and 0.3 μM NPY (Figure 2b). The concentration of the peptide used (0.1 and 0.3 μM) had only a slight contractile effect in some but not all preparations (0.1 μM NPY: 6.1 ± 0.34%, $n = 3$; 0.3 μM NPY: 11.2 ± 1.5%, $n = 5$; % relative to maximum contraction of NA).

Effect of neuropeptide Y on tritium overflow

Since NPY seemed to potentiate preferentially responses to nerve stimulation, we tested whether NPY could influence the release of NA. Electrical stimulation of the anococcygeus muscle at 2 and 4 Hz produced a rise in tritium release (Figure 3). The basal tritium efflux from anococcygeus muscle was not modified by NPY at concentrations of 0.1 and 0.3 μM.

The electrical stimulation of the muscle produced a marked rise over the basal level in tritium release. At 2 Hz the tritium overflow in S_2 and S_3 was less than that observed in S_1 but the ratios S_2/S_1 and S_3/S_1 were not significantly different ($S_2/S_1 = 0.795 \pm 0.024$; $S_3/S_1 = 0.777 \pm 0.038$). However, at 4 Hz a small decrease in the release of tritium was observed in the third period of stimulation ($S_2/S_1 = 0.80 \pm 0.06$; $S_3/S_1 = 0.56 \pm 0.08$). The addition of NPY (0.1 and 0.3 μM) to the superfusate 8 min before the electrical stimulation (2 Hz) was applied produced a slight increase in tritium overflow (Figure 3a) that did not reach a statistical significance. The release of tritium induced by stimulation at 4 Hz was also unaffected by 0.1 and 0.3 μM NPY (Figure 3b).

Responses induced by 2 Hz (S_2/S_1: 0.82 ± 0.14, $n = 4$) were dramatically reduced by 1 μM TTX (S_2/S_1: 0.1 ± 0.06, $n = 4$).

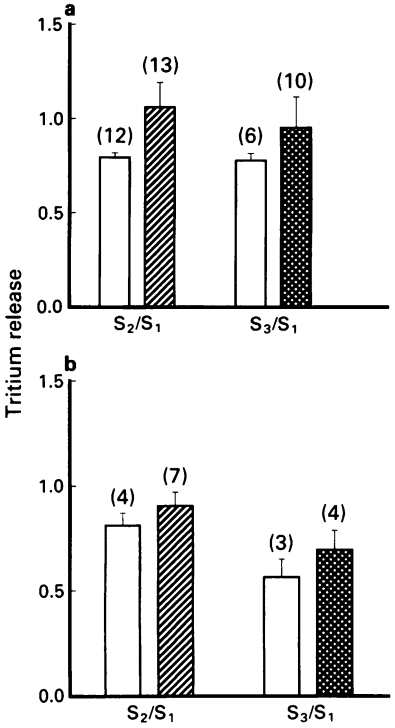

Figure 3 Effects of neuropeptide Y (NPY) on the overflow of tritium from the superfused rat anococcygeus muscle induced by electrical stimulation at (a) 2 Hz and (b) 4 Hz. The open columns represent control, the hatched columns in presence of 0.1 μM NPY and the cross-hatched columns in presence of 0.3 μM NPY. The number of experiments is indicated in parentheses above the columns; vertical bars show s.e.mean. The muscles had been loaded with [^{3}H]-NA (see Methods).

Effect of neuropeptide Y on electrical stimulation and nitrovasodilator-induced relaxations

Field stimulation of the rat anococcygeus muscle elicited frequency-response NANC relaxations of guanethidine-induced tone (Figure 4a). NPY (0.1 μM) produced a small but not significant increase in tone. However, the tone after 0.3 μM NPY (6.8 ± 0.29 g) was significantly greater ($P<0.05$ by paired Student's t test) from that observed in nontreated preparations (6.3 ± 0.3 g). The stimulation-induced relaxations were reduced by 0.1–0.3 μM NPY at all the frequencies studied (Figure 4b, c). Nevertheless, differences observed at 1–5 Hz (0.1 μM NPY) and 1 Hz (0.3 μM NPY) did not reach statistical significance. The inhibitory effect of NPY on stimulation-induced NANC relaxations was concentration-dependent (Figure 4b, c).

Cumulative addition of SNP (0.01–10 μM) produced concentration-related relaxations of guanethidine-induced tone (Figure 5). NPY (0.1 μM) and (0.3 μM) reduced the responses to low concentrations of SNP (0.01–1 μM) without modifying the responses to higher concentrations of the nitrovasodilator (Figure 5c, d). The vehicle used to dissolve the peptide was without effect on SNP-induced relaxation (Figure 5a) of guanethidine-induced tone.

Effect of L-N^G-nitroarginine on motor responses in the absence and presence of neuropeptide

The contractile responses of the anococcygeus elicited by field stimulation (0.25–1 Hz) in the absence of guanethidine were potentiated ($P<0.01$) by L-NOARG (30 μM; Figure 6a). However, the potentiation observed with higher frequencies (2.5–5 Hz) of stimulation was not statistically significant. L-Arginine (300 μM) failed to reverse the NPY-potentiated motor respones induced by electrical stimulation (results not presented). Exposure to NPY (0.1 μM) for 6 min did not modify the electrically-stimulated motor responses in presence of L-NOARG (30 μM) at the lowest frequencies (0.25–0.5 Hz, Figure 6b). There was a trend to potentiate the responses obtained at higher frequencies (1–5 Hz) of stimulation that did not reach statistical significance (Figure 6b).

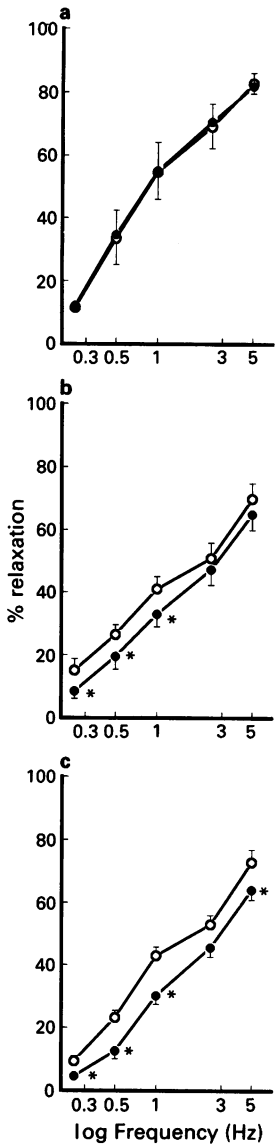

Figure 4 Frequency-response curves to field stimulation of NANC nerves at frequencies between 0.25 to 5 Hz for 10 s. Tone was induced by guanethidine (30 μM). Control responses (○) and responses in the presence (●) of (a) 0.1% BSA; (b) 0.1 μM neuropeptide Y (NPY) and (c) 0.3 μM NPY. Results are calculated as a percentage of the tone existing before stimulation. Each point is the mean of 6 experiments. The vertical bars show s.e.mean. $^{**}P<0.01$; $^{*}P<0.05$ by Student's t test for paired observations.

Comparative effects of neuropeptide Y and L-N^G-nitroarginine on NANC and noradrenergic responses

The degrees of inhibition and potentiation of stimulation (0.5–2.5 Hz)-induced noradrenergic and NANC responses by NPY (0.1–0.3 μM) or L-NOARG (3–10 μM) are shown in Figure 7.

A clear relationship between inhibition of NANC responses and potentiation of motor responses seems to exist for NPY (Figure 7a, c) but not for L-NOARG (Figure 7b, d) at 0.5 Hz. However, with a higher frequency of stimulation (2.5 Hz) the increase in potentiation induced by both com-

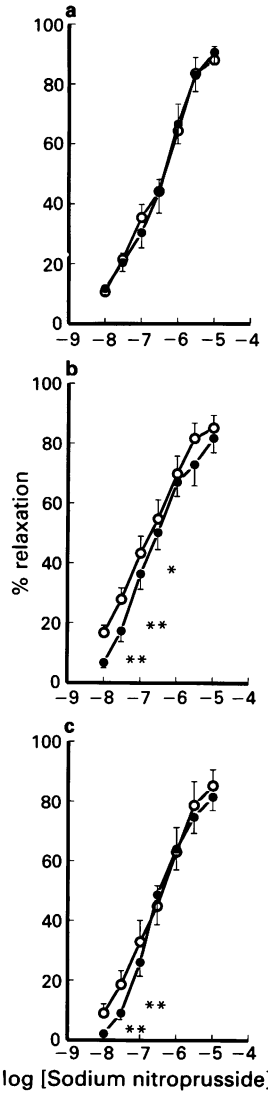

Figure 5 Concentration-response curve to sodium nitroprusside. Tone was induced by guanethidine (30 μM). Control responses (○) and responses in the presence (●) of (a) 0.1% BSA, (b) 0.1 μM neuropeptide Y (NPY) and (c) 0.3 μM NPY. Results are calculated as a percentage of the tone existing before adding the nitrovasodilator. Each point is the mean of 6 experiments. The vertical bars show s.e.mean. $^{**}P<0.01$, $^{*}P<0.05$ by Student's t test for paired observations.

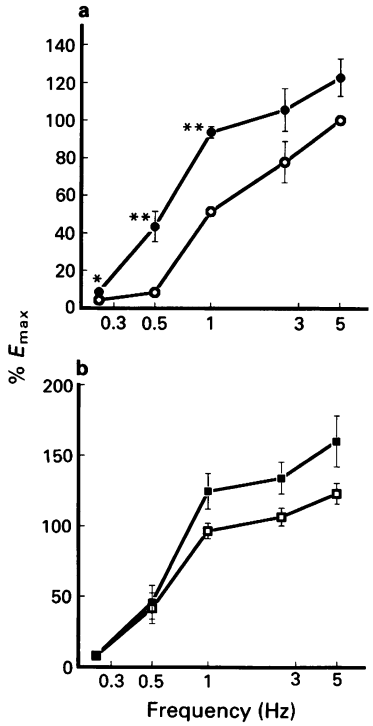

Figure 6 Frequency-response curve for the contractile responses of the anococcygeus muscle to field stimulation (a) in presence of saline (○) or 30 μM L-N^G-nitroarginine (L-NOARG, ●), (b) in presence of 30 μM L-NOARG (□) or 30 μM L-NOARG plus 0.1 μM neuropeptide Y (■). Results are expressed as a percentage of the response obtained with the highest frequency of stimulation in the first curve. Each point is the mean of 6 experiments. The vertical bars show s.e.mean. $^{**}P<0.01$; $^{*}P<0.05$ by Student's t test for paired (a) or unpaired (b) observations.

pounds was not parallel to the degree of inhibition achieved (Figure 7).

Discussion

This study, though indirect, suggests that the greater potentiation of field stimulated motor responses by NPY compared with those induced by NA in the rat anococcygeus muscle is mainly due to the blockade of NANC relaxation responses induced by electrical stimulation.

It has been demonstrated that subthreshold concentrations of NPY markedly enhanced the responses to NA and to electrical stimulation in different vascular preparations (Ekblad *et al.*, 1984; Lundberg *et al.*, 1985; Pernow *et al.*, 1986; Edvinsson *et al.*, 1989). Most of these studies seem to indicate that the potentiation of the electrical stimulation induced responses is due to a postjunctional effect, mainly via α_1-adrenoceptors. Nevertheless, in other blood vessels, subthreshold concentrations of NPY enhanced the stimulation-induced responses, but failed to modify the responses to exogenous NA (Hanko *et al.*, 1986; Cheung, 1991). On rat vas deferens, a lack of effect of NPY on the responses to exogenous NA has also been demonstrated (Lundberg *et al.*, 1982).

Our results in the anococcygeus show that NPY, at concentrations that do not contract the muscle, clearly potentiate the contractile responses induced by electrical stimulation. However, the peptide only enhanced the responses of very low doses of NA without modifying the pD_2 values. It has recently been demonstrated that NPY neuromodulates cotransmission in the guinea-pig vas deferens by inhibiting the release of ATP and NA and that these effects predominate over the postjunctional enhancement (Ellis & Burnstock, 1990). In addition, in the guinea-pig saphenous artery, NPY potentiates neurally-induced contraction by a specific action on the purinergic response without modifying the noradrenergic contractile response (Cheung, 1991). Furthermore, DMI failed to modify the NPY potentiation of motor responses to electrical stimulation. These results are in good agreement with findings on rabbit femoral (Wahlestedt *et al.*, 1985) and rat tail arteries (Vu *et al.*, 1989) where cocaine did not influence the enhancement by NPY of transmural nerve stimulation responses, indicating that the mechanism responsible for the potentiation effect of NPY is other than a blockade of neuronal uptake.

The fact that NPY failed to modify the contractile responses to tyramine, a drug that releases NA by a mechanism different (Thoa *et al.*, 1975; Marín & Sanchez, 1980) from nerve stimulation, give further support to a hypothetical mechanism for the potentiation effect of NPY linked to nerve stimulation rather than a postjunctional enhancement of NA responses via α_1-adrenoceptors.

In our experiments the possibility that NPY could facilitate the release of NA via a prejunctional mechanism could

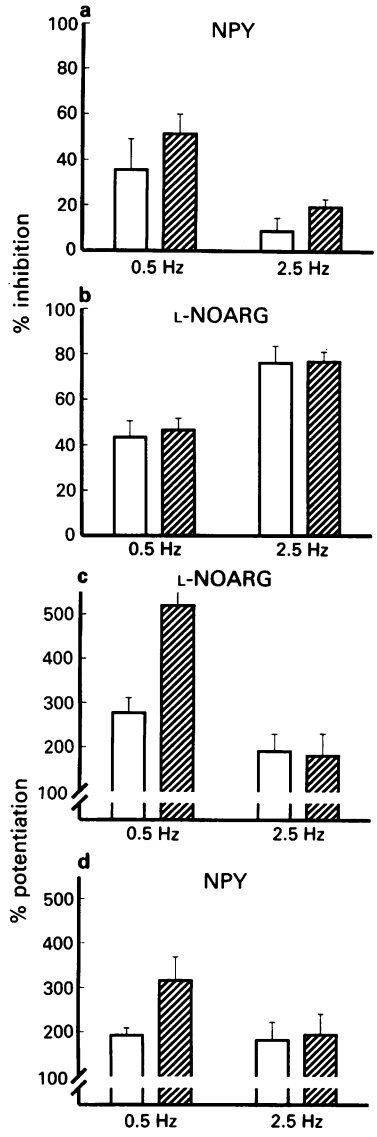

Figure 7 Percentage inhibition of NANC responses (upper panel) and potentiation of motor responses (lower panel) to 0.5 and 2.5 Hz by neuropeptide Y (NPY, a,c) and L-N^G-nitroarginine (L-NOARG, c,d). Results are expressed, for each frequency, as a percentage of their own control response. Each point is the mean of 6 experiments. The vertical bars show s.e.mean. NPY (0.1 μM) and L-NOARG (3 μM) (open columns); NPY (0.3 μM) and L-NOARG (10 μM) (hatched columns).

be excluded because NPY failed to modify the basal as well as the stimulation-induced tritium release at 2 and 4 Hz. These results are consistent with those obtained on the rat tail artery (Vu *et al.*, 1989) where NPY produced a greater potentiation on nerve stimulation-induced responses than on NA-induced vasoconstriction without altering the release of tritium. Moreover, NPY decreases the release of tritium and the contractions of rat (Lundberg & Stjärne, 1984) and guinea-pig (Ellis & Burnstock, 1990) vas deferens. The fact that TTX, a known blocker of nerve activity, almost abolished the tritium overflow, indicated that our system is able to detect changes during electrical stimulation, results that correlate with previous studies in blood vessels (Balfagón & Marín, 1989).

The relaxation responses induced by electrical stimulation were decreased by NPY. The substance released by electrical stimulation of NANC nerves in the rat and mouse anococcygeus seems to be NO (Gillespie & Sheng, 1990; Gibson *et al.*, 1990). The endothelium derived relaxing factor (EDRF), a powerful smooth muscle relaxant is almost certainly NO and its precursor is L-arginine (Palmer *et al.*, 1987). It has been clearly demonstrated that L-N^G-monomethyl arginine (L-NMMA), a competitive inhibitor of L-arginine that blocks the synthesis of NO, attenuates the NANC responses in the rat and mouse anococcygeus (Gillespie *et al.*, 1989; Gibson *et al.*, 1990) and increases the motor responses in rat anococcygeus and bovine retractor penis (Li & Rand, 1989; Liu *et al.*, 1990). In addition, L-NOARG a more potent inhibitor of EDRF (Moore *et al.*, 1990), completely inhibits the NANC responses and potentiates the motor responses of mouse anococcygeus (Gibson *et al.*, 1990). The effects of L-NMMA and L-NOARG on the relaxation and contraction of rat anococcygeus and bovine retractor penis are reversed by L-arginine (Liu *et al.*, 1991). We have observed an increase of stimulation induced motor responses in the rat anococcygeus by L-NOARG, results that are consistent with studies on mouse anococcygeus (Gibson *et al.*, 1990) and bovine retractor penis (Liu *et al.*, 1991) and that suggest that the potentiation of stimulation-induced motor responses in the rat anococcygeus results from the removal of NANC relaxations during electrical stimulation. Since NPY inhibits NANC responses and potentiates the motor responses induced by electrical stimulation, it is quite possible that the differences between stimulation-induced motor responses and those induced by exogenous NA in the presence of NPY could be explained by a reduction of NANC responses by the peptide. We have also tested whether the peptide was still able to potentiate the stimulation-induced motor responses in presence of L-NOARG. Results obtained with NPY in the absence or presence of L-NOARG were similar at all frequencies of stimulation. Nevertheless, at 1–5 Hz, NPY was still able to increase further the motor responses in the presence of L-NOARG though the effect did not reach statistical significance. These results suggest that an identical mechanism of action for the decrease of NANC responses in rat anococcygeus may not be shared by NPY and L-NOARG. To test this hypothesis we studied whether L-arginine could reverse the potentiation effect of NPY on motor responses. In our hands, L-arginine was unable to modify the NPY-induced potentiation of contractile responses, giving further support to the idea that the mechanism of action of the peptide is different from that of L-NOARG and suggesting that NPY is not acting prejunctionally, as an inhibitor of NO synthesis, but rather acts postjunctionally decreasing the NANC responses. If the latter is true, relaxations induced by a nitrovasodilator drug should also be reduced by NPY. In fact, relaxations induced by low concentrations of SNP were inhibited by the peptide, results that support the postjunctional effect of NPY. Nevertheless, when the degree of potentiation is compared to the degree of inhibition for NPY and L-NOARG, the relationship is not straightforward. At 0.5 Hz, the greater the inhibition of NANC responses by NPY the greater is the potentiation of motor responses but this parallelism is lost for L-NOARG (0.5 Hz) and for both compounds at a higher frequency (2.5 Hz) of stimulation. The degree of potentiation observed for L-NOARG and NPY at 2.5 Hz was similar but the decrease of the inhibitory responses produced by L-NOARG was more pronounced. The results obtained with the NO synthase inhibitor correlate with those observed in the mouse anococcygeus muscles, where a concentration of L-NOARG that almost abolished NANC responses has been seen to produce an increase of around 140% of the control response (Gibson *et al.*, 1990). Taken together, these results suggest that NPY at concentrations devoid of contractile effect preferentially potentiates the motor responses of the rat anococcygeus muscle probably as a consequence of the decrease of NANC relaxing responses. Nevertheless, since the effects on NANC responses are rather small, we cannot exclude the possibility that NPY could also act by increasing the release of a cotransmitter which does not contract, by itself, the anococcygeus muscle but potentiates the actions of NA. Moreover, it is also possible that in addition to the postsynaptic effect observed with SNP, the

peptide could also be acting presynaptically on the NANC nerves to inhibit the release mechanism. Further studies should be pursued in order to clarify the mechanism of action of NPY on rat anococcygeus muscle.

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Albumin inhibits platelet-activating factor (PAF)-induced responses in platelets and macrophages: implications for the biologically active form of PAF

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1 Platelet-activating factor (PAF) binds with high affinity to albumin leading Clay *et al.* (1990) to suggest that the active form of PAF is the albumin-PAF complex.

2 In the present study the proposal that albumin-bound, rather than monomeric PAF, is the active form of PAF at PAF receptors was critically evaluated by examining the effect of albumin on the potency of PAF in isolated platelets and macrophages.

3 Bovine serum albumin inhibited concentration-dependently PAF-induced responses in platelets and macrophages. The most probable explanation of this finding is that BSA reduced the concentration of free PAF.

4 Thus, we conclude that free PAF, rather than the albumin-PAF complex is the active form. Consequently, local concentrations of albumin will influence profoundly the potency of endogenously released PAF. Moreover, estimates of the affinity of PAF for PAF receptors made in buffers containing BSA, underestimate the true affinity of PAF for its receptors by approximately 3 orders of magnitude.

Keywords: Platelet-activating factor; albumin; platelet; macrophage; pharmacodynamics; receptors.

Introduction

Platelet-activating factor (PAF) is a phospholipid-derived mediator of allergy and inflammation (Braquet *et al.*, 1987). It is now established that PAF acts by stimulating stereospecific, high affinity receptors on a wide range of cell types including smooth muscle, leukocytes, endothelium and neurones (Hwang, 1988).

Albumin acts as a carrier of a wide range of substances in the blood, including a number of fatty acids (Spector, 1975). Albumin has a number of binding sites for fatty acids and exerts complex effects on the synthesis and metabolism of prostaglandins and PAF (Heinsohn *et al.*, 1987). PAF is known to be influenced by albumin in two ways: firstly, the presence of albumin is essential to the detection of the platelet-stimulating activity of PAF released from rabbit basophils (Benveniste *et al.*, 1972) and for PAF release and continued PAF synthesis in human neurotrophils (Ludwig *et al.*, 1985); secondly, the binding of PAF to albumin and other proteins has been demonstrated in a number of studies (Cabot *et al.*, 1985; Banks *et al.*, 1988; Clay *et al.*, 1990) and albumin greatly reduces the potency of PAF in activating platelets (Tokumura *et al.*, 1987).

Clay *et al.* (1990) have suggested that PAF binds to 4 sites on albumin with relatively high affinity ($K_d = 0.1\ \mu M$). The PAF-albumin complex is the most abundant form of PAF, even when the albumin concentration is well below physiological levels (e.g. 0.25% w/v) and hence, it was concluded that the albumin complex may be the form of PAF that is active at PAF receptors (Clay *et al.*, 1990). We have made a detailed study of the influence of a range of BSA concentrations on the potency of PAF in platelets and macrophages in order to evaluate the proposal that PAF complexed to albumin is active at PAF receptors. Consideration of the impact of BSA on the potency of PAF is of importance, since BSA is most commonly used as a vehicle for pharmacological studies of PAF. BSA concentration-dependently inhibited PAF-induced aggregation of platelets and superoxide anion (O_2^-) generation by macrophages in a manner that is not consistent with PAF acting as an albumin complex.

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Methods

Macrophage isolation

Male Dunkin-Hartley guinea-pigs (400–800 g) were killed by a sharp blow to the head and rapidly exsanguinated. Macrophages were isolated by peritoneal lavage: a 50 ml volume of heparinized (50 u ml^{-1}), phosphate-buffered saline was injected into the peritoneum which was gently massaged for ~1 min. The lavage fluid was aspirated and the cells were isolated by centrifugation (1000 *g*, 4°C, 10 min) (Stewart & Dusting, 1988).

Following isolation by centrifugation, cells were resuspended in RPMI 1640 at a concentration of $10^6\ ml^{-1}$, dispensed (0.5 ml) into plastic culture plates (Greiner, 24 well) and allowed to adhere for at least 2 h at 37°C. At the end of this period, non-adherent cells were removed and the remaining cells (80–90% of the total) were washed twice. This method consistently provided cells which were greater than 98% viable as assessed by trypan blue exclusion and more than 90% were macrophages as defined by non-specific esterase and Giemsa staining.

Platelet isolation

Adult rabbits (2–4 kg) were anaesthetized by intravenous administration of Saffan (alphaxalone and alphadalone). Blood was collected via a cannula placed in a carotid artery and was immediately mixed with trisodium citrate (0.38% w/v) and centrifuged at 150 *g* for 20 min at ambient temperature (20–24°C) (Stewart & Dusting, 1988). Prostacyclin (PGI_2) was added to a final concentration of 300 ng ml^{-1} to inhibit platelet activation during the washing procedure. The platelets were resuspended at a concentration of $2 \times 10^8\ ml^{-1}$ in Tyrode buffer (Stewart & Dusting, 1988) containing 1.8 mM Ca^{2+} with 0.0025, 0.25, 1 or 4% w/v BSA and allowed to stand at room temperature for at least 3 h before use.

Superoxide anion generation

Superoxide anion generation was determined by a colorimetric assay based on the reduction of cytochrome C (Johnston *et al.*, 1978). The medium overlaying adherent macrophages was removed and replaced with Tyrode buffer containing 0.0025, 0.25, 1 or 4% w/v BSA and cytochrome C (1 mg ml^{-1}) and pre-incubated for 15 min (Stewart & Dusting, 1988). Superoxide anion generation was measured as the increase in cytochrome C reduction (detected by an increase in absorbance at 550 nm and measured in an Hitachi U-2000 spectrophometer) relative to that in an aliquot of cells that were pretreated with superoxide dismutase (60 u ml^{-1}). The data from these experiments are expressed as percentages of the maximum response to formyl-Met-Leu-Phe (FMLP, 100 nM) as a normalization procedure to control for the inhibitory effect of BSA on the maximum superoxide anion generation.

Platelet aggregation

Aggregation studies were carried out at 37°C in a dual chamber aggregometer (Chrono-Log, Aggro-meter Model 540) using the spectrophotometric technique. The data are presented as percentage light transmission.

Model for the interaction of PAF with PAF receptors and specific binding sites on BSA

In the following model we have used the affinity constant of PAF for albumin ($K_d = 0.1\ \mu M$) that was reported by Clay *et al.* (1990) and each of the 4 binding sites on the albumin has been considered to be independent (actual BSA concentrations have been multiplied by 4 to obtain the concentration of PAF binding sites). It has been assumed that there are no other significant sites of loss for PAF. The experimentally-determined (arbitrary choice of those determined at 0.25% w/v BSA) apparent affinity constants (log EC_{50} values) of PAF for platelet and macrophage PAF receptors have been used to calculate a value for K_1. However, it is important to note that a value of free PAF was calculated from the EC_{50}, the affinity of PAF for BSA and the total number of binding sites according to equation 3 below. The model for PAF interacting with PAF receptors and BSA simultaneously is described by the following formulae:

$$\underset{\text{inactive}}{PAFBSA} \overset{K_2}{\rightleftharpoons} BSA + \underset{\text{active}}{PAF_{free}} + R \overset{K_1}{\rightleftharpoons} PAFR \ \alpha \ \text{response}$$

$$[PAF]_{total} = [PAFBSA] + [PAF]_{free} + [PAFR]$$

assumption: $[PAFR] = 0$

hence $[PAFBSA] = [PAF]_{tot} - [PAF]_{free}$

$$K_1 = \frac{[PAF]_{free}[R]}{[PAFR]} \quad (1)$$

$$K_2 = \frac{[PAF]_{free}[BSA]}{[PAFBSA]} \quad (2)$$

substitute for [PAFBSA] in equation (2)

$$K_2 = \frac{[PAF]_{free}[BSA]}{[PAF]_{tot} - [PAF]_{free}}$$

solve for $[PAF]_{free}$

$$[PAF]_{free} = \frac{[PAF]_{tot}}{([BSA]/K_2) + 1} \quad (3)$$

Fractional receptor occupancy (F_r)

$$F_r = \frac{[PAF]_{free}}{K_1 + [PAF]_{free}} \quad (4)$$

substitute (3) for [PAF]free in equation (4)

$$F_r = \frac{[PAF]_{tot}/(([BSA]/K_2) + 1)}{(K_1 + [PAF]_{tot})/(([BSA]/K_2) + 1)} \quad (5)$$

Since at $Fr = 0.5$, $K_1 = [PAF]_{free}$ in the absence of BSA, K_1 was determined by substituting equation (3) with $[PAF]_{total}$ equal to the log EC_{50} obtained in platelets or macrophages suspended in 0.25% w/v BSA. The assumption that $0.5\ Fr = EC_{50}$ is well justified by the many studies that indicate that the affinity of PAF for PAF binding sites on platelets ~0.5 nM (Hwang, 1990), is close to the apparent pD_2 values obtained from aggregation studies 0.1 nM (Grigoriadis & Stewart, 1991).

$$K_1 = \frac{[PAF]_{tot}}{(([BSA]/K_2) + 1)}$$

$$= 0.32\ \text{pM (platelets)}$$
$$= 0.83\ \text{pM (macrophages)}$$

The log EC_{50} values for PAF activation of platelets and macrophages at various concentrations of BSA were calculated by substituting the calculated values of K_1 in equation 5. These values have been compared to the experimentally-derived values in Figure 1. In addition, the family of curves generated by the model are shown in Figure 2.

Loss of PAF upon dilution

[^{3}H]-PAF (0.1 mCi ml^{-1}, 39.5 Ci $mmol^{-1}$) was added to 100 μM PAF solution containing either 0.25% or 0.0025% w/v BSA, to achieve a solution of PAF containing approximately 10^6 d.p.m. [^{3}H]-PAF ml^{-1}. Following the one in ten

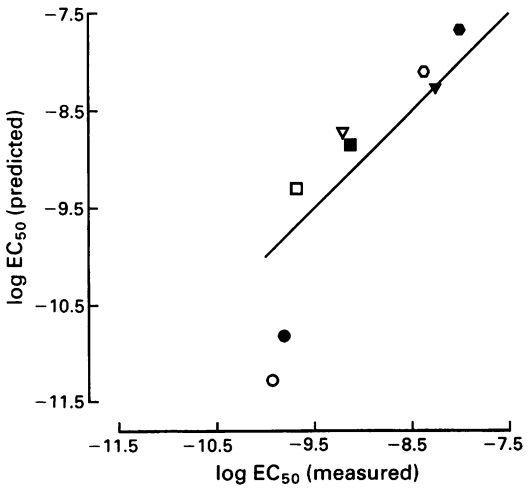

Figure 1 Comparison of measured versus predicted log EC_{50} values for PAF-induced activation of platelets (open symbols) and macrophages (filled symbols) in experiments carried out at indicated concentrations of BSA. Predicted log EC_{50} values were obtained as described in the text. BSA 0.0025%: platelet (○), macrophage (●); BSA 0.25%: platelet (□), macrophage (■); BSA 1.0%: platelet (▽), macrophage (▼); BSA 4.0%: platelet (○), macrophage (●).

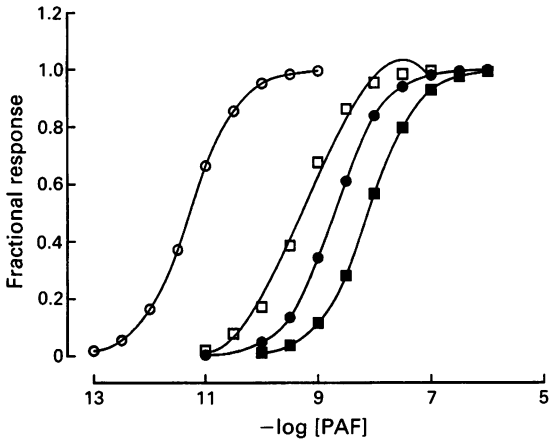

Figure 2 Concentration-response relationships for PAF activation of platelet PAF receptors predicted by the model derived in the methods section based on the interaction of free PAF with the PAF receptor. BSA concentrations: 0.0025% (○), 0.25% (□); 1.0% (●); 4.0% (■).

dilution (in either Tyrode solution containing 0.25% BSA or Tyrode solution without BSA, in duplicate) a portion of the solution (100 μl) was added to 4 ml emulsifier-safe scintillant and counted in a Packard 300C liquid scintillation spectrophotometer.

Statistical analyses

The data are presented as means and standard errors of the mean (s.e.mean) of *n* observations. Concentration-response lines have been analysed by linear regression of responses between 10 and 90% of the maximum response and the log EC_{50} values have been obtained by interpolation. Predicted log EC_{50} values were calculated as described in detail above. All figures were plotted using the Biosoft graphics package Fig P. Concentration-response curves were fitted to a sigmoid curve (excluding Figure 4: linear regression analyses).

Materials

All reagents and solvents used in this study were of analytical or higher grade. RPMI 1640 and FCS were obtained from CSL (Australia) and Flow Laboratories, respectively. Chemicals were obtained from the following sources: BSA, grade 5, essentially fatty acid-free; cytochrome C (horse heart type III); formyl-methionyl-leucyl-phenylalanine (FMLP); Giemsa stain; α-naphtyl acetate esterase kit; superoxide dismutase; Tryode salts (Sigma Chemical Co.); 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine (PAF) ($C_{26}H_{54}NO_7P.*H_2O$) (novabiochem:Product Number, 08-74-001); NET-668 hexadecyl-2-acetyl-sn-glyceryl-3 -phosphorylchloine-1- O-[hexadecyl-1′, 2′-^{3}H(N)] (Du Pont); HEPES (N-2-hydroxy ethylpiperazine-*N*-2-ethane sulphonic acid (BDH chemicals); heparin (Fisons Pty. Ltd).

Results

Effect of BSA on superoxide anion generation in PAF-stimulated macrophages

To examine the possible effect(s) of BSA on O_2^- generation, concentration-response curves to PAF were constructed in the presence of increasing concentrations of BSA (0.0025–4% w/v) (Figure 3a). BSA concentration-dependently inhibited O_2^- generation in macrophages. Increasing BSA concentrations induced a rightward shift in the concentration-response curve without a suppression of the maximum response (relative to that of FMLP) indicative of competitive antagonism.

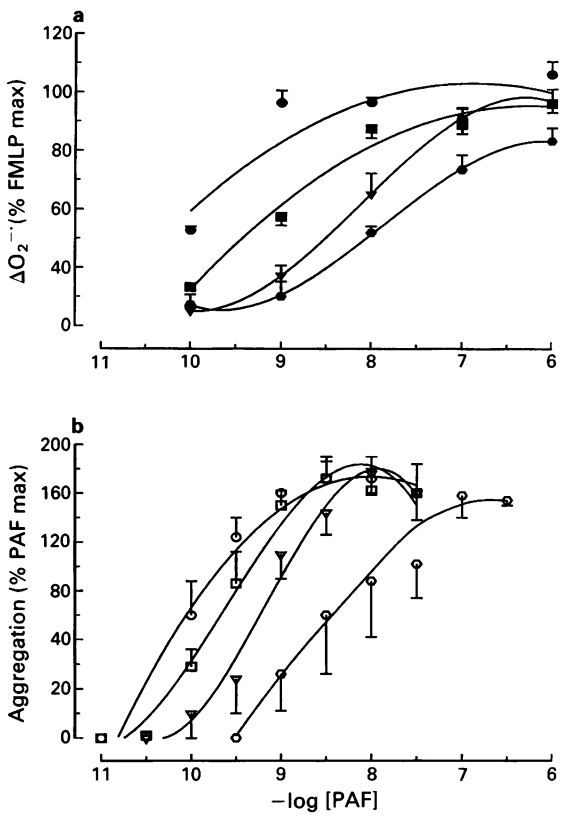

Figure 3 PAF-induced activation of guinea-pig macrophage superoxide anion (O_2^-) generation (a) and stimulation of rabbit platelet aggregation (b). Data are presented as means (s.e.mean shown by vertical bars) of a minimum of 4 observations. Concentration of BSA (solid symbols in a, open symbols in b): 0.0025% (●, ○); 0.25% (■, □); 1% (▼, ▽); 4% (◒, ◓).

The log EC_{50} increased from −9.81 in 0.0025% w/v BSA to −8.00 in the presence of 4% w/v BSA.

Effect of BSA on aggregation in PAF-stimulated platelets

The aggregation of rabbit platelets by PAF was examined in the presence of various concentrations of BSA (0.0025–4% w/v). Increasing concentrations of BSA shifted the concentration-response curve for PAF to the right (Figure 3b). The log EC_{50} increased progressively from −9.93 in 0.0035% w/v BSA to −8.37 in the presence of 4% w/v BSA.

Comparison of PAF potency in platelets and macrophages, with potency predicted for PAF acting as a 'free' monomer

The concentration-response curves for activation of rabbit platelets have been calculated based on the seed value of the log EC_{50} for PAF activation in the presence of 0.25% w/v BSA and the formulae (1–5, which assume that free PAF rather than that bound to BSA is the form that interacts with receptors) derived in the methods section (Figure 2). It is apparent that the predicted effect of BSA is similar to that observed (contrast with Figure 3b) with the exception of the effect of increasing BSA concentration from 0.0025% w/v to 0.25% w/v, for which the actual shift was considerably less than that predicted by the model.

A plot of the actual potency of PAF against the predicted potency of PAF (assuming that it acts as a monomer) reveals that there is a close agreement over the range of BSA concentrations between 0.25% and 4% w/v. However, as noted above, at the lowest concentration of BSA (0.0025% w/v), PAF was considerably less potent in either platelets or

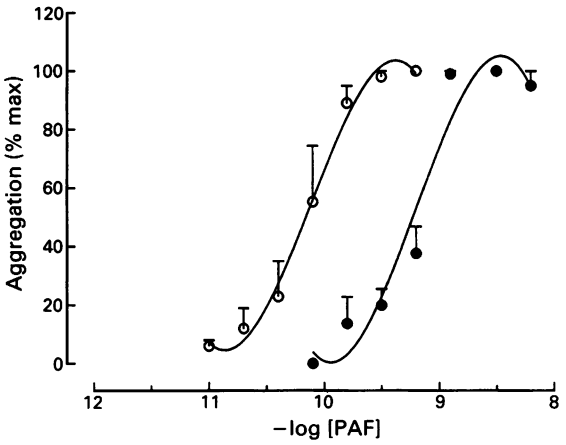

Figure 4 Comparison of the potency of PAF in activation of platelet aggregation with PAF prepared in Tyrode solution containing 0.25% w/v BSA (○) and with PAF prepared in the absence of BSA (●). The platelets were washed twice in Tyrode solution to remove any residual albumin following their isolation from plasma. Data are presented as the mean and s.e.mean (vertical bars) of 3 observations.

macrophages than predicted by the model. Nevertheless, the correlation co-efficients for measured versus predicted log EC_{50} values were significant for platelets ($r^2 = 0.725$, $0.05 < P < 0.1$) and for macrophages ($r^2 = 0.914$, $P < 0.05$).

Experiments with [^{3}H]-PAF were carried out to determine whether any losses occurred due to adsorption. PAF was prepared at a concentration of 100 μM containing either 0.25% or 0.0025% w/v BSA and 10^6 d.p.m. ml^{-1} of [^{3}H]-PAF. Upon dilution of PAF to 10 μM with 0.25% w/v BSA, the level of radioactivity was 94,920 d.p.m. ml^{-1}, indicating that no significant losses occurred in the presence of 0.25% w/v BSA. In contrast, when PAF was diluted to 10 μM from a Tyrode solution containing 0.0025% w/v BSA into Tryode without any BSA, the level of radioactivity was 68,711 d.p.m.

PAF stimulates platelet aggregation in the absence of BSA

The potency of PAF was compared in platelets that were incubated in the presence of BSA to that in platelets that were washed twice in the absence of albumin in order to ensure its removal. PAF was prepared in Tyrode buffer containing BSA (0.25% w/v) or in the case of platelets suspended in an albumin-free Tyrode solution, the PAF was prepared in Tyrode solution. In the absence of albumin, the apparent potency of PAF was reduced by approximately 1 log unit relative to that in the presence of 0.25% w/v BSA (Figure 4).

Discussion

It has been suggested that PAF bound to BSA is the active receptor stimulant (Clay *et al.*, 1990), and by implication, that free PAF may not be active at PAF receptors. The results of our systematic study of the effects of a wide range of BSA concentrations indicated a concentration-related inhibition of PAF-mediated responses in platelets and macrophages which is incompatible with the notion that PAF acts as a complex bound to BSA.

Studies of the actions of PAF invariably use BSA as a suspending agent, since non-specific adsorption leads to losses in more dilute solutions (Cabot *et al.*, 1985). We confirmed this observation in the present study, since PAF suspended in Tyrode solution was considerably less active than PAF suspended in even very low albumin concentrations (0.0025% w/v). Nevertheless, this observation indicates that PAF is able to stimulate platelet PAF receptors in the absence of albumin, suggesting that albumin is not an absolute requirement for activity. Superficially, the apparent potentiation of PAF by BSA could be seen as supporting the suggestion that the PAF-albumin complex is the active species of PAF. However, it is well established that PAF is more readily solubilized in albumin-containing solutions and that losses due to adsorption and uptake into cells are greatly reduced by albumin. The dilution experiments described here further support this contention.

The reported affinity of PAF for BSA is 0.1 μM which is approximately 3 log units less than its apparent affinity for platelet receptors. Nevertheless, even the lowest concentration of BSA used in our study represents 0.4 μM (1.6 μM binding sites) giving approximately 10^{15} binding sites per millilitre, whereas the number of PAF binding sites per platelet is 2×10^4 (Inarrea *et al.*, 1984) giving a total of 4×10^{12} PAF receptor binding sites per millilitre of platelets (suspension of 2×10^8 ml^{-1}). Thus, the excess of binding capacity of BSA compared with platelets would result in significant competition for PAF between these sites, despite the considerably higher affinity of PAF for its functional receptors. This difference in affinity is greater than indicated by apparent affinity constants determined in the presence of BSA, since these are underestimated due to the reduction in the concentration of free PAF. For example, at a total PAF concentration of 1 nM in 0.25% w/v BSA, 99.94% would be bound to BSA (as determined from equation 3). Increasing the concentration of BSA to 1% w/v would only increase the concentration of the BSA-PAF complex by 0.04%, but the concentration of free PAF would decrease from 0.6 pM to 0.2 pM. The latter decrease in free PAF concentration is the most likely explanation of the observed decrease in potency of PAF. However, it could be argued that the increase in BSA molecules not binding PAF could act as competitive antagonists of a PAF-albumin complex at the PAF receptor. Although difficult to test experimentally, this proposal seems inherently unlikely.

There was good agreement between our predicted log EC_{50} values and those obtained experimentally at concentrations of 0.25–4% w/v BSA. The log EC_{50} values obtained at 0.0025% w/v were higher than those predicted by the model. It is likely that the observed discrepancy at low BSA concentrations results from a failure of the assumption that BSA is the only significant site of loss of PAF. At low BSA concentrations, the amount of PAF bound to PAF receptors and taken up into platelets and macrophages or lost through adsorption, as was observed between concentrations of 0.25 and 0.0025% w/v BSA, may be significant in relation to the number of sites on the BSA.

It is evident that increasing the concentration of albumin reduces the concentration of free PAF, but has little effect on the concentration of BSA-bound PAF. Thus, we suggest that the decreased sensitivity to PAF of platelets or macrophages suspended in increasing concentrations of BSA (Tokumura *et al.*, 1987; present findings) indicates that free PAF, rather than BSA-complexed PAF, is active at PAF receptors. The practical implications of these findings are two fold: both binding studies and pharmacological estimates of the affinity of PAF for its receptors provide values that underestimate the true affinity of PAF; the local concentration of albumin is a prime consideration in assessing the biological activity of PAF released *in vivo*.

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MDL 26,479: a potential cognition enhancer with benzodiazepine inverse agonist-like properties

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1 The present study investigated biochemical, electrophysiological and behavioural properties of the novel cognition enhancer, MDL 26,479 (5-(3-fluorophenyl)-2,4,-dimethyl-3H-1,2,4-triazole-3-thione).

2 The 5-aryl-1,2,4-triazole, MDL 26,479, potently (0.22 ± 0.05 mg kg^{-1}) inhibited [^{3}H]-flumazenil (Ro15-1788) binding in mouse cortex but was ineffective *in vitro* at displacing radioligand binding to the $GABA_A$ receptor complex.

3 Parenteral administration of MDL 26,479 (1 mg kg^{-1}) or the benzodiazepine (BZD) inverse agonist methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) (0.3 mg kg^{-1}) increased cortical *ex vivo* binding of [^{3}H]-hemicholinium-3 ([^{3}H]-HC-3), a marker for cholinergic activation. This effect of MDL 26,479 was blocked by pretreatment with the antagonist flumazenil (1 mg kg^{-1}).

4 MDL 26,479 (20 μM) and DMCM (1 μM) increased excitation in the hippocampal long-term potentiation (LTP) slice preparation; however, unlike DMCM, the effect of MDL 26,479 was not blocked by flumazenil.

5 In behavioural studies, MDL 26,479 did not exhibit adverse properties characteristic of drugs associated with the $GABA_A$ receptor complex. It lacked convulsant, anxiogenic, anxiolytic, or depressant effects. Since MDL 26,479 lacks activity with the BZD receptor *in vitro* we suggest that it acts via the $GABA_A$ receptor complex at another site on this receptor or in an as yet undefined manner or an active metabolite is formed *in vivo*.

6 Previous work showed that MDL 26,479 enhances learning acquisition in animal models. The present study suggests that at least some of the cognition enhancing properties are due to the enhancement of cortical and hippocampal cholinergic function and LTP.

Keywords: Hemicholinium-3; $GABA_A$ receptor; long term potentiation (LTP); benzodiazepine inverse-agonist

Introduction

Drugs which interact at the $GABA_A$ receptor complex affect cholinergic function in the hippocampus and cerebral cortex (Richter *et al.*, 1982; Wenk, 1984; Miller & Richter, 1985; 1986) and cholinergic pathways play an important role in cognition and memory (Rauca *et al.*, 1980; Sherman *et al.*, 1981; Mouton *et al.*, 1988; Toumane *et al.*, 1988). In a preliminary report the 5-aryl-1,2,4-triazole, MDL 26,479 (5-(3-fluorophenyl)-2,4,-dimethyl-3H-1,2,4-triazole-3-thione) (Figure 1) was shown to interact with the $GABA_A$ receptor complex (Dudley *et al.*, 1990). Recently, MDL 26,479 has been shown to reverse scopolamine or delay induced deficits in learning acquisition in rats (Moran *et al.*, 1992). In this paper we have examined its properties in a number of assay systems regulated by γ-aminobutyric acid (GABA) or benzodiazepines (BZD).

BZD inverse agonists may enhance cognition because of their ability to disinhibit selectively cholinergic neurones in the basal forebrain (Sarter *et al.*, 1988b; 1990). Cholinergic activity has previously been estimated *ex vivo* by examining the high affinity choline uptake site (HACU) in synaptosomal preparations (Richter *et al.*, 1982; Miller & Richter, 1986; Miller & Chmielewski, 1990). It is thought that HACU measured *in vitro* reflects the activity of cholinergic neurones *in vivo* (Simon *et al.*, 1976). Therefore we have examined the effects of MDL 26,479 on the binding of [^{3}H]-hemicholinium-3 ([^{3}H]-HC-3) to the HACU site in rat brain membranes and in sections using quantitative autoradiography (QAR). We have compared these results with those of methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), a benzodiazepine receptor inverse-agonist.

Another system thought to model some of the processes involved in memory and learning is long-term potentiation (LTP). This is a long lasting (hours to weeks) enhancement of synaptic transmission in response to high frequency stimulation of afferent fibres in the hippocampus (Bliss & Lomo, 1973) and in other CNS regions (see Andersen & Hvalby, 1986). The features of LTP (i.e. its duration, anatomical substrate, etc.) are such that it is now widely regarded as a useful model of physiological processes which involve learning. Furthermore, compounds which enhance memory in behavioural models also potentiate synaptic transmission in the hippocampus in a manner similar to the induction of LTP (Olpe & Lynch, 1982; Ito *et al.*, 1988; Tanaka *et al.*, 1989). To characterize further the mechanism of MDL 26,479, we have compared its effects with those of DMCM on LTP in the CA1 region of the hippocampal slice preparation.

In addition to examining the properties that may relate to memory enhancement, we have also tested MDL 26,479 for additional BZD activity. A major liability of GABA enhancing drugs is the production of sedation or ataxia, while compounds which inhibit GABA function are often anxiogenic or convulsant. Compounds which act as BZD antagonists can precipitate seizures following BZD agonist

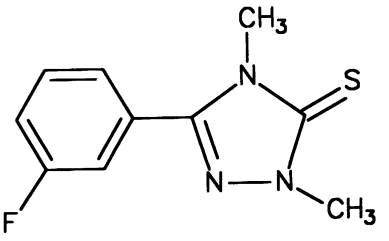

Figure 1 Structure of MDL 26,479 (5-(3-fluorophenyl)-2,4,-dimethyl-3H-1,2,4-triazole-3-thione).

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withdrawal. We have examined MDL 26,479 in several behavioural assays designed to measure these potential adverse effects.

Methods

Animals

Unless otherwise noted the animals used in these studies were male Sprague-Dawley rats or CD-1 mice (Charles River). The animals were given food and water *ad libitum* and were maintained on a 12 h light/12 h dark cycle.

In vivo *receptor binding*

In vivo binding of the benzodiazepine antagonist [^{3}H]-flumazenil to the BZD receptor was performed as described by Miller *et al.* (1988*a*). Animals were treated with MDL 26,479 (0.1 to 30 mg kg^{-1}, i.p.) and killed after 1 h. [^{3}H]-flumazenil (3 μCi, 72.8 Ci nmol^{-1}, DuPont, NEN) was given by the tail vein (i.v.) 20 min before the animal was killed. Non-specific binding of [^{3}H]-flumazenil was defined as the binding after the administration of a 5 mg kg^{-1} (i.p.) dose of clonazepam, 30 min prior to the administration of [^{3}H]-flumazenil.

GABA receptor membrane binding in vitro

Brain tissue from adult male Sprague-Dawley rats (200–250 g) was used for the GABA receptor binding assays. The methods used have been described previously: [^{3}H]-flunitrazepam (Braestrup & Squires, 1978), [^{3}H]-muscimol (DeFeudis *et al.*, 1980), [^{35}S]-*t*-butylbicyclophosphorothionate ([^{35}S]-TBPS) (Trullas *et al.*, 1987) and [^{3}H]-baclofen (Bowery *et al.*, 1985). *In vitro* binding of the benzodiazepine inverse agonist, [^{3}H]-methyl-β-carboline-3-carboxylate ([^{3}H]-β-CCM) was performed as follows. Washed membranes were prepared from cortical and hippocampal tissue as described by Murphy *et al.* (1987). On the day of the assay, the membranes were thawed at room temperature, centrifuged at 4°C for 10 min at 44,000 *g* and resuspended in an equal volume of 50 mM Tris-HCl buffer (pH 7.4). The centrifugation/resuspension procedure was repeated. The tissue was incubated with [^{3}H]-β-CCM, 8 nM, in 50 mM Tris-HCl buffer (pH 7.4), with varying amounts of MDL 26,479 for 30 min at 22°C. Non-specific binding was defined as the binding in the presence of 10 μM clonazepam. The assay was terminated by filtration using a Brandel Cell Harvester and GF/B glass fibre filters presoaked in 0.1% polyethylenimine (PEI) and washed twice with 5 ml of ice-cold buffer.

Diazepam withdrawal

Adult male CD-1 mice (Charles River, 22–33 g) were administered diazepam twice a day for nine consecutive days by the method of Patel *et al.* (1988). Briefly, the initial dose was 50 mg kg^{-1} (i.p.) and was increased by 50 mg kg^{-1} each day so that on day nine the animals were given two doses of 450 mg kg^{-1}. On day 10 (approximately 22 h after the last dose of diazepam), the mice were divided into groups of 9, and given either flumazenil (2.5 to 20 mg kg^{-1} i.p.) or MDL 26,479 (1.0 to 30 mg kg^{-1}, i.p.) and observed for the next 30 min for seizures.

Separation-induced ultrasonic vocalizations

Ultrasonic vocalizations were measured as previously described (Kehne *et al.*, 1991). Briefly, QMC Bat Detectors (QMC Instruments, Cambridge, England) were used to detect separation-induced vocalizations (SIV) in 10 day-old rat pups treated with drug or vehicle and monitored 30 min after drug administration. The audible detector output was fed into a computer and the number of vocalizations were automatically quantitated. Eight sound-proof chambers housed individual rat pups and an ultrasonic detector such that no sounds (audible or ultrasonic) from the room or adjacent chambers could be detected.

Measurement of startle reflexes

Acoustic and tactile startle reflexes were measured with a computerized apparatus and procedures that have been previously described (Kehne & Davis, 1985; Kehne *et al.*, 1992). Briefly, a stabilimeter equipped with a movement-sensitive transducer measured the amplitude of a startle reflex elicited by an acoustic stimulus (50 ms, 120 dB while noise burst) or by a tactile stimulus (50 ms, 4 PSI air puff, delivered to the rat's back). Startle amplitude was defined as the average rectified intensity over a 250 ms window (from stimulus onset) and was expressed in arbitary units from 0 to 4095.

Because of individual differences in startle amplitude, a matching procedure was used to divide rats into control and experimental groups (Kehne & Davis, 1985). For drug testing, at least one day after matching, acoustic and tactile startle reflexes were measured by use of a crossover design (Kehne & Davis, 1985). On the first test day, groups of matched rats ($n = 8$ in each group) received a pretreatment (flumazenil, MDL 26,479, or vehicle) and a treatment (diazepam or vehicle). Prior to the treatment injection, rats were tested in the startle apparatus for 30 min and then removed, injected with diazepam or vehicle, and immediately tested for an additional 60 min. One week later, this procedure was repeated, with the exception that rats that had received diazepam on week 1 now received vehicle, and *vice versa.*

Drugs were dissolved in dimethylsulphoxide (DMSO) and were given i.p. DMSO was the control vehicle. MDL 26,479 dosing parameters were based on the results of the *in vivo* binding studies (above). Diazepam and flumazenil doses and pretreatment times were derived from pilot studies and previous reports (Davis & Gallager, 1988).

Membrane [^{3}H]-hemicholinium-3 binding

The β-carboline, DMCM (1 mg kg^{-1}, i.p.), was dissolved in saline (pH 4.0) and administered to animals 30 min before they were killed as described by Miller & Chmielewski (1990). MDL 26,479 was suspended in saline (pH 7.0) by sonication and administered i.p. (1 mg kg^{-1}) 60 min before death. For all animals the cortex and hippocampus were removed, and a crude washed membrane preparation (1 g tissue in 20 ml of buffer) was prepared as previously described (Miller *et al.*, 1991). Binding of [^{3}H]-HC-3 (148–164 Ci mmol^{-1}, DuPont, NEN) was performed in 0.5 ml of isotonic Tris buffer (pH 7.4). [^{3}H]-HC-3 was present at concentrations which ranged from 0.2 to 20 nM. Nonspecific binding was defined as the binding in the presence of 10 μM unlabelled HC-3 corrected for displaceable binding to the filter in the absence of tissue. After 60 min, binding was terminated by the addition of 3 ml of ice-cold Tris buffer followed by rapid filtration through Whatman GF/B filters which had previously been soaked in 0.3% PEI using a Brandel Cell Harvester (Brandel Labs, Gaithersburg, MD, U.S.A). Protein content was determined by the method of Lowry *et al.* (1951). DMCM was purchased from Research Biochemicals Inc. (Natick, MA, U.S.A.).

Quantitative autoradiography

Quantitative autoradiography (QAR) was performed on sagittal sections of brains from rats treated with either saline or MDL 26,479 (1 mg kg^{-1}) as described above. The tissue was prepared as previously described (Miller *et al.*, 1991) with the following modification. Glass slides were previously coated with a solution of 0.5% gelatin and dried at 100°C; slides were then soaked in a solution of 0.3% PEI for 60 min and allowed to dry at room temperature before use. The

Table 1 Inhibition of various GABA receptor complex ligands in rat cortical membranes by MDL 26,479

	IC_{50} (μM)				
Compound	*[^{3}H]-muscimol*	*[^{3}H]-flunitrazepam*	*[^{3}H]-β-CCM*	*[^{35}S]-TBPS*	*[^{3}H]-baclofen*
MDL 26,479	>100	87.2 ± 7.5	29.1 ± 2.8	>100	>100
GABA	0.020				
Diazepam		0.003			
Flumazenil			0.279		
Picrotoxin				0.279	
Baclofen					0.100

[^{3}H]-β-CCM = [^{3}H]-methyl-β-carboline-3-carboxylate; [^{35}S]-TBPS = [^{35}S]-*t*-butylbicyclophosphorothionate.

sections were stored at −70°C until assayed. Binding of [^{3}H]-HC-3 was performed in 5 ml of isotonic Tris buffer (pH 7.4) for 60 min at 22°C. [^{3}H]-HC-3 was present at a concentration of 20 nM. Nonspecific binding was defined as the binding in the presence of 10 μM HC-3. The sections were removed from the incubation buffer and the reaction was terminated by rinsing twice in ice cold Tris buffer for 3 min. This was immediately followed by a 3 s wash in ice cold deionized water followed by rapid drying. After exposing the slides to Hyperfilm (Amersham/Searle, Des Plaines, IL, U.S.A.) for two weeks the films were developed. Quantitation of the autoradiograms followed previously described procedures (Miller *et al.*, 1988b; 1991). Sections of rat brain were stained in a solution of 0.2% Cresyl Violet and the anatomical areas were identified from a stereotaxic rat brain atlas (Paxinos & Watson, 1982).

Long-term potentiation

Hippocampal slice preparation techniques were modified from Mueller *et al.* (1981). Briefly, hippocampal sections (400 μm) were made with a McIlwain tissue chopper (Brinkman Instruments Inc., Westbury, NY, U.S.A.) and incubated on the interface of 34°C artificial CSF buffer (composition, mM: NaCl 124, KH_2PO_4 4.9, $MgSO_4$ 2.4, $CaCl_2$ 2.5, $NaHCO_3$ 25.6 and glucose 10) for at least 1 h. Warm, moist 95% O_2/5% CO_2 was passed over the incubation chamber. Recordings were taken from slices submerged in warmed, oxygenated media which had a flow rate of 2 ml min^{-1}. The stimulus (10 μs pulse) was applied at 30 s intervals to the stratum radiatum of the CA3 region of the hippocampus in order to activate synapses on the CA1 pyramidal neurones. The evoked waveform was digitized with a Computerscope interface board (RC Electronics, Inc., Santa Barbara, CA, U.S.A.) and stored for analysis with Neuroscope software (Levin & Associates, Denver, CO, U.S.A.) running on an IBM-AT. The viability of the slice was determined by applying the three criteria described by DiScenna (1987), and only preparations that met these were used. After twenty or more test stimuli were delivered to establish a stable baseline, drug solutions were applied for 20 min to determine drug effects on the baseline. The slice was then given a tetanizing stimulus (three 100 Hz pulses at 3 s apart) to induce LTP. This was followed by test pulses every 30 s to determine the effect of the drug on the size of the LTP population spike.

MDL 26,479 and DMCM were prepared in 0.25% DMSO/artificial CSF and flumazenil was dissolved in Krebs buffer. Drugs were applied at the following concentrations: MDL 26,479, 10–50 μM; DMCM, 1–10 μM; flumazenil, 0.1 μM. Control slices received 0.25% DMSO/artificial CSF.

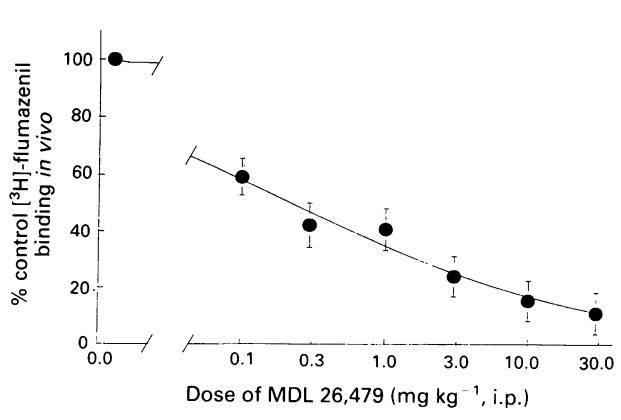

Figure 2 Binding of [^{3}H]flumazenil, given *in vivo* (3 μCi), in mouse cortex after pretreatment with varying doses of MDL 26,479 (i.p.) 6 h prior to the radioligand. The ED_{50} value was 0.22 ± 0.05 mg kg^{-1}. Values are mean (with s.e.mean shown by vertical bars), $n = 6$.

Results

Characterization of MDL 26,479 interactions with the $GABA_A$ receptor complex

In vivo *receptor binding* MDL 26,479 inhibited *in vivo* binding of the BZD antagonist [^{3}H]-flumazenil to the $GABA_A$ receptor of mouse cerebral cortex with an ED_{50} value of 0.22 ± 0.05 mg kg^{-1}, i.p., when given 6 h before the radioligand (Figure 2). In a time course study, MDL 26,479 (1 mg kg^{-1}, i.p.), achieved maximum inhibition (binding was reduced to 40% of control level) with a pretreatment time of 1 h and was maintained for pretreatment times as long as 6 h (data not shown). These data suggest that MDL 26,479 interacts with the $GABA_A$/benzodiazepine receptor, at least under the conditions *in vivo*, or alternatively, a metabolite might be responsible for this activity.

In vitro *receptor binding* MDL 26,479 at concentrations up to 100 μM did not displace the binding of [^{3}H]-muscimol or [^{35}S]-TBPS from the $GABA_A$ receptor complex nor [^{3}H]-baclofen from the $GABA_B$ receptor (Table 1). The binding of the BZD, [^{3}H]-flunitrazepam and the benzodiazepine inverse agonist, [^{3}H]-β-CCM, were inhibited weakly by MDL 26,479 with IC_{50} values of 87.2 ± 7.5 μM and 29.1 ± 2.8 μM, respectively (Table 1). These data suggest that MDL 26,479, in comparison to other BZD inverse agonists, has a very weak interaction with these sites at the $GABA_A$ receptor under these conditions *in vitro*.

Behavioural anaylsis of potential GABA-ergic activities

Diazepam withdrawal Unlike the BZD antagonist, flumazenil, MDL 26,479 did not precipitate convulsions in mice chronically treated with diazepam (Table 2). Flumazenil given at approximately 22 h after the final dose of diazepam produced seizures in animals dose-dependently with an ED_{50} of 5.83 mg kg^{-1}. MDL 26,479 in doses ranging from 1 to 30 mg kg^{-1} did not precipitate seizures or other behavioural effects in this model. Additionally, naive mice treated acutely with MDL 26,479 did not exhibit seizures at doses up to 200 mg kg^{-1} (data not shown). Under these conditions MDL 26,479 did not appear to exert BZD antagonist or inverse agonist like activities.

Table 2 Effect of flumazenil or MDL 26,479 on chronic diazepam-induced withdrawal in mice

Treatment	*Dose* (mg kg^{-1}, i.p.)	*No. convulsed/ No. tested*	*% effect*
Flumazenil	2.5	2/9	22
	5.0	4/9	44
	10.0	6/9	67
	20.0	8/9	89
MDL 26,479	1.0	0/9	0
	3.0	0/9	0
	10.0	0/9	0
	30.0	0/9	0

All mice were dosed with diazepam (50 mg kg^{-1}, i.p.) twice daily, increasing by 50 mg kg^{-1} each day for nine consecutive days. Test compounds were given about 22 h after the last dose of diazepam. The mice were monitored for seizures for 30 min after administration of either flumazenil or MDL 26,479.

Table 3 Effects of MDL 26,479 or pentylenetetrazol on separation induced vocalizations in 10 day old rats

	MDL 26,479			*Pentylenetetrazol*	
Group	*SIV*	*% control*	*Group*	*SIV*	*% control*
Vehicle	272 ± 39	100	Vehicle	445 ± 68	100
0.125	267 ± 40	98	2.5	512 ± 61	115
0.25	312 ± 58	115	5.0	420 ± 62	94
0.5	227 ± 38	83	10.0	638 ± 57	143
1.0	308 ± 150	113	20.0	617 ± 86	139
2.0	229 ± 77	84	40.0	824 ± 72*	185
4.0	291 ± 94	107			
8.0	286 ± 72	105			

SIV values given ± s.e.mean.
* Significantly different from vehicle injected controls, $P<0.05$, Newman-Keuls multiple range test.
Following injection of MDL 26,479 (0.125–8.0 mg kg^{-1}, 30 min pretreatment, $n = 7$–24) or pentylenetetrazol (2.5–40.0 mg kg^{-1}, 30 min pretreatment, $n = 11$–12) pups were returned to the litter and, after the indicated pretreatment time, they were separated from the litter and placed in the test chambers for 30 min. Each point represents the total number of vocalizations averaged over two 3 min recording periods: 0–3 and 27–30 after separation. Statistical analyses revealed an overall effect of pentylenetetrazol, $F(5,65) = 4.80$, $P<0.001$, but not of MDL 26,479, $F>1$. Subsequent individual comparisons (Newman-Keuls) revealed a significant effect of 40 mg kg^{-1} pentylenetetrazol.

Ultrasonic vocalizations Consistent with reports of anxiogenic activity, the $GABA_A$ antagonist, pentylenetetrazol (PTZ), increased SIV produced when pups were removed from the litter (Table 3). In contrast, MDL 26,479 was without effect (Table 3), indicating that it did not exhibit activity expected of a $GABA_A$ antagonist or BZD inverse agonist.

Startle reflexes MDL 26,479 was next tested in a behavioural model (startle reflex) which is sensitive to the depressant effects of benzodiazepines. The benzodiazepine agonist, diazepam (5.0 mg kg^{-1}, i.p., 30 min pretreatment) consistently depressed acoustic and tactile startle reflexes and this effect was completely blocked by the benzodiazepine antagonist, flumazenil (5.0 mg kg^{-1}, i.p., 1 min pretreatment) (Table 4). In contrast, MDL 26,479 (10 mg kg^{-1}, 0.5 or 3 h pretreatment; or 30 mg kg^{-1}, 6 h pretreatment) did not significantly alter the depressant effect of diazepam on startle (Table 4), nor did it alter baseline startle. Thus, under the present experimental conditions, MDL 26,479 did not affect startle reflexes in a manner characteristic of a classical benzodiazepine agonist or antagonist.

Activity in assays sensitive to compounds with cognition enhancing potential

[^{3}H]-HC-3 binding While apparently not acting like a BZD agonist or antagonist in the withdrawal, vocalization and startle reflex models, further experiments showed that MDL 26,479 given *in vivo* affected [^{3}H]-HC-3 binding with some similarities to the BZD inverse agonist, DMCM. When given *in vivo*, both the β-carboline DMCM and MDL 26,479 stimulated cortical membrane binding of [^{3}H]-HC-3 whereas, only DMCM stimulated hippocampal binding measured *in vitro* (Figure 3). However, when the effects of MDL 26,479 (1 mg kg^{-1}) on [^{3}H]-HC-3 binding were examined by autoradiography, significant stimulation of binding was seen in both the stratum radiatum of the CA1 and CA3 regions of the hippocampus (Table 5 and Figure 4). The binding of [^{3}H]-HC-3 in the dentate gyrus was not significantly different from the control (Table 5).

The stimulation of [^{3}H]-HC-3 binding produced by MDL 26,479 was significantly attenuated by pretreatment with the BZD antagonist, flumazenil (1 mg kg^{-1}, i.p.) (Table 6). This dose of flumazenil had no effect on [^{3}H]-HC-3 binding by itself (Table 6) although doses of 5 mg kg^{-1} had a slight inhibitory effect on the binding, consistent with the partial agonist activity of flumazenil (data not shown). All of the effects on [^{3}H]-HC-3 binding to membranes were on the B_{max} values. The K_d of [^{3}H]-HC-3 binding (see Table 6) was unaffected by either treatment with MDL 26,479 or DMCM. These experiments suggest that MDL 26,479, or a metabolite, is acting like a BZD inverse agonist at a site on the BZD receptor but with greater regional specificity than DMCM. Although we can only speculate, it might be that MDL

Table 4 The effecs of MDL 26,479 or flumazenil on diazepam depression of tactile startle reflexes in rats

	Mean amplitude tactile startle			
Pretreatment	*Vehicle treatment*	*Diazepam treatment*	*Change from control*	*% change*
Vehicle	735.2	257.0*	−478.2 ± 137.7	−65%
Flumazenil	493.4	446.1*	−47.3 ± 86.9†	−10%
Vehicle	543.8	309.9*	−233.9 ± 64.7	−43%
MDL 26,479	644.8	374.9*	−269.9 ± 100.3	−42%

* Significantly different from vehicle-treated controls, $P<0.05$ (paired t test).
† Significantly different from vehicle pretreated controls, $P<0.05$ (unpaired t test).
Following injection with MDL 26,479 (10 mg kg^{-1}, 30 min pretreatment) or flumazenil (5 mg kg^{-1}, 1 min pretreatment) rats were assigned to acoustic and tactile startle reflexes following treatment with either diazepam (5 mg kg^{-1}, 1 min pretreatment) or vehicle (DMSO). Acoustic and tactile startle means represent the average startle amplitude that occurred over the 60 min test period that immediately followed injection of diazepam or vehicle. Subsequently, a change score (vehicle minus diazepam) was calculated for each rat and the mean ± s.e.mean for each pretreatment condition is shown. This score defined the magnitude of the diazepam depression, with a more negative value indicating a greater diazepam depression. Statistical tests used were paired t tests (to determine the presence of diazepam depression within a given pretreatment group) and unpaired t tests (to compare baseline and to compare the magnitude of diazepam depression between different pretreatment groups). No effects of pretreatment were found on baseline startle. Statistically significant depressant effects of diazepam relative to vehicle injection were seen for all four groups ($P<0.05$). The magnitude of diazepam depression was significantly blunted relative to controls only in the flumazenil pretreated groups, $t(30) = 2.65$, $P<0.05$. Similar results were seen with acoustic startle measurements, and for groups of rats under the following conditions: 10 mg kg^{-1} MDL 26,479, 3 h pretreatment; 30 mg kg^{-1} MDL 26,479, 6 h pretreatment (data not shown).

Table 5 Effects of *in vivo* MDL 26,479 (1 mg kg^{-1}, i.p.) on [^{3}H]-hemicholinium-3 ([^{3}H]-HC-3) binding *in vitro* assessed by quantitative autoradiography

Brain region	*Treatment saline*	*MDL 26,479*	*% of saline*
Hippocampal formation			
CA1 Stratum radiatum	0.46 ± 0.04	0.91 ± 0.20*	198
CA3 Stratum radiatum	0.41 ± 0.10	0.88 ± 0.06*	216
Dentate gyrus granular layer	1.24 ± 0.11	1.64 ± 0.31	132

Values are fmol mm^{-2} ± s.e.mean, $n = 4$.
* Significantly different from saline treatment, $P < 0.05$

Table 6 Attenuation of MDL 26,479 stimulation of *in vitro* [^{3}H]-hemicholinium-3 ([^{3}H]-HC-3) binding in rat cortical membranes by flumazenil

Pretreatment (5 min prior to drug)	*Drug treatment*	B_{max} (fmol mg^{-1} protein)	K_d (nM)
Saline	Saline	9.7 ± 1.0	2.8 ± 0.5
Flumazenil (1 mg kg^{-1})	Saline	9.8 ± 1.3	3.9 ± 0.9
Saline	MDL 26,479 (1 mg kg^{-1})	20.6 ± 4.3*	2.6 ± 0.5
Flumazenil (1 mg kg^{-1})	MDL 26,479 (1 mg kg^{-1})	11.4 ± 1.3	3.8 ± 0.6

$n = 4$–8 values are given ± s.e.mean.
* Significantly greater than all other treatments, $P < 0.05$.

26,479, or a metabolite, has a more selective activity at the BZD_1 subtype, compared with the selectivity of DMCM (Braestrup *et al.*, 1983b). Alternatively, MDL 26,479 may interact at a site different from the classical BZD binding site which has different functional characteristics from that of BZD agonists and inverse agonists.

Long-term potentiation MDL 26,479 was further tested for cognition enhancing potential and mechanism of action on the *in vitro* hippocampal slice preparation, which has the advantage of allowing an assessment of the parent compound independent of metabolites. All slices used for LTP displayed a relatively constant population spike in response to a constant test voltage for the duration of the control recording period (10 min). In 9 control slices, DMSO (0.25%) had no significant effect on the basal population spike amplitude (data not shown). Control slices averaged 179% greater response after LTP than the response given prior to LTP. This potentiation of the response was stable for the duration of the experiment (30 min).

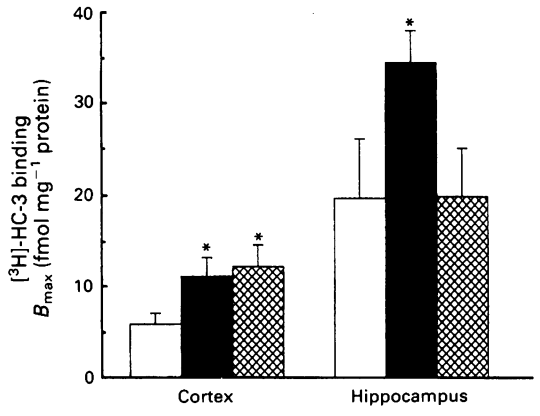

Figure 3 [^{3}H]-hemicholinium-3 ([^{3}H]-HC-3) binding (B_{max}, fmol mg^{-1} protein) in rat cortical and hippocampal membranes *in vitro* following i.p. administration of either saline (open columns), methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (solid columns) or MDL 26,479 (cross-hatched columns) (mean with s.e.mean shown by vertical bars, $n = 4$ to 6). *Significantly different from saline treated ($P < 0.05$, ANOVA and LSD).

Both MDL 26,479 and DMCM caused a dose-dependent increase in the amplitude of the basal population spike before the induction of LTP when they were superfused through the recording chamber (Figure 5). These effects achieved statistical significance ($P < 0.05$, ANOVA) with the 5 and 10 μM concentrations of DMCM and 20 and 50 μM concentrations of MDL 26,479. DMCM was more potent than MDL 26,479 in augmenting the pre-LTP population spike amplitude but both drugs demonstrated similar efficacies, augmenting the population spike amplitude by 60–70%. The BZD antagonist, flumazenil, at a concentration of 100 nM produced a slight decline in the basal population spike amplitude and an equivalent small decline in the amplitude of the LTP produced by the tetanizing stimulus (Figure 6). The combination of flumazenil (100 nM) and DMCM (5 μM) attenuated the effects of DMCM on both the basal population spike amplitude and on LTP (Figure 6). When slices were pretreated with flumazenil followed by MDL 26,479 (20 μM) the effects of MDL 26,479 were not attenuated and may have been slightly enhanced (Figure 6). Neither treatment with DMCM nor MDL 26,479 had any statistically significant effect on the population spike amplitude after the induction of LTP.

Discussion

The results of this study indicate that MDL 26,479 interacts with the $GABA_A$ receptor complex *in vivo* but not *in vitro*. The overall profile resembles in certain aspects the profile of a β-carboline inverse agonist, yet MDL 26,479 does not exhibit the potential side effects (convulsions, anxiogenesis) often associated with these agents.

MDL 26,479 potently inhibited the *in vivo* receptor binding of [^{3}H]-flumazenil which suggests that MDL 26,479 is interacting with the $GABA_A$ receptor. However, *in vitro* examination of the ability to displace the binding of several compounds known to interact with the $GABA_A$ (muscimol, flunitrazepam, β-CCM and TBPS) or the $GABA_B$ (baclofen) receptors revealed that MDL 26,479 had no or very weak activity. This suggests that MDL 26,479 acts at the $GABA_A$ receptor in a novel manner, acts at a site distinct from the $GABA_A$ receptor or is metabolized to a compound which is active at the $GABA_A$ receptor. To address the question further we examined the effects of MDL 26,479 on several other systems both *in vitro* and *in vivo*.

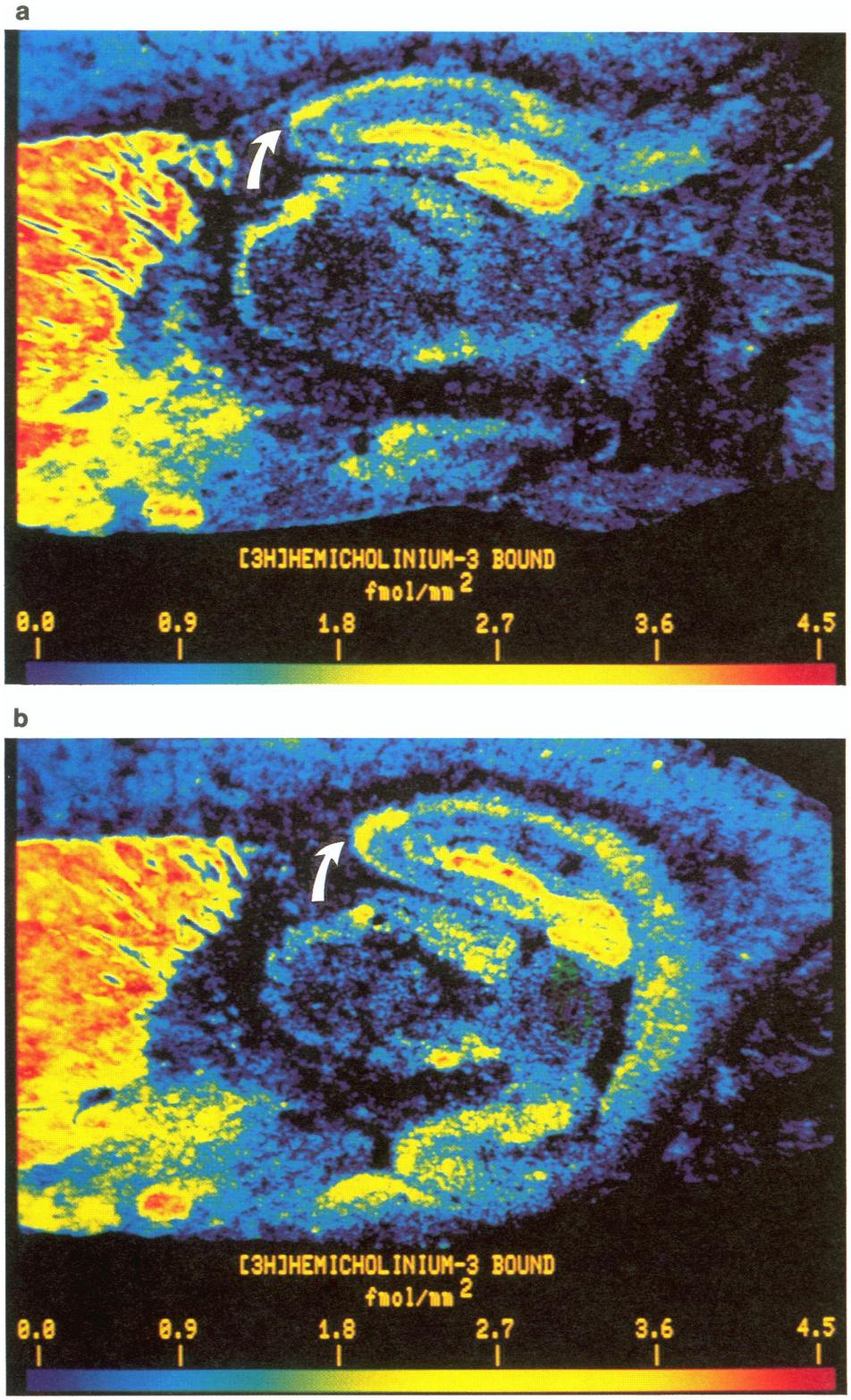

Figure 4 Binding of [^{3}H]-hemicholinium-3 ([^{3}H]-HC-3) in rat brain sagittal sections in the hippocampal region taken from rats treated either with saline (a) or MDL 26,479 (1 mg kg^{-1}, i.p.) (b). Increased binding is seen in the CA3 region of the hippocampus (arrow) in the MDL 26,479-treated rat.

Earlier studies of MDL 26,479 suggested that it had antidepressant like activities (Kane *et al.*, 1988; Sorensen *et al.*, 1990). However, it was demonstrated that MDL 26,479 lacked any effect on the uptake of noradrenaline or on monoamine oxidase activity (Kane *et al.*, 1988). In additional binding studies we have found that MDL 26,479 does not interact ($IC_{50} > 10\,\mu M$) with α_1-, α_2-, or β-adrenoceptors, D_2-dopamine, 5-HT_{1a}, 5-HT_2, $GABA_B$, H_1-histamine, muscarinic or NMDA receptors (data not shown).

A series of behavioural studies assessed whether MDL 26,479 had activities that are suggestive of agonism or antagonism of $GABA_A$ receptor function. Unlike the benzodiazepine agonist, diazepam, MDL 26,479 did not depress startle baseline, nor did it reverse the diazepam depression of startle. The benzodiazepine antagonist flumazenil, but not MDL 26,479, precipitated convulsions in mice that were chronically treated with diazepam. By itself MDL 26,479 did not produce seizures in doses up to 200 mg kg^{-1} in mice.

A number of reports have indicated that BZD inverse-agonists can produce anxiety in a number of animal models (File *et al.*, 1982; Braestrup *et al.*, 1983a; Crawley *et al.*, 1985; Insel *et al.*, 1984). However, some inverse agonists may lack anxiogenic properties or be inactive in certain models for anxiety (Crawley *et al.*, 1984). In addition, the anxiogenic activity of BZD inverse-agonists may not be mediated through a BZD mechanism (File & Lister, 1983).

SIV have been used by a number of investigators to assess potential anxiolytic activity of compounds (Gardner, 1985; Mos *et al.*, 1988; Insel *et al.*, 1988; Kehne *et al.*, 1991). Also, anxiolytic benzodiazepines suppress SIV (Gardner, 1985; Insel *et al.*, 1986) whereas anxiogenic agents such as the GABA antagonist, PTZ, or β-carboline inverse agonists increase SIV. In the present study, PTZ, but not MDL 26,479, increased SIV. These data indicated that MDL 26,479 does not have the side-effect profile of compounds that act at any of the several sites associated with the $GABA_A$ receptor complex.

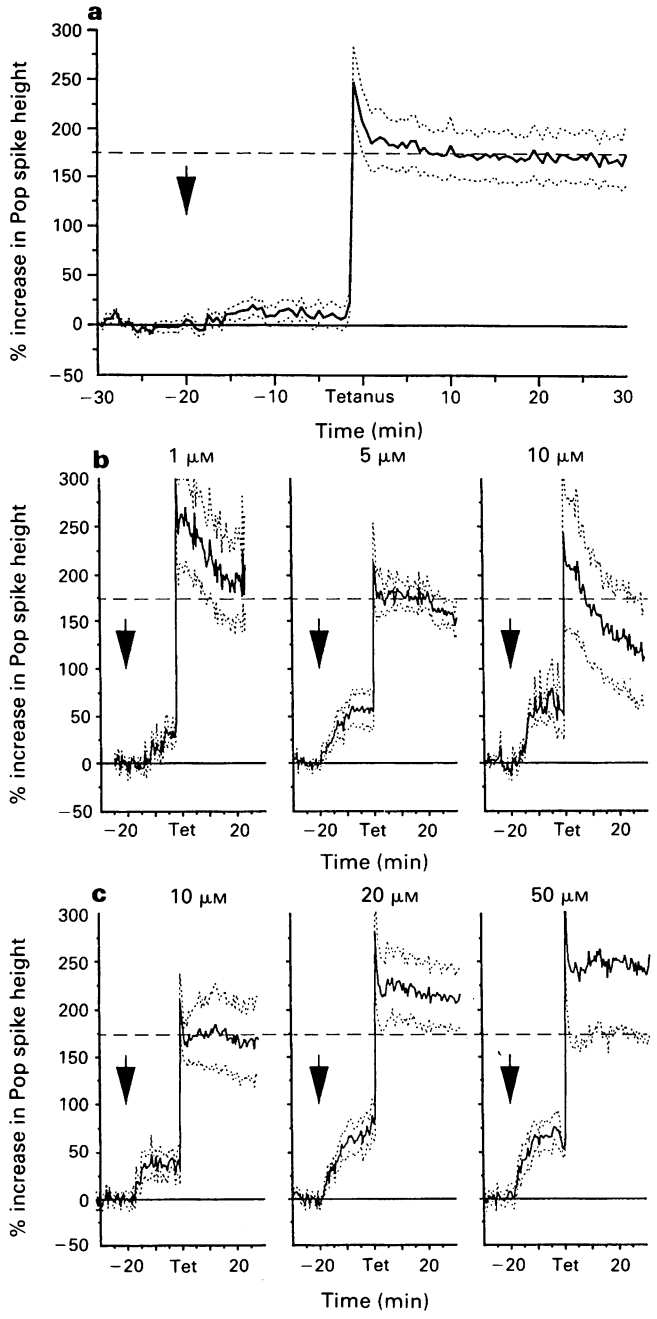

Figure 5 Stimulation of the amplitude of the basal population spike by vehicle (DMSO 0.25%) (a), methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) (b) and MDL 26,479 (c). The start of drug treatment (at arrow) began 20 min prior to the tetanizing stimulus (Tet = 0 on the time scale). The percentage increase in the population spike height is calculated from an average baseline elicited prior to drug treatment. The dotted lines represent the s.e.mean ($n = 5$ to 7 for each curve).

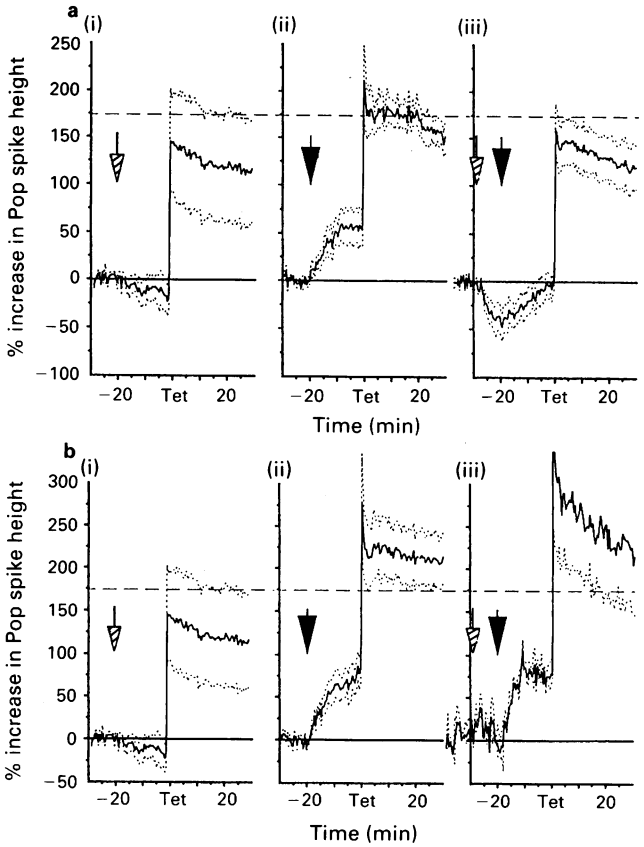

Figure 6 (a) Flumazenil (100 nM) application (small arrow, a(i)) has a slight inhibitory effect on long term potentiation (LTP) in the hippocampal slice by itself. Flumazenil pretreatment (small arrow, a(iii)) blocks effect of methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM, 5 μM) treatment (large arrow, a(ii) and a(iii)). (b) Flumazenil treatment (small arrows in b(i) and b(iii)) fails to block the effect of MDL 26,479 (20 μM) (large arrows in b(ii) and b(iii)) enhancement of the basal population spike. The dashed line represents the LTP for control slices. The dotted lines represent the s.e.mean ($n = 5$ to 7 for each curve).

It has been suggested that inverse agonists at the benzodiazepine receptor may enhance cognition because of their ability to selectively disinhibit cholinergic neurones in the basal forebrain (Sarter *et al.*, 1988b; 1990). Several studies (Shih & Pugsley, 1985; Spignoli *et al.*, 1986; Nakahiro *et al.*, 1988; Lorez *et al.*, 1988) report that 'nootropic' drugs (e.g. pramiracetam, oxiracetam and pantoyl-GABA) stimulate cortical or hippocampal HACU after *in vivo* administration. Benzodiazepine inverse agonists also have been reported to enhance cognition (Venault *et al.*, 1986; Lorez *et al.*, 1988; Sarter *et al.*, 1988a; Sarter & Stecker, 1989) and these compounds stimulate HACU after *in vivo* drug treatment (Miller & Chmielewski, 1990). The triazole, MDL 26,479, appears to emulate, in some ways, the activity of classical benzodiazepine inverse agonists in several of our experiments. However, MDL 26,479 does not display some of the undesirable effects shown by many inverse agonists such as anxiogenesis and convulsant or proconvulsant effects.

MDL 26,479 stimulated cholinergic activity, as assessed by [^{3}H]-HC-3 binding in the cerebral cortex. Unlike the BZD inverse agonist, DMCM, which is a potent convulsant, MDL 26,479 does not possess convulsant properties in the dose range (1 to 5 mg kg^{-1}) that stimulated cholinergic function. Stimulation of cortical or hippocampal HACU has been

reported for several potential nootropic agents (Shih & Pugsley, 1985; Spignoli *et al.*, 1986; Nakahiro *et al.*, 1988) suggesting that this may be a common mechanism for cognition enhancement among a diverse number of compounds. The stimulating effect of MDL 26,479 on [^{3}H]-HC-3 binding combined with its low convulsant profile suggest a possible therapeutic role for cognitive deficits especially in Alzheimer's disease, Korsakoff's disease and possibly in normal aged individuals, since all three groups have decreased cholinergic function (Bowen *et al.*, 1976; Arendt *et al.*, 1983). The mechanism by which MDL 26,479 is acting to enhance [^{3}H]-HC-3 binding is either directly or indirectly via the $GABA_A$/BZD receptor complex since its activity is attenuated by pretreatment with flumazenil.

The assays for increased [^{3}H]-HC-3 binding in cortical membranes compare well with the binding measured in sections by QAR. However, in the hippocampus this is not the case. Since the binding of [^{3}H]-HC-3 in the dentate gyrus is approximately 50 to 300% greater than the binding in the rest of the hippocampal formation, it is likely that the lack of any significant increase in binding in hippocampal membranes is partly due to a masking effect of the unchanged binding in the dentate gyrus. The more discreet stimulation of [^{3}H]-HC-3 binding in the hippocampus by MDL 26,479, as shown by membrane binding and QAR, may relate to the lack of convulsant activity of this triazole relative to the β-carbolines. The selective stimulation of hippocampal regions by MDL 26,479 vis-a-vis DMCM also indicates that MDL 26,479 may be a selective inverse agonist at the $GABA_A$ receptor complex. The ability of the benzodiazepine antagonist, flumazenil, to block the effects of MDL 26,479 on [^{3}H]-HC-3 binding combined with the inhibition of [^{3}H]-flumazenil binding by MDL 26,479 again suggests a GABA-ergic mechanism.

Several studies have demonstrated that drugs with cognition enhancing potential can enhance LTP in the hippocampus (Olpe & Lynch, 1982; Ito *et al.*, 1988; Tanaka *et al.*, 1989; Pugliese *et al.*, 1990). It has been suggested that inhibition of interneurones may underlie increased excitability in the hippocampus (Bilkey & Goddard, 1985). MDL 26,479 and DMCM both enhanced the basal postsynaptic neuronal excitability in the hippocampal CA1 region. The experiments on LTP in the hippocampal slice preparations follow a similar potency profile as that seen in the [^{3}H]-HC-3 binding assay, with DMCM being slightly more potent than MDL 26,479. The rapid response and the limited potential for metabolism within the *in vitro* slice preparation suggests that these effects are the result of MDL 26,479 acting directly and are not dependent on metabolism of the parent compound. However, since the enhancement of LTP by MDL 26,479 was not reversed by flumazenil, as was DMCM, it may be that this effect is not via the same mechanisms as that seen with the *in vivo* inhibition of [^{3}H]-flumazenil binding and the stimulation of [^{3}H]-HC-3 binding which is attenuated by flumazenil pretreatment. Therefore, it is still possible that an active metabolite is responsible for the *in vivo* activity and that these effects are separate from those seen in the hippocampal slice *in vitro*. Future experiments will attempt to locate, identify and characterize the activity of metabolites of MDL 26,479 from rat blood plasma.

In summary, actions of MDL 26,479 on [^{3}H]-HC-3 binding and LTP suggest a possible mechanism for its cognition enhancing properties in behavioural paradigms reported by Moran *et al.* (1992). The lack of seizure and anxiogenic liabilities indicate a large therapeutic index for MDL 26,479. Although MDL 26,479 appears in some systems to possess BZD inverse agonist activity it interacts with GABA-ergic systems in a novel manner by an unknown mechanism.

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Diazoxide-sensitivity of the adenosine 5′-triphosphate-dependent K^+ channel in mouse pancreatic β-cells

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1. In mouse pancreatic β-cells the regulation of the diazoxide-sensitivity of the adenosine 5′-triphosphate-dependent K^+ channel (K-ATP-channel) was examined by use of the patch-clamp technique.

2. In intact β-cells incubated at 37°C in the presence of 3 mM D-glucose, diazoxide did not affect the single channel conductance but stimulated channel-opening activity. Diazoxide produced half-maximal effects at 82 μM and 13 fold activation at maximally effective concentrations (300–400 μM). The response to diazoxide (300 μM) was not completely suppressed by saturating tolbutamide concentrations (1 or 5 mM).

3. Inside-out patch-clamp experiments were carried out using an experimental protocol favouring phosphorylation of membrane proteins. Under these conditions diazoxide was ineffective in the absence of any nucleotides, weakly effective in the presence of MgATP (26 or 87 μM) and strongly effective in the presence of the Mg complexes of adenosine 5′-diphosphate, 2′-deoxyadenosine 5′-diphosphate or guanosine 5′diphosphate (MgADP, MgdADP or MgGDP).

4. In inside-out patches exposed to nucleotide-free solutions, saturating concentrations of tolbutamide did not cause complete block of K-ATP-channels. When the channels were activated by MgdADP (48 μM), tolbutamide was even less effective. Sensitization of MgdADP-induced channel activation by diazoxide further weakened the effects of tolbutamide.

5. Diazoxide (50 or 300 μM) prevented the complete channel block induced by saturating tolbutamide concentrations in the presence of Mg^{2+} and ADP (1 mM).

6. In the presence of Mg^{2+}, the K-ATP-channel-blocking potency of cytosolic ATP decreased in the order inside-out > outside-out > whole-cell configuration of the patch-clamp technique.

7. It is concluded that the K-ATP-channel is controlled via four separate binding sites for inhibitory nucleotides (e.g. free ATP and ADP), stimulatory nucleotides (MgADP, MgdADP, MgGDP), sulphonylureas and diazoxide. Strong inhibition of the channel openings by sulphonylureas results from occupation of both sites for nucleotides. Diazoxide is only effective when the site for stimulatory nucleotides is occupied.

Keywords: ATP-dependent K^+ channel; pancreatic β-cell; diazoxide; tolbutamide; MgADP; MgdADP

Introduction

Initiation of insulin release requires depolarization of the pancreatic β-cell to the threshold potential at which voltage-dependent Ca^{2+}-channels are activated (for reviews see Henquin & Meissner, 1984; Matthews, 1985). Insulin releasing fuels (e.g. glucose) depolarize the β-cell by inhibiting an adenosine 5′-triphosphate-dependent K^+ channel (K-ATP-channel) in the plasma membrane (Ashcroft *et al.*, 1984; Cook & Hales, 1984; for recent reviews see Ashcroft & Rorsman, 1989; Dunne & Petersen, 1991). It is believed that the cytosolic ATP/ADP ratio is the second messenger linking fuel metabolism and K^+ channel inhibition. There seem to exist at least three sites by which cytosolic nucleotides control the activity of the K-ATP-channel in β-cells (Ashcroft & Rorsman, 1989; Dunne & Petersen, 1991): (1) ATP and some structurally related nucleotides decrease the activity of the K-ATP-channel; this effect does not require the presence of Mg^{2+}. (2) The Mg complexes of ADP (MgADP) and of some other nucleoside diphosphates increase the activity of the K-ATP-channel. (3) The Mg complex of ATP (MgATP) prevents or slows considerably the rapid decline in channel activity (channel run-down) observed in inside-out membrane patches in the absence of MgATP (Ohno-Shosaku *et al.*, 1987); MgATP might act by serving as substrate for one or several protein kinases closely associated with the β-cell plasma membrane. However, run-down of K-ATP-channel activity was almost abolished in Mg^{2+}-free cytosol-like solution (Kozlowski & Ashford, 1990).

Tolbutamide or other sulphonylureas inhibit the K-ATP-channel by direct interaction with the plasma membrane of insulin-secreting cells (Sturgess *et al.*, 1985; Ashcroft & Rorsman, 1989; Dunne & Petersen, 1991). The potency of sulphonylureas is much lower in excised membrane patches than in intact cells, unless the internal side of the plasma membrane is exposed to ADP in the presence of Mg^{2+} (Zünkler *et al.*, 1988b; Panten *et al.*, 1990). This effect of ADP appears to be due to the simultaneous occupation of the aforementioned inhibitory and stimulatory receptors for cytosolic nucleotides (Schwanstecher *et al.*, 1992a). Test solutions supplemented with ADP and Mg^{2+} contain both MgADP which activates the channel and free ADP which inhibits the channel, but less effectively than free ATP.

Diazoxide inhibits insulin secretion by increasing the activity of the K-ATP-channel (Trube *et al.*, 1986). Channel inhibition induced by occupation of the inhibitory receptor for cytosolic nucleotides is reversed by diazoxide (Trube *et al.*, 1986; Dunne *et al.*, 1987; Sturgess *et al.*, 1988; Zünkler *et al.*, 1988b; Kozlowski *et al.*, 1989; Gillis *et al.*, 1989; Dunne, 1989). As this effect of diazoxide requires the hydrolysis of

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cytosolic MgATP, it has been suggested that phosphorylation of the K-ATP-channel or a closely associated regulatory protein is involved in the mechanism of action of diazoxide (Kozlowski *et al.*, 1989; Dunne, 1989). However, the interaction between diazoxide and cytosolic nucleotides activating the K-ATP-channel has not been studied.

The aim of the present investigation was to gain further insight into the control of the diazoxide-sensitivity of the pancreatic β-cell. For that purpose the K^+ channel-activating potency of diazoxide in intact β-cells was compared with that in excised inside-out membrane patches exposed to channel-activating and channel-inhibiting nucleotides. In inside-out patches we also examined the role of cytosolic nucleotides in the antagonism between diazoxide and tolbutamide. Some of the results of this study have been presented in abstract form (Schwanstecher *et al.*, 1991a).

Methods

Isolation and culture of pancreatic β-cells

Pancreatic β-cells were isolated from male albino mice (NMRI, 11–15 weeks old, fed *ad libitum*) and cultured for 1–4 days as previously described (Panten *et al.*, 1990). RPMI 1640 tissue culture medium was supplemented with 10 mM D-glucose except for cell-attached and whole-cell patch experiments (cultured in 5 mM D-glucose).

Electrophysiological recording and analysis

The cell-attached, inside-out, outside-out and whole-cell configurations of the patch-clamp technique (Hamill *et al.*, 1981) were used to record currents flowing through K-ATP-channels as previously described (Zünkler *et al.*, 1988a; Panten *et al.*, 1990). Pipettes were pulled from borosilicate glass and had resistances between 3 and 8 MΩ when filled with pipette solution. Inside-out, outside-out and whole-cell patch-clamp experiments were performed at room temperature (20–22°C). In cell-attached patch experiments, cells and the surrounding medium were maintained at 37°C using the LU-CB-1 Culture Bath System and the TC-102 Temperature Controller (Medical Systems, Greenvale, N.Y.) as previously described (Panten *et al.*, 1990). The bath was perfused continuously at 2 ml min^{-1}. Unless stated otherwise in the Results section, the pipette potential was held at 0 mV in cell-attached patch experiments, at + 50 mV in inside-out patch experiments, at 0 mV in outside-out patch experiments and at − 70 mV in whole-cell patch-clamp experiments. Inward and outward membrane K^+-currents are indicated as downward and upward deflections, respectively, in all current traces.

Cell-attached patch experiments were carried out as previously described (Panten *et al.*, 1990). Data samples of 1–2 min duration were analyzed with the half-amplitude threshold technique (Colquhoun & Sigworth, 1983) using an interactive graphics-based analysis programme (pCLAMP 5.5.1, FETCH series). Amplitude histograms of sampled cell-attached currents were formed to calculate the mean single-channel current amplitudes (i). Channel activity was defined as the product of N, the number of functional channels and P_o, the open state probability and was determined by dividing the total time the channels spent in the open state by the total sample time. In case of superpositions of channels, the channel activity was calculated by summing the activity for each different current level. The mean of the channel activity during the control periods (presence of 0–10 mM D-glucose or 3 mM D-glucose + 300 μM diazoxide) before and after application of test substances (3–400 μM diazoxide or 1–5000 μM tolbutamide) was set at 1.0, and the channel activity in the presence of test substances was normalized to this value. Only one concentration of glucose, diazoxide or tolbutamide was tested per cell-attached patch and data were sampled during the last 1–2 min of a test period of 3–6 min duration. Data sampling during the control periods was started 2 min before and 4–6 min after the test period.

In inside-out patch experiments the cytoplasmic face of the membrane patch was exposed to test pulses of bath solution (supplemented with or without test substances) for 15 s intervals alternating with 45 s pulses of bath solution containing 1 mM ATP applied by a microflow system (Ohno-Shosaku *et al.*, 1987). Using the experimental design shown in Figure 3 (a,b) the test pulses of bath solution contained various nucleotides and/or drugs. Before and after application of test substance-containing bath solution, there were periods during which the same solution was applied except that the test substances were omitted. The mean of the amplitudes of the current responses (current amplitudes) during application of test substances was normalized to the mean current amplitude during the nucleotide- and drug-free control periods in each single experiment. The single-channel current amplitudes of the K-ATP-channels were not changed by tolbutamide (1–5000 μM), diazoxide (10–400 μM) or the tested nucleotide concentrations.

In outside-out patch experiments the mean current during the 90 s interval starting 2–3 min after establishing the outside-out configuration was determined using pipette solution containing no nucleotide, ATP (0.03–1 mM) or adenylyl-imidodiphosphate (AMP-PNP) (0.01–0.3 mM).

Measurement of whole-cell currents was carried out as described by Zünkler *et al.* (1988a). In brief, hyper- and depolarizing voltage pulses of 10 mV amplitude and 200 ms duration were applied alternately every 2 s. Current amplitudes always increased during the first minutes of recording due to wash-out of ATP from the cytoplasm. After reaching a maximum, the currents decreased again due to spontaneous run-down. Maximum current amplitudes (in response to depolarizing pulses) observed in experiments with pipette solution containing no nucleotide or ATP (0.3–3 mM) were used to construct the concentration-inhibition relation.

Recordings were made with an LM-EPC 7 patch clamp amplifier (List Electronic, Darmstadt, Germany). Current signals were stored on magnetic tape (Store 4, Racal Recorder, Hythe, UK) at 7.5 in s^{-1} (band width 2 kHz, − 3 dB point, cell-attached currents) or at 1⅞ in s^{-1} (band width 0.5 kHz, − 3 dB point, inside-out, outside-out and whole-cell currents) or digitized by use of an A/D converter (Instrutech, New York, U.S.A.) and stored on a video cassette recorder (cell-attached currents). Prior to analysis, the data stored on video tape were low-pass filtered at 2 kHz with a 4-pole Bessel filter (AF 173, Lötscher Elektronik, Andelfingen, Germany). For analysis, taped data were digitized at 10 kHz for cell-attached currents and at 2 kHz for inside-out, outside-out and whole-cell currents using an Axolab 1100 computer interface (Axon Instruments, Foster City, CA, U.S.A.) and stored in a microcomputer. Analysis of the data was performed with the computer programme pCLAMP 5.5.1 (Axon Instruments). For Figures 1(a), 2(a) and 3(b) taped data were replayed into a chart recorder (220, Gould, Cleveland, OH, U.S.A.). The single channel currents shown in Figure 2(b–d) were displayed using the programme pCLAMP 5.5.1 and a laser printer (Kyocera).

Chemicals and solutions

Tolbutamide, nifedipine and Na_2-2′-deoxyadenosine 5′-diphosphate (dADP) were obtained from Sigma (St. Louis, MO, U.S.A.). Na_2-ATP and Li_4-AMP-PNP were from Boehringer (Mannheim, Germany). All other chemicals were obtained from sources described elsewhere (Panten *et al.*, 1989). Stock solutions of tolbutamide and diazoxide were prepared daily in 50–100 mM NaOH or KOH.

The solution at the cytoplasmic side of the membrane (bath solution in inside-out experiments, pipette solution in whole-cell and outside-out experiments) contained (concentrations in mM): KCl 140, $CaCl_2$ 2, $MgCl_2$ 1, ethylene glycol

bis (β-aminoethyl ether)-N,N,N'N'-tetraacetic acid (EGTA) 10 and 4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid (HEPES) 5 (titrated to pH 7.15 with KOH) (free $[Ca^{2+}] = 0.05\ \mu M$). The free Mg^{2+} concentration was held close to 0.7 mM by adding appropriate amounts of $MgCl_2$ to the nucleotide-containing solutions. The required amounts of $MgCl_2$ and the composition of the solutions for pH 7.15 were calculated with a computer programme using the stability constants detailed previously (Schwanstecher *et al.*, 1992a). After addition of 1 mM Na_2-ATP this solution was also used for filling the U-shaped polythene capillary of the microflow system used in inside-out patch experiments (Ohno-Shosaku *et al.*, 1987). In whole-cell experiments this solution was supplemented with ATP (0–3 mM). The bath solution in cell-attached, outside-out and whole-cell patch experiments contained (concentrations in mM): NaCl 140, KCl 5.6, $CaCl_2$ 2.6, $MgCl_2$ 1.2, HEPES 10 (titrated to pH 7.40 with NaOH). Unless stated otherwise in the Results section, the pipette solution in cell-attached and inside-out experiments contained (in mM): KCl 146, $CaCl_2$ 2.6, $MgCl_2$ 1.2, HEPES 10 (titrated to pH 7.40 with KOH). In cell-attached experiments D-glucose and nifedipine were added to bath and pipette solutions at the concentrations stated in the Results section. Nifedipine was used to prevent action potentials and opening of Ca^{2+}-dependent K^+ channels. For the experiments in the presence of 10 mM D-glucose we selected patches which showed a channel activity > 0.01 in the presence of 10 mM D-glucose alone.

The pH of all solutions was determined after adding D-glucose, nifedipine, tolbutamide, diazoxide or any nucleotides and was readjusted if necessary.

Treatment of results

Values are presented as mean ± s.e.mean. Significances were calculated by the two-tailed U-test of Wilcoxon, Mann and Whitney. $P < 0.05$ was considered significant. The concentration-inhibition relationships for tolbutamide or nucleotides (Figures 4–7) were analyzed by fitting the function

$$(1)\quad E = a\left(1 - \frac{[A]^n}{EC_{50}{}^n + [A]^n}\right) + b$$

to the experimental data by a non-linear least-squares routine where a = maximal current amplitude in the absence of test substance, b = current amplitude in the presence of maximally effective concentrations of test substance, E = normalized effect in the presence of test substance, [A] = concentration of test substance, EC_{50} = half-maximally effective concentration and n = slope parameter (Hill coefficient). Relations beteween diazoxide concentration and stimulatory effects (Figures 1 and 3) were analyzed by fitting the function

$$(2)\quad E = a\left(\frac{[A]^n}{EC_{50}{}^n + [A]^n}\right) + b$$

to the experimental data where a = current amplitude in the presence of 300 or 400 μM diazoxide, b = current amplitude in a diazoxide-free bath solution supplemented with D-glucose (0–10 mM) or nucleotides as stated in the Results section, E = normalized effect in the presence of diazoxide, [A] = concentration of diazoxide, EC_{50} = half-maximally effective concentration and n = slope parameter (Hill coefficient).

Results

Diazoxide-sensitivity of the K-ATP-channel in cell-attached patches

We have previously observed that the slope conductance of the single K-ATP-channels of β-cells was 88 pS in cell-attached patches exposed to 150 mM K^+ at 37°C (Panten *et al.*, 1990). In the present study we found slope conductance of 91.1 ± 2.4 pS ($n = 6$), 89.8 ± 3.0 pS ($n = 7$) or 87.9 ± 1.9 pS ($n = 7$) when the bath solutions contained diazoxide (400 μM), diazoxide (400 μM) + tolbutamide (0.1 mM) or none of these drugs, respectively (single-channel current-voltage relationships not shown, data points for pipette potentials from 60 to − 60 mV at 37°C). The bath (5.6 mM K^+, 144 mM Na^+) and pipette (150 mM K^+, no Na^+) solutions contained D-glucose (3 mM) + nifedipine (10 μM). Thus, these compounds did not significantly alter the conductance of the K-ATP-channels, since diazoxide and tolbutamide reached the patch via lateral diffusion in the membrane (Trube *et al.*, 1986). The single channel slope conductance was decreased to 24.1 ± 1.5 pS ($n = 13$) at 37°C, when both pipette and bath solution contained 5.6 mM K^+, 144 mM Na^+, 3 mM D-glucose and 10 μM nifedipine (single-channel current-voltage relationship not shown, data points for pipette potentials from − 80 to − 20 mV). This decrease of the slope conductance reflects the K^+ selectivity of the observed channels.

Figure 1a shows a typical chart recorder trace demonstrating the responses of the single K-ATP-channel currents to diazoxide in the presence of 10 mM D-glucose. On changing to a bath solution containing 300 μM diazoxide, channel activity increased by 26 fold within less than 2 min. Reversal of channel activity to control values took about 3 min after changing back to diazoxide-free solution. Single channel current amplitudes were − 5.4 pA and − 6.1 pA during the control and test periods, respectively. The concentration-response curve for diazoxide in the presence of 10 mM D-glucose (Figure 1b, filled circles) shows half-maximal channel activation at 43 μM (Hill coefficient = 3.4) and about 30 fold

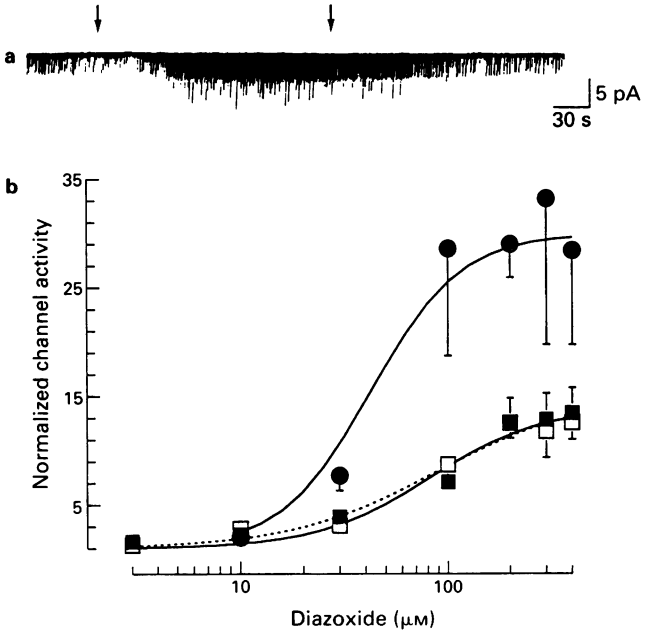

Figure 1 Concentration-response curves for diazoxide upon single-channel current activity of the K-ATP-channels in cell-attached patches of mouse pancreatic β-cells at 37°C. The pipette potential was 0 mV. The pipette solution contained 150 mM K^+. (a) Continuous chart recorder trace demonstrating the responses of single K-ATP-channel currents when changing (arrows) from a bath solution containing 10 mM D-glucose to one containing 10 mM D-glucose and 0.3 mM diazoxide and then back to a 10 mM D-glucose solution. (b) Using the experimental design shown in (a) channel activity during the test period was normalized with respect to the mean activity during the control periods before and after application of diazoxide in each single experiment. The pipette solutions and all bath solutions contained 10 μM nifedipine and no D-glucose (□, dotted line; $n = 4–11$), 3 mM D-glucose (■, continuous line; $n = 4–8$) or 10 mM D-glucose (●; $n = 4–9$). During the test period the bath solution contained 0.003–0.4 mM diazoxide (logarithmic scale). The curves through the channel activity data points were fitted as described in the Methods section. Symbols indicate means and the vertical lines the s.e.mean (when larger than symbols).

activation at maximally effective concentrations. In the presence of a low glucose concentration (3 mM) or in the absence of glucose, diazoxide produced half-maximal effects at 82 μM (Hill coefficient 1.59) or 87 μM (Hill coefficient = 1.24), respectively, and 13 fold activation at maximally effective concentrations (Figure 1b). Since the channel activity varied widely between individual cell-attached patch experiments, we did not observe significant differences between the mean values of absolute channel activity during the control periods with 0 or 3 mM D-glucose. Patches showing a level of activity high enough for reliable analysis of the control periods were selected for the experiments with 10 mM D-glucose. Therefore, significant differences were also not observed for the activity during the control periods with 0 or 10 mM D-glucose.

In order to determine whether the response to maximally effective diazoxide concentrations depended on the extracellular K^+ concentration, cell-attached patch experiments with pipette solution containing 5.6 mM K^+ were performed, as exemplified in Figure 2. As it was difficult to maintain depolarized cell-attached patches at 37°C, the experiments were started soon after seal formation. In the beginning of some experiments the seal still improved as reflected in the beginning of the current trace (a). This did not affect the single channel current amplitudes. When setting the mean of the channel activity during the control periods (presence of 3 mM D-glucose alone) before and after application of diazoxide to 1.0, the activity of the 24 pS channel in the presence of both 300 μM diazoxide and 3 mM glucose was 11.4 ± 1.2 ($n = 5$). The mean single channel current amplitude was 1.65 ± 0.03 pA and 1.68 ± 0.07 pA during the control and test periods, respectively. When no diazoxide was added during the test periods the normalized channel activity during the test periods was 0.98 ± 0.06 ($n = 5$). The difference between channel activities during the test periods of the experiments with or without diazoxide (300 μM) was significant ($P < 0.05$).

Diazoxide-sensitivity of the K-ATP-channel in inside-out patches

Since the role of the Mg complexes of nucleoside diphosphates in diazoxide-induced activation of the K-ATP-channel of β-cells was unclear, we performed inside-out patch experiments. In the presence of Mg^{2+}, but in the absence of nucleotides, diazoxide (10–400 μM) did not affect channel activity (Figure 3c (▽)). We then tested diazoxide in conjunction with dADP. In the presence of Mg^{2+} this nucleoside diphosphate (up to 200 μM) is a pure activator of the K-ATP-channel (Schwanstecher *et al.*, 1992a). The example in Figure 3b demonstrates that addition of 20 μM total dADP (9.6 μM MgdADP) enhanced channel activity by 82%. After further addition of a maximally effective diazoxide concentration (400 μM), the channel activity was trebled as compared to the control activity. In the presence of 20 μM total dADP, diazoxide was half-maximally effective at 32.9 μM (Hill coefficient = 1.55) (Figure 3c). At a concentration of 20 μM dADP exerted only about 10% of its maximum channel activating capacity (Schwanstecher *et al.*, 1992a). The channel activity observed in the presence of both Mg^{2+} and 200 μM dADP increased by 2.2 fold in response to 100 μM diazoxide (data not shown).

Both, MgADP and MgGDP have been shown to increase the activity of the K-ATP-channel (Ashcroft & Rorsman, 1989). In the presence of ADP (48% MgADP) the following EC_{50} values and Hill coefficients were obtained for diazoxide (Figure 3c): 29 μM and 1.59 (20 μM total ADP), 18.8 μM and 1.45 (1 mM total ADP). Maximally effective diazoxide concentrations enhanced the channel activity by 88% or 183% in the presence of 20 μM or 1 mM total ADP, respectively (Figure 3c). The channel activity observed in the presence of 150 μM GDP (48% MgGDP; data not shown) was enhanced

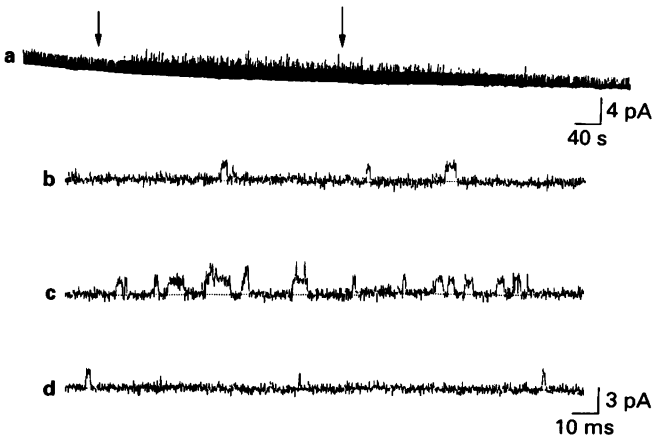

Figure 2 Effect of diazoxide on the activity of the K-ATP-channels in a cell-attached patch from a mouse pancreatic β-cell at 37°C and physiological extracellular K^+ concentration. The pipette potential was −70 mV. The pipette was filled with diazoxide-free bath solution (5.6 mM K^+; supplemented with 10 μM nifedipine and 3 mM D-glucose). All bath solutions contained 10 μM nifedipine and 3 mM D-glucose. (a) Continuous chart recorder trace demonstrating the responses of the single-channel currents when changing (arrows) from a diazoxide-free bath solution to one containing 0.3 mM diazoxide and then back to diazoxide-free solution. (b–d) Single-channel currents recorded at 2 kHz during the sampling intervals before (b), during (c) and after (d) application of diazoxide. Dotted lines denote the current level when all channels are closed. Up to 2 channel superpositions are seen in trace (c).

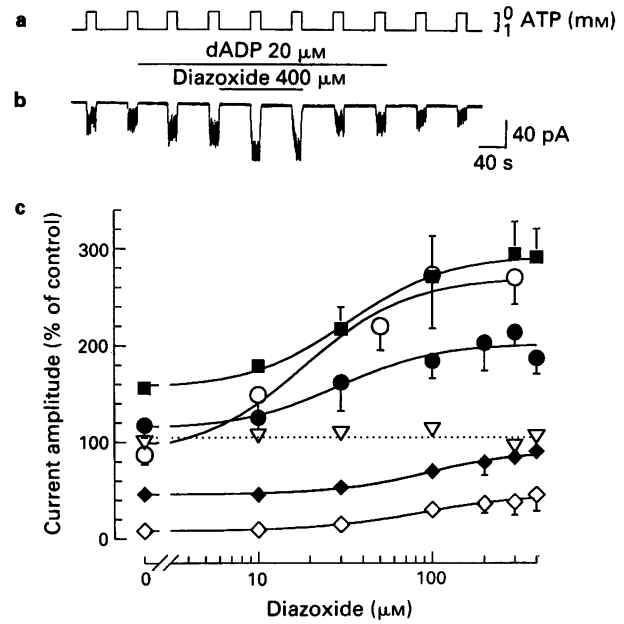

Figure 3 Activation of K-ATP-channels in inside-out patches of mouse pancreatic β-cells by diazoxide. (a) Schematic protocol of solution exchange at the inside of the patch. ATP (1 mM) was present at the inside as represented by the lower level and absent at the upper level of this trace. (b) Current trace obtained from an inside-out patch exposed to 15 s pulses of bath solution with or without test substances. Horizontal bars above trace (b) indicate application of dADP (20 μM) alone or together with diazoxide (400 μM). (c) Curves representing the relationship between normalized current amplitude and diazoxide concentration (logarithmic scale) during pulses of bath solution containing no nucleotide (▽), ATP (30 μM; ◆, 100 μM; ◇), ADP (20 μM; ●, 1 mM; ○) or dADP (20 μM; ■). Using the experimental design shown in (b) the mean current amplitude during application of diazoxide (0–400 μM) was normalized to the mean current amplitude during the nucleotide-free control periods before and after application of diazoxide in each single experiment. The curves through the current amplitude data points were fitted as described in the Methods section. Symbols indicate the mean of 4–9 experiments and the vertical lines the s.e.mean (when larger than symbols).

by 74 ± 21% after further addition of 100 μM diazoxide ($n = 7$).

In the presence of Mg^{2+} and 30 or 100 μM total ATP (26 or 87 μM MgATP) the channel activity amounted to 46 or 8% of the activity during nucleotide-free control periods (Figure 3c). Further addition of maximally effective concentrations of diazoxide restored 91 or 46% of the control activity, respectively. The following EC_{50} values and Hill coefficients were obtained for diazoxide: 92 μM and 1.66 (in the presence of 30 μM total ATP); 82.2 μM and 1.47 (in the presence of 100 μM total ATP). These EC_{50} values do not provide evidence for a competitive interaction between ATP and diazoxide at a common receptor site. In the presence of AMP-PNP (100 μM), diazoxide (100 μM) did not affect the opening activity of the K-ATP-channels ($n = 4$, data not shown).

Interaction between diazoxide and tolbutamide

The data presented so far indicate that diazoxide was more effective in conjunction with a channel-activating nucleoside diphosphate than with a channel-inhibiting nucleotide. Moreover, potent inhibition of the K-ATP-channel by sulphonylureas required the simultaneous presence of channel-activating and channel-inhibiting nucleotides (Schwanstecher *et al.*, 1992a). Therefore, we examined the role of cytosolic nucleotides in the antagonism between diazoxide and tolbutamide. In accordance with previous results (Zünkler *et al.*, 1988b; Sturgess *et al.*, 1988), even very high tolbutamide concentrations did not cause complete block of the K-ATP-channels in the absence of nucleotides; tolbutamide was half-maximally effective at 17 μM (Hill coefficient = 0.51; Figure 4). Addition of 300 μM diazoxide did not alter the response to tolbutamide ($EC_{50} = 14.1$ μM; Hill coefficient = 0.59); 5 mM tolbutamide suppressed channel activity by 81% (Figure 4). When the K-ATP-channels were activated by 100 μM dADP, saturating tolbutamide concentrations caused a reduction of channel activity to 60% of the control value (absence of any nucleotides, diazoxide and tolbutamide); tolbutamide was half-maximally effective at 8.9 μM (Hill coefficient = 0.99; Figure 4). The combined effects of dADP (100 μM) and diazoxide (50 or 300 μM) induced strong channel activity amounting to 257 ± 30% ($n = 3$; 50 μM diazoxide) or 333 ± 27% ($n = 6$; 300 μM diazoxide) of the activity of the control (absence of any nucleotides, diazoxide and tolbutamide). These high activities were lowered to 57 or 133%, respectively, by saturating concentrations of tolbutamide which were half-maximally effective at 43 (Hill coefficient = 0.95) or 55 μM (Hill coefficient = 1.30), respectively (Figure 4). ATP (4 mM) decreased the high channel activity observed in the presence of both dADP (100 μM) and diazoxide (300 μM) to 6.2 ± 5.5% of the activity in the absence of any nucleotides, diazoxide and tolbutamide ($n = 4$). Addition of AMP-PNP (300 μM) to bath solution supplemented with dADP (100 μM), diazoxide (50 μM) and tolbutamide (1 mM) reduced the current amplitudes during the test periods to 1.6 ± 0.7% of the current amplitudes in the absence of any nucleotides, diazoxide and tolbutamide ($n = 6$).

In the presence of 1 mM ADP, tolbutamide was very potent ($EC_{50} = 4.0$ μM; Hill coefficient = 0.90) and caused complete channel block at saturating concentrations (Figure 5; Zünkler *et al.*, 1988b). However, in the presence of both 1 mM ADP and 50 or 300 μM diazoxide 22 or 110%, respectively, of the control activity was not suppressed by saturating concentrations of tolbutamide. Under these conditions tolbutamide was half-maximally effective at 12 μM (Hill coefficient = 1.27; 50 μM diazoxide) or 47 μM (Hill coefficient = 1.06; 300 μM diazoxide) (Figure 5). Thus, in the presence of nucleotides the Hill coefficients ranged between 0.9 and 1.3, but were only half as high in the absence of nucleotides. This might reflect heterogeneity of binding sites for tolbutamide in the absence of nucleotides.

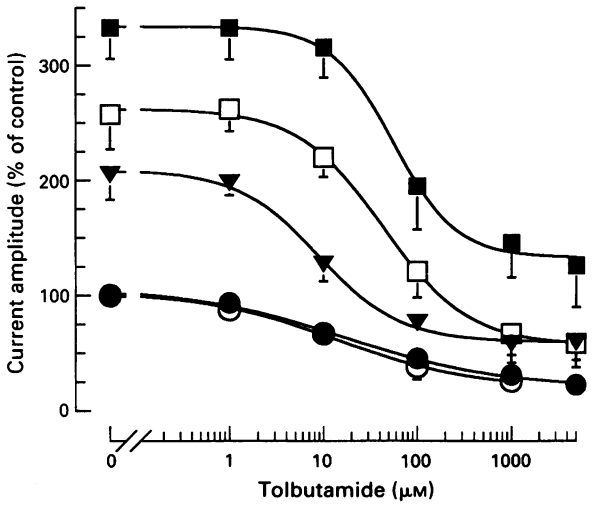

Figure 4 Effects of dADP on the interaction between diazoxide and tolbutamide in inside-out patches of mouse pancreatic β-cells. The curves represent the relationships between normalized current amplitude and tolbutamide concentration (logarithmic scale) during pulses of bath solution containing no diazoxide (●), 300 μM diazoxide (○), 100 μM dADP (▼), 100 μM dADP + 50 μM diazoxide (□) or 100 μM dADP + 300 μM diazoxide (■). Experimental design, normalization of the current amplitudes (control periods free of nucleotides, diazoxide and tolbutamide) and curve fitting were as described in Figure 3. Symbols indicate the mean of 4–8 experiments and the vertical lines the s.e.mean (when larger than symbols).

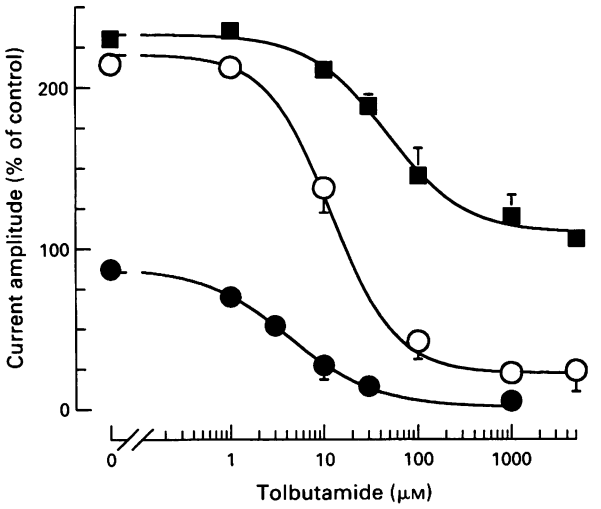

Figure 5 Effects of ADP on the interaction between diazoxide and tolbutamide in inside-out patches of mouse pancreatic β-cells. The cruves represent the relationships between normalized current amplitude and tolbutamide concentration (logarithmic scale) during pulses of bath solution containing 1 mM ADP (●), 1 mM ADP + 50 μM diazoxide (○) or 1 mM ADP + 300 μM diazoxide (■). Experimental design, normalization of the current amplitudes (control periods free of nucleotides, diazoxide and tolbutamide) and curve fitting were as described in Figure 3. Symbols indicate the mean of 5–12 experiments and the vertical lines the s.e.mean (when larger than symbols).

Figure 6 shows the inhibitory effect of tolbutamide in intact β-cells exposed to 3 mM D-glucose and a maximally effective concentration of diazoxide (300 μM). Tolbutamide was half-maximally effective at 13.9 μM (Hill coefficient = 0.61). The concentration-response relationship suggests incomplete channel block at saturating tolbutamide concentrations.

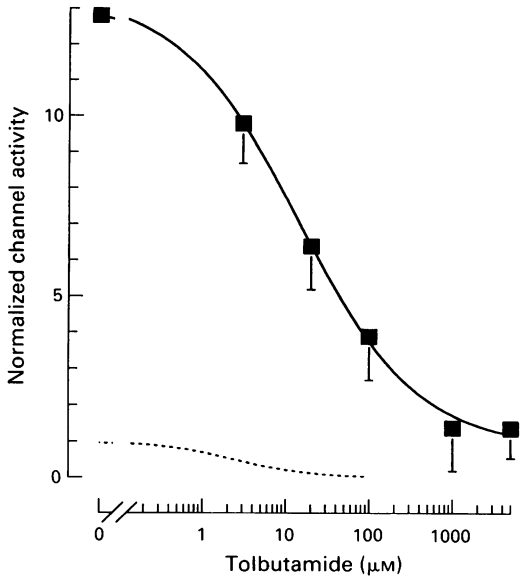

Figure 6 Tolbutamide-induced inhibition of K-ATP-channel activity in cell-attached patches of mouse pancreatic β-cells at 37°C in the presence of 300 μM diazoxide (■). The pipette potential was 0 mV. The pipette solution contained 150 mM K^+. The pipette solution and all bath solutions contained nifedipine (10 μM), D-glucose (3 mM) and diazoxide (300 μM). The experimental design was similar to that of Figure 1. The channel activity during the test period was related to the mean activity during the control periods before and after application of tolbutamide in each single experiment. According to Figure 1 (curve with filled squares) the channel activity in the absence of tolbutamide was set to 12.8 (control). The curves through the channel activity data points were fitted as described in the Methods section. Symbols indicate means of 4–8 experiments and the vertical lines the s.e.mean (when larger than symbols). To facilitate comparison, the concentration-dependent effects of tolbutamide in cell-attached patches of β-cells exposed to D-glucose (3 mM) in the absence of diazoxide are shown as a dashed line (data taken from Figure 3 in Panten *et al.*, 1990).

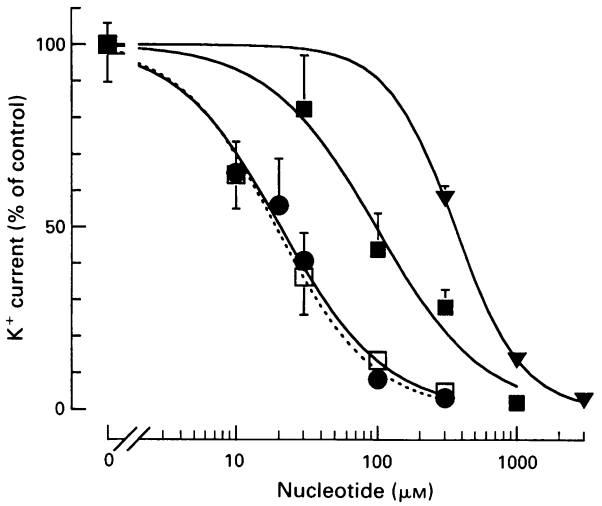

Figure 7 Inhibition of ATP-dependent K^+ currents in mouse pancreatic β-cells by ATP (filled symbols, continuous lines) and AMP-PNP (open squares, dotted line) in the inside-out (●), outside-out (■, □) or whole-cell clamp configuration (▼) of the patch-clamp technique. The values in the presence of the indicated nucleotide concentrations (logarithmic scale) were expressed as percentages of values in the absence of nucleotide (control). Control for outside-out experiments was 4.6 ± 0.5 pA ($n = 20$) and represents the mean current during the 90 s interval starting 2–3 min after establishing the outside-out configuration. Control for whole-cell clamp experiments was 27.5 ± 1.6 pA/pF ($n = 85$) and represents the maximum current amplitudes in response to depolarizing pulses divided by the cell capacitance. Control for each single inside-out experiment was the mean of the amplitudes of the current response before and after application of nucleotide. Further details are described in the Methods section. Symbols indicate the mean and the vertical lines the s.e.mean (when larger than symbols).

Potency of cytosolic ATP in inhibiting ATP-dependent K^+ currents

Inside-out patches are membrane-covered blebs of cytoplasm (Sokabe & Sachs, 1990; Ruknudin *et al.*, 1991). Therefore, the response of the K-ATP-channels to ATP-containing bath solution might be modulated by ATP-consuming reactions yielding MgADP. This possibility was examined by comparing the channel-inhibiting potencies of ATP in different configurations of the patch-clamp technique. Electron microscopic studies of the patches (Ruknudin *et al.*, 1991) suggest that the amounts of cytoplasm and ATP-consuming enzymes located between the plasma membrane and the bath or pipette solution increase in the order inside-out < outside-out < whole-cell. Figure 7 shows that this order correlated with decreasing potency of ATP. The following EC_{50} values and Hill coefficients were obtained: 20.6 μM and 1.19 ($n = 4–16$; inside-out); 97.3 μM and 1.15 ($n = 7–20$; outside-out);357 μM and 1.79 ($n = 34–85$; whole-cell). However, the channel-blocking potency of the nonhydrolyzable ATP-analogue AMP-PNP did not appear to depend on the amounts of cytoplasm attached to the plasma membrane. In outside-out patches the EC_{50} value for AMP-PNP (19.1 μM; Hill coefficient = 1.28; $n = 4–10$; Figure 7) was not higher than in inside-out patches (26 μM; Panten *et al.*, 1990).

