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Interaction of 1-methyl-4-phenylpyridinium ion and tyramine with a site putatively involved in the striatal vesicular release of dopamine

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The neurotoxin MPP^+ potently inhibited the striatal binding of [^{3}H]-tyramine, a putative marker for the vesicular transporter of dopamine, and provoked a massive *in vivo* release of striatal dopamine. Tetrabenazine, an established ligand for the vesicular catecholamine carrier, potently inhibited [^{3}H]-tyramine binding, tyramine-provoked striatal efflux of dopamine and the fast component of MPP^+-induced dopamine release. It is concluded that MPP^+ in the striatum, besides interacting with additional intracellular targets, avidly binds at a vesicular site functionally involved with the outward transport of dopamine.

Keywords: 1-methyl-4-phenylpyridinium ion (MPP^+); [^{3}H]-tyramine binding; vesicular markers; dopamine release; striatal microdialysis

Introduction MPP^+ (1-methyl-4-phenylpyridinium ion), the oxidative metabolite of the Parkinsonian neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) accumulates in dopaminergic neurones via the cell-surface dopamine transporter (Javitch *et al.*, 1985). There has previously been no evidence that synaptic vesicles are a target for intraneuronal MPP^+, though this might be expected, in view of the purported similarity of catecholamine neurones and adrenomedullary chromaffin cells where, in fact, MPP^+ is extensively transported and stored (Daniels & Reinhard, 1988). Tyramine has been shown to associate mostly in the rat striatum with a reserpine-sensitive site putatively involved with the vesicular transport of dopamine (Vaccari, 1986; Vaccari & Gessa, 1989). Both tyramine and MPP^+ infused into the caudate nucleus via microdialysis procedures cause a massive release of dopamine from nigrostriatal terminals (Rollema *et al.*, 1988; Fairbrother *et al.*, 1990). We have here ascertained that MPP^+ potently affects the [^{3}H]-tyramine binding process in striatal homogenates, and that both MPP^+- and tyramine-evoked, *in vivo* striatal release of dopamine, are sensitive to the inhibitory action of a vesicular ligand.

Methods Male, Sprague-Dawley rats (200–250 g) were used for binding and microdialysis experiments.

[^{3}H]-tyramine binding to striatal homogenates Tissue preparation and the binding assay for [^{3}H]-tyramine were performed as described previously (Vaccari, 1986). Binding parameters were calculated with the EBDA-LIGAND programme.

In vivo *release of dopamine from the striatum* Rats were implanted with the microdialysis fibre (Hospal AN69 HF, France) transversely through the limbic region of the striatum (ventral striatum, coordinates A: 1.7; V: 6.5 from bregma) under chloral hydrate (400 mg kg^{-1}, i.p.) anaesthesia. Microdialysis was performed as detailed elsewhere (Carboni *et al.*, 1990) on conscious, freely moving animals 24 h after surgery. After a stable release of dopamine was attained, drugs were added to the perfusing, modified Krebs buffer, pH 7.2 (composition, mM: NaCl 147, KCl 3.0, $CaCl_2$ 2.2, $MgSO_4$ 1.2, KH_2PO_4 0.4).

Materials Drugs used were [^{3}H]-tyramine (NEN-Dupont, Boston, U.S.A.), MPP^+ and MPTP (RBI, Wayland, U.S.A.), *p*-tyramine (Sigma Chem., St. Louis, U.S.A.), tetrabenazine (Fluka, Buchs, Switzerland), debrisoquine (Zambon, Milano, Italy). Concentrated solutions of all drugs were prepared immediately before use in the appropriate buffers, or 50 µl of acetic acid (tetrabenazine), and then further diluted in buffer.

Results *Influence of MPP^+ on the [^{3}H]-tyramine binding process* MPP^+ and tetrabenazine, a known marker of the dopamine vesicular transporter (Henry & Scherman, 1989) potently inhibited the specific binding of [^{3}H]-tyramine, with K_i values in the low nanomolar range (Table 1). MPTP and debrisoquine, an inhibitor of MPP^+ binding to monoamine oxidase-A (MAO-A) sites in the mouse brain (Del Zompo *et al.*, 1990), displaced bound [^{3}H]-tyramine at micromolar concentrations, while cocaine, a classical marker for the plasma membrane catecholamine transporter, was a very poor competitor. The Hill coefficients for tyramine, MPP^+ and tetrabenazine differed from unity (Table 1).

In vivo *striatal release of dopamine* The perfusion of tyramine or MPP^+ through the dialysis probe induced a concentration-dependent efflux of striatal dopamine (Figure 1a and 1b). The presence of tetrabenazine 10^{-5} M in the perfusing medium 60 min beforehand, potently inhibited tyramine-provoked (2×10^{-5} M) dopamine release (Figure 1c), while tetrabenazine 10^{-4} M only partially inhibited the MPP^+-induced (5×10^{-5} M) dopamine efflux (Figure 1d). Lower concentrations of tetrabenazine were ineffective (not shown). A larger concentration of MPP^+ (1 mM) was then per-

Table 1 Inhibition of specific [^{3}H]-tyramine binding to rat striatal homogenates by 1-methyl-4-phenylpyridinium ion (MPP^+) and related drugs

Drug	K_i(nM)	*Hill n*	n
p-Tyramine	13.5 ± 2.1	0.81	5
MPP^+	34.6 ± 3.0	0.56	5
Tetrabenazine	30.9 ± 4.5	0.38	5
MPTP	4217 ± 566	0.70	5
Debrisoquine	4013 ± 591	0.72	4
Cocaine	>100,000	—	2

Triplicate membrane aliquots (approx. 100 µg protein) were incubated for 10 min at 37°C with 4 nM [^{3}H]-tyramine in the absence or presence of 10 concentrations of competing drugs. Dopamine 10 µM was used for measurement of non specific binding. A K_D value of 10 nM was used in the EBDA-LIGAND calculation of K_i coefficients. Values are means ± s.e.mean of *n* experiments.

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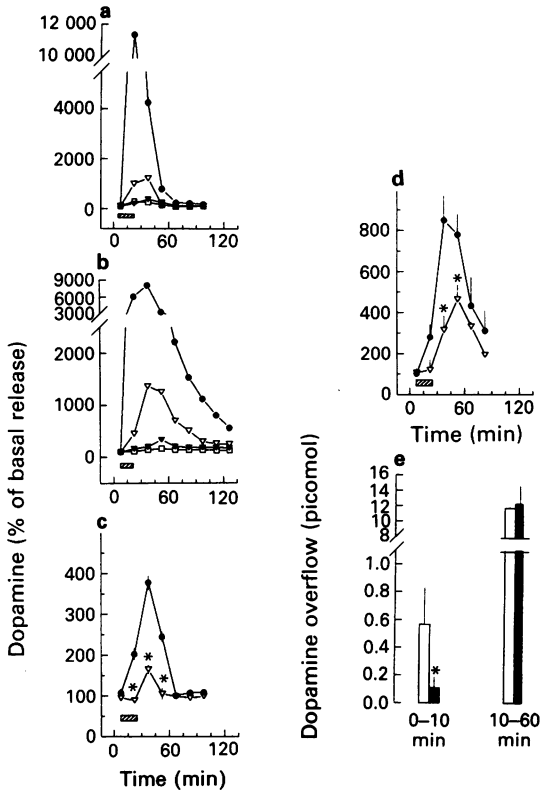


Figure 1 (a and b) Effect of the intra-striatal perfusion of tyramine (a) and 1-methyl-4-phenylpyridinium ion (MPP^+) (b) on *in vivo* release of dopamine. Drugs were added to the perfusing medium at the following concentrations (M): 10^{-6} (□), 10^{-5} (▼), 10^{-4} (▽), 10^{-2} (●), and perfused for 15 min (hatched bar). (c and d) Inhibition of tyramine (c) and MPP^+ (d)-induced dopamine efflux (●), by intrastriatal perfusion of tetrabenazine (▽) 10^{-5} M and 10^{-4} M, respectively. Tyramine (2×10^{-5} M) or MPP^+ (5×10^{-5} M) were added to the perfusing medium 60 min after tetrabenazine, and perfused during 15 min (hatched bar). (e) Effect of the intrastriatal perfusion of tetrabenazine (solid columns) on the absolute striatal overflow of dopamine induced by MPP^+ 1 mM *in vivo*; control, open columns. MPP^+ was added to the medium 60 min after tetrabenazine (10^{-4} M), and perfused for 5 min. Columns represent the total efflux of dopamine released in the first 5-min samples (left) and the last 10 samples (right), respectively. Each point is the mean of 3–6 experiments; vertical bars show s.e.mean. * $P < 0.05$, ANOVA, followed by Student-Newman-Keuls test, where appropriate.

fused for 5 min, and extracellular dopamine was measured with a higher time-resolution (5 min samples), in order to assess whether the weaker inhibition by tetrabenazine was due to a differential effect on the two components of MPP^+-evoked dopamine efflux (Rollema *et al.*, 1988). Tetrabenazine 10^{-4} M significantly inhibited the MPP^+-induced dopamine efflux collected in the first two 5 min fractions (fast component), and did not significantly affect the total dopamine efflux measured from 10 min up to 1 h after MPP^+ infusion (Figure 1e).