Discussion

In previous studies with intact cells the potency of diazoxide in activating the K-ATP-channel was determined by monitoring the ^{86}Rb efflux from insulin-secreting tumour cells (HIT-T-15; Niki *et al.*, 1989) or human medulloblastoma cells (Daniel *et al.*, 1991). The diazoxide concentrations giving 50% stimulation of ^{86}Rb efflux were found to be 45 and 100 μM, respectively. In the present work, similar values were observed when recording the activity of the K-ATP-channels in pancreatic β-cells using the cell-attached configuration of the patch-clamp technique at 37°C. Application of a maximally effective diazoxide concentration (300 μM) at normal or high K^+ concentrations in the pipette solutions revealed that at least 91–92% of all K-ATP-channels in the β-cells were already closed by 3 mM glucose alone. Increasing the glucose concentration to 10 mM caused closure of more than 97% of all K-ATP-channels. These findings support the previous conclusion that only a small percentage of the K-ATP-channels remain open at the threshold for stimulation of insulin release (Cook *et al.*, 1988; Panten *et al.*, 1990). In the presence of 10 mM glucose the half-maximally activating concentration of diazoxide was lower than in the presence of 3 mM glucose (43 and 82 μM, respectively). Hence, we did not find evidence that glucose metabolism produced an inhibitory cofactor or metabolite which competitively interacted with diazoxide.

It has been suggested that protein phosphorylation is involved in diazoxide-induced activation of the K-ATP-channels since hydrolysis of MgATP was necessary for channel activation by diazoxide (Dunne, 1989; Kozlowski *et al.*, 1989). The inside-out experiments in our present study were carried out using an experimental protocol favouring protein phosphorylation. A saturating concentration of MgATP was present as substrate for protein kinases except for 15 s test periods. However, diazoxide was ineffective in the absence of any nucleotides during the test periods and weakly effective in the presence of 26 or 87 μM MgATP. These observations suggest that phosphorylation of the K-ATP-channels and/or regulatory proteins is not sufficient for diazoxide to activate the channels in pancreatic β-cells. This view is supported by

the strong channel activation caused by diazoxide in the presence of MgADP, MgdADP or MgGDP. Thus, effectiveness of diazoxide appears to require occupation of the receptor for cytosolic nucleotides activating the K-ATP-channel. MgATP might occupy the same site and induce a channel conformation less susceptible to diazoxide than the conformation induced by the Mg complexes of nucleoside diphosphates. Alternatively, it is conceivable that MgATP serves as substrate for formation of MgADP by enzymes in the inside-out membrane and the attached bleb of cytoplasm. In the presence of Mg^{2+}, the K^+ channel-blocking potency of ATP, but not of its nonhydrolyzable analogue AMP-PNP, decreased with increasing amounts of cytoplasm located between the K-ATP-channels and the ATP-containing bath or pipette solutions (see Results).

Sulphonylureas and cytosolic nucleotides (e.g. ATP) exert their inhibitory effects on the K-ATP-channels of β-cells by binding to different sites (Schwanstecher *et al.*, 1992b). In insulin-secreting cells, diazoxide probably does not bind to the site for sulphonylureas (Schwanstecher *et al.*, 1992c). In inside-out patches, there was no evidence for interaction of diazoxide and ATP at a common site (Figure 3), in accordance with cell-attached patch experiments (see above). Moreover, diazoxide and Mg complexes of nucleoside diphosphates activate the K-ATP-channel via separate binding sites since their combined effects in inside-out patches were over-additive (see Results). Finally, binding studies and patch-clamp experiments favour the view that Mg complexes of nucleoside diphosphates activate the K-ATP-channel by a site not identical with the sites mediating channel inhibition by sulphonylureas and cytosolic nucleotides (Schwanstecher *et al.*, 1992a,b). It is therefore suggested that the K-ATP-channel is controlled via four separate binding sites, for ATP (and related inhibitory nucleotides), Mg complexes of nucleoside diphosphates, sulphonylureas and diazoxide.

The antagonism between diazoxide and sulphonylureas depends on the presence of cytosolic nucleotides (Figures 4 and 5) and is discussed using the scheme in Figure 8. It is assumed that the separate binding sites for the four classes of ligands are located on the proteins organizing the K-ATP-channel. However, it may well be that one or several binding sites are located on regulatory proteins not permanently associated with the K^+ channel. Since the present experiments were performed under conditions favouring protein phosphorylation all conformational states of the K-ATP-channel are supposed to be phosphorylated. The opening activity of the ground state is reflected in the current amplitudes during the test pulses with ligand-free solutions. Cytosolic ATP is able to induce a completely blocked state in the absence of any other ligand (state not shown in Figure 8). A state showing higher opening activity (activated) than the ground state is stabilized by MgADP, MgdADP or MgGDP. In addition to these Mg complexes, free nucleoside diphosphates are also present in the cytosol-like test solutions. Free ADP blocks the K-ATP-channel ($EC_{50} = 49\ \mu M$; Panten *et al.*, 1990) by binding to the same site occupied by free ATP ($EC_{50} = 4\ \mu M$; Ashcroft & Kakei, 1989; see also Results section) whereas free dADP and GDP are ineffective up to 0.1 or 0.5 mM, respectively (Schwanstecher *et al.*, 1992a). The effect of MgADP or its analogues is sensitized by diazoxide which is ineffective when tested alone (Figure 3).

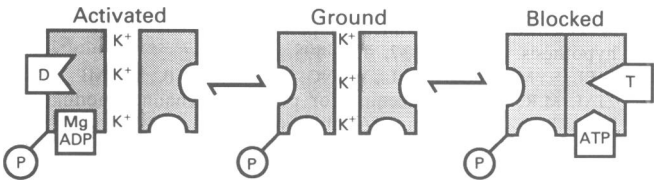

Figure 8 Simplified model for regulation of the K-ATP-channel by nucleotides, tolbutamide (T) and diazoxide (D). The protein assembly organizing the K-ATP-channel is thought to exist in at least three phosphorylated (P) conformational states. In the ground state, the channel opens less frequently than in activated states stabilized by MgADP and diazoxide. Nonconductive blocked states are stabilized by free ATP and tolbutamide.

Incomplete block at saturating concentrations (Figure 4) may be characteristic of the conformational states induced by binding of sulphonylureas alone or together with Mg complexes of nucleoside diphosphates (states not shown in Figure 8). Sensitization of MgdADP-induced channel activation by diazoxide further weakened the effects of saturating tolbutamide concentrations, probably due to the failure of tolbutamide to bind to the conformational states resulting from binding of diazoxide (Schwanstecher *et al.*, 1991b). However, high concentrations of ATP nearly completely suppressed the combined actions of MgdADP and diazoxide (see Results). This might have been due to a shift of the equilibria between the conformational states of the K-ATP-channel towards a blocked state.

It was shown previously that K-ATP-channels exposed to the combined actions of inhibitory (free ATP or ADP) and stimulatory (e.g. MgADP) nucleotides were most sensitive to sulphonylureas (Schwanstecher *et al.*, 1992a). Therefore, saturating tolbutamide concentrations completely blocked the K-ATP-channel in the presence of Mg^{2+} ions and 1 mM ADP (Figure 5). One possible explanation could be that the simultaneous binding of an inhibitory nucleotide and tolbutamide induced a blocked state unable to bind a stimulatory nucleotide. However, in the presence of both 1 mM ADP and diazoxide, tolbutamide could not completely block the K-ATP-channel (Figure 5). This finding might indicate that the concentration of inhibitory free ADP was not high enough to prevent appearance of the activated state sensitized by binding of diazoxide and unable to bind tolbutamide. In the intact β-cell, too, the concentrations of inhibitory nucleotides appear to be limiting for tolbutamide-induced inhibition of K-ATP-channels activated by maximally effective concentrations of diazoxide (Figure 6).

In summary, the present study supports the view that the K-ATP-channel is controlled via four separate binding sites for inhibitory nucleotides, stimulatory nucleotides, sulphonylureas and diazoxide. Occupation of the sites for nucleotides is required for sensitive regulation of the K-ATP-channel by sulphonylureas and diazoxide.

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Reversal by cysteine of the cadmium-induced block of skeletal neuromuscular transmission *in vitro*

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1 Neuromuscular transmission in isolated nerve-muscle preparations was blocked by exposure to Cd^{2+} for less than 30 min or more than 2 h. The abilities of cysteine, Ca^{2+} or 3,4-diaminopyridine (3,4-DAP) to reverse the blockade induced by Cd^{2+} were studied.

2 On the mouse hemidiaphragm preparation, exposure to Cd^{2+} (10 μM) for 10 to 20 min induced a blockade which was easily reversed by increasing the extracellular Ca^{2+} concentration (5–10 mM) or by 3,4-DAP (100 μM). Exposure to Cd^{2+} (3–10 μM) for over 2 h led to a blockade which was not reversed by Ca^{2+} (5–15 mM) or 3,4-DAP (100 μM). Cysteine (1 mM) was able to reverse completely the blockade induced by both brief and prolonged exposures to Cd^{2+}.

3 In chick biventer cervicis preparations, Cd^{2+} (100 μM) decreased the twitch height of indirectly stimulated preparations without affecting responses to exogenously applied acetylcholine, carbachol or KCl. Cysteine (1–3 mM) had no appreciable effect on twitch responses to indirect stimulation or to exogenously applied agonists but fully reversed the blockade induced by Cd^{2+} (100 μM).

4 In mouse triangularis sterni preparations, Cd^{2+} (1–30 μM) depressed the evoked quantal release of acetylcholine. Concentrations of Cd^{2+} which completely blocked endplate potentials (e.p.ps) were without significant effect on miniature endplate potential (m.e.p.p.) amplitude and frequency or time constant of decay. Cysteine (1–10 mM) alone had no effect on e.p.ps or m.e.p.ps, but completely reversed the blockade induced by Cd^{2+}.

5 Extracellular recording of perineural waveforms from triangularis sterni preparations revealed that Cd^{2+} was able to block the long-lasting positive component that is sensitive to Ca^{2+} channel blockers and the delayed negative deflection that is related to the Ca^{2+}-activated K^+ current ($I_{K,Ca}$) seen in the presence of 3,4-DAP. Cysteine by itself had no effect on any component of the perineural waveform, but promptly reversed the blockade induced by Cd^{2+}.

6 In addition to the competitive blocking action of Cd^{2+} at the prejunctional Ca^{2+} channels, long exposure to Cd^{2+} leads to a blockade that is not competitive. This probably involves binding of Cd^{2+} at an extracellular thiol site on, or close to, voltage-operated Ca^{2+} channels.

Keywords: Cadmium; cysteine; calcium channels; acetylcholine release; perineural waveform; neuromuscular transmission

Introduction

Cadmium ions (Cd^{2+}) are known to block voltage-operated calcium channels in different types of excitable cells (e.g. Hagiwara & Byerly, 1981; Tsien, 1983; Penner & Dreyer, 1986). Cd^{2+} also inhibits acetylcholine release from motor nerve endings, presumably as a consequence of the block of Ca^{2+} channels (Toda, 1976; Forshaw, 1977; Lin-Shiau & Fu, 1980; Cooper & Manalis, 1984). The Ca^{2+} channel blockade induced by brief exposures to Cd^{2+} appears to be competitive as it is easily reversible by increasing the extracellular Ca^{2+} concentration (Cooper & Manalis, 1984; Guan *et al.*, 1987). However, prolonged exposure to Cd^{2+} (for over 2 h) leads to a blockade that is not reversed by Ca^{2+} (Braga *et al.*, 1991b), suggesting a noncompetitive block that is time-dependent. Thiol compounds such as cysteine and glutathione can form a complex with Cd^{2+} (Matsumoto & Fuwa, 1986). Since cysteine and glutathione reversed the neuromuscular block induced by brief exposure to Cd^{2+}, it was postulated that Cd^{2+} binds to sulphydryl groups on membrane proteins to decrease the influx of Ca^{2+} across cell membranes (Toda, 1976; Lin-Shiau & Fu, 1980). Moreover, it has been proposed that the thiol-oxidizing agent, diamide, increases transmitter release, at the frog neuromuscular junction, directly via an oxidation of sulphydryl groups on membrane proteins to form disulphide bonds (Werman *et al.*, 1971; Kosower & Werman, 1971; Carlen *et al.*, 1976a,b; Publicover & Duncan, 1981). Hence, there may be an important role of membrane disulphide bonds and sulphydryl groups in regulating processes which control transmitter release.

The purpose of the present study was to investigate the mechanism through which cysteine, a thiol compound, reverses the neuromuscular blockade produced by brief exposure to Cd^{2+} and whether cysteine or the thiol-oxidizing agent diamide could reverse the Ca^{2+}-insensitive blockade induced by prolonged exposure to Cd^{2+}. By use of extracellular and intracellular recording techniques, the effects of the compounds on nerve terminal action currents and acetylcholine release could be estimated.

Methods

Chick biventer cervicis preparations

Experiments were performed at room temperature (20–25°C) using biventer cervicis nerve-muscle preparations from 3–14 day old chicks (Ginsborg & Warriner, 1960). The dissection, solutions and recording techniques were identical to those described in detail by Braga *et al.* (1991a).

Mouse diaphragm nerve-muscle preparations

Twitch tension experiments were performed at room temperature (20–25°C) on the nerve-muscle hemidiaphragm preparations isolated from 15–25 g male mice (Balb C strain).

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The dissection, solutions and recording techniques were identical to those described in detail by Braga *et al.* (1991a).

Mouse triangularis sterni preparations

Experiments were performed on the left triangularis sterni nerve-muscle preparation (McArdle *et al.*, 1981) isolated from 15–25 g male mice (Balb C strain). Experiments were carried out at room temperature (20–25°C). The dissection, solutions, stimulation parameters and recording techniques were identical to those described previously (Braga *et al.*, 1991a).

To prevent muscle twitching, 15 μM tubocurarine (for extracellular recording) or 2–5 mM Mg^{2+} or 0.2 μM μ-conotoxin (for intracellular recording) was added to the bathing solution. In all Mg^{2+}-paralysed experiments, the Ca^{2+} concentration was reduced to 0.5 mM, and a 20 min equilibration period allowed. In order to investigate the effects of Cd^{2+} and cysteine on waveforms related to Ca^{2+} currents, perineural waveforms were recorded with an extracellular electrode in the presence of maximally effective concentrations of 3,4-diaminopyridine (3,4-DAP, 400 μM, to block voltage-operated K^+ channels in motor nerve terminals) or in the presence of 4 mM tetraethylammonium (TEA) (to block Ca^{2+}-activated K^+ channels in motor nerve terminals) plus 3,4-DAP (400 μM), and the Ca^{2+} concentration in the physiological solution was increased to 6 mM (for details see Penner & Dreyer, 1986). For perineural waveforms recorded in the presence of 3,4-DAP (400 μM) plus TEA (4 mM), the intercostal nerves were stimulated once every 60 s with pulses of 50 μs duration and supramaximal voltage.

Analysis and statistics

The recording system had an overall passband of d.c.-10 kHz and signals were digitized at 25 kHz, more than twice the filter cut off frequency (Nyquist criteria) and analysed with a suite of purpose-designed programmes (Dempster, 1988) running on an IBM-compatible microcomputer (Vanilla, 286-12A).

Values in the text are mean ± s.e. of at least four experiments, unless otherwise stated. Differences between means were tested by the Mann-Whitney U-test, $P<0.05$ being taken as significant.

Materials

Compounds were obtained from Sigma Chemical Co., Poole, Dorset, except for μ-conotoxin GIII B which was from the Peptide Institue, Inc., Osaka, Japan.

Results

The effects of cadmium and cysteine on chick biventer cervicis nerve-muscle preparations

Cd^{2+} (100 μM) induced a 75 ± 7% ($n = 4$) reduction in twitch height of indirectly stimulated preparations in around 20 min, without affecting responses to exogenously applied acetylcholine, carbachol or KCl (data not shown). In contrast, cysteine (1–3 mM) had no appreciable effect on twitch responses to indirect stimulation and to exogenously applied acetylcholine, carbachol or KCl. Cysteine (1 mM), in 5 min, fully reversed the blockade induced by 20 min exposure to Cd^{2+} (100 μM).

The effects of cadmium and cysteine on mouse diaphragm nerve-muscle preparations

Cysteine (1–10 mM) had no effect on the twitch height induced by indirect stimulation. As previously reported (Satoh *et al.*, 1982), Cd^{2+} (10–300 μM) induced a concentration-dependent reduction of the twitch height of indirectly stimulated preparations. The effects of 10–20 min exposure to Cd^{2+} (i.e. time for 10 μM Cd^{2+} to block 70% of the twitch height) were completely reversed by increased Ca^{2+} concentration (5–10 mM), 3,4-DAP (100 μM) or cysteine (1 mM), as shown in Figure 1a. However, after prolonged exposure (2–3 h) to Cd^{2+} (3–10 μM), increased Ca^{2+} concentration (5–10 mM) and 3,4-DAP (100 μM) failed to reverse Cd^{2+}-induced blockade. Under these conditions, cysteine (1 mM) completely reversed the blockade produced by Cd^{2+} within 2 min (Figure 1b).

Intracellular recording in mouse triangularis sterni nerve-muscle preparations

In Mg^{2+}-paralysed preparations, control m.e.p.ps were in the range 0.3–0.6 mV in amplitude and had time constants of decay of 1.2–1.8 ms and the e.p.ps were about 2.5–7.0 mV in amplitude with time constants of decay of 1.0–1.3 ms at a resting membrane potential of −60 to −85 mV. Cd^{2+} (1–30 μM) gradually reduced the amplitude of the evoked e.p.ps in a concentration-dependent manner with no significant change in e.p.p time course. Up to 300 μM, brief exposure to Cd^{2+} had no significant effect on the amplitude, time course of decay or frequency of m.e.p.ps. Therefore, Cd^{2+} reduced quantal content of e.p.ps (Figure 2). However, after 3 h in the presence of Cd^{2+} (300 μM) the m.e.p.p. frequency was around 4 times higher than control level.

Cysteine (1 mM) rapidly restored the e.p.p. amplitude to control levels, and reversed the blockade of quantal content induced by Cd^{2+} (data not shown). However, cysteine (1–10 mM) alone had no effect on quantal content, e.p.p. amplitude, or on amplitude, time course or frequency of m.e.p.ps: for example, in the presence of 1 mM cysteine, quantal content was 96.6 ± 0.5% of control, e.p.p. amplitude was 93.0 ± 8% of control and m.e.p.p. amplitude 97.0 ± 7% of control ($n = 3$).

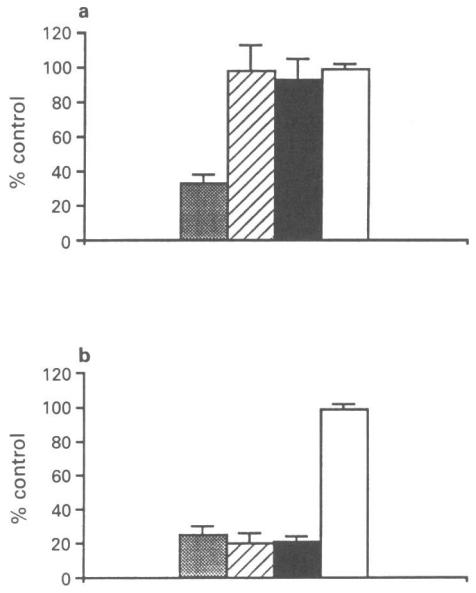

Figure 1 Reversal of (a) brief and (b) prolonged exposures to Cd^{2+} on mouse diaphragm preparations by Ca^{2+}, 3,4-diaminopyridine (3,4-DAP), and cysteine. Stippled columns: 10 μM Cd^{2+} for 10–20 min in (a) or over 2 h in (b); hatched columns: effects of 10 mM Ca^{2+}; solid columns: effects of 100 μM 3,4-DAP; open columns: effects of 1 mM cysteine. Indirect muscle stimulation at 0.1 Hz. Columns represent means of 4 experiments, and s.e. are indicated by the bars.

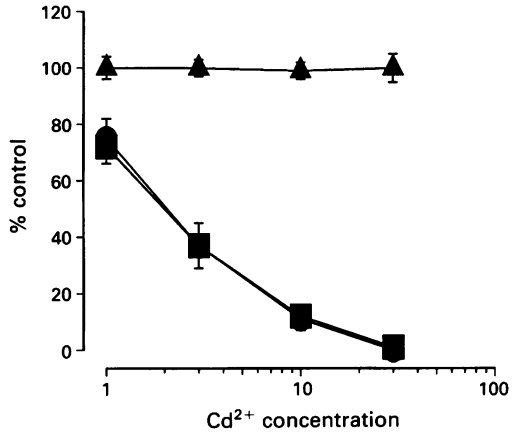

Figure 2 Effects of Cd^{2+} on e.p.p. amplitude (●), m.e.p.p. amplitude (▲) and the mean quantal content (■) of e.p.ps in mouse triangularis sterni preparations. Points are means of 3 experiments, and s.e. are indicated by the bars unless smaller than symbols.

Effects of diamide and A23187 on m.e.p.p. frequency after prolonged exposure to Cd^{2+}

In an attempt to localize the site responsible for the Ca^{2+}-insensitive blockade induced by prolonged exposure to Cd^{2+}, transmitter release was stimulated by agents (diamide and A23187) that do not require the functioning of endogenous Ca^{2+} channels. The thiol-oxidizing agent diamide has been shown to stimulate m.e.p.p. frequency by an intracellular action, probably on release sites (Werman *et al.*, 1971; Publicover & Duncan, 1981). Diamide (1 mM) induced a marked increase in m.e.p.p. frequency both in control preparations and in preparations that had been exposed to Cd^{2+} (300 μM) for 2 h (Figure 3a).

The calcium ionophore A23187 (20 μM) enhanced the frequency of m.e.p.ps in control experiments by about 100%. After 2 h of exposure to Cd^{2+} (300 μM), A23187 still increased m.e.p.p. frequency (Figure 3b). Moreover, the increase in m.e.p.p. frequency induced by A23187 was greater in the presence of Cd^{2+} than that in control preparations (Figure 3b).

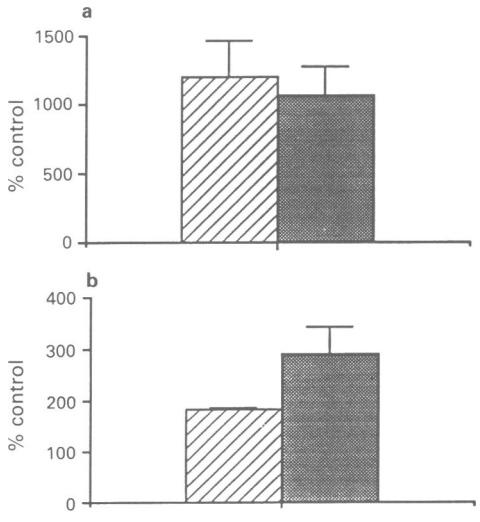

Figure 3 Increase of m.e.p.p. frequency by diamide (a) and by A23187 (b) in mouse triangularis sterni preparation. (a) Hatched column, response to 1 mM diamide in control preparations; stippled column, response to 1 mM diamide after 2 h of exposure to Cd^{2+} (300 μM). (b) Hatched column, response to 20 μM A23187 in control preparations; stippled column, response to 20 μM A23187 after 2 h of exposure to Cd^{2+} (300 μM). Preparations were exposed only once to diamide or A23187. Points are means of 3 experiments, and s.e. are indicated by the bars. M.e.p.p. frequency before exposure to drugs was 3.2–5.0 Hz.

Effects of cadmium and cysteine on perineural waveforms

Normal waveforms The perineural waveform is predominantly made up of two negative deflections, usually preceded by a small positivity as the action potential arrives near the nerve endings. The first negative deflection is associated with the inward movement of Na^+ ions at the nodes of Ranvier and at the heminode. The second negative deflection is associated with the local circuit current that is generated by the movement of both K^+ (outward) and Ca^{2+} (inward) at the motor nerve terminals (see Brigant & Mallart, 1982; Penner & Dreyer, 1986; Anderson *et al.*, 1988).

Neither Cd^{2+} (10 μM–3 mM) nor cysteine (1–10 mM) had any effect on the first or second negative deflections. However Cd^{2+} at concentrations higher than 3 mM blocked the perineural waveform completely and, under these conditions, cysteine (10 mM) failed to reverse the blockade.

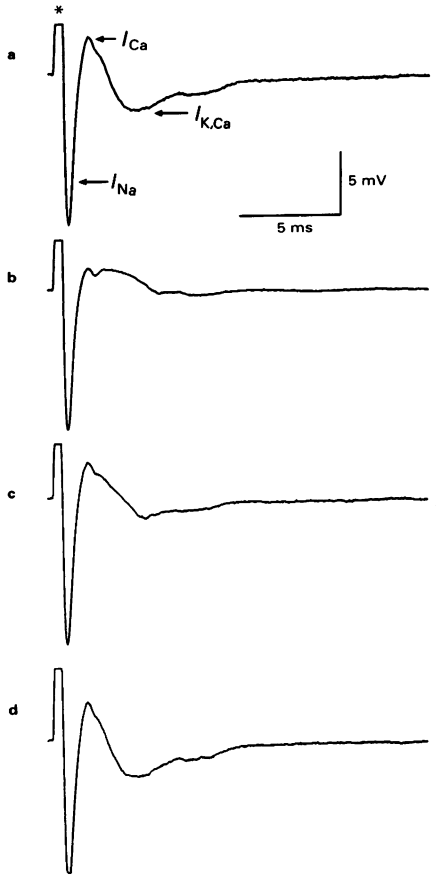

Figure 4 Presynaptic waveforms recorded by electrode placed inside perineural sheath of motor nerve innervating mouse triangularis sterni preparations. In the presence of 3,4-diaminopyridine (3,4-DAP, 400 μM), the first negative deflection (I_{Na}) is associated with the movement of Na^+ ions (inward) at the nodes of Ranvier in the parent axon; the second negative deflection is Ca^{2+}-dependent and is abolished by tetraethylammonium (TEA) suggesting that it is a type of $I_{K,Ca}$; and the positive component that is enhanced in the presence of TEA represents an inward Ca^{2+} current at the nerve terminals (I_{Ca}). (a) Averaged control waveform recorded in the presence of 3,4-DAP (400 μM) and Ca^{2+} (6 mM); (b) averaged waveform after addition of Cd^{2+} (300 μM); (c) averaged waveform after cysteine (300 μM); and (d) averaged waveform after cysteine (1 mM) in the continued presence of 300 μM Cd^{2+}. Note that Cd^{2+} has a marked effect on the delayed negative component and that this is reversed by cysteine. * = Stimulus artefact.

Effects on calcium-activated potassium currents ($I_{K,Ca}$) and calcium currents In the presence of 3,4-DAP (400 μM), there is a short-lasting positive component followed by another negative component of the perineural waveform. This negative deflection is small in amplitude, delayed in onset and dependent on the Ca^{2+} concentration in the bathing medium (Figure 4a). Similar observations had been described previously (Mallart, 1985; Anderson *et al.*, 1988). In the presence of 3,4-DAP, the addition of TEA (3–4 mM) suppressed the delayed negative deflection and enhanced the short-lasting positive component, giving rise to a large long-lasting positive deflection that could be reduced by the addition of Ca^{2+} channel blockers (see Figure 6). Thus, the delayed negative deflection seen in the presence of 3,4-DAP corresponds to a TEA-sensitive, Ca^{2+}-dependent K^+ current ($I_{K,Ca}$) of motor nerve terminals (Mallart, 1985b; Anderson *et al.*, 1988; Braga *et al.*, 1991a). The positive component that is enhanced in the presence of TEA is associated with inward Ca^{2+} currents at the nerve terminals (Brignant & Mallart, 1982; Penner & Dreyer, 1986; Anderson *et al.*, 1988).

In tubocurarine paralysed preparations exposed to 400 μM 3,4-DAP, Cd^{2+} (1–30 μM) reduced the amplitude of the delayed negative deflection (i.e. equivalent to $I_{K,Ca}$) in a concentration-dependent manner, but had no effect on the amplitude of the first negative deflection and little effect on the short-lasting positive component of the perineural waveform (Figure 5). At higher concentrations of Cd^{2+} (100–300 μM), the blockade in the amplitude of the delayed negative deflection of the perineural waveform was accompanied by a reduction of the short-lasting positive component (Figure 4b). Cysteine (0.3 mM) reversed the blockade of the short-lasting positive component but only partially reversed the blockade of the delayed negative deflection (Figure 4c). At 1 mM, cysteine completely reversed the effects of Cd^{2+} (Figure 4d). In contrast, cysteine alone (1–10 mM) had no effect on the amplitude of the delayed negative deflection of the perineural waveform observed in the presence of 3,4-DAP, nor indeed on any other component of the waveform (data not shown).

A combination of 3,4-DAP (400 μM) and TEA (4 mM) gave rise to a large positive deflection (Figures 6a and 7a) which seems to consist of two components, a fast one of around 6 ms duration and a prolonged one of around 60 ms duration. In the presence of Cd^{2+} (10–100 μM) there is a marked reduction in the amplitude and duration of the long-lasting positive waveform. However, the short-lasting positivity is only partially blocked (Figure 6). Concentrations of cadmium higher than 100 μM markedly diminished the fast short-lasting component (Figure 7b). The blockade of the long-lasting positive component was only partially reversed by increased Ca^{2+} concentrations (5–20 mM) as shown in Figure 6, but cysteine at 300 μM partially, and at 1 mM, completely reversed the effects of Cd^{2+} (Figure 7c and d). However, cysteine by itself had no effect on this long-lasting waveform, even at concentrations as high as 10 mM (data not shown). As previously shown by Penner & Dreyer (1986), verapamil (10 μM) also reduced the long-lasting positive waveform. However, cysteine (1–10 mM) was unable to reverse the verapamil-induced blockade (data not shown).

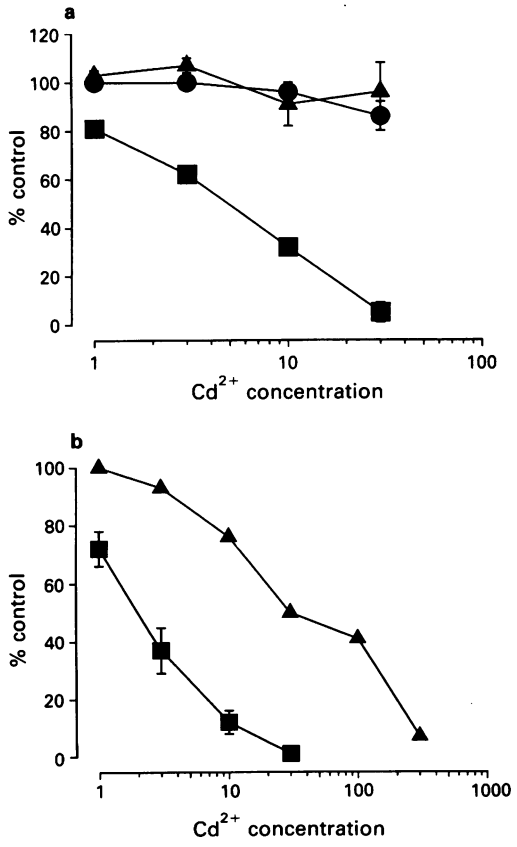

Figure 5 (a) Effects of Cd^{2+} on I_{Ca} (●), I_{Na} (▲) and $I_{K,Ca}$ (■) of the perineural waveforms. (b) Effects of Cd^{2+} on quantal content of Mg^{2+}-paralysed preparations (■) and on e.p.p. amplitude of a μ-conotoxin-paralysed preparation (▲). Points are means of 3 experiments (except for the μ-conotoxin paralysed experiment, $n = 1$), and s.e. are indicated by the bars unless smaller than symbols.

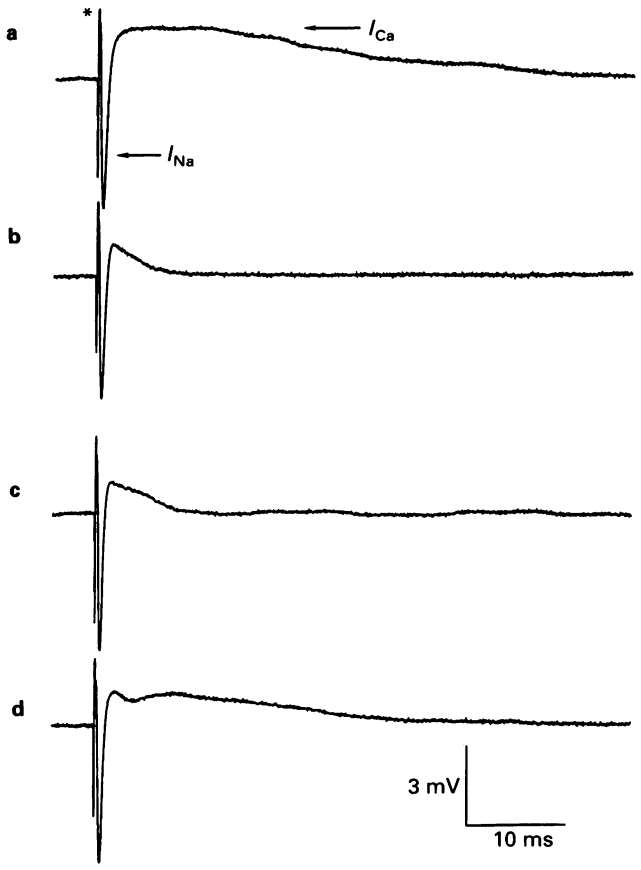

Figure 6 Effects of Cd^{2+} on the long-lasting positive component of the perineural waveforms (I_{Ca}) recorded from mouse triangularis sterni preparations in the presence of 3,4-diaminopyridine (400 μM) and tetraethylammonium (4 mM). (a) Averaged control waveform; (b) averaged waveform after addition of Cd^{2+} (100 μM); (c) averaged waveform after increasing the Ca^{2+} concentration from 2.5 to 5 mM; and (d) averaged waveform after Ca^{2+} at a final concentration of 20 mM. Note the partial reversal induced by Ca^{2+} on Cd^{2+}-induced blockade. * = Stimulus artefact.

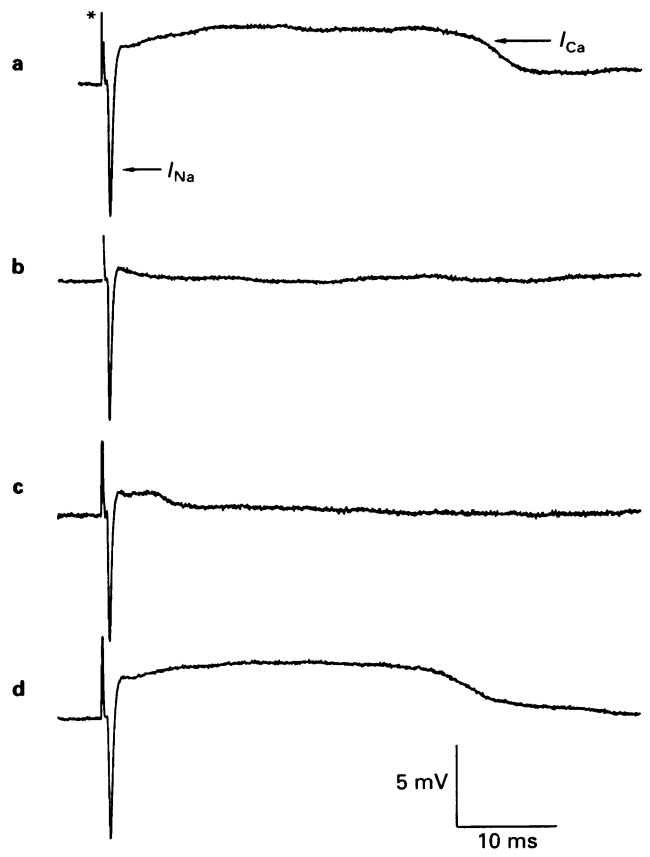

Figure 7 Effects of Cd^{2+} and cysteine on the long-lasting positive component of the perineural waveforms (I_{Ca}) recorded from mouse triangularis sterni preparations in the presence of Ca^{2+} (6 mM), 3,4-diaminopyridine (400 μM) and tetraethylammonium (4 mM). (a) Averaged control waveform; (b) averaged waveform after addition of Cd^{2+} (300 μM); (c) averaged waveform after cysteine (300 μM); and (d) averaged waveform after cysteine (1 mM). Note the complete reversal induced by cysteine on Cd^{2+}-induced blockade. * = Stimulus artefact.

Since the amplitude of the delayed negative deflection is dependent on inward Ca^{2+} currents at the motor nerve terminal and because it is difficult to study these Ca^{2+} currents in isolation under physiological conditions, we investigated whether the more easily measured delayed negative deflection of the perineural waveform could be correlated with Ca^{2+} influx and transmitter release. In Mg^{2+}-paralysed preparations the reduction of quantal content induced by Cd^{2+} paralleled the reduction of the delayed negative deflection of the perineural waveform induced by Cd^{2+} (Figure 5). Moreover, one experiment was carried out where the preparation was paralysed with μ-conotoxin (0.2 μM, which blocked Na^+ channels of the muscle fibre preferentially to Na^+ channels of the nerve: Cruz *et al.*, 1985; Hong & Chang, 1989) and e.p.ps were recorded under very similar conditions as those for the delayed negative deflection of the perineural waveform, i.e. in the presence of 3,4-DAP (400 μM) and 6 mM Ca^{2+}. Under these conditions, the blockade induced by Cd^{2+} in the e.p.p. amplitude also paralleled the block of the delayed negative deflection of the perineural waveform (Figure 5).

Discussion

Previous reports on the actions of Cd^{2+} ions at the neuromuscular junction have shown that Cd^{2+} inhibits acetylcholine release by blocking Ca^{2+} entry into nerve terminals in a competitive manner (Cooper & Manalis, 1984; Guan *et al.*, 1987). Others have reported that Cd^{2+} itself can promote spontaneous release in addition to its blocking action (Nilson & Volle, 1976; Cooper & Manalis, 1984; Nishimura *et al.*, 1984; Guan *et al.*, 1987; Molgo *et al.*, 1989). Our results reveal that the neurmuscular blockade induced by Cd^{2+} is biphasic, depending upon time of exposure. Only the effects of a brief exposure to Cd^{2+} are reversible by Ca^{2+}, whereas cysteine was able to reverse completely the blockades induced by both brief and prolonged exposures to Cd^{2+} .

In order to investigate how cysteine reverses the neuromuscular blockade induced by Cd^{2+}, extracellular recordings of perineural waveforms from motor nerve terminals were carried out. Neither Cd^{2+} nor cysteine had any effect on normal perineural waveforms. Cd^{2+}, however, blocked the long-lasting positive deflection that is related to Ca^{2+} currents and the delayed negative deflection that represents the $I_{K,Ca}$ of motor nerve terminals. Cysteine by itself had no effect on these components of the waveform, but promptly reversed Cd^{2+} blockade. Hence, cysteine probably reverses the blockade of neurotransmitter release induced by Cd^{2+} by reversing the block of Ca^{2+} currents induced by Cd^{2+}. This is probably due to the ability of cysteine to chelate Cd^{2+} (Toda, 1976; Forshaw, 1977), rather than cysteine acting to increase quantal content, as cysteine was unable to increase quantal content in Mg^{2+}-paralysed preparations or reverse the blockade of Ca^{2+} currents induced by verapamil.

In order to localize the site responsible for the Ca^{2+}-insensitive blockade induced by prolonged exposure to Cd^{2+}, diamide and A23187 were used to stimulate transmitter release without involving presynaptic Ca^{2+} channels. Diamide increased m.e.p.p. frequency even after prolonged exposure to Cd^{2+}, indicating that the binding site for Cd^{2+} is different from that of diamide. Diamide acts intracellularly and does not require external Ca^{2+} to induce an increase in transmitter release (Werman *et al.*, 1971; Kosower & Werman, 1971; Publicover & Duncan, 1981). Similarly, after prolonged exposure to Cd^{2+}, the Ca^{2+} ionophore A23187 was still able to increase m.e.p.p. frequency. Hence, the nerve terminal was still responding to an increase in the level of intracellular calcium with a increase of transmitter release, indicating that the sites of acetylcholine release were not affected by prolonged exposure to Cd^{2+}. Moreover, the effect of A23187 in the presence of Cd^{2+} was greater than in control preparations. This could be due to the ability of Cd^{2+} to permeate the ionophore A23187 and activate the transmitter release process by itself. These data are in agreement with those of Molgo *et al.* (1989).

In conclusion, in addition to its competitive blocking action at Ca^{2+} channels, prolonged exposure to Cd^{2+} leads to a blockade of Ca^{2+} channels that is not competitive. This probably involves binding of Cd^{2+} at an extracellular thiol site on, or close to, voltage-operated Ca^{2+} channels.

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Suramin inhibits excitatory junction potentials in guinea-pig isolated vas deferens

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1. Intracellular microelectrode recording techniques were used to investigate the action of the putative P_2-purinoceptor antagonist, suramin, on sympathetic neurotransmission in the guinea-pig isolated vas deferens.

2. The resting membrane potential of the control cells was 67.4 ± 0.7 mV ($n = 48$). Field stimulation of the sympathetic nerves innervating the vas deferens produced excitatory junction potentials (e.j.ps) which reached a mean magnitude of 8.5 ± 0.8 mV ($n = 23$) when fully facilitated at a stimulation frequency of 0.5 Hz.

3. Introduction of suramin 1–100 μM produced no change in the resting membrane potential of the smooth muscle cells, but gradually reduced e.j.p. magnitude. Suramin, 20 μM, reduced the mean magnitude of the fully facilitated e.j.ps to 1.4 ± 0.3 mV ($n = 18$).

4. After suramin-induced inhibition of e.j.ps, nerve stimulation at 1–8 Hz resulted in summation of e.j.ps to a subthreshold level. Subsequent introduction of the α-adrenoceptor antagonists, prazosin or phentolamine (1 μM) did not reduce the magnitude of the summated e.j.ps.

5. The results support the proposal that e.j.ps in vas deferens are mediated by adenosine 5'-triphosphate, and not by noradrenaline, and confirm that suramin can antagonize responses mediated via P_2-purinoceptors.

Keywords: Suramin; ATP; purinergic; purinoceptors; junction potentials; vas deferens; sympathetic; cotransmitter

Introduction

The study of adenosine 5'-triphosphate (ATP) as a sympathetic co-transmitter has been hampered by the lack of a suitable competitive antagonist of postjunctional purinoceptors on effector tissues, such as smooth muscle. Dunn & Blakeley (1988) provided the first evidence that the trypanocidal compound, suramin, was a selective antagonist of P_2-purinoceptors. They showed that in mouse vas deferens 100 μM suramin inhibited the contractile response to the selective P_2-purinoceptor agonist, α,β,methylene-ATP, but did not reduce contractions evoked by exogenous noradrenaline or the muscarinic agonist, carbachol. Suramin has now been tested in a variety of other smooth muscle preparations in which contraction to ATP is thought to be mediated via P_{2X}-purinoceptors and there is general agreement that, at concentrations of suramin up to 300 μM, it has a selective inhibitory action against ATP, without reducing responses to other agonists (von Kugelgen & Starke, 1989; Schlicker *et al.*, 1989; Hoyle *et al.*, 1990; Urbanek *et al.*, 1990; Leff *et al.*, 1990). Suramin seems to have an inhibitory action not only in those tissues where ATP produces contraction via P_{2X}-purinoceptors, but also where ATP mediates smooth muscle relaxtion, via P_{2Y}-purinoceptors. For example, in guinea-pig taenia caeci the relaxation to exogenous ATP but not that to noradrenaline was blocked by suramin (Hoyle *et al.*, 1990). It has also been shown that 300 μM suramin was able to block the P_{2Y}-mediated hyperpolarization of guinea-pig taenia caeci with little effect on the α_1-adrenoceptor-mediated response (Den Hertog *et al.*, 1989). The same group have also provided evidence that suramin is able to inhibit ATP-induced membrane currents in cultured vas deferens smooth muscle cells (Hoiting *et al.*, 1990). These findings would suggest that suramin should be able to inhibit excitatory junction potentials (e.j.ps) in vas deferens, which are thought to be due to the action of ATP released, as a co-transmitter from sympathetic nerves, acting on P_{2X}-purinoceptor on the smooth muscle cells (Sneddon *et al.*, 1982; 1984; Sneddon & Westfall, 1984; Sneddon & Burnstock, 1984; Stjarne & Åstrand, 1987; Allcorn *et al.*, 1987). I have now investigated the effect of suramin on e.j.ps to confirm the purinergic origin of the junction potentials and gain more information on the mode of action of suramin at the electrophysiological level.

Correspondence line

Methods

Albino male guinea-pigs (250–300 g) were killed by stunning and exanguination. The vas deferens was removed and cleaned of connective tissue. Intracellular recordings were made with glass microelectrodes of 20–40 MΩ resistance. The signal was recorded on a storage oscilloscope (Tektronix) and tape recorder (Racal) via a preamplifier (Cell Explorer 800, Dagan). Recordings were made only from cells with stable resting membrane potentials greater than −60 mV. E.j.ps were evoked at a frequency of 0.5 Hz with 0.5 ms pulse width at a voltage below that required to initiate an action potential. All quantitative data regarding measurement of resting membrane potential and e.j.ps are based on comparisons from the same cell, before and after introduction of the drug. In each case the mean of at least five fully facilitated e.j.ps is used for calculation of e.j.p. magnitude.

Statistics

Values in the text refer to mean ± s.e.mean. Statistical comparison of the results was tested by Student's *t* test for paired or unpaired data, as appropriate. Differences were considered significant when $P < 0.05$.

Drugs

Suramin (Bayer, UK) was dissolved in distilled water and kept frozen as a stock solution at 10^{-1} M, before dilution into the physiological salt solution. Other drugs, phentolamine hydrochloride and prazosin hydrochloride were obtained from Sigma Chemical Company and Pfizer UK respectively.

Results

Effect of suramin on membrane potential and e.j.ps

In the guinea-pig isolated vas deferens the control resting membrane potential of the smooth muscle cells was 67.4 ± 0.7 mV ($n = 48$). Field stimulation at 0.5 Hz produced excitatory junction potentials which grew steadily in magnitude due to the process of facilitation. In control cells the mean value of the fully facilitated e.j.ps was 8.5 ± 0.8 mV ($n = 23$). Stimulation at 1 or 2 Hz produced a greater degree of facilitation, so that e.j.ps reached the threshold for firing action potentials, which in most cells was estimated to be about − 55 mV. It was difficult to make an exact estimate of the threshold potential in a cell since a propagated action potential may be conducted from another cell, and penetration of the cell by the microelectrode was often lost due to the resultant contraction of the smooth muscle. Therefore, all control experiments were performed using stimulation at 0.5 Hz, and all drug effects were examined on the fully facilitated e.j.ps.

Suramin produced a dose-dependent reduction in e.j.p. magnitude. No significant reduction was seen in the presence of 1 μM suramin, whilst 10 μM suramin produced a profound reduction in e.j.p. size. Only a slight further reduction was observed on subsequent addition of 100 μM suramin (Figure 1). Concentrations beyond 100 μM were not investigated. The washout of the effect of suramin was extremely slow. After over 1 h washout of 20 μM suramin, e.j.p. magnitude had still not fully recovered to the original control level.

Introduction of suramin (1–100 μM) did not change the resting membrane potential of the smooth muscle cells, even during prolonged exposures of 30 to 60 min. Figure 2 shows the effect of suramin 20 μM for 30 min on resting membrane potential and e.j.p. magnitude. Notice that there is a steady decline in e.j.p. size over the period shown, but no change in resting membrane potential. In several such experiments using 20 μM suramin, e.j.ps were significantly reduced to a mean magnitude of 1.4 ± 0.3 mV ($n = 18$), whilst the resting membrane potential of the cells was not significantly different from the control value (68.2 ± 0.9 mV, $n = 18$).

After 30 min in 20 μM suramin, e.j.ps in all cells were reduced to very low levels. Under these conditions trains of stimuli at higher frequencies could be applied before threshold for an action potential was reached. Figure 3 shows an example of a cell from a tissue treated with suramin in which e.j.ps were evoked at 2–8 Hz. Notice that at 2, 4 and 6 Hz, individual e.j.ps summate, to produce a maintained level of depolarization, the magnitude of which depends on the frequency of stimulation. At 8 Hz depolarization reduced the resting membrane potential from − 71 mV to − 56 mV, which evoked an action potential in the muscle. This result suggests that suramin does not prevent action potential generation in the smooth muscle.

Effect of α-adrenoceptor antagonists on e.j.ps after suramin

Once e.j.p. magnitude had been greatly reduced by suramin, it was possible to determine whether noradrenaline contributed to the summated e.j.ps observed at higher stimulation frequencies by investigating the effect of subsequent addition of α-adrenoceptor antagonists such as prazosin or phentolamine.

The magnitude of the maintained depolarization produced by stimulation at 4 Hz in the presence of suramin was 4.2 ± 0.4 mV ($n = 5$). After prazosin, it was 5.2 ± 0.6 mV ($n = 5$), which was not a statistically significant change. Similar results were observed with phentolamine, which slightly increased the fully summated e.j.p. magnitude at 6 Hz from 5.5 ± 0.4 mV to 6.4 ± 0.6 ($n = 4$), although this increase was not statistically significant.

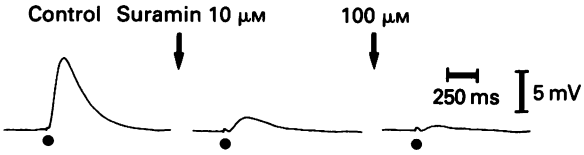

Figure 1 The effect of suramin on the magnitude of fully facilitated e.j.ps at 0.5 Hz in the guinea-pig vas deferens. The control e.j.p. in this cell was about 8 mV. After 30 min in 10 μM suramin the e.j.p. magnitude was reduced to about 2 mV. Subsequent introduction of suramin 100 μM further reduced e.j.p. magnitude to less than 1.0 mV. Each stimulus (●) produced a small stimulus artifact which precedes each e.j.p. on the trace. The resting membrane potential of the cell was − 68 mV.

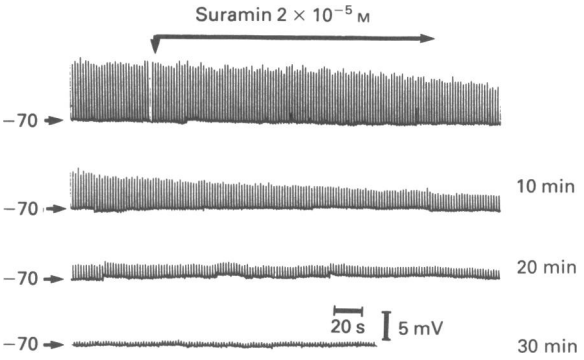

Figure 2 The effect of suramin on membrane potential and e.j.ps in guinea-pig vas deferens. Notice that over the 30 min period shown, the resting membrane potential of the cell was constant, at − 70 mV. The magnitude of e.j.ps, however, gradually declined due to the effect of suramin. In this experiment e.j.ps were evoked at 0.5 Hz throughout the recording period.

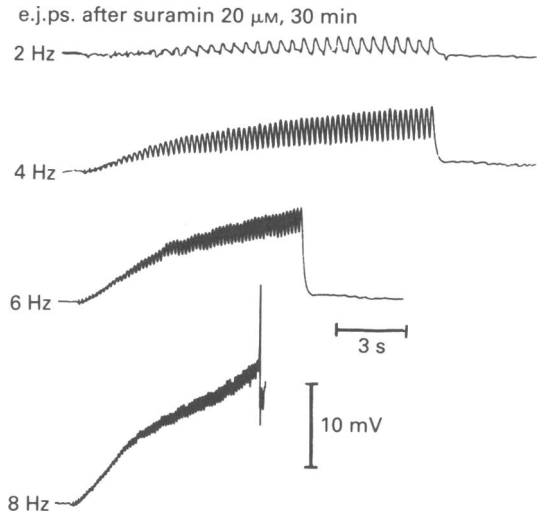

Figure 3 After treatment with suramin, nerve stimulation at 2, 4 and 6 Hz produced summation of e.j.ps to subthreshold levels of maintained depolarization. Stimulation at 8 Hz reduced the membrane potential from its resting value of − 71 mV to − 56 mV, which initiated an action potential. Immediately after the action potential the electrode was expelled from the cells, presumably due to contraction of the muscle.

Discussion and conclusions

There is now considerable evidence which supports the proposal that sympathetic nerves innervating many smooth muscles release ATP and noradrenaline to act as co-transmitters (for recent review see von Kugelgen & Starke, 1991). In the vas deferens of various species, including rat, rabbit, guinea-pig and mouse, the contractile response to sympathetic nerve stimulation is biphasic. The first phase is thought to be mediated by ATP acting on P_{2X}-purinoceptors, and the second component by noradrenaline acting on α_1-adrenoceptors. The main evidence supporting the role of ATP as a co-transmitter in vas deferens comes from experiments using either arylazidoaminopropionyl-ATP (ANAPP$_3$) to block the P_{2X}-purinoceptors (Fedan *et al.*, 1981; Sneddon *et al.*, 1982; 1984; Sneddon & Westfall, 1984) or from experiments using the stable analogue of ATP, α,β, methylene-ATP to produce desensitization of the P_{2X}-purinoceptor (Meldrum & Burnstock, 1983; Allcorn *et al.*, 1987). Both these procedures were found to reduce the initial, phasic components of the contraction, but not the secondary, maintained phase, which is blocked by α_1-adrenoceptor antagonists. It has also been demonstrated that both ANAPP$_3$ and α,β,methylene-ATP (but not α-adrenoceptor antagonists) inhibit e.j.ps in guinea-pig vas deferens (Sneddon *et al.*, 1982; Sneddon & Westfall, 1984; Sneddon & Burnstock, 1984; Stjarne & Åstrand, 1984; Allcorn *et al.*, 1987), indicating that e.j.ps are mediated by ATP acting on P_{2X}-purinoceptors. The present study demonstrates that suramin is also able to block e.j.ps in vas deferens, and therefore supports the view that e.j.ps are mediated by ATP in this tissue.

The finding that α-adrenoceptor antagonists did not reduce e.j.p. magnitude, even at stimulation frequencies of up to 8 Hz, confirms that noradrenaline does not appear to contribute to e.j.ps in this preparation. It is however also possible that the postjunctional blocking action of the antagonists is obscured by prejunctional α_2-adrenoceptor mediated enhancement of transmitter release.

It was also found that addition of suramin did not alter the resting membrane potential of the smooth muscle cells, even during prolonged exposures which reduced e.j.ps to very low levels. In guinea-pig vas deferens E_{Na}, E_{Ca} and E_{Cl} are much more positive than E_m, whereas E_K is much more negative. If suramin had a blocking effect on any one of these ion channels then a change in the resting membrane potential would be expected. The fact that no change was seen in resting potential supports the view that suramin acts as a receptor antagonist, and does not reduce e.j.ps by a non-selective effect on the electrical properties of the cells.

These studies on the effect of suramin on e.j.ps seem to correlate well with previous studies examining the effect of suramin on mechanical responses of the vas deferens of various species. For example, in mouse vas deferens, which gives a biphasic contraction to a single stimulus of the sympathetic nerves, von Kugelgen *et al.* (1989) reported that suramin (300 μM) selectively inhibited the initial, purinergic phase of the contraction, with little effect on the secondary adrenergic component, which was abolished by prazosin (0.1 μM). A similar effect of suramin has also been demonstrated in the rat vas deferens (Mallard *et al.*, 1989).

In conclusion, suramin is able to inhibit e.j.ps in the guinea-pig vas deferens, which confirms the suggestion that ATP and not noradrenaline mediates these excitatory electrical responses. The results on both electrical and mechanical responses of the vas deferens support the view that suramin can be used as an antagonist of ATP-mediated responses.

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Alteration of the L-type calcium current in guinea-pig single ventricular myocytes by heptaminol hydrochloride

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1 The effects of heptaminol on calcium current amplitude and characteristics were studied in single ventricular myocytes of guinea-pig by use of the whole cell configuration of the patch clamp technique.

2 A concentration-dependent decrease in I_{Ca} amplitude was observed. At heptaminol concentration as low as 10^{-6} M, this effect was observed in only two cells ($n = 6$). At 10^{-5} M the reduction of I_{Ca} was of $30 \pm 15\%$ ($n = 11$).

3 The current recovery from inactivation at -40 mV holding potential (HP) seemed less sensitive to perfusion with heptaminol ($> 10^{-6}$ M). However, at -80 mV HP the overshoot of the recovery curve was decreased by heptaminol.

4 Both at -40 mV and -80 mV HP, heptaminol (10^{-5} M) significantly increased the steady state inactivation of I_{Ca}.

5 As previously proposed by others to explain the effects of membrane active substances, the effects of heptaminol may result from alterations in cell membrane properties and possibly from an increase in intracellular free calcium ion concentration.

Keywords: Heart; calcium current; patch clamp; heptaminol; membrane active substances; ventricular myocytes

Introduction

Since the early 1950's (Loubatieres, 1951), heptaminol (6-amino 2-methyl heptanol) has been described as a cardiotonic molecule, mainly in cardiac ischaemic preparations and in drug-induced hypotension. Although some indications have been given about the mechanisms underlying these effects (Coraboeuf & Boistel, 1953; Garrett *et al.*, 1962; Berthiau *et al.*, 1989; see also Allard *et al.*, 1991 on skeletal muscle), the action of heptaminol on ionic currents remains less understood. Due to the structure of its molecule, heptaminol is related both to alcohol and amine hydrocarbon derivatives. The effects of hydrocarbons including n-alkanols and amines are widely studied in excitable cells but there is no common view on the mechanisms of action of these molecules. Changes in ionic channels activity (Haydon & Urban, 1983a,b; Mongo & Vassort, 1990), in ionic cotransport (Michaelis & Michaelis, 1983; Philipson, 1984; Haworth *et al.*, 1989), in enzymatic activities (Ohnishi *et al.*, 1984; Chatelain *et al.*, 1986; Swann, 1990), in $[Ca^{2+}]_i$ (Vassort *et al.*, 1986; Daniell & Harris, 1988; Davidson *et al.*, 1990) and in $[H^+]_i$ (Vassort *et al.*, 1986) have been shown to occur in various tissues as a consequence of the interaction of these hydrocarbons with biological membranes. In cardiac muscle, general and local anaesthetics (amines, straight chain alcohols and inhalation compounds) are known to alter most of ionic currents including Ca current (I_{Ca}) which is decreased (Ikemoto *et al.*, 1985; Hiroto *et al.*, 1988; Terrar & Victory, 1988; Mongo & Vassort, 1990). The myocardial activity was also shown to be depressed by these compounds (see review by Rusy & Komai, 1987). However, their underlying mechanisms of action are not completely established.

The aim of the present work was to study the effects of heptaminol on the main characteristics of the voltage-dependent L-type calcium current in single ventricular cells of guinea-pig heart. Maximum calcium current was reduced as a function of the applied heptaminol concentration. The steady state inactivation of I_{Ca} was slightly accentuated, while, the current recovery from inactivation seemed unchanged at the drug concentrations studied. Some mechanisms underlying these observations are discussed.

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Methods

The method used to obtain single myocytes from guinea-pig ventricles has been previously described (Le Guennec *et al.*, 1990). Briefly, guinea-pigs (250–350 g) were killed by cervical dislocation, the heart was quickly removed and a Langendorff retrograde perfusion of the isolated heart was performed. All solutions were derived from Tyrode solution of the following composition (in mM): NaCl 140, KCl 5.4, $CaCl_2$ 2, $MgCl_2$ 1, glucose 11.1, HEPES 10 and NaH_2PO_4 0.33, pH 7.4 adjusted with NaOH. The heart was successively perfused with free calcium Tyrode solution for 5 to 6 min and free calcium Tyrode solution with added collagenase 30 mg 50 ml^{-1} (Boehringer) and protease type XIV 7 mg 50 ml^{-1} (Sigma) for 40 to 50 min. Then the heart was washed with the low calcium (0.2 mM) and normal Tyrode solution. These solutions were kept at 37°C and perfused at a rate flow of 10 ml min^{-1}. After perfusion was completed the ventricles were placed in a Petri dish containing about 10 ml of Tyrode solution at room temperature (22–26°C). Small pieces of tissue were removed from either right or left ventricle and kept up to 7 h in Petri dishes containing Tyrode solution. Single cells were then obtained by gentle agitation of these pieces of ventricle. I_{Ca} was recorded by the whole cell patch-clamp technique, using an extracellular patch system (Biologic RK 300, France). Potassium currents were eliminated by bathing cells in a K-free Tyrode solution with substitution of 20 mM $CsCl_2$. To minimize sodium current, cells were maintained at a holding potential of -40 mV. In some experiments, Na^+ ions were substituted by an equivalent concentration of TEA^+ in order to suppress I_{Na} completely. Heptaminol (a gift from Delalande) was dissolved directly in the control solution to obtain the desired concentration. All the solutions were applied extracellularly by means of microcapillaries (Tygon microbore tubing, U.S.A.). Patch electrodes (1–5 MΩ) were prepared from glass micropipettes (Drummond, U.S.A., 100 µl microcaps) and were filled with a solution containing (mM): CsCl 130, HEPES 10, disodium EGTA 10, ATP-Mg 5 and disodium phosphocreatine 5 with the pH adjusted to 7.1 with CsOH.

After breaking the membrane patch under the suction pipette tip, the cell was depolarized for 300 ms from

− 40 mV to OmV every 8 s to elicit maximum I_{Ca}. The current amplitude was estimated as the difference between the peak inward current and the current at the end of the pulse. Current records were low-pass filtered through a 3 kHz 5-pole Tchebicheff and sampled at 5 kHz by an analogic to digital converter (Tecmar Labmaster, U.S.A.).

Stimulation protocols, data acquisition and analysis were performed by use of pClamp (Axon Instruments) software by a 386 IBM compatible computer.

The statistical analyses were performed with Wilcoxon matched pairs test. Some results are expressed as mean ± s.d.

Results

Effects of heptaminol on peak I_{Ca}

The data in Figure 1 illustrate the time course of peak I_{Ca} before, during, and after application of 10^{-5} M heptaminol to the cell. With brief periods of perfusion, the effect of 10^{-5} M heptaminol was reversible (as can be seen in Figure 1), if one takes into account the normal run down of I_{Ca} that generally occurs over such a period of recording (Belles *et al.*, 1988). The upper part of Figure 1 shows the original records of I_{Ca} before, during and after perfusion with the drug. This is a typical experiment out of 11 cells from different hearts in which this concentration of heptaminol induced a mean decrease in I_{Ca} amplitude of 30 ± 15%. A dose-response study of the effects of heptaminol on I_{Ca} amplitude was undertaken and Figure 2 summarizes the results of such experiments. Although the I_{Ca} amplitude was mainly reduced as a function of heptaminol concentration, variable observations were made with heptaminol concentration as low as 10^{-6} M. At this concentration, the current was increased by 25% ($n = 2$), decreased by 31% ($n = 2$) or unchanged ($n = 2$). In most cells, exposure to 10^{-4} M heptaminol rapidly led to contracture and cell death; thus we were able to achieve a steady state effect at this concentration in only one cell in which the I_{Ca} amplitude was reduced by approximately 90% of its control value.

Effects of heptaminol on I_{Ca}*-voltage relationship and steady state inactivation*

Changes in I_{Ca} characteristics (current-voltage relationship, steady-state inactivation and recovery from inactivation) in the presence of heptaminal were analysed at 10^{-5} M. Figure 3a shows the same reduction of I_{Ca} amplitude during perfusion with heptaminol for all membrane depolarizations that elicited a significant current. No modification in the shape of the current-voltage relationship curve nor any shift of this curve in respect to the membrane potenials could be observed. These observations suggest that the effects of heptaminol show little if any voltage-dependence (Murrell *et al.*, 1991 and see also Figure 3b of the present study).

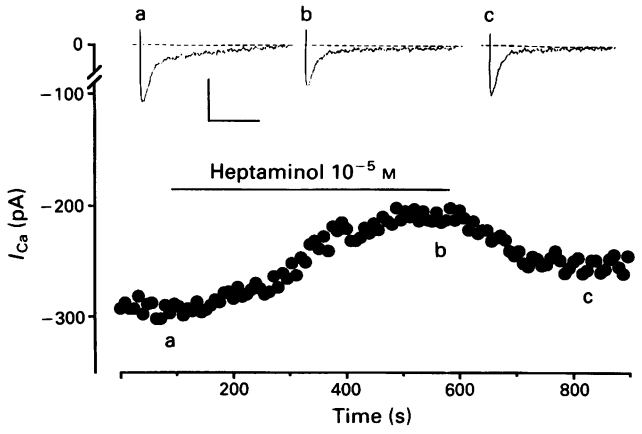

Figure 1 Time course of reduction of peak I_{Ca} by 10^{-5} M heptaminol. The current amplitude was estimated as described in Methods and was plotted as a function of time (before, during and after heptaminol perfusion). The upper part shows original records of I_{Ca} under control conditions (a), in the presence of drug (b) and during drug washout (c). Letters in the time course graph correspond to records shown in the upper part. Horizontal bar = 100 ms; vertical bar = 200 pA.

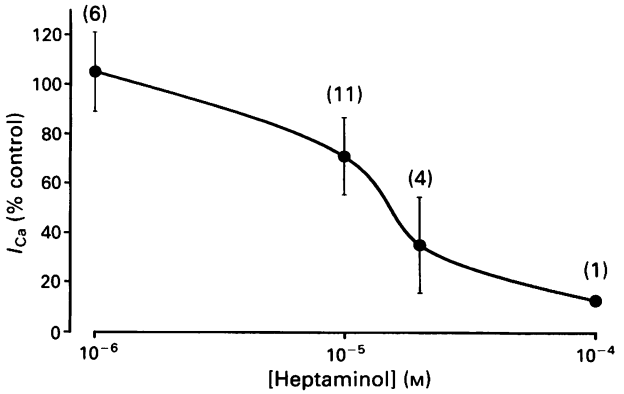

Figure 2 Dose-response curve of the effects of heptaminol on I_{Ca} amplitude. The concentrations were 10^{-6} M, 10^{-5} M, 2×10^{-5} M, 10^{-4} M. Except at 10^{-6} M (as mentioned in the text), heptaminol induced a decrease in I_{Ca} amplitude. Vertical bars indicate the standard deviation and the number of cells is given in parentheses.

In Figure 3b, the I_{Ca} steady-state inactivation was studied with double pulse protocol (see legend) and was plotted as a function of prepulse potential. One can see that the U-shape of the I_{Ca} availability curve was not changed by 10^{-5} M heptaminol nor was the degree of inactivation for membrane potentials up to + 20 mV. At more positive membrane potentials ($\geqslant$ + 30 mV), current availability was significantly reduced ($P < 0.02$, $n = 8$) suggesting an accentuation of the steady-state inactivation of I_{Ca}. A marked steady-state inactivation in the right part of the I_{Ca} availability curve can result from an increase in Ca-dependent component of I_{Ca} inactivation (Mentrard *et al.*, 1984). The membrane potential for half current inactivation was quite the same in the absence and the presence of 10^{-5} M heptaminol and ranged between − 25 and − 20 mV. This again supports the lack of voltage-dependence of the effects of heptaminol on I_{Ca} characteristics. In the presence of 10^{-6} M heptaminol, I_{Ca} availability for strong positive depolarizations was increased (steady-state inactivation was less marked) when the peak current increased and was reduced when the peak current decreased (not shown).

In another series of experiments, cells were bathed in a standard sodium-free solution, with an equivalent amount of tetraethylammonium (TEA) ions substituting for sodium ones. Under these conditions, the membrane holding potential was set at − 80 mV and cells were depolarized for 300 ms to 0 mV. Although the elicited I_{Ca} was larger under these conditions, its steady state inactivation was similar to that observed at − 40 mV HP (see also Schouten & Morad, 1989). Heptaminol (10^{-5} M) had similar effects on the current-voltage relationship and on its availability (not illustrated) as those observed at − 40 mV HP and shown in Figure 3a,b.

Effects of heptaminol on I_{Ca} *recovery from inactivation*

Another characteristic of I_{Ca} which may depend on cell membrane potential and free intracellular calcium ions concentration is its recovery from inactivation. As described by others (Argibay *et al.*, 1988; Tseng, 1988; but see also Schouten & Morad, 1989) and shown in Figure 4, I_{Ca} recovery kinetics depended on the membrane holding potential. They were more rapid as the HP was more negative (half

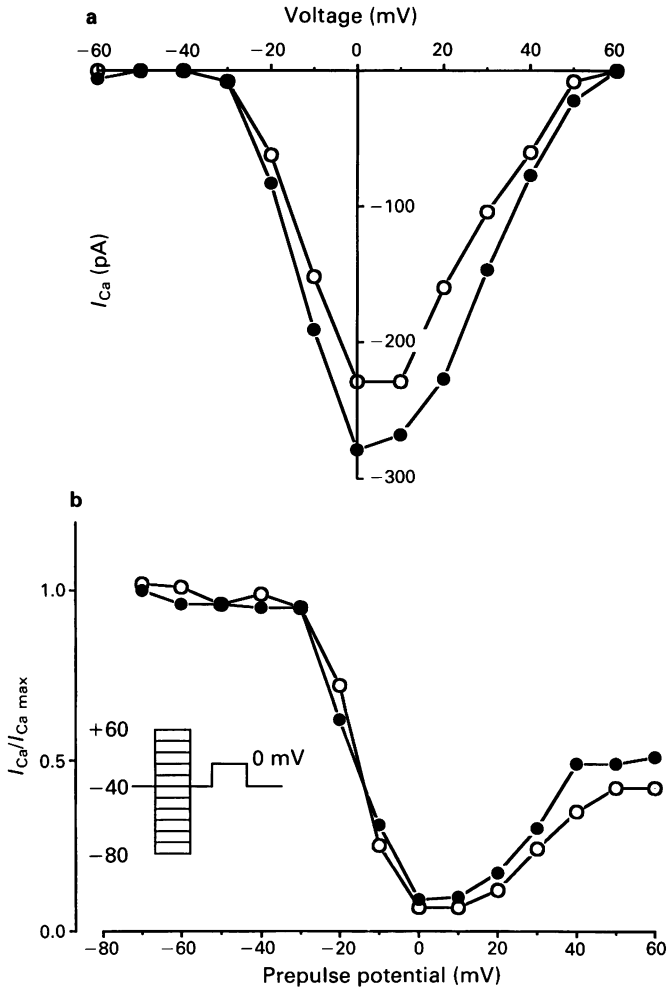

Figure 3 Effects of 10^{-5} M heptaminol on I_{Ca} current-voltage relationship and on its steady-state inactivation; (●: control; ○: heptaminol). (a) The reduction of peak I_{Ca} for all membrane potentials during perfusion with heptaminol. There is no shift of the curve in respect to membrane potentials. The curves were obtained by plotting the current elicited by a prepulse to the voltage of the corresponding prepulse. The protocol is shown as an inset in (b). (b) Increase in steady state inactivation of I_{Ca} under perfusion with the drug. The inset shows the protocol used to study the current-voltage relationship and the steady-state inactivation. A 300 ms prepulse from − 40 mV (HP) to variable amplitudes (from − 80 mV to 60 mV) was followed by a 300 ms test pulse to 0 mV. The two pulses were separated by 5 ms return to − 40 mV. The inactivation curve is a plot of the ratio of I_{Ca} obtained by the test pulse for a given prepulse to that elicited by the test pulse in the absence of the prepulse to the voltage of the corresponding prepulse. This is a typical experiment out of 8 in which a significant increase in steady-state current inactivation was observed ($P < 0.02$).

time of recovery ≈150 ms at − 80 mV and 500 ms at − 40 mV). The addition of heptaminol (10^{-5} M) to the perfusing solution had no significant effect on the I_{Ca} recovery when the HP was set at − 40 mV (Figure 4a). The half time of recovery was quite similar in the presence and the absence of heptaminol (≈500 ms). At more negative values of HP the rapid recovery of I_{Ca} is usually associated with the well known phenomenon of 'overshoot or I_{Ca} facilitation' (see Argibay *et al.*, 1988; Tseng, 1988; Mongo & Vassort, 1990). This is illustrated in Figure 4b, were the recovery curves obtained in a cell under control conditions and in the presence of 10^{-5} M heptaminol are shown. It can be seen that heptaminol significantly reduced this overshoot. Similar results were obtained in 5 other cells. Figure 4c is an expanded graph of the first 500 ms of Figure 4b normalized to maximum value and it shows that, despite the marked decrease of the overshoot, there was no change in the half time of current recovery in the presence of heptaminol 10^{-5} M, even though the membrane potential was held at − 80 mV (half time of recovery ≈80 ms). This lack of effect of heptaminol perfusion on I_{Ca} recovery was confirmed by the exponential analysis of the recovery kinetics. When the holding potential was set at − 40 mV, the mean recovery curves ($n = 6$, Figure 4a) (the complete process) were well fitted by the sum of two exponentials either under control or under heptaminol perfusion conditions. For these two curves the calculated time constants for the two exponentials were 192.5 ms and 1426 ms for control and 201.5 ms and 1650.8 ms during heptaminol perfusion. Since the recovery curve showed an overshoot at − 80 mV HP, the analysis was restricted only to the first 500 ms of the curve. This part was fitted by a single exponential under control and heptaminol perfusion condi-

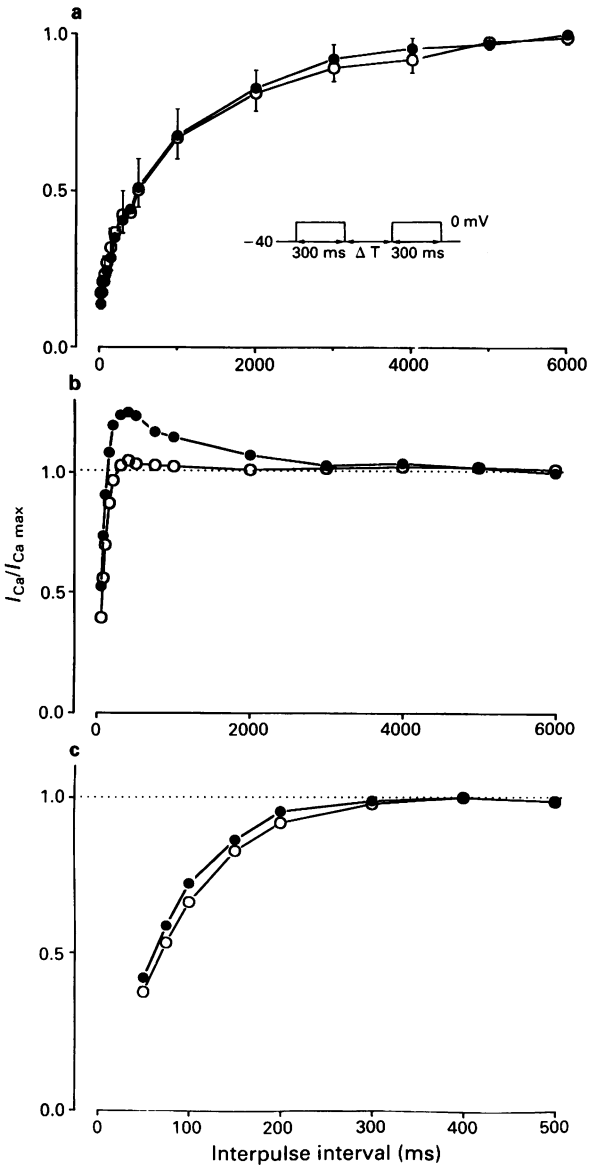

Figure 4 Heptaminol (10^{-5} M) application and I_{Ca} recovery from inactivation (●: control; ○: heptaminol). A double pulse protocol was also used for this study (see inset). Two pulses of 300 ms from either − 40 mV or − 80 mV HP to 0 mV were separated by a variable interpulse interval (ΔT = 25 to 6000 ms). The ratio of I_{Ca} obtained by the test pulse to that elicited by the prepulse is plotted as a function of the interpulse interval. (a) Mean recovery curves ($n = 6$) obtained at the holding potential of − 40 mV. (b) Recovery curves obtained in one cell at the holding potential of − 80 mV. Similar results were obtained in 5 other cells. (c) An expanded graph of the first 500 ms of (b) normalized to the maximum value. Despite the marked decrease in the overshoot, the kinetics of recovery were not significantly modified by heptaminol.

tions and the time constants were 80.6 ms and 89.2 ms respectively. The little difference between these two values show that this effect was not significant.

Discussion

The present study shows that heptaminol is a membrane active agent which mainly reduced the calcium current amplitude and altered its characteristics. However, at heptaminol concentrations as low as 10^{-6} M, variable results (increase, decrease or no change in current amplitude) were obtained. This may be due to a lack of effect of the drug at this concentration. On the other hand, such concentration-dependent effects has also been reported in single ventricular myocytes of frog and guinea-pig for other membrane active compounds (L-palmitoylcarnitine by Meszaros & Pappano, 1990 and ethanol by Mongo & Vassort, 1990).

Although, the molecular basis of the action of membrane active substances is not well understood, it is generally admitted that two pathways, one hydrophobic the other hydrophilic, are used by these compounds to reach their site of action and this depends on the fact that molecules are or not amphiphile. The hydrophobic pathway can use the hydrophobic pockets on sensitive proteins as targets (Richards *et al.*, 1978; Franks & Lieb, 1987; Tas *et al.*, 1990; Murrell *et al.*, 1991) and/or lipophilic regions of the membrane (Seeman, 1972; Haydon & Urbam 1983a,b). Whatever the pathway followed, these agents are known to disturb the membrane bilayers lipid and protein composition and/or conformation as a result of an interaction with membrane lipids and/or membrane proteins (see Tas *et al.*, 1990; Murrell *et al.*, 1991). The effects of heptaminol on I_{Ca} amplitude described in this work are qualitatively similar to those reported by others for general and local anaesthetics in various tissues including cardiac muscle cells (Ikemoto *et al.*, 1985; Hirota *et al.*, 1988; Sanchez-Chapula, 1988; Terrar & Victory, 1988; Murrell *et al.*, 1991). As discussed by Mongo & Vassort (1990) for the effects of straight chain alcohols and halogenated compounds, the decrease in peak I_{Ca} by heptaminol may result from changes in the membrane calcium channel activity as a consequence of: (1) an interaction of heptaminol molecule with membrane lipids and/or proteins, and (2) its alteration of intracellular free calcium ion concentration. An increase in $[Ca^{2+}]_i$ has been observed in various cell types during perfusion with membrane active substances (Daniel & Harris, 1988; Davidson *et al.*, 1990). This increase in $[Ca^{2+}]_i$ may result from: (1) a release of membrane bound Ca^{2+} (Vassort *et al.*, 1986); (2) an inhibition of Na^+-Ca^{2+} exchange mechanism (Michaelis & Michaelis, 1983; Philipson, 1984; Haworth *et al.*, 1989) and of sarcolemmal and intracellular membrane Ca-ATPases (Salama & Scarpa, 1983; Ohnishi *et al.*, 1984). In order to explain their effects on I_{Ca} characteristics, Mongo & Vassort (1990) have proposed the participation of an increase in $[Ca^{2+}]_i$ as a mechanism of action of n-alkanols and halogenated compounds in addition to those previously described (see Introduction and also Meszaros & Pappano, 1990).

Since heptaminol, like other membrane-active compounds may disturb cell membrane properties (see above), a possible explanation of the alterations found in I_{Ca} characteristics is a direct effect of this drug on the properties of the L-type calcium channel.

An alternative explanation of the effects could be an increase in $[Ca^{2+}]_i$ (Mentrard *et al.*, 1984; Argibay *et al.*, 1988; Tseng, 1988) during perfusion with heptaminol. This increase in $[Ca^{2+}]_i$ can, at least in part, explain the decrease in current amplitude. The decrease in I_{Ca} availability is consistent with a marked Ca-dependent component of I_{Ca} current inactivation (Mentrard *et al.*, 1984). This reduction, although significant ($P < 0.02$, $n = 8$), appeared to be not very pronounced; this may be due to the use of EGTA (10 mM) in the patch pipette to buffer intracellular Ca^{2+} to avoid cell contraction. However, it has been shown that with EGTA in the patch pipette, Ca^{2+} ions are not completely buffered and addition of BAPTA slowed the I_{Ca} inactivation more than did EGTA alone (Argibay *et al.*, 1988; Lipp & Pott, 1991). Thus, one should not exclude a rise in the intracellular Ca^{2+} ion concentration just at the level of the internal face of the membrane or in the vicinity of the internal channel mouth. As $[Ca^{2+}]_i$ increases, I_{Ca} inactivation becomes more marked and its amplitude decreases.

The recovery of the current from inactivation is a complex process modulated by a number of factors including membrane potential and intracellular free Ca^{2+} ion concentration. Our observations confirm the dependence of I_{Ca} recovery on the membrane holding potential (Schouten & Morard, 1989). Since the effects of hepatminol are not voltage-dependent, the HP dependence of I_{Ca} recovery was not modified by heptaminol application. The control of I_{Ca} recovery by $[Ca^{2+}]_i$ is less clear. Both potentiation and inhibition of the current by $[Ca^{2+}]_i$ have been reported and this may be related to the presence or not of the overshoot in the recovery curve (for more details, see Argibay *et al.*, 1988; Tseng, 1988). Some schemes have been proposed to explain the cellular mechanism of the overshoot but they are as yet poorly established. As suggested by others and reported by Tseng (1988), if we assume that intracellular calcium exerts dual actions of the L-type calcium channels activity: enhancement at moderately or transiently elevated levels and inhibition at higher or sustained elevated levels, it seems likely that a sustained increase in intracellular free calcium ions is, at least in part, responsible for the suppression of the current recovery overshoot during perfusion with heptaminol. Intracellular Ca^{2+} concentration increase may also accentuate I_{Ca} 'rundown' (Belles *et al.*, 1988); this could, in part, explain the diminished reversibility of the heptaminol effect during washout.

Other mechanisms have been proposed to explain the cardiotonic effects of heptaminol: (1) the cardiovascular actions of heptanolamines were attributed to an indirect sympathomimetic action due to release of endogenous catecholamines (Garrett *et al.*, 1962). This cannot explain our observations, since we are concerned with single isolated cells and the results described so far show mainly a reduction of peak I_{Ca}. (2) Berthiau *et al.* (1989), in the moderately ischaemic rat isolated heart, have suggested a contribution of an intracellular realkalinization through the Na^+-H^+ exchange process. Recently, the stimulation of the Na^+-H^+ antiport by heptaminol has been evoked to explain its ability to stop the decline in tension in frog isolated twitch muscle fibre (Allard *et al.*, 1991). An alkalinization has also been reported and shown to be concomitant with $[Ca^{2+}]_i$ increase in squid giant axon during perfusion with n-alkanols and amines (Vassort *et al.*, 1986). In any case, such an alkalinization could not account for the observed current reduction during heptaminol perfusion; however, such an effect could be evoked to explain I_{Ca} potentiation, since lowering internal H^+ increases I_{Ca} (Kurachi, 1982). Even if the internal H^+ were buffered by addition of 10 mM HEPES in the patch pipette, any change in these ion concentrations could not be excluded or confirmed, since no measure of this concentration was undertaken in this study.

Other possible explanations of peak I_{Ca} increase appearing during perfusion with low heptaminol concentrations (10^{-6} M) may arise from: (1) an effect of heptaminol on the interaction between adenylate cyclase and the guanyl nucleotide regulatory protein as shown by Chatelain *et al.* (1986) for n-alkanols and leading to an increased adenosine 3':5'-cyclic monophosphate (cyclic AMP) production; (2) a slight $[Ca^{2+}]_i$ increase-induced potentiation of I_{Ca} (Tseng, 1988; Gurney *et al.*, 1989) and (3) membrane effects like those proposed for L-type calcium channels agonists and antagonists (Hess *et al.*, 1984; McDonald *et al.*, 1989).

In conclusion, the reduction of calcium current amplitude and the changes in its characteristics induced by heptaminol may result from: (1) an alteration of membrane-related func-

tions (ionic channels and enzymatic activities) as a consequence of the interaction of the drug either with membrane lipids, or proteins or with both; (2) an increase in intracellular free calcium ions which are are known to regulate calcium channels activity. An increase in $[Ca^{2+}]_i$ may also contribute to the described cardiotonic properties of the heptaminol molecule.

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Vasopressin-stimulated [^{3}H]-inositol phosphate and [^{3}H]-phosphatidylbutanol accumulation in A10 vascular smooth muscle cells

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1 The characteristics of vasopressin-stimulated phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P_2) and phosphatidylcholine (PtdCh) hydrolysis were examined in A10 vascular smooth muscle cells (VSMC), by assessing the formation of [^{3}H]-inositol phosphates ([^{3}H]-IP) and the accumulation of the phospholipase D (PLD) specific product, [^{3}H]-phosphatidylbutanol ([^{3}H]-PtdBuOH).

2 Vasopressin ([Arg^8]-VP) and a number of related analogues stimulated the accumulation of [^{3}H]-IP and [^{3}H]-PtdBuOH with similar EC_{50} values, generating the same rank order of potency for each response (Arg^8-VP = vasotocin = Lys^8-VP $\gg$ oxytocin).

3 Inhibition of vasopressin-stimulated [^{3}H]-IP and [^{3}H]-PtdBuOH accumulation by the V_{1a} receptor antagonists, Des-Gly^9[β-mercapto-β,β,-cyclopentamethylene propionyl, O-Et-Tyr^2,Val^4,Arg^8]-vasopressin generated similar IC_{50} values suggesting that both these responses are mediated through the activation of a single V_{1a} receptor subtype.

4 The onset of vasopressin-stimulated inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3) mass formation preceeded [^{3}H]-PtdBuOH accumulation indicating that PtdCh hydrolysis was activated subsequent to PtdIns(4,5)P_2 breakdown.

5 The protein kinase C (PKC) activator, tetradecanoylphorbol acetate (TPA) also stimulated [^{3}H]-PtdBuOH accumulation. Preincubation with the PKC inhibitor Ro-31-8220 abolished both TPA- and vasopressin-stimulated [^{3}H]-PtdBuOH, suggesting that the intermediate activation of protein kinase C is involved in the regulation of PLD by vasopressin.

6 Pretreatment of the A10 VSMC with Ro-31-8220 (100 μM) also potentiated vasopressin-stimulated Ins(1,4,5)P_3 mass formation. Therefore stimulation of PKC may have opposing roles in the regulation of agonist activation of PLC and PLD.

7 Preincubation of the cells with EGTA, verapamil, or the receptor-operated calcium channel antagonist, SK&F 96365, reduced vasopressin-stimulated [^{3}H]-PtdBuOH accumulation by approximately 30%, suggesting that influx of calcium has a significant role to play in the regulation of vasopressin-stimulated PLD activity.

Keywords: Vasopressin; phosphatidylinositol hydrolysis; phosphatidylcholine hydrolysis; protein kinase C; phospholipase D

Introduction

A number of vasoconstrictors stimulate the phospholipase C (PLC) catalysed hydrolysis of phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P_2) to generate the two second messengers inositol -1,4,5-trisphosphate (Ins(1,4,5)P_3) and sn-1,2-diacylglycerol (DAG). These molecules are thought to be involved in different stages of the contractile process. Ins(1,4,5)P_3 stimulates the initial release of calcium from internal stores, whilst DAG activates protein kinase C (PKC) (Berridge & Irvine, 1989; Nishizuka, 1984). PKC is thought to play a role in the maintenance of contraction, through the phosphorylation of proteins such as caldesmon and myosin light chain when $[Ca^{2+}]_i$ has returned to near basal levels (Rasmussen *et al.*, 1987).

In smooth muscles cells, it has been established that, for a number of agonists such as endothelin-1, angiotensin II and vasopressin, PtdIns(4,5)P_2 hydrolysis is rapidly desensitized and $[Ca^{2+}]_i$ levels fall, whereas DAG levels remain elevated (Greindling *et al.*, 1986; Sunako *et al.*, 1990). Sustained DAG is thought to be derived from the hydrolysis of another phospholipid phosphatidylcholine (PtdCh) (see review by Billah & Anthes, 1990). Phosphatidylcholine hydrolysis has been demonstrated in a number of systems including vasopressin-stimulated hepatocytes (Augert *et al.*, 1989), gonadotrophin releasing hormone-stimulated ovarian granulosa cells (Liscovitch & Amsterdam, 1989), bombesin- and vasopressin-(Cook & Wakelam, 1991) and platelet derived growth factor-stimulated (Plevin *et al.*, 1991) Swiss 3T3 fibroblasts. However, in vascular smooth muscle cells little is known about the manner in which this process is regulated, in particular with regards the potential roles of PKC and extracellular calcium, both of which have been suggested to regulate PtdCh hydrolysis in other tissues (Billah *et al.*, 1989; MacNulty *et al.*, 1990). It has also been proposed that in some tissues agonist-stimulated PtdCh hydrolysis may be regulated by a specific G-protein or possibly through activation of different receptor subtypes (Boccking *et al.*, 1987). We therefore sought to examine the possible pathways by which vasopressin-stimulated PtdCh hydrolysis in vascular smooth muscle cells.

A preliminary account of some of these findings has been presented to the British Pharmacological Society (Plevin *et al.*, 1991).

Methods

A10 rat vascular smooth muscle cells (VSMC) were maintained in Dulbecco's modified Eagle's medium (DMEM) con-

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taining 15% foetal calf serum (FCS) at 37°C in a humidified atmosphere of air/CO_2 (19:1). For experiments cells were seeded in 24-well plates and labelled for 72 h with either [^{3}H]-inositol (Sp.Act. 20.0 kBq mmol^{-1}) in inositol free-DMEM containing 3% dialysed FCS, in DMEM containing 3% FCS with [^{3}H]-palmitic acid (20.7 kBq mmol^{-1}) or [^{3}H-methyl]-choline chloride (17.8 kBq mmol^{-1}). On the day of the experiments the labelling medium was removed and the cells were washed twice with 0.5 ml of Hank's buffered saline, pH 7.4, containing 1% (w/v) bovine serum albumin (fraction V) and 10 mM-glucose (HBG), and incubated for 30 min in this buffer. For inositol phosphate experiments the cells were incubated for 10 min with HBG containing 10 mM-LiCl (HBG/LiCl) and the stimulation was then carried out in a final volume of 0.25 ml for 30 min at 37°C. The reaction was terminated by the addition of 50 µl 10% (v/v) $HClO_4$. For the measurement of intracellular choline formation, the cells were incubated with agonist for the times indicated, the media aspirated and the reaction terminated by the addition of 0.5 ml ice cold methanol. For the assay of PLD-stimulated PtdBuOH accumulation, cells prelabelled with [^{3}H]-palmitic acid were preincubated with 30 mM butanol for 5 min before initiation of the reaction. The reaction was terminated by rapid aspiration followed by the addition of 0.5 ml of ice cold methanol.

For analysis of total labelled inositol phosphates, the cell extracts were scraped, washed with a further 100 µl of 10% (v/v) $HClO_4$ and then transferred to vials. The samples were neutralised with 1.5 M KOH/60 mM HEPES and the water-soluble inositol phosphates assayed by batch chromatography on Dowex-1 formate columns as previously described (Plevin *et al.*, 1990). For measurement of choline metabolites the cells were scraped, washed (0.25 ml methanol) and the extracts transferred to plastic vials. Chloroform (0.3 ml) was added and the samples allowed to extract at room temperature for 30 min. The phases were split by the addition of chloroform (0.5 ml) and water (0.5 ml) followed by centrifugation. An aliquot of the upper aqueous phase was analysed for glycerophosphocholine, choline phosphate and choline by cation exchange chromatography upon Dowex-50W-H^+ columns (Cook & Wakelam, 1989). For measurement of PtdBuOH formation the cell extracts were treated as above except glass vials were used throughout. The aqueous phase was removed and the lower phase dried down under vacuum. The sample was dissolved in chloroform/methanol 19:1 (v/v) and applied to Whatman LK5DF plates as described by Randall *et al.* (1990). The resolving solvent consisted of the upper phase of 2,2,4,-trimethylpentane/ethyl acetate/acetic acid/H_2O (50/110/20/100, v/v). The location of the peak was established by co-migration with a [^{3}H]-Ptd-butanol standard (R_F value 0.4). Preliminary experiments confirmed the dose-dependency of [^{3}H]-PtdBuOH formation upon butan-1-ol concentration (results not shown).

For Ins(1,4,5)P_3 mass measurements, unlabelled cells were grown to confluency on either 1 or 6 cm diameter dishes. Cells were incubated with agonist in a final volume of 300 µl for the times indicated and the reaction terminated by the addition of 25 µl 20% (v/v) $HClO_4$. The cells were treated as outlined above and the sample of the neutralised extract (200 µl) was assayed for Ins(1,4,5)P_3 by the method Palmer *et al.* (1989) using competitive displacement of [^{3}H]-Ins(1,4,5)P_3 binding to adrenal cortex microsomes quantified by a standard curve.

Dose-response curves were fitted to a logistic equation by an iterative fitting procedure (Delean *et al.*, 1980). Statistical analysis was performed using Student's *t* test. Results are given ± s.e.mean.

All radiolabelled compounds were obtained from Amersham International (Amersham, Bucks). All other compounds were of the highest grades commercially available. Ro-31-8220 and SK&F 96365 (1-{β-[3-(4-methoxyphenyl) propoxy]-4-methoxyphenethyl}-1H-imidazole hydrochloride) were gifts from Roche Products Ltd. and Dr J.E. Merritt, Smith Kline & Beecham Ltd respectively.

Results

Initial experiments showed that vasopressin-stimulated PtdCh hydrolysis in A10 smooth muscle cells, as measured by the formation of water soluble choline in cells prelabelled with [^{3}H]-choline. An approximate 2-3 fold increase in intracellular [^{3}H]-choline formation was obtained upon stimulation with 100 nM of the peptide ((d.p.m. ± s.e.mean) control = 385 ± 21; vasopressin = 1221 ± 57, $n = 5$). In contrast, there was no significant increase in choline phosphate formation (control = 10889 ± 1594 d.p.m.; vasopressin = 9549 ± 534 d.p.m., $n = 5$) suggesting that vasopressin stimulated PtdCh hydrolysis by a PLD-catalysed mechansim. This was confirmed by measuring vasopressin-stimulated formation of phosphatidylbutanol in cells preincubated with 30 mM butanol. A 6–8 fold increase in [^{3}H]-PtdBuOH accumulation was obtained over a 5 min incubation period (control = 1130 ± 73 d.p.m.; vasopressin = 8248 ± 999 d.p.m., $n = 5$). Vasopressin also stimulated the accumulation of [^{3}H]-IP in A10 VSMCC. Over a series of experiments vasopressin stimulated a 4–6 fold increase in [^{3}H]-IP accumulation over a 30 min period (control = 796 ± 141 d.p.m.; vasopressin = 3129 ± 543 d.p.m., $n = 6$). In order to generate sufficient radioactivity associated with [^{3}H]-IP and [^{3}H]-PtdBuOH respectively, times of 30 and 5 min were selected for pharmacological studies. The potential cellular toxicity of butanol also necessitated measuring [^{3}H]-PtdBuOH at relatively short time points. Ins(1,4,5)P_3 mass levels were measured in the absence of LiCl since in a number of high performance liquid chromatography (h.p.l.c.) studies inclusion of lithium resulted in a biphasic accumulation of [^{3}H]-Ins(1,4,5)P_3 (results not shown). Butanol was without effect upon the onset of vasopressin-stimulated Ins-(1,4,5)P_3 accumulation and was thus omitted from further experiments (results not shown).

The pharmacological characteristics of vasopressin ([Arg^8]-vasopressin)-stimulated PLC and PLD activity were examined. Vasopressin stimulated both [^{3}H]-IP and [^{3}H]-PtdBuOH formation in the low nanomolar range and the EC_{50} values for [^{3}H]-IP and [^{3}H]-PtdBuOH accumulation were similar for a number of vasopressin analogues (see Table 1). A rank order of potency of [Arg^8]-vasopressin = vasotocin = [Lys^8]-vasopressin >> oxytocin was obtained in each case. In addition to being less potent than other analogues, oxytocin was found to be a partial agonist, only eliciting 60–70% of the maximum vasopressin response. The receptor specificity was further examined by assessing the effect of the V_{1a} antagonist on vasopressin-stimulated [^{3}H]-IP and [^{3}H]-PtdBuOH accumulation (Figure 1). Similar IC_{50} values were obtained for the inhibition of both responses ([^{3}H]-IP = 37.2 ± 12 nM; [^{3}H]-PtdBuOH = 20.6 ± 9 nM, $n = 3$). These results indicated that both PLC and PLD are controlled through activation of a single V_{1a} subtype.

The kinetics of vasopressin-stimulated Ins(1,4,5)P_3 mass [^{3}H]-IP accumulation and [^{3}H]-PtdBuOH accumulation is shown in Figures 2 and 3. Ins(1,4,5)P_3 formation increased rapidly in response to vasopressin, reaching a peak between 10–20 s at 6–7 fold greater than basal (Figure 2). Stimulated levels then fell rapidly between 30–120 s, returning to basal values within 5 min. By comparison, vasopressin-stimulated [^{3}H]-PtdBuOH formation was only detectable after a lag of some 10–15 s. Accumulation then increased in a linear manner before reaching a maximum between 2–3 min. Stimulated PtdBuOH levels remained constant for a further 60 min suggesting that PLD activity was transient (Figure 3a). This contrasted with the time course of [^{3}H]-IP accumulation (Figure 3b). Following a lag time of approximately 2 min, inositol phosphate accumulation increased in a linear manner for between 45–60 min after which no further accumulation was observed (Figure 3b).

The PKC activating phorbol ester, tetradecanoylphorbol acetate (TPA), also stimulated PLD activity in a dose-dependent manner (Figure 4) with an EC_{50} value of 10.96 ± 3.16 nM ($n = 3$). This value is in close agreement with that

Table 1 EC_{50} values for stimulated [^{3}H]-inositol phosphate ([^{3}H]-InsP) and [^{3}H]-phosphatidylbutanol ([^{3}H]-PtdBuOH) formation in A10 VSMC in response to vasopressin analogues

Analogue	*EC_{50} (nM)*	
	[^{3}H]-InsP	*[^{3}H]-PtdBuOH*
[Arg^8]-vasopressin	3.37 ± 0.53	1.84 ± 0.64
Vasotocin	2.49 ± 0.21	2.78 ± 1.3
[Lys^8]-vasopressin	3.47 ± 0.43	2.78 ± 1.5
Oxytocin	626.00 ± 17.0	668.00 ± 18.0

Cells prelabelled as outlined in the methods section were incubated with increasing concentrations of analogue for 30 min or 5 min, then assayed for [^{3}H]-InsP or [^{3}H]-PtdBuOH accumulation respectively. Each value represents the mean ± s.e.mean obtained from at least 3 separate experiments performed in triplicate ($n = 3-5$). Basal values for [^{3}H]-IP accumulation were between 700–1250 d.p.m. and stimulation were routinely 4–600% of basal values. Basal values for [^{3}H]-PtdBuOH accumulation were between 1200 and 1860 d.p.m. Vasopressin stimulations were 5–700% of basal value

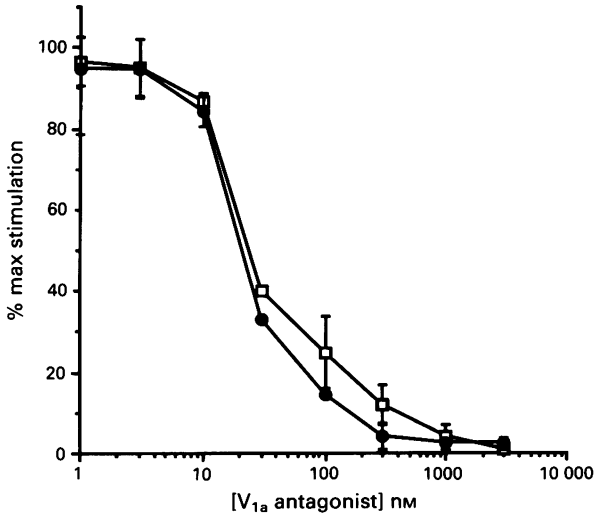

Figure 1 The effect of the V_{1a} receptor antagonist on vasopressin-stimulated [^{3}H]-inosoitol phosphate ([^{3}H]-IP) and [^{3}H]-phosphatidylbutanol ([^{3}H]-PtdBuOH) accumulation in A10 cells. Cells prelabelled as in the method section were incubated with 10 nM vasopressin in the presence of increasing concentrations of Des-Gly^9-[β-mercapto-β,β-cyclopentamethylene-propionyl^1, O-Et-Tyr^2, Val^4, Arg^8]-vasopressin as described in the legend to Table 1, and then assayed for [^{3}H]-IP (●) and [^{3}H]-PtdBuOH (□). Each point is the mean (s.e.mean shown by vertical bars) where $n = 3$. Basal values varied between 1000–1300 d.p.m. for [^{3}H]-PtdBuOH accumulation and 650–900 d.p.m. for [^{3}H]-IP accumulation. Vasopressin stimulated a 3–5 fold increase in [^{3}H]-IP accumulation and a 5–7 fold increase in [^{3}H]-PtBuOH accumulation.

obtained for TPA activation of PKC in a number of tissues (Nishizuka, 1984). The effect of preincubation with the PKC inhibitor Ro-31-8220 (Davis *et al.*, 1989), upon both TPA- and vasopressin-stimulated PLD activity is shown in Figure 5. TPA-stimulated PtdBuOH accumulation was completely abolished by low μM concentrations of Ro-31-8220, with an IC_{50} value of 1.85 ± 0.79 μM ($n = 3$). Pretreatment with Ro-31-8220 also abolished vasopressin-stimulated [^{3}H]-PtdBuOH accumulation. However, agonist stimulation was approximately 15 fold less sensitive to Ro-31-8220 pretreatment, with full inhibition being obtained at approximately 100 μM ($IC_{50} = 27.5 \pm 0.65$ μM, $n = 3$). The effect of the PKC inhibitor on vasopressin-stimulated Ins(1,4,5)P_3 formation was also examined (Figure 6). Preincubation with 100 μM Ro-31-8220 significantly enhanced maximal vasopressin-stimulated Ins(1,4,5)P_3 formation by approximately 50%, but was without effect upon the basal level (control = 2.16 ± 0.49 – 9.1 ± 1.3 pmol; Ro-31-8220 = 2.06 ± 0.46 – 15.5 ± 1.0 pmol at 20 s). In addition, stimulated values returned to basal in the presence of Ro-31-8220 more slowly than in control cells. A two fold increase in Ins(1,4,5)P_3 levels was still observed after 5 min following preincubation with this compound (1.85 ± 0.28 vs 3.6 ± 0.3 pmol).

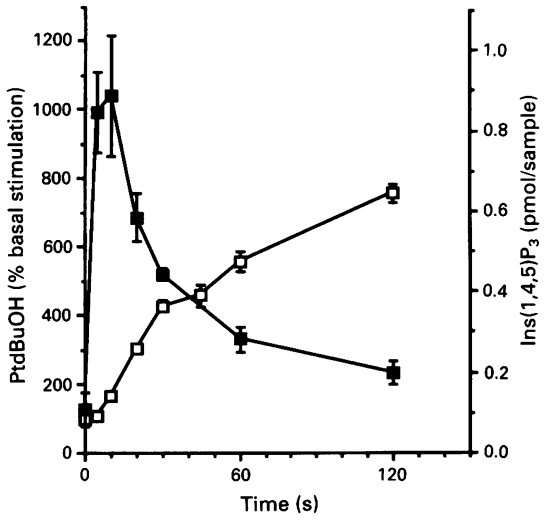

Figure 2 The onset of vasopressin-stimulated inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3) formation and [^{3}H]-phosphatidylbutanol ([^{3}H]-PtdBuOH) accumulation in A10 cells. Cells were incubated with vasopressin (100 nM) for the times indicated then Ins(1,4,5)P_3 mass (■) measured; [^{3}H]-palmitate labelled cells were stimulated at the times indicated and the formation of [^{3}H]-PtdBuOH determined (□). Each point represents the mean (s.e.mean shown by vertical bars) where $n = 4$ (12 observations). Basal values for [^{3}H]-PtdBuOH accumulation were 950–1500 d.p.m. over 4 experiments.

The effect of removing extracellular calcium on vasopressin-stimulated PLD activity is shown in Table 2. Reducing the external calcium concentration to 100 nM by buffering with EGTA resulted in an approximate 30% decrease in vasopressin-stimulated accumulation of [^{3}H]-PtdBuOH (Table 2). TPA-stimulated [^{3}H]-PtdBuOH formation was unaffected by this treatment. The calcium ionophore A23187 also stimulated the accumulation of [^{3}H]-PtdBuOH in control cells. However, this was only approximately 30% of the vasopressin response and virtually abolished by calcium chelation with EGTA. The protein kinase C inhibitor, Ro-31-8220, abolished the A23187 response, suggesting that calcium regulation of PLD activity is mediated through an action upon PKC. Ro-31-8220 (30 μM) also reduced vasopressin-stimulated PtdBuOH accumulation by approximately similar amounts (50 and 66% respectively). In a number of additional experiments the PKC inhibitor was also found to be equally effectively against vasopressin-stimulated PLD activity in both control and in calcium-free conditions (IC_{50} values (μM) control = 28.51 ± 7.2; 100 mM Ca^{2+} = 31.1 ± 6.3, $n = 3$) suggesting that the agonist activation of PKC is not significantly compromised by the removal of extracellular calcium. A number of calcium channel antagonists were also without effect upon vasopressin-stimulated PLD activity (Table 2). This included the voltage-dependent calcium channel inhibitors, nifedipine and verapamil, and the putative receptor operated calcium antagonist, SK&F 96365 (Merrit *et al.*, 1990). Only at high micromolar concentrations of the drug was any significant inhibition observed and at these concentrations the drug was equally effective against K^+-

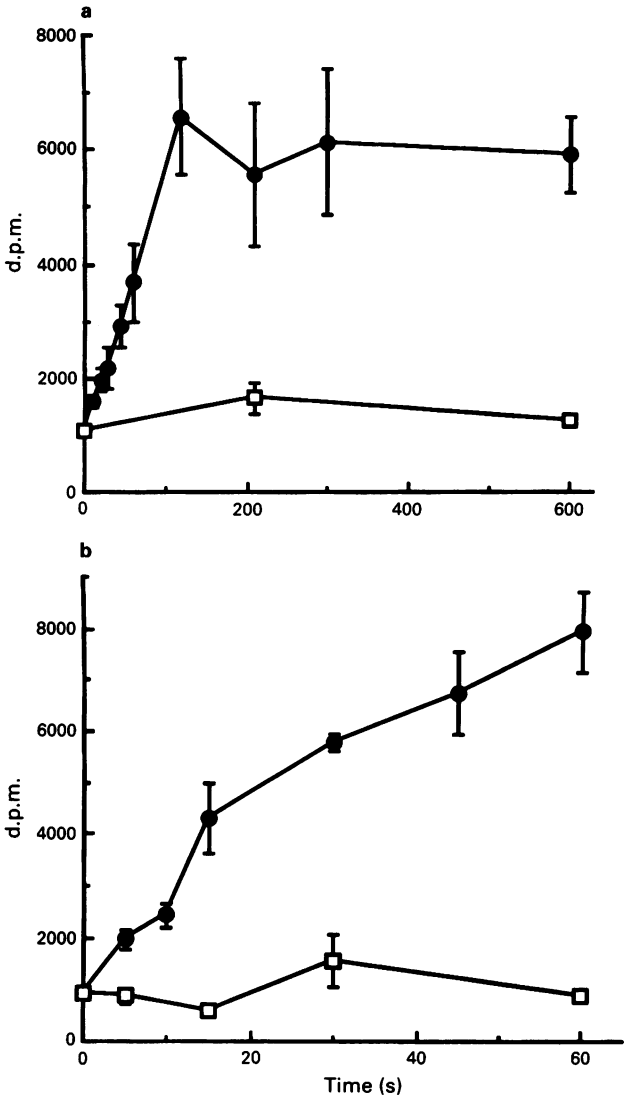

Figure 3 The kinetics of vasopressin stimulated [^{3}H]-phosphatidylbutanol ([^{3}H]-PtdBuOH) and [^{3}H]-inositol phosphate ([^{3}H]-IP) accumulation in A10 smooth muscle cells. Cells prelabelled as outlined in the Methods section were incubated with vehicle (□) or 100 nM vasopressin (●) for the time indicated then assayed for PtdBuOH (a) or [^{3}H]-IP accumulation (b). Each point represents the mean (s.d. shown by vertical bars) of triplicate determinations from a single representative experiment were $n = 3$.

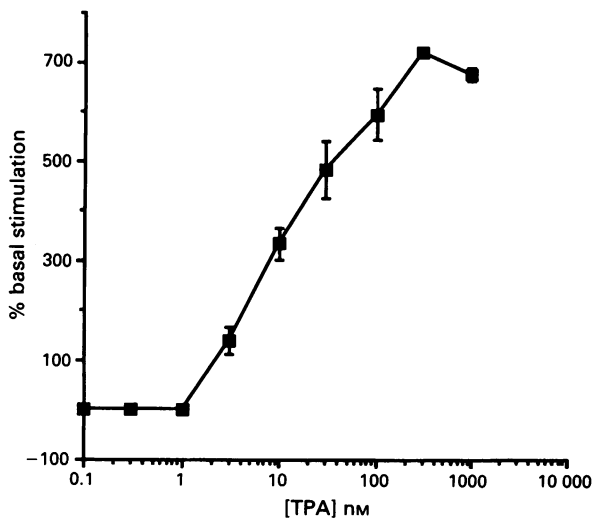

Figure 4 Tetradecanoylphorbol acetate (TPA) dose-dependent stimulation of [^{3}H]-phosphatidylbutanol ([^{3}H]-PtdBuOH) accumulation in A10 smooth muscle cells. Cells preincubated as outlined in the methods section were incubated with increasing concentrations of TPA for 15 min, then assayed for [^{3}H]-PtdBuOH formation. Each point represents the mean (s.e.mean shown by vertical bars) where $n = 3$. Basal values varied from 1100–1650 d.p.m.

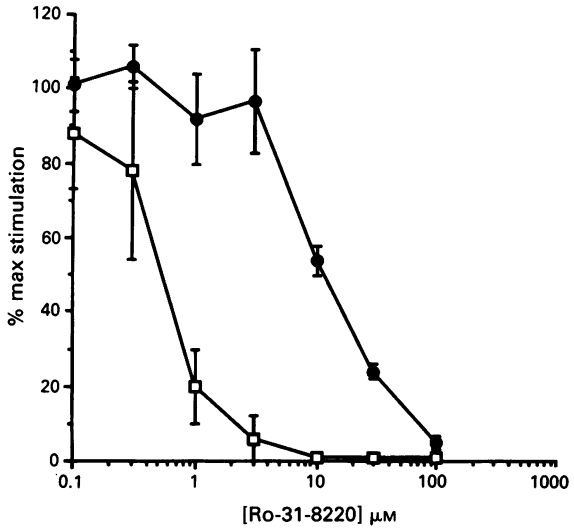

Figure 5 The effect of Ro-31-8220 on tetradecanoylphorbol acetate (TPA) and vasopressin-stimulated [^{3}H]-phosphatydlbutanol ([^{3}H]-PtdBuOH) accumulation in A10 cells. Cells prelabelled as outlined in the methods section were preincubated with increasing concentrations of Ro-31-8220 for 5 min, stimulated with 100 nM TPA (□) or vasopressin (●) for 15 min and then [^{3}H]-PtdBuOH formation determined. Each point represents the mean (s.e.mean shown by vertical bars) where $n = 4$. Basal values varied between 850–1600 d.p.m. and stimulated values ranged between 5–7 fold for vasopressin and 9–14 fold for TPA.

stimulated PLD activity. The time course of vasopressin-stimulated Ins(1,4,5)P_3 formation in control and reduced [Ca^{2+}] conditions is shown in Table 3. Preincubation with EGTA had no effect upon either the magnitude or duration of vasopressin-stimulated Ins(1,4,5)P_3 formation in A10 cells.

Discussion

In this study we examined the mechanisms by which PLD-catalysed PtdCh hydrolysis is regulated by vasopressin in the A10 smooth muscle cell line. Although this cell line has limited application as a model for smooth muscle contraction due to the loss of actin and myosin, the early events associated with this process such as calcium transients appear to be intact. Few studies have examined the mechanisms of agonist-stimulated PtdCh hydrolysis in smooth muscle, although both primary and secondary products of this pathway have been implicated in the initiation and maintenance of both smooth muscle contraction and proliferation (Ohanian *et al.*, 1990). Initial experiments measured the accumulation of the PLD-specific product phosphatidylbutanol. Although this assay is used routinely to measure agonist-stimulated PtdCh hydrolysis it is possible that PLD may catalyse the hydrolysis of other phospholipids such as phosphatidylserine and phosphatidylethanolamine to give the same product. However, in a number of fibroblast cell lines we have shown that [^{3}H]-palmitate is incorporated into these

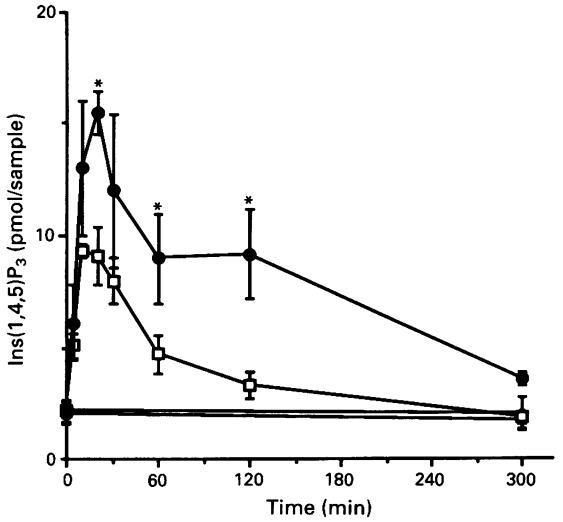

Figure 6 The effect of Ro-31-8220 on vasopressin-stimulated inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3) mass formation in A10 cells. Unlabelled cells were preincubated with vehicle (□) or 100 μM Ro-31-8220 (●) for 10 min then incubated with vasopressin (100 nM) for the times indicated and Ins(1,4,5)P_3 mass measured as described in the methods. Each point represents the mean (s.e.mean shown by vertical bars) where $n = 3$. *$P < 0.005$ vs control stimulation with vasopressin.

Table 2 The effect of extracellular calcium concentration reduction and calcium channel inhibition on [^{3}H]-phosphatidylbutanol ([^{3}H]-PtdBuOH) accumulation in A10 vascular smooth muscle cells.

	% basal stimulation	
	1.26 nM [Ca^{2+}]	*100 nM [Ca^{2+}]*
A. VP (100 nM)	540 ± 41	396 ± 23*
TPA	1033 ± 66	995 ± 50
A23187	238 ± 11	125 ± 8*
VP + Ro-31-8220 (30 μM)	248 ± 18	294 ± 17
A23187 + Ro-31-8220 (30 μM)	114 ± 4	ND
B. VP (100 nM)	568 ± 17	
VP + verapamil (10 μM)	548 ± 12	
+ nifedipine (5 μM)	571 ± 12	
+ SK&F 96365 (10 μM)	605 ± 26	
+ SK&F 96365 (100 μM)	358 ± 34*	
K^+ (80 mM)	338 ± 31	
+ SK&F 96365 (10 μM)	302 ± 22	
+ SK&F 96365 (100 μM)	184 ± 21*	

Prelabelled cells were preincubated with either HBG or HBG containing Ca^{2+} and 0.3 mM EGTA (free [Ca^{2+}] = 100 nM) or calcium channel antagonists for 15 min before addition of TPA (100 nM), vasopressin (VP) (100 nM), A23187 (5 μM) or 80 mM K^+ for 5 min. Each d.p.m. value represents the mean ± s.e.mean where $n = 4$ (12 observations). *$P < 0.05$ vs 1.26 mM [Ca^{2+}] stimulated with VP (**A**) or vs VP or K^+ stimulation (**B**). Basal values (d.p.m.), experiments (**A**) 1.26 mM [Ca^{2+}] = 787 ± 57; 100 nM [Ca^{2+}] = 874 ± 65; experiment (**B**) = 943 ± 88. For abbreviations, see text. ND = not done.

phospholipids to only a minor extent (Cook & Wakelam, unpublished results) and a similar finding has been obtained by Welsh *et al.* (1990) in A10 VSMC. Additional preliminary experiments also suggested that PtdCh was a major substrate for vasopressin-stimulated, PLD-catalysed hydrolysis, as measured by the formation of water soluble choline metabolites.

Table 3 The effect of extracellular calcium removal on vasopressin-stimulated inositol-1,4,5-trisphosphate (Ins(1,4,5) P_3) formation in A10 cells

	Ins(1,4,5)P_3 (pmol/sample)	
	1.26 mM [Ca^{2+}]	*100 nM [Ca^{2+}]*
Control (10 s)	2.40 ± 0.24	1.96 ± 0.45
Vasopressin (10 s)	11.60 ± 1.89	10.10 ± 2.0
Control (5 min)	1.96 ± 0.2	2.33 ± 0.2
Vasopressin (5 min)	3.20 ± 0.58	2.80 ± 0.75

Cells were preincubated in HBG or in HBG containing 0.3 mM EGTA (free [Ca^{2+}] = 100 nM) for 15 min then incubated with 100 nM vasopressin or vehicle for the times indicated. Ins(1,4,5)P_3 was measured as outlined in the methods. Each value represents the mean ± s.e.mean where $n = 3$.

We initially sought to characterize pharmacologically vasopressin-stimulated [^{3}H]-IP and [^{3}H]-PtdBuOH formation since it is believed that at least two vasopressin-V_1 receptor subtypes are expressed in mammalian tissues (Penit *et al.*, 1983; Jard *et al.*, 1986). In A10 and primary vascular smooth muscle cells a V_{1a} receptor subtype has been identified as mediating vasopressin-stimulated calcium influx and inositol phosphate formation (Doyle & Ruegg, 1985; Aiyar *et al.*, 1986). However, athough much attention has focused on the possibility that PLD and PLC may be regulated by distinct G-proteins, little attempt has been made to determine if PLC and PLD may be achieved by occupation of different receptor subtypes. This does not appear to be the case in A10 cells since the order of agonist potency was the same for both responses (Table 1) and the V_{1a} antagonist (Des-Gly^9-[β-mercapto-β,β-cyclopentamethylene-propionyl^1, O-Et-Tyr^2, Val^4, Arg^8]-vasopressin) yielded similar IC_{50} values in each case (Figure 1). There was also no indication of a high affinity receptor for oxytocin, indeed this peptide behaved as a weak, partial agonist, with about 60% of the efficacy of vasopressin (Table 1). Thus, both PLC and PLD appear to be stimulated by occupation of a V_{1a} receptor; however, an accurate receptor subtype definition can only be achieved by a more extensive comparison of agonist and antagonist potency orders (Jard *et al.*, 1986).

The kinetics of vasopressin stimulated Ins(1,4,5)P_3 accumulation were transient suggesting that the activation of PLC was not sustained. This is supported by a number of additional experiments which have shown a transient decrease in PtdIns(4,5)P_2 mass levels in response to vasopressin (Plevin & Wakelam, 1992). This finding argues strongly against the rapid removal of Ins(1,4,5)P_3 by 5-phosphatase and 3-kinase activity being the sole mechanism underlying the rapid return of Ins(1,4,5)P_3 accumulation to basal values. However, it is also possible that an agonist-stimulated flux through PtdIns and PtdIns(4)P may lead to a new steady state level of PtdIns(4,5)P_2. Therefore the possible sustained nature of PtdIns(4,5)P_2 hydrolysis may be impossible to delineate under the present assay conditions.

The apparent transient nature of PtdIns(4,5)P_2 hydrolysis argues against this lipid being a substrate for PLD. In a vast number of studies examining the hydrolysis of PtdIns(4,5,)P_2 the main $InsP_2$ isomers formed following agonist stimulation are Ins(1,4)P_2, Ins(1,3)P_2 and Ins(3,4)P_2 (see Berridge & Irvine, 1989) but not Ins(4,5)P_2 which would be the product of a PtdIns(4,5)P_2-PLD activity. Furthermore, PLD activity was also transient as judged by a number of criteria including the transient accumulation of PtdBuOH (Figure 3) and [^{3}H]-choline (Plevin & Wakelam, 1992).

Vasopressin-stimulated [^{3}H]-PtdBuOH accumulation was found to be subsequent to Ins(1,4,5)P_3 formation in A10 smooth muscle cells. This result is similar to that obtained in other peptide receptor systems such as endothelin-1 (ET-1)-

stimulated Rat-1 fibroblasts (MacNulty *et al.*, 1990) and bombesin-stimulated Swiss 3T3 fibroblasts (Cook *et al.*, 1990) and suggests that in this tissue PLC is activated before PLD. This argues in favour of a sequential pathway involving the activation of some type of intermediate. This is potentially PKC, since TPA stimulated the accumulation of PtdBuOH in the concentration-range consistent with its activation of PKC. Also both TPA- and vasopressin-stimulated [^{3}H]-PtdBuOH accumulation were completely inhibited by preincubation with the PKC inhibitor Ro-31-8220. These findings are consistent with results obtained in other tissues where down regulation of PKC by chronic phorbol ester pretreatment prevented subsequent activation of PLD (Cook & Wakelam, 1989; Martinson *et al.*, 1989; MacNulty *et al.*, 1990).

However, recent studies have shown that in some tissues agonist-stimulated PLD is only partially inhibited by PKC inhibitor pretreatment (Billah *et al.*, 1989; Liscovitch & Amsterdam, 1989; Cook *et al.*, 1991). This has implicated a PKC-independent component in the regulation of the PtdCh response, which has been suggested may involve a G-protein (Bocckino *et al.*, 1987; Martin & Michaelis, 1989). The construction of full inhibition curves for the effect of the PKC inhibitor, Ro-31-8220, upon agonist- and PMA-stimulated PLC activity yielded different IC_{50} values with the inhibitor being 15 fold more potent against the phorbol ester response (Figure 4). This result would suggest that agonist-stimulated PLD activity is not solely mediated by the prior activation of PKC and, since Ro-31-8220 is an ATP site inhibitor, PLD activation may involve the stimulation of additional kinases. However, Figure 5 demonstrates that pretreatment with Ro-31-8220 potentiates vasopressin-stimulated Ins(1,4,5)P_3 formation and thus the generation of DAG from PtdIn(1,4,5)P_2. Since in a number of previous studies it has been shown that the principal site of PKC-mediated negative feedback is at the level of the receptor/G-protein interface (e.g. Plevin *et al.*, 1990), it is reasonable to suggest that Ro-31-8220 may act to prolong PtdIns(4,5)P_2 hydrolysis in response to an agonist whose receptor is coupled to the effector PLC, by a G-protein. Indeed in Swiss 3T3 cells, vasopressin-stimulated [^{3}H]-InsP_3 accumulation is enhanced and prolonged in conditions where protein kinase C is inactive (Brown *et al.*, 1990). Although it is also possible that PKC may activate Ins-(1,4,5)P_3 kinase and/or phosphatase we have also shown previously that preincubation with Ro-31-8220 enhances vasopressin-stimulated [^{3}H]-IP accumulation in A10 VSMCs (Plevin & Wakelam, 1992). This argues against an effect on the rate of Ins(1,4,5)P_3 removal as the sole mechanism of action of PKC since this would have no effect on the accumulation of total [^{3}H]-IP. Thus, it remains possible that the maintenance of a PtdIns(4,5,)P_2-derived DAG signal may activate a form of PKC which requires a greater concentration of Ro-31-8220 to be fully inhibited.

In vascular smooth muscle cells, calcium influx is important in the regulation of a number of intracellular events (Zschauer *et al.*, 1987; Ruegg *et al.*, 1989). The results in this study suggest a partial involvement of extracellular calcium in the regulation of vasopressin-stimulated PLD activity in A10 smooth muscle cells. Buffering with EGTA resulted in a small but significant reduction (approximately 30%) in the vasopressin-stimulated response, whilst pretreatment with a series of calcium channel antagonists was without effect. This includes the L-type voltage dependent calcium antagonists, nifedipine and verapamil and also SK&F 96365 which it has been proposed inhibits receptor mediated calcium influx (Merrit *et al.*, 1990). This compound was only effective at high micromolar concentrations where it acted in a non-specific manner, being equally effective against K^+-stimulated PLD activity. Indeed the original studies by Merrit *et al.* (1990) have suggested that some smooth muscle cells may contain receptor-operated calcium channels insensitive to this type of compound.

The largely calcium-independent nature of vasopressin-stimulated PLD activity suggests that agonist activation of PKC (and thus PLD activity) can be almost fully achieved at resting levels of intracellular Ca^{2+}. This was confirmed by analysing the dose-response relationship for Ro-31-8220 inhibition of vasopressin-stimulated [^{3}H]-PtdBuOH accumulation in calcium-free conditions. This parameter was not significantly affected, which suggests that the sensitivity of agonist-stimulated [^{3}H]-PtdBuOH accumulation to inhibition by Ro-31-8220 is not changed in the absence of extracellular calcium. Furthermore, the finding that A23187-stimulated PLD activity is completely abolished by PKC inhibitor pretreatment once again emphasizes the central role PKC may play in the regulation of PLD in this cell. These findings are consistent with that obtained for ATP-stimulated endothelial cells (Martin & Michaelis, 1989), vasopressin-stimulated hepatocytes (Bocckino *et al.*, 1987) and with a recent report that showed no effect of chelation with 10 mM EGTA upon PLD activity in A10 smooth muscle cells in culture (Welsh *et al.*, 1990). However, in other tissues, agonist-stimulated PLD activity is reduced substantially upon extracellular calcium removal (Pai *et al.*, 1988; Lassegue *et al.*, 1991; Wakelam *et al.*, 1991). In these systems Ca^{2+} influx may also directly regulate PLD activity in addition to modifying PKC activity.

In this study we have examined the characteristics of vasopressin-stimulated PtdIns(4,5)P_2 and phosphatidylcholine hydrolysis in A10 vascular smooth muscle cells. The results indicate that the hydrolysis of both lipids are regulated by interaction with the same receptor subtype. Vasopressin stimulation of PLD is achieved through a sequential pathway, subsequent to the initial PtdIns(4,5)P_2 hydrolysis and involving the intermediate activation of PKC. Calcium entry appears to have a small but significant regulatory role in vasopressin-stimulated PLD activity in this tissue.

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The effects of calcitonin gene-related peptide on formation of intra-articular oedema by inflammatory mediators

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1 The temporal and quantitative effects of inflammatory mediators on plasma extravasation in the rat knee were investigated by use of a perfusion technique.

2 Intra-articular perfusion of substance P (SP), bradykinin or histamine over a 5 min test period produced rapid-onset and prolonged plasma extravasation in a dose-dependent fashion. The rank order of potency was bradykinin > SP > histamine.

3 Calcitonin gene-related peptide (CGRP) did not induce plasma extravasation but enhanced substance P-induced plasma extravasation in a dose-dependent fashion. A 5 min co-perfusion of the two agents produced short-term enhancement lasting 10 min while continuous co-perfusion produced enhancement for the duration of the perfusion.

4 A 5 min perfusion of CGRP enhanced plasma extravasation when co-perfused with bradykinin but not histamine. However, when CGRP and histamine were continuously co-perfused over a 20 min test period, an enhanced response was apparent.

5 The results indicate that intra-articular perfusion of CGRP enhances synovial plasma extravasation induced by agents that increase vascular permeability, but suggest that the response is not uniform and is critically dependent on the duration of perfusion within the joint.

Keywords: Plasma extravasation; joint inflammation; neurogenic inflammation; neuropeptides; calcitonin gene-related peptide (CGRP); substance P; bradykinin

Introduction

The nervous system exerts a profound effect on the inflammatory response. Antidromic stimulation of sensory nerves results in localized vasodilatation and increased vascular permeability (Jancso *et al.*, 1967) and a similar response is observed following injection of peptides known to be present in sensory nerves (Foreman *et al.*, 1983). From this and other data it is postulated that peptides released from sensory nerve endings mediate many inflammatory responses, at least in the early stages (Foreman, 1987; Kidd *et al.*, 1989).

There is evidence that neuropeptides modulate the actions of other inflammatory agents. Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide produced by the alternative processing of the calcitonin gene and is present in up to 50% of primary sensory nerves (Gibson *et al.*, 1984). It is a potent vasodilator capable of inducing a protracted increase in microvascular blood flow when injected extra-vascularly (Brain *et al.*, 1985). Although CGRP has not been shown to affect plasma extravasation directly, it potentiates intradermal oedema induced by established mediators of increased vascular permeability, including substance P (SP) (Brain & Williams, 1985; Gamse & Saria, 1985).

The synovial tissues of human and animal joints are richly innervated with peptide-containing sensory nerves (Mapp *et al.*, 1990). However, in contrast to cutaneous nerves, sensory nerves from synovial tissues are inactive under normal conditions (Grigg *et al.*, 1986). It has therefore remained unclear whether CGRP could modify synovial reactions to inflammatory mediators in a similar fashion to that demonstrated within skin. Indeed, a recent study of late synovial responses following an intra-articular injection of SP failed to show CGRP-enhancement of plasma extravasation (Lam & Ferrell, 1991). In this study we used a knee perfusion technique to examine the temporal and quantitative effects of inflammatory mediators on synovial plasma extravasation and to determine whether exogenous CGRP could enhance these effects.

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Methods

The experiments were performed on male Wistar rats (240–300 g) which were deeply anaesthetized with sodium pentobarbitone (65 mg kg^{-1}, i.p.). Plasma extravasation into the perfused knee joint was measured by a modification of the technique described by Coderre *et al.* (1989). Evans blue (100 mg kg^{-1}) was injected into the penile vein. The right knee was cannulated and perfused with physiological saline at a constant rate of 100 $\mu l\ min^{-1}$; 30 G and 27 G needles, inserted through the joint capsule with a spacing of approximately 3 mm, served as the inlet and outlet cannulae respectively. The perfusion rate was maintained with syringe driver pumps (Graseby Medical, Watford, U.K.). A clear perfusate with a constant flow rate indicated that the cannulae were satisfactorily positioned.

After a 40 min equilibration period, during which a stable baseline was established, the knee was perfused for 5 min with various agents and then for a further 25 min with saline. The agents perfused in this experiment were SP, CGRP (Cambridge Research Biochemicals), bradykinin and histamine (Sigma). The perfusate was collected as 1 ml fractions at 10 min intervals throughout the experiment. If any fraction was observed to contain red blood cells the results from that experiment were rejected. Evans blue binds to plasma proteins which are largely retained within the vasculature and its presence within the perfusate is indicative of plasma extravasation into the synovial space. The concentration of dye present in the perfusate was calculated by measuring the absorbance at 620 nm (Titertek Multiscan, Flow) and calibrating this against a standard curve prepared with known concentrations of Evans blue. The results are presented as the increase in Evans blue above the baseline level.

A dose-response curve was established for each of the

mediators. The ability of rat CGRP to potentiate plasma extravasation was then examined. From the dose response data a concentration of SP (1.0 μM) which induced a small but significant increase in plasma extravasation was selected. By simultaneously perfusing this concentration of SP with CGRP (1.0–10.0 μM) a dose-response curve for enhancement of SP-induced plasma extravasation by CGRP was established. The ability of CGRP to potentiate extravasation induced by bradykinin (0.1 μM) and histamine (10.0 μM) was also assessed.

A further investigation was conducted in which the ability of CGRP to enhance plasma extravasation induced by the constant perfusion of SP or histamine was assessed. In these experiments 1.0 μM CGRP was continuously co-perfused with 1.0 μM SP or 10.0 μM histamine over a 20 min period.

Results

The ability of SP, CGRP, bradykinin and histamine to induce plasma extravasation within the synovial cavity was assessed. CGRP at any dose did not induce significant plasma extravasation. SP, bradykinin and histamine all induced dose-dependent increases in plasma extravasation during the initial, and subsequent, test periods (Figure 1). The rank order of potency for inducing plasma extravasation was bradykinin > substance P > histamine.