Discussion Our results show that the neurotoxin MPP^+ is equipotent with the vesicular catecholamine transporter ligand tetrabenazine (Henry & Scherman, 1989) in displacing specifically bound [^{3}H]-tyramine in striatal homogenates, with K_i values approx. 2.5 fold higher than those of *p*-tyramine itself (Table 1). The failure of cocaine, a recognized blocker of the neuronal uptake of catecholamines, to inhibit [^{3}H]-tyramine binding further supports the purported attachment of tyramine at the vesicular transport system for dopamine (Vaccari, 1986; Vaccari & Gessa, 1989), thus implying that intraneuronal MPP^+ may label a vesicular site in dopaminergic regions of the brain. Of course, MPP^+ may reach additional intraneuronal targets, as inferred from Hill coefficients different from unity. Debrisoquine, an inhibitor of [^{3}H]-MPP^+ binding at MAO-A (Del Zompo *et al.*, 1990) had only micromolar affinity for tyramine sites. Consistent with the need for MPTP to be metabolized into MPP^+ in order to exert neurotoxicity at target nerve endings, MPTP was a poor inhibitor of tyramine binding.

These findings recall the reported storage of MPP^+ in adrenergic granules of bovine adrenomedullary chromaffin cells via a process involving the vesicle catecholamine transporter (Daniels & Reinhard, 1988). Previously, however, the relative insensitivity of the adrenal gland to MPTP toxicity (Reinhard *et al.*, 1987), compared with the high vulnerability of basal ganglia, obscured the probability that striatal and adrenal vesicles could similarly bind MPP^+.

Both MPP^+ and tyramine, infused into the caudate nucleus via a microdialysis procedure, provoked a concentration-dependent release of dopamine from the striatum, thus confirming previous observations (Rollema *et al.*, 1988; Fairbrother *et al.*, 1990). The monophasic, tyramine-induced efflux of dopamine, predominantly vesicular in nature (Fairbrother *et al.*, 1990), was potently inhibited by previous intrastriatal perfusion of tetrabenazine. Tetrabenazine, however, could only inhibit the initial, fast component of MPP^+-induced dopamine efflux, while leaving unchanged the later sustained, long-lasting release of dopamine, a putative toxic response to MPP^+ (Rollema *et al.*, 1988).

In conclusion, the tetrabenazine-sensitive, fast component of MPP^+-provoked dopamine release might well reflect the attachment of the toxin at a site to which tyramine also binds with high affinity, and which is functionally involved in triggering the vesicular release of dopamine.

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Changes in brain striatum dopamine and acetylcholine receptors induced by chronic CDP-choline treatment of aging mice

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1 Spiroperidol binding (dopamine D_2 receptors) and quinuclidinyl benzilate binding (muscarinic receptors) in striata of 19-month old mice was analyzed for animals that had received chronic administration of cytidine 5'-diphosphocholine (CDP-choline) incorporated into the chow consumed (100 or 500 mg kg^{-1} added per day) for the 7 months before they were killed.

2 Treated animals displayed an increase in the dopamine receptor densities of 11% for those receiving 100 mg kg^{-1} and 18% for those receiving 500 mg kg^{-1} as compared to the control aged animals that had received no CDP-choline. Control animals showed, from 2 months to 19 months of life, a 28% decrease in the receptor density. No change in the affinity of the receptors for spiroperidol was found in the treated or untreated animals.

3 Muscarinic acetylcholine receptor densities were also partially recovered by the same treatment in aged animals that showed a 14% decrease of these receptors in this case. The muscarinic receptor density increased 6% for the animals that received 100 mg kg^{-1} and 17% for the animals that received 500 mg kg^{-1} without any change in the affinity of the receptor for quinuclidinyl benzilate.

4 Aged animals displayed a slight increase in brain membrane fluidity as indicated by a decrease in the polarization value of the non-polar fluorophore 1,6-diphenyl-1,3,5-hexatriene. Interestingly, in the treated animals a greater increase in membrane fluidity was determined and found to be very similar for the two doses.

5 It is concluded that chronic administration of CDP-choline to aged animals promoted a partial recovery of the striatum dopamine and acetylcholine receptor function normally reduced with aging, which might be explicable in terms of mechanisms involving fluidity of the brain neuronal membrane.

Keywords: Muscarinic receptor; dopamine D_2 receptor; aging; striatum; choline donor; CDP-choline (cytidine 5'-diphosphocholine); membrane fluidity

Introduction

Cytidine 5'-diphosphocholine (CDP-choline) has been widely used for the treatment of different diseases of the central nervous system (Manaka *et al.*, 1974; Salvadorini *et al.*, 1975; Cohadon *et al.*, 1982; Lecuire & Duplay, 1982). Membrane diffusion of this compound, after intravenous or oral administration, requires splitting of the molecule into CMP and phosphocholine, which are later metabolized to cytidine and choline (Yashima *et al.*, 1975; Lopez-G. Coviella *et al.*, 1987). These two major circulating products are then incorporated into their respective cellular pools and their increased concentrations would account for the pharmacological properties of CDP-choline.

Choline, as phosphocholine, is a precursor in the synthesis of phosphatidylcholine (PC), and cytidine, as CTP, is also an intermediary in the synthesis of PC and other membrane phospholipids. Administration of CDP-choline reportedly affects brain phospholipid biosynthesis (Dorman *et al.*, 1983), membrane structure (Horrocks *et al.*, 1981) and, both *in vitro* and *in vivo*, the metabolism and release of certain brain and peripheral neurotransmitters (Martinet *et al.*, 1981; Agut *et al.*, 1984; Lopez-G. Coviella *et al.*, 1986).

The question of how neurotransmitter binding can be affected by this treatment seemed of primary importance in understanding the mechanisms of the pharmacological action of CDP-choline. Some reported therapeutic effects of choline donors on several diseases that go together with aging such as Parkinson's disease (Agnoli *et al.*, 1982), Huntington's chorea (Growdon *et al.*, 1977) or Alzheimer's disease (Etienne *et al.*, 1979), focused our attention on the neurotransmitters dopamine and acetylcholine as good markers of possible neurotransmission changes in these cases. Several authors have reported that the density of either dopamine receptors (Marquis *et al.*, 1981; Morgan *et al.*, 1987) or muscarinic cholinoceptors (Biegon *et al.*, 1988) decreases with aging of animals without any change in affinity. Morgan *et al.* (1987) have found that the decrease in dopamine receptor density is mostly found in the D_2 type, which led us to analyze a D_2 ligand such as spiroperidol. Furthermore, in man, Seeman *et al.* (1987) have reported a decrease of D_1 and D_2 dopamine receptors of 3.2% and 2.2% per decade respectively.

It has been shown that the phospholipid content of various regions of the human brain diminishes with age and that the cholesterol content displays, in these same samples, an even more dramatic decrease that results in a decrease in the cholesterol/phospholipid ratio (Söderberg *et al.*, 1990). In another context, measurements of phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine have been shown to increase by 15% to 20% in membrane preparations of aging mice treated with CDP-choline (Lopez-G. Coviella *et al.*, 1988). These changes in the lipid composition of the membrane may cause modifications in the membrane fluidity according to descriptions of several authors, showing that methylation of membrane phospholipids affect the membrane fluidity (Mio *et al.*, 1984).

In this context it seemed of interest to determine in aging animals the effect of the administration of CDP-choline on the possible modulation of the number of receptors as well as on their brain membrane fluidity.

Methods

Animals

Female CD-1 mice were housed at constant room temperature (21°C), relative humidity (60%) and with 12:12 h light:dark cycle, with free access to food and water. At 12 months of age animals were assigned randomly to one of three

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dietary groups and fed either regular (0 mg kg^{-1} day^{-1}), or CDP-choline supplemented 100 mg or 500 mg kg^{-1} day^{-1}) chow for another 7 months. From the average animal weight and food intake, amounts of CDP-choline to be added to the diet were calculated to adjust a mean dose and the adjustment was repeated every two weeks. A group of young animals consisting of untreated two month-old mice kept under the same conditions were used for comparison. The group of aged animals fed with the regular diet was used as control animals.

Tissue preparation

Mice were decapitated, and the corpus striatum was immediately removed and immersed in 50 mM Tris-HCl buffer (pH 7.1) containing (mM): NaCl 120, KCl 5, Ca_2Cl 2, $MgCl_2$ 1 and pargyline 10 μM. The striatum of each mouse was homogenized in 2 ml of buffer for 15 s with a Polytron (PCU Kinematica Model GmbH) at setting 7. The homogenate was then centrifuged at 27,000 g for 20 min and the pellet resuspended in the same volume of buffer, and centrifuged twice more. The final pellet was resuspended in 2.5 ml of the same buffer.

When the preparation was used for the measurement of membrane fluidity, cerebral hemispheres were homogenized in 2 ml of 10 mM Tris-HCl (pH 7.4) and the homogenate centrifuged at 12,000 g for 30 min. The pellet was discarded and the supernatant centrifuged at 48,000 g for 30 min. The pellet, resuspended in the homogenizing buffer, was used as plasma membrane fraction for use in fluidity measurements.

Binding assay

The D_2 receptor densities were measured by saturating the tissue with increasing concentrations of [^{3}H]-spiroperidol. The binding of [^{3}H]-spiroperidol was determined by a modification of the assay described by Leysen *et al.* (1978). Membranes from the striatum of the mice were added to test tubes containing [^{3}H]-spiroperidol. Chlorpromazine at 10 μM concentration was used to define specific binding. Concentration kinetics of the binding were performed at concentrations of [^{3}H]-spiroperidol ranging from 24 pM to 820 pM.

Muscarinic cholinoceptor densities were measured by saturating the tissue with increasing concentrations of [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB). The binding of [^{3}H]-QNB was measured by a modification of the assay described by Baron & Siegel (1989). The test tubes contained [^{3}H]-QNB in Tris-buffered Krebs (pH 7.4) containing (mM): NaCl 118, KCl 5, $MgCl_2$ 2, $CaCl_2$ 1.9, Tris 25 and glucose 10. Nonspecific binding was defined as that remaining in the presence of 10 μM atropine. Concentration kinetics were performed between 75 pM and 3026 pM of [^{3}H]-QNB.

Binding assays were carried out in duplicate at 25°C for 40 min and membrane-bound ligand was collected *in vacuo* onto GF/B filter strips with a Brandel Cell Harvester, followed by three washes of 5 ml of cold buffer. Radioactivity in each filter was determined after 24 h in 5 ml of scintillation fluid consisting of 0.8% 2,5-diphenyl oxazole in toluene at a counting efficiency of 35–40%.

Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin used as standard.

Fluorescence polarization

Fluorescence polarization to estimate membrane fluidity was performed as described by Wilson *et al.* (1988) except that crude membrane preparations contained 80 μg of protein that were diluted to 2 ml of the original homogenizing buffer were used. This suspension was added to a test tube with the non-polar fluorophore, 1,6-diphenyl-*trans*-1-3,5-hexatriene (DPH) at a DPH to lipid molecular ratio of 1:400.

Statistical analysis

Saturation binding data were obtained by Scatchard plot. The data are reported as arithmetic means ± s.e.mean. Significance tests were performed by Student's t test. Differences between means were regarded as statistically significant when $P < 0.02$.

Chemicals

[^{3}H]-spiroperidol (72.9 Ci mmol^{-1}) was obtained from New England Nuclear, U.K. and [^{3}H]-QNB (44.3 Ci mmol^{-1}), from Amersham, U.K. Chlorpromazine, pargyline and DPH were from Sigma, St. Louis, MO. U.S.A. Atropine was purchased from Merck, Darmstadt, Gemany and CDP-choline from Ferrer Internacional, S.A. Barcelona, Spain.

Results

Spiroperidol binding

Our reference values of spiroperidol binding of striata showed, as has already been reported (Morgan *et al.*, 1987), clear differences between the groups of young and old animals. In our experimental conditions the receptor density of old animals presented a significant 28% decrease ($P < 0.01$) compared to the value obtained with young animals (Table 1). The K_d displayed a slightly increased value for the old animals as compared to the young ones (non-significant).

The B_{max} values obtained with treated animals always lay between the reference values of the young and old animals. As shown in Table 1, the average value determined for the group of animals treated with 100 mg kg^{-1} of CDP-choline was 11% higher than that of the group of non-treated aged animals. The average value for the group of animals treated with 500 mg kg^{-1} was even higher, with an increase reaching 18% of the average value corresponding to the group of non-treated aged animals. This second value differed significantly ($P < 0.02$) from that of the aged control group. The average values of the K_d for both groups of treated animals were slightly higher than the average values found for both groups of untreated animals (old and young); however, the differences were not statistically significant.

Quinuclidinyl benzilate binding

Our results indicated that the difference in binding between old and young mice is smaller for QNB than for spiroperidol. The decrease, although statistically significant ($P < 0.01$), was in this case a mere 14% (Table 2). The values of K_d determined for QNB, as those found for spiroperidol, did not differ significantly, displaying a slight increase.

The data obtained with the treated aged animals followed the same trends as were found for spiroperidol. Thus the treatment of aged animals with CDP-choline at 100 mg kg^{-1} increased the B_{max} by 6% while treatment with 500 mg kg^{-1} increased the B_{max} by 17%; in this second case with a high statistical significance ($P < 0.02$). The average value of K_d corresponding to either of the two groups of treated animals dis-

Table 1 Effect of chronic administration of cytidine 5'-diphosphocholine (CDP-choline) on spiroperidol binding constants

Animal group	B_{max} (fmol mg^{-1})	K_d (pM)	n
Aged control	306 ± 14.8	49 ± 2.9	20
Aged + 100 mg kg^{-1}	340 ± 17.9	55 ± 3.4	20
Aged + 500 mg kg^{-1}	361 ± 16.8*	53 ± 2.6	16
Young	423 ± 40.7**	43 ± 9.1	5

Results are presented as means ± s.e.mean. Untreated aged animals are taken as control and the differences between means analysed by Student's unpaired test. Statistically significant differences from the control group are shown as * $P < 0.02$ and ** $P < 0.01$.

Table 2 Effect of chronic administration of cytidine 5'-phosphocholine (CDP-choline) on the quinuclidinyl benzilate (QNB) binding constants

Animal group	B_{max} (fmol mg^{-1})	K_d (pM)	n
Aged control	1871 ± 63	299 ± 19	12
Aged + 100 mg kg^{-1}	1988 ± 61	306 ± 25	12
Aged + 500 mg kg^{-1}	2190 ± 70*	286 ± 29	11
Young	2168 ± 66*	332 ± 30	12

Results are presented as means ± s.e.means. Untreated aged animals are taken as control and the differences between means analysed by Student's unpaired test. Statistically significant differences from the control group are shown as * $P < 0.01$.

played no difference when compared to the reference value of the non-treated animals (Table 2).

Membrane fluidity

Studies of brain membrane fluidity were performed by measuring the polarization changes of the non-polar fluorophore DPH used as a probe in our membrane preparations. Membrane fluidity for old animals was shown to be slightly higher than that corresponding to membranes of young animals as indicated by the decrease of 0.55 in the steady state polarization value (Table 3). Chronic administration of CDP-choline clearly diminished the polarization and hence increased the membrane fluidity. The values determined differed significantly from those of the aged control animals regardless of the dose administered, with a change of 1.13 ($P < 0.02$) for the animals treated with 100 mg kg^{-1} of CDP-choline and a change of 1.08 ($P < 0.005$) for the animals treated with 500 mg kg^{-1}. No clear distinction could be established between the two groups of treated animals, which had a very close polarization average value.

Discussion

Phospholipid methylation and PC membrane enrichment has been correlated with increases in β-adrenoceptors by Hirata & Axelrod (1980). Increase in receptor densities by administration of CDP-choline has also been reported for the cholinergic system in young animals (Petkov & Popova, 1987). In this paper we demonstrate that D_2 dopamine receptor densities, as indicated by the spiroperidol binding, which are normally reduced with aging (Seeman *et al.*, 1987; Morgan *et al.*, 1987) are partially recovered by administration of a choline and cytidine donor such as CDP-choline, known to increase the membrane levels of PC (Lopez-G. coviella *et al.*, 1988). Similarly, the decline in muscarinic cholinoceptors also found with aging is clearly blunted by CDP-choline treatment.

In the two cases studied, changes in receptor densities as a consequence of CDP-choline administration seemed not to affect the affinity constant of the receptor for their ligands, which is consistent with the fact that in general, aging induced a decrease in receptor densities without any change in affinity (Pradhan, 1980). Thus, CDP-choline treatment seems to improve the dopamine receptor and also the muscarinic cholinoceptor function through increases in the receptor number.

With the information available to date, it has been difficult to propose a molecular mechanism to explain this phenomenon, especially for the simultaneous effect on both systems. Some authors have tried to assign the parallel modification of dopamine and choline receptors by CDP-choline administration to a specific mechanism of increase in dopamine content of the striatum after treatment, which would cause a decline in the acetylcholine content leading to an increase in the cholinoceptor densities (Petkov & Popova, 1987). The observations described here of spiroperidol and QNB binding modification could also be explained by a less specific effect of the membrane structure on the receptor function, either through receptor availability or through changes in receptor conformation (Loh & Law, 1980; Henis, 1989).

It is well known that in most of the regions of the human brain, including the striatum, the phospholipid content declines with age and that cholesterol declines even more, resulting in an important decrease in the cholesterol to phospholipid ratio (Söderberg *et al.*, 1990). It has also been shown that the decrease in cholesterol content of the membranes enhances their fluidity (Wilson *et al.*, 1988). Therefore, aging would lead to an increase in membrane fluidity, as is indicated by our experiments showing a lower polarization value (higher membrane fluidity) for the old animals compared to the young animals. As CDP-choline administration increases brain phospholipid content (Lopez-G. Coviella *et al.*, 1988), the cholesterol to phospholipid ratio would also decrease with the corresponding membrane fluidity increase (see Table 3). How these changes in membrane structure modify receptor function cannot be unambiguously explained at present. It should be borne in mind that receptor density measurements are a consequence of receptor number and optimal receptor structure and that the interaction of membrane phospholipids and proteins with receptor protein may affect receptor mobility as well as receptor structure.

Chronic treatment of aged mice with CDP-choline recovers the reduced density of dopamine and acetylcholine receptors of the striatum. This beneficial effect of the choline donor might perhaps be related, through more or less complex mechanisms, to an increase in brain membrane fluidity observed in these animals.

Table 3 Effect of chronic administration of cytidine 5'-phosphocholine (CDP-choline) on the fluorescence polarization value of cerebral membranes

Animal group	*Polarization × 100*	n
Aged control	30.63 ± 0.28	17
Aged + 100 mg kg^{-1}	29.50 ± 0.37*	14
Aged + 500 mg kg^{-1}	29.54 ± 0.19**	16
Young	31.18 ± 0.19	17

Results are presented as means ± s.e.mean. Untreated aged animals are taken as control and the differences between means analysed by Student's unpaired test. Statistically significant differences from the control group are shown as * $P < 0.02$ and ** $P < 0.005$.

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Amantadine and sparteine inhibit ATP-regulated K-currents in the insulin-secreting β-cell line, HIT-T15

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1 The effects of pharmacological agents that potentiate insulin release were studied on ATP-regulated K-currents (K-ATP currents) in the insulin-secreting β-cell line HIT-T15 by use of patch-clamp methods.

2 The tricyclic drug, 1-adamantanamine (amantadine), reversibly inhibited both whole-cell currents (with a K_i of 120 μM) and single channel currents in inside-out patches. This effect was principally due to an increase in a long closed state which reduced the channel open probability. The related compound, 1-adamantanol, in which the amino group is substituted by a hydroxyl one, did not inhibit K-ATP currents substantially.