The time course of the response varied with the mediator used (Figure 2). The plasma extravasation induced by SP peaked during the first 10 min perfusion period and then slowly subsided. The perfusion of histamine resulted in continuous plasma extravasation over a 20 min period followed by a slow decline. In contrast the response to bradykinin peaked during the second 10 min test period. Following the induction of a significant increase in plasma extravasation by an individual mediator the levels of Evans blue within the perfusate remained above baseline for the entire test period (i.e. 30 min).

The ability of CGRP to modulate plasma extravasation was assessed by co-infusing CGRP over a 5 min test period with SP (1.0 μM), bradykinin (0.1 μM), or histamine (10.0 μM). CGRP was found to enhance SP-induced plasma extravasation in a dose-dependent fashion (Figure 3). Co-infusion of 1.0 μM CGRP also significantly enhanced bradykinin- but not histamine-induced plasma extravasation (Figure 4). The effect was only noted during the first 10 min collection period and no enhancement was noted thereafter.

When continuously co-perfused over a 20 test min period, 10 μM CGRP significantly enhanced the plasma extravasation induced by 10 μM histamine as shown in Figure 5. This enhancement was evident in both the 10 and 20 min collection periods. A similarly enhanced response was observed when 10 μM CGRP was continuously co-perfused with 1.0 μM SP.

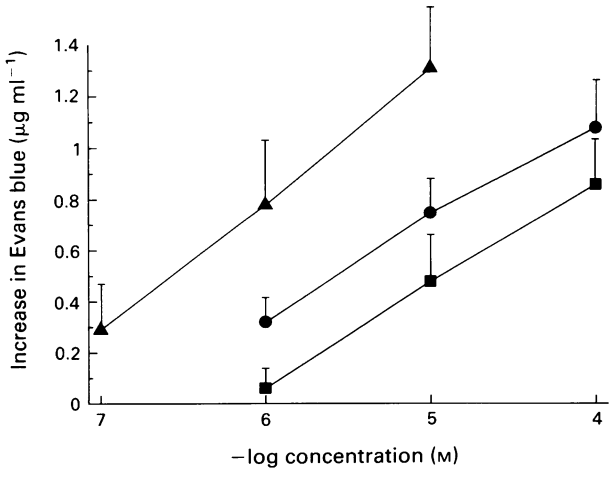

Figure 1 The dose-response curves for plasma extravasation into rat knee joints induced by a 5 min perfusion of substance P (●); bradykinin (▲); and histamine (■). Points are the mean results from 4 animals; vertical bars show s.e.mean.

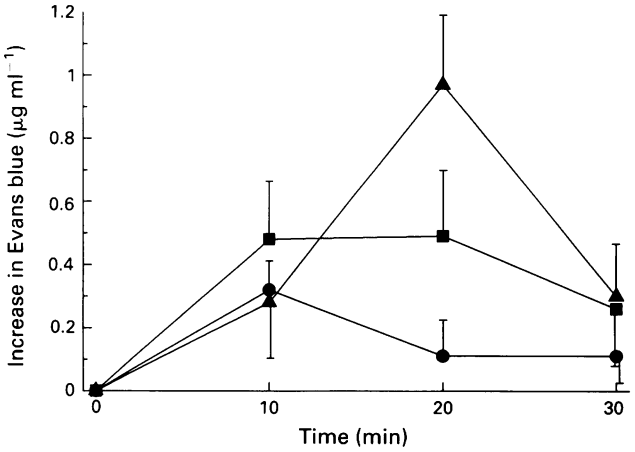

Figure 2 Time-course for plasma extravasation into rat knee joints following a 5 min perfusion of bradykinin (0.1 μM) (▲); substance P (1.0 μM) (●); and histamine (10.0 μM) (■). Points are the means results from 4 animals; vertical bars show s.e.mean.

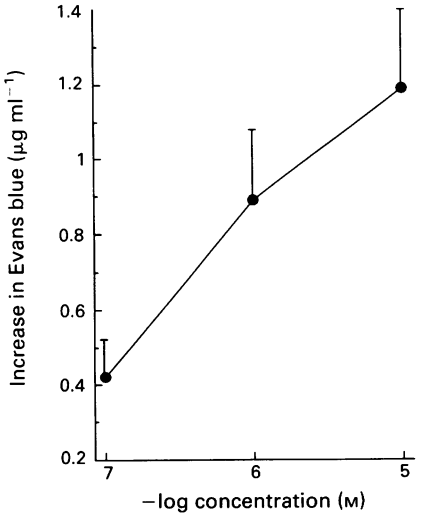

Figure 3 Dose-response curve for enhancement of substance P-induced plasma extravasation into rat knee joints. Calcitonin gene-related peptide (CGRP) (1.0–10.0 μM) was co-perfused with 1.0 μM substance P for 5 min, test fractions were collected after 10 min. Points are the mean results from 4 animals; vertical bars show s.e.mean.

Discussion

The present study demonstrates that CGRP enhances SP-, bradykinin-, and histamine-induced plasma extravasation in the rat knee joint. This confirms and extends previous observations of synergism between CGRP and inflammatory mediators in both rabbit and rat skin (Brain & Williams, 1985; Gamse & Saria, 1985) but contrasts with a recent study of the rat knee which was unable to show such an effect (Lam & Ferrell, 1991).

The study by Lam & Ferrell (1991) measured plasma

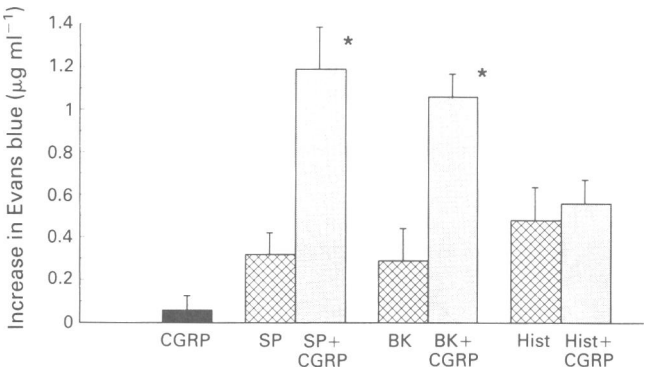

Figure 4 Plasma extravasation into rat knee joints induced by a 5 min perfusion of 1.0 μM substance P (SP), 0.1 μM bradykinin (BK) or 10 μM histamine (Hist) and effects of co-perfusion with 1.0 μM calcitonin gene-related peptide (CGRP). Data are the mean results from 4 animals; vertical bars show s.e.mean. $*P<0.05$, paired t test.

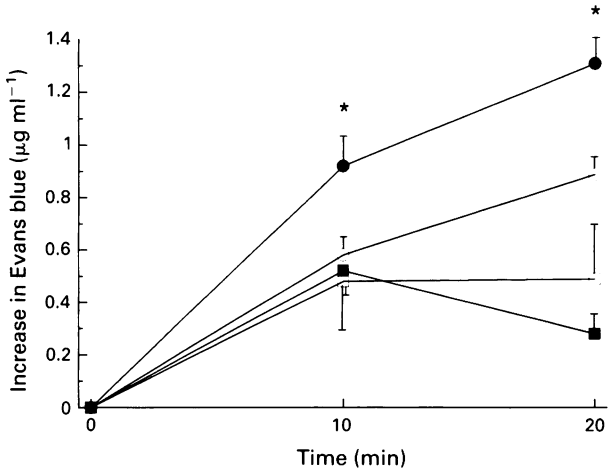

Figure 5 Time course of plasma extravasation into rat knee joints induced by: 5 min perfusion 10 μM histamine (□); 5 min perfusion 10 μM histamine + 10 μM calcitonin gene-related peptide (CGRP) (■); 20 min perfusion 10 μM histamine (○); 20 min perfusion with 10 μM histamine + 10 μM CGRP (●). Data are the mean results from 4 animals; vertical bars show s.e.mean. $*P<0.05$, paired t test.

extravasation in biopsy specimens taken from the knee 4 h after intra-articular injection. The technique measured the cumulative inflammatory response and the 4 h time point was chosen as the course of neurokinin-induced plasma extravasation in the joint was not known. Early or transient effects could therefore have been easily missed and it is significant that the synergistic effects of CGRP in skin were observed in biopsies taken at 15 and 30 min (Brain & Williams, 1985; Gamse & Saria, 1985). The technique used in the present study assessed plasma extravasation during the first 30 min and had the additional advantage of showing both the time sequence and duration of response.

Three of the agents used in the present study increased synovial permeability in a dose-dependent fashion with bradykinin being the most potent followed by SP and histamine respectively. The duration of the response was prolonged and it was noteworthy that a 5 min perfusion of each agent induced plasma extravasation lasting in excess of 30 min. CGRP did not induce plasma extravasation but significantly increased SP-induced plasma extravasation in a dose-dependent fashion. In skin, the potentiating activity of CGRP has been shown to correlate with its vasodilator activity as does the potentiating activity of other vasodilators, such as the prostaglandins (Brain & Williams, 1988; Williams & Morley, 1973) and it is assumed that a similar mechanism is operative in the joint.

A 5 min co-perfusion of SP and CGRP produced increased SP-induced plasma extravasation during the first 10 min but not thereafter. There is evidence that SP causes a loss of the prolonged vasodilator activity of CGRP by a mechanism involving proteases (Brain & Williams, 1988) and it follows that the potentiating effect of CGRP on SP-induced plasma extravasation would, in consequence, be attenuated by the same mechanism. Support for this came from the observation that perfusion of the two agents over periods likely to overcome the effects of local proteases induced a sustained increase in plasma extravasation.

The enhanced response following a 5 min co-perfusion of bradykinin and CGRP was apparent only for the first 10 min and suggests the presence of a similar attenuating mechanism involving proteases. The need for a longer co-perfusion of histamine and CGRP before a significant enhancement of extravasation was observed may indicate that this combination has a greater susceptibility to proteolytic attack: this awaits further investigation.

A number of human arthropathies appear to be influenced by neurogenic mechanisms, the most obvious example being the synovitis that accompanies reflex sympathetic dystrophy. On the basis of the findings of the present study it could be argued that potentiation of inflammatory mediators by neurogenically derived CGRP may play a role in the development of synovial inflammation. Inhibition of neurogenic mechanisms might therefore modify the course of disease and prove therapeutically useful.

In conclusion, this study shows that intra-articular perfusion of SP, bradykinin or histamine, but not CGRP, produces prolonged synovial plasma extravasation in a dose-dependent fashion. CGRP significantly enhances SP-, bradykinin- and histamine-induced extravasation but the response is not uniform and is critically dependent on the duration of perfusion within the joint.

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Pharmacological characterization of adenosine A_1 and A_2 receptors in the bladder: evidence for a modulatory adenosine tone regulating non-adrenergic non-cholinergic neurotransmission

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1 The nerve-evoked contractions elicited by transmural electrical stimulation of mouse urinary bladders superfused in modified Krebs Ringer buffer containing 1 μM atropine plus 3.4 μM guanethidine were inhibited by adenosine (ADO) and related nucleoside analogues with the following rank order of potency: **R**-phenylisopropyladenosine (**R**-PIA) > cyclohexyladenosine (CHA) > 5'N-ethylcarboxamido adenosine (NECA) > ADO > **S**-phenylisopropyladenosine (**S**-PIA). Tissue preincubation with 8-phenyltheophylline (8-PT) displaced to the right, in a parallel fashion, the NECA concentration-response curve.

2 The contractions elicited by application of exogenous adenosine 5'-triphosphate (ATP) were also inhibited by ADO and related structural analogues. The rank order of potency to reduce the motor response to ATP was: NECA >2-chloroadenosine (CADO) > **R**-PIA > ADO > CHA > **S**-PIA.

3 The ADO-induced ATP antagonism was of a non-competitive nature and was not specific. Tissue incubation with 10 μM NECA not only reduced the motor responses elicited by ATP, but also 5-hydroxytryptamine, acetylcholine and prostaglandin $F_{2\alpha}$. The action of NECA was antagonized following tissue preincubation with 8-PT. The inhibitory action of NECA was not mimicked by 10 μM CHA.

4 The maximal bladder ATP contractile response was significantly increased by tissue preincubation with 5–30 μM 8-PT.

5 The 0.15 Hz evoked muscular twitch was significantly increased by 8-PT while dipyridamole consistently reduced the magnitude of the twitch response. These results are consonant with the hypothesis that an endogenous ADO tone modulates the bladder neurotransmission.

6 A working model is proposed suggesting the presence of ADO-A_1 and A_2 receptors in the mouse urinary bladder. The A_1 receptor subpopulation is probably of presynaptic origin whereas the smooth muscle membranes contain a population of the A_2 receptor subtype.

Keywords: Non-noradrenergic non-cholinergic neurotransmission; purinergic transmission; adenosine receptors; adenosine A_1, A_2 mechanisms; adenosine modulation; urinary bladder

Introduction

It is more than 20 years since Burnstock hypothesized that adenosine 5'-triphosphate (ATP) or a related purine might participate in neurotransmission (Burnstock, 1972; Burnstock *et al.*, 1972). Within the past years, it has become generally accepted that ATP is probably the non-adrenergic non-cholinergic (NANC) transmitter (Burnstock *et al.*, 1978 b). Furthermore, neurochemical studies identified ATP in adrenergic and cholinergic synaptic vesicles, from where the nucleotide is co-released with either noradrenaline or acetylcholine (for a recent review see Burnstock, 1990). These findings pioneered the concept of cotransmission, where two neurochemicals simultaneously released from the same neurone, act in a concerted fashion at a common target cell (Campbell, 1987). Pharmacological studies aimed at characterizing the nature and mechanism of the extracellular ATP actions, concluded that ATP activates selective receptors, distinguishable from those of adenosine (ADO). Furthermore, these sites could be classified into two subtypes according to the nature of the ATP responses: P_{2x} and P_{2y} (Burnstock & Kennedy, 1985). In these studies, the use of α, β methylene ATP, a slowly degradable ATP analogue has played a pivotal role in ATP receptor classification. This synthetic nucleotide is a selective agonist at P_{2x} purinoceptors that, under certain conditions desensitizes specifically the P_{2x} receptor (Kasakov & Burnstock, 1983).

In an almost parallel fashion, data accumulated indicating that ADO can modulate neurotransmission. Although the source of ADO at the synapse is not as yet clearly established, there are reasonable indications that it could derive from the metabolism of ATP by ecto-ATPases and 5' nucleotidases in the surroundings of the nerve terminals (McDonald & White, 1984; Cusack & Hourani, 1984; Cusack *et al.*, 1988). There is evidence in favour of the notion that ADO, or a related compound, decreases transmitter release, thereby modulating neurotranmission in a variety of peripheral tissues. Although the inhibition of neurotransmitter release induced by purines was first described in the rat phrenic diaphragm preparation (Ginsborg & Hirst, 1972), most experiments have since been performed in sympathetically innervated organs (Hedquist & Fredholm, 1976; Clanachan *et al.*, 1977; Su, 1978; Wakade & Wakade, 1978; Hayashi *et al.*, 1978; Hedquist & Fredholm, 1979). At present, it is well recognized that there are at least two subtypes of ADO receptors: A_1 and A_2 (Burnstock & Kennedy, 1985).

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These sites have been pharmacologically characterized by use of several ADO structural analogues and antagonism by different methylxanthines (Williams, 1987).

In view of the finding that ATP may be released from bladder nerves (Burnstock *et al.*, 1978 a, b), and since it is readily metabolized by ectoenzymes to ADO in this tissue (Cusack & Hourani, 1984), we thought it of interest to investigate whether ADO may act as an endogenous regulator of the NANC neurotransmission as an extension to its well characterized regulatory action at adrenergic and cholinergic neuroeffector junctions. With this purpose, we characterized pharmacologically the mouse bladder responses to the exogenous application of ADO following transmural depolarization of bladder nerve terminals or the motor action of exogenous ATP. In addition, we tested for the existence of an ADO tone in the mouse bladder transmission, where our previous research revealed the participation of ATP as a neurotransmitter (Acevedo & Contreras, 1985; Acevedo *et al.*, 1990).

Methods

Animals

Adult albino Swiss Webster mice (28–32 g) were raised at the Animal Reproduction Laboratories of the University of Concepción. Purina chow and water were not restricted.

Bioassay and quantification of drug effects

Animals were killed by cervical dislocation; the abdomen was opened via a midline incision. The urinary bladder was removed, voided and immediately placed in Krebs-Ringer solution warmed at 37°C and bubbled with 95% O_2/5% CO_2 to maintain a pH close to 7.4. The bladder was dissected from surrounding tissues; two transverse, incomplete cuts were performed which resulted in a thin muscle strip which was tied on both ends (Acevedo & Contreras, 1985). One extreme was fixed to a hook on the 12 ml tissue chamber, the other was connected to a Grass force displacement transducer (FT 03C) in order to record isometric contractions. The tissue chamber was provided with two ring platinum electrodes 22 mm apart that were connected to a Grass S44 Stimulator. The tissues were initially placed under 0.5 g of resting tension, and adjustments were made to maintain the tension all through the experiment. The composition of the Krebs-Ringer solution was (mM): NaCl 118, KCl 5.4, $CaCl_2$ 2.5, KH_2PO_4 1.2, Na HCO_3 23.8 and glucose 11.1. In addition, 1 μM atropine plus 3.4 μM guanethidine was added to the buffer.

In most cases concentration-response experiments were performed; IC_{50} values were derived by interpolation using computerized linear regression analysis.

Determination of the potency of adenosine and related analogues at inhibiting the neuronally induced bladder muscular contractions

Following a 45–60 min equilibration period of the bioassay, the tissues were stimulated for 1 s with pulses of 0.1, 0.5, 1 and 5 Hz (1 ms duration, supramaximal voltage, circa 70 V), each separated by 60 s, so as to derive a frequency-contraction curve. To avoid preparation fatigue, the tissues were stimulated with these trains of pulses every 15 min until reproducible responses were obtained. Next, tissues were incubated for 5 min with 10 μM of either **R** and **S**-PIA, CHA, NECA or ADO prior to the application of the trains of transmural electrical stimulation. At least 4–6 separate tissues were used to study the action of ADO and related structural analogues.

In a parallel but separate experiment, the tissues were stimulated with 0.15 Hz pulses for periods of 3 min before application of varying single concentrations of NECA to obtain a concentration-response curve ($n = 8$). Once the maximal inhibition of the twitching response occurred, the electrical stimulus was stopped and tissues were rinsed. Fifteen min later, electrical stimulation was resumed and a larger concentration of the agonist was tested. At least 4 concentrations of NECA were used to define a concentration-response curve. In a subsequent experiment, separate tissues were preincubated for 5 min with 10 μM 8-PT before the addition of non-cumulative concentrations of NECA.

Determination of the potency of adenosine and related nucleosides in blocking the motor response to ATP and other contractile agonist drugs

Concentration-response experiments were performed applying non-cumulative ATP concentrations in the absence and following a 5 min tissue incubation in the presence of 10 μM NECA. To determine more precisely the potency of ADO and its analogues, a separate series of experiments was performed utilizing a fixed concentration of 25 μM ATP. Once the responses to ATP were standardized, the tissues were preincubated for 5 min with varying concentrations of either **R**- or **S**-PIA, NECA, CHA, CADO or ADO prior to the ATP challenge. The concentration of each agonist causing half maximal blockade of the ATP response (IC_{50}) was derived by interpolation from each concentration-response study.

To evaluate the specificity of the interaction, a fixed concentration of 10 μM NECA was used to examine the contractile responses induced with acetylcholine, 5-hydroxytryptamine or prostaglandin $F_{2\alpha}$ (each 10 μM). Tissues were preincubated with NECA for 5 min prior to the addition of the contractile agents.

In the next series of experiments, an acetylcholine concentration-response experiment was performed in the absence and 5 min after the application of 10 μM NECA ($n = 6$ separate tissues). In a parallel experiment, tissues were preincubated for 5 min with 20 μM 8-PT prior to the addition of 10 μM NECA. A further experiment was performed where 10 μM CHA was substituted for NECA. It must be noted that experiments were performed in buffer containing 1 μM atropine plus 3.4 μM guanethidine.

Effect of 8-phenyltheophylline on the ATP concentration-response curve

To ascertain whether the maximal contractile activity of ATP is influenced by ADO, derived presumably from extracellular tissue ATP metabolism, nucleotide concentration-response, curves were obtained in the absence and in the presence of either 5, 10 or 30 μM 8-PT ($n = 8$, 6 and 6 respectively). The ATP concentration-response curves were compared to that evoked with α, β methylene ATP ($n = 9$); separate preparations were used to test each agonist.

Effect of 8-phenytheophylline and dipyridamole on the bladder stimulated with trains of 0.15 Hz

To evaluate whether ADO receptor blockade resulted in a potentiation of the 0.15 Hz-induced twitch due to antagonism of endogenous ADO, the magnitude of the twitch response was observed prior to and following a 3–5 min addition of 10 or 30 μM 8-PT ($n = 8$ separate preparations). Likewise, to examine the influence of the adenosine uptake blocker on the contractile activity evoked by nerve stimulation, we observed the magnitude of the 0.15 Hz nerve-evoked muscular twitch prior to and 3–5 min after tissue incubation with 10 μM dipyridamole ($n = 6$ experiments).

Drugs

The drugs and their sources were as follows: adenosine 5'-triphosphate (ATP) sodium salt, α, β methylene ATP lithium

salt, acetylcholine hydrochloride, prostaglandin $F_{2\alpha}$, 5-hydroxytryptamine creatinine sulphate, dipyridamole hydrochloride, atropine sulphate (Sigma Chemical Co., St. Louis, MO, U.S.A.). **R** and **S** phenylisopropyladenosine (PIA); 2-chloroadenosine (CADO); cyclohexyladenosine (CHA); adenosine (ADO) 5'N-ethylcarboxamido adenosine (NECA) and 8-phenyltheophylline (8-PT) were purchased from Research Biochemicals Inc. (Natick, MA, U.S.A.). A stock 10 mM 8-PT solution was prepared (MeOH: 1N NaOH/4:1 v/v made up to 10 ml with distilled water); aliquots were diluted daily 100 fold in distilled water. This solvent alone proved inactive.

Statistical analysis

Paired or unpaired Student's t test was used to compare IC_{50} values or to analyse the effect of a drug before/after a pharmacological manipulation. Analysis of variance was used when applicable. In all cases statistical significance was set at a P value less than 0.05.

Results

Blockade of the bladder motor activity evoked by nerve stimulation; antagonism by 8-phenyltheophylline

Application of 10 μM ADO and related structural analogues caused a significant downward displacement of the frequency-contraction curve elicited by transmural stimulation of the bladder nerve terminals. Independent of the potency of the analogues, the inhibition of the muscular twitch was of rapid onset; maximal effect was attained within approx. 30–60 s. The inhibition was readily reversible upon drug washout. The rank order of potency of ADO and related analogues was **R**-PIA > CHA > NECA > ADO > **S**-PIA (Figure 1). These results do not allow calculation of the exact potency ratio of the PIA enantiomers.

The inhibitory action of these compounds was antagonized by 8-PT; preincubation with 10 μM 8-PT caused about a 10 fold rightward parallel displacement of the NECA concentration-response curve (Figure 2). In addition, 8-PT caused a slowly rising increase in the nerve evoked muscular twitch without affecting the basal bladder tension (Figure 2). The estimated NECA 8-PT pA_2 value, based on the method of a single concentration of the antagonist, is 5.9; the K_e value is 799 nM.

Blockade at the ATP-induced motor responses

Tissue preincubation with ADO and related analogues resulted in a non-surmountable rightward displacement of the ATP-induced concentration-response curve. Figure 3 shows the effect of 10 μM NECA; the antagonism was easily reversible upon drug washout and sensitive to 8-PT (data not shown). The rank order of potency of the ADO agonists required to reduced to one half the tension caused by 25 μM ATP was: NECA > CADO > **R**-PIA > ADO > CHA > **S**-PIA. The potency ratio between **R**- and **S**-PIA was more than 300. Table 1 summarizes the IC_{50} values obtained in these experiments.

The action of NECA was not specific to ATP since it also depressed the contractions induced by 5-hydroxytryptamine, acetylcholine and prostaglandin $F_{2\alpha}$ (Table 2). Furthermore, 10 μM NECA caused a rightward shift of the acetylcholine concentration-response curve (Figure 4). This effect of 10 μM NECA was reduced by the simultaneous incubation of the tissue with 20 μM 8-PT. In contrast, 10 μM CHA did not significantly change the tissue sensitivity to the action of acetylcholine (Figure 5) nor ATP. The low tissue sensitivity to acetylcholine is explained by the presence of 1 μM atropine in the buffer solution.

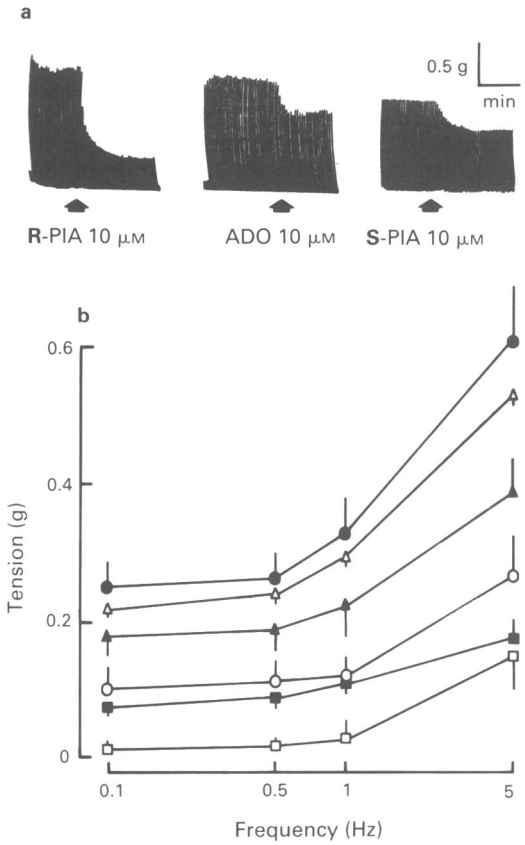

Figure 1 Adenosine (ADO) and related nucleosides-induced blockade of the muscular contractions elicited by transmural nerve stimulation frequency-response curve. Nervous stimulation of the bladder elicited non-adrenergic non-cholinergic contractions that were proportional to the frequency of stimulation; application of ADO and related drugs to the superfusion buffer containing 1 μM atropine plus 3.4 μM guanethidine reduced the magnitude of the contractions. (a) Polygraph tracings depicting the effect of 10 μM **R**-PIA, ADO and **S**-PIA on a 0.15 Hz train of pulses. (b) Frequency-response curve in the absence and in the presence of 10 μM ADO and related analogues with the number of separate experiments performed (in parentheses). Symbols indicate mean values: (●) control (14); (△) **S**-PIA (6); (▲) ADO (7); (○) NECA (6); (■) CHA (8) and (□) **R**-PIA (6). Bars show s.e.mean. For abbreviations, see text.

Evidences in support of an endogenous role of adenosine in bladder neurotransmission

In contrast to the ATP concentration-response curve that achieved a maximal contraction of about 0.5 g tension following 10 μM ATP, the tension developed after the addition of α, β methylene ATP was almost 2 g before it reached a maximal response (Figure 6). To examine whether this difference could be attributed, at least in part, to the genesis of ADO following tissue metabolism of the exogenous ATP, nucleotide concentration-response curves were obtained in the presence of 5–30 μM 8-PT. Figure 6b shows that 8-PT increased the maximal ATP contractile response without causing a significant leftward displacement of the ATP concentration-response curve. The largest concentrations of ATP produced contractions proportional to the dose of 8-PT; no evidence of a plateau response was observed. The use of 30 μM 8-PT did not displace to a larger extent the ATP concentration-response curve (data not shown).

In addition, as indicated in Figure 2, the application of 10 μM 8-PT caused a consistent 38 ± 2.2% ($n = 22$), ($P < 0.01$) increase in the twitch tension following a 0.15 Hz train of stimuli. Furthermore, tissue preincubation with 10 μM dipyridamole caused a minor, yet significant 5 ± 1% ($n = 10$), ($P < 0.01$) decrease in the twitch tension caused by the 0.15 Hz train of electrical pulses.

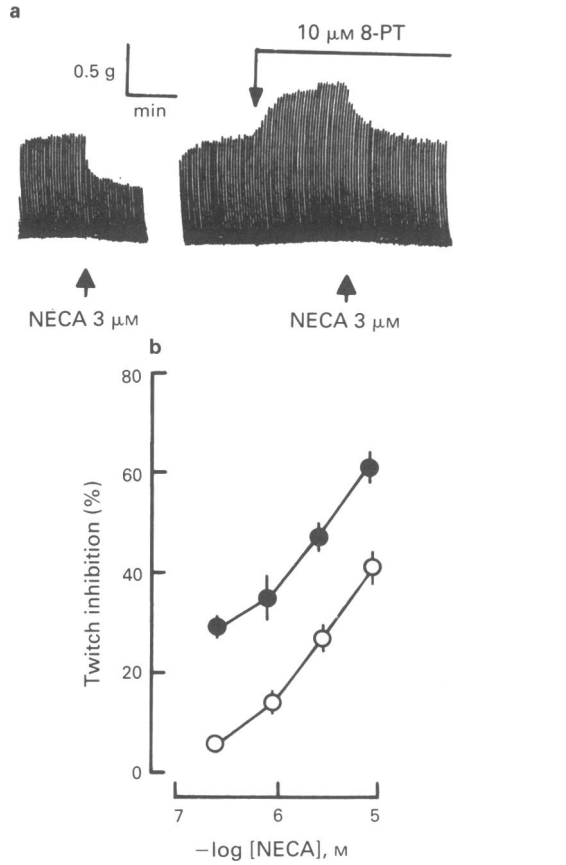

Figure 2 5'N-ethylcarboxamido adenosine (NECA)-induced blockade of the 0.15 Hz bladder twitch; antagonism by 8-phenyltheophylline (8-PT). (a) Polygraph, tracings show representative recording of the inhibitory action of NECA and its blockade by 10 μM 8-PT activity. (b) NECA concentration-response experiments performed in the absence (●) and following a 5 min preincubation with 10 μM 8-PT (○) ($n = 8$). Symbols indicate mean values, bars show s.e.mean.

Table 1 Potency of adenosine and related structural analogues in blocking the contractile activity caused by the exogenous application of 25 μM ATP to isolated superfused bladders of mice

Agonist	IC_{50} (μM)
5'N-ethylcarboxamide adenosine (7)	7 ± 1
2-Chloroadenosine (8)	48 ± 5
R-Phenylisopropyladenosine (6)	280 ± 140
Adenosine (6)	9700 ± 950
Cyclohexyladenosine (6)	57000 ± 3000
S-Phenylisopropyladenosine (6)	> 100.000

Values are given ± s.e.mean.
In parentheses, number of separate preparations used to perform these determinations.

Table 2 Lack of specificity of the 5'N-ethylcarboxamido adenosine (NECA) inhibiting action on the bladder motor activity

Agonist (10 μM)	*Control*	*+ 10 μM NECA*
5-Hydroxytryptamine (4)	0.11 ± 0.02	0.02 ± 0.007*
Acetylcholine (8)	0.08 ± 0.02	0.01 ± 0.006*
Prostaglandin $F_{2\alpha}$ (6)	0.15 ± 0.01	0.01 ± 0.007*

Values are given ± s.e.mean (g).
*$P < 0.01$ (paired Student's t test) as compared to its control before the application of NECA.

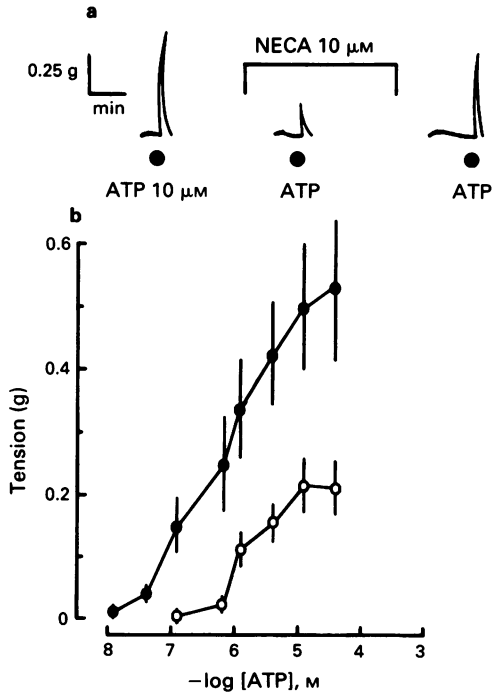

Figure 3 5'N-ethylcarboxamido adenosine (NECA) antagonism of the mouse bladder motor response to adenosine 5'-triphosphate (ATP). (a) Shows record of the effect of application of 10 μM ATP in the absence and in the presence of 10 μM NECA. (b) ATP concentration-response curves were obained in bladders prior to and following a 5 min incubation with 10 μM NECA ($n = 6$). Symbols indicate mean values: (●) control; (○) plus 10 μM NECA, $n = 6$ for both; bars show s.e.mean.

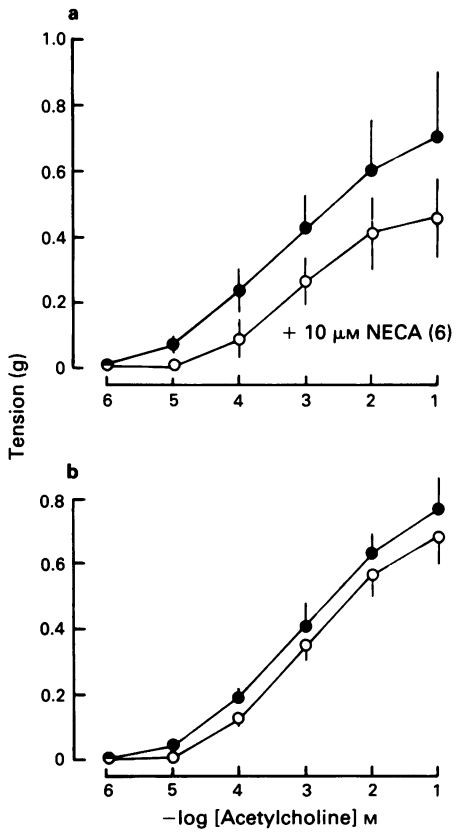

Figure 4 Acetylcholine motor concentration-response curves; inhibitory action of 5'N-ethylcarboxamido adenosine (NECA) and antagonism by 8-phenyltheophylline (8-PT) in urinary bladder. (a) Acetylcholine concentration-response curves prior to (●) and following a 5 min tissue preincubation with 10 μM NECA (○), ($n = 6$). (b) Acetylcholine concentration-response curves prior to (●) and following tissue preincubation with 10 μM NECA plus 20 μM 8-PT (○) ($n = 6$). Symbols indicate mean values; bars show s.e.mean.

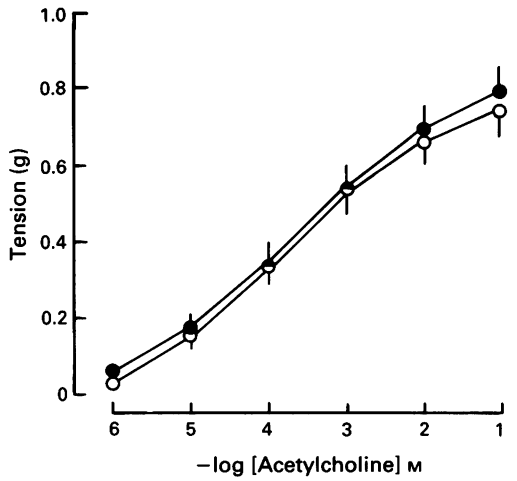

Figure 5 Lack of modification by cyclohexyladenosine (CHA) of the motor response to acetylcholine in the mouse bladder. Acetylcholine concentration-response curves were obtained prior to (●) and following a 5 min tissue incubation with 10 μM CHA (○) ($n = 6$). Symbols indicate mean values; bars show s.e.mean.

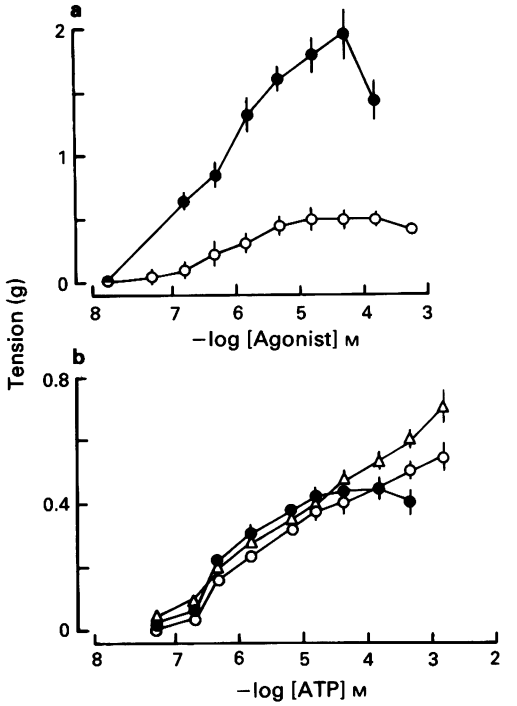

Figure 6 Influence of adenosine (ADO) on the adenosine 5'-triphosphate (ATP) motor activity in the bladder. (a) Concentration-response curves for ATP (○) ($n = 9$) and its slowly degradable analogue, α, β methylene ATP (●) ($n = 9$). (b) ATP concentration-response curves were obtained prior to (●) ($n = 14$) and following a 5 min tissue incubation with either 5 (○) ($n = 8$) or 10 (△) ($n = 6$) μM 8-phenyltheophylline. Symbols indicate mean values; bars show s.e.mean.

Discussion

The present results clearly demonstrate the presence of ADO receptors in the mouse bladder. This nucleoside and related structural analogues reduce the motor activity caused either by the exogenous application of ATP or by the neuronally released nucleotide. The effect of the nucleosides is antagonized by 8-PT, a non-selective ADO-A_1, A_2 receptor blocker. Interestingly, the rank order of potency of ADO and related ligands in blocking the nerve-evoked contractions is notably different from that required to reduce the motor effect of exogenous ATP. R-PIA and CHA are the most potent ligands in the former assay. The estimated R-PIA/S-PIA potency ratio although difficult to calculate from the present data, is appoximately 30. These data taken together are consistent with the notion that the ADO-induced blockade of the neurogenic contractions may be related to the activation of the ADO-A_1 receptor (Burnstock & Kennedy, 1985; Williams, 1987). In contrast, CHA is one of the least potent of the ADO analogues in blocking the motor action of exogenous ATP, while NECA was the most potent. These data could indicate that the blockade of the motor activity is tentatively related to ADO-A_2 receptor activation. However, this assumption is difficult to reconcile with the large PIA enantiomer potency-ratio observed.

With regard to the localization and these ADO receptor subtypes in the bladder, present results allow the interpretation that in all likelihood the ADO-A_2 receptor is postjunctional. Little information is available for the ADO-A_1 receptor. However, based on similar investigations performed in the rat vas deferens, Clanachan *et al.* (1977), and Huidobro-Toro & Parada (1989), proposed that the ADO-$_1$ receptor is of neuronal origin and operates a negative feedback mechanism regulating neurotransmitter release. It is reasonable to infer that the ADO-A_1 receptor in the mouse bladder might similarly have a neuronal, presynaptic location.

The pharmacology of the mouse bladder neurotransmission is compatible with the concept that its nerve terminals release ATP as a motor neurotransmitter (Acevedo & Contreras, 1985; Acevedo *et al.*, 1990). ATP initiates contractions following interaction with P_{2x} purinoceptors located on the smooth muscle membranes. Extracellular ATP is rapidly hydrolyzed and converted to ADO by extracellular bladder metabolism (Cusack *et al.*, 1984; 1988) and activates ADO-A_1 and A_2 receptors. The combined final result of ADO receptor activation is a reduction of the bladder excitability. These ideas are summarized in the scheme shown in Figure 7. Support for this model is found in the studies by Brown *et al.* (1979) who demonstrated that ADO reduces the carbachol-induced rat bladder contractions. However, Brown *et al.* (1979) failed to observe that theophylline potentiated the responses to either intramural nerve stimulation or ATP. There are several reasons for the differences which include, among others, species differences and the higher affinity of 8-PT for the ADO receptor.

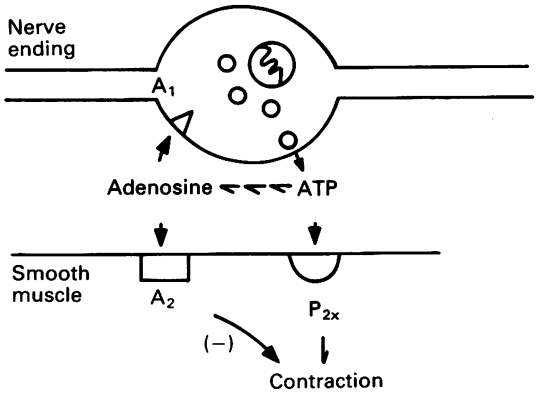

Figure 7 Hypothetical model for the organization of adenosine receptors in the mouse bladder nerve endings. Although the nature of the nerve endings releasing ATP remains undetermined, the nucleotide interacts with P_{2x} receptors to contract the muscle; rapid nucleotide hydrolysis generates adenosine which may interact at the neuroeffector junction with either adenosine-A_1 or -A_2 receptor to regulate neurotransmitter activity.

In accordance with this model, it is reasonable to expect that ADO could exert a physiological tonic influence on the bladder neurotransmission. The present results provide three lines of evidence to support this contention. First of all, ADO receptors are distributed in the bladder; pharmacodynamic studies are consistent with the existence of two ADO receptor subtypes. Furthermore, the expression of the mRNA for the ADO-A_1 and A_2 receptors is consonant with this notion (Reppert *et al.*, 1991; Weaver, personal communication). Second, blockade of ADO receptor subtypes with 8-PT results in a potentiation of the motor contractions elicited by either the exogenous application of the transmitter or via nerve-evoked release of ATP. In this regard, 8-PT does not displace leftwards the ATP concentration-response curve but increases the maximal ATP response. This finding could tentatively be explained in two ways: on the one hand, the amount of ADO generated from extracellular ATP at the neuroeffector junction, and on the other, the affinity of ADO for its receptors. Our data indicate that the potency of ADO is relatively low as compared to that of other ligands; concentrations of ADO in the range of 100–1000 μM are required for the antagonism to occur. The intense contractions elicited by α, β methylene ATP, can be accounted for at least in part, by its slow rate of enzymatic degradation to ADO (Kasakov & Burnstock, 1983). Thirdly, blockade of ADO uptake by dipyridamole (Bender *et al.*, 1981; Marangos *et al.*, 1985) produced a consistent, albeit minor, inhibitory action which may be interpreted as due to a larger concentration of ADO at the neuroeffector junction.

The model proposed for the regulatory action of ADO is not limited to the bladder nor to NANC neurotransmission. Huidobro-Toro *et al.* (1989), demonstrated similar findings in the rat vas deferens where ATP is also thought to be involved as a motor transmitter (Fedan *et al.*, 1981; Sneddon & Westfall, 1984; Sneddon & Burnstock, 1984; Stjarne & Astrand, 1984), and Sebastiao & Ribeiro (1986) demonstrated evidence for an inhibitory role of endogenous ADO at the frog neuromuscular junction. Furthermore, the inhibitory role of ADO at the central nervous system is relatively well established. Because adenosine antagonism by xanthines can increase neurotransmitter release, cell firing, and behavioural activity, it is generally agreed that ADO is normally present extracellularly and that it may fulful a homeostatic role (Williams, 1987; White, 1988; Contreras, 1990). Hence, the concept of a purinergic inhibitory tone has become accepted in the central and peripheral nervous system. It is thus possible to propose that ADO is a regulator at a variety of synapses or neuroeffector junctions, where ATP participates in neurotransmission either as the transmitter itself or as a cotransmitter, in addition to synapses where ADO might be involved as a paracrine neuromodulator.

Little is as yet known concerning the intracellular signalling mechanisms operated by ADO-A_1 and A_2 receptors for exerting the proposed regulatory action. Although initially the action of ADO was related to modulation of adenylyl cyclase activity, recent evidence favours the notion that cyclic AMP does not appear to be an obligatory second messenger at least for the electrophysiological effects of ADO (Dunwiddie & Fredholm, 1984; Williams, 1987; Fredholm *et al.*, 1990). There are some indications that the ADO-A_1 receptor-induced inhibition of neurotransmitters release may be linked to a negative regulation of Ca^{2+} fluxes (Ribeiro *et al.*, 1979; Phillis *et al.*, 1984; Fredholm & Dunwiddie, 1988), protein kinase C stimulation or the opening of K^+ channels (Fredholm *et al.*, 1990). It is possible that the ADO-A_2 receptor is concerned with blockade of Ca^{2+} currents or stimulation of adenylyl cyclase activity (Fredholm *et al.*, 1990). The multiplicity of mechanisms operated by ADO and its pre and postsynaptic localization could, perhaps, help to understand both the non-competitive nature of the ADO-induced ATP antagonism and its lack of selectivity. The combined action at these two receptor sites might explain general reduction by ADO of the bladder excitability.

In conclusion, it is suggested that ADO is actively involved in the regulation of the NANC bladder neurotransmission. We propose a novel synaptic regulatory mechanism where the action of a transmitter, or cotransmitter, may be modulated by the extracellular genesis of an active inhibitory metabolite. In the particular case of the NANC transmission, in the mouse bladder, ADO via the operation of a multiplicity of mechanisms inhibit the excitability of the neuroeffector junction which is activated by ATP. The physiology of ATP and ADO, as well as the origin of ADO and the pharmacodynamics of its receptors remain a challenge that warrants further investigation.

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Phosphodiesterase inhibition in ventricular cardiomyocytes from guinea-pig hearts

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1 The present study compared the cyclic nucleotide phosphodiesterase (PDE) activities in cardiomyocytes and ventricular cardiac tissue from guinea-pigs. The aim of the study was to determine whether PDE activities in ventricular tissue accurately reflect the isoenzymes present in cardiomyocytes.

2 In homogenates of cardiomyocytes and multicellular ventricular tissue, four distinct soluble PDE activities could be separated by DEAE-sepharose chromatography.

3 In multicellular cardiac tissue as well as in cardiomyocyte preparations, adenosine 3':5'-cyclic monophosphate (cyclic AMP) PDE isoenzymes I–IV were comparable in terms of substrate affinities, and inhibition or stimulation by guanosine 3':5'-cyclic monophosphate (cyclic GMP). However, in cardiomyocytes the V_{max} values of PDE I–IV were lower by a factor of about 2 to 7 and the basal activities were lower by a factor of about 3 to 5 as compared to multicellular cardiac tissue.

4 To investigate whether the PDE I–IV activities were similarly inhibited by PDE inhibitors in both preparations, we studied the effects of 3-isobutyl-1-methylxanthine (IBMX), UD-CG 212 Cl (2-(4-hydroxy-phenyl)-5-(5-methyl-3-oxo-4,5-dihydro-2H-6-pyridazinyl)benzimidazole HCl) and rolipram. UD-CG 212 Cl was a selective PDE III inhibitor in cardiomyocytes (IC_{50} 0.3 μmol l^{-1}) and in ventricular tissue (IC_{50} value 0.1 μmol l^{-1}). Rolipram selectively inhibited PDE IV in cardiomyocytes (IC_{50} 1.4 μmol ml^{-1}) and in ventricular tissue (IC_{50} 1.1 μmol l^{-1}) whereas IBMX was a nonselective PDE inhibitor in both preparations.

5 It is concluded that the PDE isoenzymes I–IV from multicellular ventricular tissue can be used as a representative system for investigating PDE inhibiting properties of PDE inhibitors in the myocardium since comparable PDE isoenzymes I–IV exist in guinea-pig ventricular cardiomyocytes and multicellular ventricular tissue.

Keywords: Phosphodiesterases; ventricular cardiomyocytes; multicellular ventricular tissue; guinea-pig heart; PDE inhibitors

Introduction

A number of different cyclic nucleotide phosphodiesterase isoenzymes (PDE) have been detected in various species and tissues. Five different families exist and more than 20 distinct isoenzymes are now recognized (Beavo & Reifsnyder, 1990). In the human heart four subtypes (PDE I–IV) have been defined by anion exchange chromatography (Reeves *et al.*, 1987; Bethke *et al.*, 1989; Silver *et al.*, 1990). The PDE isoenzymes vary according to substrate specificity and kinetic characteristics (Weishaar *et al.*, 1986; Reeves *et al.*, 1987; v. der Leyen, 1989).

Most of the positive inotropic agents developed in the last decade are PDE inhibitors. The PDE inhibiting effects of compounds such as milrinone and amrinone (Kariya *et al.*, 1982; Wilmshurst *et al.*, 1984; Weishaar *et al.*, 1986), adibendan (Bethke *et al.*, 1988), pimobendan (Meyer *et al.*, 1989; v. der Leyen *et al.*, 1991), saterinone (Brunkhorst *et al.*, 1988), isomazole (Bethke *et al.*, 1991a) and enoximone (Kariya *et al.*, 1982; Bethke *et al.*, 1992) have been mainly investigated in PDE isoenzymes from homogenates of entire multicellular ventricular tissue. Recently, the PDE inhibitory effects of milrinone, rolipram and some other compounds have been examined in cardiomyocytes from the rat heart (Bode *et al.*, 1991).

Since most tissues are of heterogeneous composition, homogenates of cardiac ventricular tissue contain cardiomyocytes as well as non-myocardial cells such as cells from endothelium, vascular smooth muscle or connective tissue. Therefore, it is likely that all of the above mentioned studies with myocardial PDE were conducted with a mixture of isoenzymes from several cardiac cell types. It is unknown whether the pattern of PDE activities found in the heart is specific for cardiomyocytes or whether other cell types contribute significantly to the isoenzyme pattern (Tenor *et al.*, 1987).

The aim of the study was to characterize the PDE isoenzymes in guinea-pig ventricular cardiomyocytes and to differentiate between these PDE isoenzymes and the PDE isoenzyme pattern found in multicellular ventricular tissue from the same species. In order to exclude a contamination with PDE isoenzymes from other cardiac cells, we used a purified preparation of guinea-pig ventricular cardiomyocytes containing only about 1% of non-myocardial cells (Neumann *et al.*, 1989; Behnke *et al.*, 1990). Furthermore, we studied the effects of a nonselective (3-isobutyl-1-methylxanthine, IBMX), a PDE III-selective (2-(4-hydroxy-phenyl)-5-(5-methyl-3-oxo-4,5- dihydro-2H-6-pyridazinyl) benzimidazole·HCl, UC-CG 212 Cl) and a PDE IV-selective PDE inhibitor (rolipram) on PDE I–IV activities from both preparations to elucidate possible differences in the inhibition profiles of the three compounds.

Some of these results were presented at the Autumn meeting of the Deutsche Gesellschaft für Pharmakologie und Toxikologie in Berlin, Germany (Bethke *et al.*, 1991c).

Methods

Isolation of phosphodiesterase I–IV

Phosphodiesterase isoenzymes were separated by DEAE-sepharose anion exchange liquid chromatography as describ-

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ed previously (Bethke *et al.*, 1991b) by the method of Reeves *et al.* (1987) with minor modifications. In brief, ventricular and septal muscle of guinea-pig hearts (either sex, body weight 280–450 g, 8–10 animals for each separation) were used. The guinea-pigs were killed by cervical dislocation and bled from the carotid arteries. Their hearts were quickly excised, the ventricular and septal muscles dissected and placed in ice-cold homogenization buffer (composition in mM: Bistris 20, 2-mercaptoethanol 5, benzamidine 2, EDTA 2, phenylmethylsulphonylfluoride 50, sodium acetate 50, pH 6.5). After determination of the wet weight, all subsequent procedures were carried out at 4°C. For each separation procedure 8–12 g of tissue were used. The tissue was cut into pieces with scissors and homogenized in 30 ml of homogenization buffer in a polytron instrument (type PT 10-35; Kinematica, Littau-Luzern, Switzerland) for 10 s at setting 3 and 10 s at setting 6 and with a glass Teflon homogenizer (Colora, Lorch, Germany) for 2 min at 250 r.p.m. The homogenate was centrifuged at 30,000 *g* for 20 min. The resulting supernatant was filtered through four layers of gauze and applied to a DEAE-sepharose column (40 × 1.6 cm; bed volume about 30 ml) which was equilibrated with homogenization buffer. After washing the column with 120 ml of homogenization buffer, the phosphodiesterases were eluted with a continuous 50–1000 mM sodium acetate gradient in homogenization buffer (pH 6.5, volume 400 ml, flow rate approximately 45 ml h^{-1}). Fractions of 7 ml were collected and immediately assayed for adenosine 3':5'-cyclic monophosphate (cyclic AMP) and guanosine 3':5'-cyclic monophosphate (cyclic GMP) PDE activity (substrate concentration 1 μM cyclic AMP or cyclic GMP). The cyclic AMP PDE activity was also assayed in the presence of 0.5 μM cyclic GMP (additional characterization of PDE II–IV). To differentiate further between PDE III and IV, the selective PDE IV inhibitor, rolipram (Reeves *et al.*, 1987) and the selective PDE III inhibitor, UD-CG 212 Cl (Bethke *et al.*, 1991a) were used. Appropriate peak fractions were pooled (referred to as PDE I–IV), stored at 4°C, and used for the drug experiments within 2 weeks. Kinetic parameters were determined on the second day after separation.

Isolation of cardiomyocytes

Cardiomyocytes were isolated as described by Behnke *et al.* (1990). Guinea-pigs (500–700 g) were killed by cervical dislocation. Hearts were excised rapidly, mounted on a modified Langendorff perfusion system and perfused (STA-multipurpose pump, Desaga, Heidelberg, Germany) retrogradely via cannulation of the aorta in a nonrecirculating manner at a constant rate of 10 ml min^{-1} for 3 min with a calcium-free buffer (solution A) containing (in mM): NaCl 100.0, KCl 10.0, KH_2PO_4 1.2, $MgSO_4$ 5.0, glucose 20.0, taurine 50.0, 3-(N-morpholino)propane-sulphonic acid (MOPS) 10.0, gassed with O_2 and maintained at 37°C; the pH was adjusted to 6.9. After removing blood cells by washing, the hearts were perfused with the same medium containing 0.1% collagenase in a recirculating fashion at a rate of 30 ml min^{-1} for 27 min. Following enzyme perfusion, atria were cut off and ventricles were minced and incubated for 1 h in solution B ('power soup' according to Isenberg & Klöckner, 1982) consisting of (in mM): KCl 70.0, K_2HPO_4 30.0, $MgSO_4$ 20.0, taurine 20.0, succinic acid 5.0, creatine 5.0, EGTA 1.0, β-hydroxybutyric acid 7.3, pyruvic acid 5.0, Na_2ATP 5.0, gassed with O_2 at 37°C, pH adjusted to 7.4. The cell suspension was passed through 200 μm mesh nylon gauze (Heidland, Gütersloh, Germany) and centrifuged for 3 min at 25 *g* (MSE 18, Measuring Scientific Equipment, Crewly, England). Intact rectangular rod shaped cells sedimented to the bottom of the tube. Damaged and rounded cells form a distinct layer at the surface. The pellet of intact rectangular cardiomyocytes was resuspended in solution A. Calcium was increased gradually to 0.38 mM. This cell suspension was centrifuged (MSE SK 1, Measuring Scientific Equipment, Crewly, England) for 1 min through a gradient of solution A containing 4% albumin to remove endothelial cells. The pellet containing 65 to 90% viable rod shaped-cardiomyocytes was washed twice in Tyrode solution containing (in mM): NaCl 119.8, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 22.6, Na_2EDTA 0.05, ascorbic acid 0.28, glucose 5.0, continuously gassed with 95% O_2 + 5% CO_2 and maintained at 35°C, pH 7.4) and resuspended in solution A to attain a concentration of 3×10^6 cells ml^{-1}. The amount of cardiomyocytes was determined with a haemocytometer. Finally the cells were frozen in liquid N_2 and stored at −80°C until PDE isoenzyme isolation. The amount of endothelial cells was detected according to Neels *et al.* (1983), by passing the cells through an albumin-gradient the activity of the endothelial marker angiotensin converting enzyme (Ondetti & Cushman, 1982) was reduced from 7.56 ± 0.53 to 0.87 ± 0.15 nmol mg^{-1} protein per min ($n = 5$). In addition, histological examinations of the cell preparation according to Burnstone (1962) revealed only about 1% of non-muscle cells.

Isolation of phosphodiesterase I–IV from ventricular cardiomyocytes

The procedure to separate the PDE isoenzymes from ventricular cardiomyocytes differed slightly from that described for homogenates from whole ventricular tissue. In brief, ventricular cardiomyocytes from 4–6 guinea-pig hearts (approximately 6×10^6 cardiomyocytes per heart) were homogenized in 20 ml of homogenization buffer as described above. The homogenate was centrifuged at 30,000 *g* for 90 min. The resulting supernatant was applied to a DEAE-sepharose column (20 × 1 cm; bed volume about 10 ml) which was equilibrated with homogenization buffer. After washing the column with 30 ml of homogenization buffer the phosphodiesterases were eluted from the column as described above. The flow rate was approximately 15 ml h^{-1}. Fractions of 3 ml were collected and assayed for cyclic AMP and cyclic GMP PDE activity.

Determination of phosphodiesterase activity

PDE activity was determined in a two-step procedure according to the method of Thompson & Appleman (1971). Details are given by Bauer & Schwabe (1980) and Bethke *et al.* (1988). For the determination of the PDE activity of the fractions, aliquots of the eluate were diluted in 0.1% bovine serum albumin and used in the assay. The substrate conversion was kept below 20%, in order to be in the linear range of the enzyme reaction. The reaction mixture consisted of $MgCl_2$ 5 mM, Tris-HCl 40 mM, pH 8.0, cyclic AMP 1 μM or cyclic GMP 1 μM, 20,000–30,000 c.p.m. [^{3}H]-cyclic AMP or [^{3}H]-cyclic GMP, and cyclic GMP 0.5 μM or rolipram 10 μM in some experiments. For the evaluation of the inhibitory effects of IBMX, UD-CG 212 Cl and rolipram the pooled peak fractions (PDE I–IV), diluted in 0.1% bovine serum albumin, were used. Except for the solvent dimethylsulphoxide (DMSO) all substances investigated did not interfere with the assay procedure. Due to poor water-solubility, rolipram and UD-CG 212 Cl had to be solved in 2% DMSO (v/v). This concentration inhibited PDE activities by about 10%. The calculation of the amount of PDE inhibition by rolipram and UD-CG 221 Cl was related to the basal activity of the enzyme in the presence of DMSO. Protein concentrations were determined by the Bio-Rad Protein Assay (Bradford, 1976).

Data analysis

Values presented are means ± s.e.mean. Statistical significance was estimated by Student's *t* test for paired or unpaired observations. A *P* value of less than 0.05 was considered significant. Concentrations of drugs reducing the basal

activity of PDE to 50% (IC_{50}) were determined graphically. IC_{50} values are given as geometric means with 95% confidence limits.

Materials

Drugs and reagents used were 3-isobutyl-1-methyl-xanthine (IBMX; EGA Chemie, Steinheim, Germany, stock solution: 0.01 M in H_2O; UD-CG 212 Cl (2-(4-hydroxy-phenyl)-5-(5-methyl -3- oxo-4,5-dihydro-2H-6- pyridazinyl) benzimidazole HCl) (Dr Karl Thomae GmbH, Biberach, Germany, stock solution: 0.01 M in 100% DMSO), rolipram (Schering, Berlin, Germany, stock solution: 0.01 M in 100% DMSO), DEAE-Sepharose Cl-6B (Pharmacia LKB, Freiburg, Germany), [^{3}H]-cyclic AMP and [^{3}H]-cyclic GMP (specific activity: [^{3}H]-cyclic AMP, 31.5 Ci mM^{-1}; [^{3}H]-cyclic GMP, 33.9 Ci mM^{-1}; NEN, Dreieich, Germany). All chemicals were of analytical or best commercial grade available. Deionized and twice distilled water was used throughout.

Results

Phosphodiesterase isoenzyme profiles

Four cyclic nucleotide phosphodiesterases could be resolved from homogenates of guinea-pig isolated ventricular cardiomyocytes or multicellular ventricular tissue by DEAE-sepharose chromatography. Typical elution profiles are given in Figure 1 (ventricular cardiomyocytes) and Figure 2 (multicellular ventricular tissue). The peaks were labelled PDE I–IV, according to Reeves *et al.* (1987). In both preparations, the order of elution and the sodium acetate-concentration eluting the PDE isoenzymes I–IV were comparable.

Kinetic studies revealed comparable K_m values for PDE I–IV in both preparations (Table 1). In ventricular cardiomyocytes from guinea-pig hearts, PDE I was a low K_m enzyme (K_m values for cyclic AMP 0.6 μM and cyclic GMP 0.5 μM). In ventricular multicellular tissue, PDE I revealed similar K_m values for cyclic AMP and cyclic GMP (0.5 μM each). PDE II revealed high K_m values for cyclic AMP and cyclic GMP in cardiomyocytes (51.6 μM and 26.5 μM, respectively) as well as in ventricular tissue (82.3 μM and 21.4 μM). The cyclic AMP hydrolysis by PDE II was stimulated by a low concentration (0.5 μM) of cyclic GMP in both preparations (Figures 1b, 2b) by 2.3 fold in ventricular tissue and by 2 fold in cardiomyocytes. In cardiomyocytes, PDE III had a low K_m for cyclic AMP (0.8 μM) and for cyclic GMP (0.3 μM). Similar values were obtained in ventricular tissue (K_m for cyclic AMP 0.3 μM and for cyclic GMP 0.2 μM). As previously described by Weishaar *et al.* (1986) and by Brunkhorst *et al.* (1989) the V_{max} was lower for cyclic GMP hydrolysis than for cyclic AMP hydrolysis in both cases (Table 1). The cyclic AMP PDE III activity was inhibited by 0.5 μM cyclic GMP (Figures 1b, 2b). PDE IV also revealed a relatively low K_m for cyclic AMP (1.4 μM) in cardiomyocytes as well as in ventricular tissue (1.5 μM), with virtually no cyclic GMP hydrolytic activity. Further characterization of PDE IV was achieved with the selective PDE IV inhibitor rolipram (10 μM). Rolipram inhibited cyclic AMP PDE IV only (Figures 1c, 2c). The concentration of rolipram was chosen so that the activity of the isoenzyme PDE IV was almost abolished while PDE II was not inhibited. The characteristics of PDE I–IV being demonstrated in this study for both preparations correspond to those described by Reeves *et al.* (1987) for PDE from guinea-pig hearts.

However, kinetic studies revealed lower V_{max} values for PDE I, II (by a factor of about 7 each), and III (by a factor of about 2) than in ventricular cardiomyocytes. The V_{max} of PDE IV was slightly lower by a factor of 1.5 in cardiomyocytes. Additionally, the basal activities of PDE I–IV from ventricular cardiomyocytes were lower by a factor of about 4 to 5 for PDE I and II, respectively, and by a factor of about 3 for PDE III and IV as compared to those of ventricular tissue (Table 1).

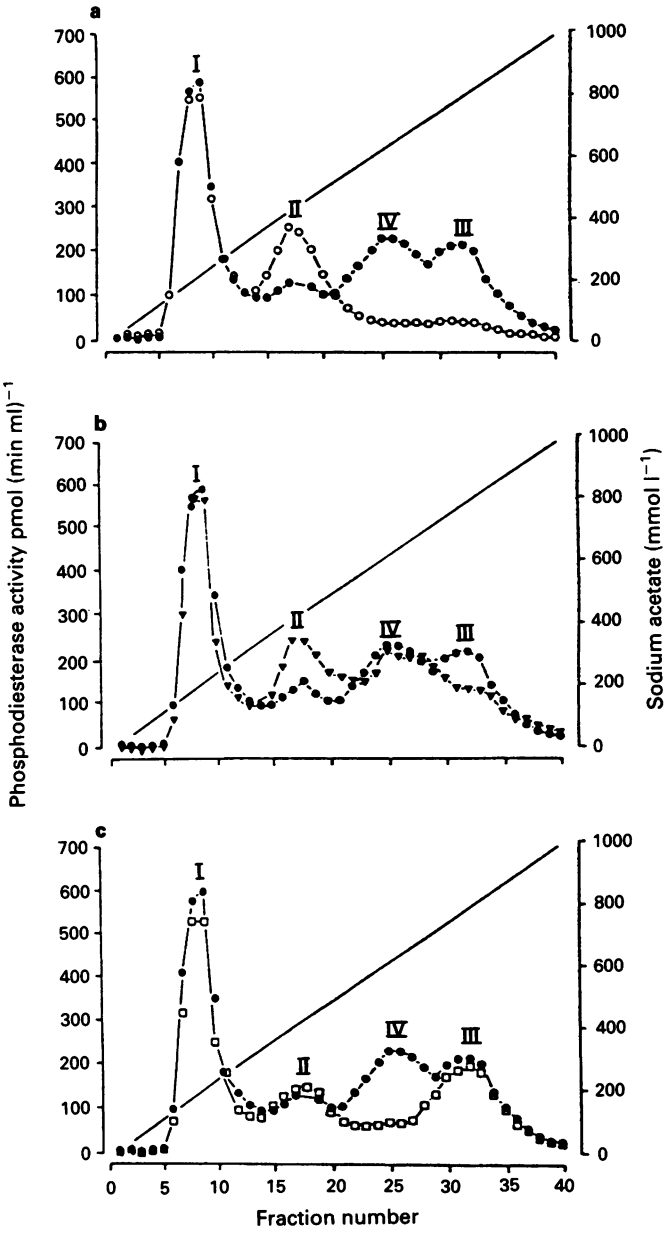

Figure 1 Typical DEAE-sepharose chromatographic profile of myocardial phosphodiesterase (PDE) isolated from guinea-pig ventricular cardiomyocytes. Four different PDE isoenzymes were separated and designated PDE I–IV according to Reeves *et al.* (1987). Fractions (3 ml each) eluted by a linear 50–1000 mM sodium acetate gradient (right ordinates) were directly assayed for PDE activity. Substrate concentrations: 1 μM cyclic AMP or cyclic GMP. (a) Cyclic AMP PDE (●) or cyclic GMP PDE activity (○). (b) Cyclic AMP PDE activity in the absence (●) or presence of 0.5 μM cyclic GMP (▼); note inhibition of PDE III. (c) Cyclic AMP PDE activity in the absence (●) or presence of 10 μM rolipram (□); note inhibition of PDE IV. Each curve is representative for similar separations from three different experiments. Left ordinates: PDE activity in pM hydrolysed cyclic AMP or cyclic GMP (min ml)$^{-1}$.

Effects of phosphodiesterase inhibitors on PDE activity

To investigate whether there are differences between the inhibition of PDE isoenzyme activities from cardiomyocytes and ventricular tissue, we studied the effects of IBMX, UD-CG 212 Cl and rolipram on PDE I–IV from both prepara-

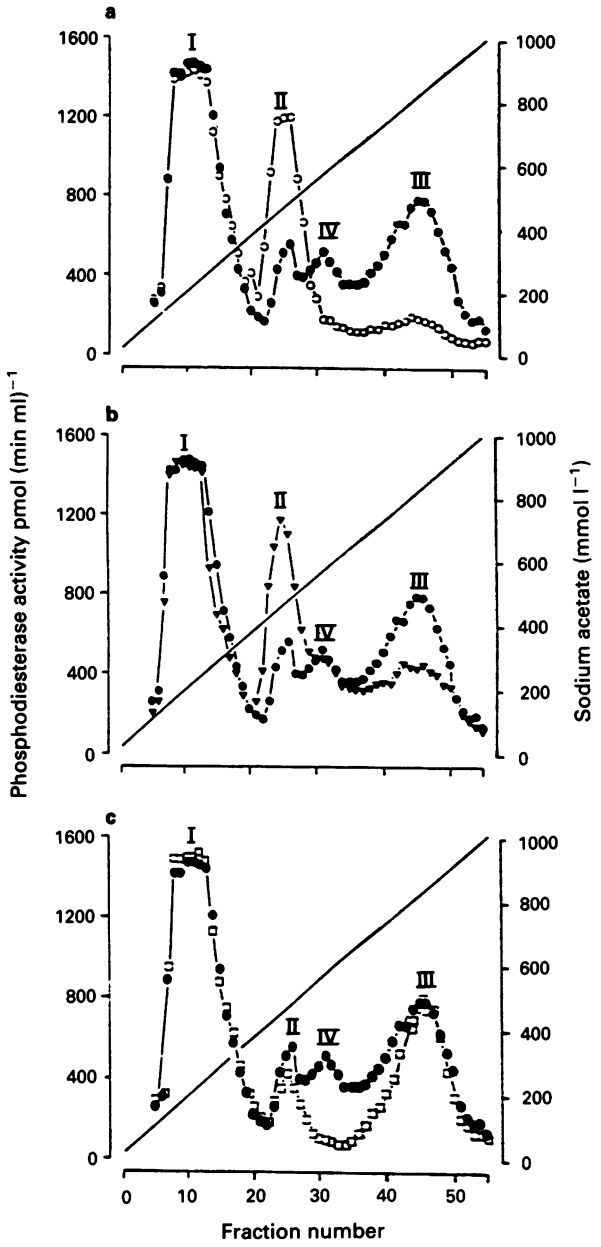

Figure 2 Typical DEAE-sepharose chromatographic profile of myocardial phosphodiesterase (PDE) isolated from multicellular guinea-pig myocardium. Four different PDE isoenzymes were separated and designated PDE I–IV according to Reeves *et al.* (1987). Fractions (7 ml each) eluted by a linear 50–1000 mM sodium acetate gradient (right ordinates) were directly assayed for PDE activity. Substrate concentrations: 1 μM cyclic AMP or cyclic GMP. (a) Cyclic AMP PDE (●) or cyclic GMP PDE activity (○). (b) Cyclic AMP PDE activity in the absence (●) or presence of 0.5 μM cyclic GMP (▼); note inhibition of PDE III. (c) Cyclic AMP PDE activity in the absence (●) or presence of 10 μM rolipram (□); note inhibition of PDE IV. Each curve is representative for similar separations from three different experiments. Left ordinates: PDE activity in pM hydrolysed cyclic AMP or cyclic GMP (min ml)$^{-1}$.

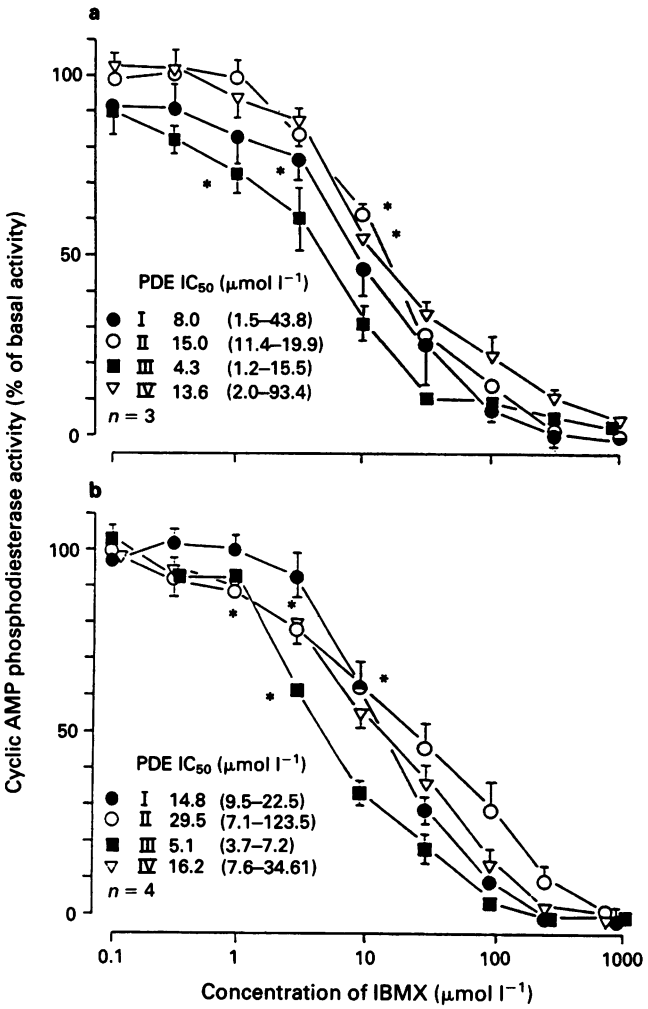

Figure 3 Effects of 3-isobutyl-1-methylxanthine (IBMX) on the activity of cyclic AMP PDE I–IV from ventricular cardiomyocytes (a) and multicellular ventricular tissue of guinea-pig hearts (b). Ordinates: cyclic AMP PDE activity as % of basal activity. Abscissae: concentration of IBMX in μM. Substrate concentration 1 μM. Incubation time 10 min at 30°C. Basal activity in pM cyclic AMP (mg prot min)$^{-1}$ are given in Table 1. In the inset IC_{50} values are given with 95% confidence limits in parentheses. * denotes the lowest value significantly different from the predrug value ($P<0.05$).

tions. IBMX concentration-dependently and nonselectively inhibited PDE I–IV in both preparations (Figure 3). IC_{50} values for PDE I–IV in cardiomyocytes were (μM): 8.0, 15.0, 4.3, 13.6 and for ventricular tissue: 14.8, 29.5, 5.1, 16.2, respectively (Table 2). UD-CG 212 Cl was a selective PDE III inhibitor (Figure 4). This agent diminished cyclic AMP PDE III activity at concentrations which were about 2–3 orders of magnitude lower than those required for the inhibition of cyclic AMP PDE I, II and IV. Similar effects could be demonstrated in cardiomyocytes (IC_{50} values for PDE III: 0.3 μM) and in ventricular tissue (IC_{50} values for PDE III: 0.1 μM). Rolipram selectively inhibited PDE IV in both preparations with an IC_{50} of 1.4 μM in cardiomyocytes and 1.1 μM in ventricular tissue (Figure 5). Rolipram reduced cyclic AMP PDE IV activity at concentrations which were almost 2–3 orders of magnitude lower than those required for the inhibition of the other isoenzymes.

Discussion

Since cardiac tissue is heterogeneous with respect to cell type, homogenates of cardiac ventricular tissue contain fragments of cardiomyocytes as well as fragments of endothelial cells, connective tissue and vascular smooth muscle cells. It seems conceivable that recent studies investigating the effects of PDE inhibitors on the PDE subtypes in homogenates from the entire multicellular ventricular tissue were conducted with a mixture of PDE isoenzymes from different cell types of the heart. Therefore, it is as yet unknown which types of PDE isoenzymes exist in guinea-pig cardiomyocytes, the target cells for positive inotropic PDE inhibitors.

Table 1 Kinetic properties and basal activities of phosphodiesterase (PDE) isoenzymes I–IV of guinea-pig ventricular cardiomyocytes (**A**) and multicellular ventricular tissue (**B**)

Enzyme	K_m *for cyclic AMP* (μM)	K_m *for cyclic GMP* (μM)	V_{max} *with cyclic AMP* (nM mg^{-1} prot·min)	V_{max} *with cyclic GMP* (nM mg^{-1} prot·min)	*Ratio:* V_{max} *cyclic AMP* / V_{max} *cyclic GMP*	*Basal activity pM cyclic AMP* mg^{-1} *prot·min*
A. *Guinea-pig ventricular cardiomyocytes* (n = 3)						
PDE I	0.6 ± 0.04	0.5 ± 0.03	1.1 ± 0.2*	1.4 ± 0.3*	0.8	+531.0 ± 72.6*
PDE II	51.6 ± 7.3	26.5 ± 9.8	46.3 ± 10.4*	69.3 ± 19.9*	0.7	+310.2 ± 44.5*
PDE III	0.8 ± 0.5	0.3 ± 0.05	1.7 ± 0.5*	0.2 ± 0.01*	8.5	+718.1 ± 144.5*
PDE IV	1.4 ± 0.1	ND	3.2 ± 0.05*	ND	ND	+746.3 ± 115.8*
B. *Guinea-pig multicellular ventricular tissue* (*n* = 4)						
PDE I	0.5 ± 0.2	0.5 ± 0.03	6.9 ± 0.9	8.2 ± 1.1	0.9	++2049.5 ± 298.3
PDE II	82.3 ± 22.0	21.4 ± 0.6	353.3 ± 33.9	257.2 ± 30.5	1.4	++1670.2 ± 231.8
PDE III	0.3 ± 0.07	0.2 ± 0.02	3.3 ± 0.3	0.7 ± 0.2	4.6	++2241.8 ± 468.6
PDE IV	1.5 ± 0.1	ND	4.7 ± 0.05	ND	ND	++1915.3 ± 135.8

ND = not determined.
* denotes significant differences vs. values obtained in multicellular ventricular tissue ($P < 0.05$); +$n = 9$; ++$n = 11$.

Table 2 IC_{50} value for inhibition of cyclic AMP phosphodiesterase (PDE) isoenzyme I–IV activity of guinea-pig isolated ventricular cardiomyocytes (**A**) and multicellular ventricular myocardium (**B**) by UD-CG 212 Cl, rolipram and 3-isobutyl-1-methylxanthine (IBMX)

	Cyclic AMP PDE, IC_{50} (μM) *guinea-pig ventricular cardiomyocytes*			
A.	*PDE I*	*PDE II*	*PDE III*	*PDE IV*
UD-CG 212 Cl	196.1 (98.0–392.1)	149.5 (76.3–293.1)	0.3 (0.01–8.3)	116.0 (13.9–967.7)
Rolipram	>1000	>1000	393.5 (216.0–716.6)	1.4 (0.6–3.2)
IBMX	8.0 (1.5–43.8)	15.0 (11.4–19.9)	4.3 (1.2–15.5)	13.6 (2.0–93.4)
B.	*Cyclic AMP PDE,* IC_{50} (μM) *guinea-pig ventricular tissue*			
UD-CG 212 Cl	269.7 (52.9–1376.1)	245.3 (52.9–1138)	0.1 (0.1–0.2)	77.2 (17.4–343.1)
Rolipram	>1000	>1000	>1000	1.1 (0.4–3.1)
IBMX	14.8 (9.5–22.9)	29.5 (7.1–123.5)	5.1 (3.7–7.2)	16.2 (7.6–34.6)

$n = 3$ for guinea-pig ventricular cardiomyocytes.
$n = 4$ for multicellular ventricular tissue from guinea-pig heart.
IC_{50} values are given as geometric means with 95% confidence limits in parentheses.

We studied the activities of the PDE isoenzymes in ventricular cardiomyocytes isolated from guinea-pig hearts in comparison with PDE I–IV from ventricular tissue. Furthermore, we investigated whether the PDE I–IV activities are similarly inhibited by PDE inhibitors in both preparations.

The most striking results of the present study are that in both preparations four PDE isoenzymes (PDE I–IV) were detected with virtually the same properties. PDE I–IV in the soluble fraction of cardiomyocytes were entirely analogous to PDE I–IV from multicellular ventricular cardiac tissue in terms of substrate affinities, sodium acetate-concentration at which the isoenzymes elute, and stimulation or inhibition by cyclic GMP. Moreover, all PDE inhibitors were similarly active in both preparations. The differences detected were a lower V_{max} of PDE I, II and III and a slightly lower V_{max} value for PDE IV in cardiomyocytes and lower basal activities in cardiomyocytes as compared to multicellular ventricular cardiac tissue. The higher V_{max} values and basal activities in the multicellular preparations, as compared to cardiomyocytes, may result from non-myocardial cells contaminating the PDE isoenzymes from ventricular tissue. The lower V_{max} values for PDE I–IV from cardiomyocytes might contribute to the lower basal activities detected in cardiomyocytes. In the rat heart, Bode *et al.* (1991) detected three peaks of PDE activities in the soluble fraction of isolated ventricular cardiomyocytes which were analogous to peak II, III and IV of multicellular ventricular myocardium from the same species. PDE I was missing in rat cardiomyocytes. Since guinea-pig cardiomyocytes contain all four isoenzymes it is very likely that there are species differences in the distribution of PDE activities in cardiomyocytes.

Most of the more recently developed positive inotropic PDE inhibitors have only been investigated in homogenates from multicellular ventricular tissue. To elucidate whether PDE inhibitors are similarly effective in inhibiting PDE activity from isolated cardiomyocytes and multicellular cardiac preparations we investigated the effects of selective (UD-CG 212 Cl, PDE III inhibitor, Bethke *et al.*, 1991a; rolipram, PDE IV inhibitors, Reeves *et al.*, 1987) and nonselective PDE inhibitors (IBMX, Bethke *et al.*, 1991b). In both preparations, similar results were obtained with the PDE inhibitors investigated. The low K_m cyclic GMP-inhibited cyclic AMP PDE III was of major interest in our investigation, since PDE III seems to be closely related to the cardiotonic effects of PDE inhibitors (Colucci *et al.*, 1986; Weishaar *et al.*, 1987; v. der Leyen, 1989). Inhibition of PDE III is probably mainly involved in positive inotropic effects of many positive inotropic agents regarded as selective PDE III inhibitors like amrinone and milrinone (Kariya *et al.*, 1982; Wilmshurst *et al.*, 1984; Weishaar *et al.*, 1986) enoximone (Kariya *et al.*, 1982; Bethke *et al.*, 1989; 1992), isomazole (Bethke *et al.*, 1991a), pimobendan (Meyer *et al.*, 1989; v. der Leyen *et al.*, 1991), adibendan (Bethke *et al.*, 1988) and saterinone (Brunkhorst *et al.*, 1988). But recent studies sug-

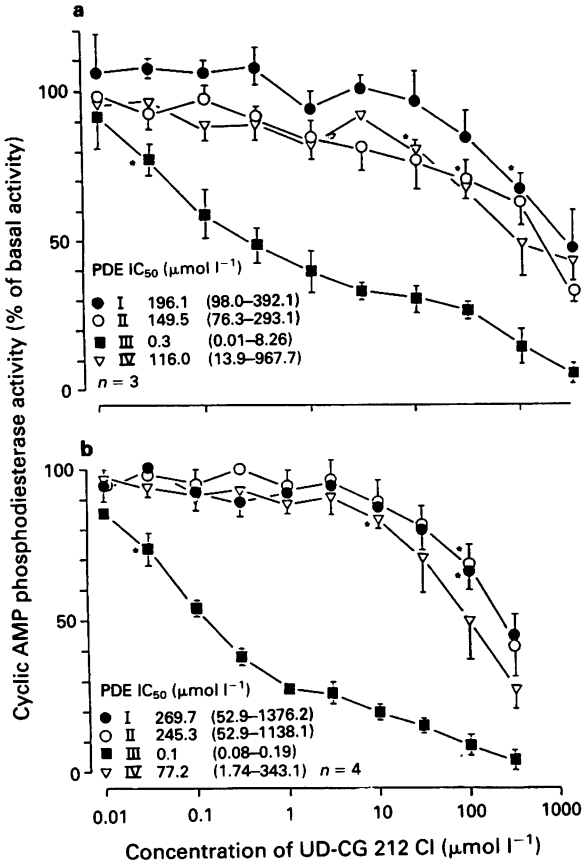

Figure 4 Effects of UD-CG 212 Cl on the activity of cyclic AMP PDE I–IV from ventricular cardiomyocytes (a) and multicellular ventricular tissue of guinea-pig hearts (b). Ordinates: cyclic AMP PDE activity as % of basal activity. Abscissae: concentration of UD-CG 212 Cl in μM. Substrate concentration 1 μM. Incubation time 10 min at 30°C. Basal activity in pM cyclic AMP (mg prot min)$^{-1}$ are given in Table 1. In the inset IC_{50} values are given with 95% confidence limits in parentheses. * denotes the lowest value significantly different from the predrug value ($P<0.05$).

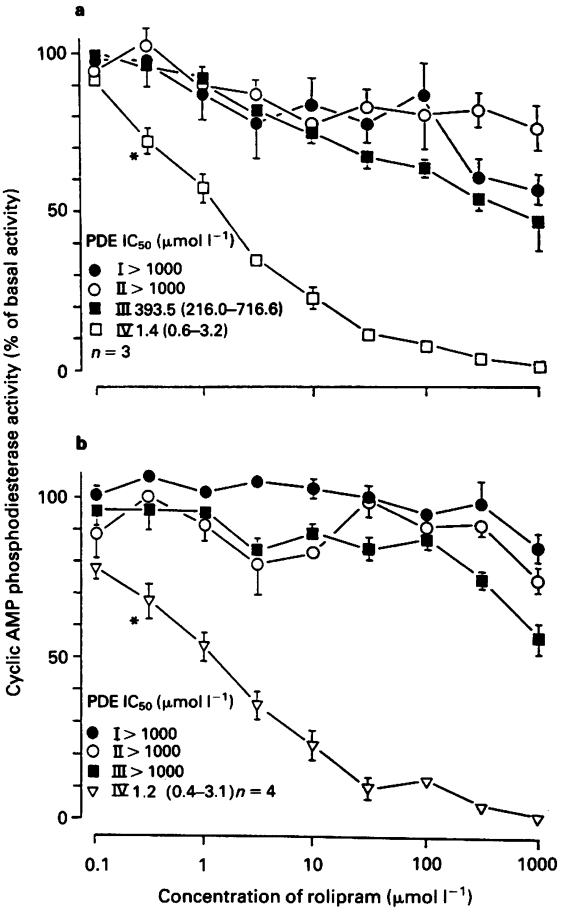

Figure 5 Effects of rolipram on the activity of cyclic AMP PDE I–IV from ventricular cardiomyocytes (a) and multicellular ventricular tissue of guinea-pig hearts (b). Ordinates: cyclic AMP PDE activity as % of basal activity. Abscissae: concentration of rolipram in μM. Substrate concentration 1 μM. Incubation time 10 min at 30°C. Basal activity in pM cyclic AMP (mg prot min)$^{-1}$ are given in Table 1. In the inset IC_{50} values are given with 95% confidence limits in parentheses. * denotes the lowest value significantly different from the predrug value ($P<0.05$).

gest that the rolipram-inhibited PDE IV might also be involved in the positive inotropic mechanism of action (Katano & Endoh, 1990; Klockow, 1990; Muller *et al.*, 1990; Shahid & Nicholson, 1990). Therefore, it was also interesting to investigate whether PDE IV is present in cardiomyocytes. PDE III and PDE IV from cardiomyocytes were similarly inhibited by the PDE inhibitors investigated as found with PDE III and IV from multicellular ventricular preparations, indicating that a contamination with PDE isoenzymes from other cardiac cell-types does not seem to be of importance with respect to PDE inhibition.

In both preparations, UD-CG 212 Cl was a very potent and selective PDE III inhibitor, diminishing cyclic AMP PDE III activity at concentrations which were markedly lower than those required for inhibition of the other isoenzymes. Similar results with this compound have been found with PDE IV from human failing heart (Bethke *et al.*, 1991a).

In isolated ventricular cardiomyocytes and multicellular cardiac tissue, PDE IV was selectively inhibited by rolipram. This compound reduced cyclic AMP PDE IV activity at concentrations which were markedly lower than those required for the inhibition of the other enzymes. Therefore, the involvement of PDE IV in the positive inotropic mechanisms as suggested (Katano & Endoh, 1990; Klockow, 1990; Muller *et al.*, 1990; Shahid & Nicholson, 1990) might even be possible at the myocardial cellular level. IBMX, regarded as a nonselective PDE inhibitor in guinea-pig ventricular tissue such as theophylline or trapidil (Bethke *et al.*, 1991b) also inhibited PDE I–IV nonselectively in cardiomyocytes.

We conclude that in guinea-pig ventricular cardiomyocytes four PDE isoenzymes exist. The specific characteristics of PDE I–IV are similar to those obtained with the PDE isoenzymes from ventricular tissue of the same species except for the V_{max} values. The PDE isoenzymes from non-myocardial cells such as connective tissue, endothelial and smooth muscle cells might not influence the PDE isoenzyme pattern of multicellular ventricular tissue to a major extent. The PDE inhibitors IBMX, UD-CG 212 Cl or rolipram reveal cyclic AMP PDE I–IV inhibiting effects in cardiomyocytes comparable to multicellular ventricular tissue, indicating that PDE I–IV from multicellular tissue can be used as a representative system for investigating the effects of PDE inhibitors on PDE activities in cardiac preparations.

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Cyclopiazonic acid, an inhibitor of the sarcoplasmic reticulum Ca^{2+}-pump, reduces Ca^{2+}-dependent K^+ currents in guinea-pig smooth muscle cells

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1 Effects of cyclopiazonic acid (CPA), a specific inhibitor of the Ca^{2+}-ATPase in sarcoplamic reticulum (SR), on membrane ionic currents were examined in single smooth muscle cells freshly isolated from ileal longitudinal strips and urinary bladder of the guinea-pig.

2 Under whole-cell clamp, CPA (1–10 μM) reduced peak outward current elicited by depolarization in a concentration-dependent manner. The concentration of CPA required for 50% decrease in the peak outward current was ~3 μM in ileal cells under these conditions. The current reduced by CPA recovered by more than 70% after washout.

3 The transient outward current elicited by application of 5 mM caffeine at a holding potential of −50 mV in Ca^{2+} free solution was almost abolished, when the preceding Ca^{2+}-loading of the cell in a solution containing 2.2 mM Ca^{2+} was performed in the presence of 3 μM CPA.

4 When the Ca^{2+}-dependent K^+ current ($I_{K\text{-}Ca}$) and Ca^{2+} current (I_{Ca}) were inhibited by addition of Ca^{2+}, the remaining delayed rectifier type K^+ current was not affected by 10 μM CPA. When outward currents were blocked by replacement of K^+ by Cs^+ in the pipette solution, the remaining I_{Ca} was not affected by 10 μM CPA.

5 CPA (10 μM) did not affect the conductance of single maxi Ca^{2+}-dependent K^+ channels or the Cd^{2+}-dependence of their open probability in both inside- and outside-out configurations.

6 These results indicate that $I_{K\text{-}Ca}$ is selectively and strongly suppressed by CPA. Its effects may be attributed to a decrease in Ca^{2+}-uptake into SR, resulting in a decrease in Ca^{2+}-induced Ca^{2+} release which is triggered by Ca^{2+} entering through voltage-dependent Ca^{2+} channels and therefore less activation of these K channels.

7 CPA may be extremely valuable pharmacological tool for investigating intracellular Ca^{2+} mobilization and ionic currents regulated by intracellular Ca^{2+}.

Keywords: cyclopiazonic acid; Ca^{2+}-dependent K^+ current; Ca^{2+}-induced Ca^{2+} release; sarcoplasmic reticulum; smooth muscle cells

Introduction

Cyclopiazonic acid (CPA), a mycotoxin from *Aspergillus* and *Penicillium*, has been described as a highly selective inhibitor of Ca^{2+}-ATPase in skeletal muscle sarcoplasmic reticulum (SR) (Goeger & Riley, 1989; Seidler *et al.*, 1989). It has also been shown that Ca^{2+}-uptake by SR vesicles prepared from skeletal muscle is inhibited by CPA (Goeger *et al.*, 1988). Very recently, effects of CPA on SR Ca^{2+}-ATPase activity in skinned fast-twitch skeletal muscle have been studied extensively (Kurebayashi & Ogawa, 1991). The possibility that CPA reduces Ca^{2+}-uptake by SR has also been suggested in intact aortic smooth muscle (Deng & Kwan, 1991). Direct evidence for the inhibition of ATP-dependent Ca^{2+}-uptake into SR by CPA and subsequent failure of Ca^{2+} release has also been obtained in skinned intestinal smooth muscle preparation (Uyama *et al.*, 1992). The effects of CPA on electrical activity or membrane ionic currents are, however, totally unknown in any type of cell.

In several types of smooth muscle cells, the membrane ionic currents are regulated by intracellular Ca^{2+} metabolism. For instance, the Ca^{2+}-dependent K^+ current ($I_{K\text{-}Ca}$) in ileal smooth muscle of the rabbit is activated by an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) via influx of Ca^{2+} and subsequent Ca^{2+}-induced Ca^{2+} release from storage sites, thought to be SR (Ohya *et al.*, 1987; Sakai *et al.*, 1988). It is also known in various smooth muscle cells that agents (e.g. caffeine) which induce pronounced Ca^{2+} release from intracellular storage sites, first transiently enhance $I_{K\text{-}Ca}$ and thereafter significantly reduce this current (Bolton & Lim, 1989; Cole *et al.*, 1989; Imaizumi *et al.*, 1989; Komori & Bolton, 1990). It is therefore expected that inhibition of the Ca^{2+}-pump in SR will change the amount of stored Ca, and result in changes in $I_{K\text{-}Ca}$.

Since $I_{K\text{-}Ca}$ is the largest outward current in many types of smooth muscle cells (Watanabe *et al.*, 1989), it may be useful as an assay of Ca storage available for Ca^{2+}-induced Ca^{2+} release. The possibility that the Ca^{2+}-pump activity in intracellular Ca storage sites can indirectly regulate smooth muscle membrane excitability, has not been examined previously, because a selective inhibitor of Ca-ATPase in SR was not known. The present study was undertaken to examine the effects of CPA on $I_{K\text{-}Ca}$ and selectivity for the current against other currents.

Methods

Smooth muscle cells were isolated from ileum and urinary bladder of the guinea-pig. Cell isolation by enzymatic digestion and recording of membrane ionic currents using whole cell patch-clamp (Hamill *et al.*, 1981) were performed as described previously (Imaizumi *et al.*, 1989; Watanabe *et al.*, 1989). The resistance of the pipette was between 2 and 5 MΩ

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for whole cell-clamp and between 7 and 15 MΩ for the patch-clamp. The seal resistance formed between cell membrane and tip under cell-attached configuration was between 10 and 50 GΩ. The composition of the solution filling the pipette (pipette solution) during recording of Ca^{2+}-dependent K^+ currents ($I_{K\text{-}Ca}$) under whole cell voltage-clamp was (in mM): K^+ 140, Na^+ 10, Mg^{2+} 4, Cl^- 148, adenosine 5'-triphosphate (ATP) 5.0, ethyleneglycol-*bis*-(β amino-ethyl ether) N,N,N',N'-tetraacetic acid (EGTA) 1.78, Ca^{2+} 0.5, HEPES 10. The pCa was approximately 7.5, when the pH was adjusted to 7.2 by KOH. When spontaneous or caffeine-induced transient outward currents were recorded, the concentration of EGTA and Ca^{2+} in the pipette solution was reduced to 0.05 mM and 0 mM, respectively. To record the voltage-dependent Ca current (I_{Ca}), CsCl was substituted for KCl and the EGTA concentration was increased to 5 mM in the pipette solution to block K^+ currents. The composition of the standard bathing solution was (in mM): Na^+ 137, K^+ 5.9, Ca^{2+} 2.2, Mg^{2+} 1.2, Cl^- 149.7, glucose 14, HEPES 10. The pH was adjusted to 7.2 with NaOH.

Single Ca^{2+}-dependent K^+ channel currents were recorded mainly in the inside-out configuration but in a few outside-out patches as well (Hamill *et al.*, 1981). The pipette solutions used during inside-out and outside-out recordings were the standard bathing solution and the pipette solution for whole cell recordings of macroscopic $I_{K\text{-}Ca}$, respectively. The bathing solution facing the intracellular site of the excised patch membrane during inside-out recordings was the standard pipette solution in which ATP was omitted and the pCa was regulated. The pCa of these solutions was adjusted to a required value with Ca-EGTA buffer (0.5 mM Ca^{2+} and adequate concentrations of EGTA) according to the methods used by Benham *et al.* (1986). Data recording under whole cell voltage-clamp and the analysis were performed using programmes kindly provided by Dr Wayne Giles (Department of Medical Physiology, University of Calgary) in the same manner as described previously (Imaizumi *et al.*, 1990). The holding current level at − 50 mV under whole cell voltage-clamp was taken as the zero current level since resting membrane potentials were around − 50 mV and the resting conductance was very low (input resistance of over 2 GΩ) in these cells. In some experiments, leakage current was corrected for. A leak was extrapolated assuming a linear relationship between voltage and current obtained in the range of − 80 and − 50 mV and subtracted from the total current. Part of the analysis of single channel current was performed with a programme developed by Dr John Dempster (Department of Physiology and Pharmacology, University of Strathclyde). Experiments were carried out at 29 ± 1°C. Pooled data were expressed as mean ± s.e.mean. Statistical significance was examined by Student's *t* test.

Results

When single smooth muscle cells obtained from urinary bladder and ileum were depolarized for 150 ms from a holding potential of − 50 to 0 mV once every 15 s, an inward current followed by transient and sustained components of outward currents were recorded (Figure 1A and B, cells from urinary bladder and ileum, respectively). The initial inward current was a time- and voltage-dependent Ca^{2+} current (I_{Ca}) as has been found in cells from rabbit intestine (Ohya *et al.*, 1986) and guinea-pig urinary bladder (Klöckner & Isenberg, 1985). The transient outward current was mainly $I_{K\text{-}Ca}$ which was activated by an increase in $[Ca^{2+}]_i$ since this current as well as I_{Ca} was suppressed by 0.3 mM Cd^{2+} (not shown, $n = 3$). As has been previously reported in various smooth muscle cells (Ohya *et al.*, 1987; Sakai *et al.*, 1988; Bolton & Lim, 1989; Cole & Sanders, 1989; Imaizumi *et al.*, 1989; Bielefeld *et al.*, 1990), the $I_{K\text{-}Ca}$ was strongly inhibited by the external application of 5 mM caffeine, 10 μM ryanodine or 2 mM tetraethylammonium (TEA), or the addition of 5 mM EGTA to the pipette solution (not shown, $n > 2$ for each).

After the application of 5 μM CPA, the peak amplitude of $I_{K\text{-}Ca}$ in urinary bladder cells decreased gradually after approximately a 1 min delay and reached a steady level within 4 min (Figure 1A). The decrease in the peak $I_{K\text{-}Ca}$ amplitude was occasionally preceded by transient enhancement of the currents by 10–30% when a low concentration (~1 μM) of CPA was applied. The decreased $I_{K\text{-}Ca}$ recovered by more than 70% after washout of CPA when concentrations lower than 10 μM were applied. In urinary bladder cells, the outward current at the end of 150 ms pulse was reduced substantially as well as the peak outward current (see Figure 1A). The decrease in $I_{K\text{-}Ca}$ was observed at potentials positive to − 10 mV (Figure 1B, (a–d), ileal cells). The current-voltage relationships obtained in ileal cells showed that 3 μM CPA decreased markedly the peak outward current but had much less effect on the sustained current measured at the end of the pulse (Figure 1B). Figure 1C denotes the relationship between the CPA concentration and the peak amplitude of outward current upon depolarization to 0 mV after the application of CPA. The amplitude of the outward current is shown as a relative value (%) of that before the application of CPA. The relationship indicates that CPA reduced the peak amplitude of the outward current in a concentration-dependent manner. Maximum decrease of peak outward current was obtained by the application of 10 or 30 μM CPA. The concentration of CPA required for a 50% decrease in the peak outward current was approximately 3 μM in ileal cells under these experimental conditions. Although an approximate 60% decrease in the peak amplitude of the outward current was obtained after application of 10 or 30 μM CPA, the transient component of the outward current was almost completely abolished and only the sustained component remained as shown in the right panel in Figure 1C. A small part of the sustained component of outward current was $I_{K\text{-}Ca}$ in ileal cells since addition of 2 mM TEA reduced this current at the end of the pulse by only about 10–20% ($n = 4$). Therefore, assuming that the major component of the sustained current is not $I_{K\text{-}Ca}$, the concentration of CPA for the half maximal inhibition of the transient component of outward current is lower than 3 μM, and from Figure 1C, is approximately 0.5 μM. A similar decrease in $I_{K\text{-}Ca}$ following the application of CPA was observed in single smooth muscle cells isolated from urinary bladder (34 and 66% decrease in peak outward current by 1 and 5 μM CPA, respectively, $n = 3$ for each).

When $I_{K\text{-}Ca}$ was inhibited by addition of 0.3 mM Cd^{2+} and 5 mM EGTA to the bathing and pipette solutions, respectively, smaller outward currents which were characterized by slower onset times and a reduced TEA-sensitivity (> 5 mM required to block) remained in both types of cells; these are delayed rectifier type K^+ currents ($I_{K\text{-}D}$) (Klöckner & Isenberg, 1985; Terada *et al.*, 1987; Cole & Sanders, 1989). The application of 10 μM CPA did not affect $I_{K\text{-}D}$ upon depolarization to + 30 mV (Figure 2A(a), ileal cell). The peak amplitude of $I_{K\text{-}D}$ after application of 10 μM CPA was 99.5 ± 4.3% of that before application ($n = 4$, $P > 0.05$ vs. before CPA application). Figure 2A(b) shows calcium current (I_{Ca}) which was activated by depolarization from − 50 to 0 mV in an ileal cell where K^+ currents were blocked using a pipette solution containing mainly CsCl. The I_{Ca} was not affected significantly by 10 μM CPA in ileal cells (93.3 ± 4.2% of that before application, $n = 3$, $P > 0.05$ vs. before application). Summarized data for the lack of effect of 10 μM CPA on $I_{K\text{-}D}$ and I_{Ca} in ileal cells at several potentials are shown as current-voltage relationships in Figure 2B. $I_{K\text{-}D}$ ($n = 3$) and I_{Ca} ($n = 2$) recorded in urinary bladder cells under the same conditions were also unaffected by 10 μM CPA. Taken together, these results indicate that CPA selectively reduces $I_{K\text{-}Ca}$.

The effects of CPA on single channel activity of Ca^{2+}-dependent K^+ channels which have a large conductance (maxi-K^+ channel) (reviewed by Latorre *et al.*, 1989) were

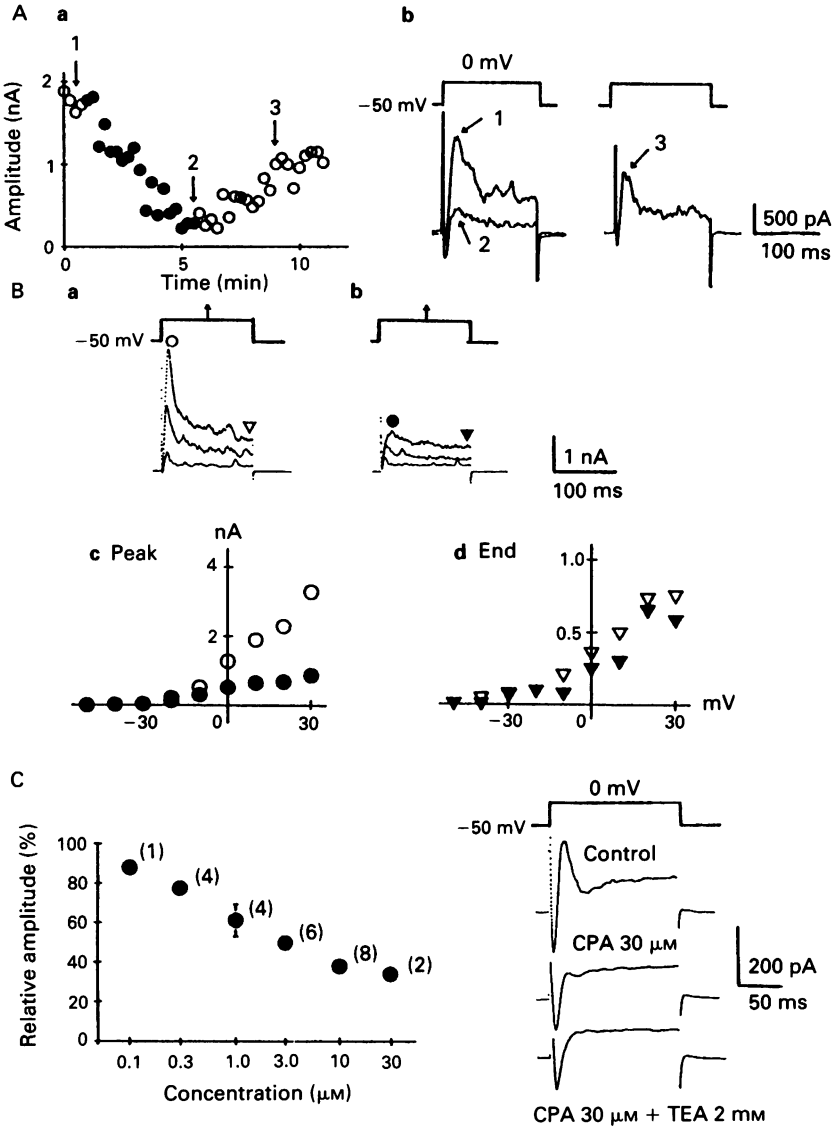

Figure 1 Effects of cyclopiazonic acid (CPA) on outward currents in smooth muscle cells from urinary bladder (A) and ileum (B and C). (A) The cell was depolarized for 150 ms from the holding potential of − 50 mV to 0 mV once every 15 s. (a) The peak amplitude of outward currents was measured and plotted against time: currents measured in the absence (○) or presence (●) of 5 μM CPA. Current recordings at times indicated by numbers in (a) correspond to those shown in (b). (1) before the application of CPA; (2) approximately 5 min after the addition of CPA; (3) 3 min after washout of CPA. Note that the effect of 5 μM CPA on the current was reversed by ~70% after washout. (B) The cell was depolarized from the holding potential of − 50 mV to potentials over the range, − 40 to + 30 mV by 10 mV steps. In (a) and (b), currents recorded at − 10, + 10 and + 30 mV are superimposed. In (a), (b) and (c) circles indicate the peak outward currents or their amplitude. Triangles in (a), (b) and (d) indicate the currents at the end of 150 ms pulse or their amplitude. Open and closed symbols indicate the currents in the absence and presence of 3 μM CPA, respectively. Note that CPA reduced the peak outward current, but not the sustained current at the end of the pulse. (C) Concentration-response curve of CPA for inhibition of $I_{K\text{-}Ca}$ in ileal cells. Experiments were performed in the same manner as shown in (A). Peak outward current upon depolarization to 0 mV was measured from the holding current level at − 50 mV and normalized as a percentage of that measured before the application of CPA. Mean values and s.e.mean are shown as circles and vertical bars, respectively. The number of observations were 1, 4, 4, 6, 8 and 2 for 0.1, 0.3, 1, 3, 10 and 30 μM CPA, respectively. The 50% decrease in peak outward current was obtained by application of approximately 3 μM CPA. The right-hand panel shows that the transient component of the outward current was almost completely abolished by application of 30 μM CPA. The sustained component of the outward current remained in the presence of 30 μM CPA and was affected little by the addition of 2 mM TEA.

examined by the inside-out patch configuration. All patches examined (ileum, $n = 25$; urinary bladder, $n = 10$) included maxi-K^+ channels and most patches contained more than three channels, as expected from the fact that $I_{K\text{-}Ca}$ is the predominant K^+ current in smooth muscle cells from ileum and urinary bladder. Figure 3A shows unitary current events and their corresponding amplitude histograms in an ileal cell due to the activity of a maxi-K channel at a holding potential of 0 mV and pCa 6.0. The open state probability of the channel was highly dependent upon pCa in the range between 7.5 and 5.0 (not shown). CPA (10 μM) applied to the bathing solution affected neither the unitary current amplitude nor the channel activity (Figure 3A(b)). For example, the open state probability of the channel under these conditions (0 mV, pCa 6.0) was not changed significantly by the application of 10 μM CPA (control: 071 ± 0.09; 10 μM CPA: 0.69 ± 0.10, $n = 5$, $P > 0.05$). Figure 3B(a) and (b) shows unitary currents at holding potentials of − 20 and 0 mV in the absence and presence of 10 μM CPA, respectively. When the pipette and bathing solutions contained 5.9 and 140 mM KCl, respectively, the averaged single channel conductance measured in the voltage-range between − 20 and + 20 mV

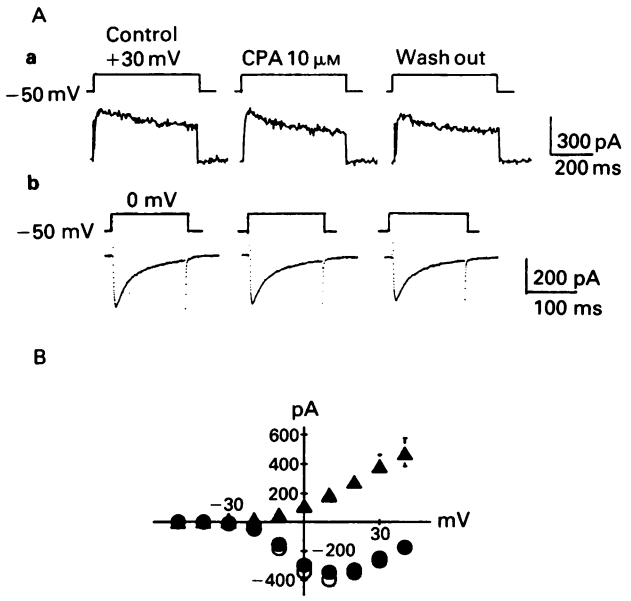

Figure 2 Effects of 10 μM cyclopiazonic acid (CPA) on the delayed rectifier K current ($I_{K\text{-}D}$) and Ca current (I_{Ca}) in ileal smooth muscle cells. (A) The three traces from the left to the right in (a) and (b) are recordings before, during the presence, and after washout of 10 μM CPA, respectively. (a) $I_{K\text{-}D}$ was elicited by depolarization from − 50 mV to + 30 mV. $I_{K\text{-}Ca}$ and I_{Ca} were blocked by simultaneous addition of 5 mM EGTA and 0.3 mM Cd^{2+} to the pipette and bathing solutions, respectively. Leak currents were subtracted on the computer. (b) I_{Ca} was elicited by depolarization from − 50 mV to 0 mV. K^+ currents were blocked by replacement of K^+ in the pipete solution with equimolar Cs^+. (B) Summarized data of the effects of 10 μM CPA on the current-voltage relationships of averaged peak $I_{K\text{-}D}$ (triangles, $n = 4$) and I_{Ca} (circles, $n = 2$). Note that 10 μM CPA affected neither $I_{K\text{-}D}$ nor I_{Ca}. (○,△) Control; (●,▲) CPA 10 μM.

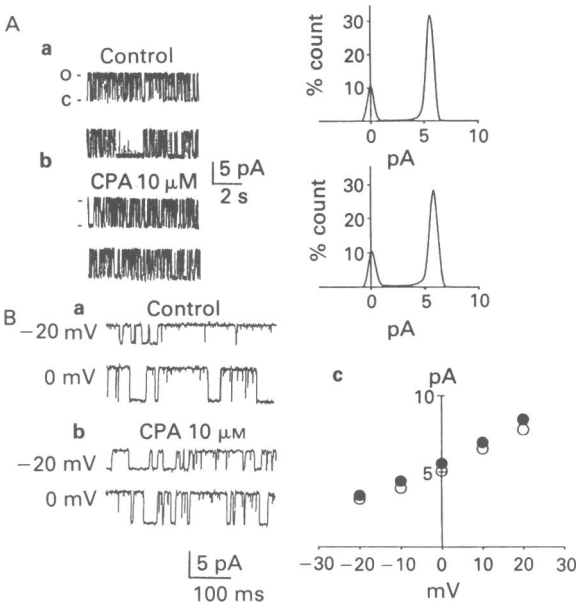

Figure 3 Effects of addition of 10 μM cyclopiazonic acid (CPA) to bathing solution on single Ca^{2+}-dependent K^+ channel current activities under the inside-out patch recording configuration in ileal cells. (A) Holding potential and pCa of the bathing solution were 0 mV and 6.0, respectively. Unitary current was 5.4 pA in the control (a) and was not affected by application of 10 μM CPA (b). Corresponding histograms of open events versus amplitude of unitary current are shown in the right panels, respectively. The ordinate scale expresses the relative time (%) spent at the corresponding amplitude in each bin (0.195 pA) versus the total recording time; 8.2 s in (a) and (b). In this particular patch, only one Ca^{2+}-dependent K^+ channel was active. The open state probability was 0.78 in (a) and 0.76 in (b), respectively. (B) Effects of 10 μM CPA on single channel conductance were examined by measuring currents at five different holding potentials (c). Recordings at − 20 and 0 mV are shown in (a) and (b). The pCa of the bathing solution was 7.5. Averaged unitary current amplitude in the absence (○) or presence of 10 μM CPA (●) ($n = 5$ for each) was plotted against potentials as current-voltage relationships in (c). S.e.means are included in the symbols. The conductance of the channel was 120 and 122 pS in the absence and presence of 10 μM CPA, respectively, and these are not significantly different ($P > 0.05$).

was 118.1 ± 3.7 pS ($n = 5$) and 121.5 ± 4.8 pS ($n = 5$, $P > 0.05$) in the absence and presence of 120 μM CPA, respectively (Figure 3B(c)). Although the effects of CPA on channel activity were not examined systematically in the outside-out patch configuration, preliminary results indicate that 10 μM CPA added to the bathing solution had no apparent effect on either unitary current amplitude or open state probability (0 mV, pCa 6.0, urinary bladder cells, $n = 2$). We concluded that under these conditions 10 μM CPA does not affect the activity of maxi-K^+ channels directly.

Caffeine enhances Ca^{2+}-release from SR and thereby increases outward currents due to activation of maxi-K^+ channel in various smooth muscle cells (Benham & Bolton, 1986; Désilets *et al.*, 1989; Imaizumi *et al.*, 1989). The Ca^{2+}-release and activation of outward currents by caffeine are transient especially in Ca^{2+}-free bathing solution, because the Ca^{2+} in the storage sites may be exhausted in a short time. Therefore, caffeine-induced transient outward currents are a convenient tool for the investigation of intracellular Ca^{2+} mobilization. The effects of CPA on Ca^{2+}-uptake by intracellular storage sites which are susceptible to caffeine were examined in these smooth muscle cells. In various types of smooth muscle cells including those of urinary bladder and ileum, caffeine-induced outward current is often observed as a transient burst of enhanced spontaneous transient outward currents (STOCs) which occur due to spontaneous Ca^{2+} release from SR in a normal solution (Benham & Bolton, 1986; Bolton & Lim, 1989; Sakei *et al.*, 1989). Therefore, effects of CPA on STOCs in the normal solution were first examined in urinary bladder cells (Figure 4A). When urinary bladder cells were clamped at − 50 mV, STOCs which had amplitude (> 25 pA) apparently larger than the noise of the recording system (~15 pA) were recorded in about 50% of cells examined ($n = 20$), whereas the amplitude and frequency of STOCs varied widely from cell to cell and also changed during a recording lasting for longer than several minutes. Application of 1 or 3 μM CPA for ~4 min did not affect STOCs (Figure 4A(a)), while higher concentrations of CPA (> 10 μM) increased the amplitude and/or frequency of STOCs for a few minutes and then tended to reduce them (Figure 4A(a)). Figure 4A(b) illustrates the summarized data regarding the effects of 3 and 10 μM CPA on STOCs. STOCs recorded for a period of 1 min after approximately 2.5 min following the application of 3 or 10 μM CPA, respectively, were integrated and expressed as a relative area versus that measured for STOCs 1 min before the application of CPA. Although 3 μM CPA did not affect STOCs significantly (109.4 ± 8.9%, $n = 5$, $P > 0.05$ vs. control), 10 μM CPA increased STOCs during this period (171.0 ± 19.6%, $n = 3$, $P < 0.05$ vs. control).

The upper trace in Figure 4B illustrates the protocol for examination of the effect of CPA on Ca^{2+}-uptake into the intracellular storage sites which are susceptible to caffeine. Caffeine was applied three times during exposure of the cell to the Ca^{2+}-free solution. Application of 5 mM caffeine induced a burst of transient outward currents for a few minutes in the Ca^{2+} free solution in cells from urinary bladder (Figure 4B(a)) and ileum (not shown) at a holding potential of − 50 mV. The caffeine-induced transient outward currents in these cells are mainly due to maxi-K^+ channel activation since 3 mM TEA almost completely

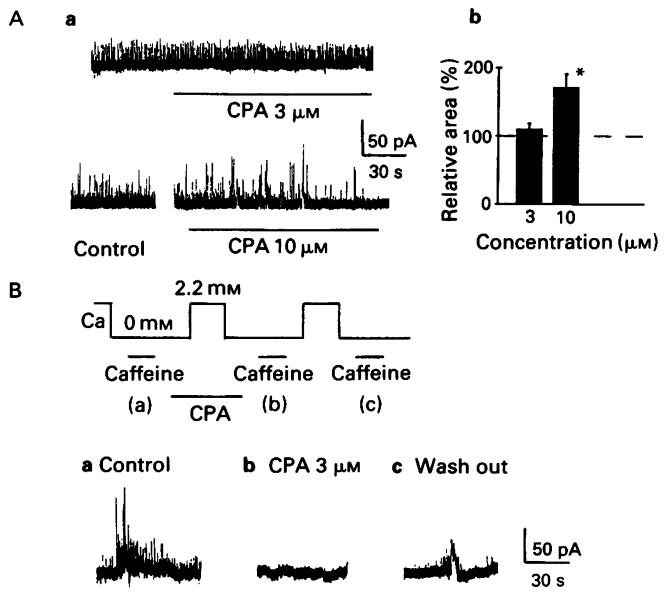

Figure 4 Effects of 3 μM cyclopiazonic acid (CPA) on spontaneous transient outward currents (STOCs) (A) and caffeine-induced transient outward currents (B) in urinary bladder cells. (A) Effects of 3 μM or 10 μM (a, upper and lower traces, respectively) CPA on STOCs were examined at holding potential of − 50 mV. Outward currents which were recorded for 1 min from just before and 2 min or 30 s after application of 3 or 10 μM CPA, respectively, were integrated from the mean level of the basal noise. Relative area (ordinate scale in (b)) indicates the integrated current after the application of CPA versus that before the application. Number of cells used was 5 and 3 for 3 and 10 μM CPA, respectively. $*P < 0.05$ vs. 100%. (B) Upper trace; protocol of the experiment. After Ca^{2+}-loading of the cell in a normal solution containing 2.2 mM Ca^{2+}, 5 mM caffeine was applied in a Ca^{2+}-free solution. This procedure was repeated three times (a, b and c). In the second trial, Ca^{2+}-loading was performed in the presence of 3 μM CPA. CPA was withdrawn before the subsequent application of caffeine. Lower panel; (a), (b) and (c) show current recordings obtained as indicated correspondingly in the protocol. Ca^{2+}-loading was performed in the absence ((a) and (c)) and presence of 3 μM CPA (b). Note that caffeine-induced outward current was not observed in (b) but partly recovered in (c).

abolished them (not shown). This has been demonstrated in other smooth muscle cells (Benham & Bolton, 1986; Ohya *et al.*, 1987). Small phasic inward currents occasionally remained after application of 3 mM TEA (not shown). In the second trial (Figure 4B(b)), Ca^{2+}-loading in a solution containing 2.2 mM Ca^{2+} was performed in the prescence of 3 μM CPA. Following this procedure, application of caffeine did not induce transient outward currents. In the third trial (Figure 4B(c)) which was performed under the same conditions as those used in the first trial, the caffeine-induced response recovered at least in part. Similar results were obtained in all cells examined (three urinary bladder cells and two ileal cells).

Discussion

Smooth muscle cells obtained from ileum and urinary bladder exhibited similar outward currents upon depolarization as has been reported previously (Klöckner & Isenberg, 1985; Ohya *et al.*, 1986; Terada *et al.*, 1987). Although the $I_{K\text{-}Ca}$ component of outward currents appeared to be larger and longer-lasting in cells from urinary bladder than those from ileum, the mechanism for activation of $I_{K\text{-}Ca}$ may be the same. The $I_{K\text{-}Ca}$ is activated upon depolarization by an increase in $[Ca^{2+}]_i$ via Ca^{2+}-induced Ca^{2+} release from SR, which is triggered by the Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Ohya *et al.*, 1987; Sakai *et al.*, 198; Kitamura *et al.*, 1989; Cole & Sanders, 1989). The delayed rectifier type outward currents which remained after the inhibition of $I_{K\text{-}Ca}$ in these cells had similar characteristics; they were reduced by higher concentration of TEA (> 3 mM) but were not affected by 4-aminopyridine (Klöckner & Isenberg, 1985; Ohya *et al.*, 1986).

The present results clearly show that CPA specifically affects $I_{K\text{-}Ca}$. The CPA concentration for the half inhibition of the peak outward current was ~3 μM. I_{Ca} and $I_{K\text{-}D}$ were not affected by 10 μM CPA. It is known that $I_{K\text{-}Ca}$ in smooth muscle cells is almost completely due to Ca^{2+}-dependent K^+ channels which have a very large conductance (so called 'maxi-K^+' or BK channel) (Inoue *et al.*, 1985; Benham *et al.*, 1986; Latorre *et al.*, 1989) and are blocked by relatively low concentrations of TEA (< 2 mM) (Inoue *et al.*, 1985; Imaizumi *et al.*, 1990) or charybdotoxin from outside the cell membrane (Green *et al.*, 1991). Neither the single channel conductance nor the open probability of maxi K^+ channels in smooth muscle cells from ileum and urinary bladder are affected by 10 μM CPA. The activity of at least two other membrane currents. Ca^{2+}-dependent chloride (Pacaud *et al.*, 1989; Amédée *et al.*, 1990) and non-specific cationic channel currents (Loirand *et al.*, 1991), strongly depends upon intracellular Ca^{2+} concentration as well as $I_{K\text{-}Ca}$. Both currents have reversal potentials at around 0 mV under the conditions used in the present study. Therefore, the contribution of these currents to the total membrane current may be minimized by measuring currents at 0 mV. Therefore, it is concluded that CPA specifically, but indirectly reduces maxi K^+ channel activities upon depolarization in these cells under these conditions.

The suppression of $I_{K\text{-}Ca}$ by CPA indicates an additional important characteristic of CPA; this substance is effective in intact smooth muscle cells and can be washed out. Recovery of Ca^{2+}-uptake from CPA-induced inhibition has been observed in skinned smooth muscle fibres (Uyama *et al.*, 1992) but not clearly shown in intact tissues (Deng & Kwan, 1991; Bourreau *et al.*, 1991). In a preliminary study, such a reversible decrease in $I_{K\text{-}Ca}$ by CPA was observed also in smooth muscle cells isolated from trachea, ureter, taenia caeci and vas deferens (Suzuki, Muraki, Imaizumi & Watanabe, unpublished observation), as well as ileum and urinary bladder. Therefore, this may be a common effect of CPA on smooth muscle cells.

Since the activtion of $I_{K\text{-}Ca}$ includes Ca^{2+}-induced Ca^{2+} release processes, the amount of Ca in storage sites may affect indirectly the $I_{K\text{-}Ca}$ amplitude and/or kinetics. It is well established that $I_{K\text{-}Ca}$ is transiently potentiated and then markedly suppressed by caffeine which enhances Ca^{2+}-release and thereby depletes stored Ca in SR (Benham & Bolton, 1986; Bolton & Lim, 1989; Imaizumi *et al.*, 1989; Bielefeld *et al.*, 1990; Sims *et al.*, 1990). It has also been reported that an intracellular Ca^{2+} mobilization elicited by agonists via IP_3-induced Ca^{2+} release from storage sites also increases $I_{K\text{-}Ca}$ transiently and then markedly reduces it (Cole *et al.*, 1989; Bolton & Lim, 1989; Sims *et al.*, 1990; Komori & Bolton, 1990). This agonist-induced suppression of $I_{K\text{-}Ca}$ can be blocked by intracellular application of low molecular weight heparin which inhibits IP_3 binding to the site on SR (Ganitkevich & Isenberg, 1990; Komori & Bolton, 1991; Takeda *et al.*, 1991). Exhaustion of releasable Ca from storage sites by Ca^{2+}-induced or IP_3-induced Ca^{2+} release has been postulated for the suppression of $I_{K\text{-}Ca}$. The suppression of $I_{K\text{-}Ca}$ by CPA in the present study mimicked that induced by caffeine or agonists. Therefore, the mechanism of CPA-induced inhibition of $I_{K\text{-}Ca}$ may be correlated with those for caffeine or agonists. After the application of CPA at a low concentration (1–3 μM), a potentiation of $I_{K\text{-}Ca}$ upon depolarization was occasionally observed prior to the CPA-induced suppression. Since the amplitude of $I_{K\text{-}Ca}$ was also markedly reduced by an increase in frequency of the depolarizing pulse, the pulse was applied at a low rate (once

every 15 s) in order to observe stable and large $I_{K\text{-}Ca}$s. Therefore, this may be part of the reason why the potentiation of $I_{K\text{-}Ca}$ by CPA prior to the suppression was not always observed.

In the present study, it is also shown that Ca^{2+}-uptake into intracellular storage sites susceptible to caffeine (lino, 1989) was almost completely abolished by treatment with 3 μM CPA during the preceding Ca^{2+}-load. This finding is consistent with the results we showed in skinned smooth muscle fibres of the longitudinal layer of the guinea-pig ileum, where the IC_{50} of CPA for inhibition of Ca^{2+}-uptake was approximately 0.6 μM (Uyama *et al.*, 1992). Based upon these results, it can be suggested that CPA easily penetrates into the cytoplasm through the plasma membrane, reduces Ca^{2+}-ATPase activity in SR/ER and can be washed away, at least in part. The mechanism may be the following; CPA decreases Ca^{2+}-uptake by the inhibition of Ca^{2+}-pump in the SR/ER, decreases the subsequent Ca^{2+} release from those storage sites and, thereby, reduces $I_{K\text{-}Ca}$.

The current elicited by caffeine at a holding potential of − 50 mV may include the Ca^{2+}-dependent Cl^- (Pacaud *et al.*, 1989; Amedee *et al.*, 1990) and non-specific cationic channel currents (Loirand *et al.*, 1991; Wang & Large, 1991). When the holding potential was less negative (e.g. − 30 mV), caffeine-induced outward currents were larger (not shown). The less negative holding potential, however, also enhanced STOCs and appeared to potentiate the CPA-induced effects of STOCs, resulting in difficulty in assessing effects of CPA on Ca^{2+}-uptake susceptible to caffeine. Effects of CPA on caffeine-induced Cl^- or non-specific cationic current were not examined systematically in the present study.

Since $I_{K\text{-}Ca}$ is responsible for the action potential repolarization and the afterhyperpolarization (Watanabe *et al.*, 1989), it is likely that Ca^{2+}-pump activity in SR may indirectly, but significantly, affect action potential shape and frequency of action potential firing in various types of smooth muscles. This may have an especially important role in gastro-intestinal smooth muscle, since $I_{K\text{-}Ca}$ also regulates the repolarization phase of slow wave potentials (Cole & Sanders, 1989; Carl *et al.*, 1990; Vogalis *et al.*, 1991). The effects of CPA on intestinal motor activity have not been examined systematically in the present study, although CPA does increase spontaneous contractions of intact ileal longitudinal strips (data not shown). Moreover, effects of CPA on STOCs were not examined systematically since amplitude and frequency of STOCs at the holding potential of − 50 mV varied so widely from cell to cell and also changed during a recording lasting for more than several minutes. STOCs, however, appeared to be affected at relatively higher concentrations of CPA (> 10 μM) or possibly after prolonged exposure to CPA. It may be worth examining whether Ca^{2+}-pump activity may control resting membrane potential via regulation of STOCs.

In conclusion, CPA, an inhibitor of the Ca^{2+}-pump in intracellular Ca storage sites (mainly SR), selectively and reversibly reduces $I_{K\text{-}Ca}$ without any direct block of the K^+ channel, in ileal and urinary bladder smooth muscle cells. Therefore, it is an extremely valuable pharmacological tool for investigation of the Ca^{2+}-pumping mechanism and intracellular Ca^{2+} homeostatis. This is the first report that Ca^{2+}-pump activity in SR/ER may indirectly regulate $I_{K\text{-}Ca}$ and thereby membrane excitability.

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GH_4ZD10 cells expressing rat 5-HT_{1A} receptors coupled to adenylyl cyclase are a model for the postsynaptic receptors in the rat hippocampus

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1 Vasoactive intestinal polypeptide (VIP) stimulated adenosine 3':5'-cyclic monophosphate (cyclic AMP) production by cultured GH_4ZD10 cells with an EC_{50} value of about 7 nM. The extracellularly recovered cyclic AMP predominated, and was reduced by co-incubation with 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) and 5-hydroxytryptamine (5-HT), whereas dopamine (0.1–30 μM) did not reduce VIP-stimulated cyclic AMP production.

2 The responses to 5-HT and 8-OH-DPAT were blocked by (−)-alprenolol and NAN 190. The antagonism by (−)-alprenolol was competitive in nature with a pA_2 value of 7.0.

3 The responsiveness of the cells to 5-HT agonists was highly dependent upon the culturing conditions used. Thus, 8-OH-DPAT inhibition of VIP (30 nM)-stimulated cyclic AMP production decreased with increasing passage number of the cells. Reduction of the zinc concentration used to promote expression of the 5-HT_{1A} receptor gene produced a greater sensitivity of the cells to 5-HT agonists.

4 Under such conditions, the following efficacies (5-HT = 100) were found: lisuride 106, (+)-lysergic-acid diethylamide 100, 5-methoxy-N,N-dimethyltryptamine 98, RU 24949 98, 5-carboxamidotryptamine 97, (±)-8-OH-DPAT 90, (+)-8-OH-DPAT 87, 1-[2-(4-aminophenyl)ethyl]-4-(3-trifluoromethylphenyl)-piperazine 86, flesinoxan 79/88, (−)-8-OH-DPAT 62, buspirone 43/50, ipsapirone 46. Spiroxatrine and spiperone had a low intrinsic activity, but reduced the response to 5-HT. These efficacies are similar to those reported in the literature for post-synaptically localized 5-HT_{1A} receptors in the rat hippocampus. Thus, the GH_4ZD10 cells serve as a useful *in vitro* model system for these receptors.

Keywords: 5-HT_{1A} receptors; adenylyl cyclase; vasoactive intestinal polypeptide; cultured cells

Introduction

In recent years, it has been established that a number of different 5-hydroxytryptamine (5-HT) receptors are found in the brain. Of these, the 5-HT_1 receptor subtypes 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D} have been shown to be coupled to inhibition of adenylyl cyclase (for review, see Schmidt & Peroutka, 1989). Recently, Albert *et al.* (1990) and Fargin *et al.* (1989) described the successful cloning and functional expression in cells of the 5-HT_{1A} receptor. Such cells have been used to explore the receptor-effector coupling of the 5-HT_{1A} receptors in the absence of other 5-HT receptor subtypes. Albert *et al.* (1990), for example, found that the [^{3}H]-8-hydroxy-2-(di-n-propylamino)tetralin ([^{3}H]-8-OH-DPAT) receptor binding profile of the rat gene expressed in mouse Ltk$^-$ cells was as expected for a 5-HT_{1A} receptor. Furthermore, expression of the rat 5-HT_{1A} receptor in rat GH_4C_1 pituitary cells produced a cell line (GH_4ZD10) where both basal and vasoactive intestinal polypeptide (VIP)-stimulated adenosine 3':5'-cyclic monophosphate (cyclic AMP) production was inhibited ~60% by 200 nM 5-HT. This inhibition was blocked either by the 5-HT receptor antagonist spiroxatrine (2 μM) or by pertussis toxin treatment of the cells (Albert *et al.*, 1990; Liu & Albert, 1991), the latter finding suggesting the mediation of the inhibitory response via G_i-proteins, consistent with animal studies (Dumuis *et al.*, 1988; Harrington *et al.*, 1988; Okada *et al.*, 1989; Zgombick *et al.*, 1989).

One of the drawbacks of the use of transfected cell lines, however, is that the degree of receptor reserve may not match the situation found in the natural state. Thus, variations in intrinsic activity of cloned human 5-HT_{1A} receptors dependent upon the level of receptor expression have recently been demonstrated by Boddeke *et al.* (1992). This is of particular importance for the 5-HT_{1A} receptor, since the degree of receptor reserve is different depending on its location in the brain and the observed efficacies of compounds can thereby vary. Thus, buspirone and ipsapirone act as partial agonists postsynaptically in the rat and calf hippocampus (De Vivo & Maayani, 1986; Dumuis *et al.*, 1988; Schoeffter & Hoyer, 1988), whereas apparent full agonist actions of these compounds are found presynaptically in the rat hippocampus, due to a very large receptor reserve (Meller *et al.*, 1990).

In the present study, the effects of a series of 5-HT agonists with different intrinsic activities have been investigated in GH_4ZD10 cells, in order to determine whether the receptor reserve in this cell line is similar to the pre- or postsynaptically localized receptor in the hippocampus.