3 The alkaloid, sparteine, reversibly inhibited both whole-cell K-ATP currents (K_i = 171 μM) and single channel currents in inside-out patches.

4 The results suggest that sparteine and amantadine can block the K-ATP channel from either side of the membrane and support the idea that at least part of the stimulatory effect of these agents on insulin secretion results from inhibition of this channel.

Keywords: K-channel; ATP-regulated K-channel; sparteine; amantadine; amantanol; insulin secreting β-cell line (HIT-T15)

Introduction

Glucose metabolism by the pancreatic β-cell causes a slow depolarization of the β-cell membrane which triggers a characteristic pattern of electrical activity and thereby initiates insulin secretion (for reviews see Henquin & Meissner, 1984; Ashcroft & Rorsman, 1989). This slow depolarization is mediated by a decrease in membrane K^+ permeability which results from the inhibition of ATP-regulated K-channels (K-ATP channels) (Ashcroft *et al.*, 1984; Rorsman & Trube, 1985; Misler *et al.*, 1986). It is believed that an increase in the cytosolic ATP/ADP ratio links glucose metabolism to channel inhibition (Cook & Hales, 1984; Ashcroft & Rorsman, 1989). K-ATP channels are also inhibited by sulphonylureas, such as tolbutamide, which are used clinically in the treatment of non-insulin-dependent diabetes mellitus (Sturgess *et al.*, 1985; Trube *et al.*, 1986).

Recently, several other moieties, unrelated to the sulphonylureas, have been found to stimulate insulin release from isolated islets in the absence of glucose, whilst concomitantly reducing resting membrane K^+ permeability (Henquin, 1990). Among these are the tricyclic compound, 1-adamantanamine (amantadine) (Garrino & Henquin, 1987) and the alkaloid sparteine (Paolisso *et al.*, 1985). It seems probable that the effect of these compounds on insulin release is also mediated via inhibition of the K-ATP channel, since this channel contributes most of the resting K-permeability of the β-cell (Ashcroft *et al.*, 1988). Amantadine is of special interest since substitution of its amine group alters its efficacy as an insulin secretagogue; thus, carboxyl substitution (1-adamantanecarboxylic acid) reduces its potency, whilst hydroxyl substitution (1-adamantanol) renders it ineffective (Garrino & Henquin, 1987). Clinically, amantadine is used in parkinsonism and as a viral prophylactic (Parkes, 1974) although its mode of action is unknown. Sparteine has been used as an anti-arrhythmic or oxytocic agent (Goodman & Gilman, 1965).

In the present paper, we use the patch-clamp technique to investigate directly the effect of amantadine, amantanol and sparteine on K-ATP channels in the insulin-secreting cell-line HIT-T15. The results demonstrate that amantadine and sparteine reversibly inhibit the K-ATP channel when applied from either side of the cell membrane, whilst amantanol has little inhibitory effect.

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Methods

Preparation

Experiments were carried out on the hamster clonal β-cell line HIT-T15 which secretes insulin in response to glucose (Ashcroft *et al.*, 1986). Cells were cultured as described previously (Ashcroft *et al.*, 1986), and used for up to 4 days after splitting and replating. All experiments were carried out on single cells.

Data recording and analysis

We used the whole-cell and inside-out configurations of the patch-clamp technique (Hamill *et al.*, 1981). Pipettes were pulled from borosilicate glass (Boralex, Rochester Scientific, U.S.A.), coated with Sylgard (Dow Corning, Seneffe, Belgium) close to their tips and fire-polished immediately before use. They had resistances between 2 and 5 MΩ when filled with intracellular solution. Currents were recorded with a List EPC 5 or EPC 7 patch-clamp amplifier (List Electronic, Darmstadt, F.R.G.) and stored on FM or video tape for later analysis.

We used a pulse protocol similar to that described by Trube *et al.* (1986) to monitor the effects of drugs on the whole-cell K-ATP current: the membrane potential was held at −70 mV and 10 ms pulses of +10 mV or −10 mV were applied alternately every 100 ms. For single channel recordings, the membrane was held at −70 mV: at this potential, K-ATP currents were inward under the ionic conditions of our experiments. The reference potential for all measurements was the zero current potential of the pipette before establishment of the seal.

Whole-cell current amplitudes were measured when the response to several successive current pulses was stable, after redisplay on a digital oscilloscope. They are expressed as the fraction I/I_c where I_c is the control current amplitude immediately before drug application and I is the current amplitude measured in the presence of the drug. The dose-response curves were fitted to the Hill equation using a least squares method.

$$\frac{I}{I_c} = \frac{1}{1 + ([X]/K_i)^h} \quad \text{eqn. 1}$$

where [X] is the concentration of inhibitor, h is the slope parameter (Hill coefficient) and K_i is the concentration that gives half-maximal inhibition ($I/I_c = 0.5$).

For analysis of channel kinetics and open probability, single channel currents were filtered (−3 db down at 2 kHz; 8-pole Bessel filter), amplified (×10) and digitized at 5 kHz using an Axolab interface and the programme PCLAMP (Axon Instruments). They were subsequently analysed using a Dell 320LX computer with in-house software. The open probability was determined from data segments of 100–200 s duration by measuring the total time spent at each unitary current level and expressing this as a fraction of the total time.

Records shown in this paper have been replotted with a Gould 3200 chart recorder which filtered the data at 15 Hz (Gould, Cleveland, U.S.A.).

Solutions

During whole-cell experiments the bath contained extracellular solution (solution A, mM): NaCl 135, KCl 5, $CaCl_2$ 5, $MgSO_4$ 2, HEPES 5 (titrated to pH 7.4 with NaOH). The pipette was filled with an intracellular solution (solution B) containing (mM): KCl 107, $MgSO_4$ 2, $CaCl_2$ 1, EGTA 11 (0.06 μM free Ca^{2+}), HEPES 11 (pH 7.2 with KOH; additional K^+ about 30 mM). Na_2ATP, 0.3 mM, was also included to prevent run-down of K-ATP currents; this concentration of ATP blocks K-ATP currents only slightly in whole-cell recordings (Trube *et al.*, 1986).

In the case of inside-out patch recordings, the pipette was filled with a high K^+ extracellular solution in which KCl replaced NaCl (solution C). K-ATP channel activity declines with time after patch excision in inside-out patches (Niki *et al.*, 1989). We attempted to minimize this rundown in two ways. In early experiments, the bath contained intracellular solution (B) to which 0.1 mM ATP, 0.1 mM ADP and 2 mM $MgCl_2$ was added (cf. Ashcroft & Kakei, 1989; Niki *et al.*, 1989). More recently, it has been reported that Mg-free solutions abolish rundown of K-ATP channel activity in CRI-G1 β-cells (Kozlowski & Ashford, 1990). In later experiments, therefore, the bath contained (solution D, mM): KCl 140, $CaCl_2$ 4.6, EDTA 10 (about 30 nM free Ca^{2+} and 1 nM free Mg^{2+}), HEPES 10 (pH 7.2 with KOH).

1-Adamantanamine (amantadine) hydrochloride (the generous gift of Merz (F.R.G.)) and sparteine (Sigma) were dissolved in water; 1-adamantanol (amantanol, Sigma) was dissolved in dimethylsulphoxide (DMSO, final concentration <1%).

The bath solution could be exchanged within 30 s. Experiments were carried out at room temperature (22°C).

Statistics

All data in the text and figures are given as the mean of n observations ± s.e. of the mean (s.e.mean).

Results

Control conditions

Following establishment of the whole-cell configuration, there was a progressive increase in the input conductance of the HIT-T15 cell, from around 0.5 nS initially to a mean value of 7.5 ± 0.6 nS ($n = 27$) after about 5 min. This increase of conductance represents the activation of K-ATP channels caused by wash-out of ATP from the cell as it can be largely prevented by inclusion of 3 mM ATP in the pipette and it is inhibited by tolbutamide (Niki *et al.*, 1989). The effect of drugs on the whole-cell K-ATP current was tested only after the input conductance was stable.

Inside-out patches showed a variable degree of channel activity with between 2 and 40 channels open simultaneously. These were identified as K-ATP channels by the following criteria (Niki *et al.*, 1989): (i) the mean single channel conductance was 62 ± 2 pS ($n = 12$); (ii) channel activity was voltage-insensitive and (iii) was inhibited by ATP (1 mM) and by tolbutamide (100 μM). In most patches, the effect of ATP on channel activity was tested before the application of any drug.

Effect of amantadine

The inhibitory effect of amantadine on the whole-cell K-ATP current is shown in Figure 1a(i). Drug concentrations greater than 10 μM rapidly and reversibly inhibited whole-cell K-ATP currents. Figure 1b shows the relationship between the K-ATP current and amantadine concentration; the solid line is fitted to the Hill equation (eqn. 1), with a K_i of 120 μM and a Hill coefficient of 0.92.

The whole-cell K-ATP current is the product of the single channel current (i), the number of functional K-ATP channels in the cell membrane (N) and the channel open probability (P). To determine which of these parameters is affected by amantadine we tested the effect of the drug on single K-ATP channel currents in inside-out patches.