Methods

Cell culturing conditions

GH_4ZD10 cells, after treatment with geniticin to select for recombinants (Albert *et al.*, 1990), were cultured in 175 cm^2 flasks in Ham's F10 medium supplemented with 10 mM HEPES (pH 7.35), 0.15 mg ml^{-1} L-glutamine, 100 μg ml^{-1} streptomycin and 70 μg ml^{-1} benzyl penicillin K plus 10% heat-inactivated foetal calf serum (FCS) at 37°C and used between passages 6 and 15 after arrival at Astra, unless otherwise stated. After passage, cells (4.8–7.6 × 10^6 cells/flask) were grown for 4–6 days. For the data shown in Figures 1 and 2, the cells were treated for 16 h with 85–90 μM $ZnSO_4$ to promote expression of the 5-HT_{1A} receptor (Albert *et al.*, 1990). After the zinc incubation, the medium

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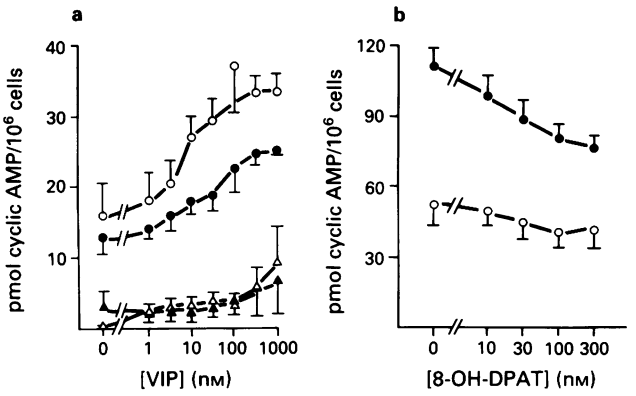

Figure 1 (a) Effect of vasoactive instestinal polypeptide (VIP) upon intracellular (Δ, ▲) and extracellular (○, ●) cyclic AMP production in zinc [85–90 μM]-treated GH_4ZD10 cells in the absence (open symbols) and presence (closed symbols) of 200 nM (±)-8-hydroxy-2-(di-n-propylamino)tetralin ((±)-8-OH-DPAT). Data are means with s.e.mean shown by vertical bars, $n = 3–4$. Two-way ANOVA gave values of intracellular: $F_{7,43}$ (VIP) = 2.15 ($0.10 > P > 0.05$), $F_{1,43}$ (8-OH-DPAT) = 0.13 (NS), $F_{7,43}$ (VIP × 8-OH-DPAT) = 0.31 (NS); extracellular: $F_{7,44}$ (VIP) = 7.15 ($P < 0.001$), $F_{1,44}$ (8-OH-DPAT) = 23.15 ($P < 0.001$), $F_{7,44}$ (VIP × 8-OH-DPAT) = 0.62 (NS). Post-hoc t tests for the extracellular data indicated that significant ($P < 0.05$) reductions in cyclic AMP production in the presence of (±)-8-OH-DPAT were found at VIP concentrations of 10, 30 and 100 nM. (b) Effect of (±)-8-OH-DPAT upon basal (○) and 30 nM VIP (●) stimulated cyclic AMP production in zinc-treated GH_4ZD10 cells. The intra- and extracellular fractions were combined. Data are means with s.e.mean shown by vertical bars, $n = 4$ (basal) or $n = 6$ (VIP-stimulated). Two-way ANOVA of the data gave values of $F_{1,40}$ (VIP) = 91.91 ($P < 0.001$), $F_{4,40}$ (8-OH-DPAT) = 3.17 ($P < 0.025$), $F_{4,40}$ (VIP × 8-OH-DPAT) = 0.71 (NS).

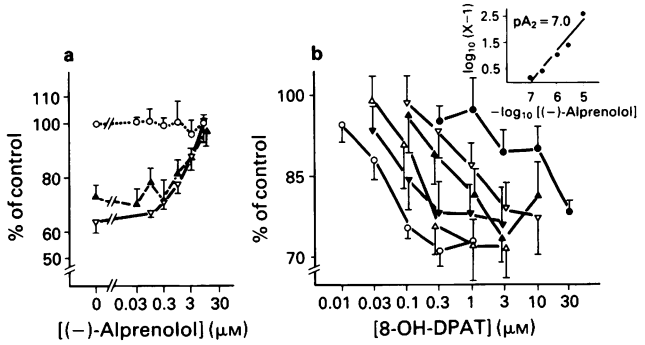

Figure 2 (a) Effect of (−)-alprenolol on the cyclic AMP production in zinc [85–90 μM]-treated GH_4ZD10 cells in the presence of 30 nM vasoactive intestinal polypeptide (VIP) alone (○), 30 nM VIP + 1000 nM 5-hydroxytryptamine (▽) and 30 nM VIP + 1000 nM (±)-8-hydroxy-2-(di-n-propylamino)tetralin ((±)-8-OH-DPAT) (▲). (b) Effect of 0 (○), 0.1 (▼), 0.3 (Δ), 1 (▲), 3 (▽) and 10 (●) μM (−)-alprenolol on the inhibition by (±)-8-OH-DPAT of the cyclic AMP response to 30 nM VIP. Data are means with s.e.mean shown by vertical bars, $n = 3–4$. The inset in (b) shows the secondary plot of $\log_{10}$ (dose ratio − 1) vs $\log_{10}$ [(−)-alprenolol] (Arunlakshana & Schild, 1959) calculated from the mean data using a % of control value of 85.

was aspirated and replaced with standard medium for 24 h before assay of adenylyl cyclase activity. For the data shown in Table 1 and Figure 3, cells ($2.1–5.0 \times 10^6$/flask) were grown for 4–6 days after passage in medium containing geniticin (700 μg l^{-1}) and then incubated in geniticin-free medium containing a lower zinc concentration (50 μM) coupled with a longer incubation time (36–40 h). The cells were then used for assay.

Adenylyl cyclase assay

The assay used was based on the method of Dorflinger & Schonbrunn (1983). Briefly, medium was removed and each flask was washed 1–2 times with either phosphate buffered saline (Figure 1), with EBSS without calcium and magnesium containing 1 mM EDTA (Table 1, Figure 3) or with FCS-free Ham's medium. The cells were harvested and suspended in 30–50 ml FCS-free Ham's medium. The suspension was centrifuged at ~250 g for 4–10 min at room temperature and resuspended to a density of 1×10^7 cells ml^{-1} in Ham's F-10 medium containing 0.01% ascorbic acid and 1 mM IBMX. The cells were incubated for 1 h at 37°C and then diluted with the same medium to a final density of $0.5–2.0 \times 10^6$ viable cells ml^{-1}. For the experiments using the higher zinc concentration, the cell viability, determined by trypan blue exclusion, was generally around 50%. This value is considerably lower than the ⩾95% viability found by Dorflinger & Schonbrunn (1983) for GH_4C_1 cells (from which the present cell line is derived), and presumably reflects the toxic effects of the zinc/IBMX treatment.

Aliquots (0.8 ml, except in Table 1 and Figure 3 where 0.4 ml was used) of the cell suspension were added to Eppendorf tubes containing 0.2 ml (0.1 ml in Table 1 and Figure 3) VIP (30 nM final concentration unless otherwise stated) + test compounds and incubated for 20 min at 37°C. The test compounds, with the exception of 5-HT, which was made up fresh for every experiment, were diluted from stock solutions kept frozen for ⩽ 10 days. Reactions were stopped by placing the assay tubes in an ice-water bath for 10 min. For the experiments shown in Figures 1b and 2, the assay tubes were vortexed gently and then heated for 7 min at 100°C to lyse the cells. For the experiments shown in Table 1 and Figure 3, the reaction mixtures were placed directly in the boiling water bath after the incubation period. The lysates were then centrifuged at 2000 g for 10 min at 4°C, and the cyclic AMP levels determined in duplicate 50 μl aliquots by use of an Amersham cyclic AMP assay kit. For the experiments shown in Figure 1a, the suspensions were centrifuged at 800 g × 10 min and the supernatant removed for assay of extracellular cyclic AMP. The cells were then resuspended in the Ham's medium, lysed and treated as described above to determine the intracellular cyclic AMP concentrations. Results are given either as pmol cyclic AMP produced during the 20 min incubation phase/10^6 cells, or as % of control, as appropriate.

Materials

GH_4ZD10 cells were obtained from Dr O. Civelli, Vollum Institute for Advanced Biomedical Research, The Oregon Health Sciences University, Portland, Oregan, U.S.A. Ham's F10 medium was obtained from Flow Laboratories, Irvine Scotland. Eagle's balanced salt solution (EBSS) without calcium and magnesium, foetal calf serum and L-glutamine were obtained from Gibco Ltd., Paisley, Scotland. Cyclic AMP assay kits (TRK 432) were obtained from Amersham International plc, Amersham, U.K. VIP, 3-isobutyl-1-methyl-xanthine (IBMX), geniticin, streptomycin sulphate, 5-methoxy-N,N-dimethyltryptamine (5-MeODMT) and 5-HT were obtained from the Sigma Chemical Co., St Louis, MO, U.S.A. 1-(2-Methoxyphenyl)-4-[4-(2-phthalimido)butyl]piperazine hydrobromide (NAN 190), 1-[2-(4-aminophenyl)ethyl]-4-(3-trifluoromethylphenyl)-piperazine (PAPP) and spiroxatrine were obtained from Research Biochemicals Incorporated, Natick, MA, U.S.A. Other agents used included buspirone (Bristol-Myers), flesinoxan (Duphar), 5-carboxamidotryptamine (5-CT) (Glaxo), L-(−)-alprenolol hydrogen tartrate monohydrate (Hässle), spiperone (Janssen), 5-methoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H indole (RU 24969) (Roussel-Uclaf), lisuride (Schering), ipsapirone (Troponwerke) (+)-lysergic acid diethylamide ((+)-LSD) (USP inc.) and benzyl penicillin K (Astra). (±)-, (+)- and (−)-8-

Table 1 Effects of 5-hydroxytryptamine (5-HT) receptor agonists and antagonists upon 30 nM vasoactive intestinal polypeptide (VIP)-stimulated cyclic AMP production in zinc [50 μM]-treated GH$_4$ZD10 cells

	% of control at concentration (nM) *of:*							
	10	100	1000	10000	1000+ 1000 nM 5-HT	1000+ 10000 nM (−)-Aprenolol	*Efficacy*[a]	*Est. IC$_{50}$*[b]
Series I								
5-HT	94 ± 6	60 ± 5	43 ± 4	37 ± 2	—	91 ± 2	100	60
5-MeODMT	—	—	48 ± 5	38 ± 4	—	—	98	—
5-CT	55 ± 5	42 ± 3	39 ± 5	39 ± 5	—	—	97	<10
(±)-8-OH-DPAT	78 ± 5	52 ± 1	43 ± 1	44 ± 1	45 ± 2	84 ± 3	90	20
(+)-8-OH-DPAT	87 ± 3	53 ± 4	45 ± 3	47 ± 4	41 ± 2	102 ± 12	87	30
Flesinoxan	75 ± 4	58 ± 4	—	50 ± 4	55 ± 5[c]	57 ± 6[c]	79	~10
(−)-8-OH-DPAT	93 ± 3	74 ± 3	61 ± 2	62 ± 3	47 ± 5	104 ± 7	62	40
Buspirone	—	84 ± 3	75 ± 2	73 ± 1	64 ± 2[d]	81 ± 6[d]	43	~100
Series II								
5-HT	93 ± 4	62 ± 3	52 ± 2	48 ± 3	—	89 ± 3	100	40
Lisuride	54 ± 4	47 ± 3	46 ± 3	45 ± 4	49 ± 2	51 ± 3	106	<10
(+)-LSD	77 ± 2	55 ± 3	48 ± 4	52 ± 4	52 ± 3	76 ± 3	100	~10
RU 24949	96 ± 4	69 ± 4	55 ± 4	49 ± 3	53 ± 3	96 ± 4	98	60
(±)-8-OH-DPAT	82 ± 0.5	60 ± 3	56 ± 3	53 ± 3	53 ± 2	80 ± 4	90	20
Flesinoxan	77 ± 3	60 ± 3	54 ± 2	55 ± 4	53 ± 3	73 ± 3	88	~10
PAPP[e]	88 ± 5	65 ± 5	57 ± 4	55 ± 7	63[d] (n = 2)	82 ± 6	86	30
Buspirone	96 ± 2	89 ± 4	75 ± 3	74 ± 4	76[d] (n = 2)	98 ± 3	50	150
Ipsapirone	94 ± 2	84 ± 3	79 ± 4	76 ± 4	79[d] (n = 2)	97 ± 2	46	40
Spiroxatrine[e]	95 ± 4	87 ± 5	84 ± 4	88 ± 6	73[d] (n = 2)	93 ± 3	—	—
Spiperone[e]	92 ± 5	95 ± 5	85 ± 5	90 ± 2	86 ± 3	85 ± 2	—	—
(−)-Alprenolol	—	—	—	94 ± 6	—	—	—	—

The compounds for each experimental series were run concurrently to allow internal comparison. Data are means ± s.e.mean, $n = 4$ (except spiperone, where $n = 3$). The absolute activity of the control samples was for Series I 310 ± 40 and for Series II 160 ± 7 pmol cyclic AMP formed during the incubation/10^6 viable cells. The variability in the absolute activity is not related to the zinc concentration or to the passage number used, and highlights the importance of expressing data as % of control from the same experiments.

[a] Efficacy is estimated as the maximum observed mean inhibition as a percentage of the mean inhibition produced by 10000 nM 5-HT.

[b] Estimated IC_{50} was determined graphically as the concentration producing half the maximum observed inhibition.

[c] Flesinoxan concentration used was 3000 nM.

[d] Concentration of test compound was 10000 nM.

[e] Some precipitation of the compounds noted at 10000 nM.

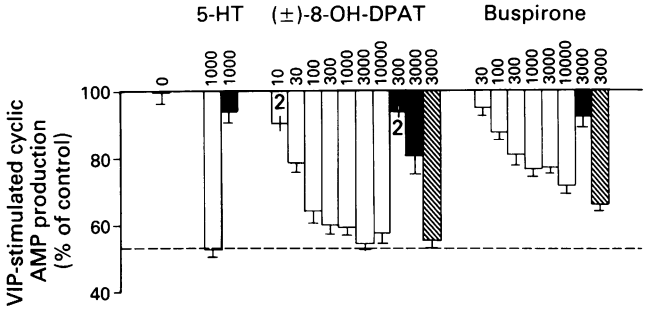

Figure 3 Effects of (±)-8-hydroxy-2-(di-n-propylamino)tetralin ((±)-8-OH-DPAT) and buspirone upon cyclic AMP production in zinc [50 μM]-treated GH$_4$ZD10 cells. The agonist concentrations used (in nM) are indicated on the abscissa scale. The compounds were treated in the absence (open columns) or presence of either 10000 nM (−)-alprenolol (solid columns) or 1000 nM 5-hydroxytrypamine (5-HT) (hatched columns). Data are means with s.e.mean shown by vertical bars, $n = 9$, except for 10 nM (±)-8-OH-DPAT and 300 nM (±)-8-OH-DPAT + 10000 nM (−)-alprenolol, where data are mean and range, $n = 2$.

hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT) were synthesized at the Department of Organic Pharmaceutical Chemistry, University of Uppsala, Sweden. 5-HT was made up fresh every day in Ham's medium; the other compounds were made up in stock solutions of either distilled water or dimethylsulphoxide.

Statistical methods

Statistical significance of the effects of VIP and (±)-8-OH-DPAT upon cyclic AMP production in the experiments shown in Figure 1 were determined by two-way ANOVA with post-hoc *t* tests where appropriate.

Results

Effects of (±)-8-OH-DPAT on basal and VIP-stimulated adenylyl cyclase in zinc-treated GH$_4$ZD10 cells

In their original paper, Albert *et al.* (1990) used a zinc concentration of 100 μM to induce expression of the 5-HT$_{1A}$ receptor. In our hands, however, this concentration was found to be toxic to the cells, and a concentration range of 85–90 μM was used for the initial experiments described here.

Concentration-response curves for the effects of VIP upon intra- and extracellular cyclic AMP production in zinc-treated GH$_4$ZD10 cells are shown in Figure 1a. VIP had minor effects on the intracellularly recovered cyclic AMP, but increased the extracellularly recovered cyclic AMP about 2 fold, with an EC_{50} value of about 7 nM. Concentration-response curves for VIP in the presence of 200 nM (±)-8-OH-DPAT were determined concomitantly (Figure 1a). The extracellular VIP concentration-response curve was significantly affected by this concentration of (±)-8-OH-DPAT. In the remaining experiments, VIP (30 nM) was used as a stimulatory agent, and the intra- and extracellular recovered cyclic AMP fractions were assayed together.

Concentration-response curves for the effect of (±)-8-OH-DPAT upon basal and 30 nM VIP-stimulated cyclic AMP production in zinc-treated GH_4ZD10 cells are shown in Figure 1b. Two way ANOVA indicated significant inhibition of the cyclic AMP by (±)-8-OH-DPAT. There was a large variability from experiment to experiment in the absolute activity of the cells (expressed as pmol cyclic AMP produced during the incubation period/10^6 viable cells). In order to minimize the effect of this variability, data were expressed as % of control. With this method of analysis, (±)-8-OH-DPAT (1000 nM) produced a reduction in the VIP-stimulated cyclic AMP production of about 30–35%, whereas 5-HT (1000 nM) produced a reduction of about 40% (Figure 2). Dopamine had no effect on VIP-stimulated cyclic AMP production: in the presence of 30 nM VIP, cyclic AMP concentrations of 111 ± 20, 123 ± 16, 133 ± 22, 117 ± 23, 117 ± 24, 116 ± 21 and 108 ± 21 (means ± s.e.mean, n = 3) pmol/10^6 cells were found for 0, 0.1, 0.3, 1, 3, 10 and 30 μM dopamine.

Effect of (−)-alprenolol

The effects of (−)-alprenolol upon (±)-8-OH-DPAT and 5-HT-induced reductions in VIP-stimulated cyclic AMP production were determined. Initial experiments indicated that a 10 min preincubation phase *per se* reduced the subsequent responsiveness of the cells to the 5-HT agonists, so that in consequence the agonists and antagonists were added simultaneously. Under these conditions, (−)-alprenolol antagonized the inhibitory effects of 5-HT and (±)-8-OH-DPAT upon VIP-stimulated cyclic AMP production, without affecting the response to VIP alone (Figure 2a). The antagonism produced by (−)-alprenolol was competitive in nature with a pA_2 value of 7.0 (Figure 2b). NAN 190 (10 μM) was also found to antagonize the inhibitory effects of 5-HT and (±)-8-OH-DPAT: thus, the % inhibition of 30 nM VIP-stimulated adenylyl cyclase in the absence and presence, respectively, of NAN 190 was for 100 nM 5-HT 14 ± 2 and 3 ± 6 and for 300 nM (±)-8-OH-DPAT, 16 ± 1 and 2 ± 1 (means ± s.e. mean, $n = 5$).

Influence of the culturing conditions used upon the responsiveness of GH_4ZD10 cells to 5-hydroxytryptamine agonists

The above experiments were undertaken using cells passaged less than 15 times after arrival and with a zinc concentration of 85–90 μM. Further experiments with (±)-8-OH-DPAT indicated that the degree of inhibition of VIP-stimulated cyclic AMP production decreased with increasing passage number. Thus although significant reductions in VIP-stimulated cyclic AMP production were found for zinc-treated GH_4ZD10 cells used between passage numbers 6 and 15 after arrival at Astra, the response was lost at passage numbers greater than 17 (data not shown).

Given the toxic nature of zinc, experiments were also undertaken using a longer induction time (36–40 h) with a lower (50 μM) zinc concentration. Under these conditions, a greater (albeit with the same inter-experimental variability) cyclic AMP production by the cells and greater effects of 5-HT and (±)-8-OH-DPAT were seen (see Table 1). This concentration of zinc was therefore chosen for the evaluation of the effects of different 5-HT agonists (see below).

Comparison of the effects of 5-HT agonists upon VIP-stimulated cyclic AMP production in GH_4ZD10 cells

Dose-response curves for (±)-8-OH-DPAT and buspirone are shown in Figure 3. In the case of (±)-8-OH-DPAT, the compound inhibited cyclic AMP production by 45% (i.e. to a similar extent as that seen with 1000 nM 5-HT), half maximal inhibition being seen at a concentration of 30 nM. Addition of 1000 nM 5-HT to 3000 nM (±)-8-OH-DPAT gave the same inhibition as seen with (±)-8-OH-DPAT alone. The inhibition produced by 300 and 3000 nM (±)-8-OH-DPAT was reduced by 10000 nM (−)-alprenolol. For buspirone, the maximum inhibition observed was 28%, with an IC_{50} value of about 100 nM. The presence of 3000 nM buspirone reduced the inhibition found with 1000 nM 5-HT, suggesting that buspirone is acting as a partial agonist in this system. The effect of 3000 nM buspirone was antagonized by 10000 nM (−)-alprenolol.

The effects of a series of compounds active at the $5\text{-}HT_{1A}$ receptor upon VIP-stimulated cyclic AMP production in GH_4ZD10 cells are shown in Table 1. The efficacies of the compounds were expressed relative to that of 5-HT. For the first series of experiments, 5-MeODMT and 5-CT acted as full agonists, whereas the two enantiomers of 8-OH-DPAT showed different efficacies. In the second series, full agonist actions of lisuride, (+)-LSD and RU 24949 were seen, whereas buspirone and ipsapirone acted as partial agonists. Flesinoxan showed a similar efficacy to (±)-8-OH-DPAT. Spiroxatrine and spiperone showed a low intrinsic activity, but reduced the response to 5-HT (Table 1).

Discussion

In 1983, Dorflinger & Schonbrunn showed that VIP stimulates cyclic AMP accumulation in GH_4C_1 rat pituitary cells with an EC_{50} of about 3 nM. These authors also reported that the levels of cyclic AMP recovered extracellularly were higher than those recovered intracellularly, due to its concentration-dependent extrusion (Barber & Butcher, 1981). Similar results were found in the present study using the GH_4ZD10 cells (Figure 1a).

In the present study, the pharmacological properties of the $5\text{-}HT_{1A}$ receptors expressed in GH_4ZD10 cells have been investigated, particularly with regard to the receptor-effector coupling efficiency and the sensitivity to the culturing conditions used. Initial experiments confirmed the finding by Albert *et al.* (1990) that stimulation of these receptors inhibits the cyclic AMP response to VIP-stimulation. This differs from the situation for the parent GH_4C_1 cells, where 5-HT has no effect upon the cyclic AMP response to VIP stimulation (Albert *et al.*, 1990).

The inhibitory effects of 5-HT and (±)-8-OH-DPAT were blocked by NAN 190, which has $5\text{-}HT_{1A}$ receptor antagonist properties (Glennon *et al.*, 1988). $5\text{-}HT_{1A}$ receptors can also be blocked by compounds such as (−)-pindolol and (−)-alprenolol (see Middlemiss *et al.*, 1985; Oksenberg & Peroutka, 1988), and the pA_2 value for (−)-alprenolol found in the present study (7.0, Figure 2b) is in line both with the pA_2 values reported for brain tissue preparations using other β-blockers of this type and with the pIC_{50} value (6.93) for the inhibition by (−)-alprenolol of [^{3}H]-8-OH-DPAT binding to rat frontal cortical $5\text{-}HT_{1A}$ receptors (Middlemiss *et al.*, 1985). The inhibitory action of (−)-alprenolol is not likely to be secondary to its action upon β-adrenoceptors, since these latter receptors stimulate adenylyl cyclase. Thus an effect of this compound upon some sort of tonic β-adrenocreptor activity would be seen as a decreased basal activity and thereby an augmentation of the effects of 5-HT and (±)-8-OH-DPAT, which is clearly not the case (Figure 2a).

Whilst the cells express $5\text{-}HT_{1A}$ receptors coupled to inhibition of adenylyl cyclase, as originally shown by Albert *et al.* (1990), the responsiveness in our hands initially was lower than that found by these authors. However, this difference presumably reflects the sensitivity of cells to the culturing conditions used, such as has been shown for other receptor systems (see e.g. Fowler & Brännström, 1990). Indeed, a reduction of the zinc concentration of 50 μM together with a longer induction time gave cell preparations showing sensitivities to 5-HT well in line with those reported by Albert *et al.* (1990) (Table 1). A second demonstration of the 'fragility' of the expressed receptors was the finding that the degree of

inhibition of VIP-stimulated adenylyl cyclase produced by (±)-8-OH-DPAT decreased quite rapidly as the passage number increased. The mechanism behind this reduction of response with passage number is unclear. The 5-HT$_{1A}$ receptor gene may be excised from the DNA after a number of passages, possibly by a back-reaction of the initiation mechanism. Alternatively, constant stimulation of the receptors by low levels of 5-HT present in the FCS may result either in a desentisization mechanism involving the transcription process, or select for recombinants not expressing the 5-HT$_{1A}$ receptor.

One of the drawbacks of the use of cloned cells for investigation of receptor-effector mechanisms is that the expression of the gene product in a novel environment may result either in an inappropriate coupling mechanism (i.e. to the wrong effector system) or to the correct effector system but with a different receptor-effector coupling efficiency from that seen for the native receptor. Differences in coupling efficiency of the human 5-HT$_{1A}$ receptor expressed in HeLa cells dependent upon the degree of receptor expression have recently been reported by Boddeke *et al.* (1992). Both these effects would severely compromise the usefulness of the cells. Whilst Albert *et al.* (1990) and Liu & Albert (1991) demonstrated that in GH$_4$ZD10 cells the expressed 5-HT$_{1A}$ receptors couple to inhibition of adenylyl cyclase in a pertussis toxin-sensitive manner, no information was provided concerning the receptor-effector coupling efficiency.

In the present study, the relative effects of a series of compounds active at the 5-HT$_{1A}$ receptor have been determined. The pattern of efficacies (e.g. affinity but low efficacy of spiperone, partial agonist character of buspirone and ipsapirone, full agonist efficacies of 5-MeODMT, 5-CT, RU 24949, PAPP, (+)-LSD, lisuride) found agree well with the observed values for the native postsynaptic 5-HT$_{1A}$ receptor in rat hippocampal slices and primary neuronal cultures (De Vivo & Maayani, 1986; Dumuis *et al.*, 1988). The slightly lower efficacy of (±)-8-OH-DPAT than 5-HT is also consistent with the literature, since 8-OH-DPAT consists of two enantiomers, one of which has full intrinsic activity while the other has partial agonist character with respect to inhibition of forskolin-stimulated cyclic AMP production in hippocampal slices (Cornfield *et al.*, 1991). Thus these data indicate that the receptor-effector coupling efficiency of the 5-HT$_{1A}$ receptor expressed in GH$_4$ZD10 cells is likely to be similar to that of the post-synaptic receptor in the rat hippocampus, and different from the presynaptic receptor, where a large receptor reserve has been demonstrated (Meller *et al.*, 1990).

In conclusion, the relative instability of the 5-HT$_{1A}$ receptors in the GH$_4$ZD10 cells found under the culturing conditions used here restricts the useful passage range of these cells and necessitate the presence of positive controls (e.g. 1000 nM 5-HT, 1000 nM 8-OH-DPAT and 3000 nM buspirone) in each experiment. Despite this drawback, the cells provide an excellent *in vitro* model for the hippocampal postsynaptic 5-HT$_{1A}$ receptors in the absence of other 5-HT receptor subtypes.

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Characterization of adrenoceptors involved in the electrogenic chloride secretion by cultured rat epididymal epithelium

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1 Short-circuit current (SCC) technique was used to study the adrenoceptors involved in the electrogenic chloride secretion by cultured cauda epididymal epithelium of rats. Stimulation of the epithelium with noradrenaline (primarily β_1-adrenoceptor selective agonist), salbutamol (β_2-adrenoceptor selective agonist) and adrenaline (non-selective β-adrenoceptor agonist) led to a rise in SCC. At a low chart-speed (2 mm min^{-1}), the response profile to these agonists consisted of a peak followed by a sustained response considerably higher than the basal SCC.

2 The EC_{50}s (doses of agonist producing 50% maximum response) of noradrenaline, salbutamol and adrenaline were 300, 115 and 10 nM respectively. Pretreating the tissues with 1 μM atenolol (β_1-selective antagonist) and 10 μM butoxamine (β_2-selective antagonist) shifted the dose-response curves of noradrenaline (shifted EC_{50} = 4000 nM) and salbutamol (shifted EC_{50} = 1050 nM) to the right. Atenolol (1 μM) and butoxamine (10 μM) shifted the dose-response curve of adrenaline to the right with new EC_{50}s of 30 nM and 115 nM, respectively.

3 The rapidly rising phase of the SCC response to noradrenaline and adrenaline observed at low chart-speed consisted of a brief and transient retraction followed by a rebound increase in SCC. At a high chart-speed (1 mm s^{-1}), the retraction and rebound phenomenon manifested as a fast initial spike which could be blocked by phentolamine (non-specific α-adrenoceptor antagonist) in a dose-dependent fashion. Similar initial spikes were observed when the tissues were stimulated with phenylephrine (α_1-selective agonist) but not with isoprenaline (non-selective β-agonist) or forskolin (activator of adenylate cyclase). The response of the initial spike triggered by noradrenaline was dose-dependent and the EC_{50} was 2000 nM.

4 The present study showed that the electrogenic chloride secretion by rat epididymis could be stimulated by α_1-, β_1- and β_2-adrenoceptor agonists. The α_1-mediated response had a faster onset and more transient action than the β-counterpart. It is postulated that epididymal chloride secretion might be regulated by neural (noradrenaline-mediated) and humoral (adrenaline-mediated) controls and that the stimulus-secretion coupling mechanisms might involve both Ca^{2+} (α_1-mediated response) and adenosine 3':5'-cyclic monophosphate (β-mediated response) as intracellular second messengers.

Keywords: Adrenoceptors; rat epididymis; chloride secretion; cell culture

Introduction

Transepithelial chloride secretion in the epididymis has been shown to be stimulated by adrenoceptor agonists (Wong & Chan, 1988). Activation of adrenoceptors on epithelial cells triggers cascades of biochemical reactions leading to increased concentrations of intracellular second messengers like adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Wong & Huang, 1990) and/or Ca^{2+} (Pfeilschifter *et al.*, 1991). These messengers act on different components of the secretory pathway to stimulate chloride secretion (for review see Donowitz & Welsh, 1986). It is believed that signal transduction mechanisms are specific to the adrenoceptors involved (Rasmussen, 1990). For instance, activation of β-adrenoceptors increases the intracellular concentration of cyclic AMP (Levitzki, 1988) whereas activation of α_1-receptors leads to a rise in intracellular Ca^{2+} concentration (Rooney *et al.*, 1989).

It has been demonstrated that the epithelium of the cauda epididymidis is richly innervated by noradrenergic fibres (El-Badawi & Schenk, 1967) which might serve a secretomotor function (Wong *et al.*, 1992). On the other hand, the threshold dose of adrenaline required to stimulate chloride secretion by the epididymis (Wong & Chan, 1988) was close to the circulating adrenaline concentration (Eisenhofer *et al.*, 1985). It is generally believed that target tissues respond to nerve and circulating catecholamine stimulation through α- and β-adrenoceptors respectively (Bevan *et al.*, 1980). The present study aimed to characterize the adrenoceptors involved in chloride secretion by cultured rat epididymal epithelium with a view to understanding the stimulus-secretion coupling mechanisms and the neural and humoral regulation of chloride secretion by the epididymis.

Methods

Tissue culture techniques

The procedures of tissue cultures have been described previously (Cuthbert & Wong, 1986; Wong, 1988a). Male Sprague-Dawley rats weighing 210 to 230 g were used as the source of tissue. The rats were killed by a blow to the head followed by cervical dislocation. The lower abdomen was opened and the caudal part of each epididymis was separated from the rest of the organ. The tissue was finely chopped with scissors and was then digested with 0.25% (w/v) trypsin followed by 0.1% (w/v) collagenase I. The primary cultures were grown on Millipore filters floating on Eagle's Minimum Essential Medium (EMEM) completed with 10% foetal calf serum and other supplements (Wong, 1988a). Cultures were incubated for 4 days at 32°C in 5% CO_2. Thereafter, the monolayers reached confluency and were ready for the measurement of short-circuit current.

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The short-circuit current measurement

The short-circuit current (SCC) measurement has been described previously (Cuthbert & Wong, 1986; Wong, 1988a). Confluent monolayers of rat epididymal cells were clamped vertically between the two halves of the Ussing Chambers. The tissues were short-circuited (transepithelial potential difference clamped at zero) by use of a voltage-clamp amplifier (DVC 1000; World Precision Instruments Inc., Florida, U.S.A.) and the short-circuit current was displayed on a pen recorder (Kipp and Zonen, Delft, The Netherlands). To measure the transepithelial resistance, the transepithelial potential was sometimes clamped intermittently at a value slightly different from zero (0.05–0.3 mV). The resulting current change allowed calculation of the resistance from the Ohmic relationships. The two channels of the amplifier were mostly used simultaneously on parallel monolayers so that studies could be made under control and experimental conditions. At the end of the experiments, the area of the monolayers was measured with an IBM compatible computer equipped with a digitizer (Hipad, Houston Instruments, Austin, Texas, U.S.A.) and the software Autocad. In most experiments, monolayers were incubated in Krebs-Henseleit solution (for composition see below).

Drug addition

Chemical agents used in the present study were added directly to the bathing solution on the basolateral aspect of the monolayers. To study the effects of adrenoceptor antagonists, the tissues were pretreated with the antagonists for 5–10 min before stimulation with the agonists to allow even distribution of the former within the tissues. In all experiments, addition of antagonists alone did not affect the basal short-circuit current. To avoid receptor desensitization (Hausdorff *et al.*, 1990), each monolayer was stimulated with a particular agonist only once (either in the absence or presence of antagonists).

Calculation of pK_B values

The pK_B is a measure of the affinity of a competitive antagonist for its receptors. It is determined from experiments in which the tissue response to various concentrations of a receptor agonist is inhibited by a fixed concentration of the corresponding antagonist. In the present study, the dose-response curves for noradrenaline (primarily β_1-selective agonist) or salbutamol (β_2-selective agonist) were obtained in the absence and presence of atenolol (β_1-antagonist) or butoxamine (β_2-antagonist) respectively. The dose-ratio (DR) was calculated from the EC_{50} in the presence of antagonist divided by that in its absence. The pK_B was calculated from DR according to the equation

$$pK_B = \log (DR-1) - \log B,$$

where B was the concentration of the antagonist expressed in M.

Although in the present study pK_B was determined from one concentration of antagonist only, it provided an estimate of the affinity of the antagonist for the receptors thereby indicating the specificity of its effects.

Solutions and materials

The normal Krebs-Henseleit (K-H) solution had the following composition (mM): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.1. The solution was gassed with 5% CO_2/95% O_2 to give a pH of 7.4. Eagle's Minimum Essential Medium (EMEM), foetal calf serum and non-essential amino acids were purchased from Gibco Laboratories (New York, U.S.A.). Penicillin/streptomycin, Hank's Balanced Salt Solution, sodium pyruvate, 5 α-dihydrotestosterone, trypsin, collagenase I, atenolol, butoxamine, noradrenaline and phenylephrine were from Sigma Chemical Co. (St. Louis, U.S.A.). Adrenaline and salbutamol were purchased from the David Bull Laboratories (Victoria, Australia) and Glaxo (Greenford, England) respectively.

Statistical analysis

Results are expressed as means ± standard error of the mean (s.e.mean). Comparisons between groups of data were made by Student's unpaired *t* test. A *P* value of less than 0.05 was considered statistically significant.

Results

Effects of β-adrenoceptor agonists on SCC

When bathed in normal Krebs-Henseleit solution, the epididymal monolayers exhibited a transepithelial potential difference of 3.3 ± 0.12 mV ($n = 258$ cultures), a basal short-circuit current (SCC) of 6.4 ± 0.21 μA cm^{-2} ($n = 256$ cultures) and a transepithelial resistance of 390.3 ± 15.8 Ω cm^2 ($n = 232$ cultures). Figure 1 shows the effects of β-adrenoceptor agonists on SCC. Addition of noradrenaline (primarily a β_1-selective agonist), salbutamol (β_2-selective agonist) and adrenaline (non-selective β-agonist) to the basolateral bathing solution caused a rapid rise in SCC which attained a maximum within 1 min after stimulation. This was followed by a plateau phase considerably higher than the basal SCC. For quantitative studies, the SCC response to these agonists was defined as the peak increase in SCC after stimulation (see below).

Effects of β-adrenoceptor antagonists

Figure 2 shows the dose-response relationships of noradrenaline in the absence and presence of a β_1-selective antagonist, atenolol. The dose-response curves exhibited a sigmoidal profile. The threshold and EC_{50} of noradrenaline were about 1 and 300 nM respectively and the maximum SCC response was attained at 1 mM. Pretreatment with atenolol (1 μM) shifted the dose-response curve to the right with an EC_{50} of 4000 nM. The dose ratio (DR) was thus 13.3 and the pK_B of atenolol was calculated to be 7.1 (See Methods).

Figure 3 shows the dose-response relationships of salbutamol in the absence and presence of a β_2-selective antagonist, butoxamine. The dose-response curve of salbutamol was sigmoidal and the threshold and EC_{50} were 1.7 and

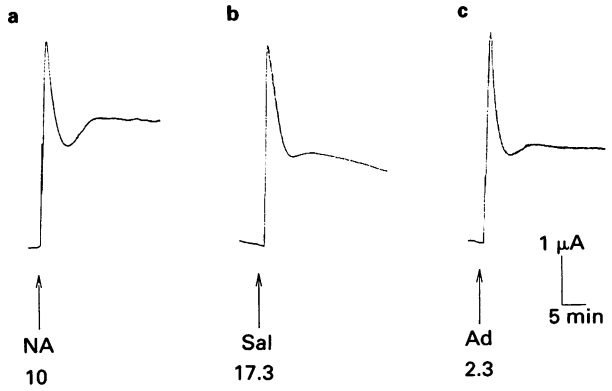

Figure 1 Short-circuit current measurement in three separate monolayers (area 0.4–0.6 cm^2). The tissues were stimulated with (a) noradrenaline (NA, 10 μM), (b) salbutamol (Sal, 17.3 μM) and (c) adrenaline (Ad, 2.3 μM). The agonists were added to the basolateral side of the tissues. The arrows indicate the time at which the drugs were added. Each record is representative of at least six separate experiments.

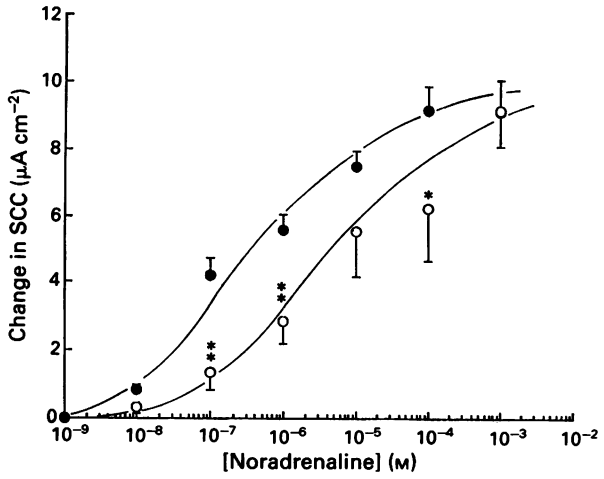

Figure 2 Effects of varying the concentration of noradrenaline on the short-circuit current (SCC) response in the absence (●) and presence (○) of 1 μM atenolol. Each data point is the mean of 4 to 6 separate experiments and each error bar represents one s.e.mean. Asterisks represent the level of significance when the SCC response to a particular dose of noradrenaline in the presence of atenolol was compared with that in its absence (*$P<0.05$; **$P<0.01$).

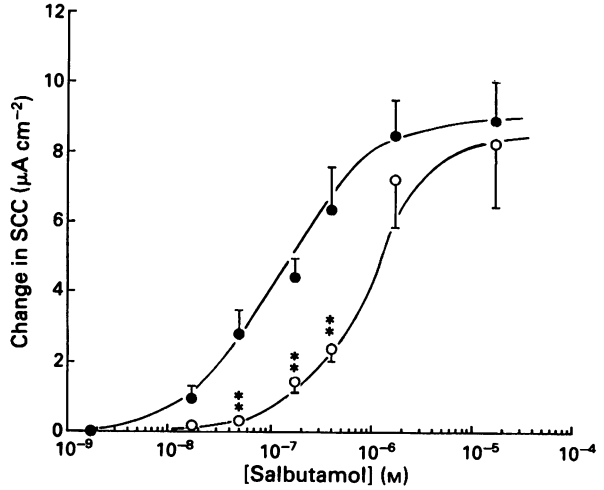

Figure 3 Effects of varying the concentration of salbutamol on the short-circuit current (SCC) response in the absence (●) and presence (○) of 10 μM butoxamine. Each data point is the mean of 4 to 6 separate experiments and each error bar represents one s.e.mean. Asterisks represent the level of significance when the SCC response to a particular dose of salbutamol in the presence of butoxamine was compared with that in its absence (**$P<0.01$).

115 nM respectively. Maximum effect was attained at 17.3 μM. Pretreatment with butoxamine (10 μM) shifted the dose-response curve to the right with an EC_{50} of 1050 nM. The DR was 9.1 and the pK_B of butoxamine was calculated as 5.9.

Figure 4 shows the dose-response relationships of adrenaline in the absence and presence of atenolol or butoxamine. The threshold and EC_{50} of adrenaline were 0.23 and 10 nM respectively and the maximum response was reached at 2.3 μM. Pretreatment with atenolol (1 μM) or butoxamine (10 μM) shifted the dose-response curves to the right with EC_{50}s of 30 and 115 nM respectively. The DR for atenolol was 3 and that for butoxamine was 11.5. The pK_B of atenolol and butoxamine were 6.3 and 6.0 respectively.

Effects of α-adrenoceptor stimulation

During the rapidly rising phase of the SCC response to noradrenaline, a brief and fast retraction followed by a rebound increase in SCC was observed (see Figure 1a). Figure 5a shows the effect of noradrenaline (a non-specific α-agonist in addition to its β_1-selective action) on SCC at expanded time-scale. The retraction and rebound phenomenon manifested as a fast initial spike followed by a delayed and relatively prolonged rise in SCC. The latter corresponded to the peak observed at low chart-speed (see Figure 1a). The initial spike lasted for 8–10 s and was succeeded by the second component before SCC had returned to the basal level. Similar experiments were performed with other adrenoceptor agonists. Figure 5b and c shows the effects of phenylephrine (an α_1-selective agonist) and adrenaline (a non-specific α-agonist in addition to its β-action) respectively on SCC at expanded time-scale. Initial spikes were observed with both agonists. Figure 5d and e show the effects of isoprenaline and forskolin respectively on SCC. Isoprenaline is a pure β-agonist and forskolin is an activator of adenylate cyclase, which forms an integral part of the signal transduction pathway triggered by β-adrenoceptor stimulation. The initial spike was not observed upon stimulation with either of these substances.

Figure 6 shows the effects of noradrenaline (10 μM) on SCC in the absence (a) and the presence of 0.1 μM (b) and 0.2 μM (c) phentolamine, a non-specific α-antagonist. It can be seen that phentolamine at 0.1 μM reduced and at 0.2 μM completely abolished the initial spike triggered by noradrenaline.

Figure 7 shows the dose-response relationships of noradrenaline measured as the maximum SCC reached by the initial spike and the second phase of response. Both curves exhibited a sigmoidal profile. The EC_{50} for the initial spike was 2000 nM while that for the second component was 300 nM. The threshold doses required to elicit the initial spike and the second component were 10 and 1 nM respectively.

Discussion

The present study demonstrated that the electrogenic chloride secretion by cultured rat epididymal epithelium could be stimulated by β_1-, β_2- as well as α_1-adrenoceptor agonists. The response triggered by α_1-stimulation (i.e. the initial spike) had a faster onset and more transient action than the β-counterpart (see Figure 5). The β_1- and β_2-mediated chloride secretion were competitively blocked by atenolol and butoxamine respectively, whereas the α_1-component was inhibited by phentolamine in a dose-dependent fashion.

Short-circuit current was used as a measure of electrogenic chloride secretion across the epididymal epithelium. Although chloride fluxes across the epithelium have not been measured, previous studies with specific chloride channel blockers (Wong, 1988b) as well as bilateral (Wong, 1988a) and unilateral chloride replacement (Leung & Wong, 1992) have shown that the short-circuit current response to secretagogues could be attributed to the opening of apical chloride channels and the subsequent exit of chloride from the cytosol into the apical solution. Like other secretory epithelia, chloride diffuses across the apical membrane along its electrochemical gradient, which is maintained by the active accumulation of chloride inside the cells by the Na^+/K^+ ATPase, the $Na^+/K^+/2Cl^-$ symport and the K^+ channels on the basolateral membrane (Young & Cook, 1987). It has been reported in the epididymal (Wong & Chan, 1988) and tracheal epithelium (Al-Bazzaz & Cheng, 1979) that chloride secretion could be increased upon stimulation of β-adrenoceptors. The present study shows that both β_1- and β_2-receptors are involved in the process. To investigate the β-adrenoceptor subtypes, noradrenaline and salbutamol were used as selective β_1- and β_2-adrenoceptor agonists respectively

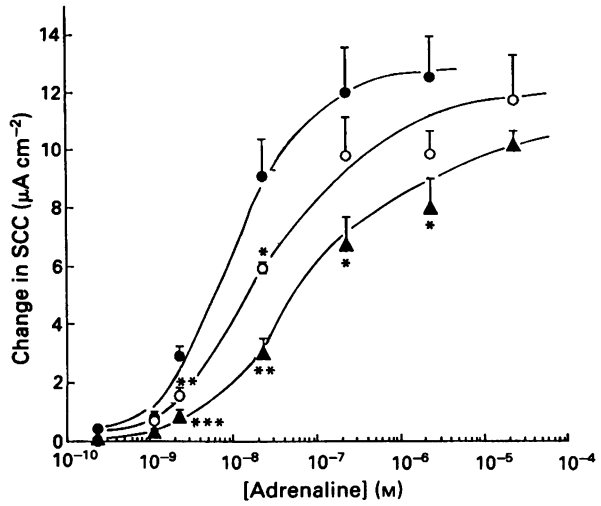

Figure 4 Effects of varying the concentration of adrenaline on the short-circuit current (SCC) response in the absence (●) and presence of 1 μM atenolol (○) or 10 μM butoxamine (▲). Each data point is the mean of 4 to 6 separate experiments and each error bar represents one s.e.mean. Asterisks represent the level of significance when the SCC response to a particular dose of adrenaline in the presence of atenolol or butoxamine was compared with that in their absence (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

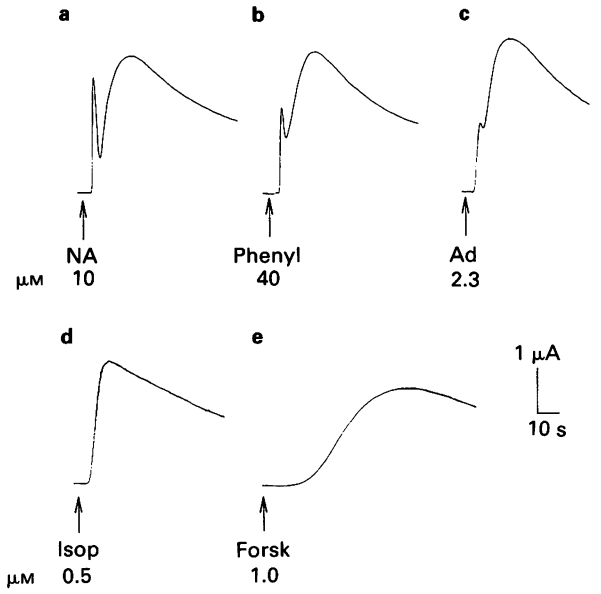

Figure 5 Short-circuit current measurement in five separate monolayers (area 0.4–0.6 cm²) at expanded time-scale. The tissues were stimulated with (a) noradrenaline (NA, 10 μM), (b) phenylephrine (Phenyl, 40 μM), (c) adrenaline (Ad, 2.3 μM) (d) isoprenaline (Isop, 0.5 μM) and (e) forskolin (Forsk, 1.0 μM). The agonists were added to the basolateral side of the tissues. The arrows indicate the time when the drugs were added. Each record is representative of at least six separate experiments.

(for review see Nahorski, 1981). Although a detailed Schild plot analysis has not been performed, the pK_B values of 7.1 and 5.9 determined from a single concentration of atenolol (1 μM) and butoxamine (10 μM) respectively were comparable to those reported in studies on atrial tissues (Leclerc *et al.*, 1981; O'Donnell & Wanstall, 1983). The results suggested that the inhibitory effects of atenolol and butoxamine could be due to the specific inhibition of β_1- and β_2-adrenoceptors

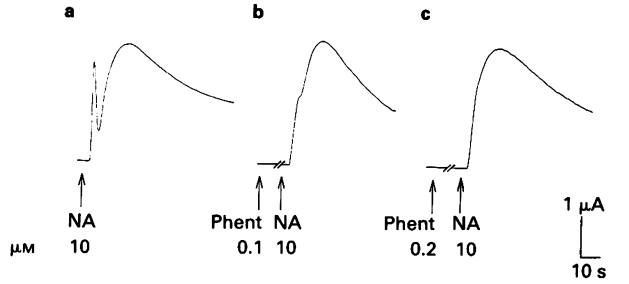

Figure 6 Short-circuit current measurement in three separate monolayers (area 0.4–0.6 cm²) at expanded time-scale. The tissues were stimulated with noradrenaline (NA, 10 μM) in the (a) absence and presence of (b) 0.1 μM or (c) 0.2 μM phentolamine (Phent). The agonists were added to the basolateral side of the tissues. The arrows indicate the time when the drugs were added. Each record is representative of at least six separate experiments.

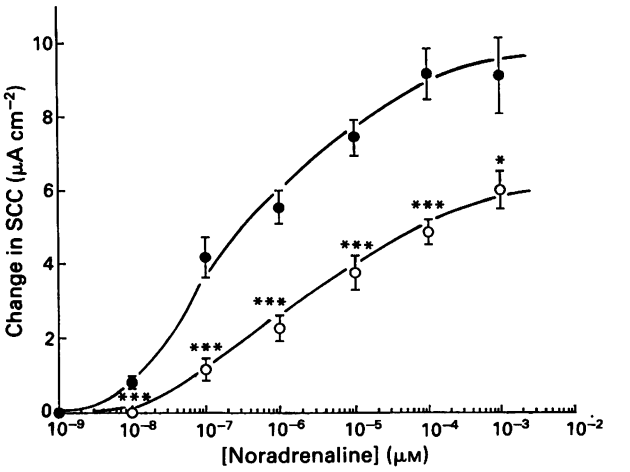

Figure 7 Effects of varying the concentration of noradrenaline on the short-circuit current (SCC) response expressed as the maximum SCC reached by the initial spike (○) and the second peak (●). Each data point is the mean of 4 to 6 separate experiments and each error bar represents one s.e.mean. Asterisks represent the level of significance when the maximum SCC reached by the initial spike at a particular dose of noradrenaline was compared with that reached by the second peak (*$P<0.05$, ***$P<0.001$).

respectively. It was also found that the SCC response to adrenaline could be inhibited by atenolol or butoxamine, suggesting that it could be mediated by both β_1-and β_2-adrenoceptor stimulation.

The present study also provides evidence for the existence of α_1-receptors in the epididymal epithelium. Stimulation of the tissues with an α_1-selective agonist phenylephrine, as well as non-selective α-agonists noradrenaline and adrenaline, gave rise to an initial spike which could be blocked by phentolamine, a non-selective α-antagonist. Recent work in our laboratory has shown that the initial spike could be abolished by agents which perturbed the intracellular Ca^{2+} homeostasis, like thapsigargin and the calcium ionophore A23187 (Wong *et al.*, unpublished). This was not unexpected as α_1-mediated cellular responses are related to changes in intracellular Ca^{2+} concentration (Exton, 1985). The effects of phentolamine on the initial spike could not easily be quantified because as the initial spike was reduced by phentolamine, it was usually masked by the β-mediated second component. It is interesting that in addition to the α_1-mediated initial spike, the SCC response to phenylephrine exhibited a prominent second component which could be reduced by propranolol, a non-selective β-antagonist (Wong

et al., unpublished). This suggested that in the epididymis, phenylephrine might possess a significant β-adrenoceptor effect in addition to its specific α_1-action. The β-effect of phenylephrine on SCC measurement has also been reported in tracheal epithelium (Bainbridge et al., 1989).

The demonstration of both β- and α-mediated chloride secretion by the epididymis may give a clue to the stimulus-secretion coupling mechanisms. Stimulation of β-adrenoceptors in the epididymal (Wong & Huang, 1990) and tracheal epithelium (Smith et al., 1982) has been shown to increase intracellular cyclic AMP concentration, causing a subsequent rise in chloride secretion. On the other hand, the presence of α_1-adrenoceptors in the epididymal epithelium suggests that intracellular Ca^{2+} may also be involved in chloride secretion because α_1-adrenoceptor agonists are prototypes of Ca^{2+} mobilizing agents (Exton, 1985). Measurement of intracellular Ca^{2+} in Fura-2 loaded epididymal cells has demonstrated a transient rise in intracellular Ca^{2+} concentration upon stimulation with noradrenaline (Wong et al., unpublished). Moreover, stimulation of the tissues with the calcium ionophore A23187 has been found to increase Cl^- secretion across the epithelium (Wong, 1988a). However, in the present study the effector processes responsible for the cyclic AMP- and Ca^{2+}-mediated chloride secretion were not investigated. Cyclic AMP has been shown to activate apical chloride channels in the epididymal (Pollard et al., 1991) as well as the colonic (Halm et al., 1988) epithelium. It is believed that cyclic AMP activates protein kinase A and that the subsequent phosphorylation and activation of chloride channels increase chloride secretion (Huang et al., 1992). An increase in intracellular Ca^{2+} concentration has been found to open potassium channels on the basolateral membrane (Petersen & Maruyama, 1984). It has been suggested that the resulting membrane hyperpolarization increases the driving force for chloride exit across the apical membrane and hence chloride secretion (McCann & Welsh, 1990). However, recent studies have also demonstrated the existence of Ca^{2+}-activated chloride conductance in the epididymal (Huang et al., unpublished observations) as well as T-84 colonic tumour cells (Cliff & Frizzell, 1990).

The demonstration of α_1-, β_1- and β_2-adrenoceptor-mediated chloride secretion suggests that the secretory functions of the cauda epididymidis might be under both neural and humoral controls. The cauda epididymidis has been shown to be innervated by intra-epithelial noradrenergic fibres (El-Badawi & Shenk, 1967). Noradrenaline released from nerve terminals may act on α_1- and β_1-adrenoceptors on the epithelium to stimulate chloride secretion. Chloride secretion could also be stimulated by adrenaline (Wong & Chan, 1988) and the threshold dose required to elicit a response was close to the adrenaline concentration in the general circulation (Eisenhofer et al., 1985). Circulating adrenaline may activate various adrenoceptors, including β_2-receptors (Davis et al., 1990), on the epithelium to trigger chloride secretion from the blood to the lumen.

The neurohumoral control of chloride secretion may play a role in the maintenance of a unique and specific microenvironment in the epididymis on which the maturation and storage of spermatozoa depend (Jenkins et al., 1980). It has also been proposed that adrenergic stimulation of chloride secretion in the epididymis may be involved in the emission reflex (Wong & Chan, 1988). Secretion of electrolytes and fluid, coupled with tubular smooth muscle contraction, may facilitate the passage of spermatozoa during ejaculation. Disruption of the control mechanism by, for instance, sectioning the sympathetic nerve supply to the epididymis has been shown to affect adversely sperm transport and motility (Billups et al., 1990a). It was suggested that sympathetic denervation might lead to functional tubular obstruction (Billups et al., 1990b), a reflection of defective fluid transport in the epididymis (Wong, 1990).

The present study demonstrated the co-existence of α_1-, β_1- and β_2-adrenoceptors in a single exocrine tissue. It is believed that mammalian tissues can co-express both β_1- and β_2-adrenoceptors (Nahorski, 1981) and that, under certain circumstances, they may mediate the same physiological response (Carlsson et al., 1972). On the other hand, co-expression of α_1- and β_2-adrenoceptors has been reported in the MDCK cells (Slivka & Insel, 1987). It is interesting that the α_1- and β-mediated chloride secretion in the epididymis follows different temporal profiles. The α_1-response has a faster onset and more transient action while the β-response has a relatively delayed onset but more prolonged effects. A similar temporal sequence of α_1- and β-mediated cellular responses has been found in dissociated kidney epithelial cells (MDCK cells) (Breuer et al., 1988). The present study did not establish whether both responses are mediated by a single epithelial cell, as in MDCK cells (Breuer et al., 1988) or by two separate epithelial cell types with different signal transduction mechanisms.

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Effects of cromakalim on the membrane potassium permeability of frog skeletal muscle *in vitro*

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1 The effects of the potassium channel opener, cromakalim, and its active enantiomer, lemakalim, have been investigated in frog skeletal muscle.

2 Cromakalim (30–300 μM) increased ^{86}Rb efflux from muscles loaded with the isotope, hyperpolarized the fibres and reduced membrane resistance.

3 These effects were inhibited by the sulphonylureas, glibenclamide and tolbutamide. The IC_{50} for glibenclamide inhibition of ^{86}Rb efflux was ca. 8 nM.

4 Phentolamine (300 μM) (which blocks responses to cromakalim in smooth muscle and inhibits ATP-sensitive K^+ channels in pancreatic β-cells) had no effect on the reduction in membrane resistance caused by 100 μM lemakalim.

5 Diazoxide (600 μM) had no effect on ^{86}Rb efflux.

6 The similarities of the K^+ channel activated by cromakalim in frog skeletal muscle to the channel acted on in smooth muscle and to the ATP-sensitive K^+ channel of β-cells are discussed.

Keywords: Cromakalim; skeletal muscle; glibenclamide; potassium permeability

Introduction

Cromakalim is a smooth muscle relaxant belonging to the class of agents known as 'potassium channel openers' which are of interest for potential use in hypertension and asthma. Its ability to relax smooth muscle follows from its action in increasing the potassium permeability of the cell membrane and so reduce electrical excitability (Hamilton & Weston, 1989). Since the effects of cromakalim in smooth muscle are inhibited by sulphonylureas (e.g. glibenclamide) (Quast & Cook, 1989) which appear to be selective inhibitors of adenosine 5'-triphosphate (ATP)-sensitive K^+ (K_{ATP}) channels (Sturgess *et al.*, 1985; Ashcroft, 1988) it has been proposed that the actions of cromakalim are on this type of channel. Cromakalim has indeed now been shown to activate identified K_{ATP} channels in excised membrane patches of ventricular myocytes (Escande *et al.*, 1988) and the important requirement for this hypothesis that smooth muscle possesses K_{ATP} channels has now been demonstrated (Standen *et al.*, 1989; Kajioka *et al.*, 1991).

Skeletal muscle is also known to possess K_{ATP} channels (Spruce *et al.*, 1985) which show sulphonylurea-sensitive opening under conditions of ATP depletion (Castle & Haylett, 1987). This suggested that it would be valuable to examine the actions of cromakalim in this tissue. Additional experiments were conducted to explore the actions of phentolamine, which has been reported to block the actions of cromakalim in smooth muscle (McPherson & Angus, 1989) and to inhibit K_{ATP} channels in pancreatic β-cells (Plant & Henquin, 1990), and diazoxide which is a well known activator of K_{ATP} channels in β-cells (e.g. Trube *et al.*, 1986; Garrino *et al.*, 1989).

In concurrent studies, Spuler *et al.* (1989) have shown cromakalim to increase K^+ conductance in human skeletal muscle and Weik & Neumcke (1990), using patch recording, have shown that cromakalim can increase the open probability of single K_{ATP} channels of mouse skeletal muscle.

Some of these results have already appeared in abstract form (Benton & Haylett, 1990).

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Methods

Experiments were carried out on sartorius muscles from adult *Rana temporaria*. The frogs were killed by a blow to the head followed by destruction of the brain and spinal cord. Except for electrophysiological recordings, the tissue was bathed in a Ringer solution of the following composition (mM): NaCl 116, KCl 2.5, $CaCl_2$ 1.8, NaH_2PO_4 0.25 and Na_2HPO_4 1.75 (pH 7.2). All experiments were conducted at room temperature.

^{86}Rb efflux experiments

Both sartorii were removed and tied to frames made of stainless steel tubing. The tissue was loaded with ^{86}Rb by incubation in Ringer solution containing the tracer at an activity of 0.1 MBq ml^{-1} for 90 min. At the end of the loading period the muscles were washed in a large volume of Ringer in order to remove superficial and much of the extracellular tracer. After 30 min (when the rate constant for ^{86}Rb efflux had become relatively steady) the tissue was transferred, at 2 min intervals, through a series of 15 test tubes. Each contained 5 ml of solution which was gassed via the tubular frame with 100% oxygen to provide mixing. The muscles were exposed to cromakalim or diazoxide for a 10 min period beginning 40 min after removal from the load solution. At the end of the experiment the muscles were dissolved in concentrated HNO_3 to allow the measurement of remaining radioactivity. The radioactivity lost into the washout solutions and in the muscle extract were determined by Cêrenkov counting in a scintillation counter (Beckman LS1801), a quench correction for the muscle extract being determined by internal standardization (spiking). The rate constant for ^{86}Rb efflux was expressed as the fractional loss of tracer per min. The increase in efflux stimulated by cromakalim was calculated by comparing the average rate constant during the 2nd, 3rd and 4th drug application periods with the average value for the three periods immediately preceding drug application. In experiments investigating the effect of glibenclamide one muscle from each pair was exposed to 100 μM cromakalim alone while the other was exposed to glibenclamide for 20 min before, and during exposure to the same concentration of cromakalim.

Intracellular recording

The effects of cromakalim and lemakalim on membrane potential and resistance were assessed by conventional electrophysiological techniques and employing microelectrodes filled with 3 M KCl. Fibres were impaled with two microelectrodes, one being used to measure membrane potential, the other to pass current. Changes in membrane resistance (in terms of input resistance) were detected by measuring the amplitude of electrotonic potentials caused by the injection of current pulses (400 ms, 10–20 nA). An Axoclamp 2A (Axon Instruments) was used for the measurement of membrane potential and for current passing. Trigger pulses were provided by a Digitimer D4030 (Digitimer Ltd) and recordings were made on a Devices chart recorder (MX4).

Muscles were mounted in a perspex recording chamber and superfused with Ringer solution at 4–5 ml min^{-1}. For these experiments a Cl^--free Ringer solution was used in the expectation that the removal of the large resting Cl^- conductance (Hodgkin & Nakajima, 1972) would allow changes in K^+ conductance to be observed more easily. The solution had the composition (mM): Na isethionate 116, K_2SO_4 1, $CaSO_4$ 2.4, NaH_2PO_4 0.25, Na_2HPO_4 0.75 (pH 7.2). In some experiments tetrodotoxin (10^{-7} M) was included to prevent the twitching which was sometimes induced in the Cl^--free Ringer solution.

Materials

^{86}Rubidium was supplied by New England Nuclear. Sodium isethionate was purchased from Fluka. Other reagents were of analytical grade. Tolbutamide was purchased from Sigma and the following were gifts: cromakalim and lemakalim (Beecham's Research Laboratories), glibenclamide (Hoechst), phentolamine (ICI) and diazoxide (Allen & Hanburys). Cromakalim, lemakalim, glibenclamide and tolbutamide were prepared as stock solutions in dimethylsulphoxide.

Statistics

Results are presented as means ± standard error of the mean. Differences were considered significant if P was less than 0.05 by Student's t test.

Results

^{86}Rb efflux

Cromakalim (30–300 μM) produced an increase in the rate constant for ^{86}Rb efflux. Figure 1 presents some results with 100 μM cromakalim and indicates that the response generally peaked between 2 and 6 min and showed some decline before the end of the drug exposure. Figure 2 gives information on the concentration-dependence of the response. The low potency of cromakalim, and our desire to avoid other effects of cromakalim at excessive concentrations (e.g. Ca^{2+}-channel block, Ito *et al.*, 1990) restricted our investigation of the upper part of the concentration-response curve. However, in those experiments ($n = 3$) where comparison was made within a single experiment, (and animal), 300 μM cromakalim did not produce a significantly greater increase in ^{86}Rb efflux than 100 μM. It is concluded that the maximal increase in ^{86}Rb efflux is unlikely to be much more than 200%. In three different muscles, diazoxide (600 μM) had no significant effect on ^{86}Rb efflux, the change in rate constant being − 5 ± 3%.

Glibenclamide inhibited the response to cromakalim, as shown in Figure 1. Concentrations of glibenclamide between 3 and 100 nM were tested for their ability to inhibit the response to 100 μM cromakalim, the concentration-inhibition curve being shown in Figure 3. The fitted curve provided an estimate of 8.0 ± 1.7 nM for the IC_{50}. Glibenclamide had no effect on the resting rate of efflux.

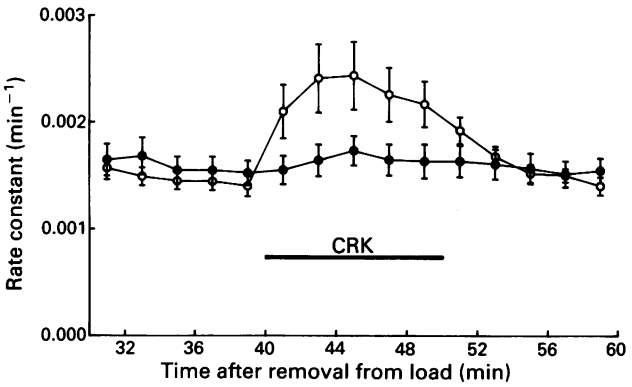

Figure 1 Effect of cromakalim (CRK) (100 μM), applied from 40 to 50 min after removal from the load solution, on ^{86}Rb efflux from frog sartorius muscle: (●) indicate the rate constants observed in experiments where glibenclamide (30 nM) was applied between 20 and 50 min; means with s.e.mean for 5 pairs of muscles are shown.

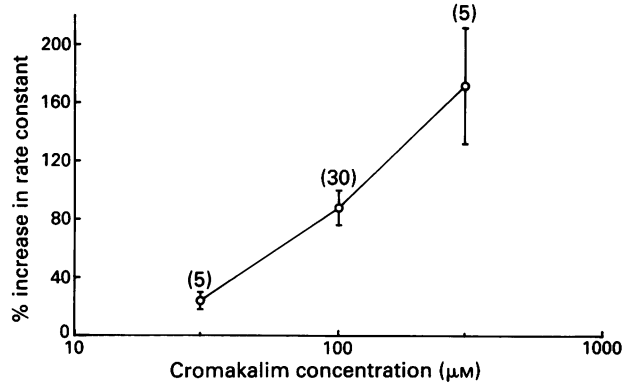

Figure 2 Concentration-response relationship for the increase in ^{86}Rb efflux rate constant produced by cromakalim (means with s.e.mean shown by vertical bars, n indicated in parentheses).

Intracellular recording

Cromakalim (100–300 μM) and lemakim (100 μM) caused cell membrane hyperpolarization and a reduction in input resistance which could be maintained for up to 10 minutes (Figure 4). Although (as previously noted) the results presented were obtained in Cl^--free Ringer these effects could be observed also in normal Ringer solution. Resting membrane potentials (uncorrected for any change in tip potential) averaged 91.5 mV (s.d. 6.1 mV, $n = 14$) and 200 μM cromakalim increased this by 7.9 ± 1.5 mV (s.e.mean, $n = 10$) and reduced 'input resistance' by 42 ± 4% ($n = 10$). In 5 fibres (from 3 frogs) 100 μM lemakalim caused a hyperpolarization of 6.6 ± 2.9 mV and reduced resistance by 49 ± 5%. It should be noted that the measurement of changes in input resistance is qualitative in that the current-passing and voltage-recording electrodes were usually 3–5 fibres diameters apart. The input resistance will therefore be underestimated but the measured percentage change in input resistance with cromakalim is likely to be increased as the space constant is reduced.

In 4 experiments, 300 μM tolbutamide completely blocked the actions of either 200 μM cromakalim or 100 μM lemakalim. Glibenclamide was more potent, 1 μM proving sufficient to abolish the response to cromakalim ($n = 3$).

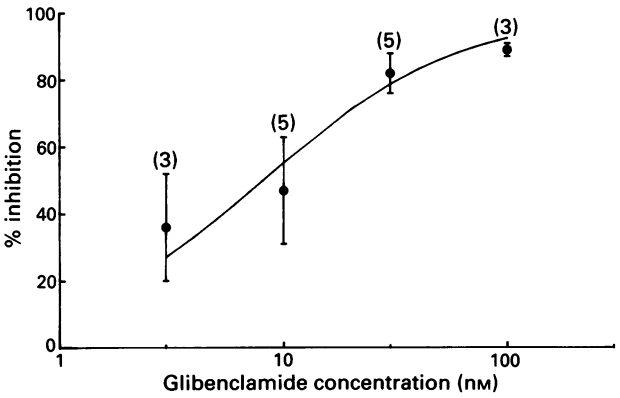

Figure 3 Relationship between glibenclamide concentration and inhibition of the ^{86}Rb efflux response to 100 μM cromakalim (means with s.e.mean shown by vertical bars, *n* indicated in parentheses). The fitted curve (non-linear least squares) is for simple competition with a Hill coefficient of unity.

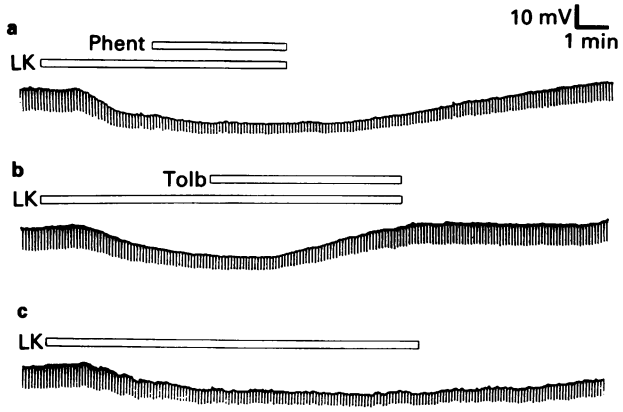

Figure 4 Effects of phentolamine and tolbutamide on the changes in membrane potential and input resistance produced by lemakalim (LK). Record (c) shows a control response to 100 μM lemakalim. In (a) 300 μM phentolamine (Phent) was added to the perfusate for the period indicated; (b) shows the reversal of the action of lemakalim by 300 μM tolbutamide (Tolb). These records are from the same fibre in the sequence (a), (b), (c). The records are broken for 17 min between (a) and (b) and for 15 min between (b) and (c). Note: the lag between the time at which drug application is indicated and any observed effect is largely attributed to the time taken for the drug to reach the muscle.

Phentolamine, tested in 3 fibres at 300 μM and 1 fibre at 100 μM, had no effect on the response to 100 μM lemakalim. Figure 4 demonstrates, in a continuous recording from a single fibre the lack of effect of phentolamine and complete block by tolbutamide.

Discussion

The increase in ^{86}Rb efflux, decrease in membrane resistance and hyperpolarization produced by cromakalim in frog skeletal muscle are each consistent with the opening of K^+ channels. As expected, lemakalim, which is the more active enantiomer of cromakalim (Hof *et al.*, 1988) reproduced these actions at a lower concentration. All of these effects could be inhibited by either glibenclamide or tolbutamide, which as described in the introduction provides *prima facie* evidence for involvement of K_{ATP} channels.

Although the electrophysiological experiments did not give a quantitative measurement of changes in specific membrane resistance, the magnitude of the change in input resistance is broadly in keeping with the 2–3 fold increase in ^{86}Rb efflux. It is worth noting that these changes in input resistance and ^{86}Rb efflux are much less than those produced by metabolic exhaustion (Fink & Luttgau, 1976; Castle & Haylett, 1987), and which are likely to involve the activation of K_{ATP} following ATP depletion. If it is supposed that K_{ATP} channel activation underlies both responses, what is the reason for the larger changes seen on poisoning? Firstly we can note that in exhausted muscle a component of the increase in ^{86}Rb efflux is not blocked by glibenclamide (Castle & Haylett, 1987), suggesting that K^+ channels other than the K_{ATP} type may be activated additionally under these conditions. Secondly, the gating of K_{ATP} channels by cromakalim is ATP-dependent. Thus Weik & Neumcke (1990) have demonstrated in mouse skeletal muscle that cromakalim will activate K_{ATP} channels that have been blocked by 0.1 mM ATP but has no effect on the open probability if ATP is raised to 1 mM. In non-poisoned frog muscle the intracellular concentration of ATP is of the order of 4 mM (Fink *et al.*, 1983) which may be sufficient to impair the effect of cromakalim.

The concentrations of cromakalim needed to increase P_K vary quite markedly between tissues, suggesting differences in the K^+ channels or their regulation. Cromakalim is most potent in smooth muscle where the EC_{50} for the increase in ^{86}Rb efflux is around 1 μM (Quast & Cook, 1989). In cardiac muscle the EC_{50} is in the range 5–30 μM (Osterrieder, 1988; Escande *et al.*, 1988; Sanguinetti *et al.*, 1988) whilst in insulin-secreting cells cromakalim is relatively ineffective (Garrino *et al.*, 1989; Dunne *et al.*, 1990). Our results in frog skeletal muscle, like those of Spuler *et al.* (1989) in human muscle suggest a potency between those in cardiac muscle and insulin-secreting cells. The relative activity of diazoxide to increase P_K in these tissues is quite different. In smooth muscle it is 100 × less potent than cromakalim (Quast & Cook, 1989) whereas in the β-cell it is more potent (Garrino *et al.*, 1989). In cardiac muscle diazoxide can *block* $P_{K(ATP)}$ (Faivre & Findlay, 1989). We have found diazoxide to have no effect on ^{86}Rb efflux in frog muscle which may thus resemble mouse skeletal muscle where diazoxide (400 μM) has no effect on K_{ATP} channel opening (Weik & Neumcke, 1990).

The complex pharmacology of cromakalim responses and K_{ATP} channels is further illustrated by our observations with phentolamine. Although phentolamine can abolish responses to cromakalim in vascular smooth muscle (McPherson & Angus, 1989) and inhibit the opening of K_{ATP} channels in β-cells (Plant & Henquin, 1990; Dunne, 1991) in the present study it was quite unable to block the change in input resistance and membrane potential produced by lemakalim.

From the results presented it is clear that cromakalim can cause the opening of K^+ channels in frog skeletal muscle. In view of the inhibition of this effect by glibenclamide and the positive identification of K_{ATP} channels in this tissue it seems likely that the ATP-sensitive K^+ channel is the target channel for cromakalim. It is also clear that the pharmacological properties of the channel differ from those of the channel acted on in other tissues.

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The effects of β_2-adrenoceptor agonists and a corticosteroid, budesonide, on the secretion of inflammatory mediators from monocytes

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1 The *in vitro* effects of the β_2-adrenoceptor agonists (1×10^{-9}–10^{-5} M), terbutaline, salmeterol, and formoterol, on the release of inflammatory mediators, i.e. the eicosanoids leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2) and the cytokine interleukin-1β (IL-1β), were assessed in cultures of human blood monocytes. For comparison, the effects of a 5-lipoxygenase inhibitor, BW A4C (1×10^{-9}–10^{-5} M), and a corticosteroid, budesonide (1×10^{-10}–10^{-5} M) were also examined. Sotalol was used to investigate whether the actions of β_2-agonists were mediated through β-adrenoceptors.

2 Terbutaline, like budesonide, had no significant effect on LTB_4 release, whereas BW A4C (IC_{50} = 2×10^{-8} M) was a potent inhibitor. All concentrations of formoterol approximately halved the LTB_4 secretion, whereas high concentrations (1×10^{-7}–10^{-5} M) only, of salmeterol, inhibited release. Only salmeterol, at high concentrations ($> 1 \times 10^{-6}$ M), lowered the secretion of PGE_2 in monocyte cultures. Formoterol and salmeterol reduced the secretion of IL-1β only at the highest dose (1×10^{-5} M). In contrast, budesonide ($\geqslant 1 \times 10^{-9}$ M) was a potent suppressant of this secretion.

3 Treatment of monocyte cultures with sotalol (1×10^{-5} M) did not significantly antagonize the inhibitory effects of salmeterol and formoterol. These results suggest that the inhibitory action of these β_2-agonists on the release of eicosanoids or IL-1β, is not mediated via β_2-adrenoceptors.

4 This study does not support a therapeutic importance of the anti-release effects of β_2-agonists since high concentrations were generally required. Furthermore, the anti-secretory action of β_2-agonists was distinct from that of corticosteroids.

Keywords: Terbutaline; sotalol; formoterol; salmeterol; interleukin-1β (IL-1β); leukotriene B_4 (LTB_4); prostaglandin E_2 (PGE_2); glucocorticoid

Introduction

Recently, the existence and possible importance of anti-inflammatory effects of β_2-adrenoceptor agonists has been widely discussed at international meetings on asthma and its treatment. Protective effects of β_2-agonists, which are additional to the relaxation of airway smooth muscle, support the possibility that they might help to control inflammation in asthma (Persson, 1990; Löfdahl, 1990; Fuller & Baker, 1990; Whelan & Johnsson, 1990). Not all of these effects are reversed by β-blockers, suggesting that not all are mediated by β_2-agonistic activity (Fuller & Baker, 1990).

The significance of macrophages in asthma was suggested originally by pathological studies that demonstrated the presence of these cells in the airway lesions in patients who died from asthma (Bullen, 1952; Dunnill, 1960). These histological observations of the involvement of macrophages in asthma are supported by a number of studies concerning activation of airways macrophages (AMs) and their precursors, blood monocytes, obtained from patients with asthma (for review see Martin & Goodman, 1990). AMs have two important functions in the airways: the phagocytosis of inhaled particles and antigens and the production of mediators that influence the bronchoalveolar environment. Thus, AMs produce a variety of protein and lipid products that may be important in the pathogenesis of asthma (Martin & Goodman, 1990). These products include arachidonic acid metabolites and cytokines.

The biological activities of arachidonic acid metabolites, such as increased vascular permeability, smooth muscle contraction, leucocyte chemo-attraction and activation, have led to suggestions that they have an important proinflammatory role in several lung diseases, including asthma (Henderson, 1987). The AM has a high membrane content of arachidonic acid as compared to other inflammatory cells such as the neutrophil, and has the ability to respond to a wide variety of soluble and particulate stimuli by releasing large amounts of eicosanoids (Chensue & Kunkel, 1983). Human peripheral blood monocytes, generally believed to be precursor cells of AMs (Blusse Van Oud Alblas *et al.*, 1983; Bitterman *et al.*, 1984), can also metabolize arachidonic acid to several important types of eicosanoids, including leukotriene B_4 and C_4 (LTB_4 and LTC_4), and prostaglandin E_2 (PGE_2; Pawlowski *et al.*, 1983; Goldyne *et al.*, 1984).

Interleukin-1 (IL-1), is a cytokine that mediates many host defence adaptations to environmental and infectious stress (Dinarello, 1985). Blood monocytes and AMs are main sources of this cytokine (Dinarello, 1985). Since IL-1 plays a major role in the initiation of the inflammatory reaction, and since it has been demonstrated that bronchoalveolar lavage (BAL) from asthmatics contains higher amounts of IL-1 than that from normal control subjects (Mattoli *et al.*, 1991), the prevention of its production by macrophages and monocytes may be of therapeutic importance.

Although mature mononuclear phagocytes, e.g. AMs, differ from blood monocytes in several ways, both cell types produce arachidonic acid metabolites as well as IL-1β, as mentioned above. Furthermore, blood monocytes are more susceptible to drugs which inhibit through membrane receptors linked to adenylyl cyclase (Fuller *et al.*, 1988) and corticosteroids (Strieter *et al.*, 1989), than autologous AMs. Moreover, the collection of blood monocytes is much less laborious than the sampling of AMs. Hence, it may be advantageous, to use blood monocytes in studies on the pharmacological modulation of e.g. arachidonic acid metabolism and secretion of IL-1. Furthermore, monocyte activation observed during asthmatic reactions (for review see, Carroll & Holgate, 1989) implies a direct involvement of this cell in

the pathophysiology of bronchial asthma.

β_2-Adrenoceptor agonists have previously been shown to inhibit antigen-induced LTC_4, LTD_4 and PGD_2 release from human lung *in vitro* (Hughes *et al.*, 1983; Butchers *et al.*, 1987). These effects were antagonized by pretreatment with β_2-selective antagonists. Although the cellular composition of lung tissue is very complex, it is probable that the authors were studying effects of β_2-agonists on lung mast cells, since these drugs are potent inhibitors of mast cell mediator secretion (Church & Hiroi, 1987). Recently, it has been suggested that the mast cell stabilizing action of β_2-agonists may be deleterious in asthma by inhibiting the release of heparin, which would counteract the effects of eosinophil cationic proteins (Page, 1991). Therefore, in the present study a different cell population, namely human blood monocytes, was used in order to verify the suggested role of β_2-adrenoceptors in the modulation of the inflammatory mediator release in asthma. Effects of β_2-adrenoceptor agonists, terbutaline, salmeterol and formoterol, alone or in combination with sotalol, a β-receptor antagonist, on the secretion of LTB_4, PGE_2 or IL-1β in human monocyte cultures were investigated. Moreover, the effects of β_2-agonists were compared with the effects of a potent corticosteroid, budesonide (Dahlberg *et al.*, 1983) and a potent 5-lipoxygenase inhibitor, BW A4C (Payne *et al.*, 1988).

Methods

Monocyte cultures

Peripheral venous blood samples were obtained from healthy volunteers. None of the subjects was using β-agonists or corticosteroids. Mononuclear cells were isolated from EDTA-treated blood by centrifugation on Mono-Poly Resolving Medium (Flow Lab, Scotland) for 35 min at 400 *g*. The mononuclear cell layer was removed and the cells were washed 3 times with phosphate-buffered saline (PBS). The cells were then resuspended in growth medium RPMI 1640 (Flow Lab, Scotland) supplemented with 5% foetal calf serum and gentamicin (50 $\mu g\,ml^{-1}$) and aliquots of cell suspension (5×10^6 cells in 2 ml) were seeded in tissue culture multidishes (24 wells, Nunc, Denmark). The cells were incubated for 3 h at 37°C and the non-adherent cells were then removed by washing with Ca^{2+} and Mg^{2+}-free PBS. The adherent cells were further incubated in fresh medium overnight. The final yield of monocytes (May-Grünwald Giemsa stain) was 97–99%, and the cell viability (Trypan blue exclusion) was 98 ± 1%.

Drug effects on leukotriene B_4 and prostaglandin E_2 secretion

Following overnight incubation, the cells were stimulated with human serum opsonized zymosan (1 $mg\,ml^{-1}$) suspended in Hank's buffer in presence or absence of β_2-agonists (1×10^{-9} -10^{-5} M), or with a combination of β_2-agonists and sotalol (1×10^{-5} M), or in presence of budesonide (1×10^{-9}–10^{-5} M), or BW A4C (1×10^{-9}–10^{-5} M) for 90 min at 37°C. When the effects of budesonide were studied, the drug was added to the cell cultures 20 h prior to stimulation while all other drugs were added simultaneously with zymosan. Cell viability was not altered after drug treatment (Trypan blue exclusion). The incubation was stopped by rapid cooling to 4°C and the culture supernatants were removed and stored at − 70°C.

The LTB_4 and PGE_2 in culture supernatants were assayed by specific radioimmunoassay (Advanced Magnetic Inc, U.S.A., and NEN, Sweden, respectively) and related to the cell number by means of DNA content in the monocyte cultures. The DNA was analysed by means of the bisbenzimidazole method, essentially as described by Labarca & Paigen (1980). Drug effects on secretion of LTB_4 and PGE_2 were expressed as a percentage of secretion in control cultures, i.e. cells incubated in Hank's buffer, supplemented with the appropriate vehicle.

Drug effects on interleukin-1β (IL-1β) secretion

The monocytes were cultured in RPMI 1640 (Flow Lab, Scotland) supplemented with 5% foetal calf serum and gentamicin (50 $\mu g\,ml^{-1}$). The cell cultures wre stimulated with lipopolysaccharide (LPS; 10 $\mu g\,ml^{-1}$) from *Escherichia coli*, serotype 026:B6 (L 2762, Sigma) and incubated in presence or absence of β_2-agonists, or with combination of β_2-agonists and sotalol, or in presence of budesonide, for 20 h at 37°C.

The IL-1β in culture supernatants was assayed by specific radioimmunoassay (Amersham Int, U.K.) and related to the cell numbers in monocyte cultures as described above. Drug effects on secretion of IL-1β were expressed as a percentage of secretion in control cultures, i.e. cells incubated in RPMI 1640 medium, supplemented with respective vehicle.

Drugs

Terbutaline, formoterol, salmeterol and budesonide were synthesized at Astra Draco AB, Sweden. Sotalol was obtained from Astra Hässle AB, Sweden, and BW A4C (N-(3-phenoxycinnamyl) acetohydroxamic acid) was generously supplied by Wellcome Research Laboratories, Kent.

For studies of effects on LTB_4 and PGE_2 secretion, terbutaline and sotalol were dissolved directly in Hank's buffer, while salmeterol, formoterol and BW A4C were first dissolved in dimethylsulphoxide (DMSO, final concentration 0.01%) and then diluted in Hank's buffer. Budesonide was first dissolved in ethanol (final concentration 0.05%) and then diluted in Hank's buffer.

For studies of effects on IL-1β secretion, terbutaline and sotalol were dissolved directly in RPMI 1640 medium, while salmeterol and formoterol were first dissolved in DMSO (final concentration 0.01%) and then diluted in RPMI medium. Budesonide was first dissolved in ethanol (final concentration 0.05%) and then diluted in RPMI 1640 medium.

Statistics

Each drug was studied in five separate experiments (i.e. with blood from separate donors) and the values are expressed as mean ± s.e.mean. Statistical comparisons between control and drug-treated cell cultures were made by paired *t* test. A $P < 0.05$ was considered significant.

Results

The β_2-receptor agonists terbulatine, salmeterol and formoterol, were evaluated for their ability to inhibit secretion of LTB_4 in human isolated monocyte cultures stimulated with opsonized zymosan (Figure 1a-c). Salmeterol (1×10^{-7} -10^{-5} M) treatment of monocytes caused a concentration-dependent inhibition of LTB_4 release. In contrast, treatment with terbutaline (1×10^{-9}–10^{-5} M) caused no significant inhibition of LTB_4 release. The inhibition of LTB_4 secretion by formoterol was, unlike that of salmeterol, partial and not concentration-dependent at the dose range studied. Budesonide had no effect on the LTB_4 release while BW A4C was the most potent of the tested drugs (Figure 1d).

The inhibitory effect of salmeterol and formoterol on the release of LTB_4 was not significantly antagonized by treatment with a β-receptor antagonist, 1×10^{-5} M sotalol (Figure 1b and c). This concentration of sotalol alone had no significant effect on LTB_4 secretion (data not shown).

Salmeterol, but not the other β_2-agonists studied, partially inhibited the secretion of PGE_2. This inhibition was not significantly reversed by simultaneous treatment of monocyte cultures with sotalol (Table 1).

IL-1β levels in the LPS stimulated monocyte cultures were

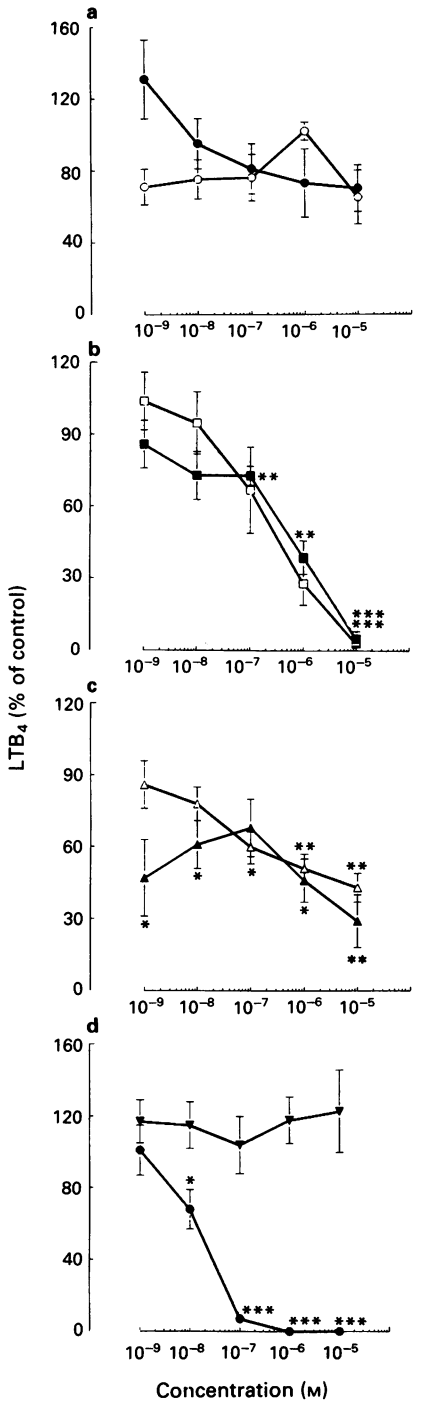

Figure 1 Effects of terbutaline (a), salmeterol (b) and formoterol (c) alone (all solid symbols) or in combination with sotalol (1×10^{-5} M) (open symbols); budesonide (▼) or BW A4C (●) (d) on the secretion of leukotriene B_4 (LTB_4) in human monocyte cultures stimulated with opsonized zymosan (1 mg ml^{-1}). LTB_4 secretion (% of control) is presented as mean ± s.e.mean (vertical bars) and represent five experiments performed on monocytes obtained from five separate individuals. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ compared with control cultures incubated with Hank's buffer and vehicle only (absolute control value for LTB_4 was 113 ± 25 pg μg^{-1} DNA).

partly inhibited by β_2-agonists (Figure 2). Salmeterol and formoterol had similar inhibitory effects. Terbutaline reduced IL-1β secretion by only 14–18%. The reduction of IL-1β secretion mediated by β_2-agonists was not antagonized by sotalol (1×10^{-5} M; Table 2; same data for IL-1β inhibition as in Figure 2 are shown in order to facilitate a direct comparison with sotalol treated cultures). This concentration of sotalol alone had no significant effect on IL-1β secretion (data not shown).

The β_2-agonists studied were much less potent than a corticosteroid, budesonide, which exerted its maximal effect at a concentration as low as 1×10^{-8} M (Figure 2).

Discussion

The results of this study suggest that formoterol and salmeterol may inhibit the release of arachidonic acid metabolites and IL-1β in human monocyte cultures. The potency of formoterol and salmeterol on the inhibition of LTB_4 secretion was, however, much lower than that exerted by a potent and selective 5-lipoxygenase inhibitor, BW A4C (Payne *et al.*, 1988).

Interestingly, a potent corticosteroid, budesonide (Dahlberg *et al.*, 1983), failed to inhibit LTB_4 secretion in human monocyte cultures, despite the 20 h pretreatment of cells. Lack of inhibitory effects of corticosteroids on LTB_4 production by AMs has been previously demonstrated after *in vitro* or *in vivo* treatment (Tateson *et al.*, 1988; Sporn *et al.*, 1990). Also, corticosteroid treatment of rhinitis or asthma patients failed to lower LTB_4 in nasal lavage and serum, respectively (Freeland *et al.*, 1989; Bergstrand *et al.*, 1990; Seggev *et al.*, 1991). Thus, the well-known beneficial anti-inflammatory effects of corticosteroids in rhinitis or asthma (Schleimer, 1988) are probably not mediated through reduction of LTB_4.

The inhibitory effects of long-acting β_2-agonists on eicosanoid release were not significantly antagonized by sotalol. This is in agreement with findings of Fuller & Baker (1990), who showed that the inhibition of thromboxane B_2 secretion in AM cultures by salmeterol was not reversed by propranolol. Moreover, in the present study terbutaline, a potent β_2-adrenoceptor agonist, lacked inhibitory effect on the eicosanoid release. This further supports the concept of a non-β-adrenoceptor mediated action of formoterol and salmeterol on eicosanoid secretion from monocytes. However, the recent studies on lung tissue (Hughes *et al.*, 1983; Butchers *et al.*, 1987) demonstrate that fenoterol and salmeterol inhibition of leukotriene release can be reversed by propranolol (Hughes *et al.*, 1983; Butchers *et al.*, 1987). Different results in those studies and in the present study have been obtained probably because the effects of drugs were examined on different cell types – mast cells, in one case and monocytes in the other.

Recent evidence indicates that the synthesis and release of IL-1 is increased in the airways of asthmatic subjects (Mattoli *et al.*, 1991). Corticosteroids are potent inhibitors of IL-1 production (Knudson *et al.*, 1987). Although, formoterol and salmeterol modulated IL-1 secretion *in vitro*, high doses (1×10^{-5} M), when compared with budesonide ($\geqslant 1 \times 10^{-9}$ M), were needed. However, it should be kept in mind that during the incubation time of 20 h some compounds studied (e.g. formoterol) may undergo chemical degradation. For this reason the comparison between various β_2-agonists should be regarded more as qualitative than quantitative. Since terbutaline had almost no effect on the IL-1 secretion, and the inhibitory action of formoterol and salmeterol was significantly antagonized by sotalol, then this anti-inflammatory effect also seems to be mediated through β-receptor-independent mechanisms. Furthermore, Tannenbaum & Hamilton (1989) have reported that other agents that elevate intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels (dibutyryl cyclic AMP or cholera toxin) had no effect on the levels of IL-1 mRNA expressed by peritoneal macrophages in response to lipopolysaccharide (LPS). Also, findings reported by Fieren *et al.* (1991) indicate that PGE_2, exogenous or endogenous, failed to influence the secretion of IL-1β from LPS stimulated human macrophages, despite elevating intracellular cyclic AMP. However, PGE_2 inhibited the secretion of tumour necrosis factor-α from these cells.

Table 1 Effects of terbutaline, salmeterol or formoterol alone or in combination with sotalol (1×10^{-5} M) on the secretion of prostaglandin E_2 (PGE_2) in human monocyte cultures stimulated with opsonized zymosan

	PGE_2 secretion (% of control)				
Drug concentration	1×10^{-9} M	1×10^{-8} M	1×10^{-7} M	1×10^{-6} M	1×10^{-5} M
Sotalol					93 ± 5
Terbutaline	114 ± 11	99 ± 6	91 ± 4	97 ± 11	90 ± 5
Terbutaline + sotalol	94 ± 4	89 ± 4	95 ± 5	100 ± 3	94 ± 9
Salmeterol	96 ± 10	91 ± 7	97 ± 8	80 ± 5*	54 ± 9*
Salmeterol + sotalol	97 ± 7	94 ± 5	83 ± 10	73 ± 8*	45 ± 10**
Formoterol	90 ± 10	97 ± 11	108 ± 14	106 ± 12	99 ± 9
Formoterol + sotalol	97 ± 6	95 ± 4	86 ± 4	82 ± 4	87 ± 11

Results are presented as mean ± s.e.mean and represent five experiments performed on monocytes obtained from five separate individuals.
*$P<0.05$; **$P<0.01$ compared with control cultures incubated with Hank's buffer and vehicle only (absolute control value for PGE_2 was 44 ± 6 pg μg^{-1} DNA).

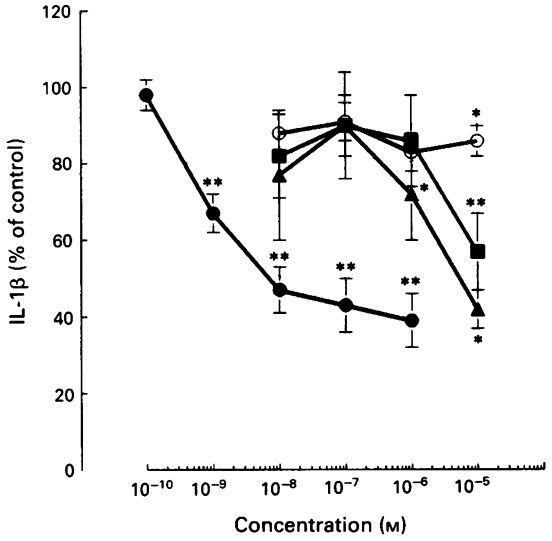

Figure 2 Effects of terbutaline (○), salmeterol (■), formoterol (▲) and budesonide (●) on the secretion of interleukin-1β (IL-1β) in the cultures of human blood monocytes stimulated with lipopolysaccharide (LPS) (10 μg ml^{-1}). IL-1β secretion (% of control) is presented as mean ± s.e.mean (vertical bars) and represent five experiments performed on monocytes obtained from five separate individuals. *$P<0.05$; **$P<0.01$ compared with control cultures incubated with RPMI 1640 medium and 0.05% ethanol only (absolute control value for IL-1β was 806 ± 66 pg μg^{-1} DNA).

Our present *in vitro* results should be considered in context with the recent report of Haahtela *et al.* (1991), demonstrating that anti-inflammatory therapy with inhaled budesonide is superior to the use of inhaled terbutaline in long-term treatment of newly detected asthma. Also, Howarth (1991) demonstrated very recently that therapy with inhaled beclomethasone reduced numbers of lavage and biopsy mast cells and eosinophils, as well as the activity of these cells, while salmeterol therapy had no effect on these parameters. Moreover, it has been suggested that stabilizing of mast cells by β-agonists may be deleterious in asthma, since these drugs inhibit the release of heparin, which would counteract the effects of eosinophil basic proteins in the asthmatic airways (Page, 1991). However, the preliminary reports on the *in vivo* inhibitory effects of inhaled salmeterol on the eosinophil activation, i.e. lowering of eosinophil cationic protein in bronchoalveolar lavage and in serum obtained from asthmatics (Dahl *et al.*, 1991; Pedersen *et al.*, 1991), indicate possible advantageous effects of this drug on the inflammatory process. Nevertheless, further research is needed to assess the potential therapeutic benefits of anti-inflammatory effects of β_2-agonists in asthma.

In conclusion, eicosanoid and IL-1 release in human monocyte cultures is inhibited by the long-acting β_2-stimulating drugs, formoterol and salmeterol. These effects are probably not mediated via action on the β_2-adrenoceptor. Since the inhibition of inflammatory mediator secretion was exerted at very high doses of β_2-agonists, the therapeutic significance of these findings in asthma treatment is questionable. Moreover, in the cell model used, the anti-secretory action of β_2-agonists was distinct from that of corticosteroids.

Table 2 Effects of β_2-adrenoceptor agonists alone or in combination with sotalol (1×10^{-5} M) on the secretion of interleukin-1 in the cultures of human blood monocytes stimulated with lipopolysaccharide

	IL-1β secretion (% of control)			
Drug concentration	1×10^{-8} M	1×10^{-7} M	1×10^{-6} M	1×10^{-5} M
Terbutaline	88 ± 5	91 ± 5	83 ± 5*	86 ± 4*
Terbutaline + sotalol	98 ± 20	120 ± 15	89 ± 21	89 ± 6
Salmeterol	77 ± 17	90 ± 8	72 ± 12	42 ± 5**
Salmeterol + sotalol	100 ± 17	89 ± 18	79 ± 17	56 ± 21
Formoterol	82 ± 11	90 ± 14	86 ± 12	57 ± 10*
Formoterol + sotalol	85 ± 12	78 ± 7*	83 ± 19	55 ± 11*

Results are presented as mean ± s.e.mean and represent five experiments performed on monocytes obtained from five separate individuals.
*$P<0.05$; **$P<0.01$ compared with control cultures incubated with RPMI 1640 medium and vehicle only (absolute control value for IL-1β was 806 ± 66 pg μg^{-1} DNA).

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H_3 receptor antagonist, thioperamide, inhibits adrenal steroidogenesis and histamine binding to adrenocortical microsomes and binds to cytochrome P450

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1 Thioperamide (TP), an imidazole and a highly potent, specific antagonist of the histamine H_3 receptor, inhibited the secretion of cortisol from bovine isolated adrenocortical cells (IC_{50} 0.20 μM) and, in the rat (5 mg kg^{-1}) prevented both basal and stress-induced secretion of corticosterone.

2 In adrenocortical microsomes, low affinity binding of [^{3}H]-histamine (K_D 27.7 μM) was potently inhibited by TP (K_i 0.33 μM).

3 In adrenocortical microsomal membranes, both histamine and TP yielded type II difference absorption spectra, characteristic of the interaction between imidazole and cytochrome P450 enzymes. Dissociation constants for binding to P450, calculated from spectral data, were 15.9 μM and 1.5 mM for histamine, and 0.3 μM and 3.7 μM for TP.

4 In view of previously reported evidence for an intracellular mediator role of histamine in platelets, the present findings suggest a physiological role for histamine in the modulation of adrenal P450 monooxygenases that generate adrenocortical steroids.

5 The results suggest that direct adrenocortical inhibition by thioperamide at a non-H_3 intracellular site must be taken into account in studies designed to elucidate functional roles of H_3 receptors.

Keywords: Histamine; H_3 antagonist; thioperamide; adrenocorticoids; cytochrome P450; adrenal cortex; histamine receptors; imidazoles; second messengers

Introduction

Collaborative studies by our laboratory have centred on the physiological role of intracellular binding sites (H_{IC}) for histamine (Brandes *et al.*, 1990). A low affinity (μM) site in microsomes has been implicated in a second messenger role for histamine in platelets (Saxena *et al.*, 1989); additional high and low affinity nuclear sites, of different ligand specificity, appear to play a growth-modulatory role in lymphocytes (Brandes *et al.*, 1990; 1991). H_{IC} exhibits strong preference for certain novel, antioestrogen-like compounds, lesser affinity for H_1 ligands and very low affinity for H_2 ligands (Brandes *et al.*, 1990). An important locus for H_3 receptors is presynaptic nerve terminals; histamine and other H_3 agonists reportedly interact at these receptors to suppress the synthesis and release of histamine (van Der Werf *et al.*, 1989). We show here that the H_3 antagonist, thioperamide (Arrang *et al.*, 1987b), is a potent competitor of histamine binding to low affinity microsomal H_{IC}; in adrenal microsomes at least some proportion of the microsomal binding sites represents P450 enzymes; and inhibition of adrenal steroidogenesis is a prominent pharmacological response to thioperamide.

Methods

Difference absorption spectra

Type II P450 difference spectra, characterized by a broad trough between 390 and 410 nm and a peak varying between 425 and 435 nm (Jefcoate, 1978) were obtained with a Milton Roy Spectronic 3000 Array Spectrophotometer; a Rapidscan computer software programme controlled and operated the unit, and collected and plotted the spectral data (Backes & Canady, 1981; Estabrook & Werringloer, 1978). The spectral data were analyzed with the LIGAND programme (Munson & Rodbard, 1980). The trough to peak differences (ΔA), unaffected by nonspecific binding which was set to 0, served as amount bound. Specific activity was given a value of 1 Ci $mmol^{-1}$ and the dose of ligand entered as pmol ml^{-1}. The computed K_s values differed by less than 2% from those manually calculated. The K_s and Hill values are reported with their corresponding standard errors and standard deviations, respectively. Microsomes from bovine adrenal cortex were prepared in 250 mM KPO_4, 150 mM KCl and 1 mM EDTA pH 7.25 and suspended in a 50 mM KPO_4, pH 7.25 buffer at 0.5 to 1.0 mg protein ml^{-1} in 1.3 or 3.0 ml quartz cuvettes for spectral determinations (Boobis *et al.*, 1980). Substrates diluted in KPO_4 buffer were added in 3 to 5 μl aliquots with equal volumes of buffer added to the reference. Microsomes were stored in 30% glycerol 50 mM KPO_4 buffer at 20 mg ml^{-1} protein for up to 1 month. Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (BSA) as standard.

[^{3}H]-histamine radioligand binding assay

Microsomes from bovine adrenal cortex were prepared and stored as described above. [^{3}H]-histamine binding assays (0.5 mg microsomal protein ml^{-1}) were performed in a modified Krebs buffer (composition, mM: NaCl 120, $NaHCO_3$ 25, KCl 4.8 and $MgSO_4$ 1.2, 95% O_2: 5% CO_2, pH 7.4) containing 8 nM [^{3}H]-histamine with 0.04 to 250 μM unlabelled histamine or 0.006 to 50 μM TP as competitors. Incubations were performed at 20°C for 1 h. Following a 10,000 *g*, 15 min, centrifugation the supernatant was aspirated, the pellet dissolved in NaOH and d.p.m. deter-

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mined (Brandes et al., 1987). The data were analyzed by the computer programme LIGAND (Munson & Rodbard, 1980). The K_d and Hill values are given with their corresponding standard errors and standard deviations respectively.

Cortisol secretion by adrenocortical cell monolayers

Fresh bovine adrenal glands were obtained from a local slaughterhouse and placed in Hank's Balanced Salt Solution (HBSS), pH 7.4, containing penicillin-G (100 iu ml^{-1}), streptomycin (1 mg ml^{-1}), and gentamycin (40 $\mu g\ ml^{-1}$). Upon arrival at the laboratory the medium was changed. Adrenal glands were stripped of fat and bisected longitudinally, the medulla removed, and the cortical portion was dissociated from the capsule. Cortical tissue was minced with scissors, washed once with Hank's Balanced Salt Solution, and incubated in HBSS containing additional sodium bicarbonate (24 mM), HEPES (25 mM), BSA (0.5%), collagenase (2 mg ml^{-1}), and DNase (25 $\mu g\ ml^{-1}$) for 60 min at 37°C on a gyratory shaker. The undigested fragments were allowed to settle, and the mixture was decanted through four layers of sterile cheesecloth. The cells were obtained by centrifugation at 100 *g* for 10 min and washed three times. The final pellet was resuspended in Dulbecco's Modified Eagle's Medium/HAM's F-12, 50/50, containing antibiotics (as above) and 10% foetal calf serum at 2×10^5 cells per plate. After 3 days incubation the monolayer was washed three times with HBSS and preincubated in this buffer for 1 h at 37°C. During the second hour of incubation in fresh HBSS, adrenocorticotrophic hormone (ACTH, 0.1 μM) was added in the presence and absence of TP. The ACTH-stimulated cortisol release was measured by radioimmunoassay (RIA) using cortisol antiserum purchased from Endocrine Science Products, Tarzana CA, U.S.A. (Li et al., 1989). The data were analyzed with a 4 parameter logistic fit (Rodbard, 1978; Maciel, 1985).

In vivo *studies*

Male Sprague-Dawley rats (200 ± 10 g) from the University of Manitoba Central Animal Care services were randomly assigned to groups as follows: vehicle (20% dimethylsulphoxide (DMSO)) – no stress; vehicle – stress; thioperamide (5.0 mg kg^{-1}, i.p.) – no stress; and thioperamide – stress. Stress consisted of immobilization in Fisher plexiglass restraint cages in a cold (4°C) environment for 3 h. Animals were injected with vehicle or thioperamide immediately prior to stress (or returned to the home cage for 3 h in the case of non-stressed rats). Immediately following stress, all rats were decapitated and blood from the cervical wound was collected in heparinized tubes, centrifuged at 5000 *g* and the plasma removed for assay of corticosterone by the fluorometric method of van der Vies (1961).

Materials

Thioperamide (4(1-cyclohexylaminothiocarbonyl-4-piperidyl)-1H-imidazole) was synthesized by the reaction of 4-(4-piperidyl)imidazole (Schunack et al., 1973) and cyclohexylisothiocyanate in toluene, essentially as described in the patent literature (Arrang et al., 1987a). The product obtained had melting point decomposition 170°C (from toluene), lit melting point 170°C. Microanalysis found: C, 61.73%, H, 7.99%, N, 18.95%. $C_{15}H_{24}N_4S$ requires, C, 61.61%, H, 8.26%, N, 19.5%.

Results

Histamine yielded a type II difference spectrum with adrenal microsomes (Figure 1), characteristic of ligands that interact directly with the haeme iron in cytochrome P450 (Jefcoate, 1978). Two binding components were resolved ($P<0.001$) with apparent K_ds (more precisely K_s's from the spectral data) of 15.9 ± 10.7 μM and 1.5 ± 1.3 mM with a Hill coefficient of 0.94 ± 0.01 ($n = 2$) (Figure 2). Thioperamide also yielded a type II spectrum with adrenal microsomes (Figure 3), and, again, two binding components were resolved ($P<0.001$) (Figure 4); the apparent K_s's were 0.3 ± 0.1 μM and 3.7 ± 1.3 μM with a Hill coefficient of 1.05 ± 0.13 ($n = 3$). The higher affinity interaction of thioperamide with P450 correlated with that to the [^{3}H]-histamine binding site in adrenal microsomes, where TP was much more potent (K_i 0.33 ± 0.09 μM) than histamine (K_d 27.7 ± 6.9 μM; Figure 5); potency of TP in the binding assay also corresponded to its IC_{50} value (0.2 ± .03 μM) for inhibition of cortisol secretion from isolated adrenal cells (Figure 6). A second binding site for histamine or TP was not resolved in the [^{3}H]-histamine binding studies due to variability at the higher competing doses. The Hill coefficient for histamine was 0.94 ± 0.06 ($n = 2$).

Table 1 summarizes the plasma corticosterone data. Analysis of variance indicates significant group differences ($F(3,28) = 10.23$; $P<0.01$), and *post-hoc* Tukey HSD tests reveal that thioperamide significantly reduced plasma corticosterone

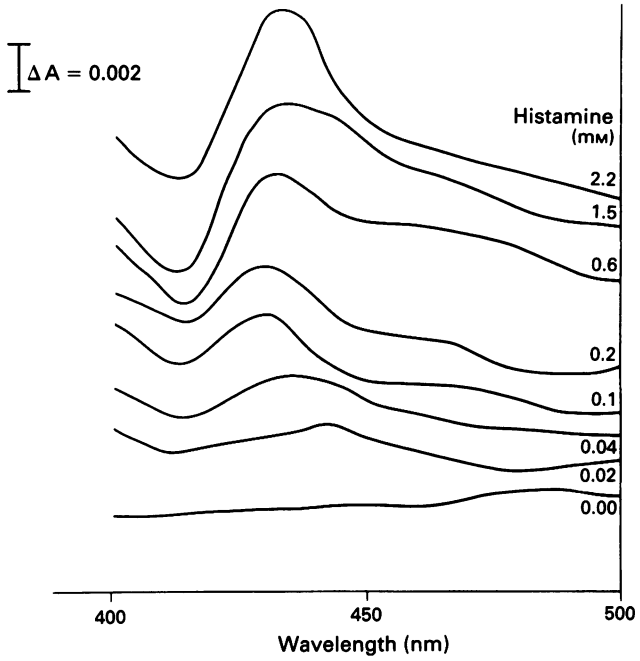

Figure 1 Representative difference spectrum obtained by the addition of histamine to adrenocortical microsomal membranes. Note in Figures 1 and 3 that the concentrations for histamine are expressed as millimolar and for thioperamide, micromolar.

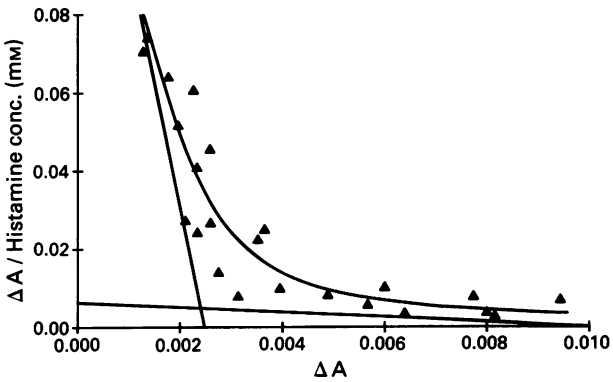

Figure 2 Scatchard representation of values from difference spectra observed in bovine adrenocortical microsomes incubated with various doses of histamine.

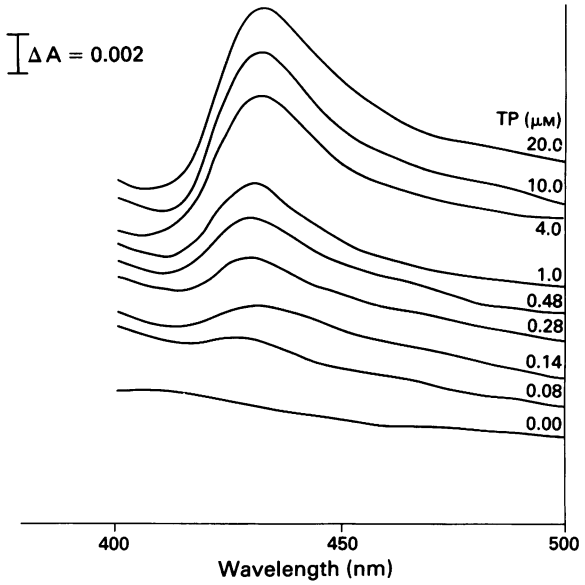

Figure 3 Representative difference spectrum resulting from interaction of thioperamide (μM) with adrenocortical microsomal membranes.

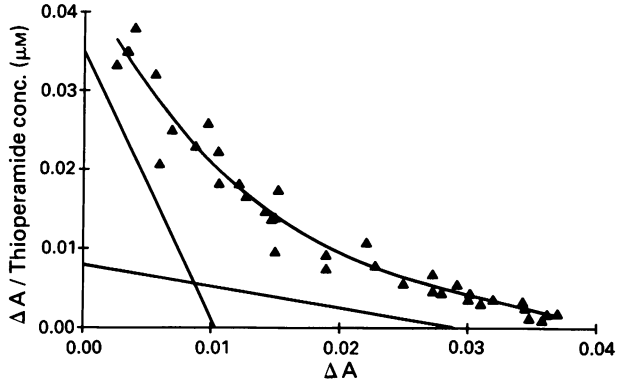

Figure 4 Scatchard representation of values from difference spectra observed in bovine adrenocortical microsomes incubated with various doses of thioperamide.

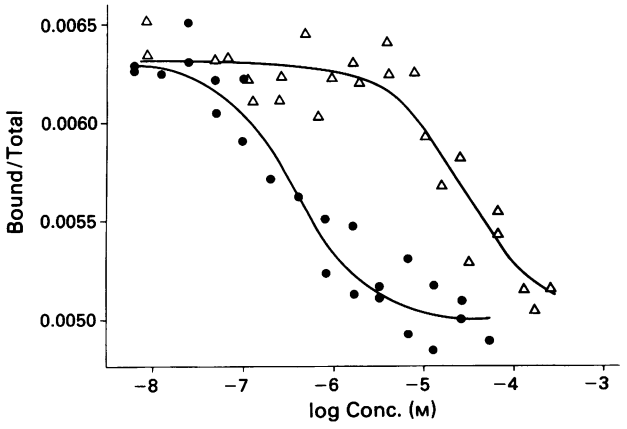

Figure 5 Competition with [^{3}H]-histamine binding to bovine adrenal microsomes by HA histamine (Δ) and thioperamide (●).

levels both in non-stressed ($P<0.01$) and stressed ($P<0.01$) rats relative to vehicle-treated controls. Tukey's Honestly Significant Difference procedure, also called the ω-procedure, is a stringent multiple comparison test and is less likely to give false significant differences (Steel & Torrie, 1960).

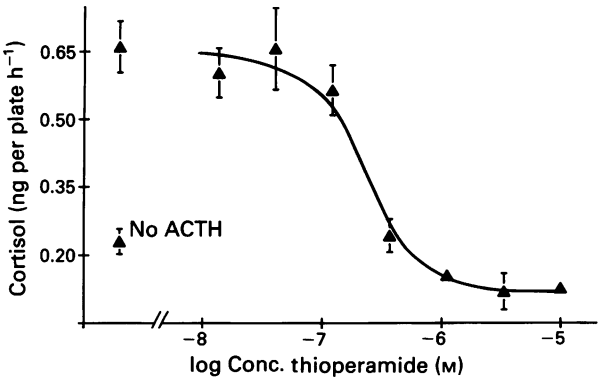

Figure 6 Inhibition by thioperamide of adrenocorticotrophic hormone (ACTH)-stimulated cortisol release from bovine adrenocortical cell monolayers.

Table 1 Effect of thioperamide on plasma corticosterone in stressed and non-stressed rats

Treatment	*No. of rats*	*Plasma corticosterone* (μmol l^{-1})
DMSO – no stress	6	9.33 ± 0.67
DMSO – stress	6	20.07 ± 1.58
Thioperamide – no stress	10	4.58 ± 0.43*
Thioperamide – no stress	10	10.86 ± 0.92*

DMSO = dimethylsulphoxide.
Values are means ± s.e.mean. Thioperamide (5 mg kg^{-1}) was given i.p.
*Significantly less than respective control group (vehicle only) $P<0.01$).

Discussion

The imidazole moiety in histamine and in a large number of drug classes interacts with P450 enzymes to yield type II binding spectra, due to the formation of a ferrihaemochrome (Testa & Jenner, 1981; Feldman, 1986; Murray, 1987; Ayub & Levell, 1989). The unhindered nitrogen in the imidazole ring binds to the catalytic haeme iron atom of the protophyrin in the enzyme, thereby precluding formation of an oxygen-haeme complex. Histamine, a naturally occurring, ubiquitous humoral imidazole, exerts a direct modulatory action (apparently competitive, but deviates from this mode at high concentrations) on liver P450-catalyzed oxidations; histamine's dissociation constant (K_s) of 111 μM determined by difference-absorption spectra agreed reasonably well with its K_i (164 μM) for one P450 reaction, but less so (K_i 243 μM) for a second reaction (Lennard *et al.*, 1986; Morris *et al.*, 1989).

In adrenocortical membranes, the high affinity binding component of thioperamide calculated from spectral data (K_s 0.3 μM) is about equal to the K_i (0.33 μM) for inhibition of [^{3}H]-histamine binding. The reasonable correspondence between these values and the potency of thioperamide to inhibit cortisol secretion *in vitro* (IC_{50} 0.2 μM) suggests a link between the histamine/P450 interaction and modulation of steroidogenesis. Since cortisol synthesis is the result of a series of enzyme-specific monooxygenations of steroid substrates, thioperamide presumably inhibits one or more of these P450-mediated reactions.

An intracellular, second messenger role for histamine at H_{IC} has been indicated in the aggregatory response of blood platelets to certain mediators (Saxena *et al.*, 1989; Brandes *et al.*, 1990). Although the intracellular mechanisms have not been fully delineated, there is good correlation between the potency of the H_{IC} antagonist, N,N-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine.HCl (DPPE) for [^{3}H]-histamine

binding sites in microsomes from several tissues and the potency to inhibit platelet aggregation (Brandes *et al.*, 1990; 1991). P450 inhibitors also block aggregation (Parnham *et al.*, 1981), suggesting a close association between H_{IC} and P450 in platelets.

An important component of the evidence in favour of an intracellular role for histamine is based on the blunting of the platelet aggregatory response by specific inhibitors of histidine decarboxylase, the enzyme that generates histamine (Saxena *et al.*, 1989). However, in our studies these inhibitors have consistently failed to block basal or ACTH-stimulated secretion of cortisol from isolated adrenal cells. It remains to be determined whether or not preformed histamine in adrenocortical cells plays a physiological role in the modulation of P450 enzymes of steroidogenesis, as we suggested for modulation of growth and proliferation of lymphocytes and cultured cells (Brandes *et al.*, 1991; Brandes & LaBella, 1992).

Finally, the direct non-H_3-mediated inhibition by thioperamide of corticoid secretion *in vivo* must be taken into consideration in studies designed to reveal the consequences of histamine H_3-receptor antagonism.

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Pharmacodynamics of the anticonvulsant effect of oxazepam in aging BN/BiRij rats

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1 The purpose of this investigation was to examine the influence of increasing age on the pharmacokinetics and the time course of the anticonvulsant response of oxazepam in BN/BiRij rats as an animal model of aging.

2 Oxazepam was administered intravenously in a dose of 12 mg kg^{-1} body weight and the anticonvulsant effect intensity was measured as elevation above baseline of a threshold for induction of localized seizure activity (TLS). Direct cortical stimulation with ramp shaped electrical pulse trains of increasing intensity was used to determine this threshold.

3 The pharmacological effect vs. time profile showed in young rats an anticonvulsant component followed by proconvulsant component which is suggestive for the occurrence of acute tolerance and/or withdrawal syndrome. With increasing age the proconvulsant component disappeared, resulting in a monophasic effect profile (anticonvulsant effect only) at the age of 35 months with significantly higher anticonvulsant effect intensity immediately following drug administration. No age-related changes in the pharmacokinetic parameters of oxazepam were observed.

4 In five animals of each age group, benzodiazepine receptor binding characteristics were determined *in vitro* with [^{3}H]-flunitrazepam as a ligand. Both receptor density and affinity did not show age-related changes. Available literature data on post-receptor events do not indicate conclusive age-related changes.

5 It is concluded, that the observed change in the pharmacodynamics of anticonvulsant effect of oxazepam can be explained by the disappearance of the tolerance/withdrawal phenomenon. This is compatible with a decreased efficiency of homeostatic control mechanisms in the elderly.

Keywords: Pharmacokinetics; pharmacodynamics; oxazepam; homeostatic reserve capacity; aging; anticonvulsant effect

Introduction

It has been frequently reported, that with increasing age there is an increase of the sensitivity of the brain to the pharmacological actions of benzodiazepines, especially for sedative effects and the effects on psychomotor function (Castleden *et al.*, 1977; Reidenberg *et al.*, 1978; Kanto *et al.*, 1981; Swift *et al.*, 1981; 1985a,b; Cook, 1986). Thus far, the influence of increasing age on the anticonvulsant effect of benzodiazepines has not been studied extensively. This may be relevant, because with increasing age there is an increase in the incidence of epilepsy and in the use of antiepileptic drugs (Hauser & Kurland, 1975; Troupin & Johannessen, 1990). Moreover, the mechanism of the anticonvulsant effect of benzodiazepines may be in part different from that of most of the other effects of these drugs, because of the involvement of micromolar affinity benzodiazepine receptors (Bowling & De Lorenzo, 1982).

The purpose of this investigation was to study the influence of increasing age on the pharmacodynamics of the anticonvulsant effect of oxazepam *in vivo*. Since changes in pharmacodynamics may occur simultaneously at different organizational levels (i.e. receptor binding characteristics, post-receptor events or homeostatic mechanisms), it was decided to study the pharmacodynamics initially *in vivo*, i.e. in the complete living system. Anticonvulsant effect intensity was determined on the basis of the threshold for induction of a localized seizure reaction upon direct cortical stimulation with ramp shaped electrical pulse trains of increasing intensity (Voskuyl *et al.*, 1989). In combination with the determination of blood concentrations and the application of simultaneous pharmacokinetic-pharmacodynamic modelling concepts, this allows the determination of concentration vs. anticonvulsant effect relationships in individual rats (Dingemanse *et al.*, 1990). In addition, benzodiazepine receptor binding characteristics were determined *in vitro* with [^{3}H]-flunitrazepam as radioligand.

Methods

Animals

Five groups of male BN/BiRij rats (TNO Institute of Ageing and Vascular Research, Leiden, The Netherlands) of different ages (4, 12, 24, 31 and 35 months; $n = 8, 8, 7, 6, 10$ respectively) were used. The 10, 50 and 90% survival ages of the male BN/BiRij rats are 38.1, 31.7 and 22.8 months, respectively. During the period in which the experiments were performed, the rats were kept solitary in Makrolon cages and in a normal 12 h light-dark cycle (light between 07 h 00 min and 19 h 00 min). The temperature was maintained at 22–23°C. They were allowed free access to water (acidified, pH 3–4) and food (Standard diet for Rat, Mouse and Hamster, AM 1410, Hope Farms, Woerden, The Netherlands).

Animals experiments

Clinical biochemical/pathological evaluation In order to be able to determine the health status of the animals, several

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clinical biochemistry parameters were measured. Blood concentrations of urea nitrogen, aspartate aminotransferase, alanine aminotransferase, glucose, total plasma protein and albumin concentration, 24 h urine production, osmolality, and creatinine clearance were determined as described before (Stijnen *et al.*, 1992).

After the pharmacokinetic/pharmacodynamic evaluation, the animals were killed, the liver was weighed and the heart, the lungs, the kidneys, the liver, the brain and macroscopically visible abnormalities were evaluated by a pathologist, who was unaware of the outcome of the pharmacokinetic/pharmacodynamic evaluation.

Pharmacokinetic/pharmacodynamic evaluation Three weeks before the experiment, two electrodes were implanted under halothane anaesthesia in the skull over the motor area of the frontoparietal cortex as described by Voskuyl *et al.* (1989). A bipolar pulse train (duration of one pulse 2 ms, frequency 50 pulses per s) of linearly increasing amplitude (0–2400 μA in 20 s), applied to the electrodes, was used as the convulsive stimulus and the threshold to induce localized seizure activity was determined, by measuring the stimulus current corresponding to the (visually determined) point at which clonic forelimb activity started. Since the convulsive threshold, which would be measured repeatedly in the experiment, decreases in the initial test sessions (Voskuyl *et al.*, 1989), this threshold was determined twice daily during the two weeks before the actual experiment until a stable baseline threshold was reached.

One day before the experiment, a polyethylene cannula was implanted in the right jugular vein under light halothane anaesthesia. On the day of the experiment the threshold to induce localized seizure activity was determined five times before the administration of oxazepam; the mean threshold value was used as the baseline level. Anticonvulsant effect intensities after administration of oxazepam were expressed as the elevation in threshold over this baseline level.

In vivo *experiment* Oxazepam, dissolved in 100 μl dimethylacetamide, was administered via the cannula in the jugular vein as an intravenous bolus dose of 12 mg kg^{-1} in about 1 min to the rats of the five different age groups. The animals of a 4-month-old control group received only the solvent. The seizure threshold was determined repeatedly (38 times) and 16 blood samples of 100 μl were collected from an incision in the tail during a period of 7 h after administration (in 5 of the 35-month-old rats the experiment was extended to a total of 11 h). Blood samples were immediately haemolyzed by addition of 500 μl of distilled water and stored at −20°C until analysis. In order to determine *in vivo* plasma protein binding, a second oxazepam bolus dose (6 mg kg^{-1}) was adminstered to the rats one day after the experiment and 1 min thereafter a blood sample of about 2 ml was withdrawn from an incision in the tail. Plasma was separated from the blood samples and stored at −20°C until analysis. A few days later the brain was removed in order to determine the *in vitro* benzodiazepine receptor binding characteristics. The brains were stored at −70°C until analysis.

Receptor binding The *in vitro* benzodiazepine receptor binding characteristics were determined at 0°C with [^{3}H]-flunitrazepam as a ligand applying the method described by Hollander-Jansen *et al.* (1989).

Drug analysis

Whole blood oxazepam concentrations were measured by a high performance liquid chromatography (h.p.l.c.) method described by Dingemanse *et al.* (1988) with slight modifications (oxazepam concentrations were measured in 100 μl whole blood instead of in 100 μl plasma and the 100 μl samples were diluted with distilled water instead of borate buffer). The h.p.l.c. system (Kratos Analytical Instruments, Ramsay, U.S.A.) consisted of a Spectroflow 400 solvent delivery system, a Promis automatic sample injector and a Spectroflow 757 u.v. detector, set at 240 nm. A Z-module containing a Radial-Pak C-18 cartridge, particle size 10 μm, was used (Waters Ass., Milford, U.S.A.). For data processing a Shimadzu C-R3A reporting integrator was applied. Protein binding of oxazepam was determined by means of ultrafiltration with the Amicon MPS-1 system (Grace B.V., Rotterdam, The Netherlands). The concentrations of oxazepam in the ultrafiltrate were measured in the same way as the blood samples.

Data analysis

Pharmacokinetics The area under the oxazepam concentration-time curve (AUC) was calculated by the linear trapezoidal rule with extrapolation to infinity, using the elimination rate constant k. This elimination rate constant was determined from the slope of the terminal phase of the log concentration vs. time profile. The elimination half life was calculated as $0.693/k$. Total body clearance was calculated as dose/AUC and the apparent volume of distribution (V_{area}) as dose/AUC*k.

In vitro *benzodiazepine receptor binding characteristics* The receptor binding data were subjected to a Langmuir-type equation (Hollander-Jansen *et al.*, 1989):

$$\text{number of receptors occupied} = \frac{B_{max} \times C}{K_D + C}$$

in which B_{max} represents the total number of specific binding sites, K_D the apparent dissociation constant and C the free flunitrazepam concentration, applying a least squares nonlinear regression algorithm (Siphar, Simed SA, Creteil, France).

Statistics

Differences between age groups were stastically evaluated by one-way analysis of variance followed by the Student t test with multiple comparison. Student's t test was applied to test whether the anticonvulsant and the proconvulsant effect in the mean effect vs. time profiles were significantly different from zero effect. P values lower than 0.05 were judged to be significant.

Chemicals

[^{3}H]-flunitrazepam (specific activity 87 Ci $mmol^{-1}$) was obtained from New England Nuclear ('s-Hertogenbosch, The Netherlands) and N,N-dimethylacetamide (DMA) from Merck Schuchardt (München, Germany).

Results

Clinical biochemical/pathological evaluation

The results of the clinical biochemical indices are shown in Table 1. The creatinine clearance and the osmolality decreased during aging. In the other parameters very few changes were found in both the mean and the individual values, except for the values for blood aspartate and blood alanine aminotransferase in the 35-month-old animals, where the high s.e.mean is caused by the extremely high individual values in one animal.

Three 35-month-old animals appeared to show pathological abnormalities. Two animals suffering from large meningeal granular cell tumours, with invasion into brain tissue in one of them and the other animals displayed a small granular cell tumour, leukaemia and liver abnormalities. These three animals were excluded from the calculations in

Table 1 Effect of age on selected clinical biochemical indices

Age (months)	*4*	*12*	*24*	*31*	*35*
Number of animals	8	8	7	6	10
Body weight (g)	243 ± 10	397 ± 21[a]	377 ± 8[a]	429 ± 14[a]	376 ± 12[a]
Liver weight (g)	8.6 ± 0.7	10.5 ± 0.6	10.9 ± 0.6 ($n = 6$)	14.1 ± 0.7[a,b]	13.0 ± 1.0[a]
Liver wt/body wt (%)	3.49 ± 0.18	2.63 ± 0.06[a]	2.90 ± 0.12 ($n = 6$)	3.29 ± 0.16	3.46 ± 0.25
Blood aspartate aminotransferase (iu l^{-1})	101.5 ± 9.7	89.9 ± 5.5	81.5 ± 4.7	81.5 ± 4.1	163 ± 79
Blood alanine aminotransferase (iu l^{-1})	38.4 ± 1.0	39.5 ± 1.0	36.7 ± 2.1	38.2 ± 4.9	68 ± 38
Plasma albumin (mg ml^{-1})	33.4 ± 0.6	32.8 ± 0.8	33.2 ± 0.9	35.2 ± 1.5	28.6 ± 2.8
Plasma total protein (mg ml^{-1})	82.8 ± 1.1	87.7 ± 1.5	88.4 ± 3.4	90.3 ± 4.7	80.9 ± 2.0
Blood glucose (mmol l^{-1})	5.0 ± 0.4	5.5 ± 0.2	5.4 ± 0.3	4.9 ± 0.4	4.8 ± 0.3
Blood urea nitrogen (mmol l^{-1})	9.5 ± 0.2	9.9 ± 0.6	8.4 ± 0.3[a]	8.5 ± 0.6	8.2 ± 0.2[a]
Creatinine clearance (ml h^{-1} kg^{-1})	297 ± 18	215 ± 11[a]	234 ± 22	158 ± 23[a]	152 ± 9[a,b,c]
24 h urine production (ml)	8.6 ± 0.5	11.4 ± 1.0	12.0 ± 0.6[a]	14.0 ± 2.7	12.7 ± 1.5
Osmolality (mOsm l^{-1})	2210 ± 110	1727 ± 53[a]	1530 ± 120[a]	1340 ± 100[a,b]	1241 ± 71[a,b]

Results are presented as mean ± s.e.mean. [a]Significantly different from 4-month value, $P < 0.05$; [b]Significantly different from 12-month value, $P < 0.05$; [c]Significantly different from 24-month, $P < 0.05$.

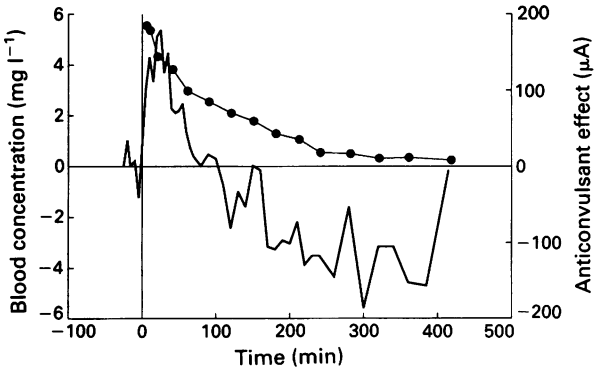

Figure 1 Blood concentration (●) vs. time and anticonvulsant effect (continuous line) vs. time profiles after administration of oxazepam as an intravenous bolus dose of 12 mg kg^{-1}, in a typical 4-month-old male BN/BiRij rat. Anticonvulsant effect intensities (μA) were expressed as the elevation in seizure threshold over baseline level. At t = 0 min oxazepam was administered.

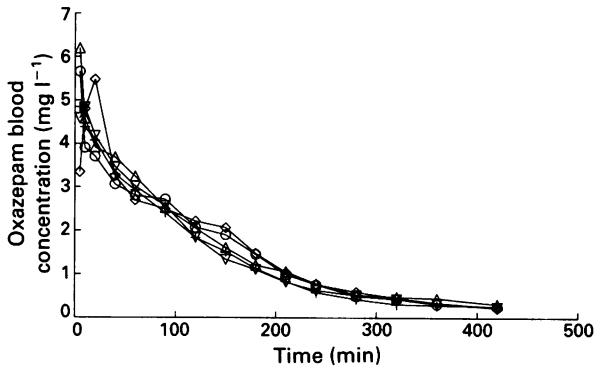

Figure 2 Total blood concentration vs. time profiles after administration of oxazepam as an intravenous bolus dose of 12 mg kg^{-1}, in male BN/BiRij rats of five different ages. The mean values of all animals in each age group are given: (+) 4-months-old, (Δ) 12-months-old, (○) 24-months-old, (◇) 31-months-old, (▽) 35-months-old.

the pharmacokinetic/pharmacodynamic evaluations on the basis of these findings.

Pharmacokinetic/pharmacodynamic evaluation

In vivo *experiment* In Figure 1 the blood concentration and anticonvulsant effect vs. time profiles after administration of oxazepam as an intravenous bolus dose of 12 mg in a typical 4-month-old male BN/BiRij rat are shown.

The mean oxazepam blood concentration vs. time profiles of the five age groups were identical (Figure 2), which is reflected in comparable pharmacokinetic parameters in the age groups (Table 2); the protein binding also showed no age-related changes (Table 2).

The anticonvulsant effect vs. time profile (Figure 1) showed a biphasic effect, first an increase in seizure threshold, later followed by a transient drop below the baseline value. The drop below the baseline was not found in the 4-month-old male control animals which received only the solvent.

Figure 3 shows the mean anticonvulsant effect vs. time profiles in the five age groups. The curves for the ages of 4, 12, 24 and 31 months are very similar: the shape is com-

Table 2 The influence of aging on pharmacokinetic parameters of oxazepam following an intravenous dose of 12 mg kg^{-1}

Age (months)	*4*	*12*	*24*	*31*	*35*
Number of animals	8	8	7	6	7
Total body clearance (ml min^{-1} kg^{-1})	20.4 ± 0.9	15.2 ± 1.7	19.4 ± 1.1	18.7 ± 1.9	20.4 ± 2.5
Volume of distribution (l kg^{-1})	2.52 ± 0.19	2.40 ± 0.22	2.27 ± 0.20	2.42 ± 0.29	2.56 ± 0.15
Elimination half-life (min)	95 ± 11	105 ± 6	81 ± 5	90 ± 7	92 ± 8
Plasma protein binding (%)	88.4 ± 1.5	91.3 ± 1.2	93.6 ± 0.5	93.1 ± 1.0	92.4 ± 0.5

The results are presented as mean ± s.e.mean.

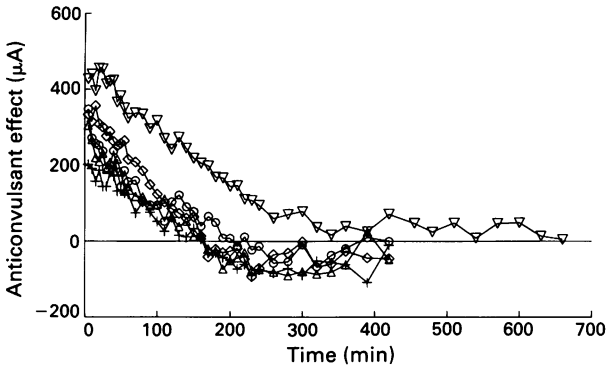

Figure 3 Anticonvulsant effect vs. time profiles after administration of oxazepam as an intravenous bolus dose of 12 mg kg^{-1}, in male BN/BiRij rats of five different ages. Anticonvulsant effect intensities (μA) were expressed as the elevation in seizure threshold over baseline level. The mean values of all animals in each age group are given: (+) 4-months-old, (Δ) 12-months-old, (○) 24-months-old, (◇) 31-months-old, (▽) 35-months-old. The last eight values for the 35-month-old rats were measured in 5 out of the total of 7 animals.

parable, but the curves tend to move to higher anticonvulsant effect values with increasing age. The curve for the 35-month-old animals shows relatively high anticonvulsant effect values and a slower decline in anticonvulsant effect with time. Moreover, the effect does not drop below the baseline as in the other age groups. The anticonvulsant effect was significantly different from zero effect in all age groups, whereas the proconvulsant part was only significant in the 4-, 12-, and 24-month-old rats.

The mean baseline seizure threshold value for all animals was 837 ± 40 μA (mean ± s.e.mean; $n = 36$). No systematic trend during aging was found for the threshold values. For all age groups the means of the highest anticonvulsant effect values in the individual animals were calculated (Table 3). These values showed an age-related tendency to increase, with the value in the 35-month-old rats significantly higher than that in the 4-month-old animals.

In all individual animals the oxazepam blood concentration at which the anticonvulsant effect crossed the baseline value was determined graphically (Table 3). In one animal in both the 4- and the 12-month-old group and three animals in both the 24- and the 31-month-old group, the effect did not drop below the baseline level but only returned to that value. In those animals the concentration at which the effect returned to the baseline was determined. No age-related change was found in the concentration at zero effect.

In vitro benzodiazepine receptor binding characteristics No age-related changes were found in the total number of specific binding sites and the apparent dissociation constant (Table 4).

Discussion

In this investigation we determined simultaneously the time course of the plasma concentration and the anticonvulsant effect intensity following a single dose of oxazepam. Of considerable interest is the observation, that at least in young rats, the time course of the pharmacological response shows a biphasic character: anticonvulsant effect followed by proconvulsant effect. This is in contrast to the situation in Wistar rats, where only an anticonvulsant effect was observed (Dingemanse *et al.*, 1990). The biphasic character of the anticonvulsant effect is highly suggestive of an acute tolerance/withdrawal syndrome. This is also confirmed by the results of some pilot experiments, in which no proconvulsant effect was observed at low concentrations of oxazepam during slow intravenous infusion. Thus it appears that initially a decrease from high concentrations to lower values is required for the proconvulsant effect to occur (Hoogerkamp *et al.*, unpublished observations). The finding of a tolerance/withdrawal phenomenon is compatibale with observations by Lister & Nutt (1986).

The pharmacological effect vs. time profile in the 35-month-old animals was quite different from that in the other age groups. Immediately following administration of oxazepam the anticonvulsant effect intensity was significantly higher and also the proconvulsant component was no longer present. Finally the anticonvulsant effect in the 35-month-old animals showed a slower decrease over time compared to the other ages. The lack of the biphasic effect profile in the older rats is compatible with the disappearance of the tolerance/withdrawal phenomenon with increasing age. The question is whether this also contributes to the initial increased effect intensity that is observed.

In theory the underlying mechanism of the observed change in anticonvulsant effect intensity can be a change in pharmacokinetics (resulting in higher concentrations at the site of action) or an increased brain sensitivity. Between the different age groups no important differences in pharmacokinetics were observed (Figure 2, Table 2). As the plasma protein binding is also unaffected by age, it is unlikely that a change in distribution is the cause of the increased effect intensity. Thus it appears that in the different age groups the concentrations of oxazepam immediately following administration are quite similar. Therefore the increase in anticonvul-

Table 3 The influence of aging on the highest anticonvulsant effect values and the oxazepam blood concentrations at which the effect crossed the baseline value

Age (months)	*4*	*12*	*24*	*31*	*35*
Number of animals	8	8	7	6	7
Highest anticonvulsant effect (μA)	262 ± 37	350 ± 37	365 ± 53	400 ± 15	549 ± 65[a]
Concentration at baseline crossing (mg l^{-1})	1.44 ± 0.23	1.52 ± 0.31	1.44 ± 0.30	1.39 ± 0.29	

The results are presented as mean ± s.e.mean. [a]Significantly different from 4-month value, $P < 0.05$.

Table 4 The influence of aging on the *in vitro* benzodiazepine receptor binding characteristics

Age (months)	*4*	*12*	*24*	*31*	*35*
Number of animals	5	5	5	5	5
Total number of specific binding sites (ng mg^{-1})	0.55 ± 0.05	0.53 ± 0.22	0.54 ± 0.08	0.44 ± 0.05	0.52 ± 0.03
Apparent dissociation constant (ng ml^{-1})	4.0 ± 0.8	4.7 ± 0.9	3.3 ± 0.8	3.5 ± 0.8	5.8 ± 1.3

The results are presented as mean ± s.e.mean.

sant effect intensity in the 35-month-old rats appears be due to a change in the pharmacodynamics of oxazepam rather than a change in its disposition, which is in agreement with observations by Kitani *et al.* (1989).

The next important question relates to the mechanism of the increase in brain sensitivity. In theory, the higher brain sensitivity we found can be caused by changes in drug-receptor interaction, post-receptor events or changes in homeostatic control mechanisms (Danhof, 1989). In the present study we did not observe age-related changes in the *in vitro* receptor binding characteristics as has been reported in other studies (Heusner & Bosmann, 1981; Barnhill *et al.*, 1990). Other data on post-receptor events (GABA receptor binding and chloride channel functioning) have not provided evidence for clear age-related changes (De Blasi *et al.*, 1982; Concas *et al.*, 1988; Ito *et al.*, 1988; Barnhill *et al.*, 1990). This leaves therefore the possibility of changes at the level of homeostatic control mechanisms as a realistic explanation for the higher brain sensitivity. Alterations in the production and or release of an endogenous ligand for the benzodiazepine receptor with inverse agonist properties have been postulated as a mechanism for the occurrence of the tolerance/withdrawal phenomena (Baldwin & File, 1988). Interestingly, the presence of endogenous n-butyl β carboline-3-carboxylate, which possesses inverse agonist properties, has indeed been demonstrated (Novas *et al.*, 1988; Medina *et al.*, 1989). In man also, the occurrence of tolerance/withdrawal phenomena with regard to the pharmacological effects of benzodiazepines appears to be ubiquitous (Bliding, 1974; Greenblatt *et al.*, 1977; 1981; Macleod *et al.*, 1977; Greenblatt & Shader, 1978; Ellinwood *et al.*, 1983; 1985; 1987; Salzman *et al.*, 1983; Ashton *et al.*, 1988). Thus a decrease in the efficiency of homeostatic control mechanisms may be a realistic explanation for the increase in sensitivity to the pharmacological actions of benzodiazepines in the elderly. This would be in line with the idea of an impaired maintenance of homeostasis in the elderly in general (Shock, 1961; 1983; Bender, 1969; Crooks, 1983). In agreement with our data in rats, data from a spontaneous reporting system maintained by the Food and Drug Administration in the U.S.A. showed a much lower frequency of withdrawal after cessation of benzodiazepine therapy in the older age group (Tanner *et al.*, 1989).

In the present investigation also detailed information on the influence of aging on the pharmacokinetcis of oxazepam was obtained. The estimates of the pharmacokinetic parameters in young male BN/BiRij rats are similar to those observed in female Wistar rats (Dingemanse *et al.*, 1988; 1990). Interestingly, the pharmacokinetics of oxazepam were found not to change with increasing age, as is also the case in man (Greenblatt *et al.*, 1980; Murray *et al.*, 1981; Ochs *et al.*, 1981). In man oxazepam is eliminated primarily by phase II metabolic reactions, which are known to be relatively insensitive to changes that occur with increasing age. In rats however, the metabolism is quite different with phase I metabolic reactions being more prominant (Sisenwine *et al.*, 1972; Greenblatt, 1981; Sisenwine & Tio, 1986). Thus it appears that the similarities in the effect of aging on the pharmacokinetics of oxazepam are merely a coincidence.

Summarising, the biphasic pharmacological effect vs. time profiles (anticonvulsant effect followed by 'proconvulsant' effect) observed in the 4- to 31-month-old BN/BiRij rats after administration of an intravenous oxazepam bolus dose of 12 mg kg^{-1} are suggestive for acute tolerance development and withdrawal. The disappearance of this biphasic profile in the 35-month-old animals and the increase in brain sensitivity are compatible with a decreased efficiency of homeostatic control mechanisms in the elderly.

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Pharmacokinetic-EEG effect relationship of midazolam in aging BN/BiRij rats

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1 The purpose of the present investigations was to determine the influence of increasing age on the pharmacokinetics and pharmacodynamics of midazolam in male BN/BiRij rats as an animal model of aging.

2 Midazolam was administered intravenously at a dose of 2.5 mg kg^{-1} and its pharmacokinetics were determined on the basis of plasma concentrations as measured by high performance liquid chromatography (h.p.l.c.). Pharmacodynamics were studied using the midazolam-induced changes in the electroencephalogram (EEG) as a measure of the pharmacological effect. Results were evaluated on the basis of stimultaneous pharmacokinetic-pharmacodynamic modelling. In an attempt to differentiate between the effects of aging and of concurrent disease, an extensive clinical biochemical/pathological examination was conducted in individual rats by an independent pathologist.

3 The pharmacokinetics of midazolam were best characterized on the basis of a two exponential model. In the 4-month-old rats the values of the clearance, volume of distribution and elimination half-life were 104 ± 13 ml min^{-1} kg^{-1} (mean ± s.e.mean), 3.4 ± 0.7 l kg^{-1} and 30 ± 3 min, respectively. With increasing age, no changes in the pharmacokinetics of midazolam were observed.

4 The pharmacodynamics of midazolam were determined on the basis of the sigmoidal E_{max} model. In the 4-month-old rats the values of the parameters relative maximum effect, midazolam concentration at half maximum effect and Hill factor were $106 \pm 10\%$, 50 ± 6 μg l^{-1} and 1.6 ± 0.3, respectively. In the group as a whole no significant changes in the pharmacodynamic parameters of midazolam were observed. However, when diseased animals were excluded from the evaluation, a tendency towards a decrease in the midazolam concentration at half maximum effect to 25 ± 14 μg l^{-1} was observed in the 36-month-old rats.

5 These findings suggest, that increasing age is associated with a tendency towards an increased brain sensitivity to midazolam, which is reflected in a parallel shift of the concentration vs. EEG effect relationship towards lower concentrations. However, it appears that factors other than age also contribute to interindividual variability in pharmacodynamics, considering the substantial interindividual variability within certain age groups.

Keywords: Pharmacokinetics; pharmacodynamics; electroencephalogram; midazolam; aging

Introduction

During recent years, midazolam has become an important intravenous sedative, because of its favourable properties, e.g. short elimination half-life, water solubility and lack of important adverse effects (Dundee *et al.*, 1984). Due to the cardiorespiratory stability after its administration, midazolam is especially preferable above other anaesthetics for induction of anaesthesia in poor-risk, elderly and cardiac patients (Dundee *et al.*, 1984). Also in the treatment of sleep disorders in the elderly, midazolam was reported to be effective and well-tolerated, provided that it is given in a carefully titrated, appropriate dose (Lachnit *et al.*, 1983). Several authors emphasized the importance of titrating the dose, since dose requirement for midazolam is reduced in elderly patients (Kanto *et al.*, 1986; Bell *et al.*, 1987; Oldenhof *et al.*, 1988). In a study in 800 patients in which the dose of intravenous midazolam required to produce adequate sedation prior to upper gastro-intestinal endoscopy was measured, an important decrease of approximately 75% in dose requirement from the age of 15 years to the age of 85 years was observed (Bell *et al.*, 1987).

An important question is, whether the decrease in dose requirement is caused by pharmacokinetics and/or pharmacodynamic changes during aging. The purpose of the present investigations was therefore to study the pharmacokinetics and the pharmacodynamics of midazolam simultaneously, with the midazolam–induced changes in the electroencephalogram (EEG) used as a measure of the pharmacological effect. The experiments were performed in an animal model of aging (BN/BiRij rats).

Methods

Animals

Five groups of male BN/BiRij rats (TNO Institute for Ageing and Vascular Research, Leiden, The Netherlands) of different ages (4, 13, 24 and 29 and 36 months) were used. The 10, 50 and 90% survival ages of this strain are 38.1, 31.7 and 22.8 months, respectively. Water (acidified, pH 3–4) and food (Standard diet for Rat, Mouse and Hamster, AM 1410, Hope Farms, Woerden, The Netherlands) were supplied *ad libitum*. The rats were kept solitary in Makrolon cages and in a normal 12-h light-dark cycle (light between 07h 00 min and 19 h 00 min). The temperature was maintained at 22–23°C.

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Animal experiments

Clinical biochemical/pathological evaluation In order to be able to determine the health status of the animals, several clinical biochemical indices were measured (blood concentrations of urea nitrogen, aspartate aminotransferase, alanine aminotransferase and glucose, total plasma protein and albumin concentration, urine production, urine osmolality and creatinine clearance). The concentrations of creatinine in blood and urine were determined with a Reflotron autoanalyzer (Boehringer, Mannheim, Germany). The other indices were determined as described before (Stijnen *et al.*, 1992). After the pharmacokinetic and pharmacodynamic evaluation, the animals were sacrificed, the liver was weighed, and the heart, the lungs, the kidneys, the liver, the brains and macroscopically visible abnormalities were evaluated microscopically by a pathologist. Details about this evaluation were reported before (Stijnen *et al.*, 1992).

Pharmacokinetic/pharmacodynamic evaluation Cortical EEG electrodes were implanted two weeks before the experiment under halothane anaesthesia, according to the method of Mandema & Danhof (1990). Five hours before the experiments, cannulae were implanted in the femoral vein and femoral artery under halothane anaesthesia. All experiments were started around 14 h 00 min in order to minimize the influence of a possible diurnal rhythm in brain sensitivity, baseline EEG characteristics and/or metabolism rate (Roberts *et al.*, 1970). During the experiments the rats were kept in a slowly rotating drum to keep the vigilance of the animals constant.

After about 15 min baseline EEG recording, midazolam was administered as an intravenous dose of 2.5 mg kg^{-1} (dissolved in saline with an equimolar quantity of hydrochloric acid) over a period of 5 min. Arterial blood samples of 100 µl (or 200 µl near the end of the experiment) were taken throughout the whole experiment. The EEG was continuously recorded during the first 60 min after drug administration (rotating drum on). After that a scheme of 4 min rotating drum off and 6 min on was followed in order not to fatigue the old animals. Only the EEG recorded during the last 5 min of the 6 min of the 'drum on' period was analyzed. The experiment was terminated after the EEG returned to a value within 10% of the baseline level.

By applying aperiodic analysis (Gregory & Pettus, 1986) the total amplitude in the frequency area of 11.5 to 30 Hz of the fronto-central EEG lead was calculated and used as the descriptor of the drug effect, because this was shown to provide an optimal measure for the effect of midazolam on the central nervous system (Mandema *et al.*, 1991b). More detailed information on the recording and analysis of the EEG are described by Mandema *et al.* (1991a).

Drug analysis

The concentrations of midazolam in plasma were measured by the high performance liquid chromatography (h.p.l.c.)-method and apparatus described by Mandema *et al.* (1991b). Part of the samples were measured by a slightly modified method: instead of 25 ng diazepam, 37.5 ng clobazam was used as the internal standard, 5 mM octansulphonic acid was added to the eluent and the flow was reduced to 0.8 ml min^{-1}, yielding retention times of 3.8 and 6.5 min for clobazam and midazolam, respectively. Coefficients of variation were less than 5% and the detection limit was about 10 µg l^{-1}.

In order to determine the plasma protein binding in each individual animal, a second intravenous dose of 2.5 mg kg^{-1} was administered to the rat about three days after the experiment and a blood sample of 2 ml was withdrawn via an incision in the tail at 1 min following administration. The blood sample was subjected to ultrafiltration and the unbound concentration of midazolam in the ultrafiltrate was measured in the same way as the plasma samples.

Data analysis

Pharmacokinetic-pharmacodynamic modelling The midazolam plasma concentration vs. time profile was described by a two exponential equation for intravenous infusion. The total body clearance, the steady state volume of distribution, the volume of the central compartment and the elimination half-life were calculated from the coefficients and exponents of the fitted functions according to standard procedures (Gibaldi & Perrier, 1982).

The relationship between the concentrations (calculated at several time points using the pharmacokinetic model) and the increase in the total amplitude in the frequency range of 11.5–30 Hz (as the measure for the EEG-effect) was characterized by the sigmoidal E_{max} model (Mandema *et al.*, 1991b). In 44% of the animals an overshoot in the effect time profile immediately after drug administration was observed, which lasted longer than 5 min. The early peak effects were not related to the midazolam concentration. These early peak effects were omitted in the characterization by the sigmoidal E_{max} model. This was considered justified, while the concentration vs. EEG effect relationship of the animals showing early peak effects were not different from those not showing these effects.

All calculations were performed using the nonlinear least squares regression programme Siphar (Simed SA, Creteil, France).

Statistics The effect of aging on the biochemical indices, the pharmacokinetic and the pharmacodynamic parameters was tested statistically by one-way analysis of variance with multiple comparison. P values lower than 0.05 were judged to be significant.

Results

Clinical biochemical/pathological evaluation

The results of the clinical biochemical indices are shown in Table 1 for all animals. An age-related decrease was measured for osmolality and blood alanine aminotransferase, a decrease followed by an increase for blood urea nitrogen and the ratio liver weight/body weight and a tendency to decrease for the creatinine clearance.

The pathological evaluation showed that five animals were suffering from diseases, and they were excluded from the evaluation of the results of the study. One 13-month-old animal displayed meningeal periarteritis, one 29-month-old a necrotizing urothelial tumour of the renal pelvis, two 36-month-old rats showed several lesions in the liver (necrosis, focal inflammation, cysts in both with abscesses in one of the two rats and severe bile duct proliferation in the other one) and another 36-month-old with endocardial schwanoma of the heart, signs of cardiac failure and an adrenocortical carcinoma.

Pharmacokinetic/pharmacodynamic evaluation

Figure 1 shows the plasma concentration vs. time profile and the EEG effect vs. time profile in one typical rat.

Since no time delay was observed between plasma concentration and EEG effect (in all age groups), these two were directly correlated to each other and characterized by the sigmoidal E_{max} model (Figure 2). Figures 3 and 4 display the estimates of the individual animals (including the diseased ones) for the pharmacokinetic and pharmacodynamic parameters for the different age groups, respectively. The mean values in the figures and in Tables 2 and 3 were calculated including only the healthy animals.

Table 1 Effect of age on selected clinical biochemical indices

Age (months)	*4*	*13*	*24*	*29*	*36*
Number of animals	8	9	8	8	6
Body weight (g)	224 ± 10	398 ± 12[a]	398 ± 11[a]	405 ± 11[a]	388 ± 13[a]
Liver weight (g)	7.7 ± 0.4 ($n = 6$)	9.6 ± 0.7 ($n = 8$)	9.6 ± 0.4 ($n = 5$)	9.7 ± 0.5	9.5 ± 1.4
Liver wt/body wt (%)	3.68 ± 0.16 ($n = 6$)	2.36 ± 0.11[a] ($n = 8$)	2.43 ± 0.22[a] ($n = 5$)	2.40 ± 0.11[a]	2.92 ± 0.26
Blood aspartate aminotransferase (iu l^{-1})	102.3 ± 7.1	94.2 ± 6.9	76.9 ± 5.6	82.7 ± 3.4	105 ± 11
Blood alanine aminotransferase (iu l^{-1})	44.8 ± 2.5	40.8 ± 2.1	34.0 ± 1.7[a]	35.2 ± 1.5	29.6 ± 3.3[a]
Plasma albumin (mg ml^{-1})	35.9 ± 1.8	32.2 ± 1.9	29.4 ± 1.8	33.0 ± 1.3	36.3 ± 2.5
Plasma total protein (mg ml^{-1})	81.5 ± 2.8	88.6 ± 3.2	92.1 ± 1.6	94.6 ± 1.3[a]	93.2 ± 4.7
Blood glucose (mmol l^{-1})	4.7 ± 0.3	4.5 ± 1.8	4.4 ± 0.3	4.2 ± 0.2	3.9 ± 0.5
Blood urea nitrogen (mmol l^{-1})	8.3 ± 0.4	7.4 ± 0.2	6.0 ± 0.4[a,b]	6.5 ± 0.4[a]	8.2 ± 0.9
Creatinine clearance (ml h^{-1} kg^{-1})	174 ± 26	151 ± 18	157 ± 14	142 ± 14	94 ± 19
24 h urine production (ml)	8.3 ± 0.6	7.7 ± 1.1	9.3 ± 1.6	9.4 ± 1.3	11.4 ± 1.3
Osmolality (mOsm l^{-1})	1980 ± 140	1550 ± 140	1340 ± 91[a]	1174 ± 71[a]	1135 ± 75[a]

Results are presented as mean ± s.e.mean. [a]Significantly different from 4-month value, $P < 0.05$; [b]Significantly different from 13-month value, $P < 0.05$.

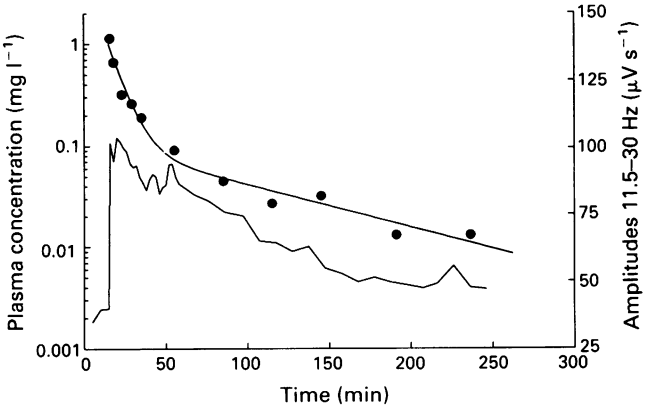

Figure 1 Midazolam plasma concentration (●) vs. time profile (the solid line through the data points represents the characterization by a two exponential equation) and EEG effect (continuous line) vs. time profile for a typical rat (4 months of age) using the total amplitude in the frequency range of 11.5–30 Hz as the descriptor of the EEG effect. The first 15 min in the effect vs. time profile represent the baseline recording. Because the 5 min infusion time is left out in this figure, time point 15 min is the end of the midazolam administration.

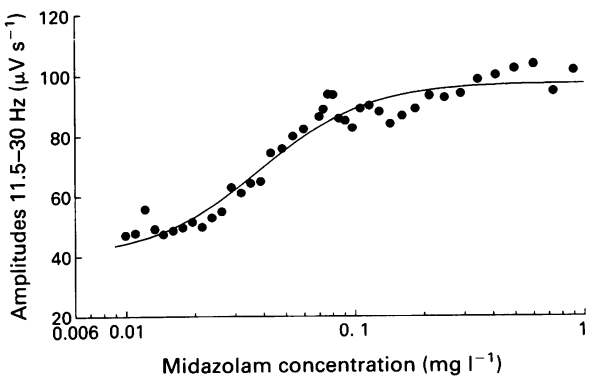

Figure 2 Plasma concentration vs. EEG effect relationship for a typical 4-month-old rat. The continuous line through the data points represents the characterization of the concentration vs. effect relationship by the sigmoidal E_{max} model.

Total body clearance, steady state volume of distribution, volume of the central compartment and elimination half-life did not show significant changes during aging (Table 2 and Figure 3). Also plasma protein binding and the fraction unbound did not change during aging (fraction unbound: 4.6 ± 0.9%, 4.6 ± 0.6%, 4.6 ± 0.7%, 3.9 ± 0.3% and 4.3 ± 0.9% (mean ± s.e.mean) for the 4, 13, 24, 29 and 36-month-old rats, respectively).

Concerning the pharmacodynamics (Table 3 and Figure 4), the EEG baseline effect, the absolute and relative maximum effect and the Hill factor did not change during aging. The total and free (unbound) midazolam concentration at half the maximum effect showed a tendency to decrease between the ages of 29 and 36 months. This tendency was found only when the diseased animals were excluded.

Discussion

In 1987 Bell *et al.* reported a remarkable age-related decrease in dose requirement of intranveous midazolam to produce adequate sedation to perform upper gastro-intestinal endoscopy. In a clinical study in about 800 patients a reduction in dose requirement of 75% between the ages of 15 and 85 years was observed. The reasons for the decrease in dose requirement were not studied; this can be relatively complex with a multitude of pharmacokinetic and pharmacodynamic factors involved. With regard to the pharmacokinetics, especially changes in the (central) volume of distribution are an important determinant (Greenblatt *et al.*, 1984); for the pharmacodynamics, changes in both E_{max} and EC_{50} can be relevant.

In a number of investigations, the influence of increasing age on the pharmacokinetics and pharmacodynamics of benzodiazepines has been studied. This has resulted in a situation, where the influence of increasing age on the pharmacokinetics of various benzodiazepines has been well documented (Danhof, 1991). The situation with regard to the pharmacodynamics is, however, much less clear. In many instances, the effects on pharmacodynamics have been characterized on the basis of psychomotor performance tests. The results of these investigations have been rather inconclusive. Complicating factors have been the occurrence of age-related changes in the baseline levels of the performance measures and the interference of various complicating pharmacokinetic factors (i.e. the role of active metabolites and changes in distribution), of which the exact contribution has often not

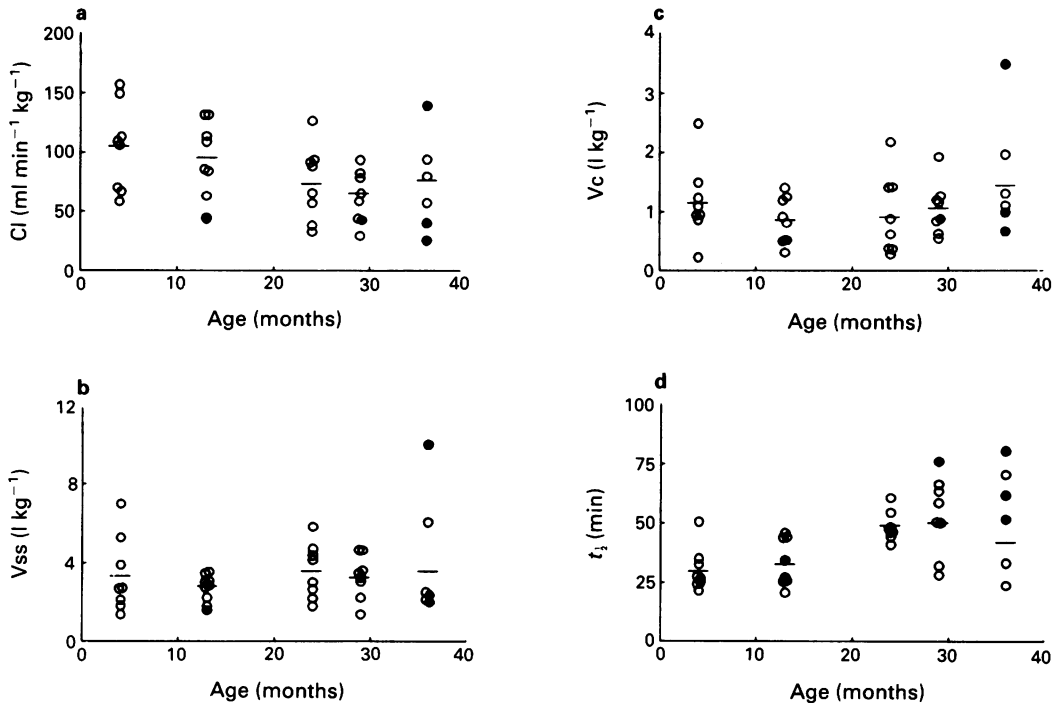

Figure 3 Individual values (of both diseased and healthy rats) and mean value (of only the healthy animals) of the pharmacokinetic parameters in the different age groups following intravenous administration of midazolam at a dose of 2.5 mg kg^{-1}: (○) healthy animals; (●) diseased animals; (–) mean value of the healthy animals. (a) Total body clearance (Cl); (b) steady state volume of distribution (Vss); (c) volume of the central compartment (Vc); (d) elimination half-life ($t_{1/2}$).

Table 2 The influence of aging on pharmacokinetic parameters of midazolam following an intravenous dose of 2.5 mg kg^{-1} (considering only healthy animals)

Age (months)	*4*	*13*	*24*	*29*	*36*
Number of animals	8	8	8	7	3
Total body clearance (ml min^{-1} kg^{-1})	104 ± 13	96 ± 11	75 ± 11	66 ± 9	78 ± 11
Steady state volume of distribution (l kg^{-1})	3.4 ± 0.7	2.9 ± 0.2	3.6 ± 0.5	3.3 ± 0.5	3.6 ± 1.3
Volume of the central compartment (l kg^{-1})	1.2 ± 0.2	0.9 ± 0.1	0.9 ± 0.2	1.1 ± 0.2	1.5 ± 0.3
Elimination half-life (min)	30 ± 3	32 ± 4	49 ± 2	50 ± 6	43 ± 14

The results are presented as mean ± s.e.mean.

Table 3 The influence of aging on the concentration vs. EEG-effect relationship parameters of midazolam (considering only healthy animals)

Age (months)	*4*	*13*	*24*	*29*	*36*
Number of animals	8	8	8	7	3
Baseline effect value (μV s^{-1})	58 ± 5	48 ± 4	45 ± 3	51 ± 4	42 ± 7
Absolute maximum effect (μV s^{-1})	59 ± 3	54 ± 4	47 ± 3	59 ± 5	55 ± 12
Relative maximum effect (% of baseline effect)	106 ± 10	116 ± 10	105 ± 5	116 ± 6	129 ± 12
Plasma concentration at half the maximum effect (μg l^{-1})	50 ± 6	45 ± 16	52 ± 10	59 ± 11	25 ± 14
Unbound plasma concentration at half the maximum effect (μg l^{-1})	2.4 ± 0.7	1.8 ± 0.6	2.6 ± 0.6	2.3 ± 0.5	1.3 ± 0.9
Shape factor	1.6 ± 0.3	1.0 ± 0.2	1.3 ± 0.1	1.3 ± 0.1	0.9 ± 0.3

The results are presented as mean ± s.e.mean.

been determined. A limitation of studies in man is, that further mechanistic studies are not possible.

In the present investigation, we studied the influence of increasing age on the pharmacokinetics and pharmacodynamics of midazolam in BN/BiRij rats as an animal model of aging. The survival curves and the age-related pathology of this rat strain are well characterized and therefore this rat strain is suitable for this type of investigation (Burek, 1978;

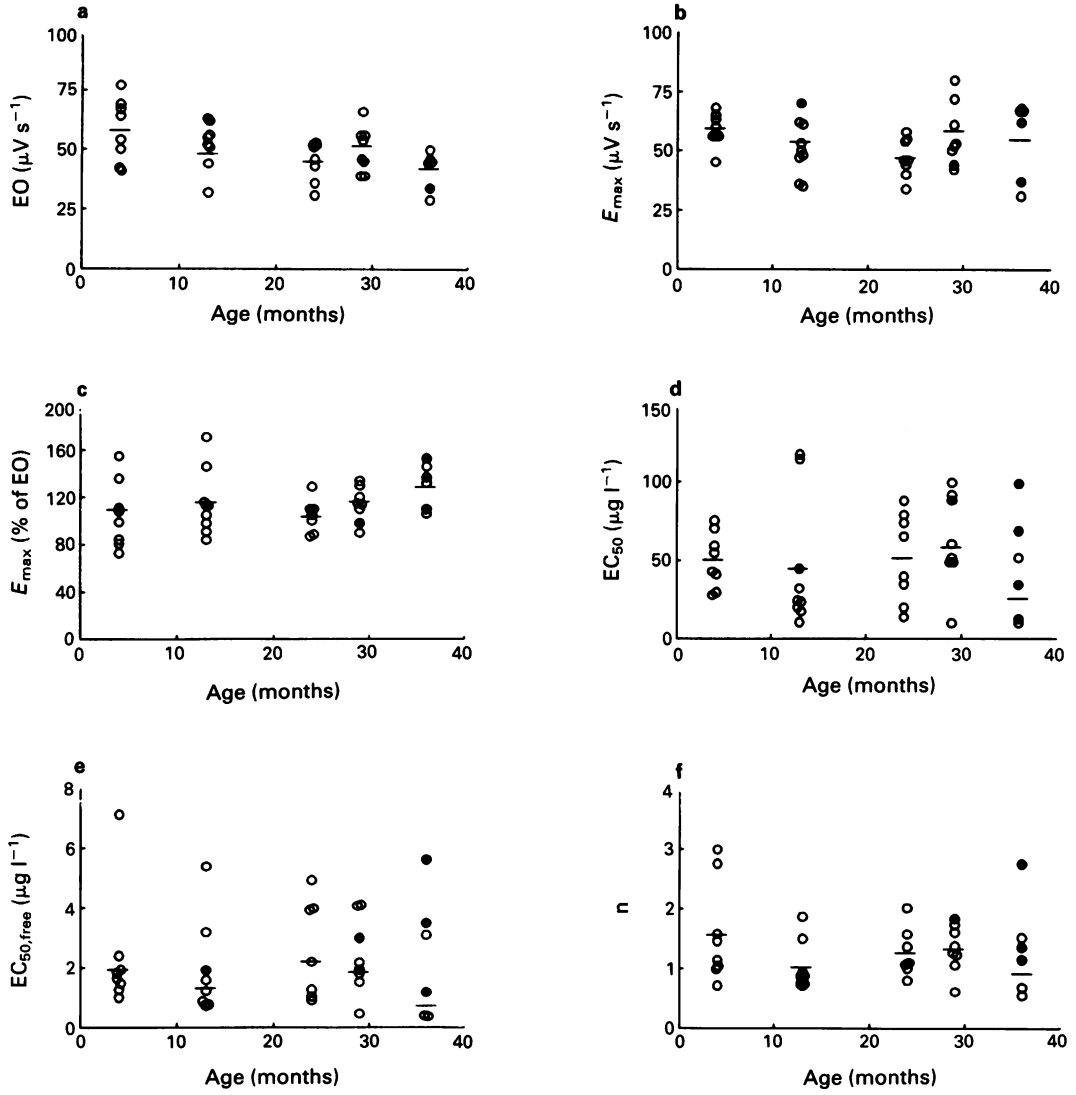

Figure 4 Individual values (of both diseased and healthy animals) and mean value (of only the healthy animals) of the sigmoidal E_{max} parameters in the different age groups following intravenous administration of midazolam at a dose of 2.5 mg kg^{-1}: (○) healthy animals; (●) diseased animals; (–) mean value of the healthy animals. (a) Baseline effect value (E0); (b) absolute maximum effect (E_{max}); (c) relative maximum effect (E_{max} expressed as percentage of E0); (d) plasma concentration at half the maximum effect (EC_{50}); (e) unbound plasma concentration at half the maximum effect ($EC_{50_{free}}$); (f) shape factor (n).

Hollander, 1979). The increase in the amplitude in the 11.5–30 Hz frequency-range of the EEG was used as a pharmacodynamics measure. This parameter is easily measured in rats (Mandema *et al.*, 1991b) and has also been found pharmacologically relevant in that it reflects the effect of benzodiazepines in GABA-ergic neurotransmission (Mandema *et al.*, 1991a). It was demonstrated, that (inter)active metabolites do not interfer with the pharmacological response to midazolam and that (acute) functional tolerance development does not occur (Mandema *et al.*, 1991b; Mandema *et al.*, unpublished observations).

The values of the pharmacokinetic parameters of midazolam in the BN/BiRij rats of the present study were found to be slightly different from those observed previously in male Wistar rats. For both the systemic clearance and the volume of distribution, larger values were observed in BN/BiRij rats relative to Wistar rats. The values of the elimination half-life were similar (Mandema *et al.*, 1991a,b). With increasing age no changes in the values of the various pharmacokinetic parameters of midazolam were observed. For both central and steady state volumes of distribution, this appears to be in line with observations in man (Greenblatt *et al.*, 1984; Smith *et al.*, 1984). This implies, that changes in the volume parameters cannot explain the decreased dose requirement reported by Bell *et al.* (1987). In man, a lower total body clearance and an increased elimination half-life were found in elderly males, but not in females (Greenblatt *et al.*, 1984; Servin *et al.*, 1987). The difference between the findings in man and those in rats might be explained by a comparable difference in the influence of age on liver weight, which decreases during aging in man (Thompson & Williams, 1965) and does not change in our rats (Table 1). In the present study, a wide interindividual variability in pharmacokinetic parameter estimates was observed, as was also reported in man (Servin *et al.*, 1987). No clear relationship with the presence of concurrent disease could be detected.

The values of the pharmacodynamic parameter estimates of midazolam in the BN/BiRij rats in the present investigation were also slightly different from those obtained in Wistar rats. The values of the maximum effect and the concentration at half the maximum effect were lower and those of the shape factor were higher than the values in Wistar rats, whereas the baseline effect values were comparable (Mandema *et al.*, 1991a).

In the group as a whole the pharmacodynamic parameter estimates in the BN/BiRij rats showed significant interin-

dividual variability, but no clear age-related changes in pharmacodynamics were observed. In man, also a high variation in pharmacological response was reported in young adults (Dundee *et al.*, 1984). In previous investigations it has been demonstrated however, that the results of pharmacodynamic investigations with drugs acting on the central nervous system can be confounded by the presence of concurrent disease (Stijnen *et al.*, unpublished observations). Therefore, an extensive pathological evaluation of the animals of this study was performed by an experienced research pathologist. This resulted in one 13-month-old animal, one 29-month-old animal and three out of the total of six animals in the 36-month-old group being found to be diseased. When these animals were excluded from the evaluation, on average a decrease in the concentration at half the maximum effect from $50 \pm 6\ \mu g\ l^{-1}$ (mean ± s.e.mean) in the 4-month-old rats to $25 \pm 14\ \mu g\ l^{-1}$ in the 36-month-old rats was observed, suggesting indeed an increased brain sensitivity to the pharmacological effects of midazolam with increasing age. Due to the limited number of healthy 36-month-old animals however, this difference did not reach statistical significance.

Baughman *et al.* (1987), upon studying the EEG effect of midazolam qualitatively, did not find differences between young (6-month-old) and old (28-month-old) rats. However, they measured an age-related increased effect of the drug on the cerebral blood flow (CBF) and the cerebral oxygen consumption ($CMRO_2$). These effects were reported to decrease coincidentally with the sedative/hypnotic effect (Hoffman *et al.*, 1986). Baughman *et al.* (1987) stated, that a depression of non-electric metabolic function by midazolam in the aged rat would be important in this respect.

Many investigators have examined the influence of aging on the pharmacodynamics of benzodiazepines generally on the basis of investigations *in vitro*. Emphasis in these studies has been on distinct aspects, as for example, the binding of benzodiazepines and γ-aminobutyric acid (GABA) to the GABA-benzodiazepine receptor complex as well as functioning of the chloride channel. However, the results of these investigations have been rather inconclusive (Pedigo *et al.*, 1981; Reeves & Schweizer, 1983; Kochman & Sepulveda, 1986; Concas *et al.*, 1988; Ito *et al.*, 1988). A relative disadvantage of these investigations *in vitro* is, that only a single aspect of the pharmacodynamics is studied. In studies *in vivo*, the entire pharmacodynamic process is studied, including binding to relevant receptors, processes that occur following binding to the receptor as well as *in vivo* homeostatic processes, that may be operative. Thus on the basis of the *in vivo* investigations, an impression of changes in the overall pharmacodynamic process is obtained.

In conclusion, the findings of the present investigation show that there is a significant interindividual variability in the pharmacodynamics of midazolam in rats. Increasing age *per se* however, appears to be only a minor factor causing this variability. In this respect, a large investigation is currently in progress on data obtained in some 150 rats where population analysis is used to identify factors that may be important in this respect. Similar studies are indicated in man and it is of interest that the EEG parameter utilized can also be applied in human studies (Mandema *et al.*, 1992).

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Effects of inhibition of the L-arginine/nitric oxide pathway in the rat lower urinary tract *in vivo* and *in vitro*

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1 The present study was performed to investigate how blockade of the L-arginine/nitric oxide (NO) pathway influences the function of the lower urinary tract *in vivo*, as studied by cystometry in conscious rats and *in vitro*, in isolated muscle preparations from the rat detrusor and urethra.

2 L-N^G-nitro arginine methyl ester (L-NAME), 10 and 20 mg kg^{-1}, administered intra-arterially, decreased micturition volume and bladder capacity, and increased spontaneous bladder contractions. D-NAME (20 mg kg^{-1}) had no effect. No changes in the urodynamic parameters were recorded if L-NAME (20 mg kg^{-1}) was administered in combination with L-arginine (200 mg kg^{-1}).

3 Cystometries performed after intra-arterial administration of sodium nitroprusside (SNP) (3 mg kg^{-1}) and 3-morpholino-sydnonimin hydrochloride (SIN-1, 2 mg kg^{-1}) showed a decrease in bladder capacity, micturition volume and threshold pressure. SIN-1, but not SNP, induced spontaneous bladder contractions.

4 Isolated precontracted urethral preparations responded to electrical stimulation with a frequency-dependent tetrodotoxin-sensitive relaxation. L-NAME (10^{-4} M), but not D-NAME, reduced the maximal relaxation to 31 ± 8% ($n = 8$) of the response prior to drug administration. The inhibition induced by L-NAME was completely reversed by L-arginine (10^{-3} M). SNP (10^{-8}–10^{-4} M), SIN-1 (10^{-6}–3×10^{-4} M) and NO (10^{-5}–10^{-3} M; present in acidified solution of $NaNO_2$), caused relaxation (93–100%) of urethral preparations. L-NAME did not affect these relaxations.

5 Detrusor strips contracted by carbachol or K^+ showed contractions in response to electrical stimulation, even when pretreated with α,β-methylene ATP and/or atropine. Small relaxations (14–41%) of detrusor strips were evoked by SNP (10^{-6}–10^{-4} M), SIN-1 (10^{-5}–3×10^{-4} M) and NO (10^{-5}–10^{-3} M). Electrically (20 Hz) induced contractions of the detrusor muscle were unaffected by addition of L-NAME (10^{-6}–10^{-4} M) or L-arginine (10^{-3} M).

6 The present results suggest that the L-arginine/NO pathway is of functional importance for the bladder outlet region, but that its role in the detrusor is questionable. They also suggest that the site of action of L-NAME for inducing bladder hyperactivity in the rat is the outlet region rather than the detrusor muscle.

Keywords: Nitric oxide; rat urinary tract; cystometry; L-N^G-nitro arginine methyl ester

Introduction

Normally, filling of the bladder occurs without any marked changes in intravesical pressure (Coolsaet, 1985). The mechanism is not known, but the behaviour has been attributed mainly to the physical properties of the detrusor muscle (Tang & Ruch, 1955; Klevmark, 1977). In addition, there may be factors which keep the detrusor muscle from being activated. Such a factor may be increased sympathetic activity and release of noradrenaline acting on the β-adrenoceptors of the detrusor (Edvardsen, 1968). However, the importance of β-adrenoceptors in human detrusor relaxation has been questioned (Klevmark, 1977; Nordling, 1983), and is not clarified.

In man, the normal pattern of voiding is characterized by an initial drop in urethral pressure, followed 5 to 15 s later by an increase in intravesical pressure (Tanagho & Miller, 1970; Asmussen & Ulmsten, 1976; Low, 1977; McGuire, 1978; Rud *et al.*, 1978). Urethral pressure variations have also been demonstrated, both in normal females and in patients with certain voiding disturbances (Ulmsten *et al.*, 1977; 1982; Kulseng-Hanssen, 1987; Low *et al.*, 1989). The mechanism(s) behind urethral relaxation and urethral pressure variations has not been clarified.

Thus, there seems to be a physiological role for a relaxation producing system in the outflow region. The involvement of such a system in voiding disturbances, such as those caused by unstable detrusor contractions may be of pathophysiological importance.

It is known that isolated, contracted urethral and trigonal smooth muscles from rabbit, pig, sheep, and man, respond to transmural stimulation of nerves with a relaxant response mediated by a non-adrenergic, non-cholinergic (NANC) mechanism (Andersson *et al.*, 1983; 1991; 1992; Klarskov *et al.*, 1983; Speakman *et al.*, 1988; Garcia-Pascual *et al.*, 1991; Dokita *et al.*, 1991; Persson *et al.*, 1991a; Persson & Andersson, 1992). This relaxant response can be reversed by N^G-L-nitro arginine (L-NOARG), but not D-NOARG, and is enhanced by L-arginine (L-Arg), suggesting involvement of the L-Arg/nitric oxide (NO) pathway (Andersson *et al.*, 1991; 1992; Garcia Pascual *et al.*, 1991; Dokita *et al.*, 1991; Persson *et al.*, 1991a; Persson & Andersson, 1992). The present study was performed to investigate how blockade of the L-Arg/NO pathway by the NO synthase inhibitor L-N^G-nitro arginine methyl ester (L-NAME; Rees *et al.*, 1990) affects micturition in the conscious rat, as reflected by cystometry, and how such blockade affects NANC-nerve-mediated relaxation of rat isolated urethral smooth muscle. A brief account of some of these results has been given previously (Persson *et al.*, 1991b).

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Methods

In vivo *experiments*

Surgical procedures Female Sprague-Dawley rats weighing 180–240 g were anaesthetized with ketamine (75 mg kg^{-1}, i.m.) and xylazine (15 mg kg^{-1}, i.m.). The abdomen was opened through a midline incision and a polyethylene catheter (Clay-Adams PE-50) with a cuff was inserted into the bladder through the dome and held in place with a purse-string suture. The catheter was tunnelled subcutaneously, and an orifice was made at the back of the animal. The abdominal incision was closed and the free end of the catheter sealed. Two days after this operation, the animals were again anaesthetized and a femoral artery was exposed through an inguinal incision and a polyethylene catheter (Clay-Adams PE-10) filled with heparinised saline (30 i.u. ml^{-1}) was inserted into the vessel and advanced proximally until the tip of the catheter reached the abdominal aortic bifurcation. Both femoral arteries were ligated to increase the amount of drug reaching the bladder. After the experiment, the position of the catheter in the abdominal aorta was confirmed in each animal.

Cystometrical investigations Cystometrical investigations were performed the day after insertion of the femoral catheter. No anaesthesia was used. The conscious rat was placed in a metabolic cage which also enabled measurements of micturition volumes by means of a fluid collector connected to a Grass force displacement transducer (FT03C). The bladder catheter was connected via a T-tube to a pressure transducer (Statham P23 DC) and an infusion pump (Microinject, Bioinvent). Saline was infused into the bladder at a rate of 10 ml min^{-1}. Intravesical pressure and micturition volume were recorded continuously on a Grass polygraph. The following urodynamic variables were investigated: basal pressure (the lowest bladder pressure during cystometry), threshold pressure (bladder pressure immediately prior to micturition), micturition pressure (the maximum bladder pressure during micturition), micturition volume (volume of expelled urine), residual volume (volume of infused saline minus micturition volume), spontaneous activity (mean amplitude and frequency of bladder pressure fluctuations during two minutes prior to micturition), and bladder compliance (bladder capacity/(threshold pressure minus basal pressure)). Urodynamic parameters were analysed as described previously (Malmgren *et al.*, 1987) during a 20 min period before and a 10 min period after each intra-arterial administration of drug.

Drugs (see below) were dissolved in 0.9% saline and administered through the intra-arterial catheter followed by a flush of 0.1 ml of heparinised saline for 5 s; 0.2 ml of heparinised saline was injected intra-arterially as control, prior to drug administration.

In vitro *experiments*

Tissue preparations and recording of mechanical activity Female Sprague-Dawley rats (200–250 g) were killed by CO_2 asphyxia, and the bladder together with the urethra were dissected out. Thereafter fat and connective tissue were removed, and the detrusor and the urethra separated by a transverse cut. Circular (intact ring segments, 2 mm wide) or longitudinal (1 × 1 × 5 mm) preparations were taken from the proximal part of the urethra. Detrusor strips (1 × 1 × 5 mm) were dissected from a ring of detrusor tissue comprising the middle third of the bladder. The ring was opened and yielded 2 to 4 preparations. The mucosa was not removed from urethral and detrusor strips.

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 urethral and detrusor 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 wave pulses (duration 0.8 ms) at supramaximal voltage. The train duration was 5 s and the stimulation interval 120 s.

Experimental procedure After the equilibration 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. Relaxant responses to electrical stimulation and to NO (present in acidified solution of $NaNO_2$), SIN-1 (3-morpholino-sydnonimin hydrochloride) and sodium nitroprusside (SNP) were studied in precontracted preparations. Contractions of detrusor preparations were evoked by carbachol 10^{-6}–10^{-5} M, and noradrenaline (NA) 10^{-5} and 3×10^{-5} M was used to induce contractions in the urethra.

In urethral preparations subjected to electrical stimulation, frequency-response (0.5–30 Hz) relations were first studied in the absence of drug treatment. Thereafter L-NAME (10^{-4} M), D-NAME (10^{-4} M) or L-NAME (10^{-4} M) + L-Arg (10^{-3} M) were given at least 15 min before the preparations were once again subjected to electrical stimulation. Control preparations were run in parallel to study the reproducibility of the relaxation. The relaxing effects of NO (10^{-5}–10^{-3} M), SIN-1 (10^{-6}–3×10^{-4} M), and SNP (10^{-8}–10^{-4} M) were studied by cumulative addition. The concentration was increased only after the response to the previous addition had reached a maximal level.

The effect of electrical stimulation on detrusor strips was studied in carbachol- or K^+ (35 mM) contracted preparations. In some experiments, α,β-methylene ATP (10^{-5} M) and/or atropine (10^{-6} M) were included. The effects of L-NAME (10^{-6}–10^{-4} M) and L-Arg (10^{-3} M) on detrusor contractions evoked by electrical stimulation were investigated at a submaximal (70–80% of max) frequency (20 Hz). When the variation between three consecutive contractions was $< 10\%$ the drug investigated was applied cumulatively.

Drugs and solutions

The following drugs were used: (−)-noradrenaline hydrochloride, atropine sulphate, carbamylcholine chloride, isoprenaline, tetrodotoxin, L-N^G-nitro arginine methyl ester (L-NAME), L-arginine hydrochloride, α,β-methylene ATP, (Sigma, USA), N^G-nitro-D-arginine methyl ester (D-NAME), (Bachem, Germany), sodium nitroprusside (Nipride, Roche, 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. Sodium nitroprusside and SIN-1 were kept in dark vessels in order to minimize light-induced degradation. To study the effects of NO, an acidified solution of $NaNO_2$ was used (Furchgott *et al.*, 1988). The $NaNO_2$ was adjusted to pH 2 by addition of HCl. Separate experiments showed that the vehicle had no relaxing effect *per se*.

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 *in vitro* 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 for paired data. A probability level <0.05 was accepted as significant. Results are given as mean values ± s.e.mean. n denotes the number of preparations, and N the number of animals. When the number of preparations and animals are identical, only n is given.

Results

In vivo *experiments*

Cystometrical investigation Repeated cystometries in the same animal gave reproducible and consistent results. No spontaneous bladder contractions were recorded between each micturition (Figure 1). Intra-arterial administration of L-NAME 10 mg kg^{-1} ($n = 6$) and 20 mg kg^{-1} ($n = 11$) induced spontaneous bladder contractions (bladder hyperactivity) and decreased bladder capacity and micturition volume (Figure 1; Table 1). L-NAME, 20 mg kg^{-1} increased the frequency of the spontaneous activity from 0.23 ± 0.12 to 1.6 ± 0.3 min^{-1} ($P<0.01$) and the amplitude from 2.7 ± 1.3 to 5.8 ± 1.3 cmH_2O ($P<0.05$). The bladder capacity and micturition volume decreased from 0.90 ± 0.08 to 0.66 ± 0.08 ml ($P<0.01$) and from 0.88 ± 0.08 to 0.59 ± 0.08 ml ($P<0.001$), respectively. In addition, a decrease ($P<0.01$) in threshold pressure and an increase ($P<0.05$) in residual urine were noted (Table 1). After administration of 10 mg kg^{-1} L-NAME, similar changes in bladder capacity, micturition volume, and in the frequency of the spontaneous activity were observed (Table 1). However, the other parameters did not achieve statistical significance at this dose. The effects of L-NAME were reversible and the cystometrical parameters were restored to the starting level within less than 1 h.

Administration of L-Arg (200 mg kg^{-1}; $n = 8$) 5 min before the injection of L-NAME (20 mg kg^{-1}) antagonized the previously described effects of L-NAME (Figure 2; Table 2). Thus, a tendency toward a decrease in the amplitude and the frequency of the spontaneous activity was found. In the presence of L-Arg, L-NAME was without effect on bladder capacity and micturition volume (Figure 2; Table 2). No significant change in any cystometrical parameter was found after administration of D-NAME (20 mg kg^{-1}; $n = 4$, Table 2).

Cystometries performed after intra-arterial administration of SNP (3 mg kg^{-1}; $n = 6$) showed a decrease in bladder capacity, micturition volume, and threshold pressure. Micturition pressure was reduced in the majority of the rats after administration of SNP, but the decrease was not significant (Table 3). SIN-1 (2 mg kg^{-1}; $n = 7$) reduced bladder capacity, micturition volume, and threshold pressure. SIN-1 also increased both frequency and amplitude of the spontaneous contractions.

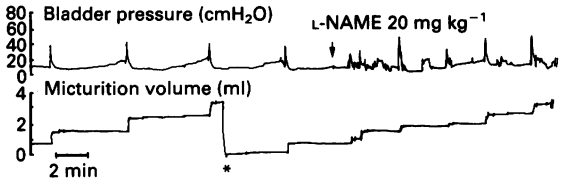

Figure 1 Original recordings of bladder pressure and micturition volume during cystometry before and after intra-arterial administration of L-N^G-nitro arginine methyl ester (L-NAME, 20 mg kg^{-1}) to an unanaesthetized rat. *indicates adjustment to baseline position.

In vitro *experiments*

Urethra All of the longitudinal urethral preparations responded to electrical stimulation with a tetrodotoxin (TTX)-sensitive relaxation. The relaxation of the longitudinal preparations was frequency-dependent. Maximal relaxation was obtained at 16 Hz and averaged 79 ± 3% ($n = 23$, N = 15) of the induced tension (Figure 3). Only 25% ($n = 12$, N = 8) of the urethral preparations taken in the circular direction showed a relaxation when stimulated electrically.

The relaxation induced by electrical stimulation of long-

Table 1 The effects of intra-arterial administration of L-N^G-nitro arginine methyl ester (L-NAME, 10 mg kg^{-1}, $n = 6$ and 20 mg kg^{-1}, $n = 11$) on cystometrical parameters in the rat

	Control	*L-NAME* (10 mg kg^{-1})
Basal pressure (cmH_2O)	10 ± 2	9.3 ± 2.0
Threshold pressure (cmH_2O)	14 ± 2	12 ± 2
Micturition pressure (cmH_2O)	51 ± 10	56 ± 10
Micturition volume (ml)	0.78 ± 0.15	0.53 ± 0.10**
Freq. Spont. activity (min^{-1})	0.32 ± 0.21	1.1 ± 0.3*
Amp. Spont. activity (cmH_2O)	2.2 ± 1.8	11 ± 4
Compliance (ml cmH_2O^{-1})	0.30 ± 0.10	0.39 ± 0.17
Bladder capacity (ml)	0.84 ± 0.17	0.60 ± 0.14**
Residual volume (ml)	0.06 ± 0.03	0.08 ± 0.04
	Control	*L-NAME* (20 mg kg^{-1})
Basal pressure (cmH_2O)	6.5 ± 0.9	6.0 ± 1.0
Threshold pressure (cmH_2O)	13 ± 2	10 ± 1**
Micturition pressure (cmH_2O)	55 ± 5	51 ± 6
Micturition volume (ml)	0.88 ± 0.08	0.59 ± 0.08***
Freq. Spont. activity (min^{-1})	0.23 ± 0.12	1.6 ± 0.3**
Amp. Spont. activity (cmH_2O)	2.7 ± 1.3	5.8 ± 1.3*
Compliance (ml cmH_2O^{-1})	0.17 ± 0.03	0.15 ± 0.02
Bladder capacity (ml)	0.90 ± 0.08	0.66 ± 0.08**
Residual volume (ml)	0.03 ± 0.01	0.08 ± 0.02*

Results are expressed as mean ± s.e.mean.
*$P<0.05$; **$P<0.01$; ***$P<0.001$.

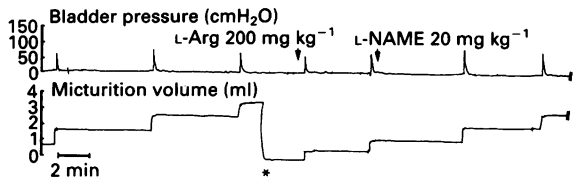

Figure 2 Original recordings of bladder pressure and micturition volume during cystometry before and after intra-arterial administration of L-arginine (L-Arg, 200 mg kg^{-1}) in combination with L-N^G-nitro-arginine methyl ester (L-NAME, 20 mg kg^{-1}) to an unanaesthetized rat. *indicates adjustment to baseline position.

itudinal preparations was reproducible. Thus, the second stimulation averaged 104 ± 6% ($n = 6$) of the maximal response during the first period of stimulation (Figure 4). In longitudinal preparations, exposure to L-NAME (10^{-4} M) significantly ($P < 0.001$) reduced the maximal relaxation to electrical stimulation to 31 ± 8% ($n = 8$) of control (Figures 4 and 5). In five of eight strips, L-NAME caused a further increase in tension (26 ± 6%) when added to the NA-induced contraction (Figure 5). L-NAME did not affect the tension when applied at baseline level. No inhibition of the electrically-induced relaxation was obtained by 10^{-4} M D-NAME ($n = 4$; Figure 4). The inhibition induced by L-NAME (10^{-4} M) was completely reversed by 10^{-3} M L-Arg (Figure 4).

Table 2 The effects of intra-arterial administration of L-arginine (200 mg kg^{-1}) + L-N^G-nitro arginine methyl ester (L-NAME, 20 mg kg^{-1}, $n = 8$) and D-NAME (20 mg kg^{-1}, $n = 4$) on cystometrical parameters in the rat

	Control	*L-Arginine* (200 mg kg^{-1}) + *L-NAME* (20 mg kg^{-1})
Basal pressure (cmH_2O)	6.5 ± 1.5	7.6 ± 2.0
Threshold pressure (cmH_2O)	11 ± 3	10 ± 2
Micturition pressure (cmH_2O)	55 ± 7	54 ± 7
Micturition volume (ml)	0.84 ± 0.08	0.86 ± 0.08
Freq. Spont. activity (min^{-1})	0.21 ± 0.08	0.09 ± 0.07
Amp. Spont. activity (cmH_2O)	1.9 ± 0.8	0.94 ± 0.66
Compliance (ml cmH_2O^{-1})	0.31 ± 0.04	0.37 ± 0.10
Bladder capacity (ml)	0.88 ± 0.08	0.90 ± 0.05
Residual volume (ml)	0.03 ± 0.01	0.04 ± 0.04
	Control	*D-NAME* (20 mg kg^{-1})
Basal pressure (cmH_2O)	6.8 ± 2.8	5.8 ± 2.2
Threshold pressure (cmH_2O)	12 ± 4	11 ± 3
Micturition pressure (cmH_2O)	55 ± 12	54 ± 16
Micturition volume (ml)	0.89 ± 0.17	0.84 ± 0.16
Freq. Spont. activity (min^{-1})	0.78 ± 0.40	0.88 ± 0.26
Amp. Spont. activity (cmH_2O)	4.5 ± 1.9	4.5 ± 0.9
Compliance (ml cmH_2O^{-1})	0.24 ± 0.06	0.23 ± 0.05
Bladder capacity (ml)	0.94 ± 0.16	0.88 ± 0.15
Residual volume (ml)	0.05 ± 0.02	0.03 ± 0.02

Results are expressed as mean ± s.e.mean.

Table 3 The effects of intra-arterial administration of sodium nitroprusside (SNP) (3 mg kg^{-1}, $n = 6$) and 3-morpholino-sydnonimin (SIN-1, 2 mg kg^{-1}, $n = 7$) on cystometrical parameters in the rat

	Control	*SNP* (3 mg kg^{-1})
Basal pressure (cmH_2O)	6.4 ± 1.3	5.2 ± 1.2
Threshold pressure (cmH_2O)	11 ± 1	7.4 ± 0.9***
Micturition pressure (cmH_2O)	49 ± 8	33 ± 6
Micturition volume (ml)	1.0 ± 0.04	0.48 ± 0.11**
Freq. Spont. activity (min^{-1})	0.46 ± 0.19	0.27 ± 0.21
Amp. Spont. activity (cmH_2O)	2.8 ± 1.0	1.7 ± 1.3
Compliance (ml cmH_2O^{-1})	0.26 ± 0.07	0.26 ± 0.08
Bladder capacity (ml)	1.1 ± 0.04	0.59 ± 0.10**
Residual volume (ml)	0.05 ± 0.01	0.12 ± 0.02
	Control	*SIN-1* (2 mg kg^{-1})
Basal pressure (cmH_2O)	5.9 ± 1.1	4.8 ± 1.0
Threshold pressure (cmH_2O)	11 ± 1	8.2 ± 1.3*
Micturition pressure (cmH_2O)	51 ± 5	46 ± 6
Micturition volume (ml)	1.0 ± 0.1	0.60 ± 0.06***
Freq. Spont. activity (min^{-1})	0.19 ± 0.11	0.69 ± 0.15**
Amp. Spont. activity (cmH_2O)	1.9 ± 1.2	5.0 ± 1.0*
Compliance (ml cmH_2O^{-1})	0.29 ± 0.06	0.24 ± 0.05
Bladder capacity (ml)	1.1 ± 0.1	0.70 ± 0.06***
Residual volume (ml)	0.07 ± 0.02	0.09 ± 0.02

Results are expressed as mean ± s.e.mean.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

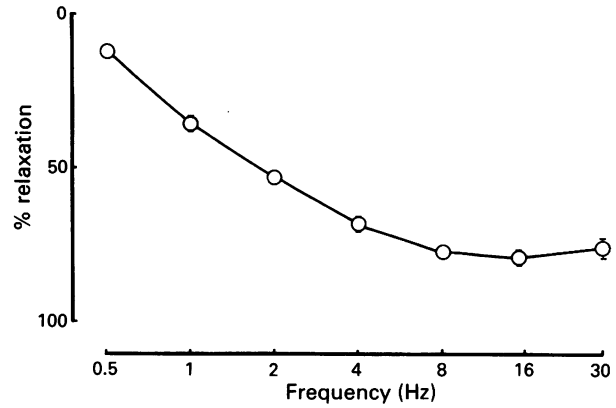

Figure 3 Frequency-response relations for electrically-induced relaxations in rat isolated urethral preparations contracted by noradrenaline (10^{-5}–3×10^{-5} M, $n = 23$, N = 15). Each point is expressed as percentage relaxation of the agonist-induced tension, and represents mean with s.e.mean (vertical bars, where bigger than the symbol).

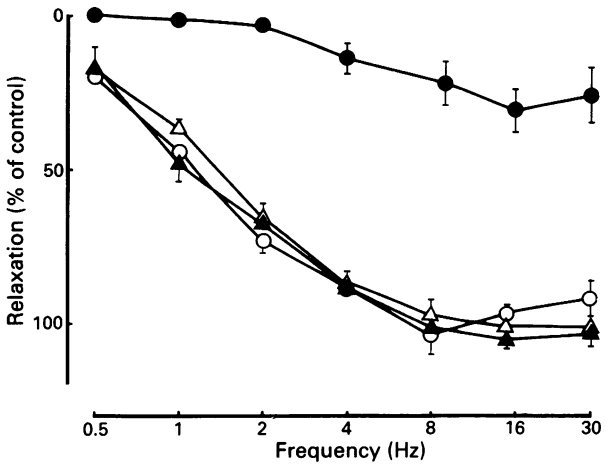

Figure 4 Frequency-response relations for electrically-induced relaxations in rat isolated urethral preparations contracted by noradrenaline (10^{-5}–3×10^{-5} M). Responses were recorded in controls (○) or after pretreatment with L-N^G-nitro-arginine methyl ester (L-NAME) 10^{-4} M (●), D-NAME 10^{-4} M (▲) or L-NAME 10^{-4} M + L-arginine 10^{-3} M (△). Each point is expressed as percentage of the maximal response before treatment, and represents mean ($n = 4$–8); s.e.mean shown by vertical bars.

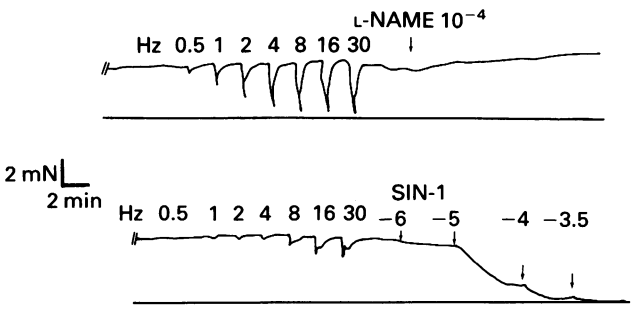

Figure 5 Original tracing showing the effect of pretreatment with L-N^G-nitro-arginine methyl ester (L-NAME, 10^{-4} M) for 15 min on relaxation induced by electrical stimulation in rat isolated urethra. Precontraction above baseline (shown as a solid line) was induced by noradrenaline (3×10^{-5} M). 3-Morpholino-sydnonimin (SIN-1, expressed as log molar concentrations) was applied at the end of the experiment.

SNP, SIN-1 and NO caused concentration-related relaxations of the longitudinal urethral preparations (Figure 6). The maximal relaxation produced by SNP, SIN-1 and NO was 99 ± 1% ($n = 6$), 100 ± 0% ($n = 6$), and 93 ± 4% ($n = 6$) of the NA-induced tension, respectively. Preincubation with L-NAME did not affect the relaxant response to the drugs.

Detrusor The detrusor preparations developed spontaneous, myogenic activity during the equilibration period. SIN-1 (10^{-4} and 3×10^{-4} M) reduced the amplitude of the spontaneous contractile activity in 5 out of 10 strips. L-NAME (10^{-6}–10^{-4} M) had no effect on spontaneous contractions. Detrusor strips precontracted by carbachol (10^{-6}–10^{-5} M; $n = 10$, N = 7) or K^+ (35 mM; $n = 7$, N = 5) showed contractions in response to electrical stimulation (Figure 7). If α,β-methylene ATP (10^{-5} M) and/or atropine (10^{-6} M) were included (in an attempt to suppress the effects of released excitatory transmitters) electrical stimulation of the detrusor still did not cause relaxation. The response to electrical stimulation in the detrusor was not dependent on the direction which the muscle strips were taken, as in the urethra, since it was shown in separate experiments ($n = 4$) that strips taken longitudinally did not behave differently from the standard preparations.

SNP, SIN-1 and NO produced small relaxations of carbachol-contracted detrusor preparations (Figure 8). The maximal relaxation evoked by SNP, SIN-1 and NO averaged 14 ± 5% ($n = 7$), 41 ± 7% ($n = 7$) and 36 ± 5% ($n = 6$) of the carbachol-induced tension, respectively (Figure 8). Isoprenaline (10^{-5} M) was added to some detrusor strips ($n = 6$) to

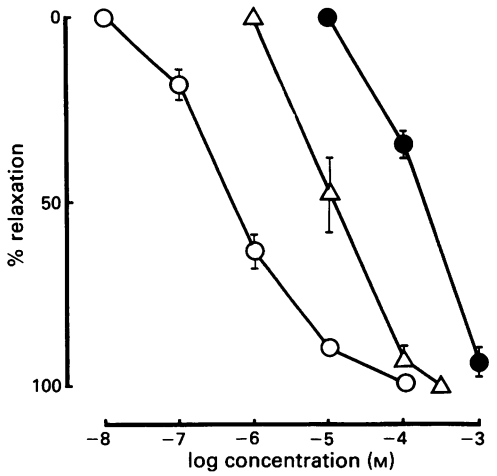

Figure 6 Concentration-response curves obtained by addition of NO (present in acidified solution of $NaNO_2$) (●), 3-morpholino-sydnonimin (△), and sodium nitroprusside (○) to rat isolated urethral preparations contracted by noradrenaline (NA) 10^{-5}–3×10^{-5} M). Each point is expressed as percentage relaxation of the NA-induced tension, and represents mean ($n = 6$) with s.e.mean shown by vertical bars.

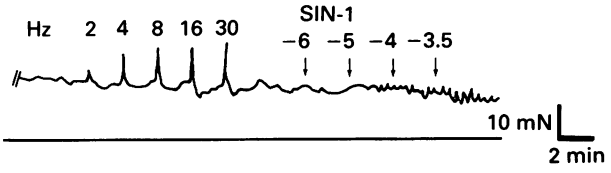

Figure 7 Original tracing showing the response to electrical stimulation in rat isolated detrusor preparations contracted above baseline (shown as a solid line) by 10^{-5} M carbachol. 3-Morpholino-sydnonimin (SIN-1) (expressed as log molar concentrations) was applied at the end of the experiment.

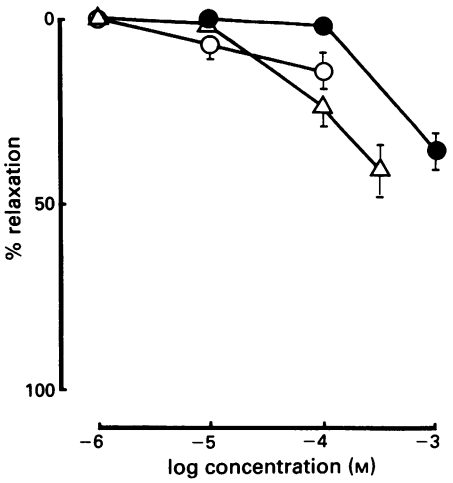

Figure 8 Concentration-response curves obtained by addition of NO (present in acidified solution of $NaNO_2$) (●), 3-morpholino-sydnonimin (Δ), and sodium nitroprusside (○) to rat isolated detrusor preparations contracted by carbachol (10^{-5} M). Each point is expressed as percentage relaxation of the carbachol-induced tension, and represents mean ($n = 6-7$) with s.e.mean shown by vertical bars.

confirm that the smooth muscle was able to relax. The relaxant response produced by isoprenaline was 56 ± 3% of the carbachol-induced tension.

Contractile responses produced by electrical field stimulation (20 Hz) of the detrusor strips were markedly depressed by TTX (10^{-6} M), but about 30–40% of the response persisted after treatment with atropine (10^{-6} M). The electrically-induced contractions were unaffected by addition of L-NAME (10^{-6} M–10^{-4} M; $n = 9$) or L-Arg (10^{-3} M; $n = 8$).

Discussion

The present study shows that inhibition of NO formation from L-Arg in the lower urinary tract of conscious rats stimulates detrusor activity. The regular micturition pattern observed prior to administration of L-NAME was changed into an irregular pattern after injection of the drug, and there was a marked bladder hyperactivity and impairment of bladder capacity. The effects of L-NAME were antagonized by L-Arg, and could not be reproduced by D-NAME, supporting that the effects are mediated by the L-Arg/NO system. It may be speculated that this system has an inhibitory function during bladder filling by acting on the detrusor, as suggested by James *et al.* (1991), or that it inhibits the micturition reflex by an effect on the trigone or the urethra (Persson *et al.*, 1991b; Persson & Andersson, 1992).

In agreement with previous findings in other species, including man (Andersson *et al.*, 1983; 1991; 1992; Klarskov *et al.*, 1983; Dokita *et al.*, 1991; Garcia-Pascual *et al.*, 1991; Persson *et al.*, 1991a; Persson & Andersson, 1992), we observed that the rat isolated urethra exhibits a NANC-nerve mediated relaxation, which can be inhibited by L-NAME. The effects of L-NAME were prevented by L-Arg and could not be reproduced by D-NAME, suggesting that as *in vivo*, the L-Arg/NO system was involved. Interestingly, there was a difference between longitudinal and circular preparations; all the longitudinal, but only 25% of the circular preparations showing relaxation when stimulated electrically. Functional differences between circular and longitudinal orientation of urethral smooth muscle have previously been demonstrated (Persson & Andersson, 1976; Hassouna *et al.*, 1983; Mattiasson *et al.*, 1990). However, the different degrees of relaxation seen in the present study may not be due solely to the orientation of the muscle fibres. Another study of the female rat urethra (Andersson *et al.*, 1990) has shown that the electrically-induced responses of the circularly oriented urethral muscle mainly reflects activation of striated rather than smooth muscle components. The importance of this difference in NANC-mediated relaxation for the function of the rat urethra is, however, not known.

Previous studies have shown that in the rabbit and sheep urethra, the relaxant response is more pronounced at high than at low tension levels (Andersson *et al.*, 1983; Garcia-Pascual *et al.*, 1991), and that spontaneous release of NO seems to occur only at high levels of tension (Andersson *et al.*, 1992). Also in this study, L-NAME increased the tension when applied to precontracted rat urethral strips, but not at baseline level. This suggests that regulation of urethral tension via the L-Arg/NO pathway dominates at high urethral pressures.

Bladder neck relaxation is thought to occur via increased activity in inhibitory nerves (de Groat & Kawatani, 1985). If these inhibitory nerves release NO, direct smooth muscle relaxation should be expected in response to NO and NO-related substances in the bladder outlet region. In fact, such a relaxant effect was evident in the rat urethra after application of NO, SIN-1 and SNP. L-NAME did not affect the relaxant response of the added drugs, i.e. it does not inhibit the smooth muscle effects of NO. It therefore seems reasonable to assume that the functional bladder changes recorded after L-NAME administration during cystometry reflects inhibition of NO synthase, and that NO is an important factor for bladder outlet smooth muscle relaxation. The present results furthermore lend support to the view that unstable detrusor contractions may be initiated from the bladder outlet region (Hindmarsh, 1983, Low *et al.*, 1989, Andersson, 1990). The fact that L-NAME administration causes bladder hyperactivity may be attributed to its ability to inhibit NO-formation in the smooth muscle of the detrusor and urethra. Vasoconstriction in the outlet region can be expected, and the amount of blood in the urethral venous plexus may induce changes in urethral pressure (Rud *et al.*, 1980). Thus, it cannot be excluded that changes in the blood supply may contribute to the observed increase in reflex activity.

In contrast to the findings in the urethra, electrical stimulation of the precontracted rat detrusor in the presence of atropine and after desensitization with α,β-methylene ATP, did not produce relaxation, but further contraction. Such a response has been observed also in pig detrusor (Persson & Andersson, 1992). However, if the pig detrusor preparations were almost maximally contracted, it was possible to induce minor relaxations of both neuronal and non-neuronal nature, which could be inhibited by L-NOARG (Persson & Andersson, 1992). This was not found in the rat detrusor. On the other hand, the experimental conditions in the rat detrusor, with its pronounced excitatory NANC-component, makes relaxation studies complicated. James *et al.* (1991) found that in K^+ (20 mM) contracted preparations of the human detrusor, electrical stimulation evoked relaxations sensitive to L-NOARG, but insensitive to TTX, and they suggested this to be an important factor for bladder relaxation during the filling phase.

The mechanisms behind detrusor relaxation during filling are not fully understood. In the cat, Edvardsen (1968) found an increased sympathetic activity during this phase. Supporting the importance of this finding, Maggi *et al.* (1985) provided evidence for a sympathetic inhibition of the reflex activation of the rat detrusor in response to physiological filling. On the other hand, no influence on the bladder function, at rest or during voiding, could be demonstrated in human patients with various lesions of the sympathetic nervous system (Nordling, 1983). Klevmark (1977) found no support for an inhibitory reflex via the sympathetic nervous system and suggested that accommodation of the bladder to increasing amount of fluid depends almost completely upon the viscoelastic properties of the detrusor muscle. Theoretically, an increased activity of NO-releasing inhibitory nerves to the detrusor, could be one factor keeping the bladder

relaxed during bladder filling. The findings *in vitro* that SNP, SIN-1, and NO were only moderately effective in relaxing the detrusor compared to their effects on the urethra, and that the drugs *in vivo* decreased bladder capacity, micturition volume, and threshold pressure do not favour this view. The increased activity in the micturition reflex seen after administration of SNP and SIN-1, could be ascribed to a drop in urethral pressure, provided that these drugs *in vivo* cause relaxation of urethral smooth muscle as they do *in vitro*. SIN-1 also increased the frequency and amplitude of spontaneous contractions. These effects may seem paradoxical, but can be explained if the drug increases the release of excitatory transmitters from nerves. In fact, such an effect of SIN-1 has been demonstrated in penile erectile tissue (Holmquist *et al.*, unpublished). Thus, NO-mediated relaxation of the detrusor smooth muscle does not seem to be of main functional importance in the rat bladder.

Taken together, the present results suggest that the L-Arg/NO pathway is of functional importance in the bladder outlet region; its role in the detrusor is still unsettled. They also suggest that the site of action of L-NAME for inducing bladder hyperactivity in the rat is the outlet region rather than the detrusor muscle.

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Cardiac α_1-adrenoceptor densities in different mammalian species

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1 α_1-Adrenoceptor densities were studied in cardiac membrane preparations from several mammalian species including human failing hearts under identical experiment conditions; the α_1-adrenoceptor antagonist, [^{3}H]-prazosin, was used as radioligand. End-stage heart failure (NYHA IV) in human hearts was due to idiopathic dilated cardiomyopathy.

2 The ventricular α_1-adrenoceptor densities were not significantly different in guinea-pig, mouse, pig, calf, and man (11 to 18 fmol mg^{-1} protein) but about 5 to 8 fold smaller than in rat (about 90 fmol mg^{-1} protein). Right and left ventricular receptor densities were similar in these species.

3 A sufficient amount of right and left atrial tissue was obtained from rabbit, pig, calf, and man only. The α_1-adrenoceptor densities in both atria of these species were found to be at the detection limit of the method used (less than 8 fmol mg^{-1} protein).

4 The equilibrium dissociation constant (K_D) was similar in all species studied ranging from 0.047 ± 0.006 to 0.063 ± 0.007 nmol l^{-1}.

5 It is concluded that differences in α_1-adrenoceptor density between atria and ventricles may exist in mammalian species. The exceptionally high density of these receptors in rat ventricles seem to be a particular feature in these animals.

Keywords: α_1-Adrenoceptor; mammalian heart; failing human heart; end-stage idiopathic dilated cardiomyopathy; radioligand binding

Introduction

The presence of α_1-adrenoceptors in mammalian hearts has been reported by several authors (see Wagner & Brodde, 1978; Benfey, 1980; 1990; Scholz, 1980; Endoh, 1982). It has been suggested that the α_1-adrenoceptor-mediated response may become important under certain pathological conditions including ischaemia (Penny *et al.*, 1985), abnormal thyroid function (Williams *et al.*, 1977), dysrhythmogenesis (Sheridan *et al.*, 1980) and hypertension (Yamada *et al.*, 1984). α_1-Adrenoceptors may also be involved in other processes such as protein synthesis (Meidell *et al.*, 1986) and cardiac hypertrophy (Simpson, 1990). Radioligand binding studies have been performed on several myocardial preparations (Williams & Lefkowitz, 1978; Rugevics & Schümann, 1987; Bevilacqua *et al.*, 1987; Steinkraus *et al.*, 1989; Shen *et al.*, 1989) revealing intra- and interspecies differences in the density of α_1-adrenoceptors which might partially be due to different experimental conditions.

The present study was designed to compare the densities of cardiac α_1-adrenoceptors and the equilibrium dissociation constants under identical experimental conditions in several mammalian species including the failing human heart.

Methods

Animals and patients

This study was reviewed and approved by the institutional animal care and use committee. Male Wistar rats (200–250 g), guinea-pigs (250–300 g), mice (50–60 g), rabbits (2500–3000 g), pietrain pigs (20–30 kg), and calves (about 400 kg) were killed by a blow on the head (rats, guinea-pigs, mice, rabbits) or captive-bolt pistol (pigs, calves) and bleeding from the carotid arteries. The hearts were quickly removed and dissected free of fat and large vessels. Ventricular and atrial tissues were weighed and removed into ice-cold incubation buffer (50 mmol l^{-1} Tris-HCl, 10 mmol l^{-1} $MgCl_2$, pH 7.5).

Eight failing human hearts were obtained from patients undergoing orthotopic heart transplantation due to end-stage idiopathic dilated cardiomyopathy (NYHA class IV, 3 female, 5 male, age: 53.2 years ranging from 46 to 61 years, left ventricular ejection fraction: 16.9 ± 1.5%, cardiac index: 1.8 ± 0.21 l min^{-1} m^{-2}). Patients were treated with nitrates, diuretics, calcium antagonists, angiotensin converting enzyme (ACE) inhibitors, and cardiac glycosides alone or in combination. Written informed consent was obtained from all patients before cardiac transplantation. The use of tissues obtained from human failing hearts was approved by the Ethical Committee of the University of Hamburg. After explantation the human hearts were transferred immediately to the laboratory in ice-cold aerated bathing solution (modified Tyrode solution) containing (mmol l^{-1}): NaCl 119.8, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 22.6, Na_2EDTA 0.05, ascorbic acid 0.28, glucose 5.0, continuously gassed with 95% O_2 plus 5% CO_2 at 35°C and pH 7.4. Tissue samples were frozen in liquid nitrogen and stored at −80°C until use.

Membrane preparation and binding assay

Crude membrane homogenates of each species were prepared according to the method of Baker & Potter (1980) and Baker *et al.* (1980) with minor modifications. Briefly, the cardiac tissues (700–800 mg) were minced and homogenized in 20

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volumes of incubation buffer with a precooled Ultra-Turrax tissue homogenizer (Janke und Kunkel, Staufen, Germany, 3 × 10 s bursts). The suspensions were diluted with an equal volume of ice-cold 1 mol l^{-1} KCl solution (Baker & Potter, 1980) and left on ice for 10 min before centrifugation at 48,000 *g* for 10 min at 4°C. Pellets were resuspended and homogenized with a glass-Teflon homogenizer (Colora, Lorch, Germany) for 1 min at setting 5 and recentrifuged. This procedure was repeated twice. The final pellets were resuspended in a volume of incubation buffer that adjusted the protein content in incubation assays to 100–200 $\mu g\ ml^{-1}$, passed through 4 layers of gauze and kept on ice under stirring. Assays were carried out in duplicate. Aliquots of 250 μl of the membrane preparations were incubated in a total volume of 1000 μl with the radioligand [^{3}H]-prazosin (250 μl, specific activity 76.6 Ci $mmol^{-1}$, New England Nuclear, Dreieich, Germany) to label myocardial α_1-adrenoceptor binding sites. For saturation binding experiments, nine different concentrations of [^{3}H]-prazosin ranging from 0.01 to 1.0 nmol l^{-1} were used. Specific binding was calculated by subtracting nonspecific binding which was defined in the presence of 5 μmol l^{-1} of the α-adrenoceptor antagonist phentolamine (phentolamine hydrochloride, Ciba Geigy, Basel, Switzerland) from total binding. The binding assays were incubated for 60 min at 25°C allowing complete equilibration of radioligands with the binding sites. Incubation was terminated by rapid vacuum filtration through Whatman GF/C filters (Maidstone, Great Britain) and two 5 ml washes with ice-cold incubation buffer. Filters were dried at room temperature and radioactivity was counted by liquid scintillation spectrometry. The protein content was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Merz und Dade, Düdingen, Switzerland) as standard. All other chemicals were of analytical or best commercial grade available. Deionized and twice distilled water was used throughout.

Statistical evaluations

Values presented are means ± s.e.mean. The maximal number of binding sites (B_{max}) and equilibrium dissociation constant (K_D) were calculated from plots according to Scatchard (1949). Statistical significance was estimated by Student's *t* test for unpaired observations. A P value of less than 0.05 was considered significant.

Results

Typical saturation experiments of [^{3}H]-prazosin binding to cardiac membranes are shown for the rat (Figure 1a) and for the guinea-pig representing the other species investigated (Figure 1b), because ventricular α_1-adrenoceptor densities were not significantly different (about 11 to 18 fmol mg^{-1} protein) in guinea-pig, mouse, rabbit, pig, calf and man (failing human hearts with end-stage idiopathic dilated cardiomyopathy) but markedly smaller than in rat (about 90 fmol mg^{-1} protein, Table 1). Preparations from all the different species were saturable, revealed the characteristics of a single-site interaction as shown by linear Scatchard plots, and had similar equilibrium dissociation constants (K_D Table 2) ranging from $4.7 \times 10^{-2} \pm 0.006$ to $6.3 \times 10^{-2} \times 0.007$ nmol l^{-1}. Right and left ventricular α_1-adrenoceptor densities (Table 1) as well as K_D-values (Table 2) were similar in the species studied. A sufficient amount of right and left atrial tissue was obtained from the species rabbit, pig, calf, and man only. Atria derived from rat, guinea-pig, and mouse were too small for determination with the method used. Right as well as left atrial α_1-adrenoceptor densities in rabbit, pig, calf and man were found to be at the detection limit of less than 8 fmol mg^{-1} protein.

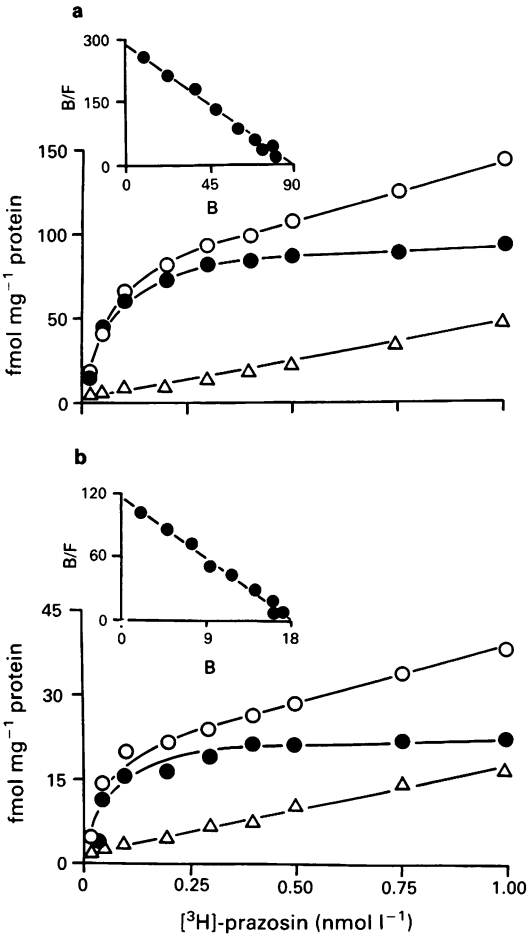

Figure 1 Typical experiments showing [^{3}H]-prazosin binding to ventricular membranes obtained from rat (a) and guinea-pig (b) showing total (○), specific (●) and non-specific (△) binding. Ordinates: [^{3}H]-prazosin specifically bound in fmol mg^{-1} protein. Abscissae: concentration of radioligand in nmol l^{-1}. Inset: linear transformation of a saturation experiment according to Scatchard (1949) as bound (B) to bound per free (B/F).

Discussion

Many contradictory results have been published on α_1-adrenoceptor density in mammalian hearts with different experimental designs (Williams & Lefkowitz, 1978; Bevilacqua *et al.*, 1987; Steinkraus *et al.*, 1989; Shen *et al.*, 1989). Studies comparing these receptors under identical experimental conditions in different species seem to be helpful to obtain insight into species differences. In the present study a marked difference in α_1-adrenoceptor density was found between rat and other species like guinea-pig, mouse, pig, calf and man only. Similar α_1-adrenoceptor densities have been reported by Guicheney & Meyer (1981) in rat, Rugevics & Schümann (1987) in guinea-pig, and Kushida *et al.* (1988) in rabbit hearts. On the other hand, several authors found a larger number of myocardial α_1-adrenoceptors in rat (Han *et al.*, 1989), guinea-pig (Karliner *et al.*, 1979), and rabbit (Mukherjee *et al.*, 1983). Furthermore, contradictory results have been reported for α_1-adrenoceptor density in the human failing hearts obtained from patients with end-stage idiopathic dilated cardiomyopathy. Kaumann (1986) found less than 10 fmol mg^{-1} protein in ventricular preparations. Also a small number of ventricular α_1-adrenoceptors has been reported by Bristow *et al.* (1988) and Böhm *et al.* (1988). In contrast, Vago *et al.* (1989) have shown an approximately 6 fold higher density in a sarcolemma-enriched subcellular fraction. However, it seems likely that different experimental

Table 1 Myocardial α_1-adrenoceptor density as shown by specifically bound (B_{max}) [^{3}H]-prazosin in right and left ventricular myocardium obtained from different mammalian species including failing human hearts (end-stage idiopathic dilated cardiomyopathy)

	α_1-Adrenoceptor density (fmol mg^{-1} protein)		
Species	*Right ventricle*	*Left ventricle*	n
Rat	85.3 ± 4.2*	91.5 ± 4.8*	9
Guinea-pig	15.9 ± 2.2	16.4 ± 2.1	9
Mouse	15.1 ± 1.9 (+)		8
Rabbit	13.3 ± 2.4	12.6 ± 1.8	8
Pig	14.1 ± 2.0	18.4 ± 2.6	7
Calf	15.3 ± 2.1	18.3 ± 2.6	6
Man	10.4 ± 1.7	10.9 ± 1.8	8

(+) Right and left ventricles were not distinguished because of the small size of the hearts. *$P < 0.05$ versus other mammalian species.

Table 2 Equilibrium dissociation constant (K_D) values in right and left ventricular myocardium obtained from different mammalian species including failing human hearts (end-stage idiopathic dilated cardiomyopathy)

	Equilibrium dissociation constant (nmol l^{-1})		
Species	*Right ventricle*	*Left ventricle*	n
Rat	$5.2 \times 10^{-2} \pm 0.007$	$4.7 \times 10^{-2} \pm 0.009$	9
Guinea-pig	$4.8 \times 10^{-2} \pm 0.007$	$5.0 \times 10^{-2} \pm 0.007$	9
Mouse	$5.0 \times 10^{-2} \pm 0.009$ (+)		8
Rabbit	$5.1 \times 10^{-2} \pm 0.006$	$5.4 \times 10^{-2} \pm 0.008$	8
Pig	$4.9 \times 10^{-2} \pm 0.006$	$4.7 \times 10^{-2} \pm 0.006$	7
Calf	$6.3 \times 10^{-2} \pm 0.007$	$5.8 \times 10^{-2} \pm 0.006$	6
Man	$6.0 \times 10^{-2} \pm 0.007$	$6.3 \times 10^{-2} \pm 0.007$	8

(+) Right and left ventricles were not distinguished because of the small size of the hearts.

conditions including different membrane preparation (Vago *et al.*, 1989), incubation temperature (Mukherjee *et al.*, 1983), and radioligand (Bristow *et al.*, 1988) may at least in part explain these discrepancies.

In a recent study, Shen *et al.* (1989) studied α_1-adrenoceptor density and mediated function under identical experimental conditions in different mammalian species demonstrating a much higher α_1-adrenoceptor density and physiological responsiveness to α-adrenoceptor agonists in rat than in baboon, calf and dog. The conclusion was drawn that myocardial contractility is regulated significantly by α_1-adrenoceptor mechanisms in the rat, but not in larger mammalian species studied. However, other studies have shown α-adrenoceptor-mediated inotropic effects in larger animals like dog (Endoh *et al.*, 1978) and calf (Brückner & Scholz, 1984).

Our conclusion is that under identical experimental conditions, differences in α_1-adrenoceptor density may exist between atria and ventricles in mammalian species. The exceptionally high density of α_1-adrenoceptors in rat seem to be a species-dependent feature, and caution should be used in extrapolating findings obtained in the rat to other mammalian species such as man. One possible explanation for this phenomenon could be a difference in the coupling between α_1-adrenoceptor and effector system (phospholipase C) which is influenced by an, as yet, incompletely characterized guanine nucleotide binding protein. Further studies are necessary to obtain more insight into the α_1-adrenoceptor-mediated signal transduction in different mammalian species.

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Effect of nucleotides on the cytosolic free calcium activity and inositol phosphate formation in human glomerular epithelial cells

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1 Glomerular epithelial cells (GEC) were cultured from human kidneys and immunologically characterized.

2 The effect of extracellular nucleotides on the cytosolic free calcium activity $[Ca^{2+}]_i$ was investigated with the fura-2 microfluorescence method. Extracellular UTP, UDP, UMP, ATP, adenosine 5'-O-(3-thio)-trisphosphate (ATP-γ-S), inosine-triphosphate (ITP), guanyltriphosphate (GTP), 2-methylthio-ATP, AMP, α,β-methylene-ATP and adenosine led to a rapid, transient, concentration-dependent increase of $[Ca^{2+}]_i$, followed by a plateau above the baseline level.

3 In a calcium-free extracellular solution, the rapid increase of $[Ca^{2+}]_i$ was still present, whereas the plateau level was abolished.

4 ATP and UTP (ED_{50} both: 10^{-5} M) stimulated inositol trisphosphate ($InsP_3$) formation in GEC.

5 The order of potency for the purine nucleotides in stimulating $InsP_3$ formation was ATP = ATP-γ-S > ADP > 2-methylthio-ATP > AMP = a,β methylene-ATP = adenosine.

6 The increase of $InsP_3$ induced by ATP (10^{-5} M) could be inhibited by the P_2 receptor blocker suramin (> 10^{-4} M). Reactive blue 2 exhibited a weak stimulating effect on the $InsP_3$ formation and only a weak inhibitory effect at a concentration of 10^{-3} M was observed.

7 Protein kinase C activation by preincubation of GEC with phorbol 12-myristate 13-acetate (PMA, 100 ng ml^{-1}, 15 min) abolished the effect of ATP (10^{-5} M) on $InsP_3$ formation. Downregulation of protein kinase C by long term incubation (18 h) with PMA had no significant effect on the phosphoinositol turnover induced by ATP.

8 The results indicate that an increase of $[Ca^{2+}]_i$ and inositol phosphate breakdown can be mediated via activation of a P_2 receptor in human GEC.

Keywords: Human glomerular epithelial cells; free cytosolic calcium activity; inositol trisphosphate; protein kinase C; adenosine 5'-triphosphate; uridine 5'-triphosphate; P_2 receptor

Introduction

Extracellular adenosine nucleotides exert important regulatory functions on many intact cells. Adenosine 5'-triphosphate (ATP) is secreted as an excitatory cotransmitter from perivascular nerves, stimulates secretion from mast cells and platelets, and has contractile or relaxant effects on blood vessels (Burnstock *et al.*, 1970; Burnstock & Kennedy, 1985; Gordon, 1986). It has been shown that vasoconstriction, induced by periarterial nerve stimulation, is primarily due to release of a purinergic transmitter, probably ATP in the rat kidney (Schwartz & Malik, 1989). On the cellular level ATP is known to enhance cytosolic free calcium $[Ca^{2+}]_i$ in established kidney cell lines such as MDCK- and LLC-PK_1 cells (Paulmichl & Lang, 1988; Weinberg *et al.*, 1989). Recently, it has been reported that besides other hormones ATP and uridine 5'-triphosphate (UTP) cause formation of inositol trisphosphate ($InsP_3$) in rat renal mesangial cells (Pfeilschifter, 1990a,b) and that ATP increases $[Ca^{2+}]_i$ in bovine glomerular endothelial cells (Marsden *et al.*, 1990). Little is known about the cellular basis of hormonal actions in human glomerular epithelial cells.

Therefore, the present study was performed to investigate the ability of GEC to respond to nucleotides and to characterize the receptor involved in this response.

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Methods

Human glomerular epithelial cell culture (GEC)

Primary cultures of GEC were established as described by Harper *et al.* (1984) with minor modifications. Glomeruli were isolated from kidneys obtained from patients undergoing tumour nephrectomy. Cortical tissue, far from the tumour zone was stripped of capsule and cut away from the medulla. The cortex was cut into small millimeter square pieces and passed through two steel sieves (Retsch, Haan, Germany) with decreasing pore sizes: 0.045 mm-pore (40 mesh) and 0.180 mm-pore (80 mesh). Glomeruli were collected on a third sieve with a pore size of 0.125 mm (120 mesh). The isolated glomeruli were rinsed in $DMEM/F_{12}$ medium with penicillin, 10^5 u l^{-1} and streptomycin 100 mg l^{-1} (Seromed, Berlin, Germany), incubated with collagenase I (120 u ml^{-1}, Sigma, Deisenhofen, Germany) for 30 min at 37°C and again washed twice in the $DMEM/F_{12}$ medium. After washing and subsequent sedimentation cycles, no particles apart from glomeruli could be detected. The glomeruli were counted under a light microscope (400 ×); encapsulated glomeruli were not observed visually. They were suspended in $DMEM/F_{12}$ culture medium, supplemented with 10% heat-inactivated foetal calf serum, glutamine 2.5 mM, sodium pyruvate 0.1 mM, HEPES buffer 5 mM, penicillin 10^5 u l^{-1}, streptomycin 100 mg l^{-1}, non-essential amino acids (100 ×) 0.1 × (all Seromed, Berlin, Germany) and insulin-transferrin-sodium selenite supplement 5 mg l^{-1} (Boehringer, Mannheim, Germany) and plated at a concentration of 10^2 glomeruli

cm^{-2} onto collagen gel coated Petri dishes (Greiner, Nürtingen, Germany). The collagen gel-coated tissue culture dishes were prepared as follows: the acid soluble collagen was mixed 8:1:1 with 10 × RPMI-1640 medium and 0.1 N NaOH (all Seromed). The glomeruli were incubated at 37°C in a moist atmosphere containing 5% CO_2 in air. After seven days, cell colonies began to sprout around the glomeruli. Cell colonies which showed the typical morphological characteristics of GEC (polygonal shape, 'cobblestone-appearance') were excised with a fine scalpel blade and incubated in a tube containing 5 ml 0.2% collagenase IV (Sigma, Deisenhofen, Germany) at 37°C for 30 min. After the collagen was solubilized the cells were washed twice and plated in 25 cm^2 culture flasks (Greiner, Nürtingen, Germany). Fibroblast contamination was excluded by growing the cell for two weeks in an L-valine free medium according to Gilbert & Migeon (1975).

Subcultures of GEC Confluent cell layers were removed with trypsin-EDTA 0.05%/0.02% in Ca^{2+}/Mg^{2+}-free PBS (Seromed, Berlin, Germany). The cells were resuspended and washed in the medium described above and seeded into tissue culture flasks, 25 cm^2 or 75 cm^2 (Greiner, Nürtingen, Germany). The subcultured cells reached confluency after four to six days. Experiments were done with cells between passages 3 and 20.

Cell characterization

Morphological studies GEC in culture could be identified by phase-contrast microscopy. They showed an epithelial morphology, a polyhedral shape and a cobblestone like appearance when confluency was reached (Kreisberg & Karnovsky, 1983).

Immunochemical studies Immunochemical characterization was performed by the peroxidase antiperoxidase (PAP) method according to Bross *et al.* (1970). Briefly, confluent cell layers were fixed with methanol at − 20°C. Cells were preincubated for 30 min with a PBS buffer containing gelatin 5% and serum albumin 22% to block nonspecific protein binding. Incubation for 30 min with antibodies to human antigens (mouse anti-human IgG) was followed by 'sandwich'-antibodies incubated for 15 min each: rabbit anti-mouse IgG, pig anti-rabbit IgG, and finally PAP-IgG established in rabbits (all Dakopatts, Hamburg, Germany). As a substrate of the PAP-complex 3'3-diaminobenzidine-tetrahydrochloride was used for staining. After staining the immunocomplex with diaminobenzidine, PAP-positive cells could be detected by light microscopy by their brown granules. Cells not incubated with the primary antibody failed to show any staining excluding nonspecific reactions. Antibodies to myosin and factor VIII did not stain, thus excluding mesangial or endothelial cells. Cells were homogeneously positive for vimentin. Therefore, GEC showed the same morphological and immunological characteristics as described recently (Torbohm *et al.*, 1990; Gröne *et al.*, 1990).

Fura-2 microfluorimetry

Fura-2 measurements were performed according to the method described by Malgaroli *et al.* (1987). Confluent cell layers were trypsinized as described above, washed and incubated for 40 min with 1 μM fura-2-AM (Sigma, Deisenhofen, Germany) in Hanks balanced salt solution (1 μM $CaCl_2$) supplemented with 0.5% albumin, 5 mM HEPES buffer and 5 mM D-glucose, pH 7.4 at 37°C (all Sigma). Cells were then washed with Hanks balanced salt solution with 5 mM HEPES and 5 mM D-glucose. Staining with trypan blue revealed information on cellular integrity and proved >98% of the cells were intact. Cells (7.5×10^5) were preincubated in a cuvette with a volume of 1.5 ml for 3 min. The cuvette was thermostated at 37°C and equipped with a magnetic stirring device. The cell suspensions loaded with fura-2 were analysed in a spectroluminescence spectrometer (Perkin-Elmer, Beaconsfield, GB). Excitation light was set with two excitation monochromators and a dual mirror chopping mechanism that allowed a rapid (10 Hz) alternating excitation of fura-2 at two wavelengths. Excitation wavelengths were set at 339 nm (peak excitation) and 364 nm (isosbestic point) and the emission wavelength at 509 nm. To ensure that the isosbestic point was valid, a scanning either in the calcium-free or the calcium-bound form of the fura-2 dye prior to each series of experiments was performed. The experiment was started by observing the base signal for 30 s. Agonists dissolved in a volume of 15 μl in Hanks balanced solution were then pipetted into the cell suspension. The experiment was observed over a time period of 3–5 min. After each experiment a calibration was performed: to obtain maximum (R_{max}) and minimum (R_{min}) fluorescence signals, cell lysis was performed with Triton X (final concentration: 0.1%) followed by the addition of EGTA 4 mM without calcium and Tris buffer 15 mM, pH 8.8, suggesting a free calcium activity of 10^{-9} M. $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz *et al.* (1985).

Measurement of phosphoinositide turnover

Cell labelling GEC were seeded in 35 mm dishes (Greiner, Nürtingen, Germany) at a density of 5×10^5 cells/dish and labelled for 72 h with *myo*-[^{3}H]-inositol (10 μCi ml^{-1}, 80 Ci mM^{-1}, Amersham Buchler, Braunschweig, Germany) in inositol-free RPMI-1640, containing 2% foetal calf serum.

Inositol phosphate extraction and separation After the labelling incubation the medium was removed and the cells were rinsed several times to remove free [^{3}H]-inositol followed by pretreatment with fresh medium containing 10 mM LiCl for 1 h at 37°C. The cells were stimulated with the different nucleotides in the presence of 10 mM LiCl. The reaction was terminated after the indicated time period by rapid aspiration of the medium and addition of ice-cold methyl alcohol (MeOH) and 0.12 N HCl (1:1) to extract the aqueous phase containing the water soluble inositol phosphates as recently described by Johnson *et al.* (1990). The dishes were put on ice for 1 h, 1.5 ml of the MeOH-HCl solution was transferred to tubes containing 6 ml H_2O, mixed and neutralized with 75 ml Tris buffer, 1.5 M. The entire volume of 7.5 ml was applied to anion exchange columns containing 1 ml of Dowex 1-X8 (200–400 mesh, formate form, Biorad, München, Germany). Free inositol and the inositol phosphates were eluted sequentially according to Berridge *et al.* (1984). The different phosphates were eluted with 16 ml each of 5 mM borax, 60 mM Na-formate for glycerophosphoinositol, 0.2 M HN_4-formate, 0.1 M acetate for $InsP_1$, 0.4 M NH_4-formate, 0.1 M acetate for $InsP_2$, and 0.8 M NH_4-formate, 0.1 M acetate for $InsP_3$ and $InsP_4$, respectively. Free inositol appeared at the beginning by rinsing the column with 30 ml H_2O. The first 8 ml of each eluated fraction (containing >80% of the amount of radioactivity) was counted in a liquid scintillation counter (Packard Instrument, Groningen, Netherlands). The peaks of $InsP_1$, $InsP_2$, and $InsP_3$ were identified with the elution profile of pure standards (Amersham Buchler, Braunschweig, Germany). Identical results were obtained by comparing this extraction procedure with the trichloroacetic acid (TCA)-ether extraction.

The following drugs were used: adenosine, adenosine 5'-monophosphate sodium salt (AMP), adenosine 5'-diphosphate sodium salt (ADP), adenosine 5'-triphosphate sodium salt (ATP), guanosine 5'-triphosphate sodium salt (GTP), α,β-methylene-ATP lithium salt, inosine 5'-triphosphate sodium salt (ITP), uridine 5'-monophosphate sodium salt (UMP), uridine 5'-diphosphate sodium salt (UDP), uridine 5'-triphosphate sodium salt (UTP), theophylline, reactive blue 2, phorbol 12 myristate 13-acetate, 4α phorbol 12,13-

didecanoate (all Sigma, Deisenhofen, Germany), ATP-γ-S tetrasodium salt (Boehringer, Mannheim, Germany), 2-methylthio-ATP tetrasodium salt, (Biotrend, Köln, Germany), suramin sodium salt (Bayer, Leverkusen, Germany).

Statistics

Data are given as arithmetic means ± standard error of the mean (s.e.mean). Statistical analysis was made by Student's *t* test and a *P* value of less than 0.05 was used as the criterion for statistical significance.

Results

Effect of purine and pyrimidine nucleotides on intracellular calcium in GEC

In the absence of extracellular nucleotides, $[Ca^{2+}]_i$ was 110 ± 1.4 nM ($n = 221$). Administration of ATP led to a rapid and transient increase of $[Ca^{2+}]_i$ followed by a period of elevation above baseline values (plateau). Figure 1a shows original traces of experiments with ATP (10^{-8}–10^{-4} M). The response to ATP was dose-dependent. Figure 2 summarizes the original data of the purine nucleotides examined. Like ATP, 2-methythio-ATP, α,β-methylene-ATP, ADP, AMP and adenosine increased the $[Ca^{2+}]_i$ in GEC. In comparison to ATP, ADP > 2-methylthio-ATP > α,β-methylene-ATP = AMP = adenosine were less effective. ATP-γ-S, a non-hydrolyzable form of ATP, induced the same $[Ca^{2+}]_i$ increase as ATP (data not shown). Preincubation with theophylline (10^{-4} M, $n = 5$) for 30 min did not inhibit the effect of adenosine. Preincubation with α,β-methylene ATP (10^{-4} M, $n = 5$) for 30 min did not abolish the effect of ATP (10^{-5} M). Not only adenosine nucleotides but also ITP and GTP increased the $[Ca^{2+}]_i$ in human GEC. ITP at lower concentrations was less active in comparison to ATP (Figure 2), the dose-response curve for GTP was similar to that for ADP (data not shown).

The pyrimidine nucleotide UTP increased the $[Ca^{2+}]_i$ in GEC dose-dependently. A transient peak value of $[Ca^{2+}]_i$ was followed by a plateau period. Figure 2 demonstrates the dose-response curves for the pyrimidine nucleotides.

Effect of ATP and UTP in the absence of extracellular calcium

To demonstrate whether ATP or UTP induced an increase of $[Ca^{2+}]_i$ by recruiting calcium from the intracellular or the extracellular space, ATP and UTP (both 10^{-5} M) were added to GEC in the absence of extracellular calcium. Again, $[Ca^{2+}]_i$ rose transiently to a peak value after addition of ATP (70 ± 2.3 mM versus 140 ± 3.4 mM, $n = 5$) and UTP (76 ± 1 versus 141 ± 1.0 mM, $n = 5$). The plateau phase was abolished in the absence of extracellular free calcium. Figure 1b shows an original recording of the response of human GEC to ATP, (10^{-5} M) in the absence of extracellular calcium.

ATP stimulation of phosphoinositol turnover in GEC

Addition of ATP or UTP to attached GEC prelabelled with [^{3}H]-inositol induced a time- and dose-dependent increase in formation of $InsP_3$, indicating stimulation of phospholipase C. Figure 3 shows the time course of the formation of $InsP_3$, $InsP_2$, and InsP. Stimulation with ATP (10^{-5} M) caused an $InsP_3$ (+ 44%) and $InsP_2$ (+ 123%) peak after 5 min, the plateau level of InsP (+ 513%) was reached after 1 h in the presence of 10 mM LiCl. ATP induced a dose-dependent elevation of $InsP_3$, $InsP_2$ and InsP as shown in Figure 4. When GEC were stimulated with ATP for 5 min a 10^{-6} M threshold concentration was required to elicit a phosphoinositol stimulation, and half-maximal responses were observed at 10^{-5} M ATP ($InsP_3$: + 1,024 ± 60 c.p.m., $InsP_2$: + 2,226 ± 114 c.p.m., InsP: + 14,558 ± 423 c.p.m., all $n = 5$, $P < 0.05$). Preincubation with α,β-methylene-ATP (10^{-4} M) for 30 min did not inhibit the effect of ATP (10^{-4} M) on the $InsP_3$ formation.

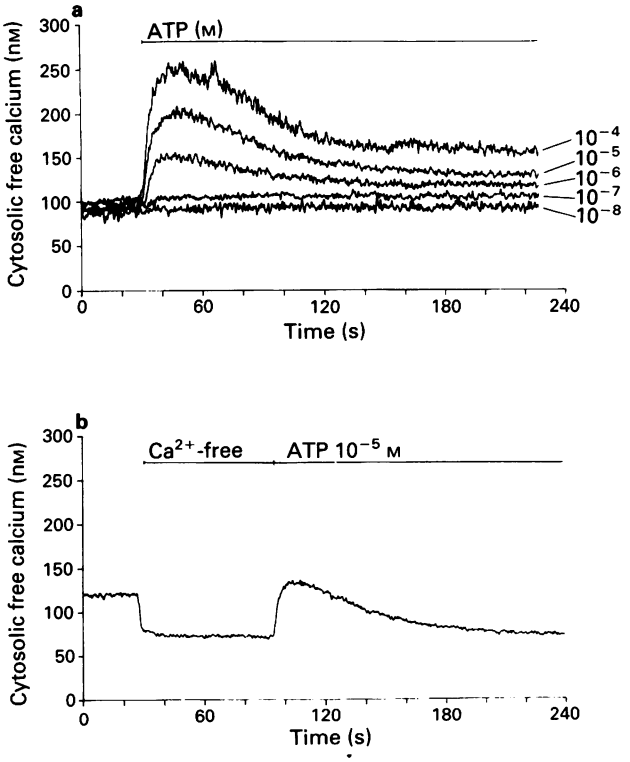

Figure 1 (a) Original recording showing the effect of ATP on $[Ca^{2+}]_i$ of fura-2 loaded human GEC. (b) Original recording showing the effect of ATP (10^{-5} M) on $[Ca^{2+}]_i$ in the nominal absence of extracellular calcium (buffered with 4 mM EGTA).

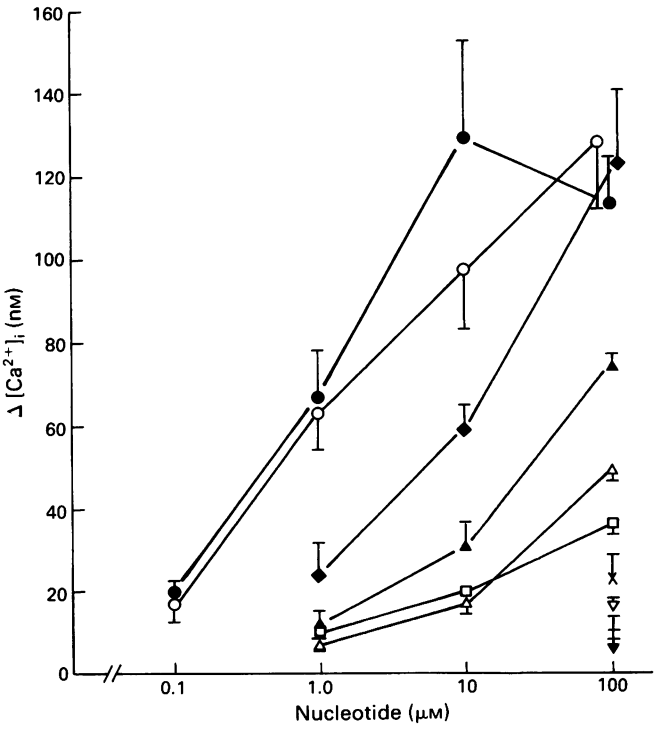

Figure 2 Dose-response curves for the $[Ca^{2+}]_i$ increase in GEC stimulated by ATP (○), ITP (◆), UTP (●), UDP (▲), ADP (△), 2-methylthio-ATP (□), α,β-methylene-ATP (X), AMP (▽), adenosine (+) and UMP (▼). Data are means of 5 experiments with s.e.mean shown by vertical bars. All results are statistically different ($P < 0.05$) from control.

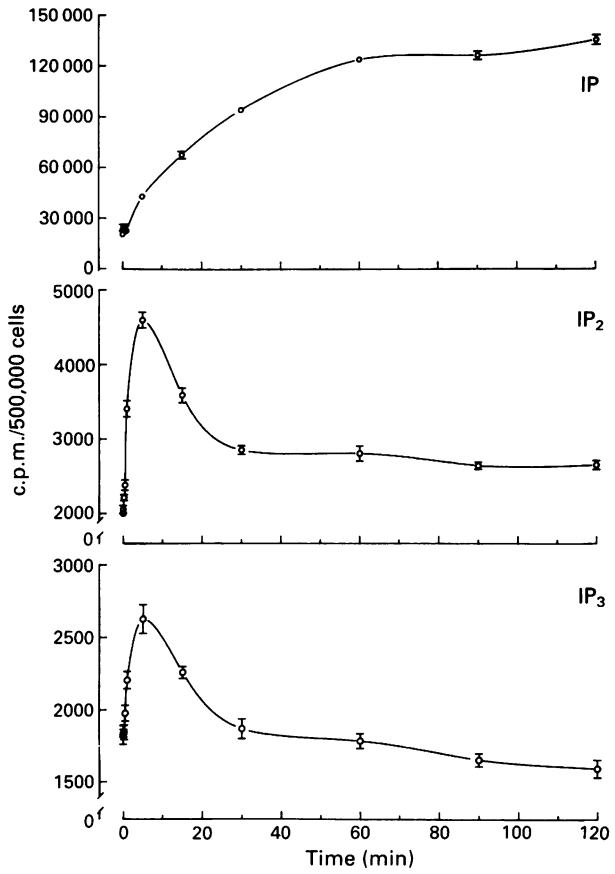

Figure 3 Time course of the effect of ATP (10^{-5} M) on intracellular levels of $InsP_3$, $InsP_2$ and InsP in human GEC. Cells were prelabelled with *myo*-[^{3}H]-inositol and stimulated with ATP for the indicated time. For symbols without error bars the s.e.mean was less than symbol size. Data are means ± s.e.mean of 5 determinations in a single representative experiment of three.

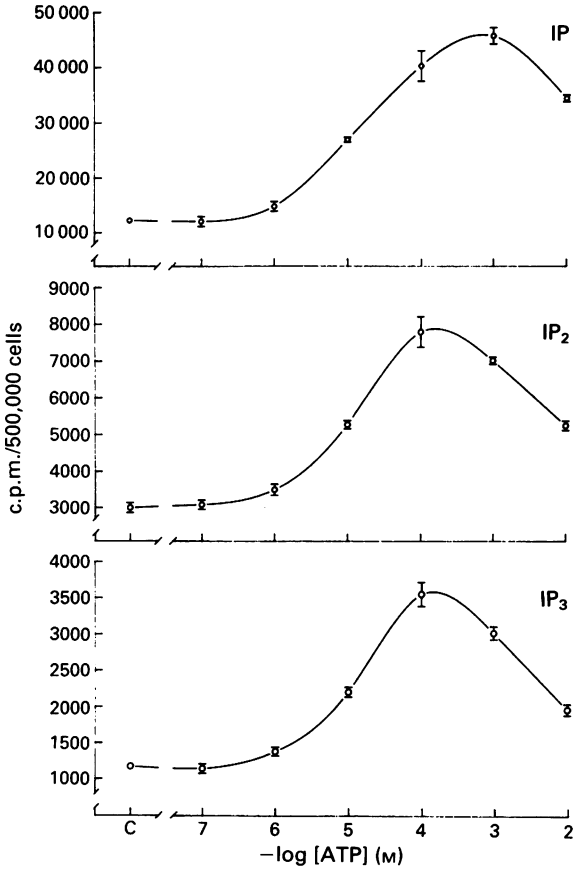

Figure 4 Dose-dependence of ATP-induced formation of $InsP_3$, $InsP_2$ and InsP in human glomerular cells. Cells were prelabelled with *myo*-[^{3}H]-inositol and stimulated with the indicated concentrations of ATP for 5 min. Data are mean values of 5 determinations in a single representative experiment of three; s.e.mean shown by vertical bars.

Figure 5 shows the rank order of potency of the purine agonists (all 10^{-5} M) in stimulating $InsP_3$ formation. The rank order was ATP-γ-S (+ 1,713 ± 80 c.p.m.) = ATP (+ 1,699 ± 65 c.p.m.) > ADP (+ 1,300 ± 63 c.p.m.) > 2-methylthio ATP (+ 557 ± 72 c.p.m.) > AMP (+ 398 ± 63 c.p.m.) = α,β methylene ATP (+ 389 ± 53 c.p.m.) = adenosine (+ 352 ± 55 c.p.m., $n = 5$ all $P < 0.05$). Like ATP, UTP was able to stimulate phosphoinositol turnover (threshold concentration: 10^{-6} M, half-maximal response: 10^{-5} M).

Effect of P_2 receptor antagonists

Preincubation with the P_2-receptor antagonist, suramin, for 30 min inhibited the effect of ATP (10^{-5} M) on the $InsP_3$ turnover (Figure 6a). Reactive blue 2 had a weak, but significant stimulating effect on the $InsP_3$ turnover even at a concentration of 10^{-6} M (+ 186 ± 46 c.p.m.). A significant inhibitory effect of reactive blue 2 (preincubation time: 30 min) on the ATP-induced phosphoinositol turnover was only seen at a concentration of 10^{-3} M. At low concentrations (10^{-6} M and 10^{-5} M) reactive blue 2 even lead to an augmentation of the ATP-dependent stimulation (Figure 6b).

Effect of phorbol ester on the ATP-induced phosphoinositol turnover

Short term preincubation with PMA (100 ng ml^{-1}) for 15 min decreased the $InsP_3$ turnover from 2,325 ± 195 to

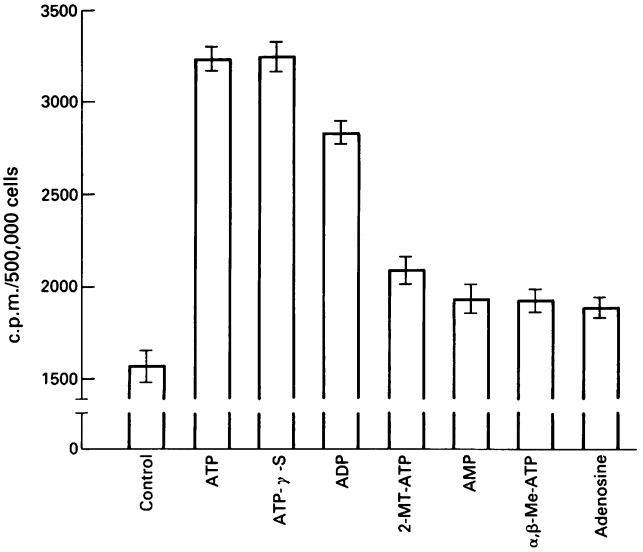

Figure 5 Effect on the $InsP_3$ formation in GEC induced by ATP, ATP-γ-S, ADP, 2-methylthio-ATP (2-MT-ATP), AMP, α,β-methylene-ATP (α,β-Me-ATP), adenosine. Cells were exposed to the agonists (all 10^{-4} M) for 5 min. Data are mean values ± s.e.mean (vertical bars) of 5 determinations in a single representative experiment of three. All results are significantly different ($P < 0.05$) from controls.

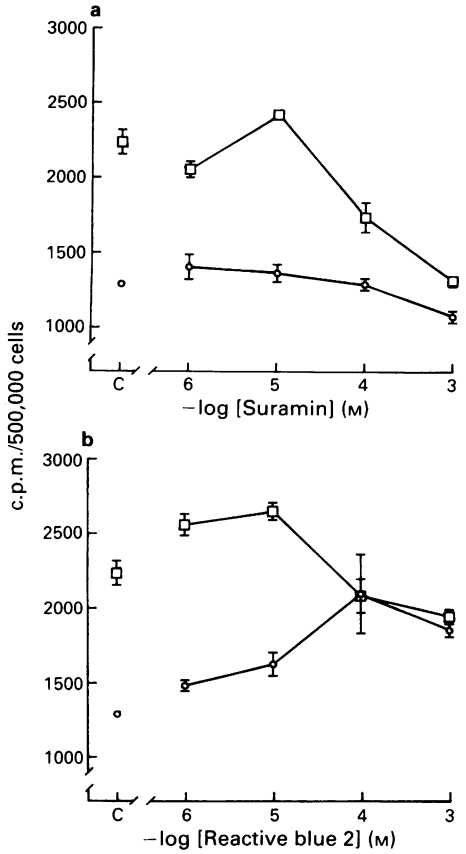

Figure 6 Effect of suramin (a) and reactive blue 2 (b) on the ATP-stimulated $InsP_3$ turnover in human GEC. Cells were pretreated for 30 min with reactive blue 2 or suramin and then stimulated with the putative antagonist or ATP, 10^{-5} M: (○) antagonist; (□) ATP + antagonist. Data are means of 5 determinations in one representative experiment of three; vertical bars show s.e.mean. C denotes controls.

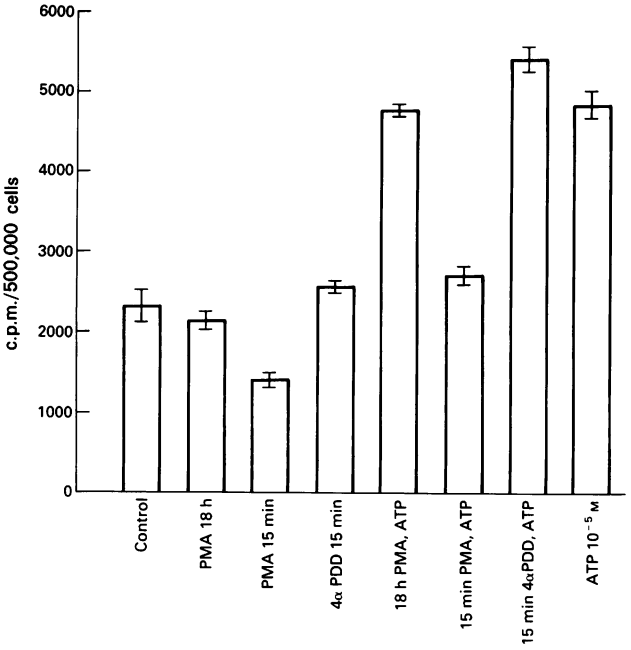

Figure 7 Effect of phorbol esters on ATP-stimulated $InsP_3$ formation. Cells were pretreated with PMA (short term, PMA or 4αPDD, 15 min; long term, PMA 18 h) and then stimulated with ATP, 10^{-5} M. Data are means with s.e.mean (vertical bars) for 5 determinations in one representative experiment of three. For abbreviations, see text.

1,408 ± 87 c.p.m. significantly and inhibited the ATP (10^{-5} M)-induced $InsP_3$ formation. Long term preincubation with PMA for 18 hours did not decrease $InsP_3$ turnover and did not reduce the ATP-induced $InsP_3$ formation significantly. The biologically inactive 4α-phorbol 12,13-didecanoate had no effect on ATP-stimulated $InsP_3$ formation (Figure 7).

Discussion

Effect of ATP on cytosolic free calcium in GEC

ATP is secreted from synaptic vesicles as a co-transmitter of the sympathetic nervous system and from purinergic nerves in the autonomous nervous system (Burnstock *et al.*, 1970). In the kidney ATP (i) influences renal haemodynamics via vasoconstriction that should be induced by direct or reflex activation of renal nerves (Dworkin *et al.*, 1984), (ii) may play a role in the regulation of glomerular filtration rate by activating phospholipase C in contractile mesangial cells (Pfeilschifter, 1989a), (iii) can induce the release of endothelium-derived relaxing factor (EDRF) from glomerular endothelial cells (Marsden *et al.*, 1990) and (iv) is able to induce chloride secretion in MDCK cells (Simmons, 1981). The data demonstrate that extracellular ATP induces a second messenger response via a P_2 receptor in GEC. Although the source of extracellular ATP is difficult to determine it is known that extracellular ATP is released from endothelial cells or platelets (Gordon, 1986). The possible function of the effect of ATP on GEC is obscure. Recently it has been demonstrated by Hulstaert *et al.* (1991) that nucleoside polyphosphatases are present in plasma membranes of rat GEC. The authors speculate that the activity of the nucleoside polyphosphatases may inhibit cytotoxic effects induced by ATP. Therefore, extracellular ATP might play an important physiological and pathophysiological role for GEC.

ATP is able to increase $[Ca^{2+}]_i$ in GEC. ATP seems to be capable of releasing calcium from intracellular stores. The observation of a biphasic pattern of $[Ca^{2+}]_i$ evident as a transient peak followed by a plateau phase, which was abolished in the absence of extracellular calcium, underlines this hypothesis. On the other hand, the sustained effect of ATP which could be abolished by removal of extracellular calcium, points to the ability of ATP to recruit calcium from the extracellular space. A calcium response to ATP has been reported for a variety of cell types including hepatocytes (Charest *et al.*, 1985). Ehrlich ascites tumour cells (Dubyak, 1985), colon carcinoma cells (Leipziger *et al.*, 1991), MDCK cells (Paulmichl & Lang, 1988), LLC-PK_1 cells (Weinberg *et al.*, 1989), rat aortic myocytes (Phaneuf *et al.*, 1987), mouse peritoneal macrophages (Pfeilschifter *et al.*, 1989), aortic endothelial (Lückhoff & Busse, 1986) and bovine glomerular endothelial cells (Marsden *et al.*, 1990).

ATP stimulates phosphoinositol turnover in GEC

It is well known that a sudden rise of intracellular calcium can be an event concomitant to $InsP_3$ accumulation (Berridge, 1984). The role of the agonist-stimulated phosphatidylinositol turnover in the glomerulus has been mostly examined in mesangial cells (Mene *et al.*, 1990). Recently, Pfeilschifter (1990a) has reported an ATP-induced phospholipase C activation in the same cell type. In rat GEC, Cybulsky *et al.* (1990) demonstrated that the extracellular matrix is able to regulate proliferation and phospholipid turnover. The experiments indicate, that ATP stimulated phosphoinositol turnover is involved in the increase of cytosolic calcium and is a possible transduction pathway that seems also to play a role in human GEC.