Addition of 0.5 ($n = 3$; Figure 2) or 1 mM ($n = 15$) amantadine to the intracellular membrane surface reversibly reduced K-ATP channel activity in all cases. In some patches a small

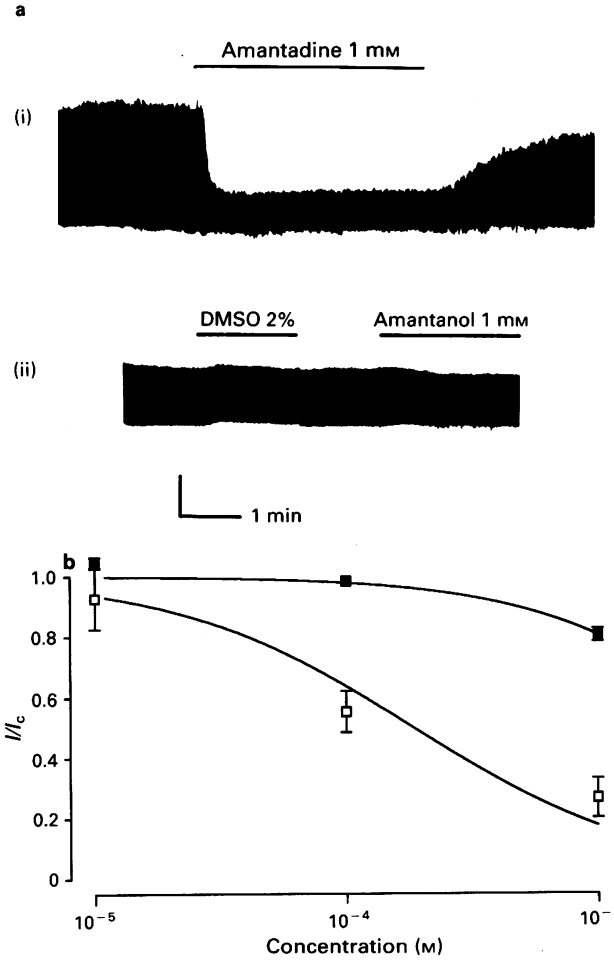


Figure 1 The effect of external application of amantadine and amantanol on the whole-cell K-ATP current. (a) (i)–(ii): Currents recorded in response to ±10 mV pulses from a holding potential of −70 mV. The time scale results in the current transients appearing as a continuous trace. Amantadine (1 mM, (i)), DMSO (2%, (ii)) and 1% DMSO + 1 mM amantanol (ii) were applied during the period denoted by the bars. Data were obtained from two different cells. The scale bar indicates 40 pA in (i) and 100 pA in (ii). (b) Dose-inhibition curve for amantadine (□) and amantanol (■). Values are given as a fraction of the control current (I/I_c). The points represent the mean of 3 or 4 cells and the vertical lines ±s.e.mean. The curve through the amantadine data (□) is fitted to eqn. 1 of the text with $K_i = 120$ μM and h = 0.92; that through the amantanol data (■) is fitted to eqn. 1 using K_i = 5 mM and h = 1.03.

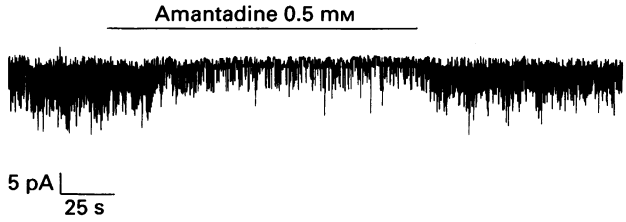


Figure 2 The effect of internal application of amantadine. Single channel currents recorded from an inside-out patch at a membrane potential of −70 mV. Amantadine (0.5 mM) was applied during the period indicated by the bar.

conductance channel was also present. This channel is probably the same as that reported in rat β-cells (Findlay *et al.*, 1985; Ashcroft *et al.*, 1988): it was not substantially affected by amantadine (Figure 2).

Figure 3 shows single channel currents and amplitude histograms obtained before and after addition of 1 mM amantadine to the intracellular solution. It is clear the drug does not alter the single channel current amplitude; nor does it affect the single channel conductance (data not shown). As we were unable to obtain a patch containing only a single active channel, we used binomial analysis to determine the effect of amantadine on N and P. An example of this analysis is shown in Figure 4, where the probability that a given number of channels are open (P_n) is plotted against the actual number of channels open (n). The binomial theorem provided a good fit to the data obtained in control solution, assuming that 3 channels are active with an individual open probability of

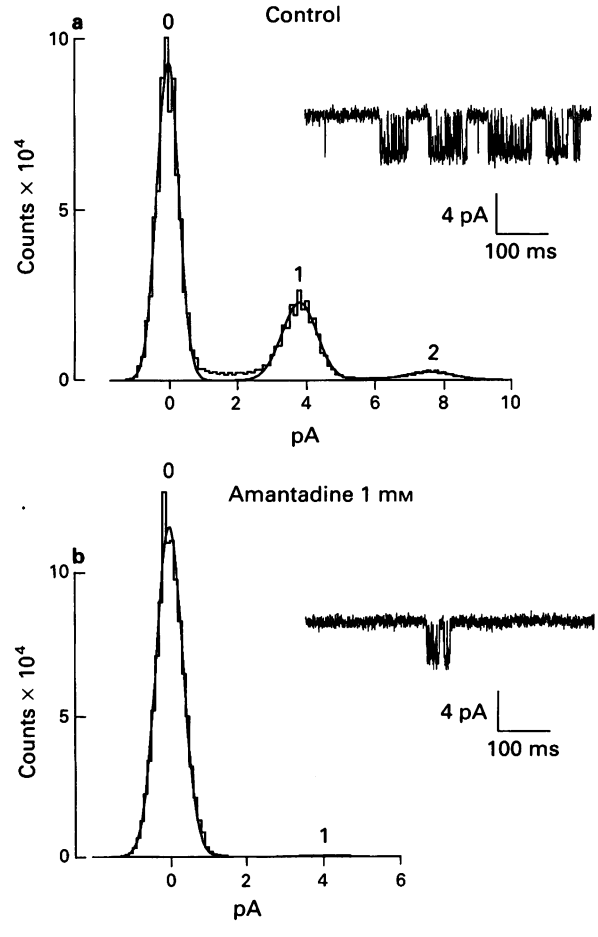


Figure 3 Amplitude histograms and single channel currents (inset) before (a) and after (b) the addition of 1 mM amantadine. Membrane potential, −70 mV. The histograms have been fit with a Gaussian function and the numbers above each peak indicate the number of channels open.

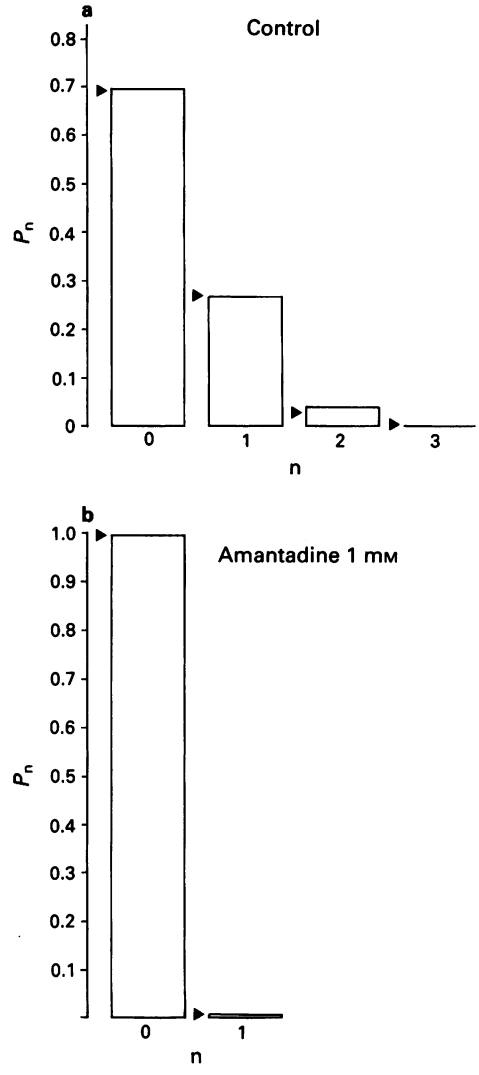


Figure 4 Binomial analysis of single channel data before (a) and after (b) addition of 1 mM amantadine to the intracellular solution. Membrane potential, −70 mV. Ordinate scale: probability that a given number of channels are open (P_n). Abscissa scale: number of channels open (n). The columns show the experimentally measured probabilities and the arrows the probabilities predicted by the binomial theorem.

0.115. In the presence of 1 mM amantadine, the data could only be fitted by assuming that a single channel was active with a markedly decreased open probability of 0.005. This suggests that the drug not only produces a decrease in the channel open probability but also causes some channels to enter a very long-lived closed state (i.e. a reduction in N). Because of the large decrease in channel activity, however, it is not possible to exclude the possibility that N is unaffected and the open probability of each channel is substantially reduced by an equal amount.

Mean values of P were 0.098 ± 0.031 in control solution and 0.005 ± 0.003 ($n = 3$) after addition of 1 mM amantadine (measured in the same patch). At lower concentration (0.5 mM), the drug also reduced the channel open probability, from 0.19 ± 0.02 (control) to 0.041 ± 0.008 ($n = 3$). Thus the open probability is decreased to approximately 21% and 5% of its control value by 0.5 mM and 1 mM amantadine respectively. This is somewhat more than is found for the whole-cell currents (Figure 1b). Whereas 1 mM amantadine substantially reduced the number of active channels in all three patches, 0.5 mM only did so in one patch.

The effect of 0.5 mM amantadine on the channel kinetics is examined in Figure 5. K-ATP channel openings occur in bursts, separated by longer closed periods (see inset Figure 3).

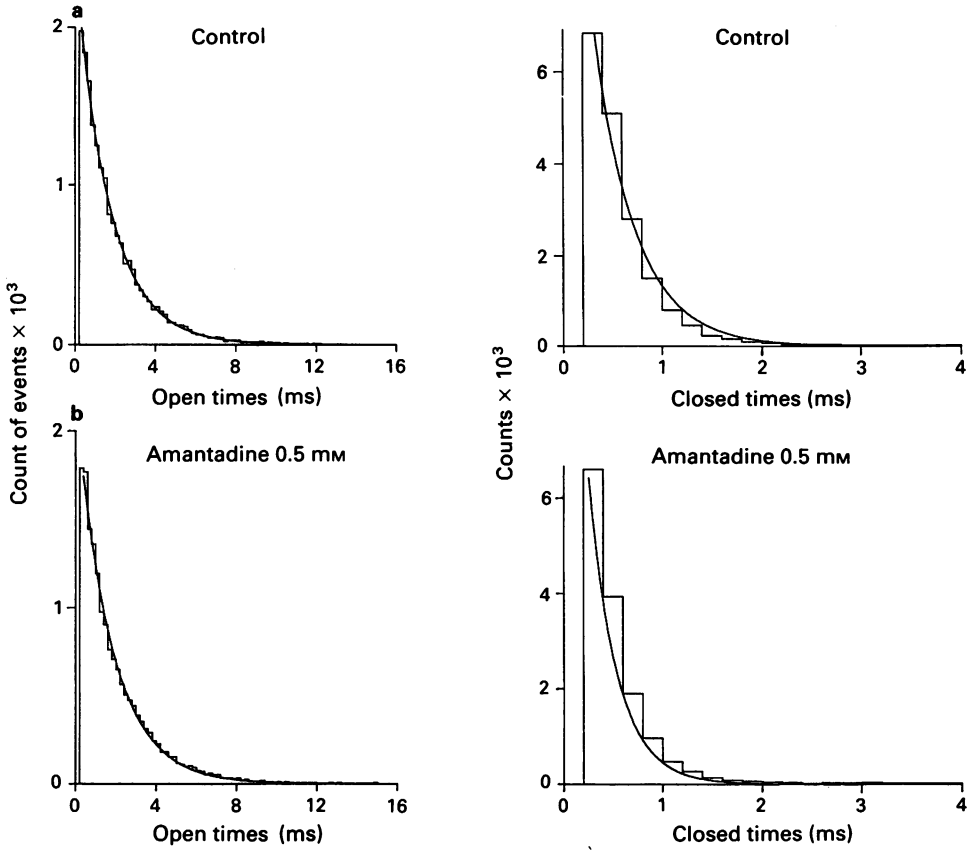


Figure 5 Distribution of open times (left) and closed times (right) during the burst before (a) and after (b) addition of 0.5 mM amantadine. Membrane potential, −70 mV. Both are fitted to a single exponential function $f(t) = A.\exp(-t/\tau_0)$ using a least squares algorithm. (a) Control: open times: A = 2440; τ_0 = 1.73 ms; closed times: A = 14711; τ_0 = 0.414 ms. Bin size displayed 0.2 ms. (b) Amantadine; open times: A = 2178; τ_0 = 1.68 ms; closed times: A = 16089; τ_0 = 0.28 ms. Bin size displayed 0.2 ms.

Our analysis is confined to the open and closed times within a burst of openings during which only a single channel was active: as we were unable to obtain any single channel patches, we do not know whether successive bursts of openings are due to the activity of the same or a different channel and a quantitative analysis of the interburst times is therefore not useful. In the absence of the drug, open times could be fitted by a single exponential with a time constant of 1.8 ± 0.1 ms (n = 5). Closed times were best described by the sum of two exponentials; the fast component, which corresponds to the intraburst closed times, comprised more than 90% of closings and had a mean lifetime of 0.37 ± 0.02 ms. These values are similar to those reported previously for HIT cells (Niki *et al.*, 1989) and mouse β-cells (Rorsman & Trube, 1985). Amantadine (0.5 mM) had little effect on either the open time or the closed time within the burst. Mean values obtained for open and closed times in the presence of the drug were 1.4 ± 0.1 ms (n = 3) and 0.39 ± 0.02 ms (n = 3), respectively. It is very clear from the records (Figures 2, 3), however, that amantadine dramatically reduces the burst duration and prolongs the interburst interval so that the channel enters a very long closed state (or states). The increase in this long closed state is responsible for the decrease in the channel open probability produced by the drug. Unfortunately, it was not possible to obtain an accurate estimate of the lifetime of the long closed state because of the very long recording periods that are necessary and the presence of channel rundown. Furthermore, we were unable to obtain a single channel patch.

The rate of onset of amantadine block of the whole-cell currents (48 ± 7 s; n = 4) was slower than that found for inside-out patches (13 ± 4 s; n = 6). The recovery of whole-cell K-ATP currents following removal of the drug was even slower (215 ± 31 s, n = 3, compared with 22 ± 6 s, n = 5, in inside-out patches). Thus we suggest that the site of action of amantadine is more accessible from the intracellular side of the membrane.

Effects of amantanol

In contrast to amantadine, application of amantanol to the bath had little effect on whole-cell K-ATP currents (Figure 1a(ii)). Even at the concentration of 1 mM, inhibition was only 20% (Figure 1b). Likewise, the activity of single K-ATP channels in inside-out patches was unaffected by 1 mM amantanol (n = 6; Figure 6). Application of DMSO at twice the maximal concentration used in experiments with amantanol was without effect (Figure 1a(ii)).

Effects of sparteine

The inhibitory effect of sparteine on whole-cell K-ATP currents is shown in Figure 7a. Currents were inhibited reversibly

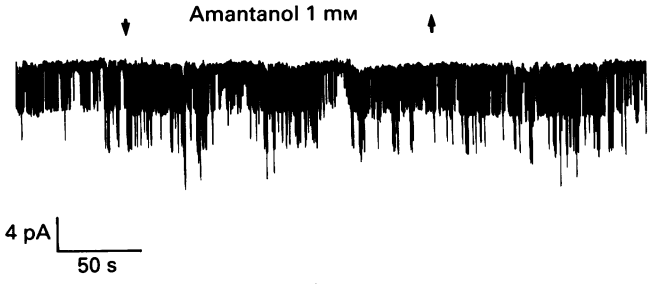


Figure 6 The effect of internal application of amantanol. Single channel currents recorded from an inside-out patch at a membrane potential of −70 mV. Amantanol (1 mM) was applied during the period indicated by the arrows.

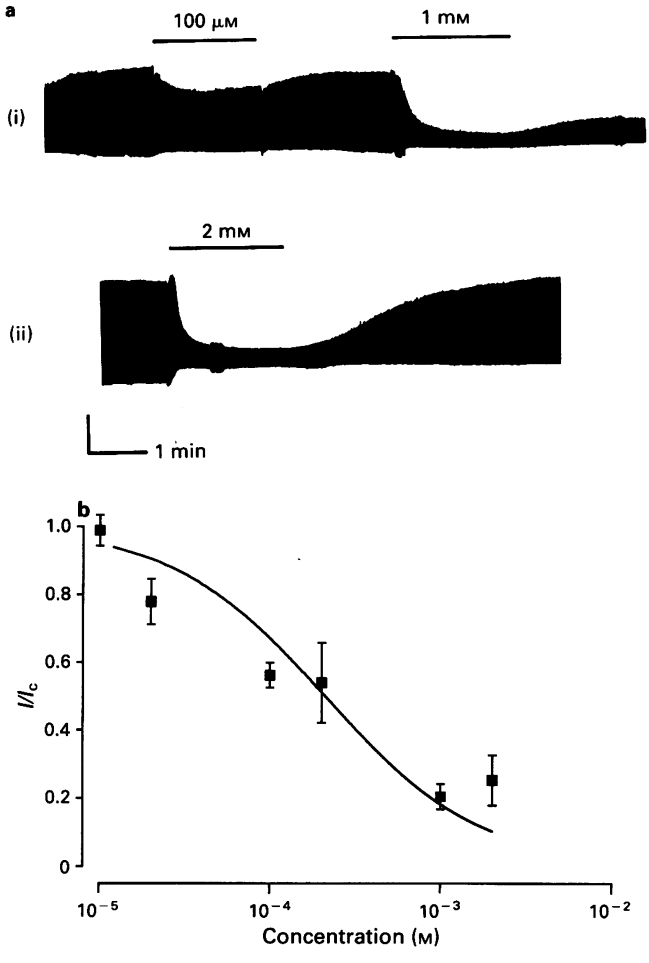


Figure 7 The effect of external application of sparteine on whole-cell currents. (a) (i)–(ii) Whole-cell currents recorded in response to +10 mV pulses from a holding potential of −70 mV in two different cells. Sparteine, at the concentrations indicated was applied during the period denoted by the bars. The scale bar indicates 100 pA. (b) Dose-inhibition curve for sparteine. Values are given as a fraction of the control current (I/I_c). The points represent the mean of 3 or 4 cells and the vertical lines ± s.e.mean. The curve is fitted to eqn. 1 of the text with K_i = 171 μM and h = 0.96.

at concentrations of sparteine between 20 μM and 2 μM in all cells tested (n = 16). Figure 7b shows the dose-response curve for sparteine inhibition; the solid line is fitted to the Hill equation (eqn. 1), with a K_i of 171 μM and a Hill coefficient of 0.96. It is apparent that sparteine is far less potent than the related alkaloid quinine, which produces almost complete inhibition of K-ATP currents at a concentration of 100 μM (Bokvist *et al.*, 1990).