Characteristics of the receptor for purine nucleotides

Since specific antagonists against purine receptors are not yet available, the classification of the receptors has been performed by use of synthetic analogues of ATP. Consequently the relative potency of a range of purines was used as a preliminary classification of purinoceptors (Kennedy, 1990). In GEC the potency sequence of purine nucleotides in increasing $[Ca^{2+}]_i$ was ATP > ITP > GTP = ADP > 2-methylthio ATP > α,β-methylene-ATP = AMP = adenosine (Figure 2). α,β-Methylene-ATP was not able to desensitize the receptor. The effect of adenosine could not be abolished by theophylline, indicating that the response to adenosine is not mediated via a P_1 receptor. ATP-γ-S, a non-hydrolyzable form of ATP was able to induce the same response like ATP, indicating that probably no phosphorylation of membrane receptor proteins occurred. The measurement of $[Ca^{2+}]_i$ in cells in suspension is an established method which has also been used to characterize the ATP response and the receptor in PC 12 cells (Fasolato *et al.*, 1990). To examine whether the characterization of the receptor by the underlying method could have influenced the results, the rank order of the purine nucleotides on the stimulation of $InsP_3$ in resident GEC was tested. Again we observed that ATP = ATP-γ-S > ADP > 2-methylthio-ATP = α,β-methylene ATP = adenosine could stimulate $InsP_3$ turnover in the same rank order of potency (Figure 5). Furthermore we investigated whether suramin, a P_{2x} and P_{2y} receptor blocker or reactive blue 2, a P_{2y} receptor blocker (for review see Kennedy, 1990) abolished the response to ATP. To avoid quenching problems with these agents during the fura-2 measurements, we tested whether they were able to abolish the ATP-induced increase of $InsP_3$ in GEC. Suramin was able to block the ATP-induced $InsP_3$ formation at a concentration $> 10^{-4}$ M (Figure 6a), whereas reactive blue 2 had only a weak inhibitory effect at 10^{-3} M (Figure 6b). However, reactive blue 2 itself had a stimulating effect on the $InsP_3$ turnover and even augmented the $InsP_3$ stimulating effect of ATP at low concentrations (Figure 6b). A tendency for an increase in the vasoconstrictor response to ATP in the presence of reactive blue 2 has been reported in the rabbit basilar artery (von Kügelgen & Starke, 1990). An intrinsic $[Ca^{2+}]_i$ increasing effect of reactive blue in a colon carcinoma cell line was recently reported by Leipziger *et al.* (1991). The effects of reactive blue 2 on the cellular level may be an explanation for the contradictory effects seen when trying to antagonize the effect of ATP with this dye (Kennedy, 1990). The data indicate that the receptor involved in the ATP response is a P_2 receptor. This receptor seems to be different from the P_{2y}, P_{2x} (Burnstock & Kennedy, 1985), P_{2Z}, P_{2T} (Gordon, 1986) or P_{2S} receptor (Wiklund & Gustafsson, 1988) as well as from the recently described purine receptor in rat mesangial cells. In these cells 2-methylthio ATP was more potent than ATP. Adenosine and GTP had no effect in increasing $InsP_3$ and reactive blue 2 could inhibit the ATP response (Pfeilschifter, 1990a,b). The purine receptor in GEC shows similarities to recently described P_2 receptors of adrenal medulla cells (Allsup & Boarder, 1990), pituitary cells (Davidson *et al.*, 1990) and human fibroblasts (Fine *et al.*, 1989). Furthermore, a similar P_2 receptor was characterized in the rabbit basilar artery (von Kügelgen & Starke, 1990). In this vessel the rank order of potency of the purine- and pyrimidine nucleotides was with little differences (ATP-γ-S was more potent than ATP, 2-methylthio-ATP and α,β-methylene-ATP were equipotent) similar to that found in human GEC. Additionally, α,β-methylene ATP was also unable to desensitize the P_2 receptor and reactive blue was not able to abolish the vasoconstriction induced by ATP in the rabbit basilar artery. In summary, the results demonstrate that the P_2 receptor in GEC is neither a P_{2x} nor a P_{2y} receptor. Because the rank order of potency of the P_2 receptor found in GEC is similar to the recently proposed nucleotide receptor (O'Connor *et al.*, 1991) GEC might represent a receptor of that type.

Effect of pyrimide nucleotides in GEC

It is known that extracellular UTP, like ATP, is an effective activator of cell functions. In some cells pyrimidines activate a receptor that is distinguished from the P_2 receptor (for review see Seifert & Schultz, 1989; Häussinger *et al.*, 1987; von Kügelgen *et al.*, 1987). On the other hand, in rat mesangial cells there is no evidence that UTP or ATP act on different receptors (Pfeilschifter, 1990b). The aim of the experimental series with pyrimidine nucleotides was not to distinguish a possible pyrimidine receptor from the identified P_2 receptor but to investigate whether pyrimidines are also able to induce a transduction response to GEC. Similar to purine nucleotides, pyrimidines were able to increase $[Ca^{2+}]_i$ in GEC. The rank order of potency was UTP > UDP > UMP (Figure 2). This rank order fits well to those found in rat liver (Häussinger *et al.*, 1987) and rabbit basilar artery (von Kügelgen & Starke, 1990). The calcium response to pyrimidine nucleotides showed, in comparison with the response to purine nucleotides, a similar appearance of the calcium transient. The transient peak could also be observed in extracellular calcium-free solution, whereas the plateau phase was abolished without extracellular calcium. α,β-Methylene-ATP was also ineffective in desensitizing the pyrimidine receptor.

Effect of phorbol ester on the ATP-induced phosphoinositol turnover

Short term treatment of cells with the protein kinase C activator has been reported to cause desensitization of several receptors coupled to phosphoinositol phosphate turnover. This is presumably caused by activation of protein kinase C (for review see Rana & Hokin, 1990). In our experiments, short term incubation with PMA (100 ng ml^{-1}) decreased the $InsP_3$ level, probably by the inhibition of a basal phosphoinositol turnover in GEC. The short term incubation of PMA caused a desensitization of the ATP stimulated formation of $InsP_3$ by about 50% (Figure 7). The biologically inactive 4α-phorbol 12,13-didecanoate had no effect on the ATP-stimulated $InsP_3$ turnover. These data indicate that activation of protein kinase C by short term treatment of GEC with PMA inhibits the ATP-stimulated phosphoinositide turnover in GEC. An inhibition of ATP induced second messenger response with phorbol esters has recently been demonstrated in rat mesangial cells (Pfeilschifter, 1990a), human fibroblasts (Fine *et al.*, 1989), hepatocytes (Charest *et al.*, 1985) and in HL 60 cells (Dubyak *et al.*, 1988). When GEC were preincubated for 18 h with PMA, no inhibition or activation of the ATP-induced $InsP_3$ turnover could be observed (Figure 7). Long term treatment of cells with phorbol esters has been reported to induce a complete downregulation of protein kinase C, apparently due to an increased rate of enzyme degradation (Young *et al.*, 1987). The results show that in the case of protein kinase C downregulation, phorbol ester-induced desensitization of the ATP response could be reversed. However, these data do not agree well with the idea of a negative feedback function of protein kinase C, because the inhibition of the negative feedback function of protein kinase C should have resulted in an increased $InsP_3$ response. Therefore, a feedback mechanism does not operate under such conditions and the desensitization of the P_2 receptor by activated protein kinase C may be due to a protein kinase C-mediated change in GEC function.

In summary, the data indicate that purine- and pyrimidine nucleotides are able to stimulate phosphoinositol turnover and to increase $[Ca^{2+}]_i$ via a P_2 receptor distinct from the P_{2x} or P_{2y} receptor in human GEC.

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Involvement of nitric oxide pathway in the PAF-induced relaxation of rat thoracic aorta

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1 The mechanism of the vasorelaxant effect of platelet activating factor (PAF) on rat thoracic aorta and the effect of aging on the PAF-induced relaxation were investigated.

2 PAF at concentrations causing relaxation induced marked increases in guanosine 3':5'-cyclic monophosphate (cyclic GMP) production, but did not induce an increase in adenosine 3':5'-cyclic monophosphate (cyclic AMP).

3 Removal of the endothelium by mechanical rubbing, and treatment with the PAF antagonists CV-3988, CV-6209 and FR-900452, the nitric oxide biosynthesis inhibitor, N^G-nitro L-arginine, the radical scavenger, haemoglobin, and the soluble guanylate cyclase inhibitor, methylene blue, inhibited PAF-induced relaxation and abolished or attenuated PAF-stimulated cyclic GMP production.

4 The relaxation was greatest in arteries from rats aged 4 weeks. With an increase in age, the response of the arteries to PAF was attenuated.

5 Endothelium-dependent cyclic GMP production also decreased with increase in age of the rats.

6 These results suggest that PAF stimulates production of nitric oxide from L-arginine by acting on the PAF receptors in the endothelium, which in turn stimulates soluble guanylate cyclase in the smooth muscle cells, and so increases production of cyclic GMP, thus relaxing the arteries. Age-associated decrease in PAF-induced relaxation may result from a reduction of cyclic GMP formation.

Keywords: PAF; relaxation; nitric oxide; cyclic GMP; N^G-nitro L-arginine (L-NNA); haemoglobin; methylene blue; aging

Introduction

It is known that platelet activating factor (PAF), besides aggregating platelets, has marked hypotensive effects (Tanaka *et al.*, 1983; Lai *et al.*, 1983; Kamitani *et al.*, 1984; Chu *et al.*, 1988), causes vasodilatation of the perfused mesenteric artery (Kamata *et al.*, 1989; Chiba *et al.*, 1990; Gerkens, 1990) and endothelium-dependent relaxation of arterial segments (Cervoni *et al.*, 1983; Kamitani *et al.*, 1984; Kasuya *et al.*, 1984; Vanhoutte & Houston, 1985). The vasorelaxant effect of PAF is thought to be mediated by endothelium-derived relaxing factor (Kamata *et al.*, 1989; Chiba *et al.*, 1990), but direct evidence for this proposal has not so far been presented.

In the present study, we examined further the mechanism by which PAF causes endothelium-dependent relaxation of rat thoracic aorta, with special reference to the contribution of the L-arginine nitric oxide guanosine 3':5'-cyclic monophosphate (cyclic GMP) pathway. We also investigated the effect of aging on the PAF-induced vasorelaxation.

Methods

Organ bath experiments

Male Wistar rats aged 4 weeks (25–31 days), 10 weeks (8–12 weeks), 20 weeks (20–22 weeks), 40 weeks (40–50 weeks) and 100 weeks (95–110 weeks) were used. Four-week-old rats were fed with a standard rat chow (Nihon Clea) until studied. The rats were killed by a blow to the head and cervical dislocation, followed by exsanguination. The thoracic aortae were rapidly removed, freed of adhering fat and connective tissues under a binocular dissecting microscope, and cut into ring segments of 3 mm length with parallel razors. Two stainless steel wires were inserted into the lumen of the aortic ring. One wire was connected to a transducer and the other U-shaped wire was anchored to a plastic holder. The holders were placed in a 10 ml organ bath at 34°C containing oxygenated (95% O_2 and 5% CO_2) Krebs bicarbonate solution of the following composition (mM): NaCl 115.3, KCl 4.9, $CaCl_2$ 1.4, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, glucose 11.1, disodium EDTA 0.027 and ascorbate. The organ bath and the holder were siliconed to prevent adsorption of PAF to surface.

The resting tension of the preparations was maintained at 0.5 g, 1.0 g and 1.3 g, respectively, for the arteries from rats of 4, 10–40 and 100 weeks old, and the preparations were equilibrated for 120 min before the start of the experiments.

For measurement of relaxation, the arteries were precontracted with phenylephrine at concentrations for inducing 80% of the maximal contraction; EC_{80} (0.3 μM for the arteries with endothelium, and 0.1 μM for preparations without endothelium). Responses were recorded isometrically with a force displacement transducer (Nihon Kohden SB 1TH).

For construction of concentration-response curves, PAF was added cumulatively to the 10 ml organ bath in a volume of 7–20 μl, and relaxations were plotted as percentages of the contractions induced by the EC_{80} concentrations of phenylephrine.

For removal of the endothelium, the lumen of the artery was gently rubbed with cotton thread and removal of the endothelium was confirmed by the loss of a relaxant response to acetylcholine.

Haemoglobin was prepared as described by Martin *et al.* (1985). Commercial haemoglobin at a concentration of 1 mM was reacted with 10 mM sodium hydrosulphite and then the reducing agent was removed by dialysis at 4°C.

Assay of cyclic GMP

After equilibration of the arterial segments for 2 h in Krebs solution bubbled with O_2 containing 5% CO_2, arteries were

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first incubated with the EC_{80} concentration of phenylephrine (0.1–0.3 μM) for 5 min, then incubated with PAF. At various times of incubation with PAF, the preparations were quickly frozen in liquid nitrogen and then homogenized in ice-cold 6% trichloroacetic acid. The homogenates were centrifuged at 1700 *g* for 15 min at 4°C, and then supernatants were extracted with 3 volumes of water-saturated ether. Cyclic GMP was measured by a radioimmunoassay procedure (Honma *et al.*, 1977) with commercially available kits, as previously described (Moritoki *et al.*, 1988).

Statistical analysis

Values are expressed as means ± s.e.mean. The statistical significance of differences was analyzed by Student's unpaired *t* test, and *P* values of less than 0.05 were considered as significant.

Materials

The drugs used were PAF (1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine, Sigma Chemical Co., St Louis, MO), FR-900452, (1-methyl -3- [1-[5-methylthiomethyl -6-oxo-3-(2-oxo-3-cyclopentan -1-ylidene) -2-piperazinyl]ethyl] -2-indolinone, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan), CV-6209 (2-[N-acetyl-N- (2-methoxy-3-octadecylcarbamoyloxypropoxycarbonyl) aminomethyl]-1-ethylpyridium chloride), CV-3988 [(RS)-2-methoxy-3-(octadecylcarbamoyloxy)propyl-2-(3-thiazolio)ethyl phosphate, gifts from Takeda Pharmaceutical Industries, Osaka, Japan), methylene blue, haemoglobin, phenylephrine hydrochloride, acetylcholine chloride (all from Sigma), N^G-nitro L-arginine and N^G-nitro D-arginine (Peptide Institute, Osaka, Japan), cilostamide (Ohotsuka Pharmaceutical Co. Ltd., Tokushima, Japan), bovine serum albumin (Sigma). Kits for radioimmunoassay of cyclic GMP were obtained from Yamasa Shoyu Co. Ltd. (Chosi, Japan).

A stock solution of 10 μM PAF in chloroform was stored at below − 20°C until use. The chloroform was evaporated under a stream of nitrogen gas, and Krebs solution containing 0.5% bovine serum albumin was added immediately before use, then the PAF was dissolved completely by sonication. Further dilution was made with Krebs solution containing 0.1% BSA.

Results

PAF-induced relaxation

Cumulative application of PAF caused a concentration-dependent relaxation of the thoracic aorta from rats of 8–10 weeks old (Figures 1 and 2). The threshold concentration of PAF for producing relaxation was 10 pM, and the − log EC_{50} value was 9.60 ± 0.09 (EC_{50} value: 0.27 ± 0.06 nM, $n = 7$). The relaxations caused by a single dose of PAF were not different from those caused by cumulative application of PAF (Figure 2). After reaching a maximum, the relaxation was partially restored with time in the continuous presence of PAF.

Desensitization

After the first determination of PAF-induced relaxation by a single dose of 0.3 nM PAF or its cumulative application, the preparation was washed several times for 2 h. On the 2nd application, PAF even at concentrations as high as 3 nM no longer relaxed the arteries (Figure 1). The desensitization to PAF, once developed, was irreversible. Under these conditions, relaxation induced by acetylcholine, ATP or nitroprusside was scarcely affected.

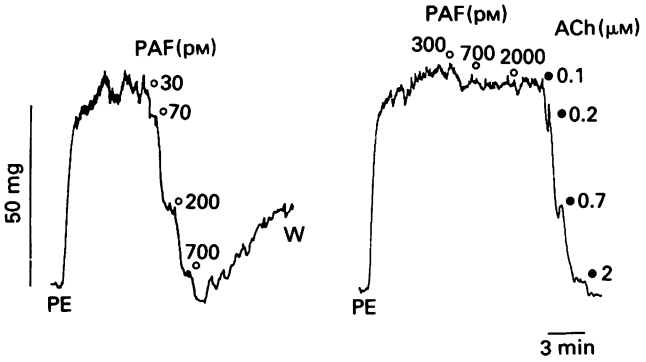

Figure 1 Typical records showing PAF-induced relaxation and self-desensitization to PAF in thoracic aorta from 10-week-old rats. The arteries were first contracted with EC_{80} concentration of phenylephrine (PE, 0.3 μM) to study relaxation. After determination of PAF-induced relaxation, the aorta was washed several times for 120 min, then the effects of PAF and acetylcholine (ACh) were determined again.

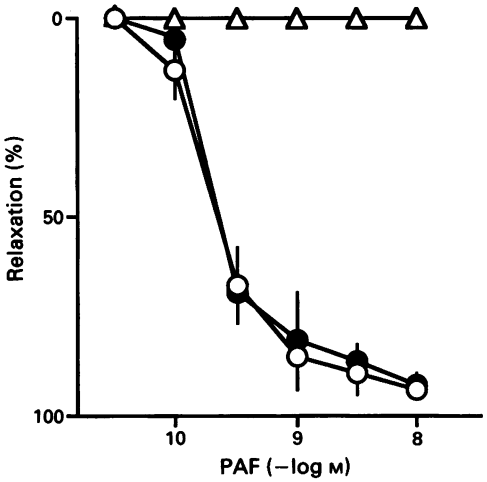

Figure 2 PAF-induced relaxation of the rat thoracic aorta from 10-week-old rats, and the effect of removal of the endothelium. (○), Relaxation induced by cumulative application of PAF; (△), removal of the endothelium. (●), Relaxation induced by application of a single dose of PAF. Experimental conditions are as for Figure 1. The ordinate scale shows the relaxation of the arteries as a percentage of the contraction induced by 0.3 μM phenylephrine. Values are means of 7 preparations; vertical lines indicate s.e.mean.

Effect of endothelium on the PAF-induced relaxation

Removal of the endothelium completely abolished PAF-induced relaxation (Figure 2). On the other hand, the relaxant effect of nitroprusside used as a reference, was scarcely altered by removal of the endothelium (data not shown).

Effect of PAF antagonists

Pretreatment of the arteries with the PAF antagonists CV-3988 and FR-900452 at a concentration of 1 μM for 30 min inhibited PAF-induced relaxation without affecting the acetylcholine-induced relaxation, the concentration-response curve for PAF being shifted about 80 fold (Figure 3). The apparent pA_2 values of CV-3988 and FR-900452 were 7.17 ± 0.23 and 7.10 ± 0.08 ($n = 5$), respectively. Essentially similar, but more potent antagonism was observed with 0.03 μM CV-6209 with an apparent pA_2 value of 8.92 ± 0.17 ($n = 2$). With an increase in the concentration of these antagonists, PAF-induced relaxations were completely abolished.

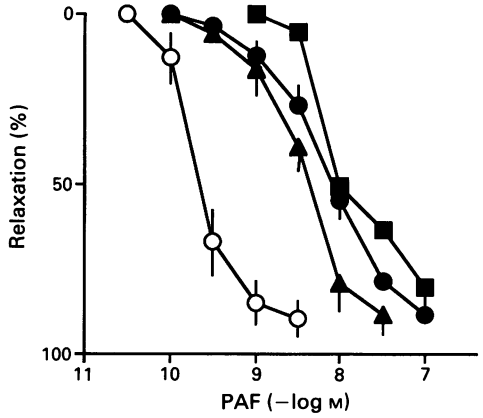

Figure 3 Selective inhibition by CV-3988, CV-6209 and FR900452 of the PAF-induced relaxation of thoracic aorta from rats that were 10 weeks old. The PAF antagonists were pretreated for 30 min before cumulative application of PAF. Controls (○); CV-3988 1 μM (●); CV-6209 0.03 μM (■); FR900452 1 μM (▲) ($n = 2$). Experimental conditions are as for Figure 1. Values are means of 5–6 preparations from 5 rats; vertical lines indicate s.e.mean.

Effect of N^G-nitro L-arginine (L-NNA), haemoglobin and methylene blue on the PAF-induced relaxation

Pretreatment of the arteries with 10 μM L-NNA for 20 min abolished PAF-induced relaxation (Figure 4). On the other hand, its D-enantiomer N^G-nitro D-arginine at concentrations of up to 100 μM did not affect the relaxation.

Pretreatment with haemoglobin for 5 min or methylene blue for 20 min had inhibitory effects similar to L-NNA on the PAF-induced relaxation.

In contrast, these agents had little effects on the relaxation caused by the cyclic AMP-phosphodiesterase inhibitor, cilostamide, used as a reference (data not shown).

Effect of aging on the PAF-induced relaxation

As shown in Figure 5, PAF-induced relaxation was greater at 4-weeks old than at 10-week-old rats, and full relaxation was observed at 1 nM with the $-\log EC_{50}$ value of 10.14 ± 0.06 (EC_{50} value: 0.07 ± 0.01 nM, $n = 5$). With an increase in age of rats to 10 and 40 weeks, the response of the arteries to PAF was attenuated. In the aorta from rats of 100 weeks old, a concentration of PAF about 10 times higher (0.3 nM) was necessary to induce relaxation, and the maximal relaxation decreased to half.

PAF-stimulated cyclic GMP formation

As shown in Figure 6, in preparations with intact endothelium, PAF ranging in concentrations necessary to induce relaxation stimulated cyclic GMP formation in a concentration-dependent manner. PAF at a concentration of 1 nM increased the level of cyclic GMP from the basal level of 1.01 ± 0.19 pmol mg^{-1} protein to 6.09 ± 1.20 pmol mg^{-1} protein ($n = 7$) in 30 s; thereafter it declined to the basal level in 5 min.

Effect of N^G-nitro L-arginine (L-NNA), haemoglobin, CV-6209 and methylene blue, and removal of the endothelium on the PAF-stimulated cyclic GMP production

Effects of L-NNA, haemoglobin, methylene blue and CV-6209 on the PAF-stimulated formation of cyclic GMP are shown in Figure 7. In preparations with intact endothelium, treatment with 10 μM L-NNA 20 min before and during incubation with PAF, besides decreasing the basal level of cyclic GMP, abolished cyclic GMP production stimulated by 1 nM PAF.

Essentially similar results were obtained with haemoglobin, methylene blue and CV-6209.

Removal of the endothelium, which lowered the basal level of cyclic GMP, abolished PAF-stimulated increase in cyclic GMP formation.

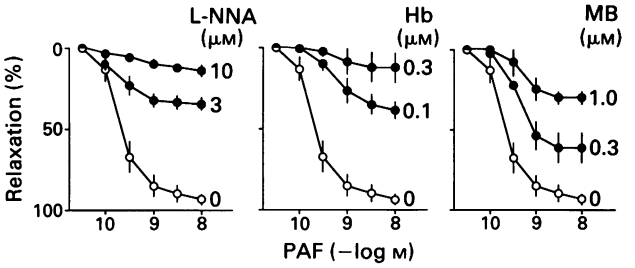

Figure 4 Inhibitory effects of N^G-nitro L-arginine (L-NNA), haemoglobin (Hb) and methylene blue (MB) on the PAF-induced relaxation. Thoracic aorta from rats of 10 weeks old were exposed to L-NNA, haemoglobin and methylene blue for 20 min, 5 min and 20 min, respectively, before and during application of PAF. Experimental conditions are as for Figure 1. Values are means of preparations from 5 rats; vertical lines indicate s.e.mean.

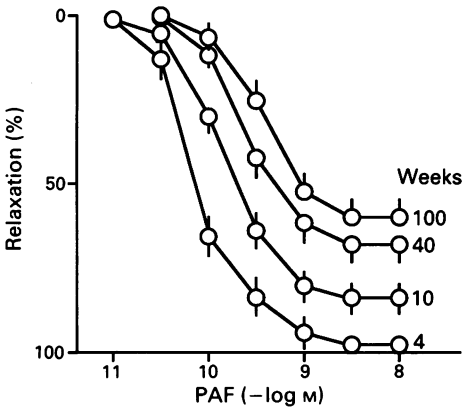

Figure 5 Effects of aging on the PAF-induced relaxation of rat thoracic aorta. Ages of rats are shown in weeks. The preparations were first contracted with the EC_{80} concentrations of phenylephrine (0.3 μM). Optimal resting tensions were 0.5 g for 4-week-old, 1.0 g for 10–40-week-old and 1.3 g for 100-week-old rats. Values are means of 6 to 10 preparations.

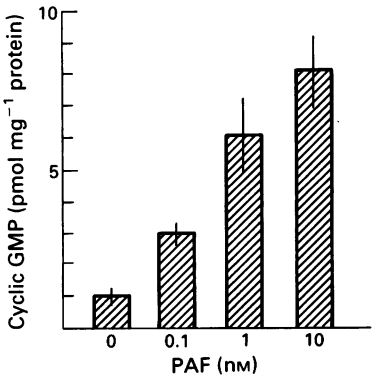

Figure 6 PAF-stimulated production of cyclic GMP in rat thoracic aorta with endothelium. Arteries from 10-week-old rats were used. Cyclic GMP was measured after incubation with the EC_{80} concentrations of phenylephrine (0.3 μM) for 5 min and then with or without PAF for 30 s. Each column represents the mean of values in 6 to 9 preparations from 7 rats. Vertical lines indicate s.e.mean.

Effect of aging on cyclic GMP formation

Next, we examined the effect of aging on the PAF-stimulated cyclic GMP production (Figure 8). Cyclic GMP formation was greater in the arteries from young rats than in those from mature rats. At 4 weeks old, 1 nM PAF increased the formation of cyclic GMP from the basal level of 1.50 ± 0.34 pmol mg^{-1} protein to 14.0 ± 2.9 pmol mg^{-1} ($n = 5$). As the age of rats increased to 10 weeks, PAF-stimulated cyclic GMP production decreased by 50% (6.09 ± 1.20 pmol mg^{-1} protein, $P < 0.05$). At 90 weeks, besides decreasing the basal level, the amount was only 15% (2.13 ± 0.36 pmol mg^{-1} protein, $P < 0.01$) of the value obtained with the arteries from rats of 4 weeks old (14 pmol mg^{-1} protein).

Discussion

PAF has been reported to induce endothelium-dependent relaxation of several vasculatures (Cervoni *et al.*, 1983; Kamitani *et al.*, 1984; Kasuya *et al.*, 1984; Kamata *et al.*, 1989; Chiba *et al.*, 1990). However, the concentration of PAF necessary to induce relaxation was so high that it was suggested that its non-specific action on the endothelial cell membrane was responsible for the relaxation (Vanhoutte & Houston, 1985). In the present experiments, PAF was found to induce relaxation of rat thoracic aorta at far lower concentrations (threshold concentration was 10 pM) than those previously reported (Cervoni *et al.*, 1983; Kamitani *et al.*, 1984; Kasuya *et al.*, 1984; Shigenobu *et al.*, 1985; Kamata *et al.*, 1989; Chiba *et al.*, 1990). It has been observed that bovine serum albumin prevents the adsorption of PAF to many surfaces (Benveniste, 1974) or interferes with uptake of PAF in the cells or tissues (Cabot *et al.*, 1982), and that albumin was necessary for PAF to induce the potent vasorelaxant effect (Shigenobu *et al.*, 1985; Gerkens, 1990). Indeed, we confirmed that in the absence of BSA, PAF induced less relaxation (data not shown), but in the presence of BSA, PAF at a concentration as low as 10 pM induced relaxation. In addition, its threshold concentration was 2–5 orders lower than those previously reported (Cervoni *et al.*, 1983; Shigenobu *et al.*, 1985; Chiba *et al.*, 1990). This discrepancy may be due to the experimental conditions used; ring segment vs. helical strip of the arteries, concentration of serum albumin, siliconing of apparatus to prevent the adsorption of PAF to the surfaces (Benveniste, 1974), difference in sub-strain of rats, or breeding conditions. In addition, the age of the rat may be an important factor affecting the sensitivity to PAF, because arteries from young rats were more sensitive to PAF than those from mature rats, as mentioned below.

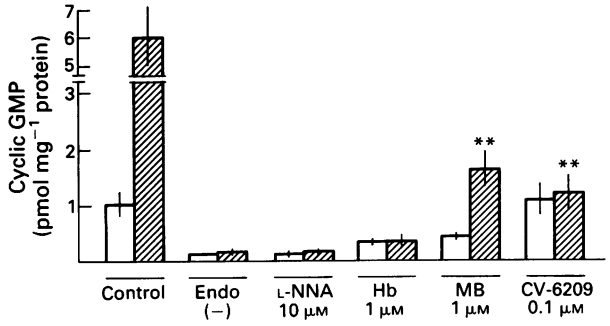

Figure 7 Effects of N^G-nitro-L-arginine (L-NNA), haemoglobin (Hb), methylene blue (MB) and CV-6209, and removal of the endothelium (Endo-) on the PAF-stimulated production of cyclic GMP in the thoracic aorta from rats of 10 weeks old. L-NNA, Hb, MB and CV-6209, when used, were applied 20, 5, 20 and 30 min, respectively, before and during incubation with 1 nM PAF. Other experimental conditions are as for Figure 6. Stippled columns, treatment with 1 nM PAF. Each column represents the mean of values in 5 to 7 preparations from 9 rats. Vertical lines indicate s.e.mean. $^{**}P < 0.01$, compared with the control value obtained in the absence of the inhibitors.

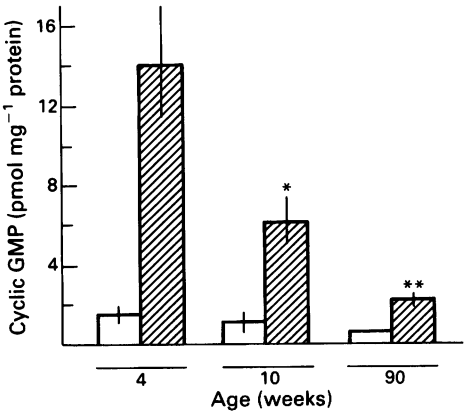

Figure 8 Effect of aging on the increase in cyclic GMP level stimulated by PAF (1 nM) in rat thoracic aorta. Experimental conditions are as for Figure 6. Stippled columns, with 1 nM PAF; open columns, control. Each column represents the mean of values in 5 to 9 preparations.
$^{*}P < 0.05$; $^{**}P < 0.01$, compared with value at 4 weeks old.

As has been reported (Chiba *et al.*, 1990), desensitization of the arteries to PAF rapidly developed once PAF was applied. However, on cumulative application of PAF, desensitization of the preparations to successive doses of PAF did not develop, but developed after the PAF had been washed out. In addition, the magnitude of the relaxation induced by a single concentration of PAF was not different from that induced by its cumulative application. We do not have any explanation for this inconsistency. When the desensitization to PAF developed, relaxations induced by acetylcholine, ATP or nitroprusside were not affected, indicating that the PAF-induced desensitization is a homologous type.

Indirect evidence supporting the idea that PAF acts on the PAF receptors in the endothelium, is that removal of the endothelium, and the PAF antagonist CV-3988 (Terashita *et al.*, 1983; Voelkel *et al.*, 1986), CV-6209 (Terashita *et al.*, 1987) and FR900452 (Okamoto *et al.*, 1986) attenuated or abolished PAF-induced relaxation and cyclic GMP formation. Furthermore, rat arteries were found to be autodesensitized by second challenge with PAF, suggesting its interaction with PAF receptors in the endothelium. In this context, several reports showing the existence of PAF receptors in the endothelial cells (Evans *et al.*, 1987; D'Humières *et al.*, 1986) have been presented. These results suggest that specific receptors for PAF are localized to the vascular endothelium.

We examined whether PAF stimulates nitric oxide formation via PAF receptors in the endothelium. Evidence for the contribution of nitric oxide to the action of PAF is that haemoglobin which binds and inactivates EDRF (Martin *et al.*, 1985) and L-NNA which inhibits biosynthesis of nitric oxide from L-arginine (Mülsch & Busse, 1990; Rees *et al.*, 1990) abolished PAF-induced, endothelium-dependent relaxation and PAF-stimulated cyclic GMP production. This idea is further supported by the finding that methylene blue which blocks the effect of nitric oxide by inhibiting soluble guanylate cyclase (Gruetter *et al.*, 1981; Martin *et al.*, 1985; Ignarro & Kadowitz, 1985; Rapoport *et al.*, 1985) inhibited the actions of PAF in both. From these results, it is suggested that PAF stimulates production of nitric oxide from L-arginine, which in turn activates soluble guanylate cyclase in the vascular smooth muscle, thus producing cyclic GMP and causing relaxation.

In the present study, we also found that aging attenuated PAF-induced relaxation. Although absolute tensions induced

by phenylephrine were greater in the arteries from old rats, it is unlikely that age-associated reduction of PAF-induced relaxation is a consequence of an increase in the initial level of phenylephrine-induced tone, because it has been reported that the tensions developed per mg wet tissue were not significantly different among the arteries from rats of several ages; rather less at 45 weeks old than at 4 weeks old (Moritoki *et al.*, 1992). A decrease in the production/accumulation of cyclic GMP which is known to be a second messenger in vascular relaxation (Rapoport & Murad, 1983), is undoubtedly the major cause of age-associated reduction of PAF-induced relaxation. Essentially similar age-dependent changes in relaxant responses and cyclic GMP production in rat thoracic aorta have been documented with histamine (Moritoki *et al.*, 1986; 1988), adenosine (Moritoki *et al.*, 1990) and ATP (Ueda & Moritoki, 1991).

Steps affected by aging may be proximal to cyclic GMP formation. Mechanisms linking PAF receptors and nitric oxide formation could be affected by aging. It is assumed that the primary defect responsible for the age-associated decrease in PAF-induced relaxation is a reduction in the number or affinity of PAF receptors, or a decrease in the ability of the endothelium to produce nitric oxide.

In addition, reduction of soluble guanylate cyclase activity may, at least in part, contribute to the age-associated changes, because aging has been found to attenuate nitroprusside-induced relaxation and cyclic GMP accumulation due to direct activation of soluble guanylate cyclase (Moritoki *et al.*, 1988; 1991; 1992). In this context, however, change in cyclic GMP-phosphodiesterase activity cannot be ruled out.

We are grateful to Takeda Pharmaceutical Industry for supplying CV-3988 and CV-6209, and to Fujisawa Pharmaceutical Co., Ltd. for supplying FR900452. This work was in part supported by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Science and Culture of Japan (63571045 and 03671054), and by the Fujisawa Foundation.

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Determination of α_1-adrenoceptor subtype selectivity by [^{3}H]-prazosin displacement studies in guinea-pig cerebral cortex and rat spleen membranes

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1 [^{3}H]-prazosin homogeneously labels α_1-adrenoceptors in guinea-pig cerebral cortex and rat spleen membranes with dissociation constants of 1.28 and 1.49×10^{-10} M respectively.

2 Phentolamine and WB 4101 displacement studies show that guinea-pig cerebral cortex contains 30% α_{1A}- and 70% α_{1B}-adrenoceptor subtypes, whereas rat spleen contains a virtually homogeneous α_{1B}-adrenoceptor subtype population. The α_1-adrenoceptor population of rat thoracic aorta is predominantly of the α_{1A}-adrenoceptor subtype, and in guinea-pig thoracic aorta it is mainly of the α_{1B}-adrenoceptor subtype.

3 Half of the compounds displacing [^{3}H]-prazosin bound to guinea-pig cerebral cortex membranes display α_{1A}-adrenoceptor selectivity. Among these compounds, WB 4101 and methoxamine are most selective, displaying selectivity ratios of $\sim$38 and $\sim$26 respectively.

4 The affinity constants of the non-selective compounds for the α_1-adrenoceptors in guinea-pig cerebral cortex membranes correlate well with the affinity constants obtained for α_{1B}-adrenoceptors in rat spleen membranes. The affinities of selective compounds for the α_{1B}-adrenoceptor subtype in guinea-pig cerebral cortex correlate very well with their affinity for α_{1B}-adrenoceptor in the rat spleen homogenate. Both regression lines coincide with the line of identity. The affinity constants of selective compounds for the α_{1A}-adrenoceptors in guinea-pig cerebral cortex only apparently correlate with the affinity for either the α_{1B}-adrenoceptors in guinea-pig cerebral cortex or in the rat spleen. Regression analyses indicate a straight line relationship ($r^2 > 0.9$) between $pK_{\alpha 1A}$ and $pK_{\alpha 1B}$ but the regression lines deviate from the line of identity.

Keywords: Radioligand binding study; α_1-adrenoceptor subtypes; [^{3}H]-prazosin; guinea-pig cerebral cortex; rat spleen; selectivity

Introduction

Although the subclassification of α_1-adrenoceptors into α_{1A}- and α_{1B}-adrenoceptor subtypes (Morrow & Creese, 1986) is now becoming more generally accepted, sometimes large differences exist in the dissociation constants reported for the α_1-adrenoceptor subtypes. These differences in absolute affinity constants could be caused by species differences or differences between central nervous system and peripheral tissues. Other possible explanations for these discrepancies could be differences in assay conditions, differences in data analysis or even the existence of more than two α_1-adrenoceptor subtypes (Schwinn *et al.*, 1990; Watson & Abbott, 1991).

To investigate the first two possibilities, we conducted [^{3}H]-prazosin displacement experiments with a large number of α-adrenoceptor agonists and antagonists using membranes from guinea-pig cerebral cortex and rat spleen. We previously showed that guinea-pig cerebral cortex contains both α_{1A}- and α_{1B}-adrenoceptor subtypes in an approximate ratio of 3:7 (Veenstra *et al.*, 1990), whereas rat spleen is reported to contain a homogeneous α_{1B}-adrenoceptor population (Han *et al.*, 1987; Minneman *et al.*, 1988; Veenstra *et al.*, 1990). Since the same assay conditions and methods of analysis are used throughout this study, no discrepancies can be attributed to these in our results.

As the number of different α-adrenoceptor compounds tested hitherto is rather limited, we determined the affinities of a large number of compounds, selecting those that are commonly used in functional studies. Examining these compounds acting on α-adrenoceptors in both preparations additionally provides information about the α_1-adrenoceptor subtype selectivity of these compounds which is of importance for future functional and biochemical studies.

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Methods

Preparation of membranes

Male guinea-pigs (Dunkin-Hartly, TNO Zeist, The Netherlands) weighing 350–450 g and Wistar rats (TNO Zeist, The Netherlands) weighing 220–280 g were killed by a blow on the head and exsanguinated. Brains, spleen and thoracic aortae were immediately removed and placed in ice-cold Sørensen buffer (Na/K phosphate 66.6 mM, pH 7.4). After the tissues had been freed from adhering tissues and endothelium was removed from the aortae by rubbing with a cotton wool stick, tissues were homogenized with a Polytron in 10 vol of ice-cold buffer and homogenized further with a glass-teflon homogenizer. After filtering the homogenate through cheese cloth the filtrate was centrifuged at 40,000 *g* for 30 min at 0–4°C. The pellets were resuspended in 2–3 vol buffer and stored in liquid N_2 until use.

Radioligand binding studies

The membranes were diluted in incubation buffer (Tris HCl 50 mM, NaCl 140 mM, $MgCl_2$ 5 mM, pH 7.4 at 37°C) and gently homogenized with a glass-teflon homogenizer. All experiments were performed for 60 min at 37°C in a final volume of 1.00 ml.

Saturation studies

Membranes, in a final concentration of approximately 5 mg ml^{-1} (cortex, wet tissue) or 8 mg ml^{-1} (spleen, wet tissue), were incubated in polystyrene tubes (0.8 × 4.2 cm) with increasing concentrations of [^{3}H]-prazosin over a range of 0.001–7 nM. The contents of the tubes were continuously

mixed by gentle shaking. After 60 min the incubation was terminated by placing the test-tubes on ice, addition of 3 ml ice-cold washing buffer (Tris HCl 50 mM, NaCl 140 mM, $MgCl_2$ 5 mM, phentolamine 1 μM, pH 7.4 at 0°C) and rapid filtration over Whatman GF/C glass-fibre filters and two additional rinses of tubes and filters with 3 ml of ice-cold buffer each.

After drying the filters in counting vials, scintillation fluid was added. Following equilibration the radioactivity trapped on the filters was counted at an efficiency of 52% in a Packard TRI-CARB scintillation counter.

Blank filter binding was determined in triplicate for each concentration of [^{3}H]-prazosin from identically treated samples in the absence of membranes. Total binding was determined in quadruplicate and total radioactivity added in duplicate.

Displacement studies

Aliquots of cerebral cortex or spleen membranes were incubated with 0.5 nM [^{3}H]-prazosin and appropriate concentrations of unlabelled ligand. Other assay conditions and sample processing were the same as those described for saturation studies. In studies with oxidizable compounds, 0.07% ascorbic acid and 10 μM EDTA was included in the incubation medium to prevent oxidation. Displacement experiments with (-)-noradrenaline, (-)-adrenaline, phenylephrine and cirazoline were performed in the presence of 0.1 mM Gpp(NH)p.

Displacement curves were constructed at least twice in triplicate, with three ligand concentrations per order of magnitude over a range of 5–7 orders. Blank filter binding was determined in quadruplicate parallel to each experiment from identically treated samples in the absence of membranes or displacing agents.

Thoracic aorta membranes were tested at an end concentration of approximately 20 mg ml^{-1} original wet tissue. Because of the paucity of aortic tissue and the low specific activity of the radiolabel, only two data points per order of magnitude were obtained.

Data analysis

Since [^{3}H]-prazosin binds non-linearly to glass-fibre filters and is displaceable by unlabelled compounds (Veenstra *et al.*, 1992), filter binding is always subtracted from total binding before analysis by the iterative non-linear curve-fitting programme LIGAND (Munson & Rodbard, 1980). The data of multiple saturation or displacement experiments were first fitted simultaneously to a one-site model and then to a two-site model and if necessary, also to a three-site model. The 'goodness of fit' of the data was then determined by the residual sum of squares test which is part of the LIGAND analysis. A two/three-site model was accepted as the most appropriate for the data set only if the residual sums of squares test showed that the two/three-site fit was statistically better than the one/two-site fit. Displacement curves were analyzed by use of the dissociation constant of [^{3}H]-prazosin obtained from the saturation studies. For the displacement of [^{3}H]-prazosin by unlabelled prazosin, it is assumed that the substitution of a hydrogen atom by a tritium atom does not influence the binding parameters of the prazosin molecule. In the fits of these data, the dissociation constants of labelled and unlabelled prazosin were shared but not fixed.

Receptor concentration, R, is expressed as fmol mg^{-1} wet tissue. The dissociation constant, K_d, as mol l^{-1} (M) and errors are the estimated standard deviations (s.d.). Pseudo Hill-slope coefficients (n_H) were estimated by the iterative non-linear curve-fitting programme Allfit, a programme based on a four-parameter logistic equation (De Lean *et al.*, 1978).

In all analyses, the level of significance was set at $P<0.05$.

Compounds used

The following compounds were used: [^{3}H]-prazosin (specific activity 30.3 Ci mmol^{-1}, New England Nuclear); (−)-noradrenaline hydrochloride (Sigma); (−)-adrenaline base, Gpp(NH)-p·Li_4 (guanosine-5′-[β,γ-imido] triphosphate) (Serva); tolazoline dihydrochloride and yohimbine hydrochloride (OPG) and WB 4101 (2-(2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4-benzodioxane; Research Biochemical Inc.). The following compounds were kindly donated: amidephrine mesylate (Bristol-Meyers Co); amosulalol hydrochloride (Yamanouchi Pharmaceutical Co); B-HT 920 dihydrochloride (6-allyl-2-amino-5,6,7,8-tetrahydro-4*H*-thiazolo-[4,5-*d*]-azepine), B-HT 933 dihydrochloride (6-allyl-2-amino-5,6,7,8-tetrahydro-4*H*-oxazolo-[4,5-*d*]-azepine), clonidine hydrochloride, St 587 nitrate (2-(2-chloro-5-trifluoromethyl phenylimino)-imidazolidine) and St 1965 hydrochloride (2-(1,3-dichloro-2-hydroxymethyl)-imidazole) (Boehringer Ingelheim); cirazoline (L.E.R. Synthélabo); doxazosin mesylate, prazosin hydrochloride and UK 14,304 tartrate (5-bromo-6-[2-imidazolin-2-ylamino]quinoxaline; Pfizer); idazoxan hydrochloride (Reckit and Colman); indanidine hydrochloride (Sgd 101/75) (Siegfried AG); labetalol hydrochloride (Glaxo); methoxamine (Burroughs Wellcome Co); naphazoline nitrate, phentolamine hydrochloride, oxymetazoline hydrochloride and xylometazoline hydrochloride (Ciba-Geigy); phenylephrine hydrochloride (Boots Co); (−)-propranolol hydrochloride (ICI Farma); timolol (MSD); verapamil (Knoll A.G.) and xylazine (Bayer). All other reagents were of highest grade commercially available.

Stock solutions of phentolamine (10 mM), naphazoline (100 mM) and WB 4101 (10 mM) were prepared in a mixture 0.9% NaCl and dimethylsulphoxide (9:1, v/v); labetalol (100 mM) in 0.9% NaCl:ethylene glycol = 1:1 (v/v) and yohimbine (10 μM) in 0.9% NaCl:methanol = 9:1 (v/v). All other compounds were dissolved in 0.9% NaCl.

Results

We first established the characteristics of the binding of [^{3}H]-prazosin to guinea-pig cerebral cortex and rat spleen membranes. Table 1 summarizes the results. As can be seen, saturation as well as displacement curves had pseudo Hill-slope coefficients not significantly different from unity and in agreement with this observation, all curves fitted best to a one receptor model. Within each preparation there is good correlation between the dissociation constant for prazosin

Table 1 Characterization of [^{3}H]-prazosin binding to guinea-pig cerebral cortex and rat spleen membranes by [^{3}H]-prazosin saturation studies and by displacement studies of [^{3}H]-prazosin by unlabelled prazosin

	Saturation	*Displacement*
	Guinea-pig cortex	
n	4	4
$K_d \pm$ s.d.	$1.28 \pm 0.17 \times 10^{-10}$	$3.51 \pm 1.37 \times 10^{-10}$
$R_{tot} \pm$ s.d.	8.33 ± 0.67	9.63 ± 0.84
$n_H \pm$ s.d.	0.98 ± 0.06	1.06 ± 0.07
	Rat spleen	
n	2	2
$K_d \pm$ s.d.	$1.49 \pm 0.43 \times 10^{-10}$	$3.13 \pm 1.90 \times 10^{-10}$
$R_{tot} \pm$ s.d.	2.67 ± 0.48	2.89 ± 0.84
$n_H \pm$ s.d.	1.02 ± 0.09	0.99 ± 0.08

n: number of quadruplicate (saturation) or triplicate (displacement) experiments; K_d: dissociation constant (M); R_{tot}: total receptor concentration (fmol mg^{-1} wet tissue); n_H: pseudo Hill-slope coefficient.

and the receptor concentration and there is no difference in the K_d of the cortex and spleen receptors but the receptor density based on tissue wet weight is approximately three times higher in guinea-pig cortex than in rat spleen.

As originally shown by Morrow & Cresse (1986) the α_1-adrenoceptor can be subdivided into at least two subtypes: the α_{1A}- and α_{1B}-adrenoceptors. Phentolamine and WB 4101 are able to discriminate between these subtypes (α_{1A}-selective) and to see whether these subtypes (co)exist in the guinea-pig cortex and rat spleen membranes we performed [^{3}H]-prazosin displacement studies with these compounds. For comparison also rat- and guinea-pig thoracic aorta membranes were used. Table 2 summarizes the results. The pseudo Hill-slope coefficients deviate only significantly from unity for the displacement curves of the guinea-pig cerebral cortex membranes. Both WB 4101 and phentolamine curves fitted significantly best to a two-site model and recognized approximately 30% high-affinity sites with dissociation constants representative for the α_{1A}-adrenoceptor subtype. Displacement curves in rat spleen membranes were monophasic and the observed dissociation constants equal the constants for the low-affinity site in guinea-pig cortex i.e. are representative for the α_{1B}-adrenoceptor subtype. Aortic membranes also contain a homogeneous receptor population. The magnitudes of the affinity constant suggest the presence of the α_{1A}-adrenoceptor in rat aorta and of α_{1B}-adrenoceptor in guinea-pig aorta.

In addition we determined the dissociation constants of 26 commonly used compounds acting on α-adrenoceptors for the α_1-adrenoceptors in guinea-pig cerebral cortex and rat spleen membranes. The results are summarized in Table 3. In guinea-pig cerebral cortex, almost all pseudo Hill-slope coefficients deviated significantly from unity. Exceptions are prazosin, doxazosin, amosulalol, St 1965 and B-HT 920.

Table 2 Dissociation constants of WB 4101 and phentolamine for α_1-adrenoceptors of guinea-pig cerebral cortex and aorta and rat spleen- and aortic membranes

	$K_{\alpha 1A} \pm$ s.d.	$K_{\alpha 1B} \pm$ s.d.	*Sel**	$\%R_{\alpha 1A}$	*n*
		WB 4101			
Guinea-pig cerebral cortex	$8.55 \pm 3.75 \times 10^{-10}$	$2.98 \pm 0.64 \times 10^{-8}$[a]	34	34.9	4
Rat spleen		$3.61 \pm 0.45 \times 10^{-8}$	0		3
Rat aorta	$4.39 \pm 2.41 \times 10^{-10}$		$\leqslant 100$	1	
Guinea-pig aorta		$1.78 \pm 0.58 \times 10^{-8}$	$\geqslant 0$		1
		Phentolamine			
Guinea-pig cerebral cortex	$9.06 \pm 3.79 \times 10^{-9}$	$1.26 \pm 0.23 \times 10^{-7}$[a]	31	13.54	
Rat spleen		$1.22 \pm 0.41 \times 10^{-7}$	0		3
Rat aorta	$8.35 \pm 5.21 \times 10^{-9}$		$\leqslant 100$	1	

[a] Significantly better fit to a two-site model; *n*: number of triplicate experiments; $K_{\alpha 1A}$ and $K_{\alpha 1B}$: dissociation constants for α_{1A}- and α_{1B}-adrenoceptors respectively (M); $\%R_{\alpha 1A}$: percentage α_{1A}-adrenoceptors of total α_1-adrenoceptor population; *Sel: selectivity = $K_{\alpha 1A}/K_{\alpha 1B}$.

Table 3 Dissociation constants of ligands for α_1-adrenoceptors of guinea-pig cerebral cortex and rat spleen membranes

		Guinea-pig cerebral cortex					*Rat spleen*	
	$K_d \pm$ s.d.	$K_{\alpha 1A} \pm$ s.d.	$K_{\alpha 1B} \pm$ s.d.	$\%R_{\alpha 1A}$	*Sel**	*n*	$K_{\alpha 1B} \pm$ s.d.	*n*
(−)-Adrenaline	$9.15 \pm 0.90 \times 10^{-6}$	$1.15 \pm 0.28 \times 10^{-6}$	$1.07 \pm 0.11 \times 10^{-5}$[a]	33	9.3	3	$1.10 \pm 0.34 \times 10^{-5}$	2
Amidephrine	$6.84 \pm 0.77 \times 10^{-5}$	$1.78 \pm 0.31 \times 10^{-5}$	$9.52 \pm 2.28 \times 10^{-5}$	39	–	2	$1.20 \pm 1.58 \times 10^{-4}$	2
Amosulalol	$1.70 \pm 0.17 \times 10^{-7}$				–	2	$3.79 \pm 0.47 \times 10^{-8}$	3
B-HT 920	$4.89 \pm 0.44 \times 10^{-5}$				–	2	$1.79 \pm 0.34 \times 10^{-5}$	2
B-HT 933	$1.14 \pm 0.44 \times 10^{-4}$				–	2	$1.25 \pm 0.72 \times 10^{-3}$	2
Cirazoline	$1.23 \pm 0.68 \times 10^{-6}$	$2.46 \pm 0.54 \times 10^{-7}$	$2.14 \pm 0.97 \times 10^{-6}$[a]	31	8.7	3	$2.62 \pm 0.52 \times 10^{-6}$	2
Clonidine	$1.01 \pm 0.06 \times 10^{-6}$	$3.67 \pm 1.00 \times 10^{-7}$	$3.71 \pm 1.15 \times 10^{-6}$[a]	39	10.1	5	$2.26 \pm 0.51 \times 10^{-6}$	2
Doxazosin	$2.82 \pm 0.23 \times 10^{-9}$				–	3	$2.23 \pm 1.41 \times 10^{-9}$	2
Idazoxan	$3.66 \pm 0.32 \times 10^{-6}$	$3.41 \pm 1.53 \times 10^{-7}$	$6.39 \pm 1.24 \times 10^{-6}$[a]	41	18.7	3	$2.10 \pm 0.70 \times 10^{-6}$	2
Indanidine	$1.76 \pm 0.13 \times 10^{-5}$	$3.96 \pm 2.32 \times 10^{-6}$	$2.86 \pm 0.98 \times 10^{-5}$[a]	27	7.2	3	$1.22 \pm 0.60 \times 10^{-5}$	2
Labetalol	$3.95 \pm 0.44 \times 10^{-7}$	$1.20 \pm 0.71 \times 10^{-7}$	$4.89 \pm 1.46 \times 10^{-7}$	23	–	2	$2.48 \pm 0.34 \times 10^{-7}$	2
Methoxamine	$3.86 \pm 0.65 \times 10^{-4}$	$1.01 \pm 0.35 \times 10^{-5}$	$2.75 \pm 0.23 \times 10^{-4}$[a]	24	27.2	2	$2.59 \pm 1.06 \times 10^{-4}$	2
Naphazoline	$3.34 \pm 0.27 \times 10^{-7}$	$1.55 \pm 0.81 \times 10^{-7}$	$2.57 \pm 1.01 \times 10^{-7}$	39	–	2	$2.83 \pm 0.45 \times 10^{-7}$	2
(−)-Noradrenaline	$1.86 \pm 0.23 \times 10^{-5}$	$3.31 \pm 0.98 \times 10^{-6}$	$2.14 \pm 0.17 \times 10^{-5}$[a]	29	6.5	4	$1.14 \pm 0.30 \times 10^{-5}$	2
Oxymetazoline	$7.09 \pm 0.68 \times 10^{-7}$	$1.68 \pm 0.71 \times 10^{-7}$	$8.01 \pm 2.04 \times 10^{-7}$[a]	40	4.8	3	$4.26 \pm 0.60 \times 10^{-7}$	2
Phenylephrine	$2.17 \pm 0.15 \times 10^{-5}$	$4.90 \pm 1.09 \times 10^{-6}$	$2.82 \pm 0.23 \times 10^{-5}$[a]	27	5.8	3	$1.89 \pm 0.32 \times 10^{-5}$	2
(−)-Propranolol	$2.62 \pm 0.31 \times 10^{-5}$	$3.67 \pm 2.01 \times 10^{-6}$	$4.16 \pm 0.85 \times 10^{-5}$[a]	22	11.3	2	$1.99 \pm 0.17 \times 10^{-5}$	2
St 587	$5.55 \pm 0.38 \times 10^{-6}$	$1.95 \pm 0.76 \times 10^{-6}$	$8.59 \pm 2.67 \times 10^{-6}$[a]	36	4.4	2	$4.46 \pm 0.33 \times 10^{-6}$	2
St 1965	$9.54 \pm 1.15 \times 10^{-6}$				–	2	$8.13 \pm 1.61 \times 10^{-6}$	2
Timolol	$4.82 \pm 0.93 \times 10^{-5}$	$6.18 \pm 2.55 \times 10^{-6}$	$2.64 \pm 0.17 \times 10^{-4}$	17	–	2	$5.60 \pm 1.94 \times 10^{-5}$	2
Tolazoline	$6.02 \pm 0.31 \times 10^{-6}$	$1.75 \pm 1.16 \times 10^{-6}$	$8.35 \pm 2.76 \times 10^{-6}$	23	–	4	$1.19 \pm 0.44 \times 10^{-5}$	2
UK 14,304	$3.18 \pm 0.22 \times 10^{-6}$	$2.09 \pm 1.04 \times 10^{-6}$	$5.75 \pm 1.99 \times 10^{-6}$	38	–	4	$4.50 \pm 1.12 \times 10^{-6}$	2
Verapamil	$8.91 \pm 1.56 \times 10^{-6}$	$9.97 \pm 5.77 \times 10^{-7}$	$1.73 \pm 0.65 \times 10^{-5}$[a]	42	17.4	2	ND	
Xylazine	$7.57 \pm 0.62 \times 10^{-5}$	$7.89 \pm 5.78 \times 10^{-6}$	$8.96 \pm 2.45 \times 10^{-5}$	15	–	2	$1.04 \pm 0.41 \times 10^{-4}$	2
Xylometazoline	$9.15 \pm 1.01 \times 10^{-7}$	$1.26 \pm 0.88 \times 10^{-6}$	$4.09 \pm 2.57 \times 10^{-6}$	40	–	3	$4.27 \pm 0.60 \times 10^{-7}$	2
Yohimbine	$2.20 \pm 0.15 \times 10^{-6}$	$1.47 \pm 0.26 \times 10^{-6}$	$3.12 \pm 1.99 \times 10^{-6}$	48	–	4	$2.74 \pm 0.47 \times 10^{-6}$	2

[a] Significantly better fit to a two-site model; *n*: number of triplicate experiments; $K_{\alpha 1A}$ and $K_{\alpha 1B}$: dissociation constants of α_{1A}- and α_{1B}-adrenoceptors (M); *Sel: selectivity = $K_{\alpha 1A}/K_{\alpha 1B}$; $\%R_{\alpha 1A}$: percentage α_{1A}-adrenoceptors of total α_1-adrenoceptor population; ND: not determined

Because of the extremely low affinity of B-HT 933, no coefficient could be determined for this compound. All other compounds could be fitted to a two-site model, although for only 14 out of 21 this resulted in a significant improvement of the fit.

In rat spleen all compounds except oxymetazoline, xylometazoline, naphazoline and UK 14,304 had a pseudo Hill-slope coefficient not significantly different from unity. Despite the lower slope for the four aforementioned compounds, all displacement curves were fitted best to a one-site model.

Linear regression analysis between the pK_d-values (-log K_d) of the non-selective compounds for the α_1-adrenoceptors in guinea-pig cortex- and rat spleen membranes show a high correlation (Figure 1). The regression line has a slope of 1 and closely follows the line of identity.

Regression analyses between the pK_d-values of selective compounds were performed with three data sets. First, the affinities of the compounds for the α_{1A}-adrenoceptors in guinea-pig cerebral cortex were correlated with the values for the α_{1B}-adrenoceptors in guinea-pig cerebral cortex (Figure 2a) and then with the values for the α_{1A}-adrenoceptor in the rat spleen (Figure 2b). In both cases the regression line is shifted to the left of the line of identity, indicating the dissimilarity of the (sub)receptors. Regression analysis between the pK_d-values for α_{1B}-adrenoceptors in guinea-pig cerebral cortex and for α_1-adrenoceptors in rat spleen membranes (Figure 2c) results in a regression line that almost completely coincides with the line of identity, confirming the high level of identity between the putative α_{1B}-adrenoceptors in guinea-pig cerebral cortex preparation and the α_1-adrenoceptors in rat spleen membranes.

Discussion

[^{3}H]-prazosin labels with equally high affinity α_1-adrenoceptors in guinea-pig cerebral cortex and rat spleen (Table 1). Although there were indications that [^{3}H]-prazosin labels two α_1-adrenoceptor populations with different affinities, this multiplicity of binding sites was probably due to displaceable non-linear filter binding (cf. Veenstra *et al.*, 1992). It is obvious that [^{3}H]-prazosin does not discriminate either between α_1-adrenoceptors from different species, or between α_1-adrenoceptors from central nervous- and peripheral tissue.

Displacement of [^{3}H]-prazosin by WB 4101 and phentolamine showed that the α_1-adrenoceptor population in guinea-pig cerebral cortex is not homogeneous: approximately 30% of total receptors are of the α_{1A}- and 70% of the α_{1B}-adrenoceptor subtype (Table 2). By contrast rat spleen contains a homogeneous population of α_1-adrenoceptors, most likely of the α_{1B}-type. This is in agreement with other results (Han *et al.*, 1987; Minneman *et al.*, 1988).

The close resemblance in the affinity constants of phentolamine and WB 4101 for α_1-adrenoceptors in the various

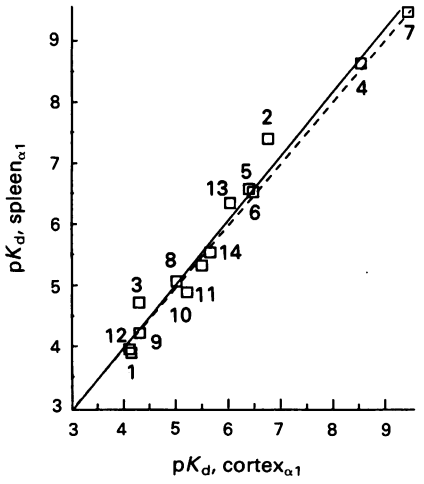

Figure 1 Linear regression analysis between pK_d-values of non-selective compounds for α_1-adrenoceptors in guinea-pig cerebral cortex and rat spleen membranes: (1) amidephrine; (2) amosulalol; (3) B-HT 920; (4) doxazosin; (5) labetalol; (6) naphazoline; (7) prazosin; (8) St 1965; (9) timolol; (10) tolazoline; (11) UK 14,304; (12) xylazine; (13) xylometazoline; (14) yohimbine. The continuous line is described by $y = (1.05 \pm 0.10) \times - 0.20$; $n = 14$, $R^2 = 0.977$ and $F = 515.11$. The dashed line represents line of identity.

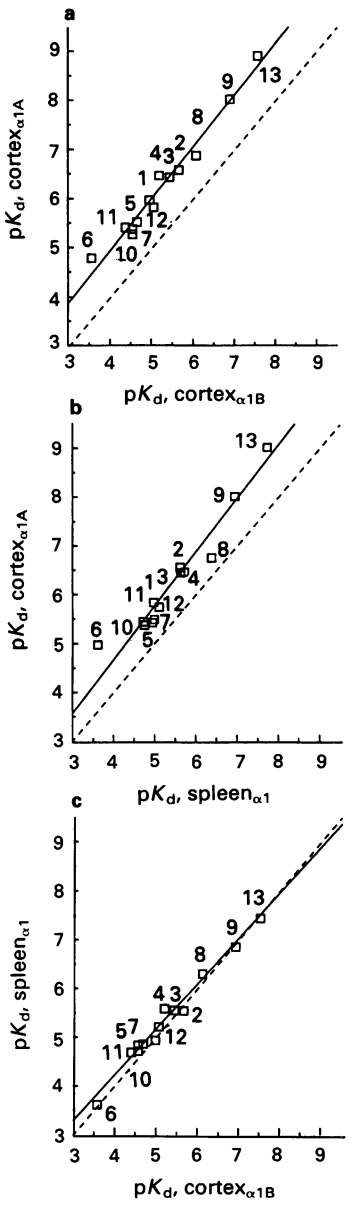

Figure 2 Linear regression analyses on pK_d-values of selective compounds for α_{1A}- and α_{1B}-adrenoceptors in guinea-pig cerebral cortex (a); α_{1A}- and α_1-adrenoceptors in guinea-pig cerebral cortex and rat spleen membranes (b); and α_1-adrenoceptors in rat spleen and α_{1B}-adrenoceptors in guinea-pig cerebral cortex membranes respectively (c). (1) (−)Adrenaline; (2) cirazoline; (3) clonidine; (4) idazoxan; (5) indanidine; (6) methoxamine; (7) (−)-noradrenaline; (8) oxymetazoline; (9) phentolamine; (10) phenylephrine; (11) (−)-propranolol; (12) St 587; (13) WB 4101. Continuous lines are described by: (a) $y = (1.06 \pm 0.17) \times + 0.70$; $n = 13$, $R^2 = 0.945$ and $F = 190.02$. (b) $y = (1.09 \pm 0.25) \times + 0.35$; $n = 13$, $R^2 = 0.896$ and $F = 195.14$ and (c) $y = (0.93 \pm 0.10) \times - 0.20$; $n = 13$, $R^2 = 0.972$ and $F = 385.73$. Dashed lines represent lines of identity.

tissues confirms the prazosin data that there are no apparent differences in α_1-adrenoceptors between the central nervous system and peripheral organs nor between different species (Table 2), at least not for the compounds tested.

In rat spleen, the displacement curves of most compounds have pseudo Hill-slope coefficients not significantly different from unity. The exceptions were oxymetazoline, xylometazoline, naphazoline and UK 14,304, compounds which are all able to displace filter-bound [^{3}H]-prazosin during the construction of a displacement curve (unpublished observations). The error introduced by this artifact is, however, small since all displacement curves obtained for rat spleen membranes fitted significantly better to the one-site model than to the two-site model.

Table 3 summarizes the affinities of 26 compounds for the α_1-adrenoceptor (subtypes) in guinea-pig cerebral cortex and rat spleen membranes. To avoid interference with possible GTP-sensitive high- and low-affinity states (Geynet *et al.*, 1981; Colucci *et al.*, 1985; Ernsberger & U'Prichard, 1987), all displacement curves of the (full) agonists were constructed in the presence of 0.1 mM Gpp(NH)p. The respective dissociation constants of the agonists will therefore probably be for the low-affinity state of the α_1-adrenoceptor (subtypes).

Displacement curves in guinea-pig cerebral cortex membranes were in most cases biphasic, with pseudo Hill-slopes smaller than unity (not shown). Although this suggested that most compounds were able to discriminate between α_{1A}- and α_{1B}-adrenoceptors, only the displacement curves of half of the compounds tested could be fitted significantly better to a two- than to a one-site model. The percentage sites with the highest affinity (presumably α_{1A}-adrenoceptors in all cases) fluctuated between 15 and 45%. The mean abundance of the α_{1A}-adrenoceptor is 30% and this figure compares well with the results of the WB 4101 and phentolamine displacements. Compounds giving highest deviations of 30% were mostly compounds with low subtype selectivity.

Table 3 also summarizes the selectivity ratios of those compounds that could be fitted best to a two receptor model (for comparison WB 4101 selectivity = 34.9). It is rather remarkable that all selective compounds show a preference for the α_{1A}-adrenoceptor. This does not mean that no α_{1B}-adrenoceptor selective compounds exist at present, but merely that there were none among those tested or that the α_{1B}-adrenoceptor selectivity is so poor that we could not prove it significantly (e.g. xylometazoline, Table 3). Actually Michel *et al.* (1989) reported that spiperone, although not a specific α_1-adrenoceptor compound, apparently displayed α_{1B}-adrenoceptor selectivity. Among the selective compounds are also the non-selective β-adrenoceptor antagonist, propranol, and the calcium entry blocker, verapamil, both displaying a relatively high affinity for the α_{1A}-adrenoceptor. If these affinities prove to be correct, these compounds should be employed with caution in functional studies. From Table 3 it follows the timolol would be a better choice in functional α_1-adrenoceptor studies to prevent interaction with β-adrenoceptors, especially since it additionally has higher affinity for β-adrenoceptors than propranolol, resulting in a more favorable α_1-:β-adrenoceptor affinity ratio.

Correlation analysis between pK_d-values of non-selective compounds for α_1-adrenoceptors in guinea-pig cerebral cortex and rat spleen (Figure 1) indicate that no apparent differences exist between the α_1-adrenoceptors in guinea-pig cortex and rat spleen. The results show a high level of identity over a range of more than 4 orders of magnitude. Correlation analysis between the pK_d-values for α_{1A}-adrenoceptors in guinea-pig cerebral cortex and either the pK_d-values for α_{1B}-adrenoceptors in the guinea-pig cerebral cortex (Figure 2a) or in the rat spleen membranes (Figure 2b), apparently indicate a high correlation as well: over a range of more than 4 orders of magnitude, the points lie close to a straight line with a slope close to unity and a correlation coefficient of 0.95 and 0.90. However, these lines do not match the lines of identity. These results emphasize once more the fundamental differences between α_{1A}- and α_{1B}-adrenoceptors.

When, however the affinities of selective compounds for the putative α_{1B}-adrenoceptors in guinea-pig cerebral cortex membranes are plotted against the affinity for α_1-adrenoceptors in rat spleen (Figure 2c), correlation is again high but now the regression line coincides with the line of identity. These results strongly support the hypothesis that the α_1-adrenoceptors in rat spleen belong to a homogeneous α_{1B}-adrenoceptor population.

In summary, the present study shows that guinea-pig cerebral cortex has 30% α_{1A}- and 70% α_{1B}-adrenoceptors, rat spleen 100% α_{1B}-, rat thoracic aorta mainly α_{1A}- and guinea-pig thoracic aorta mainly α_{1B}-adrenoceptors. All the present results show that there are no apparent differences between the α_{1B}-adrenoceptors in guinea-pig cortex and rat spleen. Functional experiments to confirm the (non)selectivity of the compounds tested are in progress at present.

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Elevation of cytosolic calcium by cholinoceptor agonists in SH-SY5Y human neuroblastoma cells: estimation of the contribution of voltage-dependent currents

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1 Muscarinic but not nicotinic receptor stimulation in SH-SY5Y human neuroblastoma cells induces a concentration-dependent increase in [^{3}H]-inositol phosphate formation and a biphasic increase in $[Ca^{2+}]_i$. The latter involves release from both an intracellular store and Ca^{2+} entry across the plasma membrane. Here we examine the possibility that this agonist-stimulated Ca^{2+} entry occurs indirectly, as a consequence of depolarization.

2 Electrophysiological characterization, by whole cell patch-clamp techniques revealed that SH-SY5Y cells possess a tetrodotoxin-sensitive inward sodium current, a dihydropyridine-insensitive calcium current and an outward potassium current which was blocked by tetraethylammonium, 4-aminopyridine and intracellular caesium ions. The outward potassium current showed voltage-dependent activation and inactivation, similar to that seen for A-currents.

3 Application of nicotinic agonists evoked an inward current in cells voltage-clamped at negative holding potentials, but this current rectified, resulting in little or no outward current flow at positive potentials. The mean amplitude at a holding potential of -60 mV was -1.14 nA. Extrapolation of the current-voltage relation gave a reversal potential of $+8$ mV, indicative of a non-specific cationic permeability.

4 Application of muscarinic agonists had no detectable effect in most of the cells tested. However, in one third of cells studied, a small slowly activating inward current was observed. The mean amplitude of this current at a holding potential of -60 mV was -8.3 pA.

5 This study confirms that SH-SY5Y cells possess voltage-dependent sodium, potassium and calcium currents. In addition, these cells are strongly depolarized by nicotinic agonists, which produce little change in $[Ca^{2+}]_i$. On the other hand, muscarinic agonists produce profound changes in $[Ca^{2+}]_i$ with only a small inward current (depolarization). The contrasting effects of these two cholinoceptor agonists strongly implies that the Ca^{2+} entry after muscarinic receptor activation is not primarily due to activation of voltage-dependent calcium channels.

Keywords: SH-SY5Y human neuroblastoma; $[Ca^{2+}]_i$; inositol phosphates; muscarinic receptors, nicotinic receptors, voltage-clamp; potassium current; sodium current; calcium current

Introduction

Stimulation of M_3 muscarinic receptors expressed by SH-SY5Y human neuroblastoma cells (Lambert *et al.*, 1989) leads to increased turnover of membrane phosphoinositides and increased intracellular ionized free Ca^{2+} (Lambert & Nahorski, 1990a). We have characterized this $[Ca^{2+}]_i$ response as biphasic, involving Ca^{2+} release from an intracellular inositol (1,4,5)P_3-sensitive site and Ca^{2+} entry across the plasma membrane (Lambert & Nahorski, 1990a). The existence of an inositol (1,4,5)P_3-sensitive Ca^{2+} pool in SH-SY5Y cells has been demonstrated by both $^{45}Ca^{2+}$ preload-release experiments and Ca^{2+}-sensitive microelectrodes (Wojcikiewicz *et al.*, 1990). In recent studies from this laboratory, inositol (1,3,4,5)P_4 has also been shown to mobilize $[Ca^{2+}]_i$ from these cells, probably by interacting with a separate receptor to that for inositol (1,4,5)P_3 (Gawler *et al.*, 1990; 1991).

The mechanism by which muscarinic receptor occupancy allows Ca^{2+} entry in these cells has been of considerable interest, with G-protein, second messenger, voltage and direct receptor-operated mechanisms being proposed (Meldolesi & Pozzan, 1987; Putney, 1990; Lambert & Nahorski, 1990b). The Ca^{2+} entry component in SH-SY5Y cells is not influenced by pertussis toxin pretreatment (in a system in which similar treatment abolished α-adrenoceptor-mediated inhibition of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) formation), indicating that the M_3 receptor-coupling to the channel is not regulated by such a G-protein (Lambert & Nahorski, 1990c). There is also indirect evidence that Ca^{2+} entry may not be regulated by inositol polyphosphates, as Ca^{2+} entry still occurs at low concentrations of agonist that do not increase either inositol (1,4,5)P_3 or inositol (1,3,4,5)P_4 mass (Lambert & Nahorski, 1990a; Lambert *et al.*, 1991).

Direct evidence for the existence of G-protein and second messenger operated Ca^{2+} channels is relatively sparse. In contrast, voltage-sensitive Ca^{2+} channels (VSCC) have been well characterized and in neuronal cells provide the major mechanism for Ca^{2+} entry (Tsien *et al.*, 1988). Whilst SH-SY5Y cells do express L- and N-type voltage-sensitive Ca^{2+} channels, they do not appear to be involved in carbachol-stimulated Ca^{2+} entry (Lambert *et al.*, 1990). On the other hand, muscarinic agonists are capable of exciting neurones, generally by inhibiting K^+-currents (Nakajima *et al.*, 1986; North, 1986) which could increase the open probability of voltage operated calcium channels. Here we present complementary second-messenger and electrophysiological data characterizing voltage-sensitive and cholinoceptor agonist-gated currents in SH-SY5Y cells.

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Methods

Cell culture

SH-SY5Y human neuroblastoma stock cultures were routinely maintained in minimum essential medium supplemented with 2 mM L-glutamine, 100 iu ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin, 2.5 $\mu g\ ml^{-1}$ fungizone and 10% foetal calf serum. Cultures were seeded into 175 cm^2 tissue culture flasks containing 30 ml of supplemented medium and maintained at 37°C in 5% CO_2/humidified air. Stock cultures were passaged at 1:6 weekly and fed twice weekly. All experimental work reported here was performed during passages 70–100.

Measurement of intracellular Ca^{2+}

Suspensions Confluent 6–7 day cultures of SH-SY5Y cells were harvested with EDTA (0.1 mM) into Krebs buffer of the following composition (in mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 4.2, HEPES 10 and glucose 10. Suspensions were loaded for 45 min at 37°C with 5 μM Fura 2/AM. Intracellular Ca^{2+} was then measured at 37°C as described previously (Lambert & Nahorski, 1990a).

Single cells SH-SY5Y cells were grown on 22 × 11 mm glass cover-slips and used 48 h after plating. The cells (on cover-slips) were loaded with 2 μM Fura 2/AM in tissue-culture medium at 37°C, then post-incubated at room temperature for a further 30 min. $[Ca^{2+}]_i$ measurements were made in single cells by standard epifluorescence microscopy (using the Photon Technology International Deltascan system) on an inverted microscope with a Peltier controlled environmental chamber (Forsythe, 1991). Single cells were optically isolated by means of manual x,y shutters fitted to the photomultiplier housing and prior to experimental determinations, background fluorescence from several single cells (unloaded) were averaged for auto-subtraction. All drugs were applied by local perfusion pipette. Intracellular Ca^{2+} was calibrated from the 340/380 nm excitation ratios using the equation of Grynkiewicz *et al.* (1985) where R_{max} (12.56), R_{min} (0.30) and sfb (16.57; the ratio of the fluorescence at baseline (380 nm) for calcium-free and calcium-replete conditions) were determined using both intracellular dye and dye in solution.

Measurement of inositol phosphate formation

Suspensions of SH-SY5Y cells (in Krebs buffer) were prelabelled with 4 $\mu Ci\ ml^{-1}$ [^{3}H]-inositol for 1 h at 37°C. The cells were subsequently test-stimulated in the presence of [^{3}H]-inositol, 5 mM Li^+ and agonist(s) for 30 min. The reaction was terminated by addition of 1 M trichloroacetic acid and inositol phosphates extracted with freon:octylamine (1:1) and neutralised with bicarbonate. Total batch [^{3}H]-inositol phosphates were separated by Dowex (chloride form) anion exchange chromatography (Rooney & Nahorski, 1986).

Data analysis

Data are expressed as mean ± s.e.mean of at least three determinations. EC_{50} (half maximum stimulation) values were obtained by computer-assisted curve fitting using ALLFIT (De Lean *et al.*, 1978). Where appropriate statistical significance was assessed by Student's *t* test and considered significant when $P<0.05$.

Patch-clamp recordings

Freshly harvested SH-SY5Y cells were re-suspended and plated onto glass cover-slips (13 mm diameter) and maintained in tissue culture for 2–4 days before use. In some experiments, the cells were induced to differentiate by treatment with 10 μM retinoic acid (Yu *et al.*, 1988), 6 days before use. On differentiation, the cells put out processes, but no change was observed in the type of currents present. Recordings were made on the stage of a Nikon Diaphot inverted microscope using a Peltier-driven environmental chamber to maintain a constant temperature of 25°C (Medical Systems Corp., PDMI-2; Forsythe, 1991). The extracellular fluid consisted of (in mM): NaCl 135, KCl 3, HEPES 10, glucose 10, $CaCl_2$ 2 and $MgCl_2$ 1. Tetrodotoxin 1 μM, was added on occasion to block sodium currents. For experiments on calcium currents the extracellular medium contained (in mM): tetraethylammonium chloride 125, HEPES 10, barium acetate 20 and tetrodotoxin 1 μM. Whole-cell patch recordings were made from the cells using patch electrodes filled with (in mM): K^+ gluconate 145, HEPES 10, EGTA 10, $CaCl_2$ 1, $MgCl_2$ 2 or in later experiments with: KCl 130, EGTA 5, HEPES 10 and $MgCl_2$ 1, ATP 5 and GTP 0.5. Patch electrodes had resistances of 3 to 7 MΩ. Potassium methyl-sulphonate, caesium methyl-sulphonate or caesium chloride were substituted for the potassium gluconate where noted in the text. Patch electrode and extracellular solutions were adjusted to pH 7.3; and the osmolarity adjusted to 310 and 320 mOsM, respectively, with sucrose. Whole-cell patch recordings were obtained from the neuroblastoma cells at least 2 days after plating. Drugs were applied by pressure ejection from a local puffer pipette which was constructed in an identical manner to the patch pipettes, but not fire polished.

An Axopatch 2A voltage-clamp amplifier (Axon Instruments) was used for recording, data were digitised using a 1401 interface (Cambridge Electronic Design, CED), stored and analysed on a Hewlett Packard or Dell 325D computer using 'Patch' software (CED). Digital leak subtraction was carried out prior to current-voltage analysis by scaling and subtraction of responses to small (5–10 mV) hyperpolarizing or depolarizing pulses.

Drugs

D/L-Muscarine chloride, carbachol chloride, nicotine hydrogen tartrate, tetrodotoxin (TTX), tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP) and Fura 2/AM were obtained from Sigma. [^{3}H]-inositol (17 Ci $mmol^{-1}$) was obtained from NEN, Dupont, U.K.

Results

Effect of cholinoceptor agonists on $[Ca^{2+}]_i$ and [^{3}H]-inositol phosphate accumulation

Both carbachol and muscarine (100 μM) caused a biphasic increase in $[Ca^{2+}]_i$ when measured in populations of cells in suspension (Figure 1a) or in single cells (Figure 1b). In suspensions, basal $[Ca^{2+}]_i$ rose from about 100 nM to approximately 500 nM, peaking 11.4 s after agonist addition (the time for a single ratio measurement in this sytem being 3.8 s). In single cells, $[Ca^{2+}]_i$ rose from about 70 nM to 750 nM in about 4 s (the time measurement of a single ratio-pair was 1 s, see Table 1). The plateau phase to carbachol and muscarine was Ni^{2+} sensitive (using cells in suspension), confirming the involvement of extracellular Ca^{2+}. In contrast to the experiments on cell populations, single cells showed a small increase in the $[Ca^{2+}]_i$ in response to nicotine, with 1 mM increasing $[Ca^{2+}]_i$ 48% over basal and 0.1 mM nicotine raising it by 46% over basal (see lower trace Figure 1b and Table 1).

Both carbachol and muscarine caused a concentration-dependent increase in total [^{3}H]-inositol phosphate formation. Maximum and half-maximum accumulation occurred at 1 mM and 13.7 ± 0.24 μM and 1 mM and 8.25 ± 1.09 μM, for carbachol and muscarine respectively. The half-maximum values did not differ significantly (Figure 2). Nicotine (1 mM) did not significantly increase total [^{3}H]-inositol phosphate formation (1.13 ± 0.19 fold over basal at 1 mM).

Characterization of voltage-sensitive currents

Whole cell patch-clamp recordings were obtained from 103 SH-SY5Y cells growing on glass cover-slips. The membrane potential was voltage-clamped over the range of −120 to +40 mV. Cell input resistances ranged from 0.35 to 3.6 GΩ in individual cells using the potassium gluconate based patch solution (mean: 1.85 ± 1.10 GΩ, $n = 10$) and were linear over a voltage range of −100 to −40 mV. Step depolarizations from a holding potential of −100 mV activated a transient inward current, followed by a more slowly activating outward current (Figure 3). On holding at −40 mV and stepping to the same potentials, the inward current was completely inactivated, while the outward current was still present (but reduced in amplitude). The inward current activated with voltage steps to around −20 mV and peaked on stepping to about 0 mV. This transient current was blocked by the addition of 1 μM tetrodotoxin to the extracellular medium (data not shown).

The outward current activated on stepping to command potentials of between −10 and 0 mV, taking 10 to 15 ms to reach a peak, before slowly inactivating during a maintained voltage step. This current was sensitive to several potassium channel blockers. Application of 10 mM TEA from a puffer pipette had a potent blocking action, as illustrated for one cell in Figure 4a. TEA (10 mM) blocked 68.4 ± 5.0% (± s.d.) of the total outward current in three cells.

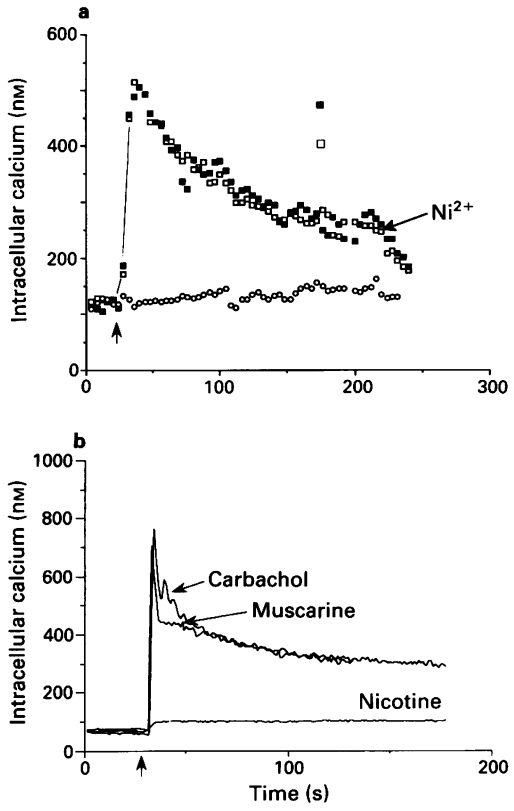

Figure 1 Muscarine and carbachol induced a biphasic increase in $[Ca^{2+}]_i$. (a) Cell suspension: muscarine (100 μM, □), carbachol (100 μM, ■) or nicotine (1 mM, ○) were added at the first arrow. Ni^{2+} (1 mM) was added at the second arrow, blocking the plateau phase of the $[Ca^{2+}]_i$ signal (Ni^{2+} did not quench the fluorescent signal). Data are from a single representative experiment (see Table 1). (b) Single cells: muscarine (100 μM), carbachol (100 μM) and nicotine (1 mM) were applied from a local puffer pipette and show essentially identical responses to the cells in suspension. Each response is from a different cell.

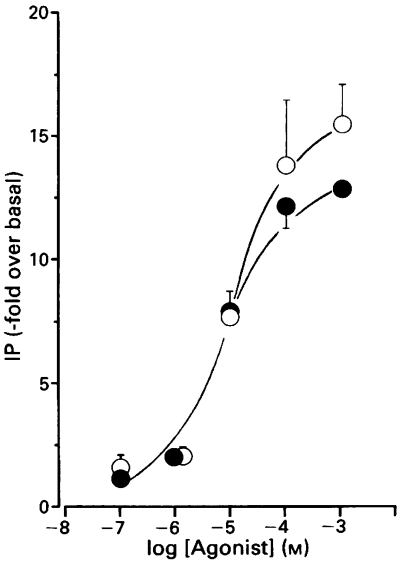

Figure 2 Muscarine and carbachol stimulate inositol phosphate formation. Cells were pre-labelled with 4 μCi ml^{-1} [^{3}H]-inositol for 1 h at 37°C. Cells were then test-stimulated in the presence of [^{3}H]-inositol, 5 mM Li^+ and the respective agonist for 30 min. Total [^{3}H]-inositol phosphates (IP) were separated by Dowex anion exchange chromatography, the concentration-response relationship is plotted for muscarine (●) and carbachol (○) stimulated IP formation. Data are mean ± s.e.mean (vertical bars) ($n = 3$).

Table 1 Effect of carbachol, muscarinic and nicotine on $[Ca^{2+}]_i$ in populations and in single SH-SY5Y cells

Agonist	$[Ca^{2+}]_i$ (nM)			
	Basal	*Peak*	*Plateau*	*After Ni^{2+}*
A				
Populations				
Carbachol (100 μM)	99 ± 13	505 ± 2*	266 ± 10*	130 ± 4
Muscarine (100 μM)	97 ± 16	487 ± 17*	265 ± 7*	104 ± 8
Nicotine (1 mM)	98 ± 18	—	—	—
B				
Single cells				
Carbachol (100 μM)	63 ± 3	766 ± 83*	349 ± 36*	—
Muscarine (100 μM)	71 ± 7	749 ± 83*	267 ± 7*	—
Nicotine (1 mM)	78 ± 3	116 ± 5*	100 ± 2**	—
Nicotine (100 μM)	62 ± 4	90 ± 5*	87 ± 5**	—

Data are mean ± s.e.mean ($n = 3$–5 for **A** and 4–6 for **B**). The plateau was taken 120 s after agonist addition in **A**. and 150 s after agonist addition in **B**. The data in the column labelled 'After Ni^{2+}' were taken 90 s after addition of 1 mM Ni^{2+}.
*$P < 0.05$ compared with basal.
**These responses were measured at 150 s after agonist addition as no distinct plateau was observed.

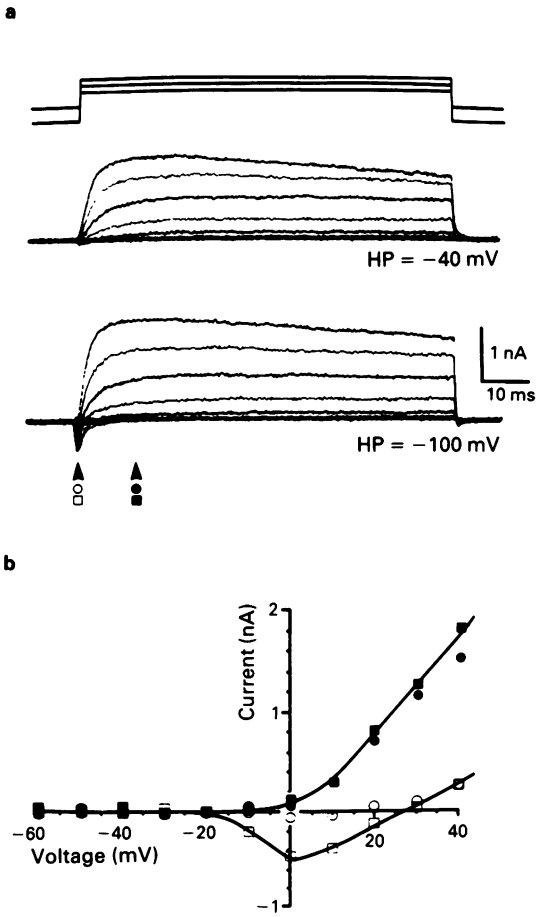

Figure 3 Voltage-sensitive currents: SH-SY5Y cells possess a fast transient inward current and a more slowly inactivating outward current. (a) Two groups of current records from the same cell are shown on stepping to voltages over a range of −20 to +40 mV from a holding potential (HP) of either −40 mV (top) or −100 mV (bottom). On stepping from −100 mV a fast inward current is evoked, which inactivates within the first 5 ms, thereafter an outward current is observed. On stepping over the same voltage range, from a HP of −40 mV, the inward current is inactivated, so the outward current is seen in isolation. (b) The current-voltage relation is plotted at the latencies indicated by the symbols in the current traces. Open symbols show the fast inward current and closed symbols show the outward current. Circles indicate the data from a HP of −40 mV and squares show that from a HP of −100 mV. The inward current activates on depolarizations beyond −20 mV but is inactivated on stepping from a HP of −40 mV. The outward current activates on depolarization beyond −10 mV but shows little inactivation over this time period (but see Figure 4).

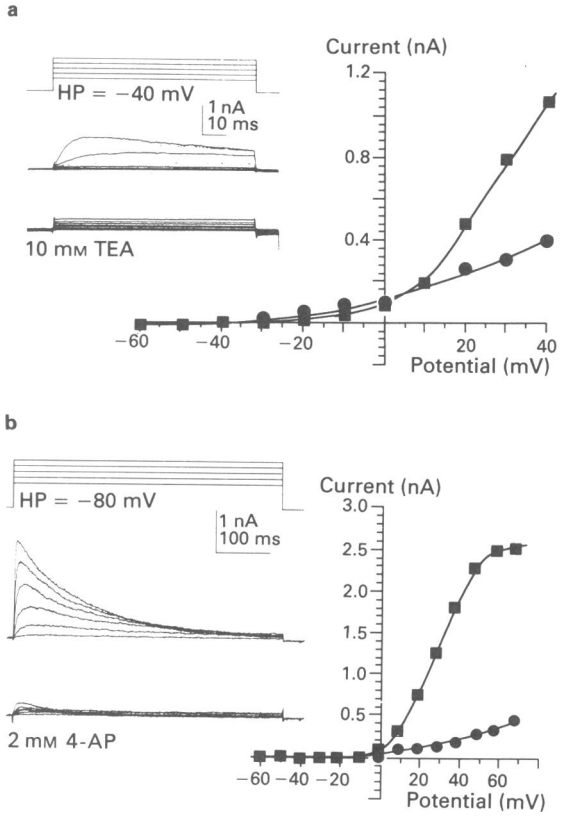

Figure 4 The transient outward current is blocked by (a) tetraethylammonium (TEA) and (b) 4-aminopyridine (4-AP). In each panel the upper records show the voltage protocol, with depolarizing steps from a holding potential of −40 or −80 mV. The currents evoked by this protocol are shown in the middle, under control conditions and the currents evoked during perfusion of the respective antagonists are shown in the lower records. Note the longer duration of the protocol in (b), illustrating the transient nature of the outward current. The respective current-voltage relations for the peak outward current is plotted on the lower right of each panel for control conditions (■) and during perfusion of the antagonist (●). All data were recorded in the presence of 1 μM tetrodotoxin.

The transient nature of this outward current is more obvious with longer duration command potentials, such as the 500 ms steps shown in Figure 4b. Puffer application of 2 mM 4-AP blocked the outward current by 80.8% ± 14.6% ($n = 4$, see Figure 4b). Substitution of K^+ in the patch pipette solution by caesium ions also blocked the outward current (data not shown). In addition, the outward current was inactivated by holding the membrane potential at progressively more depolarized levels. Examination of the activation and inactivation profile for the outward current suggests that it is an A-type current (transient outward potassium current). Both activation and inactivation curves were fitted by eye to Boltzmann equations of the form:

$$G = G_{max}/[1.0 + \exp((V\text{-}v')/k)] \quad (1)$$

Where G is the relative conductance, V is the membrane potential; v′ is the half-activation voltage and k is the slope factor. These fits show that half-inactivation (v′) was at −56.0 ± 8.9 mV, with a slope factor (k) of 11.2 ± 0.9 mV; while half-activation (v′) was at +24.6 ± 4.3 mV, and had a slope factor (k) of −10.2 ± 0.4 mV ($n = 3$, ± s.e.mean).

The effects of muscarine or carbachol (100 μM or 1 mM) were examined on the magnitude and activation of the outward potassium currents in 14 cells (11 in response to muscarine and 3 with carbachol). No changes were observed. We also checked for the presence of other voltage-gated potassium currents by running current-voltage relation protocols from holding potentials of −30 mV and −60 mV; no indication of an M-current or an inwardly rectifying potassium current were observed (data not shown). In addition, the voltage-dependent potassium currents and response to muscarine were examined in 3 cells by use of the nystatin permeabilised patch technique (Horn & Marty, 1988). No additional currents were observed. One of these cells showed an inward current on application of muscarine, which was identical to the responses observed with conventional patch recording techniques (see below).

Voltage-gated calcium currents

With physiological calcium concentrations (2 mM $[Ca^{2+}]_o$), calcium currents were too small for examination by whole cell recording techniques. Recordings were therefore made

with a caesium methyl-sulphonate based pipette solution and with barium as the extracellular charge carrier; TEA (125 mM) and TTX (1 μM) were used to block potassium and sodium currents. Under these conditions inward currents were observed on stepping from a holding potential of −100 mV to around 0 mV (Figure 5). No inward current was observed if the same protocol was repeated from a holding potential of −50 mV. The relative amount of inactivation of the current during the voltage step was quite variable from one cell to another and the current was insensitive to the dihydropyridine calcium channel antagonist, PN 200-110 (isradipine, Sandoz) when applied from a local puffer pipette at a concentration of 1 μM ($n = 3$). These findings suggest that this current is a high-voltage-threshold (N-type) calcium current. The results are broadly similar to those obtained previously in these cells by Seward & Henderson (1990).

Cholinoceptor agonist-gated currents

Whole cell recordings in current-clamp mode showed a large but transient depolarization lasting about 3 s on puffer application of 100 μM or 1 mM carbachol, as shown in Figure 6a. Further application of the agonist was without effect, indicating that the receptors desensitized, with recovery requiring about 2 min of washing. Under voltage-clamp, carbachol or nicotine produced a transient inward current at a holding potential of −60 mV (Figure 6b). These results suggest that SH-SY5Y cells possess an ionotropic nicotinic receptor. The current-voltage relation (I/V) of the nicotinic response was estimated in 7 cells on application of either carbachol or nicotine. The I/V was linear at negative holding potentials, but rectified, such that at positive potentials little outward current was observed. The mean amplitude of the inward current in 5 cells measured at a holding potential of −60 mV was -1.14 ± 0.63 nA. The mean potential at which no current was measured (reversal potential) was estimated to be $+8.1 \pm 1.5$ mV ($n = 7$, ± s.e.mean) by extrapolation of the curve at negative potentials (extrapolation was used due to the rectification).

Muscarine (100 μM or 1 mM) applied from a local puffer pipette produced no detectable current in 15 cells tested using the potassium gluconate or caesium methyl-sulphonate based patch solution (see methods), but in a second series of experiments a small inward current was observed in 11 of the 28 cells tested using potassium or caesium choloride based

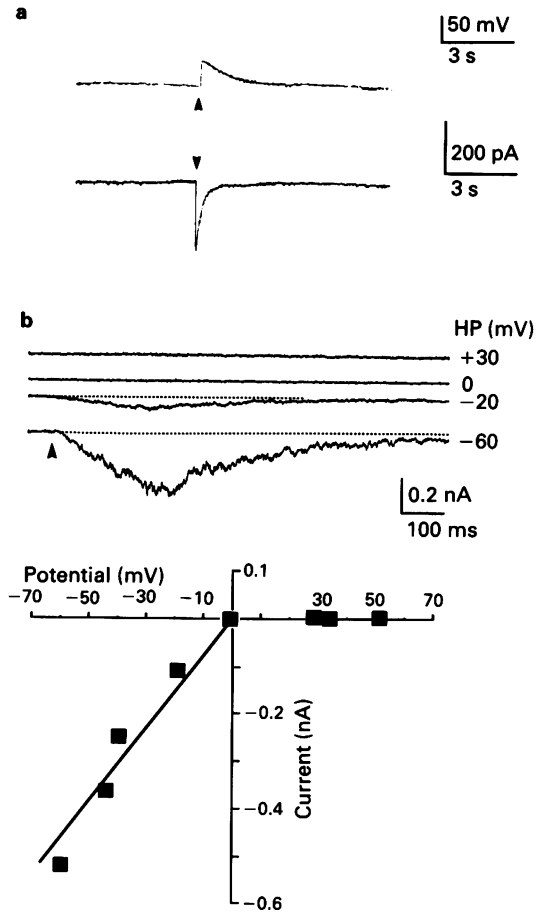

Figure 6 Electrophysiological action of nicotinic agonists. (a) In current-clamp recordings 100 μM carbachol induced a large depolarization of the SH-SY5Y cells when the resting potential was −60 mV (top trace). In complementary voltage-clamp experiments at the same potential, carbachol induced an inward current (lower trace) which desensitized for several minutes after a single application. (b) Multiple responses to carbachol are illustrated for a single cell at the holding potentials indicated by each current trace. Each 100 ms application of drug was followed by 2 min washing to permit recovery from densensitization. The current-voltage relation for these data is plotted below.

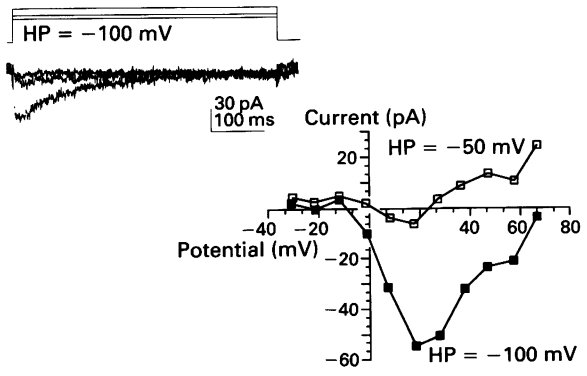

Figure 5 Voltage-dependent calcium current. Whole-cell recordings were made under conditions where sodium and potassium currents were blocked with extracellular tetrodotoxin and tetraethylammonium and with Cs^+ substituted for K^+ in the patch solution. Using barium as the extracellular charge carrier, currents due to activation of voltage-sensitive calcium channels were evoked from a HP of −100 mV on step depolarizations to around 0 mV. Example records are shown for steps to 0, 18 and 38 mV in the upper inset. The current-voltage relation is plotted below for the peak inward current in a single cell, on stepping from a holding potential of either −100 mV (■) or −50 mV (□).

patch medium. An example of the response is shown in Figure 7a, with the same response shown over a faster time-course below. Muscarine 100 μM was applied for 500 ms by pressure ejection from a pipette positioned approximately 50 μm from the cell under study. The time-course of the response was very slow, with no inward current detected until after the agonist application had ceased. The peak response occurred after 2–4 s and then decayed back to baseline over the next 30–60 s. Recovery of the response required washing for at least 3 min and each subsequent application induced a smaller current, such that a maximum of only 3 or 4 applications could be tested on one cell. The rundown made it difficult to construct a current-voltage relation to sequential responses in a single cell. No reversal of the current was observed over a voltage-range from −100 to +40 mV, but results in several different cells showed that the current declined with both hyperpolarization and depolarization from the control potential of −60 mV. The effect of changing the holding potential is shown in Figure 7c and 7d, for two different cells. The response is shown first at a holding potential of −60 mV and then after changing to a potential of either −100 or −20 mV. In both cases the current is smaller at the new voltage, with some recovery observed on returning to −60 mV, confirming that the depression was not due to

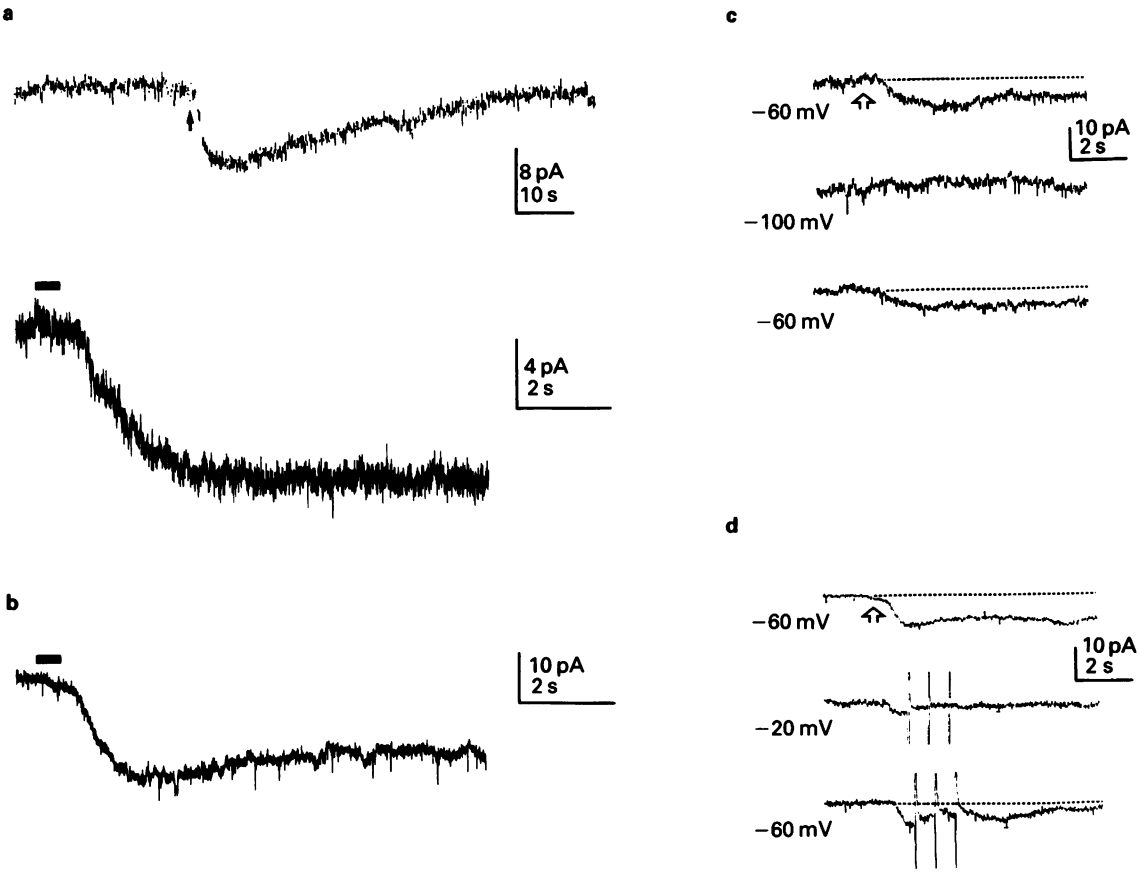

Figure 7 Electrophysiological action of muscarinic agonists. (a) Whole cell recording on application of 100 μM muscarine for 500 ms (arrow) at a HP of −60 mV. A slow inward current is evoked after a delay of about 1 s; the lower trace shows the same data plotted on a faster time base. (b) On substitution of K^+ for Cs^+ in the patch pipette, similar currents were evoked to those observed in control conditions. (c and d) The current was voltage-dependent, but no reversal was observed on changing the HP. Three consecutive applications of muscarine were made at 5 min intervals. In the cell illustrated in (c) the HP was shifted from −60 to −100 mV. In another cell the HP was changed from −60 mV to −20 mV as shown in (d) (the transients in the lower two traces are artifacts due to the application of ramp-voltage protocols). The recovery responses were recorded on return to a HP of −60 mV in each case.

rundown. Examination of current flow during a ramp voltage protocol (from +20 to −100 mV, 0.4 mV ms^{-1}) under control conditions compared with a similar ramp during agonist application also gave no reverse potential. Thus the ionic basis of this current could not be determined. Further work using a rapid perfusion system is required, in order to change the extracellular ionic composition during the response. Those responses observed in the presence of intracellular caesium were not significantly different from those recorded with potassium in the patch pipette (Figure 7b), so the data were pooled. The mean inward current at a holding potential of −60 mV was −8.3 ± 5.8 pA in the 11 cells which responded to muscarine.

Discussion

Stimulation of SH-SY5Y neuroblastoma cells with the cholinoceptor agonist carbachol results in a dramatic increase in the $[Ca^{2+}]_i$, part of which arises from calcium entry through the plasma membrane (Lambert & Nahorski, 1990a; Murphy *et al.*, 1991). Although in previous experiments we were able to eliminate the involvement of L and N Ca^{2+} channels, the evidence presented here indicates that SH-SY5Y cells possess both muscarinic and nicotinic receptors, here in view of the mixed, cholinoceptor, agonist nature of carbachol, it is conceivable that Ca^{2+} influx could occur as the result of depolarization mediated by nicotinic receptors. In a recent study, nicotinic receptors expressed by the parent SK-N-SH cell were shown to couple to Ca^{2+} influx through voltage-sensitive Ca^{2+} channels (Noronha-Blob *et al.*, 1989). However, in contrast to the muscarinic response described previously by us (Lambert & Nahorski, 1990a), the nicotinic response described here in SH-SY5Y cells is not coupled to phosphoinositide turnover. Whilst there is a nicotinic receptor-linked depolarization, only trivial increases in $[Ca^{2+}]_i$ are detectable during nicotinic receptor activation, confirming our previous demonstration that the muscarinic antagonist atropine, can suppress carbachol-induced calcium entry (Lambert & Nahorski, 1990a).

Voltage-dependent channels

Previous reports indicate that SH-SY5Y cells have resting membrane potentials in the range of −50 to −80 mV, are capable of exhibiting regenerative action potentials (Kuramoto *et al.*, 1981) and possess a transient outward potassium current (Jalonen & Akermann, 1981). Our results confirm that SH-SY5Y cells possess a TTX-sensitive inward current with a voltage-dependence and reversal potential similar to that observed in other excitable tissues.

The SH-SY5Y cells also possess a relatively slowly inactivating outward potassium current. Although preliminary pharmacological studies suggested that the outward current was a delayed rectifier, on the basis of its sensitivity to TEA (Forsythe *et al.*, 1990), the more detailed characterization presented here indicates that this current also shares several characteristics of a transient outward potassium current or

A-current (Connor & Stevens, 1971). The outward current activates relatively slowly on depolarization and inactivates during the depolarizing voltage step; it requires prior hyperpolarization for maximal activation and is blocked by extracellular 4-AP and TEA. When compared to previously reported neuronal A-currents, the activation and inactivation curves observed here are at more positive potentials (Numann *et al.*, 1987; Mayer & Sugiyama, 1988; Forsythe & Stanfield, 1989). In contrast to the neuronal A-current, which is sensitive to millimolar 4-AP (ED_{50} around 2 mM) and insensitive to up to 10 mM TEA (Segal *et al.*, 1984; Numann *et al.*, 1987), the outward current in SH-SY5Y cells is blocked by both TEA and 4-AP. Sensory ganglion cells possess a slowly inactivating transient outward current which is sensitive to micromolar 4-AP but insensitive to TEA (Stansfeld *et al.*, 1986). Thus it would appear that the transient outward current in these SH-SY5Y cells exhibit characteristics of both the delayed rectifier and A-current.

Using barium as the charge carrier, we have confirmed the presence of a high-voltage threshold, dihydropyridine-insensitive current in whole-cell recordings, as has previously been observed by Seward & Henderson (1990). It is worthy of note that depolarization with K^+ (but not muscarinic receptor stimulation) induced a Ca^{2+} influx which is mediated through a dihydropyridine-sensitive mechanism (Lambert *et al.*, 1990). Recent work using on-cell patch recording techniques has confirmed that SH-SY5Y cells do possess a dihydropyridine-sensitive calcium channel (Morton *et al.*, 1992).

Do cholinoceptor agonists depolarize SH-SY5Y cells?

Previous work with the fluorescent indicator of membrane potential, bis-oxonol, has suggested that muscarinic receptors may depolarize SH-SY5Y human neuroblastoma cells (Akermann, 1989) and could therefore activate voltage-sensitive calcium channels. In our hands these cells appear to express both L and N-type Ca^{2+} channels, in that a component of the K^+-evoked rise in $[Ca^{2+}]_i$ is sensitive to dihydropyridine and ω-conotoxin GVIA. However, carbachol responses were insensitive to these antagonists and were only suppressed by Ni^{2+} (Lambert *et al.*, 1990).

In the present experiments using voltage-clamp methods, muscarinic agonists induced a small inward current in a proportion of cells, but there was no evidence of coupling with other potassium currents (e.g. M-current, as has been reported for NG108-15 cells into which muscarinic receptors had been cloned: Fukuda *et al.*, 1988; Neher *et al.*, 1988). The muscarinic response reported here was observed in only one third of cells tested. Similar small inward currents activated by acetylcholine have been observed in mammalian smooth muscle cells (Benham *et al.*, 1985) and in substantia nigral neurones, on application of muscarine (Lacey *et al.*, 1990). We were unable to demonstrate a reversal potential for the muscarinic current, although it was voltage-dependent. At present we cannot distinguish between a mechanism involving activation of an electrogenic pump or that involving ion channels, such as activation of a calcium-dependent conductance. It is conceivable that this response may be analogous to the calcium release-activated calcium current (I_{CRAC}) observed in mast cells (Hoth & Penner, 1992). The mechanism of Ca^{2+} entry regulated by phosphoinositidase C-linked receptors is uncertain. Perhaps the strongest proposals suggest regulation by the depletion of the intracellular Ca^{2+} pool (Putney, 1990) or by the action of inositol (1,4,5) trisphosphate and/or inositol (1,3,4,5) tetrakisphosphate (Irvine, 1990). Very recent patch-clamp studies have provided direct support for the idea that both mechanisms may occur independently (Hoth & Penner, 1992; Luckhoff & Clapham, 1992). Similar approaches may reveal one or both of these mechanisms associated with muscarinic receptor-induced calcium entry in SH-SY5Y cells.

In contrast to muscarine, activation of nicotinic receptors produced a large inward current in SH-SY5Y cells, which rectified and was accompanied by desensitization. These properties are very similar to those observed for neuronal nicotinic receptors (Mathie *et al.*, 1987; Sands & Barish, 1991). The positive reversal potential of the nicotinic response implies that these channels are permeable to both K^+ and Na^+. The possibility of a significant permeability to divalent cations cannot be ruled out. Recent data from PC12 cells shows that nicotinic receptors do have a significant calcium permeability (Sands & Barish, 1991), but our fluorimetric measurement of cytoplasmic calcium in single cells showed only a trivial increase with nicotine. We can conclude that while the activation of these nicotinic receptors would cause a large depolarization, the carbachol-induced Ca^{2+} influx appears to be independent of this response and is mediated by muscarinic receptors.

These data confirm that SH-SY5Y cells possess a TTX-sensitive sodium current, a calcium current and a slowly inactivating outward potassium current. In addition, the cells possess a nicotinic receptor, linked to a cation permeable channel, and a small but significant muscarinic response. Further work using nystatin-permeabilised patches may permit a more definitive characterization of the latter current. Since the inward current flow (depolarization) during the muscarinic response is so small (especially relative to that during nicotinic receptor activation) we can conclude that $[Ca^{2+}]_i$ rise in SH-SY5Y cells is predominantly due to a mechanism other than through activation of voltage-sensitive calcium channels, since if the latter mechanism were dominant, nicotinic receptor activation should also profoundly increase $[Ca^{2+}]_i$.

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In vitro characterization of prostanoid FP-, DP-, IP- and TP-receptors on the non-pregnant human myometrium

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1 Prostaglandin F (PGF), PGD, PGI and thromboxane A_2 (TXA_2) receptors have been pharmacologically characterized on the non-pregnant human myometrium *in vitro* in accordance with the receptor classification proposed by Coleman *et al.* (1984). The tools for the classification include both natural prostanoids, synthetic, selective analogues and antagonists where available.

2 The potent excitatory actions of the natural FP-receptor prostanoid, $PGF_{2\alpha}$, and the synthetic analogue, fluprostenol, indicate the presence of FP-receptors mediating contraction on the human myometrium.

3 PGD_2 produced a biphasic response consisting of excitation followed by relaxation of spontaneous activity of the myometrium. The selective DP-receptor agonists, BW245C, produced purely inhibitory responses illustrating the presence of inhibitory DP-receptors in this tissue. The inhibitory responses of both PGD_2 and BW245C were antagonized by the competitive DP-receptor antagonist, BWA 868C, providing conclusive evidence for the existence of DP-receptors.

4 PGI_2 produced a biphasic response similar to PGD_2. Iloprost, the EP_1/IP-receptor agonist also produced a biphasic response, whilst the IP-receptor selective agonist, cicaprost, caused inhibition only, suggesting that inhibitory IP-receptors exist in the non-pregnant human myometrium.

5 The TXA_2-mimetic, U46619, produced marked stimulation of the non-pregnant human myometrium and was approximately equipotent to $PGF_{2\alpha}$ and fluprostenol in this effect. The actions of U46619 were competitively antagonized by the TP-receptor antagonist GR32191 showing that excitatory TP-receptors exist in this tissue.

6 All prostanoids tested, both natural and synthetic, had activity on the non-pregnant human myometrium *in vitro*, supporting the existence of a heterogeneous population of prostanoid receptors in this tissue. If the results from the present study are combined with those previously reported for EP-receptor agonists (Senior *et al.*, 1991), it may be concluded that excitation may occur through FP-, TP-, EP_3- and few EP_1-receptors, whereas inhibition may occur through DP-, IP- and EP_2-receptors.

Keywords: Human myometrium; non-pregnant; prostanoids; synthetic analogues; antagonists; prostanoid receptors

Introduction

Initial evidence for the existence of myometrial prostanoid receptors dates back to 1967, when Pickles proposed that up to four different types existed. Since then, extensive work has led to a systematic prostanoid receptor classification. The present prostanoid receptor classification is a simple systematic working hypothesis whereby each of the natural prostanoids has its own receptor termed a P receptor where it is at least ten times more potent than any of the other natural prostanoids. Thus the prostaglandin E_2 (PGE_2)-sensitive receptors are termed the EP-receptors, $PGF_{2\alpha}$, FP-receptors, PGD_2, DP-receptors, PGI_2, IP receptors and the thromboxane A_2 (TXA_2)-sensitive receptors, TP-receptors (Kennedy *et al.*, 1982; Coleman *et al.*, 1984). Furthermore, EP-receptors are subdivided into the EP_1-, EP_2- and EP_3-receptor subtypes on the basis of selectively acting agonists and antagonists (Coleman *et al.*, 1987a,b,c).

The existence of high and low affinity PGE_2 and PGF binding sites has been demonstrated on human myometrium (Wakeling & Wyngarden, 1974; Bauknech *et al.*, 1981; Giannapoulos *et al.*, 1985; Crankshaw *et al.*, 1986; Chegini *et al.*, 1986; Adelantado *et al.*, 1988), and the binding of these prostanoids is considered an essential step in the mechanism of their action. Some workers have investigated the occurrence of binding sites at different stages of the menstrual cycle, and found no change in PGE and PGF binding (Hofmann *et al.*, 1983; Adelantado *et al.*, 1988). Studies employing natural agonists are limited because of the low specificity of such agents. The use of [^{3}H]-sulprostone, which is a synthetic receptor-selective prostanoid, has demonstrated the presence of EP_1- and or EP_3-binding sites on human myometrium (Schillinger *et al.*, 1979).

However, there have been few other such binding studies, probably due to the lack of labelled selective ligands, and the actions of specific prostanoid analogues have been tested mostly by functional studies on human isolated myometrial preparations in order to evaluate the occurrence of receptors. By use of selective prostanoid analogues, it has been possible to demonstrate the presence of EP_1-, EP_2- and EP_3-receptor sites (Senior *et al.*, 1991).

Preliminary work with U46619 and fluprostenol has demonstrated TP- and FP-receptor sites respectively (Clayton *et al.*, 1986a,b). BW245C has been shown to relax human uterus (Sanger *et al.*, 1982); and cicaprost indicates relaxant responses in human myometrium (Dyal & Crankshaw, 1988).

The aim of the present study was to examine the effects of both natural and synthetic prostanoids and their antagonists, where available, by functional studies in order to characterize further the prostanoid receptors, other than the EP receptors, on the human myometrium *in vitro* from non-pregnant donors.

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Methods

Human myometrial samples were obtained from premenopausal non-pregnant women undergoing hysterectomy for benign disorders such as fibroids and dysfunctional menorrhagia. The specimens were taken from the anterior wall of the corpus uteri and then immediately placed in Krebs solution. Myometrial strips were set up within 60 min postoperative period and superfused with oxygenated Krebs solution (95% O_2/5% CO_2) at 37°C as previously described (Senior *et al.*, 1991). Agonists were administered as bolus doses immediately after a spontaneous contraction. It is accepted that this form of drug administration may be influenced by physiochemical and pharmacokinetic factors, and equilibrium studies are currently in progress in this laboratory. The reproducibility of the myometrial response to prostanoids has been assessed by means of time studies (Sangha, 1991), the results of which indicated that time was not a variable affecting tissue sensitivity to the agonist response.

The inherent myometrial activity is variable, which makes quantification of results difficult. Because of this, measurement of response and analysis of results were undertaken as detailed in the previous paper on EP receptors (Senior *et al.*, 1991). Briefly, excitatory responses were expressed as T/B ratios, i.e. ratio of the area of the excitatory response produced by a dose of agonist to the area of the previous spontaneous background contraction. Potency for excitatory agonists was expressed as an ED_1 value, i.e. the dose of agonist required to produce a T/B ratio equal to 1. The inhibitory actions of agonists were expressed in terms of how they modified the periodicity of spontaneous activity, measured in min. A measure of the potency of an agonist producing inhibition was obtained by calculating an inhibitory dose ID_4 value which was defined as the dose of agonist required to extend the normal period between two spontaneous background contractions by a period of 4 min.

Sample traces showing excitatory responses to $PGF_{2\alpha}$ and biphasic (excitatory followed by inhibitory) responses to PGD_2 are shown in Figure 1. The compounds used are shown in Table 1. ED_1 and ID_4 values were obtained from individual dose-effect curves and expressed as geometric means with 95% confidence limits. Where antagonists were used, potency was expressed as the pA_2 value as determined by the method of Arunlakshana & Schild (1959). Statistical comparisons were made by an unpaired Student's *t* test (Snedecor & Cochran, 1979).

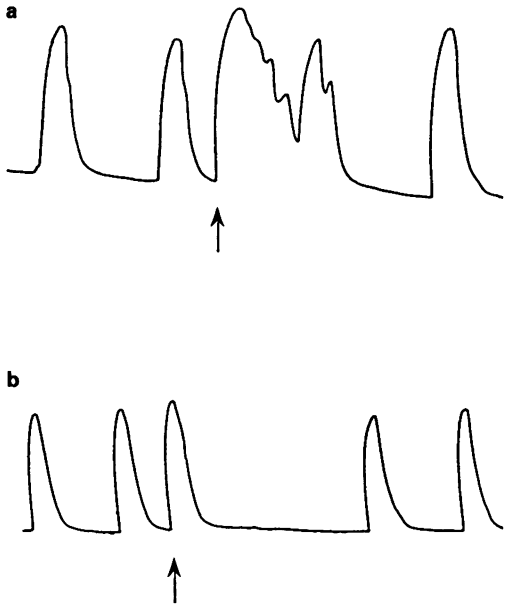

Figure 1 Sample traces showing the effect of a dose of (a) prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 1×10^{-9} mol) and (b) PGD_2 (3×10^{-8} mol) (at arrows) on the myogenic activity of human myometrium from non-pregnant donors.

Table 1 Compounds used in this study

Compound	*Stock vehicle*	*Dilutions*	*Source*
PDG_2	1% $NaHCO_3$ in 0.9% normal saline	0.9% normal saline	Glaxo
PGE_2 Dinoprostone	1% $NaHCO_3$ in 0.9% normal saline	0.9% normal saline	Upjohn
$PGF_{2\alpha}$ tromethamine salt, Lutalyse	1% $NaHCO_3$ in 0.9% normal saline	0.9% normal saline	Upjohn
PGI_2 Prostacyclin	Tris buffer pH 9 at 37°C	Tris buffer pH 8	Sigma
U46619 11α 9α-epoxymethano PGH_2	1% $NaHCO_3$ in 0.9% normal saline	0.9% normal saline	Bayer
BW245C 5-(6-carbohexyl)-1 (3-cyclohexyl-2-hydroxypropyl)-hydantoin	Ethanol	0.9% normal saline	Wellcome
ICI81008 m-trifluoromethyl-16-phenoxy 17, 18, 19 20-tetranor-$PGF_{2\alpha}$ Fluprostenol	0.9% normal saline	0.9% normal saline	ICI
Iloprost ZK 36 374 16 methyl-18-yn carbacyclin	0.9% normal saline	0.9% normal saline	Schering
Cicaprost ZK 96 480 {[1S, 5S, 6R, 7R]-3-(5-carboxy-3-Oxa-1E-penty-1 dieno)-7-hydroxy-6-(35-hydroxy-4S-methyl-nona-1,6-dinyl)- bi cyclo [3.3.0] octane}	0.9% normal saline	0.9% normal saline	Schering
BW A868C3-benoxyl-5-(6-carboxy hexyl)-1-(2-cyclohexyl -2-hydroxyethylamino) hydantoin	Ethanol	Distilled water	Wellcome
GR32191 {[1R-[1α (Z) 2β, 3β, 5α])-(+)-7-5-([1, 1-biphenyl)-4-yl) methyoxyl)-3-hydroxy-2-(1-piperidinyl) cyclopentyl]-4-heptanoic acid hydrochloride	Distilled water	Distilled water	Glaxo
Indomethacin (1-[p-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid	1% $NaHCO_3$ in 0.9% normal saline		Sigma

Results

The effect of FP receptor agonists

$PGF_{2\alpha}$ produced only an excitatory response starting at 0.014 nmol, and this increased to a maximum around 1.4 nmol. The response then declined after 14 nmol, giving the dose-response curve to $PGF_{2\alpha}$ a bell-shaped appearance (Figure 2). The response to $PGF_{2\alpha}$ was rapid and consisted of an increase in baseline tonus with frequent phasic contractions superimposed on this effect. The potency of $PGF_{2\alpha}$ was similar to that previously reported for PGE_2 (ED_1, 0.04 and 0.02 nmol respectively) and the maximum effects observed with both agonists were also similar (Table 2). The synthetic FP-receptor agonist, fluprostenol (ICI 81008) produced a response similar to $PGF_{2\alpha}$, the excitation was dose-related between 0.011–0.11 nmol, after which a further increase in dose produced little change in effect (Figure 2).

The effect of DP-receptor agonists and antagonists

PGD_2 produced a biphasic response similar to that seen with PGE_2. However, the former was less potent for both excitation and inhibition estimated by ED_1 and ID_4 values (Table 2). The excitatory component was dose-related between 0.28–14.0 nmol, whereas the inhibitory component was dose-related in the higher range of 1.4–140 nmol as shown in Figure 3a. The selective DP-receptor agonist BW245C produced only inhibition which was dose-dependent between 0.014–14 nmol (Figure 3b). Consideration of ID_4 values showed BW245C to be the most potent inhibitory agonist listed in this series of compounds (Table 2). However, it should be noted that a greater maximum effect is produced by the EP_2-receptor agonist, butaprost (Senior *et al.*, 1991).

The selective DP-receptor antagonist BW A868C (Giles *et al.*, 1989) abolished the weak inhibitory effect of PGD_2 at a concentration of 10^{-7} M. However, at the lower concentration of 10^{-8} M BW868C had no effect on the inhibitory dose-effect curve for PGD_2 (Figure 3a).

Parallel rightward displacement of the BW245C dose-response curve was observed at all concentrations of the antagonist tested 10^{-8}, 10^{-7} and 10^{-6} M (Figure 3b). Mean data from Schild analysis are shown below:

Schild regression parameter	*Value*
pA_2 (range)	8.3 (8.2–8.7)
Slope (range)	0.7 (0.6–0.84)
Regression coefficient (range)	0.97 (0.96–0.99)

All values are the geometric mean of $n = 5$ determinations.

The effect of IP-receptor agonists

The natural IP-receptor agonist, PGI_2, produced a biphasic response similar to and of similar potency to that seen with PGD_2 (Table 2). Again the excitatory component of the response occurred at lower doses than the inhibitory compo-

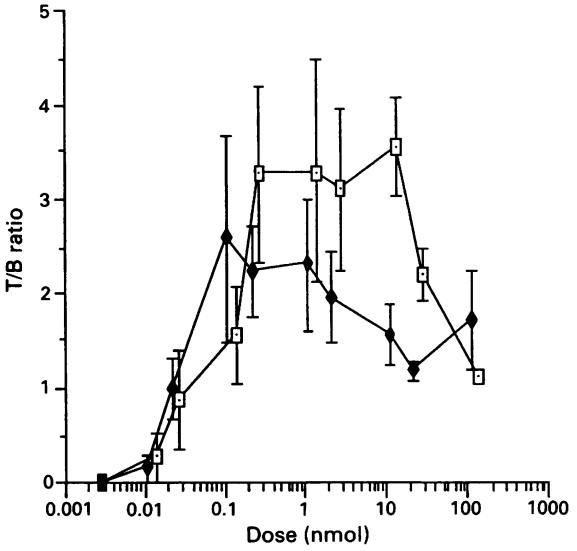

Figure 2 Mean stimulatory dose-effect curves to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, □) $n = 5$ and fluprostenol (◆) $n = 7$. Data are expressed as arithmetic means and vertical bars represent s.e.mean.

Table 2 Mean ED_1, ID_4 and maximum responses for excitation and inhibition by natural and synthetic prostanoids

	Excitation			*Inhibition*		
Prostanoid	*ED_1* (95% c.l.)	*max* (s.e.mean)	n	*ID_4* (95% c.l.)	*max* (s.e.mean)	n
PGE_2**	0.02 (0.001–0.58)	3.34 (1.69)	5	0.8 (0.2–2.9)	21.3 (4.2)	5
$PGF_{2\alpha}$	0.04 (0.01–0.14)	4.3 (0.78)	5	>140	NR	8
PGD_2	3.56 (1.25–10.2)	1.9 (0.83)	10	>1.7 (1.7>140)*	5.8 (1.5)	10
PGI_2	3.65 (0.04–31.6)	2.2 (0.48)	5	6.4 (1.2–33)	11.7 (1.9)	5
U46619	0.05 (0.02–0.10)	2.69 (0.34)	8	>140	NR	8
Fluprostenol	0.04 (0.013–0.1)	3.48 (0.75)	7	>110	NR	7
BW245C	>10	NR	7	0.05 (0.02–0.5)	39 (7)	7
Iloprost	2.74 (1.03–7.3)	2.3 (0.3)	7	0.94 (0.2–4.9)	7.6 (2.3)	7
Cicaprost	>28	NR	5	0.2 (0.02–1.0)	14.3 (1.9)	5

*PGD_2 limits for ID_4 values expressed as range.
NR = no response.
ED_1 and ID_4 values are expressed as geometric means (nmol) with 95% confidence limits in parentheses.
Maximum responses for excitation are expressed as multiples of background activity (i.e. maximum T/B ratios) and maximum responses for inhibition are expressed in min.
**Senior *et al.* (1991).

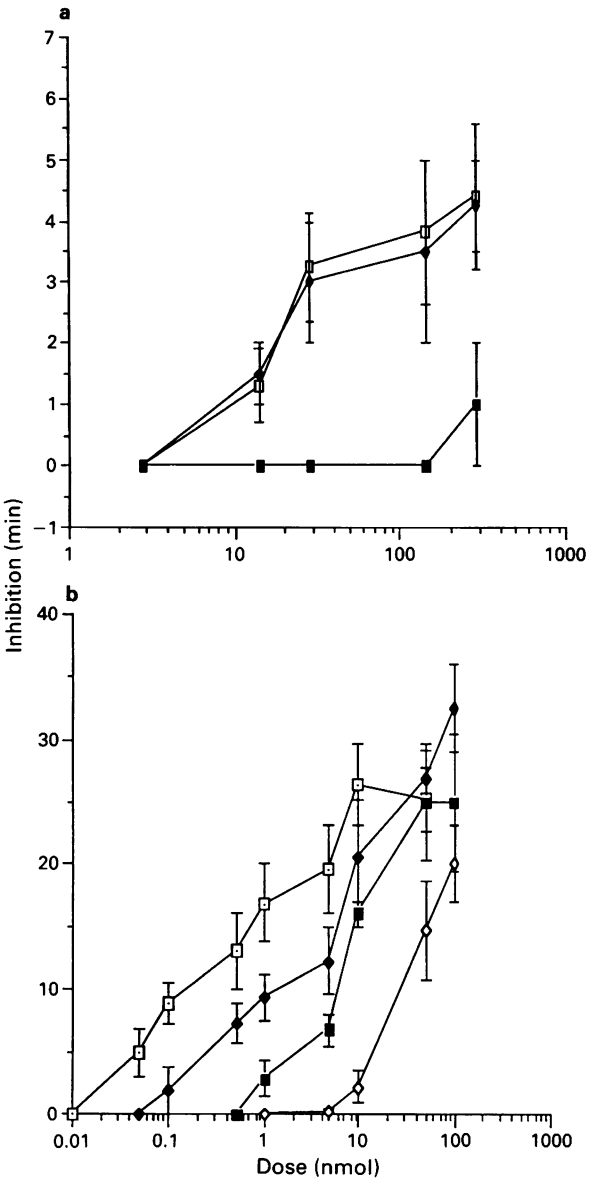

Figure 3 Mean inhibitory dose-effect curves to (a) prostaglandin D_2 (PGD_2) and (b) BW245C alone (□) and in the presence of BWA868C 10^{-8} M (◆), 10^{-7} M (■) and 10^{-6} M (◇). Data for both groups are expressed as arithmetic means of 5 determinations, and vertical bars represent s.e.mean.

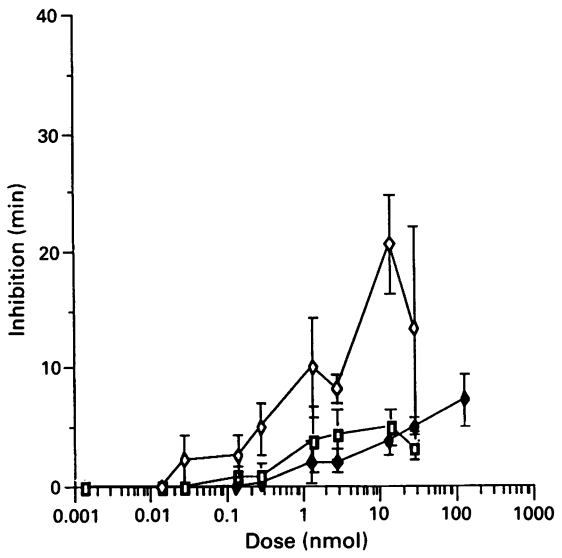

Figure 4 Mean inhibitory dose-effect curves to prostacyclin (PGI_2, ◆) $n = 5$, iloprost (□) $n = 7$ and cicaprost (◇) $n = 5$. Data are expressed as arithmetic means and vertical bars represent s.e.mean.

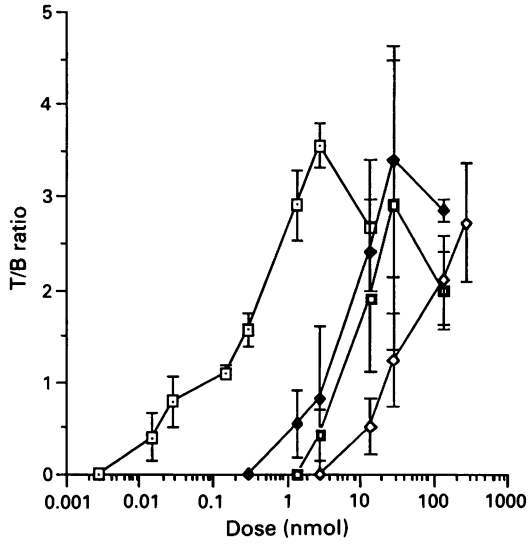

Figure 5 Mean stimulatory dose-effect curves to U46619 alone (⊡) and in the presence of GR32191 10^{-7} M (◆), 3×10^{-7} M (□) and 10^{-6} M (◇). Data are expressed as arithmetic means and vertical bars represent s.e.mean.

nent. Like PGI_2, the EP_1/IP-receptor agonist, iloprost produced a biphasic response as found previously (Senior *et al.*, 1991). The inhibition occurred at similar doses to the stimulation, i.e. between 0.14–14 nmol (Figure 4) and was dose-related within this range. As an inhibitory agonist, iloprost was more potent than PGI_2 (Table 2). In contrast, the selective IP-receptor agonist, cicaprost, produced only inhibition, similar to but less potent than BW245C. This effect of cicaprost produced a maximum inhibition of 14.3 min at 14 nmol which was the maximum dose available.

The effect of the TP-receptor agonist, U46619

The stable TP-receptor agonist U46619 produced purely contractile effects which, like those caused by $PGF_{2\alpha}$, consisted of an increase in baseline tonus with frequent phasic contractions superimposed on this effect. U46619 was a potent excitatory agonist (Figure 5) with a similar potency to both PGE_2 and $PGF_{2\alpha}$ (Table 2). The TP-receptor agonist GR32191 (Lumley *et al.*, 1989), at concentrations of 10^{-7}, 3×10^{-7} and 10^{-6} M, produced parallel rightward displacement of the U46619 dose-response curve. Mean data from Schild analysis gave the results shown below:

Schild regression parameter	*Value*
pA_2 (range)	7.1 (6.9–7.2)
Slope (range)	0.95 (0.71–1.08)
Regression coefficient (range)	0.95 (0.89–1.0)

All values are the geometric mean of $n = 5$ determinations.