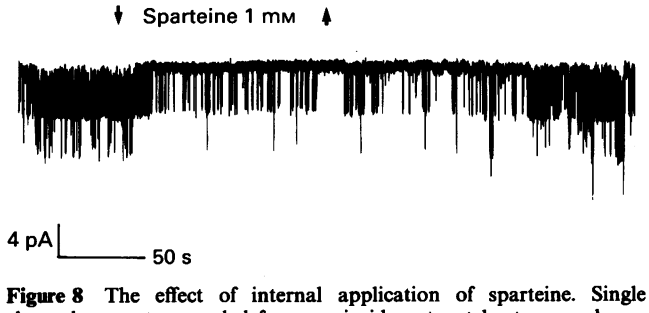


Figure 8 The effect of internal application of sparteine. Single channel currents recorded from an inside-out patch at a membrane potential of −70 mV. Sparteine (1 mM) was applied during the period indicated by the arrows.

Sparteine was also tested on single K-ATP channel currents recorded in inside-out patches. Bath concentrations of 1 and 2 mM reversibly reduced channel activity in all cases (n = 12) with no alteration of single channel current amplitude (Figure 8). Similar results have been obtained with inside-out patches from human β-cells (F.M. Ashcroft & M. Kakei, unpublished observation). The single channel current amplitude is also unaffected by quinine (Bokvist *et al.*, 1990).

Discussion

Our results show that amantadine and sparteine reversibly inhibit the K-ATP channel in HIT-T15 cells. Both these drugs have been shown to reduce the resting membrane K-permeability of β-cells and thereby elicit insulin release in the presence of non-stimulatory levels of glucose (Paolisso *et al.*, 1985; Garrino & Henquin, 1987). It seems probable that these effects of sparteine and amantadine result from their ability to inhibit K-ATP channels, since K-ATP channel activity principally accounts for the resting K-permeability of the β-cell (Ashcroft *et al.*, 1988). This hypothesis is supported by the observation that sparteine antagonizes the increase in ^{86}Rb efflux induced by diazoxide (Paolisso *et al.*, 1985), an activator of K-ATP channels (Trube *et al.*, 1986). Although a quantitative comparison is difficult, owing to the difference in experimental protocols, the concentrations reported for stimulation of insulin secretion by amantadine and sparteine (Paolisso *et al.*, 1985; Garrino & Henquin, 1987) are compatible with the K_i's we obtained for K-ATP channel inhibition.

Although our results suggest that the primary mechanism by which sparteine and amantadine increase insulin release is via inhibition of the K-ATP channel, additional effects of these drugs cannot yet be excluded. Sparteine, in particular, has been described as poorly reversible and as having only a transient effect on insulin secretion at high concentrations (2 mM, Paolisso *et al.*, 1985), effects which are less reconcilable with an action solely on the K-ATP channel. Whilst it is possible that these observations result from cellular accumulation of sparteine, the drug has been reported to affect other channels (e.g. Ohta & Narahashi, 1973; Senges & Ehe, 1973; Schauf *et al.*, 1976); furthermore, the related alkaloid quinine is known to affect β-cell metabolism (Henquin *et al.*, 1975).

The Hill coefficients obtained for inhibition by both amantadine and sparteine were close to unity, consistent with the idea that these drugs interact with their target in a one-to-one stoichiometry. Their site of action cannot be determined from the data presented here, but it is clear that both drugs can act from either side of the membrane since they are effective in inside-out patches as well as whole-cell recordings. As both amantadine and sparteine are lipid soluble to some extent, it is possible that they may reach their target site by partitioning into the lipid phase of the membrane. A similar suggestion has been made to account for the ability of the sulphonylurea tolbutamide to block the channel from both the inside and outside of the membrane (Trube *et al.*, 1986). The block by amantadine also resembles that produced by tolbutamide in that the single channel current amplitude is unchanged, the open and closed times within a burst of openings are unaffected and the principal effect is a decrease in the burst duration and an increase in the interburst interval (Gillis *et al.*, 1989).

The lack of an inhibitory effect of amantanol, compared to amantadine, is of considerable interest. Our experiments on inside-out patches allow us to discount the possibility that this difference results from the lower lipid permeability of amantanol preventing access to the inner face of the membrane (Garrino & Henquin, 1987). We still cannot exclude, however, the idea that this lower hydrophobicity may render a site within the membrane inaccessible. A more intriguing possibility is that the target site of amantadine is sufficiently specific that substitution of its amine group by an hydroxyl one renders it impotent. Our results do not allow us to determine

whether this site is on the channel protein itself or on a regulatory protein that modulates channel activity. The difference in the efficacy of amantadine and amantanol may be of use in elucidating the structure and control mechanisms of the K-ATP channel.

Amantadine is currently used in the treatment of Parkinson's disease, particularly to improve akinesia and rigidity. It is believed to augment the presynaptic release of dopamine although the mechanism by which it does so is unclear. Recent reports of K-ATP channels in substantia nigra pars compacta neurones (Röper *et al.*, 1990; Amoroso *et al.*, 1990), however, raise the intriguing possibility that the therapeutic effect of amantadine may be a consequence of inhibition of K-ATP channels in nigrostriatal neurones.

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BAY u3405, a potent and selective thromboxane A_2 receptor antagonist on airway smooth muscle *in vitro*

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1 BAY u3405 (3(**R**)-[[(4-fluorophenyl) sulphonyl]amino]-1,2,3,4-tetrahydro-9H-carbazole-9-propanoic acid) has been evaluated on airway smooth muscle, from a number of species including man, for its thromboxane A_2 (TXA_2) antagonist activity.

2 BAY u3405 was a potent, and competitive, antagonist of the TXA_2-mimetic U46619-induced contractions of human, guinea-pig, rat and ferret airway smooth muscle with pA_2 values between 8.0 and 8.9 and with no inherent contractile activity (10^{-9}–10^{-4} M).

3 The TXA_2 antagonist activity of BAY u3405 was stereoselective. Its (S)-enantiomer, BAY u3406, was approximately 50 fold less effective against U46619 on guinea-pig and human airway smooth muscle.

4 BAY u3405 also competitively antagonized contractions of guinea-pig airway smooth muscle induced by prostaglandin D_2 (PGD_2) or its metabolite 9α, 11β-PGF_2. On human and ferret airway smooth muscle it abolished contractions induced by PGD_2, $PGF_{2\alpha}$ and 16, 16-dimethyl-PGE_2.

5 A high concentration (10^{-6} M) of BAY u3405 had no effect on the contraction, or relaxation, of airway smooth muscle induced by a range of other agonists, nor did BAY u3405 have any effect on other prostanoid receptor types (DP, EP_1, EP_2, FP or IP).

6 BAY u3405, in contrast to some other TXA_2 antagonists, is a potent and selective antagonist on a wide range of airways including human. This high affinity, and the oral activity of the compound described elsewhere, suggest it may be an appropriate tool to investigate the role of prostanoids in airway diseases such as asthma.

Keywords: BAY u3405; thromboxane antagonists; PGD_2; $PGF_{2\alpha}$; TXA_2 receptor; 9α, 11β-PGF_2; human bronchial muscle; human lung strip

Introduction

It has been postulated that thromboxane A_2 (TXA_2) may be significantly involved in various diseases including asthma (Ogletree, 1987). In the past, non-steroidal anti-inflammatory drugs (NSAID) have been used as tools to investigate such a possibility but with limited success (Fairfax *et al.*, 1983, Shephard *et al.*, 1985). However such drugs inhibit the production of all cyclo-oxygenase products and consequently they have the potential to inhibit the beneficial bronchodilator effect of prostaglandin E_2 (PGE_2). It seemed possible (although never substantiated in asthmatics) that NSAID also induce shunting of arachidonic acid to the lipoxygenase pathway culminating in the synthesis of leukotrienes which are also thought to be pathological mediators of asthma. (Walker, 1980; Kuehl *et al.*, 1984). Consequently NSAID seem inappropriate tools to investigate the role of TXA_2 in asthma.

Considerable effort was assigned to the identification of more specific pharmacological tools to inhibit/antagonize TXA_2. A range of thromboxane synthase inhibitors were shown to inhibit selectively the synthesis of TXA_2 and as such represented valuable new tools in this area (Cross & Dickinson, 1987). Although such compounds inhibited TXA_2 synthesis, activation of TXA_2 receptors was still possible due to the presence of higher levels of PGH_2 and PGD_2; consequently such tools also have limited possibilities.

The identification of TXA_2 antagonists seemed a more useful target in this area. A critical prerequisite in the identification of such compounds was a general understanding of the TXA_2 receptor type(s) implicated in asthma. Evidence from some studies using platelets and smooth muscle suggested that TXA_2 receptor subtypes existed (Lefer *et al.*, 1980; Mais *et al.*, 1988). We proposed that human airway smooth muscle had more than one subtype present (McKenniff *et al.*, 1988). It seemed likely that TXA_2 antagonists which would be useful for investigation in asthma, should antagonize all TXA_2 receptor subtypes, or at least all of those present in human airways.

In this series of studies we describe for the first time extensive studies with the TXA_2 antagonist, BAY u3405. This novel indole sulphonamide (Figure 1) which is a TXA_2 antagonist on platelets and vascular smooth muscle (Rosentreter *et al.*, 1989) is also a potent and selective TXA_2 antagonist in airway smooth muscle from a wide range of species including man. Due to the poor stability of TXA_2, the TXA_2-mimetic, U46619 (Coleman *et al.*, 1981) was used in these studies.

Methods

Isolated respiratory tissues

Airway smooth muscle preparations were prepared as previously described (McKenniff *et al.*, 1988; 1989) save that

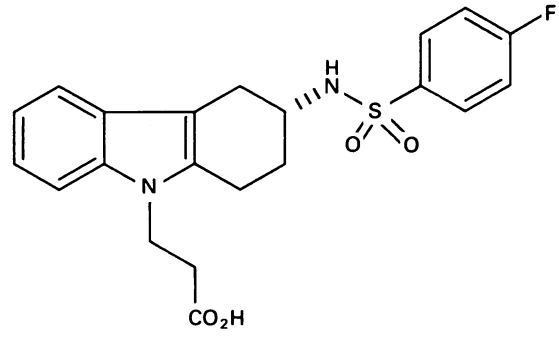


Figure 1 The chemical structure of BAY u3405: 3 (**R**)-[[(4-fluorophenyl) sulphonyl] amino]-1,2,3,4-tetrahydro-9H-carbazole-9-propanoic acid.