Discussion

All the prostanoids tested, both natural and synthetic, had some effect on the activity of myometrial strips. Previous studies, on other isolated tissues, have provided evidence that each of the natural prostanoids is at least ten times more potent at its own receptor than at any of the other pros-

tanoid receptors (Coleman, 1987). If this finding is applied to the present studies, then the equal potency of PGE_2, $PGF_{2\alpha}$ and U46619 as excitatory agonists gives a preliminary indication that EP, FP and TP receptors exist on the human myometrium. The low potency of PGI_2 and PGD_2 which are over 100 times less potent than PGE_2, for excitation suggests that their effect may be mediated by interaction with one other prostanoid receptor. The contractile effect produced by PGE_2, PGD_2 and PGI_2 was not affected by morphine (10^{-6} M), suggesting that this effect is a direct receptor-mediated event. Indeed, the data obtained with the synthetic selective agonists confirms these suggestions and will be discussed individually. In so far as inhibition is concerned, PGE_2 was the most potent of the natural prostanoids in this effect, i.e. compared to PGD_2 and PGI_2. However, all these prostanoids produced a biphasic effect, which suggests that the inhibitory responses may well be masked to some extent by the initial stimulation of the tissue by the compounds. These data suggest separate receptors for the natural prostanoids, and results with selective agonists have confirmed this.

The effect of the natural EP-receptor prostanoid, PGE_2, and selective synthetic EP-receptor prostanoids as well as the EP_1-receptor antagonist AH6809 (Coleman *et al.*, 1985) have been discussed previously (Senior *et al.*, 1991). In summary, the results of these studies indicated the existence of both contractile EP_1 and EP_3 receptors and inhibitory EP_2 receptors in the non-pregnant human myometrium *in vitro*.

$PGF_{2\alpha}$ was a potent excitatory agonist like PGE_2. This indicates that FP-receptors mediating excitation may occur in non-pregnant human myometrium but is not conclusive because of the cross-reactivity that exists between the natural prostanoids and the various receptors. In a range of tissues, $PGF_{2\alpha}$ has been shown to interact with EP and TP receptors (Coleman *et al.*, 1984). Previous studies have demonstrated that the TP-receptor antagonist, GR32191, has no effect on the $PGF_{2\alpha}$ response on the human isolated non-pregnant myometrium which indicates at most a minor involvement of TP-receptors in the $PGF_{2\alpha}$ response (Baxter, 1989). The use of the 16-phenoxy analogue of $PGF_{2\alpha}$, fluprostenol, gave support for FP-mediated contraction. Fluprostenol was as effective as $PGF_{2\alpha}$ ($ED_1 = 0.04$ nmol for both agonists) on this tissue. Fluprostenol was originally produced from structural modification of $PGF_{2\alpha}$ (Crossley, 1975) and it proved to have a potent contractile action on FP-receptor-containing preparations such as dog and cat iris sphincter muscle, where it is equipotent with $PGF_{2\alpha}$, but it is more selective than the parent compound, having no demonstrable activity at EP-, DP-, IP- and TP-receptors (Coleman *et al.*, 1987c). Due to this high selectivity of action, fluprostenol is useful in identifying FP-receptors in mixed receptor populations. Therefore, the data obtained with $PGF_{2\alpha}$ and, in particular, fluprostenol are consistent with the presence of FP-receptors in non-pregnant human myometrium. This work could not be supported by the use of antagonists because, although a wide range of compounds have been reported to antagonize various actions of $PGF_{2\alpha}$, no selective FP receptor antagonist has been developed to date. Hence the occurrence of FP-receptors in a mixed population of prostanoid receptors can only be examined by the use of selective agonists such as fluoprostenol.

PGD_2 produced a biphasic response which was qualitatively similar to that evoked by PGE_2. The low potency of PGD_2 as an excitatory agonist indicates that it is probably acting through one of the other prostanoid receptors to bring about this effect. Results with sulprostone and iloprost alone and in the presence of the EP_1-receptor blocking drug AH6809, have previously shown that few EP_1-receptors exist in this tissue (Senior *et al.*, 1991). The TP-receptor antagonist, GR32191 (10^{-6} M) (Baxter, 1989), had no effect on the contraction to PGD_2. These data therefore suggest that EP_1- and TP-receptors are unlikely to be involved in the stimulatory response of PGD_2. PGD_2 does have moderately potent activity at FP- (Beasley *et al.*, 1989) and TP-receptors (Hamid-Bloomfield & Whittle, 1986), as well as some degree of action at EP- and IP-receptors (Coleman, 1987). As far as the non-pregnant human myometrium is concerned, PGD_2 could contract this tissue by acting at EP_3- or FP-receptors. The selective DP-receptor agonist, BW245C (Giles & Leff, 1988) produced an inhibition of myometrial activity which indicates that the inhibition demonstrated by PGD_2 is DP-receptor-mediated. BW245C is a highly selective DP-agonist which appears to have little or no agonist activity at EP-, FP-, IP- or TP-receptors (Town *et al.*, 1983; Coleman *et al.*, 1984; Tynan *et al.*, 1984). In addition to its selectivity, BW245C is about thirty times more potent than PGD_2 at DP-receptors (Coleman, 1987), thereby making it a valuable tool in the identification of DP-receptors in a heterogeneous receptor population. The observation that BW245C is potent in relaxing human myometrium agrees with the data of Sanger *et al.* (1982). Indeed, the present studies reveal a high degree of potency of BW245C, supporting the existence of DP-receptors in non-pregnant human myometrium. The use of the novel, highly potent selective DP-receptor antagonist BW A868C (Giles *et al.*, 1989) adds further weight to the suggestion that relaxant DP-receptors are present in non-pregnant human myometrium. BW245C is specific in that it has no actions at the other prostanoid receptors at concentrations up to 1000 times higher than the DP-receptor affinity. Although the Schild slope was less than unity, the antagonism by BWA868C of the BW245C response appears to have some characteristics of a competitive interaction as demonstrated by the apparently parallel rightward displacements of the BW245C dose-effect curves. The inhibitory action of PGD_2 on non-pregnant myometrium was also antagonized by BW A868C at 10^{-7} M, this effect was associated with some potentiation of the contractile event. This unmasking provides further support for the contractile effect being mediated through a different receptor type. At the lower concentration of 10^{-8} M, BW A868C had no observable effect on the inhibitory dose-effect curve of PGD_2. The data obtained with PGD_2, BW 245C and BW A868C provide evidence that DP-receptors mediating inhibition of uterine activity in the non-pregnant human myometrium exist in this preparation.

Like PGD_2, PGI_2 exhibited a weak contractile effect followed by an inhibition of activity. As with PGD_2, the excitation with PGI_2 is believed to occur through another prostanoid receptor, PGI_2 is particularly active at EP-receptors (Coleman, 1987). The TP-receptor blocking drug, GR32191, at 10^{-6} M had no effect on stimulatory dose effect curves to PGI_2 (Baxter, 1989), implying that TP-receptors are not involved in this effect. The contraction could therefore be mediated through the contractile EP- or FP-receptors. As an inhibitory agonist, PGI_2 was the least potent of all those prostanoids tested that mediated this effect, and this could reflect the rapid metabolism of the compound, although in a superfused system the washout rate is high. In other tissues, PGI_2 is a potent inhibitor of platelet aggregation, and it relaxes smooth muscle (Moncada *et al.*, 1976). *In vivo* experiments in non-pregnant ovariectomized sheep have demonstrated inhibition of myometrial activity with PGI_2 (Lye & Challis, 1982). Biphasic activity has also been recorded from *in vitro* studies on non-pregnant human uterus and fallopian tubes (Omini *et al.*, 1978); this biphasic effect induced by PGI_2 was also observed when the tonus of the organ was pharmacologically increased with $PGF_{2\alpha}$.

In addition two selective synthetic IP receptor agonists, iloprost and cicaprost were tested in the present study. Iloprost also acts at EP_1-receptors as discussed previously (Senior *et al.*, 1991), the inhibition of myometrial activity is of a similar potency to PGE_2 (ID_4 0.94 and 0.8 nmol respectively), although the maximum inhibition with the former is comparatively small. The action of iloprost at IP-receptors suggests that the inhibition is most likely to be mediated through a population of IP-receptors rather than through the

contractile EP_1 receptors in non-pregnant human myometrium.

This proposal is adequately supported by the action of the IP-receptor selective prostanoid, cicaprost, which produced a potent inhibition of myometrial activity and was the most effective agent after BW245C on comparison of the ID_4 values for all the prostanoids tested ($ID_4 = 0.2$ nmol). Cicaprost was originally developed after attempts were made to improve the metabolic stability of iloprost (Stürzebecher *et al.*, 1986). It is more potent than PGI_2 or iloprost as an inhibitor of platelet aggregation, as a relaxant of bovine coronary strip and as a hypotensive agent. Unlike iloprost, it has no demonstrable action at any of the other prostanoid receptors, thereby making cicaprost the most selective IP-receptor agonist yet described (Dong *et al.*, 1986). The results obtained with PGI_2, iloprost and cicaprost suggest that prostanoid receptors of the IP type are involved in the inhibition of myometrial activity in non-pregnant human myometrial strips.

TXA_2 was not used for the study of TP-receptors in non-pregnant human myometrium because, unlike the other natural prostanoids, TXA_2 is not commerically available due to its extreme instability. However, the stable TXA_2-mimetic (11α, 9α-epoxymethano PGH_2 or U46619) is commonly used to assess the presence of TXA_2-sensitive receptors. In the present study, U46619 was equipotent with PGE_2 and $PGF_{2\alpha}$ as an excitatory agonist ($ED_1 = 0.05$, 0.02 and 0.04 nmol respectively). U46619 has been compared to TXA_2 on a range of smooth muscle preparations, including guinea-pig lung strip, dog saphenous vein, rat and rabbit aorta, which are thought to contain TP-receptors (Coleman *et al.*, 1981; Kennedy *et al.*, 1982). U46619 showed little or no activity at other prostanoid receptors; thus, it is a valuable tool for the study and characterization of TP-receptors. Further support for the presence of TP-receptors was provided by the TP antagonist, GR32191. The potent contractile action of U46619 in the myometrial strips was antagonized in a competitive manner by GR32191. The pA_2 for GR32191 on this myometrial preparation was lower than that reported by many authors, although Cuthbert *et al.* (1990) found a low pK_B (6.9) value of rat lung strip, and suggested that this preparation together with human bronchial muscle may contain a subtype of the TXA_2 receptor population. The presence of this range of both excitatory and inhibitory prostanoid receptors on human myometrium suggests that control of myometrial activity by prostanoids is complex, and that further studies are needed to elucidate the effect of mixtures of prostaglandins. It is also of interest to note that despite investigation into many species, including primates, no animal model has been found which exhibits the same complexity of prostanoid receptors as does human myometrium (studies in this laboratory to be published). The knowledge of the prostanoid receptors present in human myometrium is important in clinical conditions involving myometrial dysfunction (e.g. dysmenorrhoea) and in the development of prostanoid-mimetics for the treatment of other conditions (e.g. peptide ulceration).

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The α_2-adrenoceptors of the human retinoblastoma cell line (Y79) may represent an additional example of the α_{2C}-adrenoceptor

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1 In agreement with the literature, correlation of the ability of a series of agonists and antagonists to displace [^{3}H]-rauwolscine binding shows the α_2-adrenoceptors of HT29 cells, NG108-15 cells, OK cells and homogenates of rat sublingual gland to represent four distinct subtypes.

2 [^{3}H]-rauwolscine also bound with high affinity ($K_D = 0.30 \pm 0.10$ mM) to a human retinoblastoma cell line (Y79). Specific binding represents 73% of total binding, and a B_{max} of 38 ± 1 fmol mg^{-1} protein was determined.

3 Correlation of antagonist affinities against [^{3}H]-rauwolscine with corresponding values in the other four tissue sources showed the Y79 cells to resemble most closely the OK cells, the prototype example of an α_{2C}-adrenoceptor, with a correlation coefficient of 0.90 and a regression slope of 1.01 being obtained for 10 antagonists in these two systems.

4 Comparison of K_D values for [^{3}H]-rauwolscine also showed a similarity between the OK cells (0.19 ± 0.07 nM) and Y79 cells.

5 These data suggest that the human retinoblastoma cell line may represent an additional example of the α_{2C}-adrenoceptor subtype.

Keywords: HT29 cells; NG108-15 cells; rat sublingual gland; [^{3}H]-rauwolscine; α_2-adrenoceptor subtypes

Introduction

In the past few years, several groups have succeeded in cloning and expressing α_2-adrenoceptors from rat, human and porcine sources (Lorenz *et al.*, 1990; Guyer *et al.*, 1990; Weinshank *et al.*, 1990; Flordellis *et al.*, 1991; Harrison *et al.*, 1991; Chhajlani *et al.*, 1991; Voigt *et al.*, 1991; Lanier *et al.*, 1991). From these molecular biological studies, it is clear that there are at least three α_2-adrenoceptor subtypes, having distinct amino acid sequences. However, it is not yet clear whether the three α_2-adrenoceptor proteins isolated from rat correspond to the three isolated from human sources (Harrison *et al.*, 1991) or whether a fourth subtype has been cloned from the rat genomic library (Lanier *et al.*, 1991). Likewise, assignment of the cloned receptor proteins to the multiple α_2-adrenoceptor subtypes which have been identified by radioligand binding asays (Bylund, 1988; Uhlen & Wikberg, 1991) is still controversial.

Binding sites defined as α_2-adrenoceptors, based on their high affinity for [^{3}H]-rauwolscine, can be unequivocally subclassified into the α_{2A}- and α_{2B}-adrenoceptor subgroups, based on high (α_{2B}) or low (α_{2A}) affinity for prazosin or ARC-239. Multiple examples of both α_{2A}- and α_{2B}-adrenoceptors are known, both in tissue culture lines and in tissue homogenates from several species. Functional studies measuring the ability of prazosin and ARC-239 to inhibit adenylate cyclase activation by an α_2-adrenoceptor agonist in HT29 (α_{2A}) and NG108-15 (α_{2B}) cells support this subclassification scheme (Bylund & Ray-Prenger, 1989). Cloned and expressed α_2-adrenoceptor proteins can also be divided into two groups, having either high (<100 nM) or low (>1000 nM) affinity for prazosin (Simmoneaux *et al.*, 1991; Harrison *et al.*, 1991; Lanier *et al.*, 1991).

When the ability of a diverse series of antagonists to inhibit [^{3}H]-rauwolscine binding was compared in a variety of tissues, it became apparent that there were more than two α_2-adrenoceptor binding sites. It is currently thought that at least four α_2-adrenoceptors can be identified by this technique (Bylund, 1988; Blaxall *et al.*, 1991; Simmoneaux *et al.*, 1991). The two additional sites have been designated as α_{2C} and α_{2D}. Although the α_{2C} and α_{2D} sites resemble the α_{2B} and α_{2A}, respectively, antagonists capable of differentiating the four sites are available (Blaxall *et al.*, 1991). By examining the simultaneous interactions of several antagonists at the α_2-adrenoceptor binding site it may be possible to identify even more discrete binding sites (Uhlen & Wikberg, 1991).

The α_{2C}-adrenoceptor has only been identified thus far in the OK cell line, derived from the opossum kidney, and in tissue homogenates prepared from fresh opossum kidneys (Blaxall *et al.*, 1991), and it has been suggested that the α_{2C} and α_{2B}-adrenoceptors might represent species variants (Lorenz *et al.*, 1990). Hence the demonstration of an α_{2C} site in another tissue source, not derived from the opossum, would strengthen the case for its role as a distinct α_2-adrenoceptor subtype. In this report, we compare the ability of a series of 10 antagonists to inhibit the binding of [^{3}H]-rauwolscine to membrane homogenates from five tissues/cell lines. Tissues examined include known examples of α_{2A} (HT29 cells), α_{2B} (NG108-15 cells), α_{2C} (OK cells) and α_{2D} (rat sublingual gland), as well as a human derived retinoblastoma cell line (Y79; Reid *et al.*, 1974). Y79 cells have been shown to have α_2-adrenoceptors, which were suggested to be of the α_{2A} subtype (Kazmi & Mishra, 1989).

Methods

Cell culture

HT29 cells (American Type Culture Collection (ATCC), Rockville, MD, U.S.A.) and OK cells (kindly donated by Dr

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D.B. Bylund, University of Nebraska, Omaha, NB, U.S.A) were grown in Dulbecco's modified Eagle's medium (DMEM) containing high glucose and supplemented with 10% foetal bovine serum (FBS). NG108-15 cells (kindly donated by Dr Marshall Nirenberg, National Institutes of Health, Bethesda, MD, U.S.A.) were grown in DMEM supplemented with 10% FBS, 0.1 mM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine. Y79 cells (ATCC) were grown in suspension in RPMI 1640 medium supplemented with 15% FBS. To obtain cells for tissue preparation, HT29 and OK cells were subcultured with 0.05% trypsin containing 0.53 mM ethylenediaminetetraacetic acid (EDTA), and NG108-15 cells were gently agitated loose from the culture flask. Cells were maintained in a humidified atmosphere consisting of 5% CO_2: 95% air (10% CO_2:90% air for NG108-15) and harvested for membrane preparation after reaching confluence.

Tissue preparation

Cells were scraped, pelleted by centrifugation (300 *g*, 5 min) and washed twice in Dulbecco's phosphate buffered saline (composition in mM: KCl 2.7, KH_2PO_4 1.5, NaCl 137 and Na_2HPO_4 8.1). The pH of this solution was 7.2. The cell pellet was resuspended in ice-cold 5.0 mM Tris, 1.0 mM EDTA, pH 7.4, and homogenized on ice with a polytron (Brinkman, Westbury, NY, U.S.A.) (3 × 15 s burst on setting 6). The homogenate was centrifuged (500 *g*, 5 min). The supernatant was collected and centrifuged at 100,000 *g* for 30 min. The resulting membrane pellet was resuspended in 25 mM glycylglycine, pH 7.6, and frozen at −70°C following protein determination.

Rat sublingual glands were dissected free from the submaxillary gland (Pel Freeze, Rogers, AK, U.S.A.) and homogenized as above in ice-cold homogenizing buffer containing (mM): sucrose 250; Tris 10, EDTA 0.002 at pH 7.5 until a uniform homogenate was observed. Membranes were then prepared and stored as described above.

Protein content was determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA, U.S.A.) using the method described by Bradford (1976) with bovine serum albumin as standard.

Radioligand binding assay

All binding assays were incubated in 25 mM glycylglycine (Bylund, 1985), at 25°C for 30 min. Binding was stopped by vacuum filtration using a Brandell cell harvester through Whatman GF/B filters, with three 5 ml washes with ice-cold binding buffer. Radioactivity retained on the filter was determined by liquid scintillation counting in Readysafe scintillation fluid.

For [^{3}H]-rauwolscine saturation studies, total binding was determined in duplicate tubes containing a predetermined concentration of the radioligand and 100–250 μg membrane protein in a total volume of 300 μl. Nonspecific binding was determined in the presence of 10 μM phentolamine. Specific binding was calculated as the difference between total and nonspecific binding. The K_D and B_{max} were calculated from nonlinear regression with the aid of the AccuFit computer programme (Beckman, Fullerton, CA, U.S.A.).

For competition studies, various concentrations of α-adrenoceptor agonists and antagonists were incubated in the presence of the K_D of [^{3}H]-rauwolscine for the tissue source being examined and 100–250 μg membrane protein in a total volume of 300 μl. K_i values were determined from nonlinear regression by the Lundon$_2$ computer programme (Lundon Software Inc., Cleveland, OH, U.S.A.).

Drugs and solutions

SK&F 86466 (6-chloro,2, 3, 4, 5-tetrahydro-3-methyl-1H-3-benzazepine), SK&F 104078 (6-chloro-9- [(3-methyl-2-butenyl) oxy]-2,3,4,5-tetrahydro-3-methyl-1H-3-benzazepine), SK&F 104856 (2-vinyl-7-chloro-3,4,5,6-tetrahydro-4-methyl-thieno [4,3,2ef][3] benzazepine and BRL 44408 (2-[(4, 5-dihydro-1H-imidazol-2-yl) methyl]-2, 3-dihydro-1-methyl-1H-isoindole) were synthesized by the Medical Chemistry Department of SmithKline Beecham (King of Prussia, PA, U.S.A. or Great Burgh, Surrey, U.K.) BAM1303 (8β[2-phenylimidazol-1-yl)methyl-6-methylergoline; Maruko Pharmaceutical Co. Ltd., Nagoya, Japan), ARC-239 (2-[2,4-(*o*-methoxyphenyl)piperazin-1-yl]ethyl-4,4-dimethyl-1,3- (2H,4H) -isoquinolindione; Karl Thomae, Biberach, Germany) and prazosin (Pfizer, Groton, CT, U.S.A.) were kindly donated by their manufacturers. Sources of reagents were as follows: GIBCO, ST. Louis, MO, U.S.A. (trypsin, DMEM, DPBS, FBS); Dupont New England Nuclear, Wilmington, DE, U.S.A. ([^{3}H]-rauwolscine, 78-86 Ci mmol^{-1}); CIBA, Summit NJ, U.S.A. (phentolamine); Reseach Organics, Cleveland, OH, U.S.A. (glycylglycine); Karl Roth, Karlsruhe, Germany (rauwolscine). All other compounds and reagents were obtained from Sigma, St. Louis, MO, U.S.A.

All compounds were dissolved in the appropriate buffer with the exception of ARC 239 which was dissolved in 0.1 N HCl and immediately diluted in buffer, and BAM 1303 which was dissolved in ethanol. The maximal ethanol concentration in the assay medium was 1%. This concentration was shown to have no effect on the binding of [^{3}H]-rauwolscine.

Results

Representative saturation curves and Scatchard plots for [^{3}H]-rauwolscine in Y79 cells and rat sublingual gland are shown in Figure 1. Although characterization of α_2-adrenoceptors has previously been reported in rat submaxillary gland (Michel *et al.*, 1989), we found that when the sublingual gland was separated by dissection from the total 'submaxillary gland' obtained from the supplier, essentially all of the specific [^{3}H]-rauwolscine binding was to the sublingual component (data not shown). This may be reflected in our higher B_{max} value (301 fmol mg^{-1} protein; Table 1) compared to the value obtained by Michel *et al.* (1989) in the total submaxillary gland (137 fmol mg^{-1} protein). K_D and B_{max} values for [^{3}H]-rauwolscine binding to HT29, HG108-15 and OK cells are also presented in Table 1. From Table 1 it is apparent that (1) the receptor density in the Y79 cells is substantially lower than that in the other systems and (2) the affinity of [^{3}H]-rauwolscine for the binding sites in OK and Y79 cells is higher than in the other preparations. In all systems examined the specific binding of [^{3}H]-rauwolscine represented at least 70% of the total binding to the tissue homogenate.

The potency of a series of 10 compounds in inhibiting the binding of [^{3}H]-rauwolscine to the five tissue sources is shown in Table 2. The compounds examined included eight antagonists, plus the partial agonists, oxymetazoline and clonidine. The selectivity previously reported for prazosin, ARC 239 and SK&F 104856 in NG108-15 versus HT29 cells; and, conversely, oxymetazoline and BRL 44408 in HT29 versus NG108-15 cells is confirmed by our data. Consistent with the K_D values for [^{3}H]-rauwolscine as determined by saturation analysis, the K_i for unlabelled rauwolscine was lower in the OK and Y79 cells.

Correlation of drug affinities as inhibitors of [^{3}H]-rauwolscine binding to the five tissue sources shows a statistically significant relationship only for HT29 cells versus sublingual gland and for Y79 cells versus OK cells (Table 3).

Discussion

Our data provide additional support for the premise that α_2-adrenoceptors can be subdivided based on the ability of α-adrenoceptor agonists and antagonists to displace [^{3}H]-rauwolscine binding. As previously reported (Bylund, 1988)

the [^{3}H]-rauwolscine sites on HT29, NG108-15 and OK cells clearly represent three distinct groups. In agreement with Blaxall *et al.* (1991) we found the affinity of [^{3}H]-rauwolscine to be highest in the OK cells, although our calculated affinity (as reflected by the K_D for [^{3}H]-rauwolscine or the K_i for unlabelled rauwolscine as an inhibitor of binding) was about five fold lower than that reported by these investigators. Comparison of our data in the OK and HT29 cells with that of Blaxall *et al.* (1991) shows, with the exception of oxymetazoline and SK&F 104078 in the OK cell line, our K_i values to be 3 to 5 fold higher, suggesting the difference may be due to slight changes in experimental methodology. Nevertheless, the relative potencies in these two cell lines of the antagonists tested both by us and Blaxall *et al.* are nearly identical.

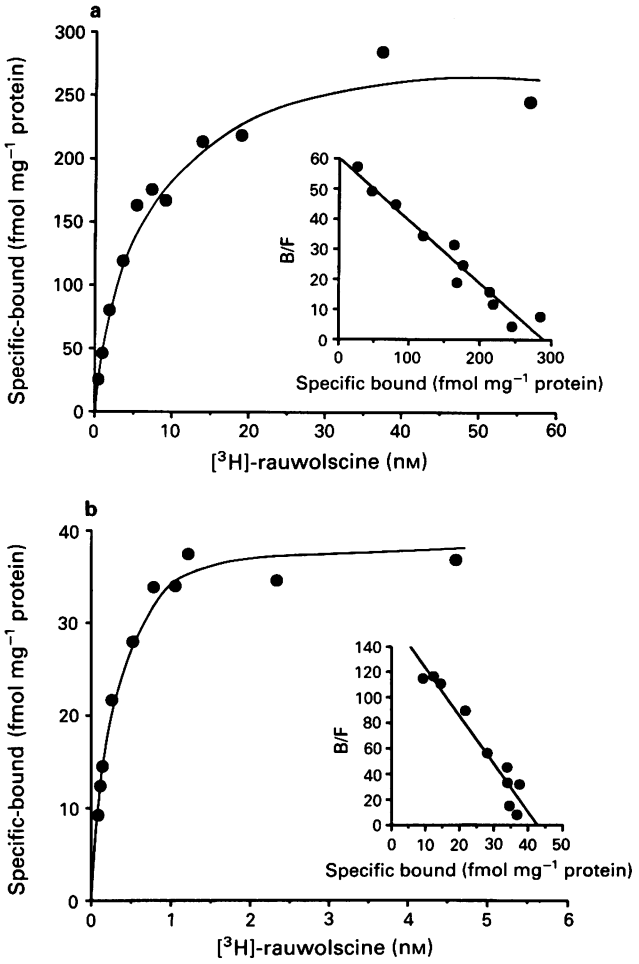

Figure 1 Representative experiments showing saturation curves and Scatchard analyses (inset) for binding of [^{3}H]-rauwolscine to membrane homogenates of rat sublingual gland (a) and Y79 cells (human retinoblastoma; b). Each point represents the mean of duplicate determinations.

Table 1 Binding parameters of [^{3}H]-rauwolscine

Tissue	K_d (nM)	B_{max}(fmol mg^{-1} protein)	*SB*[a]
HT29 cells (α_{2A})	1.10 ± 0.45	170 ± 7	87
NG108-15 cells (α_{2B})	1.37 ± 0.06	235 ± 20	81
OK cells (α_{2C})	0.19 ± 0.07	245 ± 27	93
Rat sublingual gland (α_{2D})	5.04 ± 0.37	301 ± 15	69
Y79 cells	0.30 ± 0.10	38 ± 1	73

[a]Specific binding as % of total binding.
Values represent mean ± s.e.mean, derived from at least 2 experiments, each performed in duplicate.

We found the α_2-adrenoceptor on the rat sublingual gland to be distinct from that on the NG108-15 and OK cells, but not from that on the HT29 (Table 3). Other studies have shown that the cross-subtype correlation for inhibition of [^{3}H]-rauwolscine binding is closest ($r > 0.8$ with regression slopes near unity) when α_{2D} (bovine pineal, rat submaxillary gland) are compared to α_{2A} (HT29 cells, chicken pineal) systems (Simmoneaux *et al.*, 1991). Furthermore, most of the agents examined in our study do not discriminate clearly between α_{2A}- and α_{2D}-adrenoceptors. Of the systems we examined, the affinity of rauwolscine is lowest for the receptor of the sublingual gland, as reflected either by the K_D (5 nM) or K_i(11 nM). This relatively low affinity of rauwolscine is characteristic of an α_{2D} subtype (Simmoneaux *et al.*, 1991).

Our data in the Y79 cells agrees closely with that reported by Kazmi & Mishra (1979). Although they assign the α_2-adrenoceptor in this cell line to the α_{2A} subtype, this assignment was based only on the relative potencies of prazosin and oxymetazoline. When our K_i values in the Y79 cells are compared to the corresponding values in a known α_{2A} system, the HT29 cells, the correlation is poor ($r = 0.57$, Table 3). Although the overall correlation of the Y79 and NG108-15 data is slightly better ($r = 0.65$), the three antagonists known to be selective for the α_{2B} subtype, prazosin, ARC239 and SK&F 104856 (Gleason & Hieble, 1991), all are significantly less potent in the Y79 system (Table 2). An excellent correlation is obtained when K_i values in Y79 and OK cells are compared ($r = 0.9$; Table 3). Furthermore, the best-fit regression and line of identity between these two systems are superimposable.

It is somewhat disturbing that the only compound deviating from the correlation is BAM1303, reported to be one of the most selective antagonists for α_{2C}-adrenoceptors (Blaxall

Table 2 Potency of drugs as inhibitors of [^{3}H]-rauwolscine binding

	K_i (nM)[a]				
	HT29[b]	*NG108-15*[b]	*OK*	*Rat sublingual*	*Y79*
ARC 239	466 ± 44	9.62 ± 0.81	41.3 ± 5.3	420 ± 84	60.2 ± 17.9
BAM 1303	12.7 ± 3.8	49.5 ± 6.4	1.98 ± 0.42	86.2 ± 25.2	14.8 ± 5.9
Clonidine	16.4 ± 1.4	21.8 ± 2.2	24.5 ± 8.2	10.3 ± 2.5	10.2 ± 3.7
Oxymetazoline	2.07 ± 0.76	225 ± 111	36.3 ± 11.3	11.5 ± 3.3	19.5 ± 8.1
Prazosin	779 ± 88	22.5 ± 10.6	79.9 ± 4.4	175 ± 38	125 ± 29
Rauwolscine	2.01 ± 0.66	1.47 ± 0.20	0.26 ± 0.03	11.1 ± 3.0	0.54 ± 0.24
BRL 44408	5.35 ± 2.85	205 ± 40	187 ± 24	15.7 ± 2.3	227 ± 78
SKF 86466	8.69 ± 2.01	4.91 ± 0.87	8.28 ± 1.11	5.17 ± 1.59	3.96 ± 0.53
SKF 104078	105 ± 29	71.5 ± 9.3	58.6 ± 6.7	202 ± 53	47.2 ± 7.3
SKF 104856	257 ± 52	7.77 ± 0.99	59.6 ± 19.1	117 ± 33	42.8 ± 9.9

[a]Values are mean ± s.e.mean and are derived from at least 3 experiments performed in duplicate. [b]Data from Gleason & Hieble (1991).

Table 3 Correlation of inhibitory potency between tissues

Tissue	r	*Slope*	P
HT29 cells vs NG108-15 cells (α_{2A} vs α_{2B})	0.07	0.09	<0.841
HT29 cells vs OK cells (α_{2A} vs α_{2C})	0.44	0.49	<0.173
HT29 cells vs Sublingual gland (α_{2A} vs α_{2D})	0.84	1.09	<0.001
NG108-15 cells vs OK cells (α_{2B} vs α_{2C})	0.47	0.41	<0.141
NG108-15 vs Sublingual gland (α_{2B} vs α_{2D})	0.18	0.18	<0.598
OK cells vs Sublingual gland (α_{2C} vs α_{2D})	0.22	0.25	<0.524
HT29 cells vs Y79 cells	0.57	0.72	<0.086
NG108-15 cells vs Y79 cells	0.65	0.60	<0.044
OK cells vs Y79 cells	0.90	1.01	<0.0001
Sublingual gland vs Y79 cells	0.56	0.50	<0.095

Values are derived from linear regression analysis of the correlations of the log of the drug affinities presented in Table 2.

et al., 1991). Nevertheless, in addition to the excellent overall correlation of K_i values, the OK and Y79 cells share several characteristics distinguishing them from the other systems studied: (1) a 5–10 fold lower potency of ARC 239, prazosin and SK&F 104856 *vis-a-vis* the NG-108 cells; this difference is consistent with the relative potencies for these antagonists in the OK cells compared with another α_{2B} system, the neonatal rat lung (Simmoneaux *et al.*, 1991). (2) A higher affinity for rauwolscine (3–20 fold difference for the Y79 cells compared with the NG-108 15, HT29 or rat sublingual preparations). (3) An oxymetazoline affinity intermediate between that observed in the α_{2A} and α_{2B} models. Hence, we propose that the Y79 cell line represents an additional example of an α_{2C}-adrenoceptor.

Based on the ability of mianserin enantiomers to block α_2-adrenoceptor-mediated inhibition of synaptosomal noradrenaline release (Raiteri *et al.*, 1983), it has been postulated that a functional α_{2C}-adrenoceptor may be present in the rat cerebral cortex (Blaxall *et al.*, 1991). Our results, showing these receptors to be present in a cell line derived from a human tumour (Reid *et al.*, 1974) provide further support for the premise that the α_{2C}-adrenoceptor is not limited to the opossum. Further studies, using the selective antagonists now available, will be required to establish the distribution and functional role of this receptor subtype.

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Activation of the human neutrophil 5-lipoxygenase by leukotriene B_4

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1 In the present study, we demonstrate that leukotriene B_4 (LTB_4) has the ability to activate the human neutrophil 5-lipoxygenase (5-LO).

2 Stimulation of neutrophils with 30 nM 14,15-dideuterio-LTB_4 (D_2-LTB_4) failed to induce the synthesis of LTB_4 from endogenous arachidonic acid (AA), but stimulated the formation of LTB_4 from 3.3 μM exogenous AA, as determined by GC-MS analysis.

3 The stimulatory effect of LTB_4 on 5-LO activity was further examined with an alternative substrate; LTB_4 time- and dose-dependently stimulated the 5-LO-mediated conversion of exogenous 15(S)-hydroperoxy-5,8,11,13-(Z,Z,Z,E)-eicosatetraenoate (15-HpETE) into 5(S),15(S)-dihydroxy-6,8,11,13,-(E,Z,Z,E)-eicosatetraenoate (5,15-DiHETE), with a threshold effect at 300 pM.

4 The ability of LTB_4 to activate the 5-LO showed structural specificity, since LTB_4 was found to be 100 times more potent than ω-hydroxy-LTB_4, and 300 times more potent than its Δ^6-*trans*-12-epi- isomer.

5 The LTB_4-induced 5-LO activation was effectively inhibited by MK-886 (an inhibitor of 5-LO translocation), by pertussis toxin, and by the LTB_4 receptor antagonist, LY-223982.

6 These results demonstrate that the binding of LTB_4 to its cell-surface receptor results in 5-LO activation in a process mediated by pertussis toxin-sensitive guanine nucleotide-binding proteins. Our data also suggest that the underlying mechanism involves a translocation of the 5-LO to the membrane. These findings raise the possibility that LTB_4 produced by phagocytes may positively feedback on its own synthesis.

Keywords: Arachidonate 5-lipoxygenase; 5-lipoxygenase-activating protein; translocation; guanine nucleotide-binding proteins; receptors; inflammation

Introduction

Leukotriene B_4 (LTB_4), a 5-lipoxygenase (5-LO) metabolite of arachidonic acid (AA) (Borgeat & Samuelsson, 1979), is a potent stereospecific leukocyte activator which was originally characterized as an endogenous neutrophil chemotactic factor (Ford-Hutchinson *et al.*, 1980). It has since been shown to stimulate many other neutrophil functions including degranulation, aggregation, adherence, and calcium mobilization (Naccache *et al.*, 1989a; Ford-Hutchinson, 1990). LTB_4 also acts upon other cells of the immune system, such as eosinophils, monocytes and lymphocytes (Rola-Plezczynski, 1985). Because of its well-characterized effects on various cells of the immune system, LTB_4 has become widely viewed as a potent mediator of inflammation and allergy.

Neutrophils and other phagocytes synthesize LTB_4 and related compounds in response to a variety of stimuli, notably, calcium ionophores, particulate agonists (such as urate and pyrophosphate crystals, as well as zymosan), and the soluble stimuli N-formyl-methionyl-leucyl-phenylalanine (fMLP), platelet-activating factor (PAF), interleukin-8 (IL-8) and complement component C5a (Borgeat & Samuelsson, 1979; Claesson *et al.*, 1981; Lin *et al.*, 1982; Clancy *et al.*, 1983; Poubelle *et al.*, 1987; Schröder, 1989). Synthesis of LTB_4 from endogenous AA results from the activation of two calcium-dependent events. First, the release of AA from membrane phospholipids and second, the activation of the 5-LO (Borgeat & Samuelsson, 1979; Borgeat *et al.*, 1983; Rouzer & Kargman, 1988). In the latter instance, 5-LO activation is likely to involve a calcium-dependent translocation of the enzyme to the plasma membrane (Rouzer & Kargman, 1988). Thus, the onset of LT synthesis by stimuli such as those listed above may well be related to their calcium-mobilizing properties.

The stimulatory characteristics of LTB_4 towards neutrophils resemble those of other receptor-dependent soluble neutrophil agonists, i.e. fMLP, PAF and C5a (Naccache *et al.*, 1979; Clancy *et al.*, 1983; Naccache *et al.*, 1986). These compounds bind to specific cell-surface receptors to stimulate the phospholipase C-dependent release of diacylglycerol and inositol trisphosphate, a process mediated by pertussis toxin-sensitive, guanine nucleotide-binding proteins (G proteins) (Becker *et al.*, 1985; Naccache *et al.*, 1986; Shirato *et al.*, 1988). It is through this sequence of events that soluble agonists elicit several neutrophil functional responses, such as degranulation, superoxide anion generation, adherence, locomotion, and activation of the 5-LO (McDonald *et al.*, 1991). LTB_4 elicits similar responses, but in contrast to PAF, fMLP and C5a, it has not yet been shown to stimulate 5-LO activity.

Nevertheless, such a possibility was raised in recent reports from our laboratory, in which we investigated the mechanism whereby exogenous AA activates polymorphonuclear leukocytes (PMNL) (McColl *et al.*, 1989; Naccache *et al.*, 1989b). In these previous studies, we showed that LTB_4 synthesis and/or triggering of LTB_4 receptors significantly accounted for the ability of exogenous AA to stimulate an increase in intracellular calcium mobilization and activation of the 5-LO. Exogenous AA induced a dose-dependent mobilization of calcium that was inhibited by pertussis toxin, or upon preincubation of neutrophils with LTB_4; similarly, exogenous AA-induced 5-LO product synthesis was inhibited by pertussis toxin. These observations prompted us to investigate potential stimulatory effects of LTB_4 itself on the 5-LO metabolic pathway in neutrophils, as such effects of LTB_4 could represent an important feedback amplification mechanism in the production of this potent pro-inflammatory mediator.

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Methods

Cell separation

Blood was collected by venepuncture with heparin used as an anticoagulant, and following dextran sedimentation of erythrocytes, PMNL were purified by centrifugation on Ficoll-Plaque cushions (Pharmacia, Dorval, Québec, Canada). The erythrocytes remaining in the final pellet were removed by hypotonic lysis with water (30 s), and the cells were resuspended in HBSS buffered with HEPES (10 mM, pH 7.4) and supplemented with calcium (1.6 mM) and magnesium (1 mM), at a final concentration of 5×10^6 PMNL ml^{-1} (unless otherwise stated). The entire cell separation was carried out at room temperature. The percentage of PMNL in the cell suspensions used in this study exceeded 96%, and cell viability was greater than 98%, as determined by trypan blue exclusion. The platelet/leukocyte ratio varied from 2 to 5.

Cell incubations

Cell suspensions were warmed to 37°C for 5 min before incubation with substrate and/or stimulus. All agonists, including exogenous AA and D_2-LTB_4, were dissolved in dimethyl sulphoxide (DMSO) and added to 1.0 ml aliquots of the cell suspension; the final concentration of DMSO (maximum of 0.3%) consistently failed to stimulate any detectable leukotriene synthesis. In the case of 15-HpETE, a stock solution (3 mM in ethanol) was diluted 10 fold in 0.6 mM sodium carbonate (to form the sodium salt of the fatty acid), and 10 µl of the resulting solution was added to 1.0 ml aliquots of the cell suspensions, to yield a final concentration of 3 µM. After the desired incubation time with the stimuli, the cells were denatured by the addition of 1.0 ml of an ice-cold mixture of methanol:acetonitrile (50:50 v:v) containing internal standards: 12.5 ng·ml^{-1} of prostaglandin B_2 and 19-OH prostaglandin B_2. The samples were then stored at −20°C before reversed-phase h.p.l.c. (r.p.-h.p.l.c.) analysis.

In the experiments involving pertussis toxin, the cell suspensions were pre-incubated with the toxin at a final concentration of 0.5 µg ml^{-1} for 3 h at 37°C. During this time, the cells were gently swirled in a rotary water bath (New Brunswick Scientific, Edison, New Jersey, U.S.A.); calcium and magnesium were added 20 min before stimulation. In the experiments involving MK-886 or LY-223982, the drugs were added to the cells 1 min before stimulation; both compounds were dissolved in DMSO.

Analysis of lipoxygenase products by r.p.-h.p.l.c.

Analysis of lipoxygenase products was performed by r.p.-h.p.l.c. as described previously, using an on-line extraction procedure (Borgeat *et al.*, 1990). Briefly, the denatured cell suspensions were centrifuged at 600 *g* (4°C, 10 min) to remove precipitated material, and the supernatants were directly injected onto a Radial Pak Resolve C_{18} cartridge (5 × 100 mm, 5 µm particles, Waters Millipore, Milford, Massachusets, U.S.A.) protected by Guard-Pak Resolve C_{18} and silica cartridges (Waters Millipore). The various lipoxygenase products were eluted at 1.5 ml min^{-1} using gradients of organic solvents and a change of pH. Elution was monitored using fixed-wavelength ultra-violet (u.v.) photometers at 229 and 280 nm; quantification was performed by comparing peak heights of each compound with those of calibrated standards, after correction for recovery using the internal standard, prostaglandin B_2. The lower limit of detection was 0.5 ng at 280 nm and 1 ng at 229 nm. In the experiments involving D_2-LTB_4, h.p.l.c. analysis of the samples was performed as described above, except that acetic acid was used instead of phosphoric acid to acidify the various mobile phases, and LTB_4-containing h.p.l.c. eluate fractions were collected for GC-MS analysis.

GC-MS analysis of LTB_4

The eluate fractions containing LTB_4 were evaporated under a stream of nitrogen at 40°C. The trimethylsilyl ether methyl ester derivative of LTB_4 was obtained by successive treatment with etheral diazomethane in methanol (5 min at 20°C), and with N-methyl-*N*-trimethylsilyltrifluoroacetamide and dimethylformamide (25 µl each) for 60 min at 50°C. The reagents were evaporated under a stream of nitrogen and the samples were dissolved in hexane prior to injection. GC-MS analysis (multiple ion monitoring) was performed on a VG MicroMass MM-16 (Altrincham, Cheshire) mass spectrometer coupled to a Varian 3700 GC, using a fused silica capillary column (Durabond DB-1, 0.25 mm I.D. × 15 m, J&W Scientific Inc., Folsom, California, U.S.A.) directly connected to the source. Samples were injected by an on-column injection technique and He was used as carrier gas (81 kPa). The injection temperature was 140°C, and after 2 min, the oven temperature was increased to 260°C at the rate of 10°C min^{-1}. The D_2-LTB_4 and D_0-LTB_4 derivatives co-eluted at 14.47 min. Analysis was performed in the electron impact mode. Ionizing energy was 25.0 eV, the trap current 200 µA and the source was maintained at 250°C. Ion current was recorded at m/z 463.3 and 465.3 (M-31); these ions represented 4% of the base peak (m/z 129) in both spectra. LTB_4 was quantified from a calibration curve generated from mixtures of LTB_4 (0–20 ng) and D_2-LTB_4 (12 ng) and the contribution of LTB_4 to D_2-LTB_4 at m/z 465.3 was subtracted.

Materials

PAF, fMLP, HEPES, pertussis toxin and arachidonic acid were obtained from the Sigma Chemical Company (St-Louis, Missouri, U.S.A.). Hank's Balanced Salt Solution (HBSS) was from GIBCO (Burlington, Ontario, Canada), and all solvents were high performance liquid chromatography (h.p.l.c.) grade from Anachemia (Montréal, Québec, Canada). As previously described for 13-hydroperoxy-octadecadienoic acid (Gardner, 1975), soybean lipoxygenase was used to synthesize 15-HpETE from AA. AA was purified on a silicic acid column before use for enzymatic synthesis of 15-HpETE, which was then purified by straight-phase h.p.l.c. It must be stressed that it is critical to purify AA and 15-HpETE freshly before use, because of the natural reactivity of these compounds. Leukotriene B_4, D_2-LTB_4 (14,15-dideuterio) and MK-886 were generous gifts from Dr Robert N. Young of the Merck-Frosst Centre for Therapeutic Research (Pointe-Claire, Québec, Canada).

Statistical analyses

Where mentioned, statistical significance was assessed by Student's paired *t* test.

Results

Conversion of exogenous AA into LTB_4 stimulated by D_2-LTB_4

To determine whether LTB_4 has the ability to stimulate its own synthesis from endogenous or exogenous substrate, PMNL (2×10^6 ml^{-1}) were incubated with 30 nM D_2-LTB_4 in the presence or absence of 3.3 µM exogenous AA for 2.5 min. Use of D_2-LTB_4 as a stimulus allows the quantification by GC-MS procedures of LTB_4 generated from AA. Figure 1 shows that PMNL exposed to 30 nM D_2-LTB_4 failed to synthesize LTB_4 in amounts significantly above control,

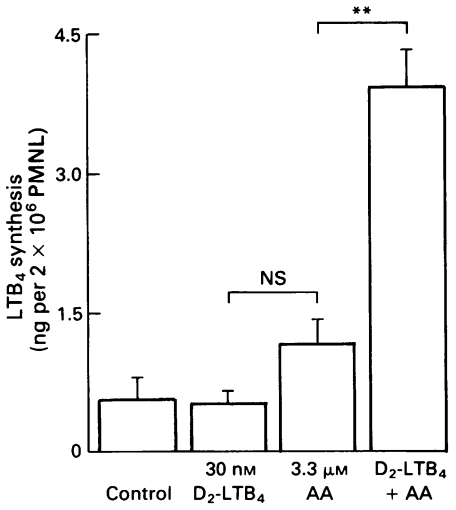

Figure 1 Stimulation by 14,15-dideuterio-leukotriene B_4 (D_2-LTB_4) of LTB_4 synthesis from exogenous arachidonic acid (AA). PMNL were incubated at 37°C for 2.5 min under the conditions indicated in Methods; samples were processed by r.p.-h.p.l.c. and LTB_4-containing fractions were collected and further analysed by GC-MS. The results are expressed as the mean ± s.e.mean of averaged triplicate incubations from 3 independent experiments. NS: not statistically significant; $^{**}P < 0.011$.

indicating that D_2-LTB_4 did not stimulate the formation of LTB_4 from endogenous AA. Consistent with these observations, no 5-HETE (5(S)-hydroxy-6,8,11,14-eicosatetraenoic acid) could be detected under these conditions. Similar results were obtained with 100 nM D_2-LTB_4 (not shown). Incubation of PMNL with 3.3 μM AA did not elicit a significant synthesis of LTB_4. In contrast, co-addition of 3.3 μM AA and 30 nM D_2-LTB_4 consistently resulted in a marked increase in the synthesis of LTB_4. Under such conditions, small amounts of 5-HETE were sometimes detected. These results indicate a stimulatory effect of LTB_4 on 5-LO activity in PMNL.

Stimulation by LTB_4 of 5-LO-dependent conversion of 15-HpETE into 5,15-DiHETE

The stimulatory effect of LTB_4 on 5-LO activity was confirmed and further characterized with an alternative substrate, 15-HpETE. The resulting product, 5,15-DiHETE, can be measured by r.p.-h.p.l.c. and u.v. detection at 229 nm. PMNL were incubated with 3.0 μM 15-HpETE in presence or absence of 100 nM LTB_4 for 15 min, the reactions were stopped, and the supernatants analysed by r.p.-h.p.l.c. When both LTB_4 and 15-HpETE were added to PMNL, 2 major absorbance peaks were detected at 229 nm (Figure 2a): 15-hydroxy eicosatetreaenoic acid (15-HETE), the reduction product of 15-HpETE, and 5,15-DiHETE, the reduced 5-LO metabolite of 15-HpETE. In contrast, when 15-HpETE only was added, a much smaller peak of 5,15-DiHETE was detected, along with the 15-HETE peak (Figure 2b). Finally, no 5,15-DiHETE could be detected in cells stimulated with LTB_4 alone (Figure 2c). Therefore, as demonstrated by this experiment, LTB_4 stimulated the 5-LO-mediated transformation of 15-HpETE into 5,15-DiHETE, thus confirming our previous observations using AA as exogenous substrate.

To determine the time course of the 5-LO transformation of 15-HpETE into 5,15-DiHETE, PMNL were incubated for up to 15 min with 3.0 μM 15-HpETE, either alone or with 100 nM LTB_4. When PMNL were incubated with 15-HpETE

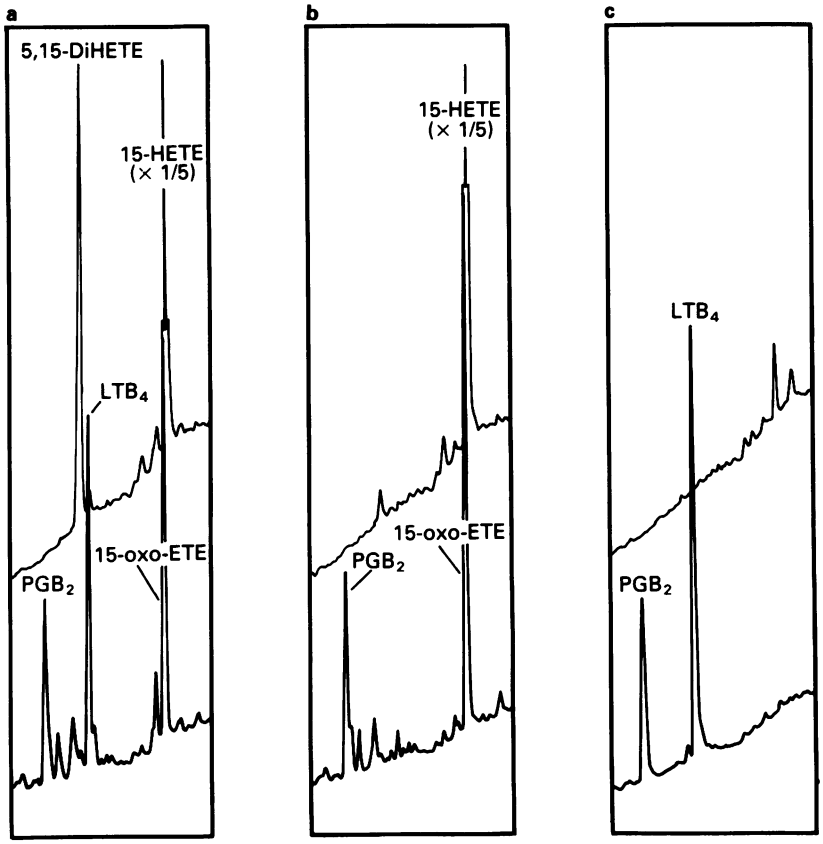

Figure 2 Stimulation by leukotriene B_4 (LTB_4) of the transformation of exogenous 15-HpETE by the 5-lipoxygenase (5-LO). PMNL were incubated at 37°C for 15 min under the following conditions: (a) 100 nM LTB_4 and 3.0 μM 15-HpETE; (b) 3.0 μM 15-HpETE alone; (c) 100 nM LTB_4 alone. This figure shows r.p.-h.p.l.c. chromatograms (upper tracings, u.v. absorbance at 229 nm with attenuation set at 0.20 a.u.f.s.; lower tracings, u.v. absorbance at 280 nm with attenuation set at 0.05 a.u.f.s.) of lipoxygenase products in the denatured incubation media.

alone, a small increase in 5,15-DiHETE synthesis occurred, which plateaued at about 3 min (Figure 3a). Co-addition of 100 nM LTB_4 led to a greatly enhanced synthesis of 5,15-DiHETE, relative to unstimulated cells, with a similar time course. The effect of increasing concentrations of LTB_4 on the 5-LO metabolism of 15-HpETE is shown in Figure 3b. Activation of the 5-LO was detected with concentrations of LTB_4 lower than 1 nM, and was nearly maximal at 1 μM LTB_4. LTB_4 alone neither stimulated the synthesis of 5,15-DiHETE, nor that of endogenous AA-derived 5-LO products (as determined by r.p.-h.p.l.c. and u.v. detection), regardless of the concentration used (not shown).

Inhibition by MK-886 of the LTB_4-induced activation of 5-LO

MK-886, an indole derivative, has recently been described as a specific LT synthesis inhibitor that blocks the A23187- or fMLP-induced 5-LO translocation from the cytosol to the plasma membrane (Kargman *et al.*, 1991). We therefore conducted experiments to determine whether pre-incubation of PMNL with this drug would result in the inhibition of the 5-LO activation induced by LTB_4. PMNL were pre-treated with increasing concentrations of MK-886 (up to 300 nM), and incubated for 15 min with 3.0 μM 15-HpETE, either alone or with 100 nM LTB_4. Figure 4 shows that MK-886 dose-dependently inhibited the LTB_4-induced 5,15-DiHETE formation, a maximal effect being reached between 100 and 300 nM MK-886. The maximal inhibition averaged 68.0 ± 3.1% (mean ± s.e.mean) in 5 independent experiments.

Structural specificity of the LTB_4-induced activation of 5-LO

We next investigated whether the LTB_4-induced activation of the 5-LO was specific, using the ω-oxidation metabolite of LTB_4, 20-hydroxy-LTB_4, and a stereoisomer of LTB_4, Δ^6-*trans*-12-epi-LTB_4. PMNL were incubated for 15 min with 3.0 μM 15-HpETE, either alone or with increasing concentrations of LTB_4, Δ^6-*trans*-12-epi-LTB_4, or 20-hydroxy-LTB_4 (Figure 5). All compounds investigated failed to stimulate the formation of 5-LO products when added by themselves, regardless of their respective concentrations (not shown). In contrast, co-incubation with 15-HpETE induced a concentration-dependent activation of the 5-LO by all compounds. However, LTB_4 was approximately 100 times more active than 20-hydroxy-LTB_4, and about 300 times more active than Δ^6-*trans*-12-epi-LTB_4. These results indicate that the activation of the 5-LO by LTB_4 shows a high degree of structural specificity (including stereospecificity).

Effect of pertussis toxin on the LTB_4-induced activation of 5-lipoxygenase

Pertussis toxin-sensitive G proteins are involved in the transmembrane signalling process whereby LTB_4 exerts its various effects on neutrophil functions. We therefore examined whether the LTB_4-induced activation of the 5-LO was also G protein-dependent. PMNL suspensions were pre-incubated with either pertussis toxin or diluent, and then exposed to 3.0 μM 15-HpETE, alone or with 100 nM LTB_4 for 15 min. Pre-incubation with pertussis toxin strongly inhibited (69.6 ± 10.7%, $n = 7$, $P < 0.001$) the LTB_4-induced 5,15-DiHETE formation from 15-HpETE, relative to control preparations. Finally, the minor 5,15-DiHETE synthesis that occurs when

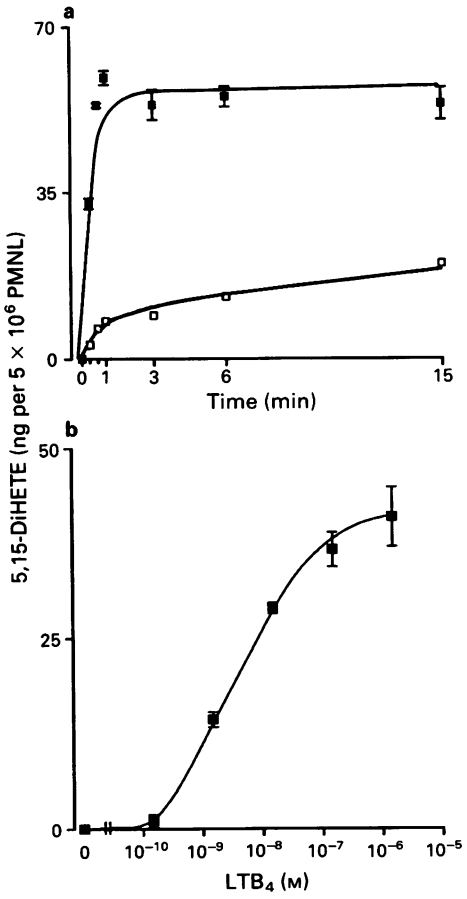

Figure 3 Time course and concentration dependency of the leukotriene B_4 (LTB_4)-induced 5-lipoxygenase (5-LO) transformation of 15-HpETE into 5,15-DiHETE. PMNL were incubated (a) for varying lengths of time at 37°C with 3.0 μM 15-HpETE, by itself or with 100 nM LTB_4; (b) for 15 min at 37°C with increasing concentrations of LTB_4, either by itself (not shown) or with 3.0 μM 15-HpETE. Note that in (b), the 5,15-DiHETE levels (17.5 ± 0.9 ng per 5 × 10^6 PMNL) resulting from exposure to 15-HpETE alone have been subtracted, so as to depict only the effect of LTB_4. Symbols: (□) 15-HpETE alone; (■) 15-HpETE and LTB_4. These experiments are representative of three (a) and eight (b); each data point is the mean with s.e.mean (vertical bars) of triplicate incubations.

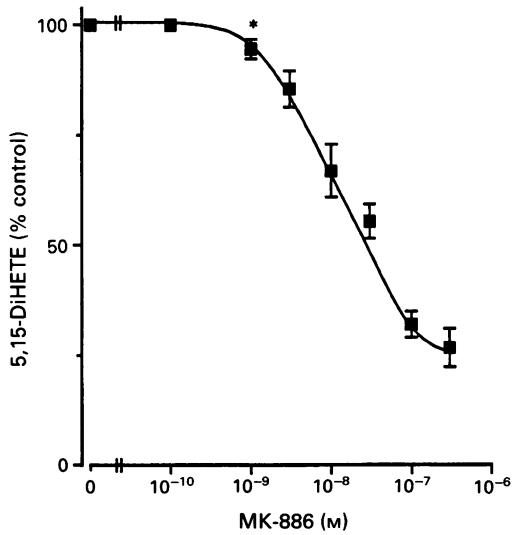

Figure 4 Inhibition of the leukotriene B_4 (LTB_4)-induced 5-lipoxygenase (5-LO) activation by MK-886. PMNL were pretreated with increasing concentrations of MK-886 (for 1 min at 37°C) and incubated for 15 min with 3.0 μM 15-HpETE, alone or with 100 nM LTB_4. The 5,15-DiHETE levels resulting from exposure to 15-HpETE (± MK-886, in the absence of LTB_4) have been subtracted, so as to only depict the effect of MK-886 on the 5-LO activation induced by LTB_4. The results are expressed as the mean ± s.e.mean (vertical bars) of averaged triplicate incubations from five independent experiments. *$P < 0.036$.

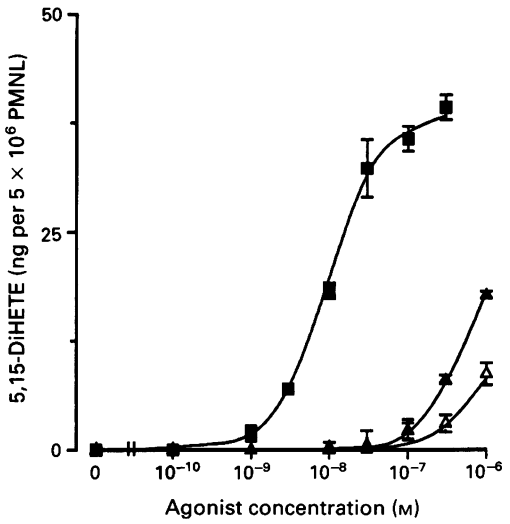

Figure 5 Structural specificity of the leukotriene B_4 (LTB_4)-induced activation of the neutrophil 5-lipoxygenase (5-LO). PMNL were incubated for 15 min at 37°C with 3.0 μM 15-HpETE, alone or with increasing concentrations of LTB_4 (■), Δ^6-*trans*-12-epi-LTB_4 (△) or 20-hydroxy-LTB_4 (▲). The 5,15-DiHETE levels (9.0 ± 0.2 ng per 5 × 10^6 PMNL) resulting from exposure to 15-HpETE alone have been subtracted, so as to depict only the effect of the compounds investigated. This experiment is representative of four; each data point is the mean ± s.e.mean (vertical bars) of triplicate incubations.

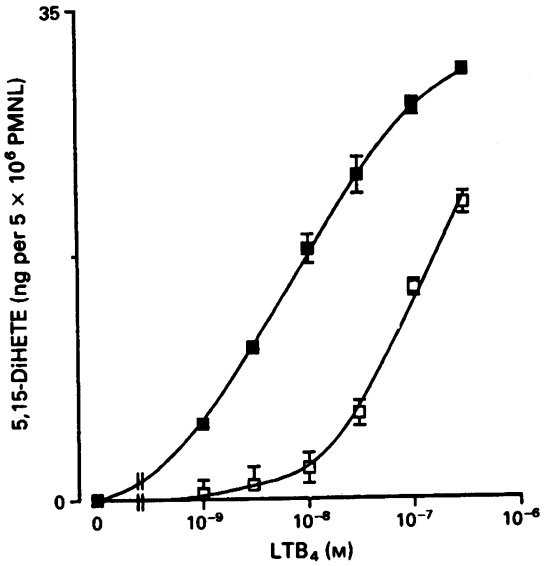

Figure 6 Inhibition by the receptor antagonist LY-223982 of the leukotriene B_4 (LTB_4)-induced 5-lipoxygenase (5-LO) activation. PMNL were incubated at 37°C for 15 min with 3.0 μM 15-HpETE and increasing concentrations of LTB_4, with (□) or without (■) prior treatment with 1.0 μM LY-223982 (1 min at 37°C). The 5,15-DiHETE levels (2.0 ± 0.2 ng per 5 × 10^6 PMNL) resulting from exposure to 15-HpETE alone (± LY-223982) have been subtracted, so as to depict only the effect of LY-223982 on 5-LO activation induced by LTB_4. This experiment is representative of three; each data point is the mean ± s.e.mean (vertical bars) of triplicate incubations.

PMNL are incubated with 15-HpETE alone was not significantly affected by pretreatment of the cells with pertussis toxin in 7 independent experiments ($P = 0.24$; data not shown).

Inhibition of the LTB_4-induced 5-LO activation by the LTB_4 receptor antagonist LY-223982

That the LTB_4-induced 5-LO activation is stereospecific and probably involves a pertussis toxin-sensitive G protein led us to investigate further this process at the level of the LTB_4 receptor. PMNL were pre-incubated with 1.0 μM of the LTB_4 receptor antagonist LY-223982 for 1 min and then exposed to 3.0 μM 15-HpETE alone, or together with increasing concentrations of LTB_4 for 15 min. LY-223982 pretreatment induced a pronounced shift to the right of the dose-response curve for the LTB_4-induced activation of the 5-LO (Figure 6). LY-223982 had no significant effect on the 5,15-DiHETE synthesis resulting from exposure of the cells to 15-HpETE alone in 5 independent experiments ($P = 0.32$; data not shown).

Discussion

It was previously observed that soluble neutrophil agonists which share many common actions with LTB_4 (namely, PAF, fMLP, C5a and IL-8) stimulate the 5-LO-dependent conversion of exogenous AA into leukotrienes in human PMNL (Lin *et al.*, 1982; Clancy *et al.*, 1983; Schröder, 1989). In a recent study, we further characterized the stimulatory effect of these neutrophil agonists on 5-LO activation through the use of an alternative substrate, 15-HpETE (McDonald *et al.*, 1991). We now show that, like the other soluble neutrophil agonists, LTB_4 activates the 5-LO in human PMNL.

First, we demonstrated that LTB_4 activates the human neutrophil 5-LO, but by itself does not appear to induce the formation of 5-LO products derived from endogenous substrate. This conclusion is based upon the observations that LTB_4 greatly increased the 5-LO-dependent metabolism of low micromolar concentrations of exogenous AA (Figure 1) and 15-HpETE (Figure 2), while it failed to induce a detectable synthesis of 5-LO products when added to the cells by itself. Although it is still possible that LTB_4 stimulated the release of endogenous AA and subsequent synthesis of LTB_4 in amounts too small to be detected by r.p.-h.p.l.c. or by our GC-MS assay (detection limit, 100 pg), our present results are in accordance with those of Prescott *et al.* (1984) who demonstrated that LTB_4 did not cause the release of [^{3}H]-AA from pre-labelled neutrophils. Similarly, Haurand & Flohé (1989) showed that LTB_4 failed to induce the formation of 5-LO metabolites from endogenous AA. Neither study however, investigated the transformation of exogenous substrate as in the present paper.

Further characterization of the stimulation by LTB_4 of the 5-LO-mediated metabolism of exogenous 15-HpETE revealed that the activation of the 5-LO by LTB_4 is a time- and concentration-dependent process (Figure 3). It must be emphasized here that the use of 3 μM 15-HpETE entails an underestimation of 5-LO activation, as this substrate concentration is suboptimal (McDonald *et al.*, 1991). Our results also demonstrated that the LTB_4-elicited 5-LO activation can be concentration-dependently inhibited by MK-886 (Figure 4). MK-886 is known to bind to the 5-LO-activating protein or FLAP (Dixon *et al.*, 1990), and has been shown to block the A23187- and fMLP-elicited translocation of the 5-LO to the plasma membrane, as well as the concomitant LT synthesis (Kargman *et al.*, 1991). Since MK-886 shows no effect on the 5-LO itself, its inhibitory effect on the LTB_4-induced 5-LO activation suggests that the mechanism whereby LTB_4 activates the 5-LO probably involves translocation of the enzyme to the plasma membrane. However, whilst our experiments with MK-886 provide plausible evidence for such a mechanism, a formal proof would require the use of more direct approaches, such as immunoblotting or ELISA determination of 5-LO levels in subcellular fractions.

In addition, our data indicate that the activation of the 5-LO by LTB_4 results from the binding of LTB_4 to specific cell-surface receptors, and that the transmembrane signal thus generated is transduced by pertussis toxin-sensitive G

proteins. This assertion is supported by the results of several experiments. First, the ability of LTB_4 to stimulate 5-LO activation was strongly inhibited by pertussis toxin, a characteristic previously reported for other receptor-dependent actions of LTB_4 in neutrophils (Molski *et al.*, 1984; Becker *et al.*, 1985). It is therefore likely that the G protein(s) involved in the activation of the 5-LO by LTB_4 is the same as that implicated in the other actions of LTB_4. Second, our finding that the 5-LO activation induced by LTB_4 is stereospecific (Figure 5) is also in good agreement with the reported effects of LTB_4 on other neutrophil responses (Naccache *et al.*, 1989a). Finally, the involvement of the LTB_4 cell-surface receptor was confirmed through the use of the LTB_4 receptor antagonist LY-223982 (Jackson *et al.*, 1988), which effectively inhibited the LTB_4-induced 5-LO activation (Figure 6).

The preceding findings suggest that the presence of LTB_4 at inflammatory foci could potentially stimulate the synthesis of LTB_4 itself, provided that exogenous AA is available to the activated cells. That these conditions are fulfilled in a number of pathophysiological conditions makes such a scenario realistic. Indeed, LTB_4 is known to be actively synthesized upon exposure of phagocytes to soluble or particulate agonists, and has been detected in many inflammatory conditions in man (Ford-Hutchinson, 1990). Similarly, micromolar concentrations of AA have been measured in inflammatory lesions (Barr *et al.*, 1984; Greaves, 1986). Moreover, AA could be supplied to activated neutrophils through cellular interactions; in this respect, it was recently reported that PMNL stimulated by soluble agonists can effectively synthesize LTB_4 using AA derived from activated platelets (Fiore & Serhan, 1990; Palmantier & Borgeat, 1991). Therefore, the generation of LTB_4 at inflammatory sites, as well as its ability to synergise with AA to produce positive feedback on its own synthesis, could represent a powerful amplification mechanism of the inflammatory reaction *in vivo*, especially when considering the potent action of the lipid mediator on phagocyte recruitment. In a similar fashion, the presence of PAF, C5a, IL-8 and formylated peptides at inflammatory foci may also enhance ongoing leukotriene synthesis by PMNL, since these compounds share with LTB_4 the ability to activate the 5-LO (Schröder, 1989; McDonald *et al.*, 1991). Studies are in progress to assess potential autocrine effects of LTB_4 on leukotriene synthesis and on other functional responses in activated PMNL undergoing leukotriene synthesis.

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Differential modulation of extracellular levels of 5-hydroxytryptamine in the rat frontal cortex by (R)- and (S)-zacopride

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1 The ability of various anxiolytic and potential anxiolytic agents to modify 5-hydroxytryptamine (5-HT) release in the frontal cortex of the rat was assessed by the microdialysis technique.

2 The benzodiazepine receptor agonist, diazepam (2.5 mg kg^{-1}, i.p.), the 5-HT$_{1A}$ receptor agonist 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT, 0.32 mg kg^{-1}, s.c.) and the 5-HT$_{1A}$ receptor partial agonist buspirone (4.0 mg kg^{-1}, i.p.) maximally reduced extracellular levels of 5-HT in the rat frontal cortex by approximately 50–60%, 70–80% and 30–40%, respectively.

3 (R)-zacopride (1.0–100 μg kg^{-1}, i.p.) dose-dependently reduced extracellular levels of 5-HT in the rat frontal cortex (approximately 80% maximal reduction) whereas the other 5-HT$_3$ receptor antagonists ondansetron (10 μg kg^{-1}, i.p.) and (S)-zacopride (10–100 μg kg^{-1}, i.p.) were ineffective.

4 In contrast to (S)-zacopride (100 nM; administered via the microdialysis probe), (R)-zacopride (1.0–100 nM; administered via the microdialysis probe) induced a concentration-dependent reduction in extracellular levels of 5-HT in the rat frontal cortex (approximately 70% maximal reduction).

5 In contrast to ondansetron (100 μg kg^{-1}, i.p.), (S)-zacopride (10–100 μg kg^{-1}, i.p.) dose-dependently reversed the (R)-zacopride (10 μg kg^{-1}, i.p.) induced reduction in extracellular levels of 5-HT in the rat frontal cortex. The highest dose of (S)-zacopride (100 μg kg^{-1}, i.p.) completely prevented the (R)-zacopride response. In addition, (S)-zacopride (100 nM; administered via the microdialysis probe) attenuated the inhibitory action of (R)-zacopride (10 nM; administered via the microdialysis probe) on extracellular levels of 5-HT in the rat frontal cortex.

6 In conclusion, the present study provides further evidence of the ability of diazepam, 8-OH-DPAT and buspirone to reduce the activity of the central 5-hydroxytryptaminergic system *in vivo*. Furthermore, the results indicate that the ability of (R)-zacopride to reduce the *in vivo* release of 5-HT in the rat frontal cortex does not correlate with its 5-HT$_3$ receptor antagonism. However, the differential affinity of (R)- and (S)-zacopride for a (S)-zacopride-insensitive (R)-zacopride site in rat cerebral cortex mirrors the relative activity of the two zacopride stereoisomers to modify the *in vivo* release of 5-HT in the frontal cortex of the rat and their ability to release suppressed behaviour in animal models of anxiety.

Keywords: 5-Hydroxytryptamine; anxiety; *in vivo* microdialysis; frontal cortex; 5-HT$_3$ receptor; 5-HT$_4$ receptor; benzamides

Introduction

A characteristic feature of many compounds that reduce the functional activity of the central 5-hydroxytryptaminergic system is the manifestation of anxiolytic-like behaviour (for reviews see Iversen, 1984; Chopin & Briley, 1987). Thus the clinical efficacy of the benzodiazepine receptor agonists and the 5-HT$_{1A}$ receptor agonist/partial agonists as anxiolytic agents has been attributed to a reduction in the neuronal release of 5-hydroxytryptamine (5-HT; e.g. Stein *et al.*, 1977; Chopin & Briley, 1987). In contrast, the anxiolytic-like activity of 5-HT$_3$ receptor antagonists (for reviews see Costall *et al.*, 1990; Barnes *et al.*, 1992a) is probably a consequence of preventing the natural agonist interacting with postsynaptic receptors.

Whilst structurally dissimilar 5-HT$_3$ receptor antagonists display anxiolytic-like activity in pre-clinical models and in preliminary trials with patients with generalized anxiety disorder (for reviews see Costall *et al.*, 1990; Barnes *et al.*, 1992a), the report that the (R)- and (S)-isomers of the benzamide derivative 5-HT$_3$ receptor antagonist, zacopride, display differential activity in animal models of anxiety remains an unexplained phenomenon (Barnes *et al.*, 1990a; Young & Johnson, 1991). Thus the (R)-isomer of zacopride possesses anxiolytic-like activity at low microgram doses whereas effective doses of (S)-zacopride are at least 4 orders of magnitude higher (Barnes *et al.*, 1990a; Young & Johnson, 1991). This potency difference, however, is not consistent with their affinities for the 5-HT$_3$ receptor since both possess considerable antagonistic activity at nanomolar concentrations (Barnes *et al.*, 1990a). The pharmacological diversity of the isomers of zacopride is further emphasized by the ability of (S)-zacopride to attenuate the anxiolytic profiles of (R)-zacopride (Barnes *et al.*, 1992b) and other pharmacologically distinct agents (Barnes *et al.*, 1992c).

In an attempt to reveal a neurochemical correlate for the ability of the isomers of zacopride to modulate suppressed behaviour, in the present studies we assess the actions of various anxiolytic and putative anxiolytic agents and the stereoisomers of zacopride, on the *in vivo* extracellular levels of 5-HT in the frontal cortex of freely-moving rats using the microdialysis technique. A preliminary account of this work has been presented to the British Pharmacological Society (Cheng *et al.*, 1992).

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Methods

Animals

Male hooded-Lister rats (300–350 g) were housed in groups of 6 in a controlled environment (temperature 21 ± 1°C) under a 12 h light/dark cycle (lights on 07 h 00 min–19 h 00 min) and were given free access to food and water.

Stereotaxic implantation of chronic indwelling guide cannulae

Rats were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and chloral hydrate (150 mg kg^{-1}, s.c.) before the insertion of 5 mm chronically indwelling guide cannulae (19 guage stainless steel tubing; Coopers Needle Work Ltd) which were positioned above the frontal cortex (final probe tip location; right hemisphere, A + 0.7, V − 7.0, L − 1.5, relative to Bregma at an angle of 45°) and secured to the skull with screws and dental cement. The guide cannulae were kept patent with stylets.

Microdialysis probe construction, implantation and collection of dialysates

Microdialysis probes were constructed 'in house'. Briefly, stainless steel 'bodies' (11 mm, 23 guage, Coopers Needle Work Ltd) and 'collars' (5 mm, 26 guage, Coopers Needle Work Ltd) were fixed into a perspex holder. Dialysis membrane (external/internal diameter 310/220 μm, molecular weight cut off 40,000; AN69, Hospal Medical) was glued to the tip of the stainless steel 'body' and the end sealed with epoxy resin (Araldite) leaving 4 mm in total length exposed to the brain area. A silica glass fibre (external/internal diameter 140/74 μm, Scientific Glass Engineering PTY Ltd) guided artificial cerebrospinal fluid (aCSF; mM: NaCl 126, $NaHCO_3$ 27.4, KCl 2.4, KH_2PO_4 0.5, $CaCl_2$ 1.1, $MgCl_2$ 1.3, $NaHPO_4$ 0.49 and glucose 7.0; pH 7.4) to the tip of the microdialysis probe, which subsequently eluted from the probe (via coiled polypropylene tubing) following passage between the outer surface of the silica glass fibre and the inner surface of the dialysis membrane.

At least 14 days after stereotaxic location of the guide cannulae, rats were immobilized by a soft-cloth wrapping technique and the microdialysis probe was gently implanted into the frontal cortex and secured with cyanoacrylate adhesive (Permabond C2).The rat was placed in a single animal cage (with free access to food and water) for 12 h before being transferred to the test cage (42 × 24 × 26 cm; length, width, height) where the animal also had free access to food and water. Following a 30 min period for the rat to habituate to the test box, the microdialysis probe was connected, via polypropylene tubing, to a microdialysis pump (CMA 100, Carnegie) and was perfused at a constant rate of 2.0 μl min^{-1} with aCSF. The dialysate collected over the first 60 min was discarded and subsequent samples were collected every 20 min for a period of up to 6 h. All samples were analysed immediately for levels of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) by high performance liquid chromatography (h.p.l.c.) with electrochemical detection (e.c.d.). At the end of the experiment, microdialysis probe placement was verified visually by coronal slicing of the brain with a freezing microtome. Data from animals where the microdialysis probes were not correctly located within the frontal cortex were not included in the present study.

H.p.l.c.-e.c.d. system for the quantification of 5-HT and 5-HIAA

For the simultaneous determination of 5-HT and 5-HIAA levels in dialysates, the h.p.l.c.-e.c.d. system comprised of an isocratic pump (Waters 510 Solvent Delivery System) which was connected to an analytical column (Hypersil 5 ODS; 150 × 4.6 mm; HPLC Technology) via an automatic injector (Waters Wisp). The analytical column was protected by a C_{18} guard column (Guard-Pak, Waters). The effluent from the analytical column was passed into an electrochemical detector (ESA Coulochem with model 5011 analytical cell, working electrode potential + 0.45 V versus a solid state reference electrode incorporated within the analytical cell). The output from the electrode was monitored with a recording/plotting integrator (Hewlett-Packard 3392A). A guard cell (ESA model 5020) was placed between the pump and injector and was set at + 0.50 V (relative to a solid state reference electrode incorporated within the cell) to reduce the background current originating from the mobile phase. The h.p.l.c.-e.c.d. system, with the exception of the integrator, was maintained at a constant temperature of 4°C inside a glass-fronted cool cabinet. The optimized mobile phase (methanol 11% v/v, disodium hydrogen orthophosphate 106 mM, citric acid 36 mM, tetraethylammonium bromide 2 mM, pH 6.2–6.3; slight adjustments to the pH and/or methanol concentration were made to overcome column to column variation) was delivered to the analytical column at a rate of 1.4 ml min^{-1}. Injections of external standards were made in order to identify and calibrate the peaks resulting from the injection of the dialysates.

Drugs

Buspirone (Mead Johnson), 8-OH-DPAT (8-hydroxy-2-(di-n-propylamino) tetralin, Research Biochemicals Inc.), ondansetron (Glaxo), pargyline (Sigma), tetrodotoxin (Sigma),

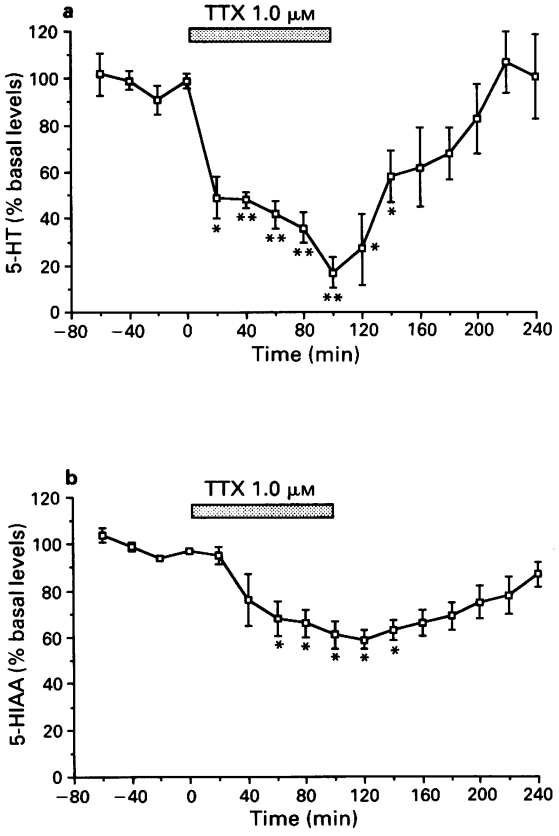

Figure 1 Effect of tetrodotoxin (1.0 μM; TTX) in the perfusing aCSF on 5-hydroxytryptamine (5-HT) (a) and 5-hydroxyindoleacetic acid (5-HIAA) (b) levels in rat frontal cortex dialysates. 5-HT and 5-HIAA levels in dialysates are expressed as the percentage of the meaned absolute amount in the 4 collections preceding the drug treatment. Data represent the mean ± s.e.mean (vertical bars) from 4 animals. ANOVA:$P<0.05$; *$P<0.05$; **$P<0.01$ (Dunnett's t test).

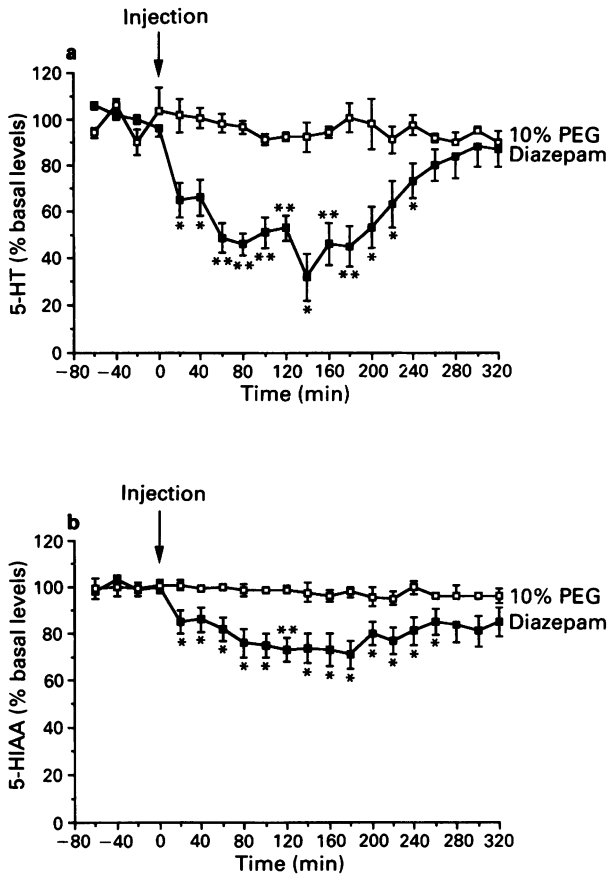

Figure 2 Effect of vehicle (10% v/v polyethylene glycol in saline, 1.0 ml kg^{-1}, i.p.; 10% PEG) and diazepam (2.5 mg kg^{-1}, i.p.) on 5-hydroxytryptamine (5-HT) (a) and 5-hydroxyindoleacetic acid (5-HIAA) (b) levels in rat frontal cortex dialysates. 5-HT and 5-HIAA levels in dialysates are expressed as the percentage of the meaned absolute amount in the 4 collections preceding the drug treatment. Data represent the mean ± s.e.mean (vertical bars) from 3–6 animals. ANOVA: $P<0.05$; $^{*}P<0.05$; $^{**}P<0.01$ (Dunnett's t test).

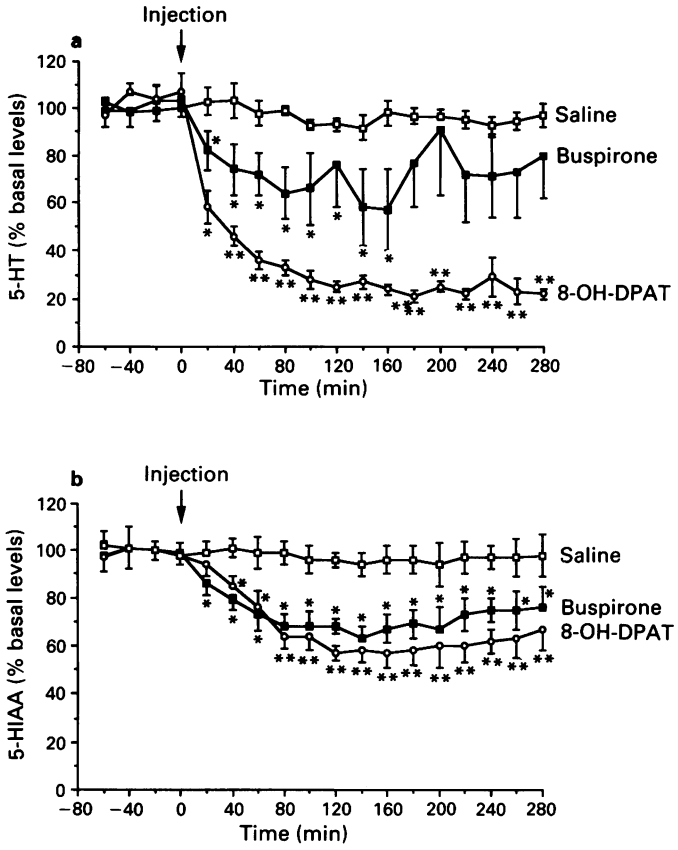

Figure 3 Effect of vehicle (saline, 1.0 ml kg^{-1}, i.p.), 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), 0.32 mg kg^{-1}, s.c.) and buspirone (4 mg kg^{-1}, i.p.) on 5-hydroxytryptamine (5-HT) (a) and 5-hydroxyindoleacetic acid (5-HIAA) (b) levels in rat frontal cortex dialysates. 5-HT and 5-HIAA levels in dialysates are expressed as the percentage of the meaned absolute amount in the 4 collections preceding the drug treatment. Data represent the mean ± s.e.mean (vertical bars) from 6–8 animals. ANOVA: $P<0.05$; $^{*}P<0.05$; $^{**}P<0.01$ (Dunnett's t test).

(R)-zacopride (A.H. Robins) and (S)-zacopride (A.H. Robins) were dissolved in distilled water and diluted to volume in saline (0.9% w/v NaCl) or aCSF. Diazepam (Sigma) was dissolved in 10% v/v polyethylene glycol (in saline). All drugs were used as received and were freshly prepared immediately before use.

Results

Methodological results

The *in vitro* recoveries of 5-HT and 5-HIAA using the 'in-house' microdialysis probes were 24 ± 1 and 20 ± 1%, respectively (mean ± s.e.mean, 6 probes). The limits of detection for both 5-HT and 5-HIAA by the h.p.l.c.-e.c.d. assay was at least 1 pg (signal to noise ratio of 3:1; injection volume 40 µl).

Validation of the neuronal origin of the 5-HT in the dialysates

The basal level of 5-HT and 5-HIAA varied between 37–63 fmol/40 µl and 1.83–2.88 pmol/40 µl, respectively, in the dialysates obtained from the frontal cortex of freely-moving rats 12 h following the implantation of the dialysis probe.

Inclusion of tetrodotoxin (1.0 µM) in the perfusing aCSF reduced the levels of 5-HT and 5-HIAA in the frontal cortex dialysates by approximately 80 and 40%, respectively. These levels recovered to basal values following the removal of tetrodotoxin (Figure 1).

Removal of $CaCl_2$ from the aCSF (and inclusion of EGTA, 0.2 mM) maximally reduced the frontal cortex dialysate levels of 5-HT by approximately 50%. The 5-HT levels, however, began to return to basal values before the re-addition of $CaCl_2$ to the perfusing aCSF (data not shown).

Effect of monoamine oxidase inhibition on the dialysate levels of 5-HT and 5-HIAA

Peripheral administration of the non-selective monoamine oxidase inhibitor, pargyline (75 mg kg^{-1}, i.p.) increased the frontal cortex dialysate levels of 5-HT by up to 800% and decreased the levels of 5-HIAA (maximally to 30–40% of basal levels) over the 320 min period following the peripheral injection (data not shown).

Effect of systemic drug administration on the dialysate levels of 5-HT and 5-HIAA

Administration of the benzodiazepine receptor agonist, diazepam (2.5 mg kg^{-1}, i.p.), reduced the frontal cortex dialysate levels of 5-HT and 5-HIAA (Figure 2). Injection of diazepam's vehicle (10% v/v polyethylene glycol in saline, i.p.) failed to alter dialysate levels of either 5-HT or 5-HIAA (Figure 2).

The 5-HT_{1A} receptor agonist, 8-OH-DPAT (0.32 mg kg^{-1},

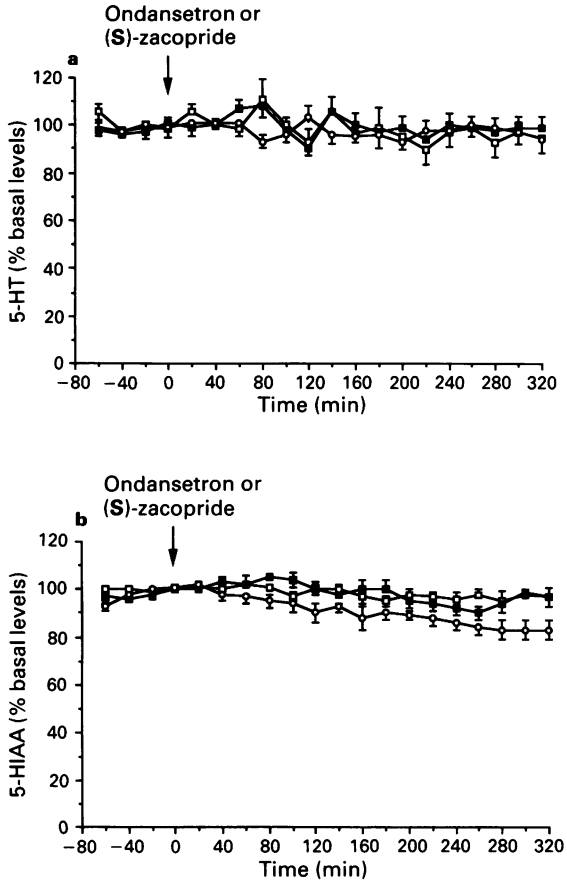

Figure 4 Effect of ondansetron (10 μg kg^{-1}, i.p. (○)), and (S)-zacopride (10 μg kg^{-1}, i.p. (□) and 100 μg kg^{-1}, i.p. (■)) on 5-hydroxytryptamine (5-HT) (a) and 5-hydroxyindoleacetic acid (5-HIAA) (b) levels in rat frontal cortex dialysates. 5-HT and 5-HIAA levels in dialysates are expressed as the percentage of the meaned absolute amount in the 4 collections preceding the drug treatment. Data represent the mean ± s.e.mean (vertical bars) from 8 animals. ANOVA: $P > 0.05$.

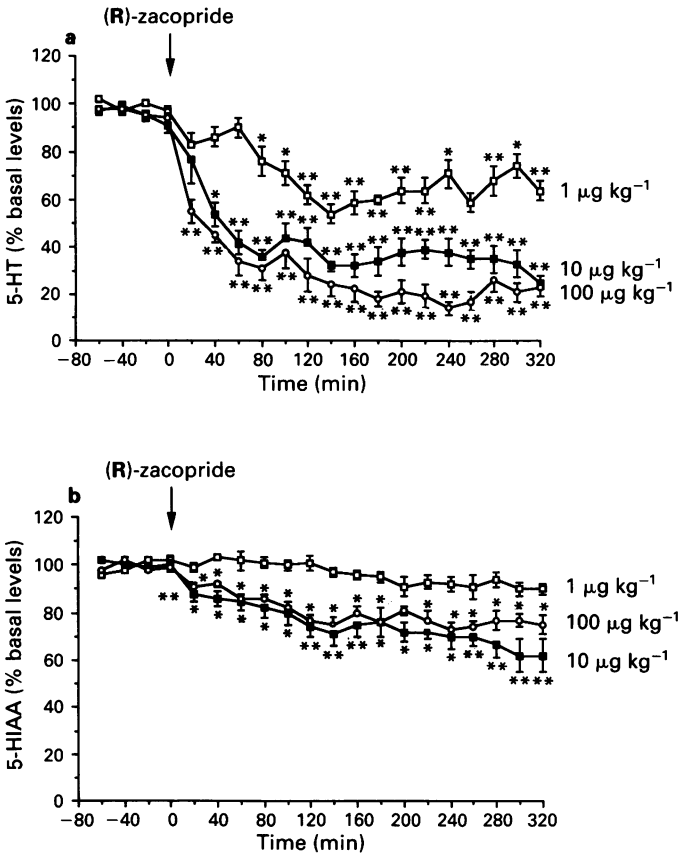

Figure 5 Effect of (R)-zacopride (1.0–100 μg kg^{-1}, i.p.) on 5-hydroxytryptamine (5-HT) (a) and 5-hydroxyindoleacetic acid (5-HIAA) (b) levels in rat frontal cortex dialysates. 5-HT and 5-HIAA levels in dialysates are expressed as the percentage of the meaned absolute amount in the 4 collections preceding the drug treatment. Data represent the mean ± s.e.mean (vertical bars) from 8 animals. ANOVA: $P < 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$ (Dunnett's t test).

s.c.) and partial agonist buspirone (4.0 mg kg^{-1}, i.p.) decreased the levels of 5-HT and 5-HIAA in the frontal cortex dialysates (Figure 3). Subcutaneous (data not shown) or intra-peritoneal injection of vehicle (saline) failed to alter dialysate levels of either 5-HT or 5-HIAA (Figure 3).

Ondansetron (10 μg kg^{-1}, i.p.) and (S)-zacopride (10–100 μg kg^{-1}, i.p.) failed to alter the levels of 5-HT in the dialysates from rat frontal cortex (Figure 4). Furthermore, of these treatments, only (S)-zacopride (100 μg kg^{-1}, i.p.) induced a small reduction in the dialysate levels of 5-HIAA (by some 15–20%) which was maximal at the end of the experiment (320 min after the injection; Figure 4).

(R)-zacopride (1.0–100 μg kg^{-1}, i.p.) reduced the dialysate levels of 5-HT from rat frontal cortex in a dose-dependent manner; the highest dose decreasing the levels by some 70–80% (Figure 5). At all of the doses tested, the maximal decreases in dialysate 5-HT levels were detected approximately 140 min following the injection and the reduced levels remained near maximal for the remainder of the experiment (320 min after the injection). (R)-zacopride (10–100 μg kg^{-1}, i.p.) also reduced the dialysate levels of 5-HIAA (by upto 40%) whilst a lower dose (1.0 μg kg^{-1}, i.p.) was without effect. The (R)-zacopride-induced reduction in 5-HIAA levels reached a maximum at approximately 120 min after the injection of 100 μg kg^{-1} (and remained so for the duration of the experiment), whilst the 5-HIAA levels continued to decrease up to 320 min following the injection of 10 μg kg^{-1} (R)-zacopride (Figure 5).

Effect of central drug administration on the dialysate levels of 5-HT and 5-HIAA

(R)-zacopride (1–100 nM) administered via the perfusing aCSF induced a concentration-dependent decrease in the dialysate levels of 5-HT and 5-HIAA from the rat frontal cortex (Figure 6). The highest concentration maximally reduced 5-HT and 5-HIAA levels by approximately 70% (Figure 6); the reduction being maximal 60 min after the introduction of (R)-zacopride (Figure 6).

(S)-zacopride (100 nM) administered via the perfusing aCSF failed to modify the dialysate levels of either 5-HT or 5-HIAA from the rat frontal cortex (Figure 6).

Effect of (S)-zacopride and ondansetron on the (R)-zacopride-induced reduction in the dialysate levels of 5-HT and 5-HIAA

(S)-zacopride (10–100 μg kg^{-1}, i.p.) administered 5 min prior to the injection of (R)-zacopride (10 μg kg^{-1}, i.p.) dose-dependently prevented the reduction in extracellular levels of 5-HT and 5-HIAA (Figure 7).

In contrast, injection of saline (0.9% w/v NaCl i.p.) or ondansetron (100 μg kg^{-1}, i.p.) 5 min prior to the injection of (R)-zacopride (10 μg kg^{-1}, i.p.) failed to attenuate the (R)-zacopride-induced (10 μg kg^{-1}, i.p.) reduction in dialysate levels of either 5-HT or 5-HIAA (Figure 7).

Following a reduction in 5-HT dialysate levels induced by (R)-zacopride (10 nM; administered via the perfusing aCSF), the addition of (S)-zacopride (100 nM) to the aCSF

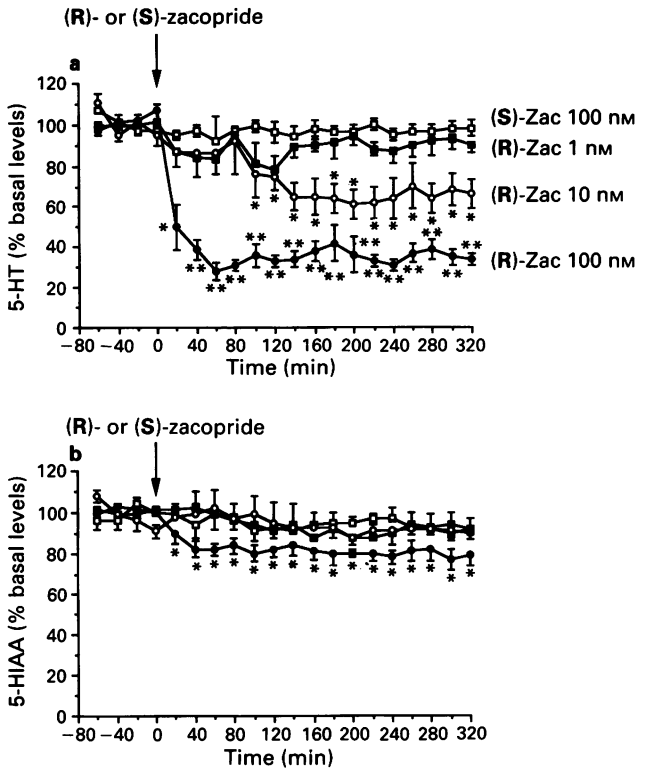

Figure 6 Effect of (R)-zacopride (1.0–100 nM; (R)-Zac) and (S)-zacopride (100 nM; (S)-Zac) administered via the perfusing aCSF on 5-hydroxytryptamine (5-HT) (a) and 5-hydroxyindoleacetic acid (5-HIAA) (b) levels in rat frontal cortex dialysates. 5-HT and 5-HIAA levels in dialysates are expressed as the percentage of the meaned absolute amount in the 4 collections preceding the drug treatment. Data represent the mean ± s.e.mean (vertical bars) from 8 animals. ANOVA: $P<0.05$; $^{*}P<0.05$; $^{**}P<0.01$. (Dunnett's t test).

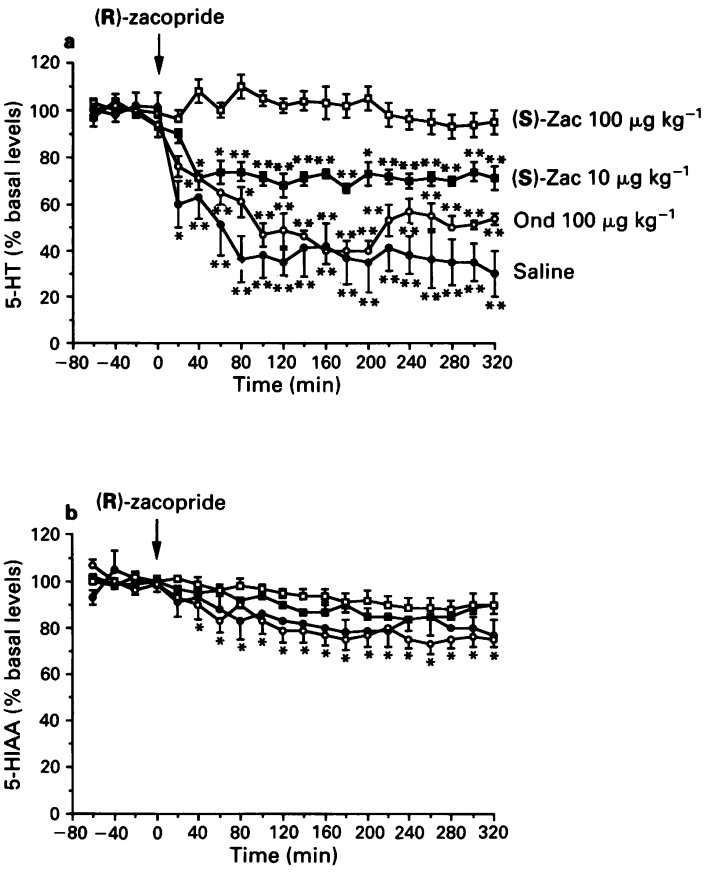

Figure 7 Ability of saline vehicle (0.9% w/v NaCl i.p.; Saline), ondansetron (100 μg kg^{-1}, i.p.; Ond) or (S)-zacopride (10–100 μg kg^{-1}, i.p.; (S)-Zac) to modify the (R)-zacopride-induced reduction in dialysate levels of 5-hydroxytryptamine (5-HT) (a) and 5-hydroxyindoleacetic acid (5-HIAA) (b) from rat frontal cortex. Saline vehicle, ondansetron or (S)-zacopride were administered 5 min prior to the injection of (R)-zacopride (10 μg kg^{-1}, i.p.). 5-HT and 5-HIAA levels in dialysates are expressed as the percentage of the meaned absolute amount in the 4 collections preceding the drug or vehicle treatment. Data represent the mean ± s.e.mean (vertical bars) from 8 animals. ANOVA: $P<0.05$; $^{*}P<0.05$; $^{**}P<0.01$ (Dunnett's t test).

attenuated the (R)-zacopride-induced reduction in dialysate 5-HT levels (Figure 8).

Discussion

In the present studies, the effect of anxiolytic and putative anxiolytic agents on 5-HT release from the frontal cortex was assessed by the microdialysis technique in freely moving rats. The finding that the dialysate 5-HT levels were sensitive to the addition of the sodium channel blocker, tetrodotoxin, to the perfusing aCSF and to the removal of $CaCl_2$ from the aCSF, indicates that a relatively large component (at least 70–80%) of the quantified 5-HT was neuronal in origin, this being consistent with previous reports (e.g. Auerbach *et al.*, 1989; Carboni & DiChiara, 1989). Likewise the response following inhibition of monoamine oxidase subsequent to peripheral administration of pargyline (e.g. Carboni & DiChiara, 1989), where a gradual increase in the levels of 5-HT and a concomitant decrease in the levels of the metabolite of 5-HT, 5-HIAA, presumably reflected a decrease in the metabolism of this monoamine.

As has been previously demonstrated in the rat frontal cortex and/or ventral hippocampus (e.g. Maidment *et al.*, 1986; Pei *et al.*, 1989; Sharp *et al.*, 1989), 5-HT release in the frontal cortex, measured by *in vivo* microdialysis, was decreased following administration of the benzodiazepine receptor agonist, diazepam, and the 5-HT_{1A} receptor agonist 8-OH-DPAT and partial agonist buspirone. Such findings are consistent with the action of benzodiazepine receptor agonists to potentiate the inhibitory action of γ-aminobutyric acid (GABA) (e.g. Enna & Mohler, 1987) and the actions of 5-HT_{1A} receptor ligands with intrinsic activity to reduce the release of 5-HT in terminal regions following interaction with somato-dendritic 5-HT_{1A} autoreceptors (Maidment *et al.*, 1986; Auerbach *et al.*, 1989; Hutson *et al.*, 1989; Sharp *et al.*, 1989). The maintenance of a maximal reduction in dialysate 5-HT levels following 8-OH-DPAT administration at the termination of the experiment (over 4 h after the subcutaneous injection) may at first appear unexpected since this compound is rapidly metabolized (half life < 30 min; Perry & Fuller, 1989). It should be noted, however, that previous studies have demonstrated that comparable doses of 8-OH-DPAT induce a maximal response for at least as long as in the present study (e.g. Maidment *et al.*, 1986).

The 5-HT_3 receptor antagonist, ondansetron (Butler *et al.*, 1988) when used at a dose known to reduce the behavioural response to an aversive situation (Costall *et al.*, 1990) failed to alter the release of 5-HT in the frontal cortex. This was not entirely unexpected since 5-HT_3 receptor recognition sites do not appear to be predominantly presynaptic in their location since their density is not reduced following selective lesion of the central 5-hydroxytryptaminergic system (Barnes *et al.*, unpublished observation). The absence of any detectable change in frontal cortex dialysate 5-HT levels by the acute administration of ondansetron also indicates that modulation by 5-HT_3 receptors of release via postsynaptic

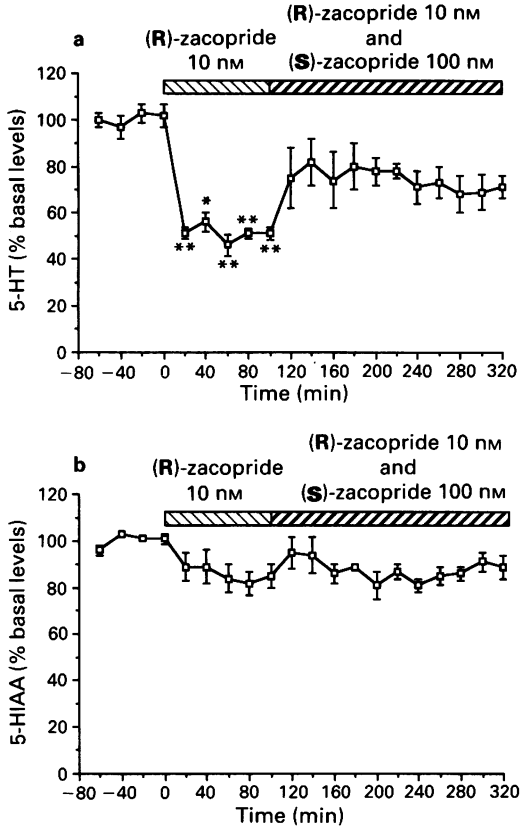

Figure 8 Ability of (S)-zacopride (100 nM) administered via the perfusing aCSF to modify the (R)-zacopride-induced reduction in dialysate levels of 5-hydroxytryptamine (5-HT) (a) and 5-hydroxyindoleacetic acid (5-HIAA) (b) from rat frontal cortex. (R)-zacopride was added to the aCSF at $t = 0$ and (S)-zacopride was added to the aCSF after an additional 100 min. 5-HT and 5-HIAA levels in dialysates are expressed as the percentage of the meaned absolute amount in the 4 collections preceding the drug treatment. Data represent the mean ± s.e.mean (vertical bars) from 8 animals. ANOVA: $P<0.05$; $*P<0.05$; $**P<0.01$ (Dunnett's t test).

feedback systems is not involved. In accordance with the results obtained with ondansetron, the structurally dissimilar 5-HT_3 receptor antagonist, (S)-zacopride (Barnes *et al.*, 1990a,b), also failed to modify the *in vivo* release of 5-HT from the rat frontal cortex. These findings therefore imply that the dose-dependent ability of (R)-zacopride to reduce extracellular levels of 5-HT in the frontal cortex was not a consequence of antagonism of 5-HT_3 receptors. However, this neurochemical response may provide the basis for the anxiolytic-like action of (R)-zacopride (see Introduction).

The ability of nanomolar concentrations of (R)-zacopride to reduce the apparent terminal release of 5-HT in the rat frontal cortex was detected when (R)-zacopride was administered via the microdialysis probe. It would therefore be predicted that the response was a probable consequence of recognition site interaction within the frontal cortex. However, the potential involvement of additional areas following systemic administration of (R)-zacopride cannot be excluded and warrants further investigation.

At present we have no evidence as to the nature of the (R)-zacopride recognition site. Whilst this site may represent a receptor, the potential for the involvement of an uptake system or an enzyme in the (R)-zacopride-induced reduction in extracellular levels of 5-HT should not be ruled out. It may be relevant, however, that (R)-zacopride displays low nanomolar affinity for a recognition site within the brain for which (S)-zacopride displays at least 3 orders of magnitude lower affinity (Barnes *et al.*, 1990a; Kidd *et al.*, 1992). Such an affinity difference is more consistent with the differential potency of these two isomers in pre-clinical models designed to assess potential anxiolytic activity (Barnes *et al.*, 1990a; Young & Johnson, 1991) and their ability to reduce extracellular levels of 5-HT within the rat frontal cortex (present studies) compared with their ability to interact with 5-HT_3 receptors (Barnes *et al.*, 1990a,b) or stimulate 5-HT_4 receptors (Eglen *et al.*, 1990; Baxter *et al.*, 1991). It must be emphasized, however, that the pharmacological knowledge relating to this (S)-zacopride-insensitive (R)-zacopride recognition site is limited (Barnes *et al.*, 1990a; Kidd *et al.*, 1992).

Since behavioural experiments in the mouse have identified that the anxiolytic-like actions of (R)-zacopride are reversed by (S)-zacopride (Barnes *et al.*, 1992b), it is of considerable interest that in the present studies, administration of (S)-zacopride prevented the (R)-zacopride-induced reduction in extracellular 5-HT levels in the rat frontal cortex. The ability of (S)-zacopride to evoke this response when administered via the microdialysis probe indicates that this pharmacological interaction probably occurs within the frontal cortex. It is unlikely, however, that (S)-zacopride is competing with (R)-zacopride for the (R)-zacopride site which may mediate the reduction in 5-HT release since radioligand binding studies have demonstrated that (S)-zacopride displays relatively low affinity for this site (Barnes *et al.*, 1990a; Kidd *et al.*, 1992). With respect to the ability of (S)-zacopride to antagonize the (R)-zacopride-induced reduction in extracellular levels of 5-HT, it is interesting that (S)-zacopride in its own right failed to enhance extracellular 5-HT levels—such a finding may be explained by the requirement to reduce 5-hydroxytryptaminergic tone before functional expression of this (S)-zacopride-induced response. Such a hypothesis remains to be explored as does the pharmacological characterization of the 'receptor' responsible for reversing the (R)-zacopride-induced reduction in extracellular levels of 5-HT.

Regardless of the precise mechanism of action, the ability of (R)-zacopride to reduce extracellular levels of 5-HT within the rat frontal cortex and the apparent failure of (S)-zacopride to elicit a similar response in the same model provides a neurochemical response which mirrors the differential activities of the zacopride isomers in animal models of anxiety (Barnes *et al.*, 1990a; Young & Johnson, 1991). Furthermore, the capacity of (S)-zacopride to prevent the (R)-zacopride-induced reduction in 5-HT release may be relevant to the ability of (S)-zacopride to antagonize the anxiolytic profile of action of (R)-zacopride (Barnes *et al.*, 1992b).

In summary, the present studies have demonstrated that extracellular levels of 5-HT in the frontal cortex of freely moving rats (the majority of which is apparently neuronal in origin) are reduced by drugs with agonistic activity at the benzodiazepine and 5-HT_{1A} receptor but not by the 5-HT_3 receptor antagonists, ondansetron and (S)-zacopride. The ability of (R)-zacopride, therefore, to reduce the extracellular levels of 5-HT, following peripheral or central administration, is unlikely to result solely from an antagonistic interaction with 5-HT_3 receptors and may be a consequence of interaction with a (S)-zacopride insensitive 'receptor'. Such an interaction may provide a neurochemical basis for the anxiolytic-like actions of (R)-zacopride.

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Evidence that 5-HT_2 receptor activation decreases noradrenaline release in rat hippocampus *in vivo*

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1 Recent electrophysiological studies have shown that 5-HT_2/5-HT_{1C} receptor agonists inhibit the electrical activity of noradrenergic neurones in the rat locus coeruleus. Here we examine the effect of various agonists and antagonists of 5-HT_2/5-HT_{1C} receptors on noradrenaline release in hippocampus of anaesthetized rats using microdialysis.

2 Subcutaneous administration of the 5-HT_2/5-HT_{1C} receptor agonist, 1-(2,5-dimethoxy-4-iodophenyl) -2-aminopropane (DOI: 0.2 and 0.5 mg kg^{-1}), caused a marked decrease (50% of pre-drug levels 60 min after injection) of noradrenaline in hippocampal dialysates which was long-lasting (> 120 min). Noradrenaline output also decreased in response to administration of the structural analogue of DOI, 1-(2,5-dimethoxy-4-bromophenyl)-2-aminopropane (DOB: 1 mg kg^{-1}, s.c.).

3 The effect of DOI on noradrenaline output was prevented by pretreatment with the 5-HT_2/5-HT_{1C} receptor antagonist, ritanserin (0.4 mg kg^{-1}, s.c.). Spiperone (0.2 and 1 mg kg^{-1}, s.c.), a 5-HT_2/dopamine D_2 receptor antagonist which has low affinity for 5-HT_{1C} receptors, also antagonized the effect of DOI (0.5 mg kg^{-1}, s.c.). Sulpiride (50 mg kg^{-1}, s.c.), a dopamine D_2 receptor antagonist did not alter the response to DOI (0.5 mg kg^{-1}, s.c.).

4 Both the non-selective 5-HT receptor agonist, quipazine (1 mg kg^{-1}, s.c.), and the 5-HT-releasing agent, *p*-chloroamphetamine (2 mg kg^{-1}, s.c.), decreased noradrenaline release in hippocampus and these effects were antagonized by pretreatment with ritanserin (0.4 mg kg^{-1}, s.c.).

5 Our data suggest that *in vivo*, noradrenaline release in hippocampus is inhibited by 5-HT_2 receptor activation. This effect is probably associated with a decrease in noradrenergic neuronal activity.

Keywords: Microdialysis; noradrenaline; 5-HT_2 receptors; 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI); ritanserin; quipazine; *p*-chloroamphetamine

Introduction

Several lines of evidence suggest that the activity of central noradrenergic neurones is regulated by the neurotransmitter 5-hydroxytryptamine (5-HT). In particular, the major source of noradrenergic innervation to the forebrain, the locus coeruleus (LC), receives a dense input of 5-HT-containing neurones (Pickel *et al.*, 1977; Leger & Descarries, 1978) and contains high levels of both 5-HT reuptake sites (Savaki *et al.*, 1985; De Souza & Kuyatt, 1987) and 5-HT binding sites (Weissmann-Nanopoulos *et al.*, 1985). In addition, both 5-HT denervation (Pujol *et al.*, 1978) and 5-HT synthesis inhibition cause an increase in noradrenaline synthesis in the LC (Crespi *et al.*, 1980; McRae-Degueurce *et al.*, 1985). Inhibition of 5-HT synthesis also results in an increase in basal firing rate of noradrenergic neurones in the LC (Ferron *et al.*, 1988). Taken together these findings support the view that central noradrenergic neurones may be under an inhibitory influence of 5-HT.

Evidence from *in vivo* electrophysiological studies suggests that the putative inhibitory influence of 5-HT on central noradrenergic neurones may be mediated by the 5-HT_2 or 5-HT_{1C} receptor. Thus, it has been shown that systemic injection of a range of 5-HT_2/5-HT_{1C} receptor agonists, including the phenylalkylamines 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (DOM) and 1-(2,5-dimethoxy-4-bromophenyl)-2-aminopropane (DOB), inhibits the firing rate of spontaneously active neurones in the rat LC (Rasmussen & Aghajanian, 1986; Gorea & Adrien, 1988). No studies to date, however, have examined whether noradrenaline release is decreased following 5-HT_2 receptor activation, as would be predicted from the latter work.

Recent studies have shown that noradrenaline release measured by *in vivo* microdialysis is neuronally derived and changes according to the level of LC neuronal activity (Abercrombie *et al.*, 1988; Kalen *et al.*, 1988). Here we have used the brain microdialysis technique to investigate further the nature of the interaction between 5-HT_2/5-HT_{1C} receptors and noradrenergic neurones. Noradrenaline release in hippocampus, a major efferent projection of the LC (Foote *et al.*, 1983), was measured following systemic administration of the 5-HT_2/5-HT_{1C} receptor agonists, DOI and DOB (Glennon *et al.*, 1988; Appel *et al.*, 1990), the non-selective 5-HT agonist, quipazine (Hoyer, 1988), and the 5-HT-releasing agent, *p*-chloroamphetamine (Pettibone & Williams, 1984). A preliminary account of these findings was presented to the British Pharmacological Society (Done & Sharp, 1991).

Methods

Microdialysis procedure

Male Sprague-Dawley rats (250–350 g; Harlan Olac, Bicester, U.K.) were maintained on a 12 h light/dark cycle with free access to food and water. Rats were anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p. with supplementary doses of 60 mg kg^{-1} as required) and a short loop microdialysis probe was stereotaxically implanted into the ventral hippocampus (coordinates: AP −4.0 mm, L −4.6 mm, DV −9.5 mm, relative to bregma and skull surface; Paxinos & Watson, 1982) and cemented in place. The probe was continuously perfused (1.2 µl min^{-1}) with artificial CSF (composition mM: NaCl 140, KCl 3, $CaCl_2$ 1.2, $MgCl_2$ 1.0, Na_2HPO_4 1.2, NaH_2PO_4 0.27 and glucose 7.2, pH 7.4) containing the noradrenaline uptake inhibitor, desipramine (5 µM). Desi-

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pramine was used to increase the basal level of noradrenaline in hippocampal dialysates to readily detectable amounts.

H.p.l.c.-e.c. analysis of noradrenaline

Noradrenaline in the perfusates was analysed as described previously (Done *et al.*, 1992; Sharp & Zetterstrom, 1992). The high performance liquid chromatography (h.p.l.c.) system utilised an h.p.l.c. column (4.6 × 150 mm containing spherical Microsorb C18, 5 μm particles, Dynamax/Rainin Inc.) and a mobile phase comprising 0.1 M NaH_2PO_4, 2 mM sodium octane sulphonate, 0.5 mM EDTA, and 12% (vol/vol) methanol (final pH 4.6, flow rate 1.1 ml min^{-1}). Electrochemical detection was by a glassy carbon electrode (BAS MF 1000, held at +0.7 V with respect to a Ag/AgCl reference electrode) connected to a BAS LC-4 potentiometer. Following collection, dialysate samples (20 μl) were injected immediately onto the analytical column using a Rheodyne 7125 injector. The approximate limit of detection was 10 fmol/sample.

Experimental design

After implantation of the dialysis probe a baseline period of 2–3 h was allowed for stabilization of noradrenaline output. At this time, injection of the α_2-adrenoceptor agonist, clonidine (1 mg kg^{-1}), decreases hippocampal noradrenaline output to 90% of pre-drug levels, within 40 min (Done *et al.*, 1992; Sharp & Zetterstrom, 1992), suggesting that a significant proportion of noradrenaline in the dialysates is of neuronal origin.

After the baseline period, drugs were administered subcutaneously and their effects were followed for 2 h. In some experiments 5-HT antagonists were injected subcutaneously 40 min before 5-HT agonists. The dose of quipazine used (1 mg kg^{-1}) induces 5-HT$_2$-mediated behaviour in rats (e.g. Wing *et al.*, 1990). The dose of *p*-chloroamphetamine used (2 mg kg^{-1}) has been shown to increase 5-HT release *in vivo* (Sharp *et al.*, 1986). Doses of ritanserin (0.4 mg kg^{-1}) and spiperone (0.2 mg kg^{-1} and 1 mg kg^{-1}) were chosen on the basis of their ability to antagonize 5-HT$_2$ receptor-mediated head shakes in rats (Peroutka *et al.*, 1981; Backus *et al.*, 1990).

Drugs

The drugs used, together with their suppliers, were as follows; DOI hydrochloride, DOB hydrobromide, quipazine maleate (all from Research Biochemicals Inc., Semat, St. Albans, U.K.), *p*-chloroamphetamine hydrochloride, spiperone, sulpiride hydrochloride (all from Sigma Chemical Co., Poole, U.K.) and ritanserin tartrate (Janssen Pharmaceuticals, Breese, Belgium). DOI, DOB, quipazine and *p*-chloroamphetamine were dissolved in saline (0.9% NaCl, w/v). Ritanserin, spiperone and sulpiride were dissolved in a few drops of glacial acetic acid and then diluted to volume with 5% glucose solution.

Data analysis

Noradrenaline in each sample is expressed as a percentage of the absolute amount of noradrenaline in the sample collected immediately before drug administration. Time points are expressed as the mean ± s.e.mean of the results obtained from 4–7 rats. Values for individual time points over the 120 min postdrug period were accumulated to give area under the curve. Area under the curve data were analysed statistically by the Kruskal-Wallis ANOVA followed by Mann-Whitney U-test. *P* values of 5% or less were considered statistically significant.

Results

Effect of DOI and DOB on noradrenaline output in rat hippocampus

Figure 1 shows that the 5-HT$_{2/1C}$ receptor agonist DOI, at doses of 0.2 and 0.5 mg kg^{-1}, caused a marked decrease of noradrenaline in hippocampal dialysates of anaesthetized rats. A dose of 0.5 mg kg^{-1} DOI decreased noradrenaline to about 50% of pre-drug levels within 60 min of injection, and this effect was long-lasting (> 120 min). A higher dose of DOI (2 mg kg^{-1}) had a similar effect on noradrenaline output compared to 0.5 mg kg^{-1} ($n = 5$, data not shown). At a dose of 0.02 mg kg^{-1}, DOI was ineffective compared to saline-injected controls. Thus, overall the slope of the dose-response curve looks steep (Figure 7).

DOB (1 mg kg^{-1}) also decreased hippocampal noradrenaline release by 50 ± 19% (60 min post-drug, $n = 3$) compared to pre-drug levels.

A dose of 0.5 mg kg^{-1} DOI was chosen for antagonist experiments, since of the doses used, this produced the most consistent and reproducible decrease in hippocampal noradrenaline output.

Effect of pretreatment with ritanserin, spiperone or sulpiride on DOI response

Pretreatment with the 5-HT$_{2/1C}$ receptor antagonist, ritanserin (0.4 mg kg^{-1}, s.c.) completely prevented the inhibitory effect of DOI (0.5 mg kg^{-1}, s.c.) on hippocampal noradrenaline output (Figure 2). Pretreatment with 0.2 mg kg^{-1} of the 5-HT$_2$/dopamine D$_2$ antagonist, spiperone, also blocked the effect of DOI (0.5 mg kg^{-1}, s.c.) although only partially (Figure 3). A higher dose of spiperone (1 mg kg^{-1}) completely prevented the effect of DOI (Figure 3). When injected alone neither ritanserin nor spiperone altered noradrenaline output over the 2 h post-drug period. Figure 4 shows that pretreatment with the D$_2$ receptor antagonist, sulpiride (50 mg kg^{-1}, s.c.), 40 min before DOI (0.5 mg kg^{-1}, s.c.), did not alter the response to DOI. Since sulpiride was dissolved in acid, this experiment also confirms that the blockade of the DOI effect by ritanserin and spiperone (which were also dissolved in acid) cannot be accounted for by vehicle alone.

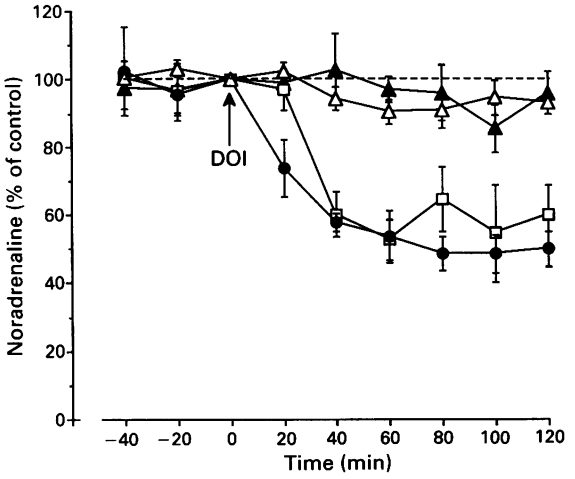

Figure 1 Effect of 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI, 0.02–0.5 mg kg^{-1}, s.c.) on noradrenaline in hippocampal microdialysates of the anaesthetized rat. Rats were injected with either saline (△) or DOI (0.02 mg kg^{-1}, ▲; 0.2 mg kg^{-1}, □; 0.5 mg kg^{-1}, ●) at the time point indicated. Each point represents the mean of data obtained from 5–7 rats; s.e.mean shown by vertical bars. The mean (± s.e.mean) basal output of noradrenaline for the above groups combined was 0.096 ± 0.006 pmol/20 min sample ($n = 26$). Statistical analysis of area under the curve data revealed that DOI (0.2 and 0.5 mg kg^{-1})-injected groups and the saline-injected controls were significantly different ($P < 0.01$).

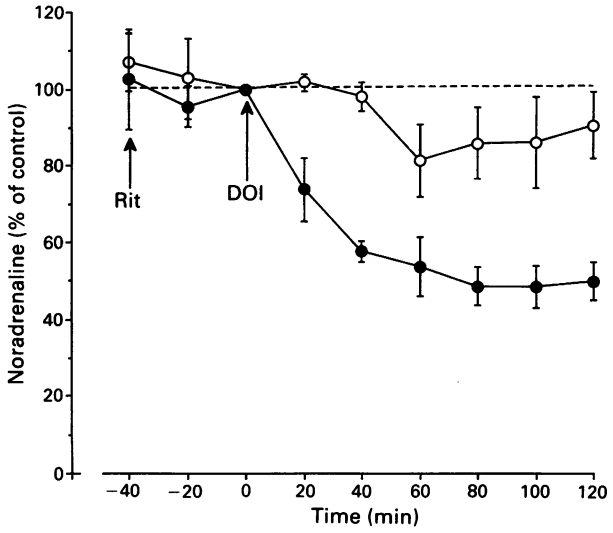

Figure 2 Antagonism of the 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) induced inhibition of hippocampal noradrenaline release by ritanserin (Rit). DOI (0.5 mg kg^{-1},s.c.) was injected into control rats (●) or rats treated 40 min previously with 0.4 mg kg^{-1}, s.c. ritanserin (○). Results are means ± s.e.mean (vertical bars) of data obtained from 5–7 rats. The mean (± s.e.mean) basal output of noradrenaline for the above groups combined was 0.106 ± 0.008 pmol/20 min sample (n = 12). Statistical analysis of area under the curve data revealed that the ritanserin/DOI and DOI alone groups were significantly different ($P<0.01$).

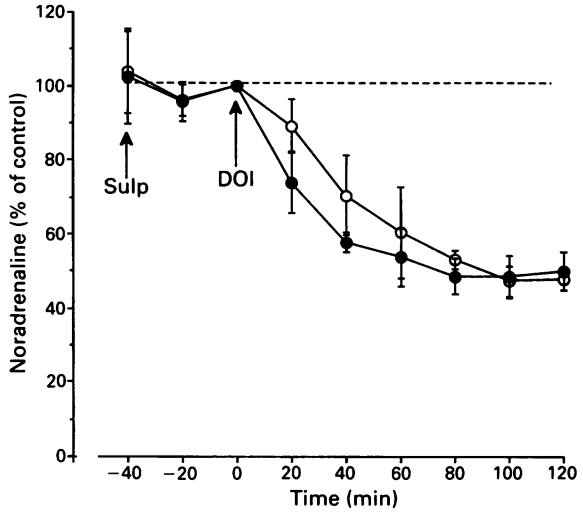

Figure 4 Lack of effect of sulpiride (Sulp) in blocking 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI)-induced inhibition of noradrenaline release in hippocampus. DOI (0.5 mg kg^{-1}, s.c.) was injected into control rats (●) or rats treated 40 min previously with 50 mg kg^{-1}, s.c. sulpiride (○). Results are means ± s.e.mean (vertical bars) of data from 4–7 rats. The mean (± s.e.mean) basal output of noradrenaline for the above groups combined was 0.104 ± 0.007 pmol/20 min sample (n = 11). Statistical analysis of area under the curve data revealed no significant difference between sulpiride/DOI and DOI alone groups.

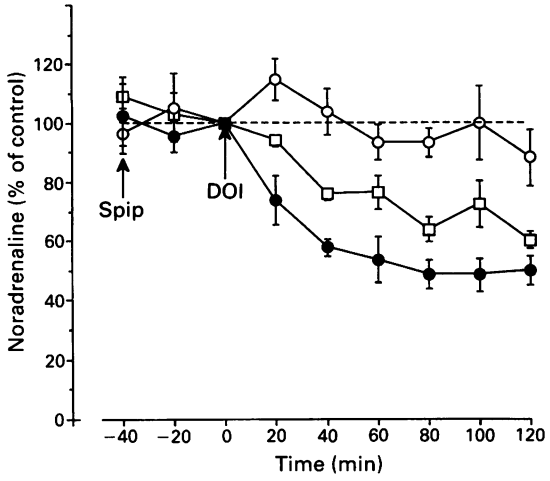

Figure 3 Reversal of 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI)-induced inhibition of noradrenaline release in hippocampus by spiperone. DOI (0.5 mg kg^{-1}, s.c.) was injected into control rats (●) or rats treated 40 min previously with either 0.2 mg kg^{-1} (□) or 1 mg kg^{-1}, s.c. (○) spiperone. Results are means ± s.e.mean (vertical bars) of data obtained from 4–7 rats. The mean (± s.e.mean) basal output of noradrenaline for the above groups combined was 0.118 ± 0.007 pmol/20 min sample (n = 16). Statistical analysis of area under the curve data revealed that the spiperone (0.2 mg kg^{-1})/DOI and DOI alone groups were significantly different ($P<0.05$) and the spiperone (1 mg kg^{-1})/DOI and DOI alone groups were significantly different ($P<0.01$).

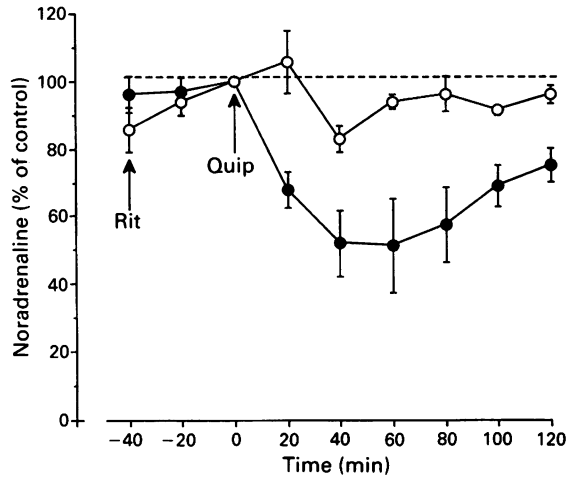

Figure 5 Effect of quipazine (Quip) on noradrenaline release in hippocampus. Quipazine (1 mg kg^{-1}, s.c.) was injected into control rats (●) or rats treated 40 min previously with 0.4 mg kg^{-1}, s.c. ritanserin (Rit) (○). Results are means ± s.e.mean (vertical bars) of data from 5 rats. The mean (± s.e.mean) basal output of noradrenaline for the above groups combined was 0.121 ± 0.008 pmol/20 min sample (n = 10). Statistical analysis of area under the curve data revealed that the quipazine-injected group and the saline-injected controls were significantly different ($P<0.01$). In addition the ritanserin/quipazine and quipazine alone groups were significantly different ($P<0.05$).

The data for DOI in combination with ritanserin and spiperone are summarized in Figure 7.

Effect of quipazine on noradrenaline output in hippocampus

Figure 5 shows that the non-selective 5-HT receptor agonist quipazine (1 mg kg^{-1}, s.c.) induced an immediate and marked decrease of noradrenaline in hippocampal dialysates. The maximum effect was a reduction of hippocampal noradrenaline to 50% of pre-drug levels, 60 min after injection. Pretreatment with ritanserin (0.4 mg kg^{-1}) antagonized the effect of quipazine (Figure 5).

The data for quipazine in combination with ritanserin are summarized in Figure 7.

Effect of p-*chloroamphetamine on noradrenaline output in hippocampus*

Figure 6 shows that the 5-HT-releasing agent *p*-chloroamphetamine (2 mg kg^{-1}, s.c.), like quipazine and DOI, induced a long-lasting decrease in hippocampal noradrenaline output, the maximum effect being about 40% reduction of pre-drug control levels. The effect of *p*-chloroamphetamine was antagonized by pretreatment with 0.4 mg kg^{-1} ritanserin (Figure 6).

The data for *p*-chloroamphetamine in combination with ritanserin are summarized in Figure 7.

Discussion

Our results show that systemic injection of the phenylalkylamine hallucinogen, DOI, which has high binding affinity for both 5-HT$_2$ and 5-HT$_{1C}$ receptors (Glennon *et al.*, 1988; Appel *et al.*, 1990), decreases levels of noradrenaline in hippocampal dialysates of anaesthetized rats. Both ritanserin and spiperone prevented this inhibitory effect of DOI. Receptor binding studies show that ritanserin has high affinity for both 5-HT$_2$ and 5-HT$_{1C}$ receptors, while spiperone has high affinity for 5-HT$_2$ but low affinity for 5-HT$_{1C}$ receptors (Hoyer, 1988). These findings strongly suggest that the inhibitory effect of DOI on noradrenaline release is mediated by the 5-HT$_2$ receptor. Indeed the complete blockade by spiperone of the DOI effect suggests that the 5-HT$_{1C}$ receptor does not influence noradrenaline release under the present experimental conditions. Although spiperone is a dopamine D$_2$ receptor antagonist (Stefanini *et al.*, 1980) pretreatment with the selective D$_2$ receptor antagonist, sulpiride, did not alter the response to DOI, suggesting that the D$_2$ receptor is not involved.

Two other drugs with 5-HT$_2$ agonist properties, quipazine and DOB (Vetulani *et al.*, 1980; Glennon *et al.*, 1983; Glennon *et al.*, 1986), like DOI, decreased hippocampal noradrenaline output. The effect of quipazine was also antagonized by ritanserin and given the above discussion, it would seem reasonable to assume that the effect of quipazine is mediated by 5-HT$_2$ rather than 5-HT$_{1C}$ receptors.

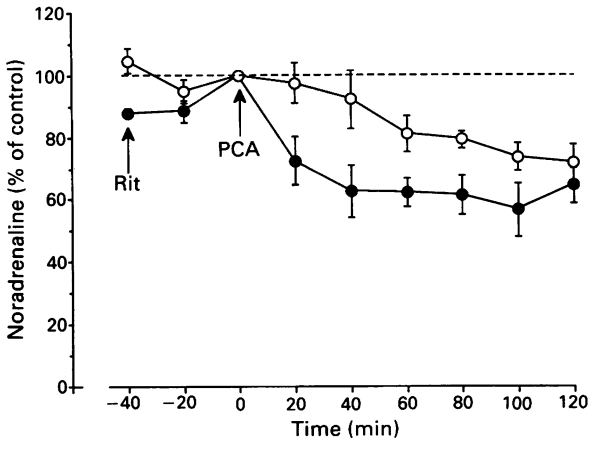

Figure 6 Effect of *p*-chloroamphetamine (PCA) on noradrenaline release in hippocampus. *p*-Chloroamphetamine (2 mg kg^{-1}, s.c.) was injected into control rats (●) or rats treated 40 min previously with 0.4 mg kg^{-1}, s.c. ritanserin (Rit) (○). Results are means ± s.e.mean (vertical bars) of data from 4–5 rats. The mean (± s.e.mean) basal output of noradrenaline for the above groups combined was 0.113 ± 0.007 pmol/20 min sample ($n = 9$). Statistical analysis of area under the curve data revealed that the *p*-chloroamphetamine-injected group and the saline-injected controls were significantly different ($P < 0.01$). In addition the ritanserin/*p*-chloroamphetamine and *p*-chloroamphetamine alone groups were significantly different ($P < 0.05$).

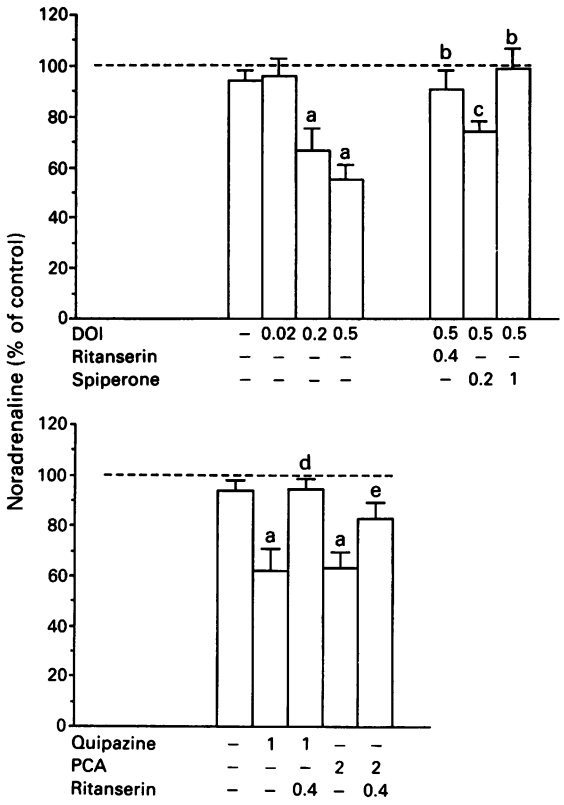

Figure 7 Summary of data expressed as means (± s.e.mean, vertical bars) of areas under the curve for the 120 min post-drug period: 100% represents no change in noradrenaline output over the 120 min post-drug period. Doses (mg kg^{-1}) are shown in the figure. [a]$P < 0.01$ versus saline-injected controls; [b]$P < 0.01$ versus 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI, 0.5 mg kg^{-1}, s.c.) alone; [c]$P < 0.05$ versus DOI (0.5 mg kg^{-1}, s.c.) alone; [d]$P < 0.05$ versus quipazine (1 mg kg^{-1}, s.c.) alone; [e]$P = 0.05$ versus *p*-chloroamphetamine (2 mg kg^{-1}, s.c.) alone. Data analysis by Kruskal-Wallis ANOVA followed by Mann-Whitney U-test.

The 5-HT-releasing agent, *p*-chloroamphetamine, also decreased hippocampal noradrenaline output, an effect blocked by ritanserin. *p*-Chloroamphetamine, at a dose similar to that used in the present study, has previously been shown to release 5-HT *in vivo* (Sharp *et al.*, 1986), which supports the idea that the effect of this drug on noradrenaline output is mediated by endogenous 5-HT. However, *p*-chloroamphetamine also releases dopamine (Sharp *et al.*, 1986), and it is therefore possible that the drug normally releases noradrenaline. It is unlikely, however, that *p*-chloroamphetamine would release noradrenaline in the present experiments since a noradrenaline uptake inhibitor, desipramine, was present in the perfusion medium. Desipramine would prevent entry of *p*-chloroamphetamine into the noradrenergic nerve terminals in the region of the microdialysis probe and thereby circumvent any indirect noradrenaline releasing action.

The 5-HT$_2$ agonist-induced decrease in hippocampal noradrenaline release may well be directly related to a decrease in firing rate of noradrenergic neurones in the LC. Thus, it has been reported previously that systemic administration of 5-HT$_{2/1C}$ agonists, including quipazine and phenylalkylamines such as DOB and DOM, inhibits the spontaneous electrical activity of noradrenergic neurones in the LC (Rasmussen & Aghajanian, 1986; Rasmussen *et al.*, 1986; Gorea & Adrien, 1988). Furthermore, the latter studies showed that the inhibitory actions of quipazine and the phenylalkylamines are antagonized by 5-HT$_{2/1C}$ antagonists.

It should be noted that whereas the phenylalkylamines and quipazine cause a near complete inhibition of firing of neurones in the LC, noradrenaline output in hippocampus

was reduced to only 50% of control values. The latter effect compares with a 90% reduction in hippocampal noradrenaline output induced by 1 mg kg^{-1} clonidine (Done *et al.*, 1992; Sharp & Zetterstrom, 1992), a drug which is well known to inhibit LC neuronal firing completely. The quantitative difference in the effects of the 5-HT$_{2/1C}$ agonists on hippocampal noradrenaline output versus LC neuronal firing may suggest that, at least in the case of 5-HT$_{2/1C}$ agonists (but presumably not clonidine), the inhibition of LC neuronal firing may be offset by compensatory changes in noradrenaline release at the nerve terminal. Alternatively, not all noradrenergic neurones innervating hippocampus may be under the inhibitory influence of 5-HT$_2$ receptors. In this respect it would be interesting to know the effects of 5-HT$_{2/1C}$ agonists on noradrenaline output in other brain areas.

Our experiments show that neither ritanserin nor spiperone influence the basal output of noradrenaline in hippocampus. This finding suggests that there is no basal 5-hydroxytryptaminergic tone at the 5-HT$_2$ receptor inhibiting noradrenaline output under the present experimental conditions. An earlier report showed that the 5-HT$_{2/1C}$ antagonist, ketanserin, has no consistent effect on LC firing (Gorea & Adrien, 1988). However, in another study both ritanserin and LY 53857 (also a 5-HT$_{2/1C}$ antagonist) were observed to cause a slight increase in LC firing (Rasmussen & Aghajanian, 1986). The latter study suggests that under certain, as yet undefined, conditions central noradrenergic neurotransmission may be under an inhibitory tone mediated by the 5-HT$_2$ receptor. We are investigating this issue at present.

Although we have no direct evidence to suggest that the 5-HT$_2$-induced reduction of noradrenaline release observed in the present study is mediated centrally, this seems likely since intracerebroventricular injection of quipazine induces a reduction of noradrenergic neuronal firing (Gorea & Adrien, 1988). Interestingly, recent electrophysiological studies indicate that the inhibitory effect of 5-HT$_{2/1C}$ agonists on noradrenergic activity is mediated, not via a direct action in the LC but involves afferents to the LC from the medulla (Gorea *et al.*, 1991). It is unlikely that 5-HT$_2$ agonists inhibit noradrenaline release by a direct action at the nerve terminal since local perfusion of DOI into hippocampus has no effect on noradrenaline release (this laboratory, unpublished observations).

In summary, this study demonstrates that DOI, DOB, quipazine and *p*-chloroamphetamine decrease noradrenaline output in hippocampus of anaesthetized rats. Furthermore our results suggest that this effect is 5-HT$_2$-mediated. These findings are consistent with several lines of evidence indicating an inhibitory influence of 5-HT, mediated via the 5-HT$_2$ receptor, in the regulation of central noradrenergic transmission.

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Pharmacological characterization of presynaptic α_2-autoreceptors in rat submaxillary gland and heart atrium

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1 The pharmacological properties of presynaptic α_2-autoreceptors were studied in rat isolated submaxillary glands and atria. Tissue pieces were preincubated with [^{3}H]-noradrenaline, then superfused with medium containing desipramine, and stimulated electrically. In one series of experiments, pEC_{30} values of 12 α-adrenoceptor antagonists were determined, i.e., negative logarithms of concentrations that increased the electrically evoked overflow of tritium by 30%. In another series, pK_D values of 9 α-adrenoceptor antagonists against the release-inhibiting effect of 5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline (UK 14304), and of 3 antagonists against the release-inhibiting effect of methoxamine, were determined.

2 In submaxillary glands, the pEC_{30} values of the antagonists correlated well with their pK_D values against UK 14304 ($r = 0.93$). The same was true for atria ($r = 0.92$).

3 In submaxillary glands, the pK_D values of 3 antagonists against UK14304 were very similar to their pK_D values against methoxamine, with a maximal difference of 0.4. The same was true for atria where the maximal difference was 0.3.

4 The pEC_{30} values obtained in submaxillary glands correlated significantly with those obtained in atria ($r = 0.81$). The same was true for the pK_D values ($r = 0.79$). However, the pEC_{30} and pK_D values also indicated consistent differences between the two tissues.

5 It is concluded that the sites of action of the imidazoline UK 14304 (α_2-selective), the phenylethylamine noradrenaline, and the phenylethylamine methoxamine (α_1-selective) are exclusively α_2-adrenoceptors. There is no indication for presynaptic α_1-adrenoceptors or for an effect of UK 14304 mediated by presynaptic imidazoline receptors. The α_2-autoreceptor population in the submaxillary gland differs from that in the atrium.

6 Comparison with studies from the literature indicates that the submaxillary autoreceptors are closely similar to the α_{2D} radioligand binding site found in the bovine pineal gland and probably the rat submaxillary gland. The atrial autoreceptors also conform best to this site, but the agreement is more limited; the atrial autoreceptors may represent a type related to, but distinct from, the α_{2D} site, or a mixture of different types.

Keywords: α_1-Adrenoceptor; α_2-adrenoceptor; imidazoline receptor; presynaptic α_2-autoreceptor; receptor classification; rat heart atrium; rat submaxillary gland; sympathetic nervous system; noradrenaline release

Introduction

Studies on presynaptic α-autoreceptors led to the view that α-adrenoceptors are not homogeneous (Starke, 1972) but comprise at least two classes, α_1 and α_2 (Langer, 1974; for review see Docherty, 1989; Ruffolo *et al.*, 1991; Wilson *et al.*, 1991). Studies on presynaptic α-autoreceptors also first indicated heterogeneity within the α_2 class (Doxey & Everitt, 1977; Dubocovich, 1979). A species difference between the rabbit and the rat, for example, was a consistent finding (Reichenbacher *et al.*, 1982; Ennis, 1985; Lattimer & Rhodes, 1985; Alabaster *et al.*, 1986; Limberger *et al.*, 1989).

More systematic attempts to subclassify α_2-adrenoceptors were based on radioligand binding techniques (Cheung *et al.*, 1982; Bylund, 1985). They have led to the differentiation of four subclasses: α_{2A}, occurring for example on human platelets and HT29 human colonic carcinoma cells and characterized, *inter alia*, by low affinity for prazosin (Bylund *et al.*, 1988); α_{2B}, occurring for example in neonatal rat lung, with relatively high affinity for prazosin (Bylund *et al.*, 1988); α_{2C}, occurring for example in an opossum kidney (OK) cell line, also with relatively high affinity for prazosin (Blaxall *et al.*, 1991); and α_{2D}, occurring in the bovine pineal gland (Simonneaux *et al.*, 1991) and perhaps some rat tissues (Michel *et al.*, 1989; Paris *et al.*, 1990), with low prazosin affinity.

To which of these subgroups, if any, do the presynaptic α_2-autoreceptors belong? From data in the literature it was suggested that the autoreceptors were generally α_{2B} (Bylund *et al.*, 1988), that they might be α_{2C} in rat cerebral cortex and submaxillary gland (Blaxall *et al.*, 1991), or that some might be α_{2D} (Simonneaux *et al.*, 1991). Four studies, to our knowledge, have addressed the question directly, measuring the release of [^{3}H]-noradrenaline or postsynaptic responses to neural stimulation. In contrast to the suggestions mentioned, the autoreceptors were classified as α_{2A} in rat vas deferens (Connaughton & Docherty, 1990), rat brain cortex (Gobbi *et al.*, 1990), rabbit brain cortex (Limberger *et al.*, 1991) and guinea-pig urethra (Alberts, 1992); only in rat atrium the autoreceptors seemed to be α_{2B} (Connaughton & Docherty, 1990). However, the classification of the rat atrial receptors as α_{2B}, i.e. prazosin-sensitive, must be viewed with caution since the sympathetic nerves of this tissue have been postulated to possess (highly prazosin-sensitive) α_1-adrenoceptors (Kobinger & Pichler, 1980; Docherty, 1984).

We describe here a functional characterization of α_2-autoreceptors in rat submaxillary glands and atria. Rat atrial autoreceptors were chosen in order to re-examine their α_{2B} classification. Rat submaxillary gland autoreceptors had been important for the concept that α_2-autoreceptors were generally α_{2B} (Bylund, 1988; Bylund *et al.*, 1988). After our experiments were finished, Smith *et al.* (1992) published a comparison of α_2-autoreceptors in rat vas deferens, atrium and submaxillary gland in which the α_{2B} character of the

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atrial receptors was confirmed and the submaxillary receptors, like those of the vas deferens, were classified as α_{2A}. Our study suggests a reinterpretation of some findings of Smith *et al.* (1992).

Methods

Experiments with submaxillary glands and atria were carried out in parallel. Male Wistar rats weighing 160 to 240 g were decapitated. Submaxillary glands and atria were dissected free and cut into pieces of 4–6 mg. Six to eight pieces were preincubated with [^{3}H]-noradrenaline 0.1 μmol l^{-1} in 2 ml medium (37°C for 30 min) and subsequently washed five times with 3 ml of [^{3}H]-noradrenaline-free medium. One piece was transferred to each of twelve 0.16 ml superfusion chambers, six for glandular and six for atrial tissue. The tissue piece was held by a polypropylene mesh between platinum wire electrodes 6 mm apart. Tissues were superfused for 210 min at 1 ml min^{-1}. There were five periods of electrical stimulation (rectangular pulses of 2 ms width and 15 V cm^{-1} voltage drop, yielding a current strength of 24 mA). The first stimulation period was given after 40 min of superfusion (180 pulses, 3 Hz); it was not used for determination of tritium overflow. The following periods were applied after 70 (S_1), 110 (S_2), 150 (S_3) and 190 min (S_4) of superfusion. The collection of successive 5 min superfusate samples began after 60 min of superfusion. At the end, the tissue was solubilized in 0.5 ml Soluene-350 (Packard, Frankfurt am Main, Germany).

The preincubation and superfusion media contained (mmol l^{-1}): NaCl 118, KCl 4.8, $CaCl_2$ 0.2, 2 or 2.5 (see below), $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, ascorbic acid 0.57, disodium EDTA 0.03 and corticosterone 0.01. The superfusion but not the preincubation medium also contained desipramine 1 μmol l^{-1}.

There were two kinds of experiment. In the first series, the effect of α-adrenoceptor antagonists on the electrically evoked overflow of tritium was determined. The concentration of $CaCl_2$ was 2 mmol l^{-1}. Each of the stimulation periods S_1 to S_4 consisted of 60 pulses/1 Hz. The antagonist under study was added at increasing concentrations from 20 min before to 20 min after the onset of S_2, S_3 and S_4 to yield cumulative antagonist concentration-response curves (see Figure 1, upper panels). In the second series of experiments, antagonist effects against the agonists 5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline (UK 14304) or methoxamine were determined. The concentration of $CaCl_2$ was 0.2 mmol l^{-1} for preincubation and 2.5 mmol l^{-1} for superfusion; the basal outflow of tritium was more stable under these conditions than at 2 mmol l^{-1} $CaCl_2$. Each of the stimulation periods S_1 to S_4 consisted of 10 trains of 6 pulses/100 Hz; the trains were delivered at intervals of 30 s; the total duration of a stimulation period, hence, was 4.5 min. The antagonist under study was present throughout superfusion, and either UK 14304 or methoxamine was added at increasing concentrations from 20 min before to 20 min after the onset of S_2, S_3 and S_4 to yield cumulative agonist concentration-response curves. One common set of concentration-response data for UK 14304, given alone, served for comparison with UK 14304 curves obtained in presence of each of the antagonists; the same was done for methoxamine. Concentration-response curves for rauwolscine were also determined under the conditions of the second series (Figure 1, lower panels).

The outflow of tritium was expressed as fractional rate (min^{-1}). The stimulation-evoked overflow was calculated by subtraction of the basal outflow and was expressed as a percentage of the tritium content of the tissue (Limberger *et al.*, 1989; 1991). For further evaluation of basal tritium efflux, ratios were calculated of the fractional rate during the 5 min before S_2, S_3 and S_4, and the fractional rate during the 5 min before S_1 (b_2/b_1, b_3/b_1, b_4/b_1). Ratios were also calculated for the electrically evoked overflow (S_2/S_1, S_3/S_1, $S_4/$

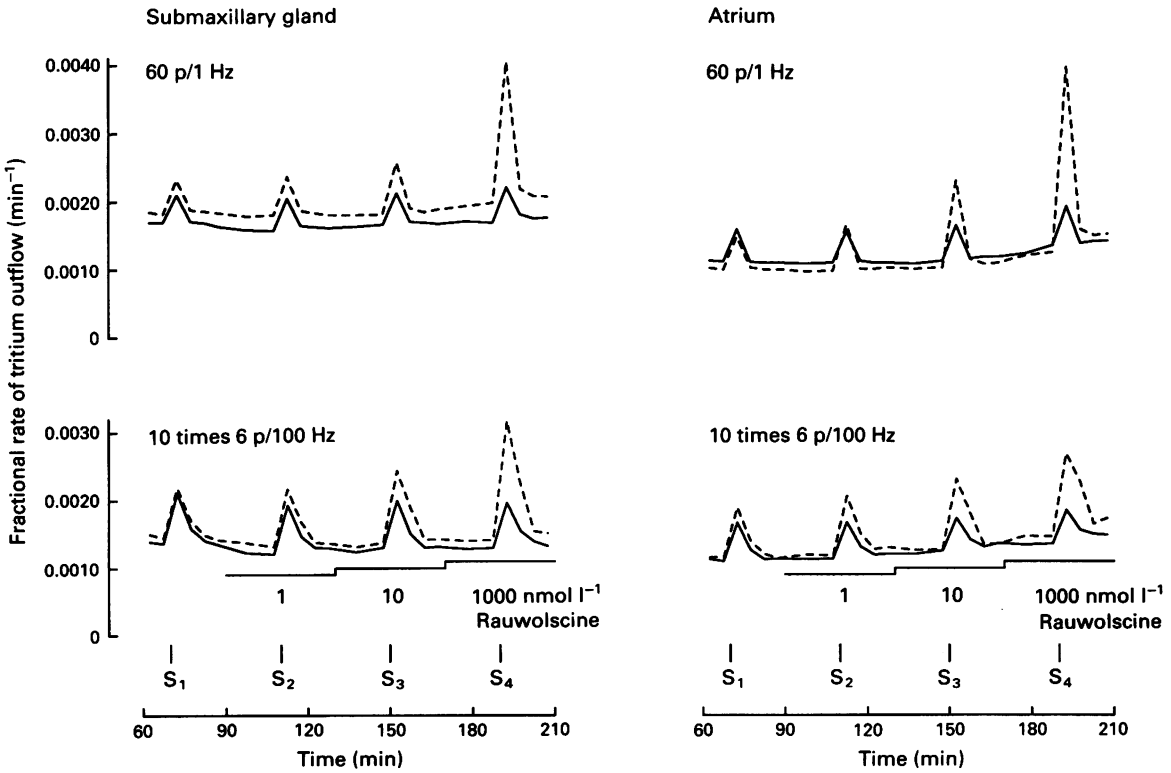

Figure 1 Time course of tritium outflow from rat submaxillary gland (left-hand panels) and atrium (right-hand panels), and effect of rauwolscine. The tissue was preincubated with [^{3}H]-noradrenaline and then superfused with medium containing desipramine 1 μmol l^{-1} and corticosterone 10 μmol l^{-1}. Each stimulation period (S_1–S_4) consisted either of 60 pulses/1 Hz (upper panels), or of 10 trains of 6 pulses/100 Hz, train interval 30 s (lower panels). Solvent (solid lines) or increasing concentrations of rauwolscine (dashed lines) were added as indicated. Abscissa scale, min of superfusion. Ordinate scale, mean fractional rate of tritium efflux. Each curve is the mean from two tissue fragments.

S_1). Moreover, the percentage change caused by an agonist or antagonist in each single piece of tissue was calculated, taking, as the reference value, the average corresponding S_2/S_1, S_3/S_1 and S_4/S_1 ratio obtained in experiments in which no drug was added before S_2, S_3 and S_4.

Antagonist pEC_{30} values (negative logarithms of concentrations that enhanced the evoked overflow of tritium by 30%) were interpolated from the averaged concentration-reponse curves. The effect of an antagonist against UK 14304 and methoxamine was quantitated as follows. A sigmoid curve was fitted to the weighted mean percentage inhibition values for the agonist given alone, and a second curve was simultaneously fitted to the weighted mean percentage inhibition values for the agonist in the presence of the antagonist, assuming a common maximal agonist effect and slope (equation No. 25 of Waud, 1976); this yielded the EC_{50} (concentration producing 50% of the maximal effect) of the agonist alone, and of the agonist in the presence of antagonist. The pair of EC_{50} values was taken to calculate the pK_D of the antagonist (Starke *et al.*, 1975), using the Gaussian law of error propagation. The common set of concentration-response data for 'agonist given alone' (see above) yielded different fitted curves, depending on which antagonist entered into the same fitting calculation. However, the differences were minimal. Concentration-response curves, EC_{50} values and maximal effects for 'agonist given alone' in Results are taken from the calculation in which rauwolscine 0.1 μmol l^{-1} was the antagonist.

Results are expressed as arithmetic means ± s.e.mean. Group means were compared with the Mann-Whitney test if Kruskal-Wallis analysis indicated a significant difference. In the case of multiple comparisons, the significance levels to be exceeded were adjusted according to Bonferroni. *n* is the number of tissue pieces.

Drugs

Purchased drugs were (-)-[ring-2,5,6-^{3}H]-noradrenaline, specific activity 56.7 Ci $mmol^{-1}$ (Du Pont, Dreieich, Germany), (±)-2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4-benzodioxane HCl (WB 4101) (Biotrend, Köln, Germany), corynanthine HCl, rauwolscine HCl and yohimbine HCl (Roth, Karlsruhe, Germany) and corticosterone (Sigma, Deisenhofen, Germany). The following drugs were kindly provided by the producers: (±)-methoxamine HCl (Burroughs Wellcome, London, U.K.); desipramine HCl and phentolamine methanesulphonate (Ciba-Geigy, Basel, Switzerland); prazosin HCl and 5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline tartrate (UK 14304) (Pfizer, Karlsruhe, Germany); (±)-idazoxan HCl (Reckitt & Colman, Kingston-upon-Hull, U.K.); (±)-imiloxan HCl (RS 21361; Syntex, Edinburgh, U.K.); (±)-2-[2H-(1-methyl-1,3-dihydroisoindole)methyl]-4,5-dihydroimidazole (BRL 44408) and (-)-1,2-dimethyl-2,3,9,13b-tetrahydro-1H-dibenzo[c,f]imidazo[1,5-a]azepine (BRL 41992) (SmithKline Beecham, Great Burgh, Epsom, Surrey, U.K.); 6-chloro-9-[(3-methyl-2-butenyl)oxy]-3-methyl-1H-2,3,4,5-tetrahydro-3-benzazepine maleate (SKF 104078) (Smith Kline and French, King of Prussia, Philadelphia, U.S.A.); 2-{2-[4-(o-methoxyphenyl) piperazin-1-yl] ethyl}-4,4-dimethyl-1,3 (2H,4H)-isoquinolinedione dihydrochloride (ARC 239) (Thomae, Biberach an der Riss, Germany). Drugs were dissolved in distilled water except WB 4101 (HCl 1 mmol l^{-1}), corticosterone (1,2-propanediol), BRL 44408 and BRL 41992 (HCl 10 mmol l^{-1}).

Results

Release-enhancing effect of antagonists

The first series of experiments examined the potency of 12 α-adrenoceptor antagonists at enhancing the release of [^{3}H]-noradrenaline. The slices were stimulated by trains of 60 pulses/1 Hz, conditions under which marked autoinhibition developed. The overflow of tritium evoked by S_1 amounted to 0.233 ± 0.004% of the tritium content of the tissue for the submaxillary gland ($n = 161$) and to 0.234 ± 0.006% for the atrium ($n = 154$). In control experiments without α-adrenoceptor antagonists, the basal outflow of tritium, when expressed as fractional rate, was approximately constant (glands) or increased slightly (atria), and the evoked overflow was similar from S_1 to S_4 (Figure 1, upper panels).

All antagonists tested increased the evoked overflow of tritium. Representative experiments with rauwolscine are shown in the upper panels of Figure 1, and concentration-response curves for rauwolscine, prazosin, SKF 104078, imiloxan and corynanthine in Figure 2. No attempt was made to obtain maximal effects, firstly because concentration-response curves of α-adrenoceptor antagonists on the release of noradrenaline often are bell-shaped, with non-specific inhibition coming into play at high concentrations (Borowski *et al.*, 1977), and secondly because high concentrations often accelerated the basal efflux of tritium (see below). The highest increases observed were by about 400%. The percentage increases caused by a given antagonist concentration often differed between submaxillary gland and atrium, some antagonists being more effective in the atrium, others in the submaxillary gland.

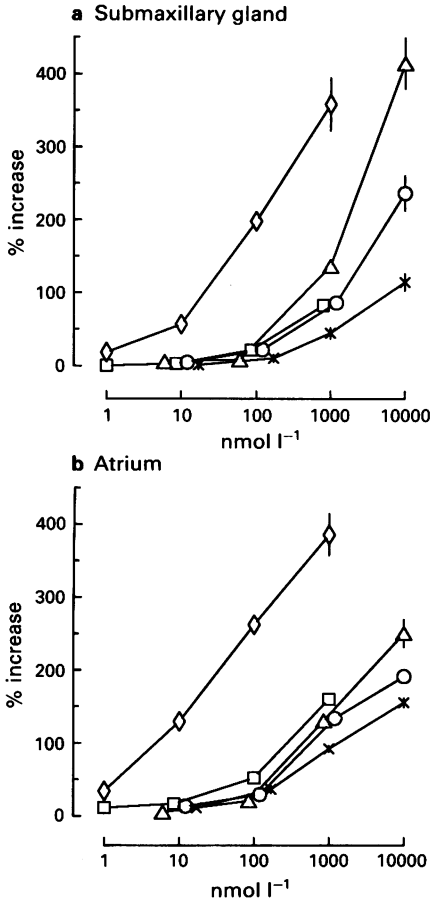

Figure 2 Effects of α-adrenoceptor antagonists on electrically evoked tritium overflow from rat submaxillary gland (a) and atrium (b). The tissue was preincubated with [^{3}H]-noradrenaline and then superfused with medium containing desipramine 1 μmol l^{-1} and corticosterone 10 μmol l^{-1}. Each stimulation period (S_1–S_4) consisted of 60 pulses/1 Hz. Rauwolscine (◇), prazosin (□), SKF 104078 (○), imiloxan (△) or corynanthine (×) was added at increasing concentrations before S_2, S_3 and S_4. Abscissae, antagonist concentration. Ordinates, percentage increase caused by antagonists, calculated from S_n/S_1 values. Means ± s.e.mean (vertical bars) of at least 4 tissues.

pEC_{30} values are summarized in Table 1, and the correlation of submaxillary with atrial pEC_{30} values is shown in Figure 3a. Imiloxan had similar pEC_{30} values in the two tissues, and the same was true for SKF 104078 and idazoxan. For other antagonists, however, the two pEC_{30} values differed by up to 0.8, with higher affinity in the submaxillary gland in some cases (points above line of identity in Figure 3a) and higher affinity in the atrium in others (points below line of identity), so that the correlation coefficient was only 0.81.

The basal outflow of tritium was not changed by most antagonists. Exceptions were increases caused by SKF 104078 10 μmol l^{-1} (by about 210%), prazosin 0.1 (by about 30%) and 1 μmol l^{-1} (by about 160%) and ARC 239 1 μmol l^{-1} (by about 30%). pEC_{30} values were calculated despite these increases, because the electrically evoked overflow remained well set off from the raised baseline; moreover, the acceleration of basal tritium efflux was small at concentrations close to the calculated EC_{30}.

Effect of antagonists against UK 14304 and methoxamine

The second series of experiments examined the potency of 9 α-adrenoceptor antagonists in attenuating the release-inhibiting effect of UK 14304, and the potency of 3 antagonists in attenuating the effect of methoxamine, under conditions of minimal autoinhibition. Autoinhibitory tone can be avoided when single electric pulses or brief high-frequency pulse trains are used (see Singer, 1988). Single pulses, however, and single brief high-frequency trains (for example 6 pulses/100 Hz) elicited too small an increase in tritium outflow for reliable measurement (data not shown). The conditions finally chosen (each stimulation period consisting of 10 trains of 6 pulses/100 Hz, train interval 30 s) were a compromise. On the one hand, the stimulation-evoked overflow peaks were sufficiently high, and on the other hand, autoinhibition was relatively minor as shown by a relatively small effect of rauwolscine (compare upper and lower panels of Figure 1); rauwolscine 1 μmol l^{-1}, for example, increased the evoked overflow of tritium by only 137.8 ± 6.6% in the submaxillary gland ($n = 6$) and 105.8 ± 11.5% in the atrium ($n = 6$) instead of the much greater increase observed with 60 pulses/1 Hz (by about 400%; Figure 2).

The overflow of tritium elicited by S_1, in the absence of

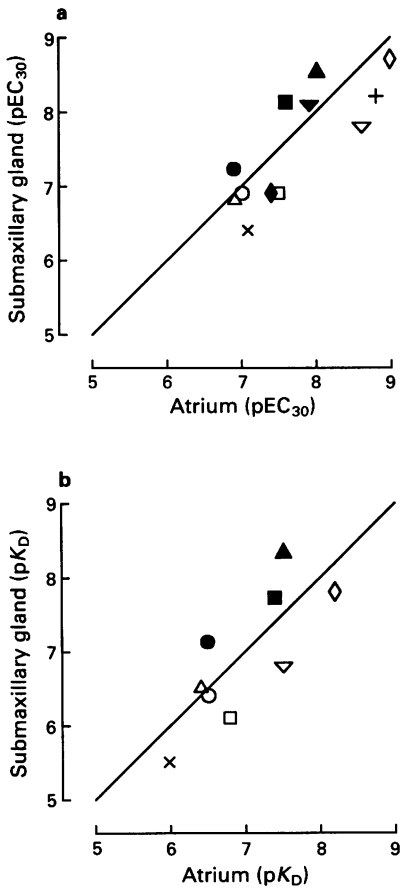

Figure 3 Correlation between antagonist affinity estimates for α_2-autoreceptors in rat atrium and submaxillary gland: (a) shows correlation between pEC_{30} values (negative logarithms of concentrations that increased evoked tritium overflow by 30%); (b) shows correlation between pK_D values (calculated from antagonism against UK 14304). Values are from Table 1. Lines are the lines of identity, not regression lines. Correlation coefficients are 0.81 (a) and 0.79 (b). Symbols: (▲) phentolamine; (◇) rauwolscine; (■) BRL 44408; (●) BRL 41992; (▽) WB 4101; (△) imiloxan; (○) SKF 104078; (□) prazosin; (×) corynanthine; (+) yohimbine; (▼) idazoxan; (◆) ARC 239.

Table 1 Potencies of α-adrenoceptor antagonists in increasing the evoked overflow of tritium (pEC_{30}) and in antagonizing the inhibitory effects of UK 14304 and methoxamine (pK_D)

α-Adrenoceptor antagonist	*Submaxillary gland* pEC_{30}	pK_D *(against UK 14304)*	pK_D *(against methoxamine)*	*Atrium* pEC_{30}	pK_D *(against UK 14304)*	pK_D *(against methoxamine)*
Phentolamine	8.5	8.3 ± 0.1	–	8.0	7.5 ± 0.1	–
Rauwolscine	8.7	7.8 ± 0.1	7.8 ± 0.6	9.0	8.2 ± 0.1	7.9 ± 0.3
BRL 44408	8.1	7.7 ± 0.1	–	7.6	7.4 ± 0.2	–
BRL 41992	7.2	7.1 ± 0.1	–	6.9	6.5 ± 0.2	–
WB 4101	7.8	6.8 ± 0.2	–	8.6	7.5 ± 0.2	–
Imiloxan	6.8	6.5 ± 0.1	6.7 ± 0.7	6.9	6.4 ± 0.2	6.5 ± 0.3
SKF 104078	6.9	6.4 ± 0.2	–	7.0	6.5 ± 0.2	–
Prazosin	6.9	6.1 ± 0.2	6.5 ± 0.3	7.5	6.8 ± 0.2	6.7 ± 0.2
Corynanthine	6.4	5.5 ± 0.2	–	7.1	6.0 ± 0.2	–
Yohimbine	8.2	–	–	8.8	–	–
Idazoxan	8.1	–	–	7.9	–	–
ARC 239	6.9	–	–	7.4	–	–

pEC_{30} values are negative logarithms of concentrations that increased evoked tritium overflow by 30%. pK_D values were calculated from antagonism against UK 14304 and methoxamine. Antagonist concentrations used to determine pEC_{30} were those of Figure 2 and 1–1000 nmol l^{-1} for other antagonists. Antagonist concentrations used to determine pK_D were those of Table 2; prazosin 0.1 μmol l^{-1} was also tested against UK 14304; prazosin 0.1 μmol l^{-1} increased basal tritium outflow only slightly and did not increase electrically evoked overflow; the pK_D against UK 14304 was 6.3 ± 0.3 in submaxillary gland and 6.7 ± 0.4 in atrium. Means ± s.e.mean. Each pEC_{30} is based on 8–16 and each pK_D on 10–12 tissues, controls and tissues that received agonist only not included.

antagonists, was higher in this series of experiments (Table 2) than in the first one (see above) despite the equal number of pulses (60). Possible reasons are the weaker autoinhibition and the slightly higher concentration of Ca^{2+} in this (2.5 mmol l^{-1}) than in the first series (2 mmol l^{-1}). In control experiments (no antagonist, no agonist), the basal outflow of tritium again remained approximately constant (submaxillary glands) or increased slightly (atria), and the evoked overflow was similar from S_1 to S_4 (Figure 1, lower panels). All antagonists, when present throughout superfusion, increased S_1 values (Table 2). As in controls, S_1 to S_4 overflow values were similar when an antagonist was present throughout superfusion, without subsequent addition of an agonist (data not shown). Prazosin 1 μmol l^{-1} markedly accelerated the basal efflux of tritium (Table 2).

UK 14304 and methoxamine, given alone, decreased the evoked overflow of tritium (experiments represented by circles in Figures 4 and 5). An asymptotic maximum of inhibition was reached in the case of UK 14304 (Figure 4). Sigmoidal curve fitting yielded EC_{50} values of UK 14304 of 6.2 ± 1.1 nmol l^{-1} (submaxillary gland) and 6.8 ± 1.2 nmol l^{-1} (atrium), and maximal inhibition values of 91.7 ± 2.2% (submaxillary gland) and 82.6 ± 3.4% (atrium) (from 14 gland and 11 atrial pieces). UK 14304 did not change the basal efflux of tritium. In contrast, no obvious asymptotic maximum of inhibition was reached for methoxamine (Figure 5). Concentrations higher than methoxamine 100 μmol l^{-1} were not used because, whereas methoxamine 100 μmol l^{-1} only slightly accelerated the basal efflux of tritium, higher concentrations caused a drastic increase. Despite the lack of a clear maximum, the curve fitting calculation converged to give EC_{50} values of methoxamine of 20.9 ± 16.2 μmol l^{-1} (submaxillary gland) and 8.0 ± 3.8 μmol l^{-1} (atrium), and maximal inhibition values of 76.0 ± 15.3% (submaxillary gland) and 75.4 ± 11.1% (atrium) (from 13 gland and 11 atrial pieces).

The antagonism of rauwolscine, imiloxan and prazosin against UK 14304 and methoxamine is shown in Figures 4 and 5, respectively–rauwolscine, imiloxan and prazosin were the three drugs tested against either agonist. pK_D values are summarized in Table 1, and the correlation between submaxillary gland and atrium is shown in Figure 3b. Three comparisons are of particular interest: between pEC_{30} and pK_D values within one tissue; between pK_D values measured against UK 14304 and those measured against methoxamine; and between submaxillary glands and atria.

A comparison of pK_D with pEC_{30} values in the same tissue

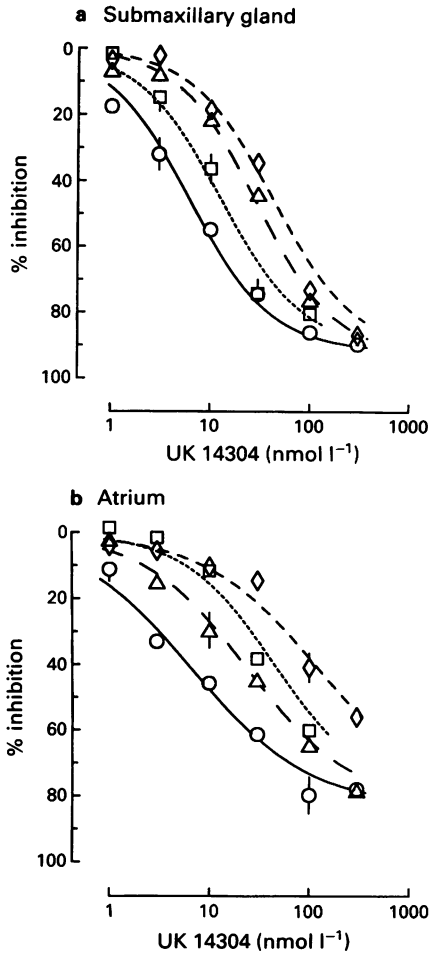

Figure 4 Effect of UK 14304 on electrically evoked tritium overflow from rat submaxillary gland (a) and atrium (b), and interaction with α-adrenoceptor antagonists. The tissue was preincubated with [^{3}H]-noradrenaline and then superfused with medium containing either desipramine 1 μmol l^{-1} and corticosterone 10 μmol l^{-1} only (○) or, in addition, rauwolscine 0.1 μmol l^{-1} (◇), imiloxan 1 μmol l^{-1} (△) or prazosin 1 μmol l^{-1} (□). Each stimulation period (S_1–S_4) consisted of 10 trains of 6 pulses/100 Hz, train interval 30 s. UK 14304 was added at increasing concentrations before S_2, S_3 and S_4. Abscissae, concentration of UK 14304. Ordinates, percentage inhibition caused by UK 14304, calculated from S_n/S_1 values. Means ± s.e.mean (vertical lines) of at least 6 tissues.

Table 2 Basal outflow (b_1) and evoked overflow of tritium (S_1) from rat submaxillary gland and atrium

α-Adrenoceptor antagonist present throughout superfusion (μmol l^{-1})		Submaxillary gland			Atrium		
		b_1 (min^{-1})	S_1 (% of tissue tritium)	n	b_1 (min^{-1})	S_1 (% of tissue tritium)	n
–		0.00161 ± 0.00003	0.439 ± 0.016	40	0.00103 ± 0.00003	0.434 ± 0.021	36
Phentolamine	0.3	0.00154 ± 0.00004	0.721 ± 0.038**	18	0.00096 ± 0.00002	0.761 ± 0.043**	22
Rauwolscine	0.1	0.00153 ± 0.00005	0.811 ± 0.031**	33	0.00100 ± 0.00002	0.704 ± 0.036**	39
BRL 44408	1	0.00159 ± 0.00008	0.650 ± 0.037**	18	0.00085 ± 0.00003**	0.707 ± 0.036**	18
BRL 41992	1	0.00142 ± 0.00003**	0.747 ± 0.040**	18	0.00102 ± 0.00003	0.584 ± 0.023**	18
WB 4101	0.3	0.00181 ± 0.00010	0.747 ± 0.046**	18	0.00103 ± 0.00002	0.807 ± 0.038**	18
Imiloxan	1	0.00148 ± 0.00004	0.767 ± 0.032**	33	0.00096 ± 0.00003	0.639 ± 0.026**	32
SKF 104078	1	0.00172 ± 0.00007	0.654 ± 0.037**	18	0.00107 ± 0.00003	0.533 ± 0.023*	18
Prazosin	1	0.00343 ± 0.00006**	0.650 ± 0.030**	30	0.00317 ± 0.00004**	0.552 ± 0.025*	36
Corynanthine	3	0.00154 ± 0.00005	0.684 ± 0.035**	18	0.00118 ± 0.00004	0.640 ± 0.022**	18

The tissue was preincubated with [^{3}H]-noradrenaline and then superfused with medium containing desipramine 1 μmol l^{-1}, corticosterone 10 μmol l^{-1} and the α-adrenoceptor antagonists indicated. Electrical stimulation (S_1) consisted of 10 trains of 6 pulses/100 Hz, train interval 30 s. b_1 represents the outflow of tritium in the collection period immediately before S_1, i.e. from 65–70 min of superfusion, expressed as fractional rate (min^{-1}); the absolute outflow averaged 2.52 ± 0.13 nCi/5 min (submaxillary gland; n = 40) and 1.65 ± 0.09 nCi/5 min (atrium; n = 36) in controls. S_1 represents the overflow of tritium elicited by the period of electrical stimulation applied after 70 min of superfusion, expressed as a percentage of tissue tritium; the absolute overflow averaged 1.40 ± 0.08 nCi (submaxillary gland) and 1.45 ± 0.12 nCi (atrium) in controls. Means ± s.e.mean from n tissues. Significant differences from control (no α-adrenoceptor antagonist): *P < 0.05; **P < 0.01.

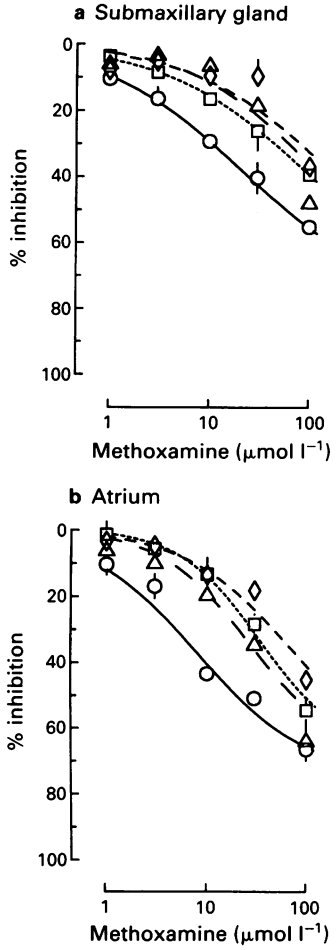

Figure 5 Effect of methoxamine on electrically evoked tritium overflow from rat submaxillary gland (a) and atrium (b), and interaction with α-adrenoceptor antagonists. The tissue was preincubated with [^{3}H]-noradrenaline and then superfused with medium containing either desipramine 1 μmol l^{-1} and corticosterone 10 μmol l^{-1} only (○) or, in addition, rauwolscine 0.1 μmol l^{-1} (◇), imiloxan 1 μmol l^{-1} (△) or prazosin 1 μmol l^{-1} (□). Each stimulation period (S_1–S_4) consisted of 10 trains of 6 pulses/100 Hz, train interval 30 s. Methoxamine was added at increasing concentrations before S_2, S_3 and S_4. Abscissae, concentration of methoxamine. Ordinates, percentage inhibition caused by methoxamine, calculated from S_n/S_1 values. Means ± s.e.mean (vertical lines) of at least 5 tissues.

shows that pK_D values were consistently lower, the difference varying between 0.1 and 1.1 (Table1). Because of the systematic character of this deviation, there was good correlation between pEC_{30} values and the pK_D values against UK 14304. The regression equation was, for the submaxillary gland: $pEC_{30} = 0.85 \times pK_D + 1.63$ ($r = 0.93$; $n = 9$ antagonists; $P < 0.001$); for the atrium: $pEC_{30} = 0.99 \times pK_D + 0.68$ ($r = 0.92$; $n = 9$ antagonists; $P < 0.001$).

In either tissue, the pK_D values of rauwolscine, imiloxan and prazosin against UK 14304 on the one hand and against methoxamine on the other hand were closely similar, with a maximal difference of 0.4 (Table 1). The pK_D values against methoxamine varied more than those against UK 14304 (s.e.mean in Table 1), presumably because the inhibition was smaller and no clear maximum was reached in the case of methoxamine (Figure 5).

The third comparison, between the two tissues, indicates that imiloxan had similar pK_D values in submaxillary gland and atrium, and the same was true for SKF 104078. However, for other antagonists the two pK_D values differed by up to 0.8, with higher affinity in the gland in some cases (points above line of identity in Figure 3b) and higher affinity in the atrium in others (points below line of identity), so that the correlation coefficient was only 0.79. Parts (a) and (b) of Figure 3 show that a given antagonist occupies approximately the same position with respect to the line of identity, irrespective of whether pEC_{30} values (Figure 3a) or pK_D values (Figure 3b) are correlated.

Discussion

The electrically evoked overflow of tritium in the present kind of experiment reflects neuronal release of [^{3}H]-noradrenaline. In accord with this view, tetrodotoxin 0.3 μmol l^{-1} abolished the evoked overflow (not shown). Two procedures were used to characterize the presynaptic α-adrenoceptors known to modulate the release of noradrenaline in rat submaxillary glands (Filinger *et al.*, 1978) and atria (Majewski *et al.*, 1981). The first method assessed the overflow-increasing potency of antagonists under conditions of pronounced α_2-autoinhibition. Since desipramine was present, the increases were not due to blockade of the reuptake of released [^{3}H]-noradrenaline but to facilitation of its neuronal release, and the pEC_{30} values of the antagonists were in fact estimates of their autoreceptor affinities. The second procedure assessed antagonist potencies against exogenous agonists and yielded pK_D values. An autoinhibition-free release of noradrenaline is optimal for such determinations (see Starke, 1987). We did not obtain an autoinhibition-free and yet reliably measurable release in the present study; however, with the protocol chosen the distortion by autoinhibition (examples in Limberger *et al.*, 1989) was minimized.

The pEC_{30} values correlated well with the pK_D values (against UK 14304) both in the submaxillary gland ($r = 0.93$) and the atrium ($r = 0.92$; $P < 0.001$). The close correlation indicates an inner consistency of the data: two independent estimations of antagonist affinity led to similar results. The pEC_{30} values were all higher than the pK_D values, presumably because less than 50% receptor occupation sufficed for a 30% increase in [^{3}H]-noradrenaline release. pEC_{30} values were also determined in rat submaxillary glands and atria by Connaughton & Docherty (1990) and Smith *et al.* (1992). Of 12 pEC_{30} values that can be compared, 10 were higher (by 0.3–1.1) in our experiments than in those of these authors; the remaining 2 were similar. Differences in methods may explain the tendency for greater antagonist effects in the present study; for instance, we used much smaller pieces of tissue, a longer exposure to antagonists and a lower frequency of stimulation.

No evidence for imidazoline receptors and α_1-adrenoceptors

The good correlation of the pK_D (against UK 14304) and pEC_{30} values (antagonism against released noradrenaline) is noteworthy for a second reason. It shows that the sites of action of UK 14304, an imidazoline, and of endogenous noradrenaline, a phenylethylamine, had the same pharmacological properties, those of an α-adrenoceptor. (The same holds true for the phenyethylamine methoxamine; see below.) Presynaptic imidazoline receptors distinct from the α-autoreceptors have been demonstrated in rabbit aorta and pulmonary artery (Göthert & Molderings, 1991; Molderings *et al.*, 1991). No indication for an effect of UK 14304 on imidazoline receptors was found in rat submaxillary gland and atrium (see also Starke, 1987; Docherty, 1989).

Rat atria were postulated to possess release-inhibiting α_1- in addition to α_2-autoreceptors. The suggestion was based on *in vivo* experiments in which noradrenaline release was estimated indirectly as an increase in heart rate (Kobinger & Pichler, 1980; Docherty, 1984). However, *in vitro* findings seemed to support the suggestion: in rat isolated atria, the α_1-selective agonist (Starke *et al.*, 1975) methoxamine

(10 μmol l^{-1}) reduced the release of [^{3}H]-noradrenaline, an effect antagonized by prazosin (0.1 μmol l^{-1}), in apparent agreement with the occurrence of presynaptic α_1-adrenoceptors (Story *et al.*, 1985). The present experiments confirm the observation of Story *et al.* (1985), but the more quantitative approach questions their conclusion. First, the pK_D value of prazosin against methoxamine (6.7; Table 1, atrium) is far too low for an α_1-adrenoceptor (range 8–11.2 in Figure 1 of Wilson *et al.*, 1991). Second, the α_2-selective antagonists, rauwolscine (Weitzell *et al.*, 1979) and imiloxan (RS 21361; Michel & Whiting, 1981; Lattimer & Rhodes, 1985), also antagonized methoxamine and their pK_D values (rauwolscine 7.9, imiloxan 6.5) are far too high for an α_1-adrenoceptor (rauwolscine range 5–7, imiloxan range 3.1–4.3 in Figure 1 of Wilson *et al.*, 1991). Third, each of the three antagonists was equipotent against methoxamine and the α_2-selective agonist (Cambridge, 1981) UK 14304 (Table 1, Figures 4 and 5), indicating that the two agonists acted at the same site. Finally, the common site was an α_2-adrenoceptor: the pK_D values of prazosin, rauwolscine and imiloxan demonstrate this (Figure 1 of Wilson *et al.*, 1991), as do other findings such as the high potencies of yohimbine and idazoxan at increasing the release of [^{3}H]-noradrenaline. α_2-Adrenoceptor agonist effects of methoxamine are known for other tissues (e.g., Guimaraes *et al.*, 1987). Generally speaking, the existence of presynaptic α_1-adrenoceptors at postganglionic sympathetic axons remains doubtful (Starke, 1987; Docherty, 1989; Shinozuka *et al.*,1991).

Properties of the α_2-autoreceptors

To which of the subgroups defined by radioligand binding do the α_2-autoreceptors belong? The comparison will be based on the pK_D values determined against UK 14304 (Table 1), but a comparison based on the pEC_{30} values leads to the same conclusions. The low absolute affinity of the autoreceptors for prazosin (pK_D against UK 14304 6.1 in submaxillary gland and 6.8 in atrium) already argues against α_{2B} properties (binding pK_D 7.6–8.3 in Table 2 of Simonneaux *et al.*, 1991). Affinity correlations as well as affinity ratios (see below) indicate that the properties of the submaxillary autoreceptors agree well with those of the α_{2D} binding site in bovine pineal gland as defined by Simonneaux *et al.* (1991). The atrial autoreceptors also resemble the α_{2D} site most; here, however, the similarity is much more limited; moreover, consistent differences between atrial and submaxillary autoreceptors indicate that they are not the same.

Correlations are summarized in Table 3. The submaxillary autoreceptor affinities correlate significantly with binding affinities to the bovine pineal gland α_{2D} site ($r = 0.90$) but not with α_{2A}, α_{2B} and α_{2C} binding affinities. Moreover, the regression coefficient is close to unity in the case of the α_{2D} correlation. For the atrial autoreceptors, the answer is doubtful. Although correlation is best with the bovine pineal α_{2D} site ($r = 0.95$), the correlation with the neonatal rat lung α_{2B} site is almost as good, and there is also significant correlation with α_{2A} and α_{2C} sites.

Ratios of antagonist K_D values, calculated from the present experiments and from binding data, are summarized in Table 4. Several ratios clearly differentiate the rat submaxillary autoreceptor from α_{2A}, α_{2B} and α_{2C}. For example, the phentolamine/rauwolscine ratio is 8.6 at α_{2A} sites (the two values of Table 4 averaged) but 0.32 at the submaxillary autoreceptor, and the prazosin/WB 4101 ratio is 240 at α_{2A} sites (on average) but 5 at the autoreceptor. On the other hand, 5 of 6 submaxillary autoreceptor ratios are relatively close to the α_{2D} ratios in bovine pineal gland (less than 5 fold difference; exception: WB 4101/phentolamine). As to atrial autoreceptors, several ratios also set them apart from α_{2A}, α_{2B} and α_{2C}. For example, the BRL 44408/BRL 41992 ratio is 131 at the neonatal rat lung α_{2B} site but 0.13 at atrial autoreceptors, a 1008 fold difference, and the BRL 44408/prazosin ratio is 49.8 at the α_{2B} site but 0.25 at the autoreceptors, a 199 fold difference. The atrial autoreceptors agree better, but less than the submaxillary autoreceptors, with α_{2D}: 3 of 6 ratios are relatively close to the α_{2D} ratios in bovine pineal gland (less than 5 fold difference), and for the remaining 3 ratios the greatest difference is 8 fold (corynanthine/phentolamine).

It has been suggested that binding sites for radiolabelled α_2-adrenoceptor antagonists in rat submaxillary gland (Michel *et al.*, 1989) and rat jejunum epithelial cells (Paris *et al.*, 1990) also represent α_{2D}-adrenoceptors (Paris *et al.*, 1990; Lanier *et al.*, 1991; Simonneaux *et al.*, 1991). Seven antagonists could be used to compare our functional autoreceptor affinities with affinities for the [^{3}H]-rauwolscine binding site in rat submaxillary gland (Michel *et al.*, 1989). The correlation between the submaxillary autoreceptors and the submaxillary [^{3}H]-rauwolscine binding site is excellent, correlation coefficient 0.98 (Table 3; see also Figure 6a). The correlation between the atrial autoreceptors and the submaxillary [^{3}H]-rauwolscine binding site, in contrast, is relatively poor, correlation coefficient 0.78 (Table 3; see also Figure 6b). Moreover, all antagonist K_D ratios for submaxillary autoreceptors are very close to those for the submaxillary [^{3}H]-rauwolscine binding site (Table 4). In the case of the atrial autoreceptors, there are differences, the largest one between a phentolamine/rauwolscine ratio of 5 for atrial autoreceptors and of 0.18 for the submaxillary binding site (Table 4). If the submaxillary binding site is α_{2D}, this com-

Table 3 Correlation between antagonist affinity estimates for α_2-autoreceptors and various α_2 binding sites

Tissue used for [^{3}H]-antagonist binding	*Submaxillary gland autoreceptors*		*Atrial autoreceptors*		*Number of antagonists*
	Correlation coefficient	*Regression coefficient*	*Correlation coefficient*	*Regression coefficient*	
Human platelets (α_{2A})[a]	0.81	0.88	0.85	1.29	5
HT29 cell line (α_{2A})[b]	0.73	0.79	0.87*	1.23	6
Neonatal rat lung (α_{2B})[b]	0.74	0.60	0.93**	1.00	6
OK cell line (α_{2C})[b]	0.50	0.58	0.85*	1.31	6
Bovine pineal gland (α_{2D})[c]	0.90*	0.92	0.95**	1.28	6
Rat submaxillary gland[d]	0.98**	0.92	0.78*	0.91	7

Autoreceptor affinity estimates are pK_D values calculated from antagonism against UK 14304 (from Table 1). Binding site affinity estimates are pK_D values calculated from inhibition of the binding of [^{3}H]-yohimbine or [^{3}H]-rauwolscine (from references quoted). Shown are correlation coefficients and slopes of the regression of binding pK_D on autoreceptor pK_D. Antagonists were phentolamine, rauwolscine, WB4101, SKF 104078, prazosin and corynanthine except for correlations with human platelet binding sites (no binding data on SKF 104078) and rat submaxillary gland binding sites (imiloxan in addition; the correlations with submaxillary binding sites are shown in Figure 6). Significant differences from 0: $*P<0.05$; $**P<0.01$.

[a] Cheung *et al.* (1982).
[b] Blaxall *et al.* (1991).
[c] Simonneaux *et al.* (1991).
[d] Michel *et al.* (1989).

Table 4 Ratios of K_D values of α-adrenoceptor antagonists

Tissue	*Phentolamine/ rauwolscine*	*Corynanthine/ phentolamine*	*Prazosin/ corynanthine*	*Prazosin/ WB 4101*	*Prazosin/ phentolamine*	*WB 4101/ phentolamine*	*BRL 44408/ BRL 41992*	*BRL 44408/ prazosin*
				Autoreceptors				
Rat submaxillary gland	0.32	631	0.25	5.0	158	31.6	0.25	0.03
Rat atrium	5.0	31.6	0.16	5.0	5.0	1.0	0.13	0.25
				Binding sites				
Human platelets (α_{2A})[a,b]	3.6	47.9	4.0	216	190	0.88	0.02	0.003
HT29 cell line (α_{2A})[c,d]	13.6	22.0	2.4	263	52.7	0.20	0.61	0.007
Neonatal rat lung (α_{2B})[b,c]	8.0	27.8	0.05	0.62	1.5	2.4	131	49.8
OK cell line (α_{2C})[c]	226	2.9	0.54	55.6	1.5	0.03	–	–
Bovine pineal gland (α_{2D})[e]	0.91	248	0.14	14.0	34.2	2.5	–	–
Rat submaxillary gland[f]	0.18	457	0.30	7.6	135	17.8	–	–

Shown are ratios for the α_2-autoreceptors of rat submaxillary gland and atrium (K_D calculated from antagonism against UK 14304; from Table 1) and for various α_2 binding sites (K_D calculated from inhibition of the binding of [^{3}H]-yohimbine or [^{3}H]-rauwolscine; from references quoted).
[a] Cheung *et al.* (1982).
[b] Young *et al.* (1989) (BRL 44408/BRL 41992 and BRL 44408/prazosin).
[c] Blaxall *et al.* (1991).
[d] Gleason & Hieble (1991) (BRL 44408/BRL 41992 and BRL 44408/prazosin).
[e] Simonneaux *et al.* (1991).
[f] Michel *et al.* (1989).

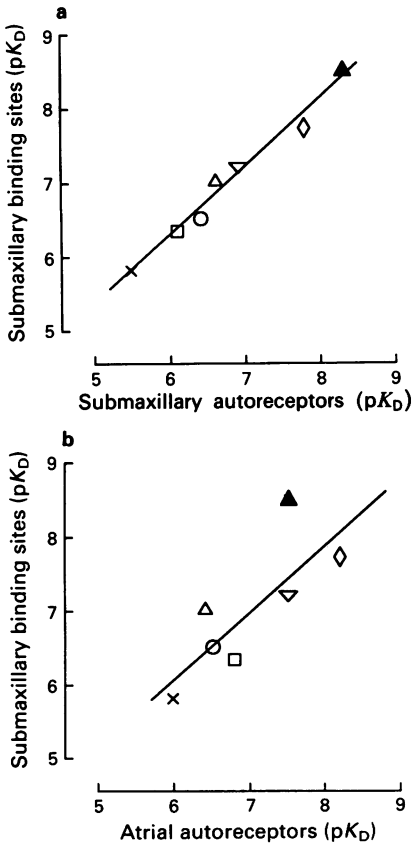

Figure 6 Correlation between antagonist affinity estimates for α_2-autoreceptors and the [^{3}H]-rauwolscine binding site in rat submaxillary gland: (a) shows correlation for rat submaxillary gland autoreceptors and (b) for rat atrial autoreceptors. Autoreceptor affinity estimates are pK_D values calculated from antagonism against UK 14304 (from Table 1). Binding site affinity estimates are pK_D values calculated from inhibition of the binding of [^{3}H]-rauwolscine (from Michel *et al.*, 1989). Symbols as in Figure 3. Regression line in (a): binding $pK_D = 0.92 \times$ autoreceptor $pK_D + 0.79$; in (b): binding $pK_D = 0.91 \times$ autoreceptor $pK_D + 0.61$.

parison confirms both the α_{2D} character of the submaxillary autoreceptors and the deviation in the case of the atrial autoreceptors.

Previous α_2-autoreceptor characterizations

Smith *et al.* (1992) have recently classified the α_2-autoreceptors of rat submaxillary gland and rat vas deferens as α_{2A} and those of rat atrium as α_{2B}. They based their conclusion on a comparison with their own binding data for α_{2A} and α_{2B} sites and did not attempt a classification in terms of α_{2C} or α_{2D}. We confirm the difference between the atrial and the submaxillary autoreceptors. The difference is real: the same similarities and differences were detected in two independent assessments of autoreceptor properties (Figure 3a versus Figure 3b). On the other hand, we would like to suggest that the results of Smith *et al.* (1992) are open to re-interpretation as far as subtype assignments are concerned. In the case of the vas deferens autoreceptor, a comparison of the pK_D (pA_2) values of Docherty's group (Connaughton & Docherty, 1989; Smith *et al.*, 1992) with the binding sites listed in our Table 3 yields the best correlation with the bovine pineal α_{2D} site ($r = 0.87$) and the submaxillary [^{3}H]-rauwolscine (α_{2D}?) binding site ($r = 0.98$). Moreover, the vas deferens autoreceptor pK_D values correlate very well with our submaxillary autoreceptor (α_{2D}) values ($r = 0.98$). These correlations suggest that the vas deferens autoreceptor is α_{2D} rather than α_{2A}; some K_D ratios point in the same direction. There are too few antagonists to compare the submaxillary autoreceptor pEC_{30} values of Smith *et al.* (1992) with literature data. However, none of their findings excludes α_{2D}, and the excellent agreement between the submaxillary and the vas deferens autoreceptors found by Smith *et al.* (1992) makes a re-interpretation as α_{2D} possible. Finally, the findings of Connaughton & Docherty (1989) and Smith *et al.* (1992) on atrial autoreceptors are not incompatible with a closer similarity to α_{2D} than α_{2B}. For example, their atrial autoreceptor pEC_{30} values correlate better with pK_D values for the α_{2D} site in bovine pineal gland ($r = 0.90$) than with pK_D values for the α_{2B} site in neonatal rat lung ($r = 0.71$) (binding studies

listed in our Table 3; see already Connaughton & Docherty, 1989).

Differences between the presynaptic autoreceptors of rat and rabbit tissues early indicated a non-homogeneity of the α_2 class (see Introduction). The difference may be due to the occurrence of predominantly α_{2A}-autoreceptors in rabbits (Limberger *et al.*, 1991) and α_{2D}-autoreceptors in rats.

In conclusion, the imidazoline adrenoceptor agonist UK 14304 inhibits the release of noradrenaline in rat submaxillary glands and atria exclusively by an effect on α_2-autoceptors and not on imidazoline receptors. The selective α_1-adrenoceptor agonist methoxamine also causes inhibition only through α_2-adrenoceptors; no evidence for presynaptic α_1-adrenoceptors was found. The pharmacological properties of the atrial α_2-autoreceptors, as assessed by two independent methods, differ consistently from those of the submaxillary α_2-autoreceptors. The properties of the submaxillary autoreceptors agree so well with those of α_{2D} binding sites that the two, apart from species differences, may be identical. The atrial autoreceptors also resemble α_{2D} binding sites most. They may belong to a subtype similar to, but not identical with, α_{2D}. However, the existence of a mixed α_2-autoreceptor population in rat atria cannot be ruled out.

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Modulation of non-adrenergic non-cholinergic inhibitory neurotransmission in rat gastric fundus by the α_2-adrenoceptor agonist, UK-14,304

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1 The influence of the α_2-adrenoceptor agonist, UK-14,304, on non-adrenergic non-cholinergic (NANC) relaxation induced by electrical field stimulation was investigated in longitudinal muscle strips of the gastric fundus of reserpinized rats.

2 In tissues where tone was raised by 3×10^{-7} M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), the inhibitory effect of 10^{-6} M UK-14,304, on the NANC relaxations induced by short train stimulation (40 V, 1 ms, 20 s) was inversely related to the stimulus frequency (1–4–16 Hz). UK-14,304 (10^{-6} M) did not influence relaxations induced by administration of exogenous nitric oxide (NO, 2×10^{-6} M–10^{-4} M). The inhibitory effect of UK-14,304 on the electrically induced relaxations was antagonized by 10^{-6} M rauwolscine but not by 10^{-6} M prazosin.

3 UK-14,304 (10^{-6} M) also reduced the amplitude of the sustained NANC relaxation, induced by electrical field stimulation (40 V, 1 ms, 4 Hz) for 5 min. The effect of UK-14,304 was also antagonized by 10^{-6} M rauwolscine but not by 10^{-6} M prazosin. UK-14,304 (10^{-6} M) did not reduce the relaxation induced by 3×10^{-9} M vasoactive intestinal polypeptide (VIP).

4 These results suggest that the release of the inhibitory NANC neurotransmitter during short train stimulation, thought to be NO, and during sustained stimulation, thought to be VIP, is inhibited by stimulation of presynaptic α_2-adrenoceptors in the rat gastric fundus.

Keywords: Rat gastric fundus; NANC neurotransmission; nitric oxide (NO); vasoactive intestinal polypeptide (VIP); presynaptic α_2-adrenoceptors

Introduction

The modulation via presynaptic receptors of noradrenaline release from noradrenergic nerve terminals in the peripheral nervous system is well established (Langer, 1981). In a similar way, presynaptic modulation of acetylcholine release via muscarinic autoreceptors or receptors sensitive to agents other than acetylcholine has been described. The concept is less well developed for peripheral non-adrenergic non-cholinergic (NANC) neurotransmission but recent studies suggest that NANC neurotransmitter release can also be influenced via presynaptic receptors. In the guinea-pig airways, NANC bronchoconstriction can be inhibited via presynaptic α_2-adrenoceptors (Grundström *et al.*, 1984; Grundström & Andersson, 1985), opioid receptors (Belvisi *et al.*, 1988; Matran *et al.*, 1989), $GABA_B$-receptors (Belvisi *et al.*, 1989), neuropeptide Y receptors (Matran *et al.*, 1989) and histamine H_3-receptors (Ichinose & Barnes, 1989). In the gastro-intestinal tract, presynaptic inhibition of NANC inhibitory responses has been shown in the guinea-pig proximal colon (via α_2-adrenoceptors; Kojima *et al.*, 1988), the rat anococcygeus muscle (via muscarinic receptors; Li & Rand, 1989) and the canine lower oesophageal sphincter (via opioid receptors; Barnette *et al.*, 1990).

In the rat gastric fundus, we previously found that clonidine did not influence the NANC relaxation induced by transmural stimulation at a frequency of 5 Hz for 45 s (Lefebvre & Bogaert, 1986) while MacDonald *et al.* (1990) reported that the selective α_2-adrenoceptor agonist UK-14,304 (Cambridge, 1981) concentration-dependently inhibited the NANC relaxation induced by transmural stimulation at a frequency of 0.5 Hz for 10 s. Both nitric oxide (NO) and vasoactive intestinal polypeptide (VIP) seem involved in inhibitory NANC neurotransmission of the rat gastric fundus (De Beurme & Lefebvre, 1988; Li & Rand, 1990; Boeckxstaens *et al.*, 1991; D'Amato *et al.*, 1992), whereby NO is released with short trains or low frequencies of stimulation, while VIP is released during sustained stimulation. Although it has not been definitely established that NO and VIP are released from the same neurones in the rat gastric fundus, the observation that immunoreactivity for NO synthase is co-localized with that for VIP in nerve cell bodies and fibres of the guinea-pig small intestine (Costa *et al.*, 1991) correlates with such an hypothesis. As it has been shown that the release of the cotransmitters noradrenaline and ATP can be differentially influenced via presynaptic receptors in the guinea-pig vas deferens (Ellis & Burnstock, 1989), we investigated the effect of UK-14,304 on relaxations induced by short and sustained trains of stimulation and by the putative respective transmitters NO and VIP. A preliminary account of part of these results has been given (Lefebvre & Smits, 1992).

Methods

Tissue preparation

Male Wistar rats (weight 150–390 g) were reserpinized (5 mg kg^{-1}, i.p.) 24 h before killing and fasted from then on. Two longitudinal muscle strips (about 15 mm long × 3 mm wide) were prepared from the gastric fundus and mounted under a load of 1 g in 5 ml organ baths, containing Krebs solution at 37°C (composition in mM: NaCl 118.5, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0 and glucose 10.1). The Krebs solution always contained 10^{-6} M atropine and was bubbled with 95% O_2:5% CO_2. Changes in length were recorded isotonically (Hugo Sachs Lever transducer B type

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368) on a Graphtec Linearcorder 8 WR 3500. Electrical stimulation (40 V, 1 ms, 0.25–16 Hz) was performed via two parallel platinum electrodes by means of a Hugo Sachs Elektronik Stimulator I type 215/I. The tissues were equilibrated for 1 h with rinsing every 15 min.

Protocols

The influence of UK-14,304 on the relaxant responses induced by 20 s train stimulation at 1, 4 and 16 Hz was studied as follows. After the equilibration, the tissues were contracted by administration of 3×10^{-7} M $PGF_{2\alpha}$. About 10 min after the beginning of the stable plateau contraction, the tissues were stimulated with 20 s trains (40 V, 1 ms, 1, 4 and 16 Hz) at 5 min intervals. The tissues were then rinsed several times during approximately 1 h. $PGF_{2\alpha}$ (3×10^{-7} M) was again administered and once the plateau contraction was reached, UK-14,304 (10^{-6} M) or its solvent were administered 10 min before stimulation. To study the influence of rauwolscine (10^{-6} M) and prazosin (10^{-6} M) on the effect of UK-14,304, the antagonists were administered in the organ bath 30 min before the second $PGF_{2\alpha}$ administration and left in contact with the tissue during the rest of the experiment. The influence of UK-14,304 (10^{-6} M) on relaxations induced by bolus administrations of exogenous NO (2×10^{-6} M, 10^{-5} M and 10^{-4} M) was studied as described for the relaxations induced by 20 s train stimulation except that only one concentration-response curve to NO was obtained per tissue, in the presence of UK-14,304 or its solvent. As NO-induced relaxations were transient, the three NO concentrations were administered at 5 min intervals without replacing the bathing solution.

Following the same protocol with 2 cycles of $PGF_{2\alpha}$ administration, the influence of 10^{-6} M UK-14,304 was also tested on the relaxant response to sustained transmural stimulation (40 V, 1 ms) with increase of the frequency (0.25–16 Hz) when the relaxation induced by a given frequency had reached a stable maximum (further indicated as cumulative increase of the frequency), to transmural stimulation (40 V, 1 ms) at 4 Hz for 5 min and to 3×10^{-9} M VIP. The influence of the antagonists rauwolscine (10^{-6} M) and prazosin (10^{-6} M) on the effect of 10^{-6} M UK-14,304 was also studied.

Drugs

The following drugs were used: atropine sulphate (Boehringer Ingelheim, Germany), 5-hydroxytryptamine creatinine sulphate (Sigma, St Louis, U.S.A.), prazosin hydrochloride (Pfizer, Brussels, Belgium), prostaglandin $F_{2\alpha}$ (Sigma), rauwolscine hydrochloride (Carl Roth, Karlsruhe, Germany), reserpine (Aldrich Chemie, Brussels, Belgium), tetrodotoxin (Janssen Chimica, Beerse, Belgium), UK-14,304 tartrate (5-bromo-6-[-2-imidazolin-2-ylamino]-quinoxaline; Pfizer, Sandwich, England), vasoactive intestinal polypeptide (CRB, Northwich, England). For reserpine, a stock solution was prepared from powder (5 mg ml^{-1} dissolved in 10% ascorbic acid). Drugs were dissolved and diluted with distilled water. Stock solutions of 10^{-3} M VIP were kept frozen at −20°C and dilutions were made the day of the experiment. A saturated NO solution (2×10^{-3} M) was prepared in distilled water as described by Kelm & Schrader (1990) from NO gas (l'Air Liquide, Belgium) for NO administration.

Statistical analysis

Results are given as means ± s.e.mean. Relaxation was expressed as percentage of $PGF_{2\alpha}$-induced tone. Results within the same tissues were compared by the Wilcoxon signed-ranks test and results in different groups of tissues by the Mann-Whitney two-sample rank test. P values of less than 0.05 were considered statistically significant.

Results

In preliminary studies in tissues contracted with 5-hydroxytryptamine, UK-14,304 (10^{-8}–10^{-5} M) concentration-dependently reduced NANC relaxations induced by 20 s train stimulation; UK-14,304 did not influence the tone of the tissues *per se* except at 10^{-5} M, which induced a small transient relaxation in some tissues. UK-14,304 10^{-6} M was selected for further experiments. When the influence of the α_2-adrenoceptor antagonist, rauwolscine, on the effect of UK-14,304 was studied in these tissues, we observed that incubation with rauwolscine clearly reduced 5-hydroxytryptamine-induced tone so that responses in its presence were difficult to interpret. This effect of rauwolscine can very probably be ascribed to its ability to antagonize competitively 5-hydroxytryptamine-induced contractions in the rat gastric fundus (Clineschmidt *et al.*, 1985). In further experiments, tone was, therefore, raised with 3×10^{-7} M $PGF_{2\alpha}$, when required.

Transmural stimulation (40 V, 1 ms) at 1, 4 and 16 Hz for 20 s induced frequency-dependent relaxations (Figure 1); these responses were abolished after incubation of the tissues with 3×10^{-6} M tetrodotoxin for 10 min ($n = 3$). When stimulating at 16 Hz, tone did not completely recover during the observation period of at least 5 min after ending stimulation. The amplitude of the contraction to the second administration of 3×10^{-7} M $PGF_{2\alpha}$ was the same as to the first administration (100.4 ± 4.6%, $n = 8$). Addition of 10^{-6} M UK-14,304 on top of $PGF_{2\alpha}$ did not change the degree of contraction, but clearly reduced the amplitude of the stimulation-induced relaxations (Figure 1). In control tissues where the solvent of UK-14,304 was administered, the relaxant responses to transmural stimulation were well maintained, while UK-14,304 significantly reduced the response at each stimulation frequency tested (Figure 2). When expressing the response in the presence of UK-14,304 as a percentage of that before addition of UK-14,304, the reduction attained was 61.1 ± 10.4% at 1 Hz, 41.2 ± 8.2% at 4 Hz and 27.1 ± 4.5% at 16 Hz ($n = 8$). Bolus administration of NO induced transient relaxations (Figure 3). Preliminary experiments revealed that the relaxant responses to a second set of NO

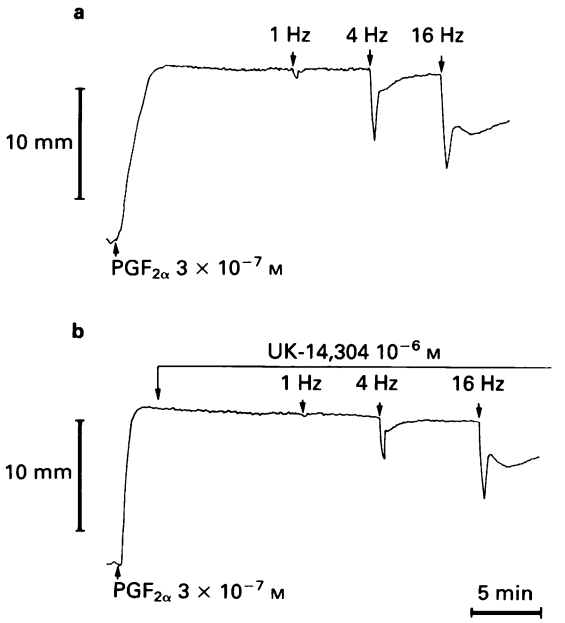

Figure 1 Original recording of a longitudinal muscle strip of the rat gastric fundus, showing the increase of tone by administration of 3×10^{-7} M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and the relaxant responses to electrical stimulation (40 V, 1 ms, 20 s) at 1, 4 and 16 Hz in the absence (a) and presence (b) of 10^{-6} M UK-14,304.

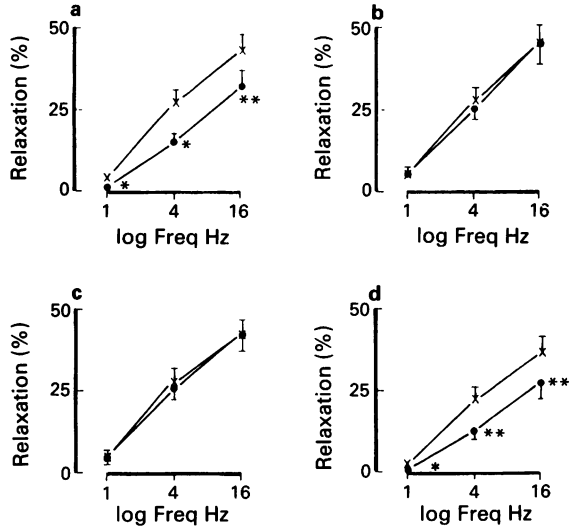

Figure 2 Responses to electrical stimulation (40 V, 1 ms, 20 s) at 1, 4 and 16 Hz in the absence (X) and in the presence (●) of 10^{-6} M UK-14,304 (a, c, d) or its solvent (b). (c and d) Tissues were incubated with 10^{-6} M rauwolscine and 10^{-6} M prazosin respectively before UK-14,304 was administered. The tone of the tissues was raised with 3×10^{-7} M prostaglandin $F_{2\alpha}$. Mean of $n = 8$; vertical bars show s.e.mean. $*P<0.05$; $**P<0.01$: significantly different from the value in the absence of UK-14,304 (Wilcoxon Signed-Ranks test).

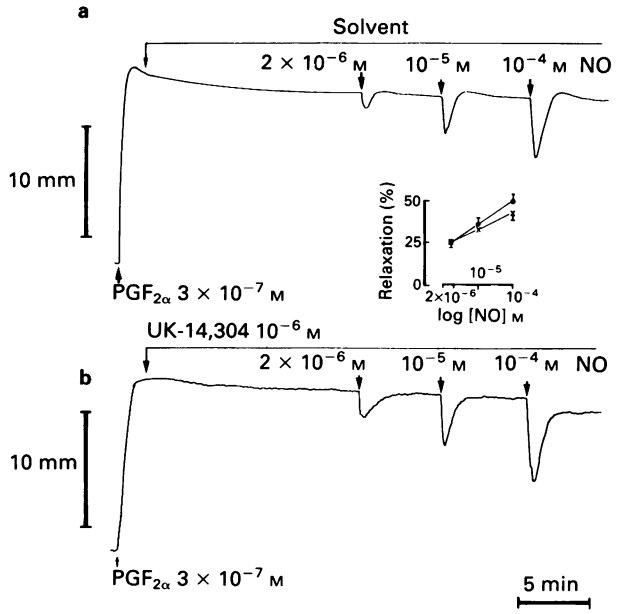

Figure 3 Original recording of 2 longitudinal muscle strips of the gastric fundus of the same rat. After increasing tone with 3×10^{-7} M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), the relaxant responses to bolus administrations of NO (2×10^{-6}, 10^{-5} and 10^{-4} M) were studied in the presence of 10^{-6} M UK-14,304 (b) or its solvent (a). Insert: Mean (± s.e.mean) relaxant responses to NO in the presence of 10^{-6} M UK-14,304 (●, $n = 7$) or its solvent (X, $n = 8$).

boluses in the same tissue clearly declined; the influence of UK-14,304 and its solvent was therefore tested in parallel tissues of the same rat, where only one concentration-response curve to NO was obtained. UK-14,304 did not influence the NO-induced relaxations (Figure 3). Incubation with 10^{-6} M rauwolscine or 10^{-6} M prazosin did not influence the amplitude of the $PGF_{2\alpha}$-induced contraction (98.9 ± 7.0%, $n = 8$ and 100.6 ± 4.2%, $n = 8$ respectively). Rauwolscine completely prevented the inhibitory effect of UK-14,304 on stimulation-induced relaxations while prazosin was not able to do so (Figure 2).

When transmural stimulation was sustained, the relaxation reached a plateau within approximately 1 min. The relaxant responses to sustained transmural stimulation with cumulative increase of the frequency were reproducible in control tissues; the responses were abolished by 3×10^{-6} M tetrodotoxin ($n = 3$). In the presence of 10^{-6} M UK-14,304, the relaxant responses were decreased (Figure 4). The inhibition was most pronounced at a stimulation frequency of 4 Hz (35.3 ± 9.8% at 2 Hz, 45.9 ± 9.2% at 4 Hz and 15.6 ± 3.4% at 8 Hz). At 16 Hz, no inhibition was observed. When the tissue was stimulated at 4 Hz for 5 min, UK-14,304 (10^{-6} M) significantly reduced the relaxation (Figure 5; from 45.3 ± 7.5% to 26.9 ± 5.4%, $n = 7$, $P<0.05$) while the response was well maintained in the control strips (51.1 ± 6.0% and 50.5 ± 6.6%, $n = 8$). In the presence of 10^{-6} M UK-14,304, the relaxation induced by 3×10^{-9} M VIP was significantly decreased from 35.4 ± 6.4% to 29.6 ± 14.9% ($n = 8$, $P<0.05$) but the same decline was observed in the control tissues, where the solvent of UK-14,304 was administered (from 43.0 ± 5.0% to 33.6 ± 4.9%, $n = 8$, $P<0.05$).

To investigate the influence of rauwolscine and prazosin on the effect of UK-14,304, four parallel groups of tissues were studied. The results of this series of experiments are shown in Figure 6. UK-14,304 (10^{-6} M) reduced the relaxation, induced by transmural stimulation at 4 Hz for 5 min, by 44%, which was significantly more pronounced than the decline in response (16%) observed in the control tissues of this series where the solvent of UK-14,304 was administered. In the presence of 10^{-6} M prazosin, UK-14,304 reduced the stimulation-induced relaxation to the same extent (43%) as when given alone. However, 10^{-6} M rauwolscine almost completely prevented the inhibitory effect of UK-14,304.

Discussion

In the rat gastric fundus, α-adrenoceptor agonists can inhibit smooth muscle cell activity directly by stimulation of postsynaptic α_1-adrenoceptors and indirectly by stimulation of α_2-like adrenoceptors on the postganglionic cholinergic neurones (Verplanken *et al.*, 1984; Kelly & MacDonald, 1990; MacDonald *et al.*, 1990). MacDonald *et al.* (1990) also suggested the presence of α_2-adrenoceptors on the inhibitory NANC neurones of the rat gastric fundus, on the basis of the inhibitory effect of the α_2-adrenoceptor agonist UK-14,304 on

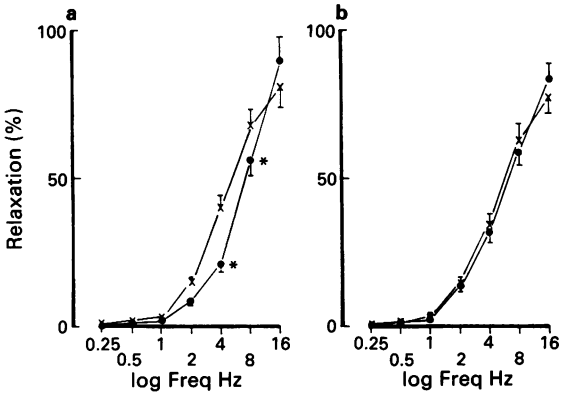

Figure 4 Responses to electrical stimulation (40 V, 1 ms, 0.25–16 Hz) with cumulative increase of the frequency in the absence (X) and in the presence (●) of 10^{-6} M UK-14,304 (a) or its solvent (b). The tone of the tissues was raised with 3×10^{-7} M prostaglandin $F_{2\alpha}$. Mean of $n = 6$ (a) and 7 (b); vertical bars show s.e.mean. $*P<0.05$: significantly different from the value in the absence of UK-14,304 (Wilcoxon Signed-Ranks test).

relaxation induced by transmural stimulation at 0.5 Hz for 10 s. As different transmitters (i.e. NO and VIP) seem to be released from the inhibitory NANC neurones during short-lasting and sustained stimulation (see Introduction for references), this study was set up to investigate further α_2-mediated modulation of inhibitory NANC neurotransmission in the rat gastric fundus. The study was performed under NANC conditions as the tissues were obtained from reserpinized rats to avoid noradrenergic responses (Lefebvre, 1986) and the bathing medium contained atropine to avoid cholinergic contractile responses (Lefebvre *et al.*, 1983).

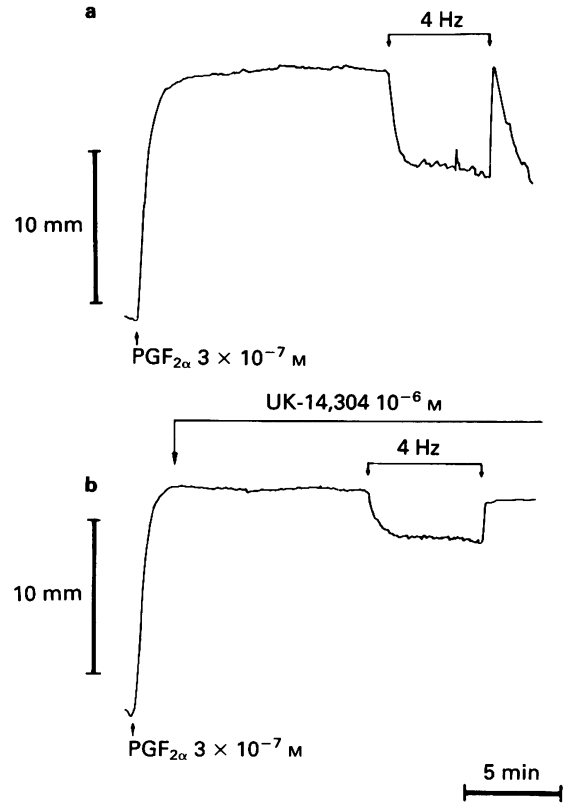

Figure 5 Original recording of a longitudinal muscle strip of the rat gastric fundus, showing the relaxant response to electrical stimulation (40 V, 1 ms, 4 Hz for 5 min) in the absence (a) and presence (b) of 10^{-6} M UK-14,304.

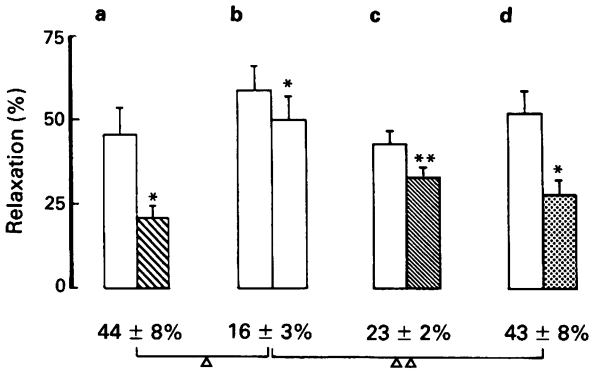

Figure 6 Responses to electrical stimulation (40 V, 1 ms, 4 Hz for 5 min) in the absence (first column) and in the presence (second column) of 10^{-6} M UK-14,304 (a, c, d) or its solvent (b). (c and d) Tissues were incubated with 10^{-6} M rauwolscine and 10^{-6} M prazosin respectively before UK-14,304 was administered. The tone of the tissues was raised with 3×10^{-7} M prostaglandin $F_{2\alpha}$. Mean (± s.e.mean) of $n = 6$ (b) and 7 (a and d) and 8 (c). The figures below the columns indicate the percentage reduction calculated as

$$\text{responses to } \frac{\text{4 Hz before} - \text{4 Hz after}}{\text{4 Hz before}} \times 100$$

$^*P<0.05$; $^{**}P<0.01$: significantly different from the value in the absence of UK-14,304 or its solvent (Wilcoxon Signed-Ranks test). $^{\Delta}P<0.05$; $^{\Delta\Delta}P<0.01$: significantly different from the reduction in the control tissues (Mann-Whitney two-sample Rank test).

The selective α_2-adrenoceptor agonist UK-14,304 inhibited NANC relaxations induced by short train stimulation, confirming the results of MacDonald *et al.* (1990), and also those induced by sustained stimulation. This suggests that the release of both the transmitter involved in relaxation induced by short train stimulation (thought to be NO; Li & Rand, 1990; Boeckxstaens *et al.*, 1991) and that involved in relaxation induced by sustained stimulation (thought to be VIP; De Beurme & Lefebvre, 1988; Kamata *et al.*, 1988; Li & Rand, 1990; D'Amato *et al.*, 1992) can be inhibited by stimulation of presynaptic α_2-adrenoceptors. It seems unlikely that the effect of UK-14,304 is due to a postsynaptic action at the smooth muscle cell level. First, up to 10^{-6} M UK-14,304 had no influence *per se* on the tone of the tissues. The transient relaxant effect observed with 10^{-5} M UK-14,304 in some tissues can probably be ascribed to activation of the postsynaptic α_1-adrenoceptors by this high concentration of UK-14,304. Second, antagonism of the NO- and VIP-induced relaxation at the smooth muscle cell level can be excluded as UK-14,304 did not influence relaxations induced by exogenous NO and VIP, of similar amplitude to those induced by electrical stimulation. Inhibition of transmitter release via presynaptic receptors is inversely related to the intensity of nerve stimulation (Duckles & Budai, 1990). The inhibitory action of UK-14,304 on NANC relaxations induced by short trains of stimulation, was inversely related to the stimulus frequency. We have no explanation as to why the inhibitory action of UK-14,304 on a frequency-response curve with sustained stimulation was more pronounced on the response at 4 Hz than on the response at 2 Hz, although in these conditions, the inhibitory action of UK-14,304 also decreased and finally disappeared with the higher frequencies of stimulation. These observations thus corroborate the conclusion that UK-14,304 is acting at the presynaptic level. This action is mediated via α_2-adrenoceptors as it is blocked by the selective α_2-adrenoceptor antagonist, rauwolscine (Weitzell *et al.*, 1979; Timmermans *et al.*, 1980) but not by the α_1-adrenoceptor antagonist, prazosin (Davey, 1980). Both agents were used in concentrations selective for α_1- and α_2-adrenoceptors respectively in other tissues (Tanaka *et al.*, 1978; Fagbemi & Salako, 1980). Our previous observation that 10^{-5} M clonidine did not influence the NANC relaxation by electrical stimulation at 5 Hz during 45 s might be due to the partial agonism and limited selectivity of this agent for α_2-adrenoceptors.

During NANC relaxation induced by sustained stimulation in the rat gastric fundus, NO is only involved in the very first phase of the response (Li & Rand, 1990; Boeckxstaens, 1991). The presynaptic inhibition of NO release by UK-14,304, as demonstrated with the responses to short train stimulation, might thus contribute to some extent to the presynaptic inhibition by UK-14,304 of the response to sustained stimulation. The main part of the latter response is however due to a transmitter other than NO, very probably VIP. In the perfused canine ileum, it was shown that release of VIP-like immunoreactivity, as measured by radioimmunoassay, is subject to presynaptic inhibition via α_2-adrenoceptors (Manaka *et al.*, 1989). If, in the rat gastric fundus, NO and VIP are released from the same neurones, which is probable in the guinea-pig small intestine (Costa *et al.*, 1991), the presynaptic influence of the α_2-adrenoceptor agonist UK-14,304, on these cotransmitters is the same. Examples of a differential influence of a presynaptic mechanism on cotransmitters have been obtained (Ellis & Burnstock, 1989; Bulloch & Starke, 1990).

It has been shown that the majority of postsynaptic sympathetic fibres in the gut end on intrinsic neurones and not

on the smooth muscle cells (Wood, 1987). Inhibition of acetylcholine output by noradrenergic nerves via α-adrenoceptors on the postganglionic cholinergic neurones seems an important mechanism by which the sympathetic nervous system contributes to gastrointestinal smooth muscle regulation (Burnstock & Wong, 1981). This mechanism might be present in the rat gastric fundus as α_2-adrenoceptors are present on the postsynaptic cholinergic neurones in this tissue (see above). In a similar way, noradrenaline released from the postganglionic sympathetic fibres might reach the α_2-adrenoceptors, present on the intrinsic inhibitory NANC neurones. Our results do not allow us to localize exactly the presynaptic α_2-adrenoceptors involved in the inhibition of NANC transmitter release (i.e. at the nerve endings or closer to the soma or at the soma). The preganglionic vagal supply to the stomach seems centrally organized in a reciprocal way: when gastric relaxation is required, the preganglionic efferents supplying the intrinsic inhibitory NANC neurones are activated, while the discharge in those supplying the intramural cholinergic neurones is suppressed; the opposite happens when gastric contraction is required (Miolan & Roman, 1974; Andrews, 1990). The sympathetic nervous system might contribute to this regulation by inhibiting the release of acetylcholine or the inhibitory NANC neurotransmitters respectively, as required.

In conclusion, in the rat gastric fundus, the release of the inhibitory NANC neurotransmitter during short train stimulation, thought to be NO, and during sustained stimulation, thought to be VIP, is inhibited by stimulation of presynaptic α_2-adrenoceptors.

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Cardiovascular effects of substituted tetrahydroisoquinolines in rats

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1 A series of substituted tetrahydroisoquinolines derived from the cleavage products of tetrandrine were found to inhibit [^{3}H]-nitrendipine binding to rat cerebral cortical membranes. Those compounds which displaced [^{3}H]-nitrendipine binding were also able to inhibit high KCl-induced contraction of rat aorta *in vitro*.

2 There was a significant correlation between the ability of these tetrahydroisoquinolines to inhibit [^{3}H]-nitrendipine binding and KCl-induced contraction ($r = 0.99$, $P < 0.001$).

3 CPU-23 (1-{1-[(6-methoxy)-naphth-2-yl]}-propyl-2-(1-piperidine)-acetyl-6,7- dimethoxy-1,2,3,4,-tetrahydroisoquinoline), one of the most potent compounds identified in this series, behaved as a simple competitive inhibitor at the [^{3}H]-nitrendipine binding site and reduced the apparent affinity but not the maximal number of binding sites in saturation analysis.

4 In contrast to nifedipine which caused hypotension and tachycardia, CPU-23 induced both hypotension and bradycardia in a dose-dependent manner in pentobarbitone-anaesthetized Sprague-Dawley rats, spontaneously hypertensive and age-matched normotensive WKY rats.

5 It is suggested that CPU-23 may exert its cardiovascular effects via interaction with the dihydropyridine binding site on the L-type calcium channel.

Keywords: Tetrandrine; rat aorta; blood pressure; heart rate; hypertension; nitrendipine; calcium channel; diltiazem; verapamil; nifedipine

Introduction

Calcium entry blockers, capable of inhibiting transmembrane influx of extracellular calcium through specific calcium channels, are useful drugs in the treatment of hypertension, angina pectoris, supraventricular arrhythmia and various cardiovascular diseases (Conti *et al.*, 1985). Currently, three distinct classes of calcium entry blockers of the L-type calcium channel are in clinical use, namely, the dihydropyridines (e.g. nifedipine and nitrendipine), the phenylalkylamines (e.g. verapamil) and the benzothiazepines (e.g. diltiazem) (Fleckenstein, 1977). With the availability of radioactive ligands of dihydropyridines, verapamil and diltiazem at high specific activity, it has become possible to study drug interactions at the L-type calcium channel by direct radioligand binding assays. Such studies indicate that these compounds interact at distinct binding sites which are allosterically linked on the channel complex and that occupation of one site affects ligand binding at other sites. For instance, the specific binding of [^{3}H]-nitrendipine to cardiac sacrolemma and brain was competitively inhibited by nifedipine, only partly inhibited by verapamil and was allosterically stimulated by diltiazem (DePover *et al.*, 1982; Murphy *et al.*, 1983).

The prototypic dihydropyridine calcium entry blocker, nifedipine, can induce relaxation of arterial smooth muscle and lower blood pressure by reduction of peripheral vascular resistance. It also produces marked reflex tachycardia which somewhat limits its clinical use. While numerous new dihydropyridine analogues are currently being developed by different laboratories to provide better benefit-to-risk ratio than nifedipine (Freedman & Waters, 1987; Ohtsuka *et al.*, 1989), we have been trying to develop novel calcium entry blockers based on lead compounds isolated from Chinese medicinal herbs (Kong *et al.*, 1986; Huang *et al.*, 1988; Hon *et al.*, 1990).

Tetrandrine is a *bis*-benzylisoquinoline alkaloid isolated from *Stephania tetrandra*. It has been used clinically in China to treat angina and hypertension with good results (Gao *et al.*, 1965; Department of Pharmacology, Wuhan Medical College and Health Department, Wuhan Textile Factory, 1979). Based on its ability to shorten the cardiac action potential and inhibit the potassium- and calcium-induced contraction of smooth muscles *in vitro* (Fang *et al.*, 1981; Hu *et al.*, 1983; Yao *et al.*, 1983; Zong *et al.*, 1983; Zheng & Bian, 1986), as well as decrease blood pressure in normotensive and hypertensive rats *in vivo* (Qian *et al.*, 1983; Fang *et al.*, 1986; Kawashima *et al.*, 1990), it has been suggested that tetrandrine may be a novel calcium entry blocker. Indeed, recent pharmacological studies have demonstrated that tetrandrine blocks inward calcium current through the voltage-sensitive L-type calcium channel (King *et al.*, 1988; Wang *et al.*, 1988). Since it inhibits [^{3}H]-diltiazem binding competitively, partly inhibits [^{3}H]-verapamil binding and stimulates [^{3}H]-nitrendipine binding to cardiac sarcolemmal membranes in a manner very similar to diltiazem, it is likely that it acts at the benzothiazepine site of the L-channel (King *et al.*, 1988).

Chemically, tetrandrine is a *bis*-benzyl-tetrahydroisoquinoline with its 2 subunits connected in a head-to-head, tail-to-tail fashion through ether linkages. Its total chemical synthesis was reported in 1969 by Inubushi and co-workers. In order to simplify the chemical structure and to provide information on the structure-activity relationship of tetrandrine, we have synthesized a series of benzyl-tetrahydroisoquinolines (half-molecules of tetrandrine) (Huang *et al.*, 1990). The present experiments were undertaken to examine their interactions with the L-type calcium channel and their cardiovascular effects in normotensive and hypertensive rats.

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Methods

[^{3}H]-nitrendipine binding assay

[^{3}H]-nitrendipine binding assays were performed essentially according to the procedure described by Gould and co-workers (1982). Briefly, Sprague-Dawley rats of either sex (250–300 g) were killed by cervical dislocation. The cerebral cortex was homogenized in 10 vol (w/v) of ice-cold 50 mM Tris-HCl buffer (pH 7.4 at 22°C) in a Brinkmann Polytron PT-10 (setting 6, 10–15 s). The homogenate was centrifuged at 45,000 *g* for 10 min and the pellet was washed three times in 20 vol of the same buffer. The crude membrane fraction was stored as a pellet at −20°C. For binding experiments, the membrane pellet was resuspended in the same Tris-HCl buffer to give a protein concentration of about 5 mg ml^{-1}. An aliquot of the membrane preparation (300 μl) was incubated with [^{3}H]-nitrendipine (0.3 nM final unless otherwise indicated) and various tested drugs at the concentrations indicated in a total volume of 500 μl in duplicate. After incubation at 22°C for 30 min in a shaking water bath, the reaction was terminated by adding 3 ml of ice-cold Tris-HCl buffer and the mixture was immediately filtered through a glass fibre filter (Whatman GF/B) under suction. Preliminary experiments indicated that specific binding has reached equilibrium under these conditions. The filter was washed three times with 3 ml aliquots of ice-cold Tris-HCl buffer. Radioactivity retained on the filter was determined by liquid scintillation spectrometry. Non-specific binding was defined as binding in the presence of 1 μM nifedipine. Specific binding was calculated by subtracting the non-specific binding from total binding. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

KCl-induced contraction of rat aortic strip

Sprague-Dawley rats of either sex (300–350 g) were killed by cervical dislocation. The aorta was taken out, cut into helical strips (2 mm wide and 20 mm long) and suspended in an organ bath containing 20 ml of a modified Krebs bicarbonate solution (mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 10). The solution in the organ bath was bubbled with a mixture of 95% O_2 and 5% CO_2 and maintained at 37°C. Aortic strips were allowed to equilibrate for 2 h at a resting tension of 2 g. After equilibration, the tissue was contracted with a high K^+-containing Krebs solution (80 mM NaCl in the modified Krebs bicarbonate solution was substituted with 80 mM KCl). After the tension reached a steady state, the tested drugs were added from low to high concentration and the relaxation was measured isometrically via a force-displacement transducer (FTO3) using a Grass polygraph (79D). Concentrations of drugs required to inhibit 50% of the high K^+-induced contractions (IC_{50}s) were determined from the respective dose-response curves by Hill transformation.

Measurements of blood pressure and heart rate in normotensive and hypertensive rats

Sprague-Dawley rats of either sex (300–350 g), male spontaneously hypertensive rats (SHR, 300–350 g) and age-matched WKY rats were used. Tracheotomy and cannulation of the right femoral vein and left carotid artery were performed under pentobarbitone anaesthesia (75 mg kg^{-1}, i.p.). Arterial blood pressure and heart rate were measured from the left carotid artery with a pressure transducer (Gould P23 ID) and recorded on a Grass Polygraph (79D). After blood pressure and heart rate had stabilized, generally 20–30 min after surgical operations, drugs were injected intravenously in a volume of 0.1 ml/100 g of body weight and their effects were monitored over the next hour. The rectal temperature was maintained at 37°C with a table lamp.

Drug sources

[^{3}H]-nitrendipine (specific activity: 84 Ci mmol^{-1}) was purchased from New England Nuclear (Boston, MA, U.S.A.). Nifedipine was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Verapamil and diltiazem were from Research Biochemicals Inc (Natick, MA, U.S.A.). Tetrandrine was a generous gift of Prof. Liang N.C. of Zhanjiang Medical College, China. Tetrahydroisoquinolines were synthesized as previously described (Huang *et al.*, 1990).

All drugs except nifedipine were dissolved in saline. Nifedipine was dissolved in acetone and stored at 4°C. It was diluted in saline before used. The final acetone concentration was 0.2%, which was found in preliminary tests to have no effect on the experiments by itself.

Statistical analysis

Data are expressed as the mean ± s.e.mean of (*n*) separate experiments. Significant differences ($P < 0.05$) between means were evaluated by Student's paired or unpaired *t* test where appropriate.

Results

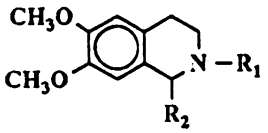
[^{3}H]-nitrendipine binding studies

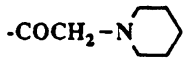
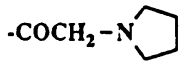
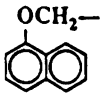
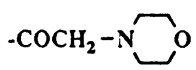
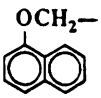
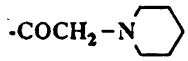
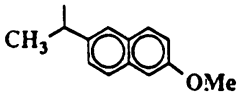
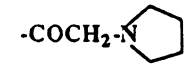
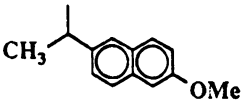
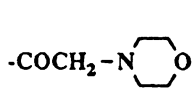
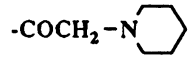
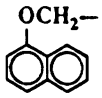
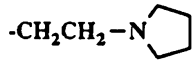
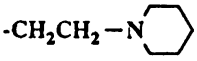
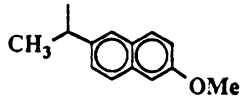
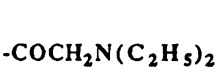
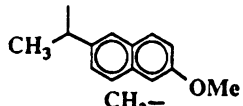
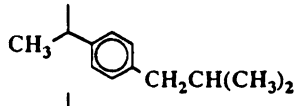
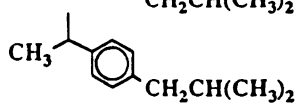
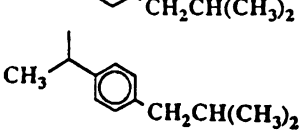
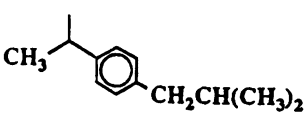
Unlike tetrandrine which stimulated [^{3}H]-nitrendipine binding, none of the substituted tetrahydroisoquinolines examined in the present study exhibited a stimulatory effect. Instead, many of them inhibited [^{3}H]-nitrendipine binding to rat cerebral cortical membranes in a concentration-dependent manner (Table 1). In our previous studies, reductive cleavage of tetrandrine yielded two benzylisoquinoline analogues, *o*-methylarmepavine and N-methylcoclaurine, both of which displayed very weak calcium antagonist activity when compared with the parent compound (Huang *et al.*, 1988). In the present study, by increasing the size of the N-alkyl substituent (R1) from an acetyl group (CPU-72) to propionyl (CPU-73), nicotinoyl (CPU-79) and finally 2-(1-piperidine)-acetyl group (CPU-80), a marked improvement in inhibitory potency against [^{3}H]-nitrendipine binding to rat cerebral cortical membranes was obtained (Table 1). To shed light on the importance of the 2-[4-(2-methylpropyl) phenyl]-propyl group (R2) in CPU-80, we kept the 2-(1-piperidine)-acetyl group as the N-alkyl substituent while changing R2 to a 1-naphthylmethyl (CPU-20), 1-naphthoxymethyl (CPU-54) or 2-[2-(6-methoxyl) naphthyl] propyl group (CPU-23). Both naphthylmethyl and naphthoxymethyl substitutions resulted in a marked decrease (> 10 fold) in inhibitory potency while the 2-[2-(6-methoxy) naphthyl]-propyl substitution slightly improved activity. On the other hand, changing the N-alkyl substituent (R1) from a 2-(1-piperidine)-acetyl group to a 2-(1-pyrrolidine)-acetyl group (compare CPU-54 with CPU-21 and CPU-23 with CPU-50), or a 2-(4-morpholino)-acetyl group (compare CPU-54 with CPU-22), or a 2-(diethylamino)acetyl group (compare CPU-23 with CPU-57), caused no loss of binding affinity. The presence of a carbonyl function in the N-alkyl substituents appears to be critical for their interaction with the dihydropyridine binding site as its absence in the corresponding 1-piperidine-ethyl (compare CPU-23 with CPU-56) and 1-pyrrolidine-ethyl (compare CPU-20 with CPU-55) analogues reduced affinity dramatically.

CPU-23 and CPU-57 were the most potent tetrahydroisoquinoline analogues identified in this series with IC_{50}s (concentration of drug whch inhibited 50% of specific [^{3}H]-nitrendipine binding) of 0.51 ± 0.08 and 0.41 ± 0.01 μM (mean ± s.e.mean), respectively. When the displacement curves were analysed with the Hill plot, the pseudo-Hill coefficients were found to be close to unity, suggesting that

they may act as simple competitive inhibitors at the nitrendipine binding site. To evaluate further the nature of their interaction with the dihydropyridine site, we analysed the effects of various concentrations (10^{-7}–10^{-6} M) of CPU-23 and CPU-57 on the K_d (equilibrium dissociation constant) and B_{max} (maximum number of binding sites) of [^{3}H]-nitren-

Table 1 Structural formula of isoquinolines and their IC_{50} in inhibiting [^{3}H]-nitrendipine binding to rat cerebral cortex membranes



Compound	R_1	R_2	IC_{50}, μM (n)
CPU-20			10.03 ± 1.31 (3)
CPU-21			4.63 ± 1.01 (3)
CPU-22			6.69 ± 0.74 (4)
CPU-23			0.51 ± 0.08 (4)
CPU-50			0.76 ± 0.16 (3)
CPU-53			>60
CPU-54			9.41 ± 1.96 (3)
CPU-55			>200
CPU-56			>200
CPU-57			0.41 ± 0.01 (3)
CPU-58			27.59 ± 12.66 (3)
CPU-72			>700
CPU-73			>700
CPU-79			>500
CPU-80			0.71 ± 0.19 (4)

dipine binding by saturation analysis. As illustrated in Tables 2 and 3, both CPU-23 and CPU-57 behaved as a simple competitive inhibitor like nifedipine and significantly reduced the apparent affinity without affecting the B_{max} of [^{3}H]-nitrendipine binding. When the data for CPU-23 in Table 2 were further analysed by the Arunlakshana & Schild (1959) plot, i.e. log (dose ratio − 1) versus log (molar concentration of CPU-23), where dose-ratio is the ratio of the K_d value measured in the presence of each concentration of CPU-23 to that measured in its absence; a slope close to unity (0.94) and a K_B value of 1.67×10^{-7} M was obtained. This K_B value agrees quite well with its K_i value (2.6×10^{-7} M) calculated from the drug displacement experiments by the Cheng-Prusoff equation: $K_i = IC_{50}/(1 + [D^*]/K_d)$, where [D*] is the concentration of [^{3}H]-nitrendipine used in the drug displacement assay.

Effects of drugs on high KCl-induced contraction of rat aortic strips

Prototypic calcium entry blockers, including nifedipine, verapamil and diltiazem, produced concentration-dependent inhibition of the contraction of rat aorta induced by 80 mM KCl (Figure 1). Substituted tetrahydroisoquinolines which were active in inhibiting specific [^{3}H]-nitrendipine binding to rat cerebral cortical membranes, were also able to inhibit the KCl-induced contraction of rat aorta (Figure 1, Table 4). The rank order of potencies for nifedipine and tetrahydroisoquinolines in both assays was the same: nifedipine > CPU-57 ≥ CPU-23 > CPU-50 > CPU-21. Indeed, a good correlation ($r = 0.99$, $P < 0.001$) exists between their potencies in inhibiting specific [^{3}H]-nitrendipine binding and KCl-induced contraction of rat aorta (Figure 2).

Effects of CPU-23 and nifedipine on blood pressure and heart rate in Sprague-Dawley rats

The time course of the cardiovascular response of pentobarbitone anaesthetized Sprague-Dawley (SD) rats to CPU-23 is shown in Figure 3 a and b. Acute administration of CPU-23 (1–10 mg kg^{-1}, i.v.) caused a rapid-onset and dose-dependent decrease in mean arterial blood pressure (MAP) and heart rate (HR). The hypotensive and bradycardic effects were rather short-lived and returned towards baseline levels within 10–12 min after drug administration at the lowest

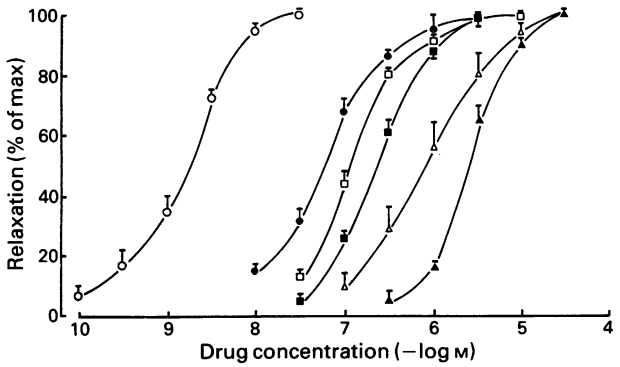


Figure 1 Concentration-dependent relaxation of 80 mM KCl-induced contraction of rat isolated aortic strips by nifedipine (○), verapamil (●), diltiazem (□), CPU-23 (■), tetrandrine (△) and CPU-21 (▲). Each point is the mean of 6 separate experiments with s.e.mean shown by vertical bars.

dose (1 mg kg^{-1}). A more pronounced and longer lasting response was observed at the higher doses (3–10 mg kg^{-1}) and both MAP and HR remained significantly depressed even at 60 min after drug administration. The maximal response was a 45% and a 40% decrease in MAP and HR, respectively. Nifedipine, at a dose of 0.3 mg kg^{-1} (i.v.) produced a comparable decrease in MAP (maximal response was a 35% decrease) as that of 10 mg kg^{-1} CPU-23 (Figure 4). However, unlike CPU-23, nifedipine did not induce bradycardia during the first 30 min after drug administration but rather caused a significant reflex tachycardia from 30–60 min after drug administration.

Effects of CPU-23 on MAP and HR of spontaneously hypertensive (SHR) and normotensive (WKY) rats

The time courses of cardiovascular responses to CPU-23 in pentobarbitone-anaesthetized spontaneously hypertensive (SHR) and normotensive WKY rats are shown in Figure 5a,b and Figure 6a,b, respectively. Similar to the observations in Sprague-Dawley rats, intravenous bolus injection of CPU-23 produced a dose-dependent hypotensive and

Table 2 Scatchard analysis of the interaction of CPU-23 with the binding of [^{3}H]-nitrendipine to rat cerebral cortical membranes

Conc. (M)	K_D (nM)	B_{max} (fmol mg^{-1} protein)	*Hill coefficient* (n_H)
0	0.31 ± 0.03 (6)	18.23 ± 1.15 (6)	0.81 ± 0.04 (6)
10^{-7}	0.48 ± 0.07 (4)*	16.49 ± 1.45 (4)	0.83 ± 0.04 (4)
3×10^{-7}	1.15 ± 0.09 (3)**	20.11 ± 2.74 (3)	0.75 ± 0.09 (3)
10^{-6}	1.81 ± 0.20 (3)**	15.47 ± 1.79 (3)	1.00 ± 0.05 (3)

[^{3}H]-nitrendipine (0.06–3 nM) was incubated with rat cerebral cortical membranes in the presence or absence of CPU-23 for 30 min at room temperature and specific binding was measured. K_D and B_{max} were determined by Scatchard analysis with the LIGAND computer programme (Elsevier-BIOSOFT). Data shown are the mean ± s.e.mean of (n) separate experiments performed in duplicate. *$P < 0.05$; **$P < 0.001$ when compared with control.

Table 3 Scatchard analysis of the interaction of CPU-57 with the binding of [^{3}H]-nitrendipine to rat cerebral cortical membranes

Conc. (M)	K_D (nM)	B_{max} (fmol mg^{-1} protein)	*Hill coefficient* (n_H)
0	0.38 ± 0.11 (4)	18.88 ± 1.62 (4)	0.83 ± 0.06 (4)
3×10^{-7}	0.86 ± 0.07 (3)*	16.96 ± 1.96 (3)	0.90 ± 0.02 (3)
10^{-6}	1.64 ± 0.50 (3)**	17.03 ± 1.53 (3)	1.02 ± 0.02 (3)

Please see legend under Table 2 for details.
*$P < 0.01$, **$P < 0.001$ when compared with control.

Table 4 Inhibition of specific [^{3}H]-nitrendipine binding to rat cerebral cortical membranes and 80 mM KCl-induced contraction of rat aortic strips by classical calcium entry blockers and tetrahydroisoquinolines

	IC_{50} (M)	
Drugs	*[^{3}H]-nitrendipine binding*	*Contraction of rat aortic strips*
Nifedipine	$1.1 \pm 0.2 \times 10^{-9}$	$1.2 \pm 0.3 \times 10^{-9}$
Verapamil	$2.1 \pm 0.1 \times 10^{-7}$	$6.3 \pm 0.5 \times 10^{-8}$
Diltiazem	Stimulation*	$1.4 \pm 0.2 \times 10^{-7}$
Tetrandrine	Stimulation*	$7.0 \pm 1.9 \times 10^{-7}$
CPU-21	$4.6 \pm 0.6 \times 10^{-6}$	$2.5 \pm 0.3 \times 10^{-6}$
CPU-23	$5.1 \pm 0.8 \times 10^{-7}$	$2.1 \pm 0.2 \times 10^{-7}$
CPU-50	$7.6 \pm 1.0 \times 10^{-7}$	$5.0 \pm 0.4 \times 10^{-7}$
CPU-57	$4.1 \pm 0.1 \times 10^{-7}$	$2.5 \pm 0.3 \times 10^{-7}$

IC_{50} is the molar concentration of drug required to give 50% inhibition of specific [^{3}H]-nitrendipine binding or 80 mM KCl-induced contraction of rat aortic strips. Results are the mean ± s.e.mean of 3 (for binding) and 6 (for bioassay) separate experiments. *Diltiazem (10^{-5} M) and tetrandrine (10^{-5} M) enhanced specific [^{3}H]-nitrendipine binding at 37°C to 168 ± 8% and 263 ± 29% of control, respectively. The same concentration of diltiazem did not stimulate [^{3}H]-nitrendipine binding at 22°C (103 ± 3%) while tetrandrine was still able to enhance binding to 154 ± 6% of control.

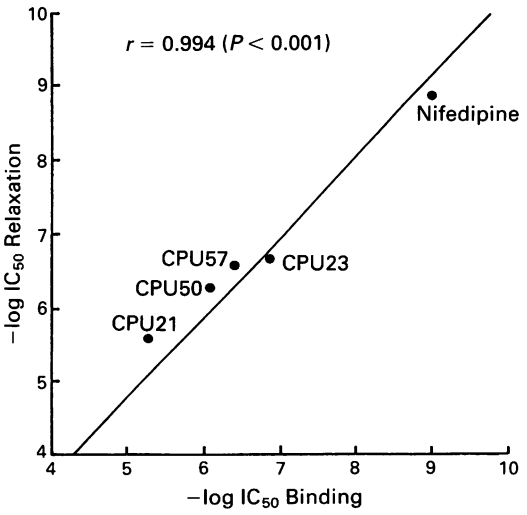


Figure 2 Correlation between antagonism of 80 mM KCl-induced contraction of rat isolated aortic strips and inhibition of specific [^{3}H]-nitrendipine binding by nifedipine and tetrahydroisoquinolines. The correlation coefficient ($r = 0.99$, $P < 0.001$) was calculated by linear regression analysis.

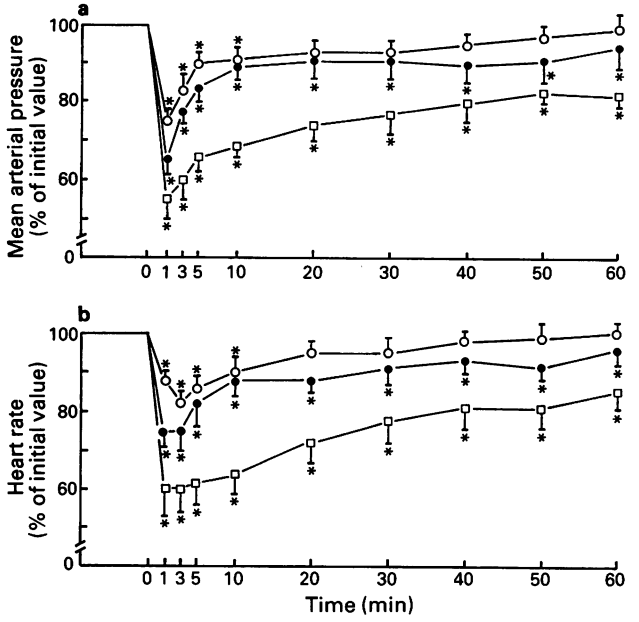


Figure 3 Effects of CPU-23 on (a) mean arterial blood pressure (MAP) and (b) heart rate in pentobarbitone anaesthetized Sprague-Dawley rats. The drug (1 mg kg^{-1} (○), 3 mg kg^{-1} (●) and 10 mg kg^{-1} (□)) was administered at 0 min. Each point represents the mean of 5 animals; vertical bars show s.e.mean. *$P < 0.05$ when compared with control at zero time. Baseline MAP = 105 ± 5 mmHg, HR = 356 ± 12 beats min^{-1}.

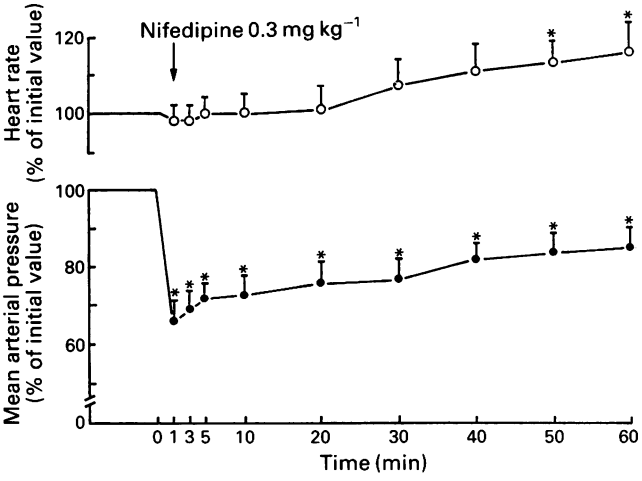


Figure 4 Effects of nifedipine (0.3 mg kg^{-1}, i.v.) on mean arterial blood pressure (MAP) and heart rate in pentobarbitone-anaesthetized Sprague-Dawley rats. The drug was administered at 0 min. Each point represents the mean of 5 animals vertical bars show s.e.mean; *$P < 0.05$ when compared with control at zero time. Baseline MAP = 98 ± 6 mmHg, HR = 343 ± 14 beats min^{-1}.

bradycardic response in both SHR and WKY rats. The magnitude of the bradycardia induced by CPU-23 was similar in SHR and WKY, although it was longer-lasting in the latter species (compare Figures 5b and 6b). The hypotensive effect of CPU-23, on the other hand, was similar in both extent and duration in both species (compare Figures 5a and 6a).

Discussion

The principal finding of this study is that by suitably modifying the reductive cleavage products of tetrandrine we have obtained novel tetrahydroisoquinoline analogues (exemplified by CPU-23 and CPU-57) which may be classified as calcium entry blockers on the basis of radioligand binding and *in vitro* as well as *in vivo* biological assays. These tetrahydroisoquinolines, unlike tetrandrine which interacted with the diltiazem site, apparently behaved as competitive ligands at the dihydropyridine site of the L-type calcium channel. Thus, in Scatchard analyses, they inhibited rather than stimulated [^{3}H]-nitrendipine binding to rat cerebral cortical membranes. Future experiments to examine the effect of these compounds on the dissociation kinetics of [^{3}H]-nitrendipine will provide a more conclusive answer to the competitive nature of this

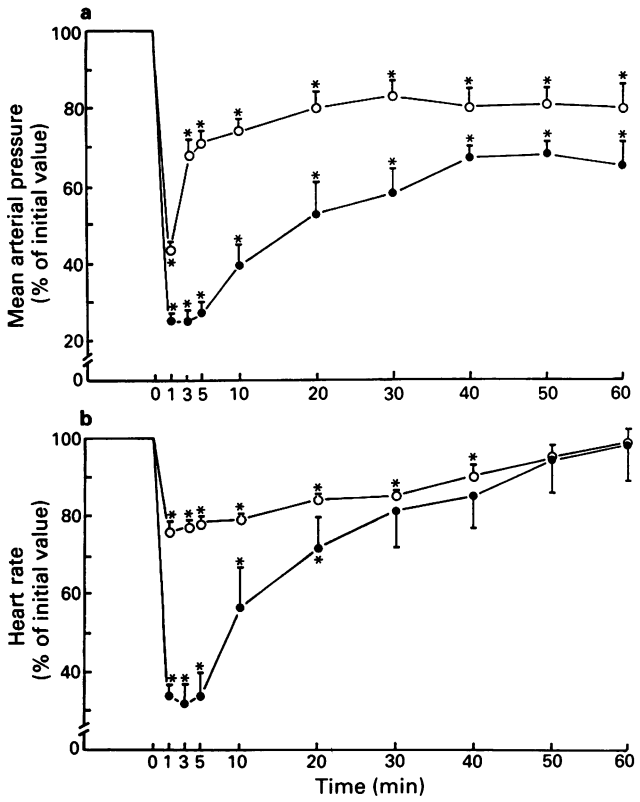


Figure 5 Effects of CPU-23 on (a) mean arterial blood pressure (MAP) and (b) heart rate in pentobarbitone-anaesthetized spontaneously hypertensive rats (SHR). The drug (3 mg kg^{-1} (○) and 10 mg kg^{-1} (●)) was administered at 0 min. Each point represents the mean of 13 animals; vertical bars show s.e.mean. *$P<0.05$ when compared with control at zero time. Baseline MAP = 172 ± 6 mmHg, HR = 388 ± 7 beats min^{-1}.

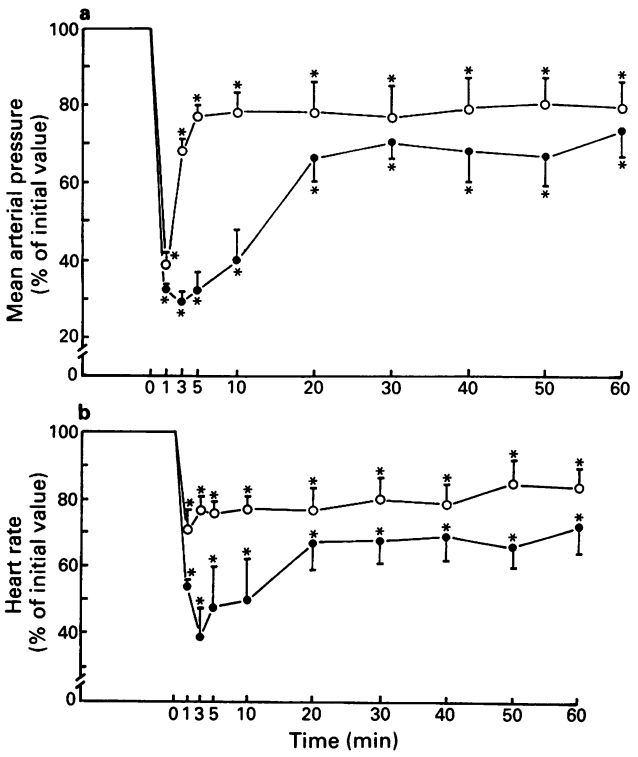


Figure 6 Effects of CPU-23 on (a) mean arterial blood pressure (MAP) and (b) heart rate in pentobarbitone-anaesthetized normotensive WKY rats. The drug (3 mg kg^{-1} (○) and 10 mg kg^{-1} (●)) was administered at 0 min. Each point represents the mean of 11 animals; vertical bars show s.e.mean. *$P<0.05$ when compared with control at zero time. Baseline MAP = 116 ± 6 mmHg, HR = 322 ± 17 beats min^{-1}.

interaction. Moreover, there is a significant correlation between the ability of tetrahydroisoquinolines in inhibiting specific [^{3}H]-nitrendipine binding and KCl-induced contraction of rat aorta ($r = 0.99$, $P<0.001$), further supporting the notion that they may relax the aorta by blocking the L-type calcium channel through interaction with the dihydropyridine site.

From the relative potencies of the substituted tetrahydroisoquinolines in inhibiting [^{3}H]-nitrendipine binding, several structure-activity features can be deduced. Firstly, it was clear that the N-alkyl substituent (R_1) can be changed from a 2- (1-piperidine) -acetyl to 2- (1-pyrrolidine) -acetyl, 2-(4-morpholino)-acetyl or 2-(diethylamino)-acetyl group with little or no effect on their binding affinity at the dihydropyridine site. Secondly, it was evident that a carbonyl function in the N-alkyl substituent is a critical requirement for activity since the corresponding analogues without a carbonyl function were unable to displace [^{3}H]-nitrendipine binding. However, in spite of its importance, the mere presence of a carbonyl function is insufficient by itself for receptor activity as the acetyl- propionyl- and nicotinoyl- analogues were also inactive. Thirdly, we have demonstrated that one can change the R_2 substituent from a 2-[4-(2-methylpropyl) phenyl]-propyl group to a 2-[2-(6-methoxy)naphthyl]-propyl group with no loss in receptor binding activity. However, the introduction of either 1-naphthylmethyl or 1-napthoxymethyl group into this position dramatically reduced receptor affinity.

On the basis of the structure-activity data obtained, we chose one of the most potent compounds, CPU-23 (1-{1-[(6-methoxy) -naphth-2-yl] } -propyl-2- (1-piperidine) -acetyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline), as a representative for further cardiovascular studies *in vivo*. CPU-23 was about 30 times less active than nifedipine in lowering blood pressure in Sprague-Dawley rats. However, it was over 500 times less active than nifedipine in inhibiting [^{3}H]-nitrendipine binding *in vitro*, suggesting that CPU-23 may have a better bioavailability than nifedipine *in vivo* or it may induce hypotension via other modes of action. Further experments to compare the pharmacokinetic behaviour of tetrahydroisoquinolines and dihydropyridines will be needed to differentiate these possibilities. CPU-23 also differed from nifedipine in its effect on the heart rate. At doses which caused a similar degree of hypotension, CPU-23 exerted a pronounced negative chronotropic action whereas nifedipine induced a reflex tachycardia. Most 1,4-dihydropyridine calcium entry blockers can cause a reflex sympathetic tachycardia, and do not cause a marked decrease in heart rate *in vivo* presumably because of their relatively weak activity on the heart (Ohtsuka *et al.*, 1989; but see Spedding *et al.*, 1987). On the other hand, diltiazem and tetrandrine which are more cardioselective than the dihydropyridines have been reported to produce either no change or a mild bradycardic effect at hypotensive doses in animals and man (Kinoshita *et al.*, 1979; Qian *et al.*, 1983; Kawashima *et al.*, 1990). The marked bradycardia produced by CPU-23 may provide insight on the tissue selectivity of calcium entry blockers and offer some myocardial protection in treating hypertensive conditions accompanying with high circulatory concentration of catecholamines (Todd *et al.*, 1986).

Since calcium entry blockers have been reported to exert a stronger inhibition of the noradrenaline-induced contraction of rat arterial muscle in essential hypertensive man and spontaneously hypertensive rats than in normotensive man and rats (Aoki *et al.*, 1982), we have also compared the antihypertensive effect of CPU-23 in normotensive WKY rats

and spontaneously hypertensive rats (SHR). However, we found no significant difference in the hypotension and bradycardia in response to CPU-23 in SHR and WKY rats.

In summary, we have examined the structure-activity relationship of a series of N-alkyl substituted tetrahydroisoquinolines in their inhibition of [^{3}H]-nitrendipine binding and KCl-induced contraction of rat aorta in the present study. One of the most potent compounds, CPU-23, has been studied in some detail. The results indicate that CPU-23 may relax KCl-induced contraction of rat vascular smooth muscle by blocking the L-type calcium channel through an interaction with the dihydropyridine site. However, unlike nifedipine, the prototypic dihydropyridine calcium entry blocker, CPU-23 lowered both blood pressure and heart rate in pentobarbitone-anaesthetized Sprague-Dawley, spontaneously hypertensive and normotensive WKY rats.

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Erratum

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I.S. de la Lande. Evidence for a 5-HT_1-like receptor mediating the amplifying action of 5-HT in the rabbit ear artery.

During preparation of the above article for printing, a transposition occurred such that the pages which appeared as pp. 551–554 were an incorrect sequence of pages, although their pagination ran consecutively. The complete article is reproduced in its correct form on the following pages.

The publishers accept full responsibility for the error and offer their sincere apologies to the author.

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