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indomethacin (3×10^{-6} M) was used rather than flurbiprofen. In brief, lung strips were taken from the periphery of the lung and set up under a resting tension of 0.5 g. Tracheae were set up as single rings with a resting tension of 1.0 g.

Rat lung strips from male Wistar rats (250–300 g) were prepared as described for the guinea-pig and human lung strip and set up at a resting tension of 1.0 g. In studies with leukotriene C_4 (LTC_4) and LTD_4 the bathing solution also contained 4.5×10^{-2} M L-serine borate and 10^{-2}M L-cysteine to inhibit the metabolism of these leukotrienes. All tension changes were measured isometrically.

Experimental design

Preparations were left to equilibrate for 1 h with washes every 15 min. Tissue viability was determined by addition of KCl (5×10^{-2} M). After washing and 30 min re-equilibration, antagonists were added and left in contact with the preparation for 60 min before the cumulative addition of increasing concentrations of the agonist. Only one agonist concentration-response curve was obtained from each preparation and KCl (5×10^{-2} M) was then added to obtain a maximal contractile response for normalisation of responses. Consequently paired preparations (i.e. one preparation treated with a vehicle control and the other with the test antagonist) were used to determine the degree of antagonism relative to the control agonist response.

Selectivity at other receptor types

Non-prostanoids The effect of BAY u3405 (10^{-6} or 10^{-5} M) was evaluated by the same procedure as described above against the following contractile agonists on the airway smooth muscle preparation indicated. Cumulative concentration-response curves to the test agonist were constructed in each case; guinea-pig trachea: carbachol, histamine, 5-hydroxytryptamine, LTC_4 and LTD_4; human lung strip: LTD_4; rat lung strip: carbachol. The effect of BAY u3405 on the isoprenaline-induced relaxation of guinea-pig tracheal rings contracted with histamine was also determined.

Prostanoids The effect of BAY u3405 (10^{-6} M) was also examined against a range of prostanoids on the following tissues. The setting up of tissues and the procedures used were the same as those described by the following groups:— PGI_2, PGD_2 and BW 245C-induced relaxations of rabbit jugular veins (Giles *et al.*, 1989), 16,16-dimethyl-PGE_2 and $PGF_{2\alpha}$-induced contractions and butaprost-induced relaxation of guinea-pig trachea (McKenniff *et al.*, 1988) and finally $PGF_{2\alpha}$- and fluprostenol-induced contractions of rat colon (Eglen & Whiting, 1988).

Spasmogenic activity on airway smooth muscle

Human and guinea-pig airway smooth muscle was prepared as described above, save that studies were also carried out with indomethacin omitted from the bathing solution. After the response to KCl had been determined and resting tension re-established a single concentration of BAY u3405 between 10^{-9} and 10^{-4} M, was added to each preparation to assess whether any tension changes resulted. To assess whether BAY u3405 was a bronchodilator, guinea-pig tracheal rings were contracted with carbachol and increasing concentrations of BAY u3405 added up to 10^{-4} M. Isoprenaline (10^{-6} M) was then added to determine the maximal relaxant response.

Data analysis

Data were expressed as arithmetic or geometric means with 95% confidence limits. Student's *t* test was used for paired, or unpaired, data as appropriate. Linear regression was employed to calculate EC_{50} values. Dose-ratios were calculated from the ratio of EC_{50} values for the test agonist. The method of Arunlakshana & Schild (1959) was used to determine pA_2 values. The pA_2 values were calculated with the slope constrained to unity after linear regression had shown the slope not to be significantly different from unity. If a parallel shift of the agonist concentration-response curve was only observed at a single antagonist concentration a $pK_B = \log$ (dose ratio $-$ 1)/[antagonist]) was determined at that concentration.

Drugs

BAY u3406 and BAY u3405 (respectively 3(S) and 3(R)-[[(4-fluorophenyl)sulphonyl]amino-1,2,3,4-tetrahydro-9H-carbazole-9-propanoic acid]) were supplied by Dr U. Rosentreter (Institute of Chemistry, Bayer AG, Wuppertal, Germany). Stock solutions of 10^{-2} M were prepared by dissolving BAY u3405 or u3406 in dimethylsulphoxide and serial dilutions were prepared in phosphate-buffered saline. Other drugs were dissolved as previously described.

Drugs were obtained from the following suppliers:— PGD_2, 16,16-dimethyl- PGE_2, U46619 (11α,9α-epoxymethano- prosta-5Z,13E-dienoic acid) PGI_2, carbachol, indomethacin, isoprenaline and 5-hydroxytryptamine (Sigma); LTC_4 and LTD_4 (Dr T.S. Abram, Bayer UK, Bucks); 9α,11β-PGF_2 (Salford Ultrafine Chemicals, Manchester), I-BOP ((1S-[1α,2α(Z), 3β(1E,3S,5S)4α])-7-(3-[3-hydroxy-4-{4-iodophenoxy}-1-butenyl]-7-oxabicyclo [2,2,1]hept-2-yl)-5-heptenoic acid) (Cayman Chemicals, Ann Arbor, MI, U.S.A.). $PGF_{2\alpha}$ (Medimpex, Budapest, Hungary), butaprost (methyl (11α, 13(E),16(S))-11,16-dihydroxy-9-oxo-17,17-(spirobutyl)prosta-13-enoate) (Bayer AG), fluprostenol (5Z, 11α, 13E,15(S))-11,15-dihydroxy 16-(3-trifluromethylphenoxy)-17,18,19,20-tetranor-9-oxo prosta-5,13-dienoic acid (ICI, Macclesfield, Cheshire), GR32191 ((Z)-1(R), 2(R), 3(S), 5(S))-7-[5-(biphenyl-4-ylmethoxy)-3-hydroxy-2-piperidinocyclopentyl]hept-4-enoic acid hydrochloride) (Glaxo, Ware, Herts.) and BW245C (5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropyl)hydantoin) (Wellcome, Beckenham, Kent). All salts used were of AnalaR grade.

Results

Intrinsic activity

BAY u3405 at concentrations between 10^{-9} M and 10^{-4} M in the presence, or absence, of indomethacin had no direct contractile effect on the airway smooth muscle of any of the species examined. Guinea-pig and human airways were examined in the presence or absence of indomethacin, whilst rat and ferret airways were examined solely in the presence of indomethacin. On human airway tissue, small reductions in tone were often observed but did not appear to be dose-related, although a small relaxation was consistently observed at 10^{-4} M. Against carbachol-induced tone on guinea-pig trachea, BAY u3405 (10^{-6} to 10^{-4} M) produced a small dose-related relaxation (20% of the isoprenaline maximum at 10^{-4} M BAY u3405).

Antagonism at thromboxane A_2 receptors

BAY u3405 produced rightward shifts in the U46619 concentration-response curves in airway smooth muscle from all four species examined. The effects of BAY u3405 were examined over at least a hundred fold concentration range, between 10^{-9} and 10^{-6} M, and produced progressive, concentration-related, displacements of the U46619 concentration-response curves (Figures 2 and 3). Schild analysis indicated that, in each case, the slope of the regression line did not differ significantly from unity (Table 1) and the pA_2 values were then calculated (Table 1). Similarly when another TXA_2 mimetic, I-BOP, was used as the agonist

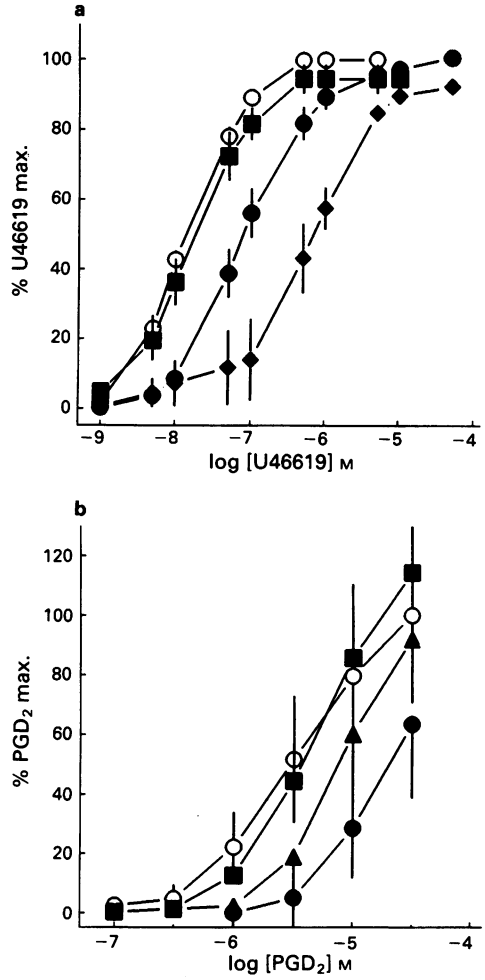


Figure 2 The effect of BAY u3405 on contractions of guinea-pig trachea induced by (a) U46619; control (○); BAY u3405 10^{-9} M (■), 10^{-8} M (●) and 10^{-7} M (◆). Mean from 6 preparations; vertical lines show s.e.mean; and (b) PGD_2; control (○); BAY u3405 10^{-9} M (■), 3×10^{-9} M (▲) and 10^{-8} M (●). Mean from 8 preparations; vertical lines show s.e.mean.

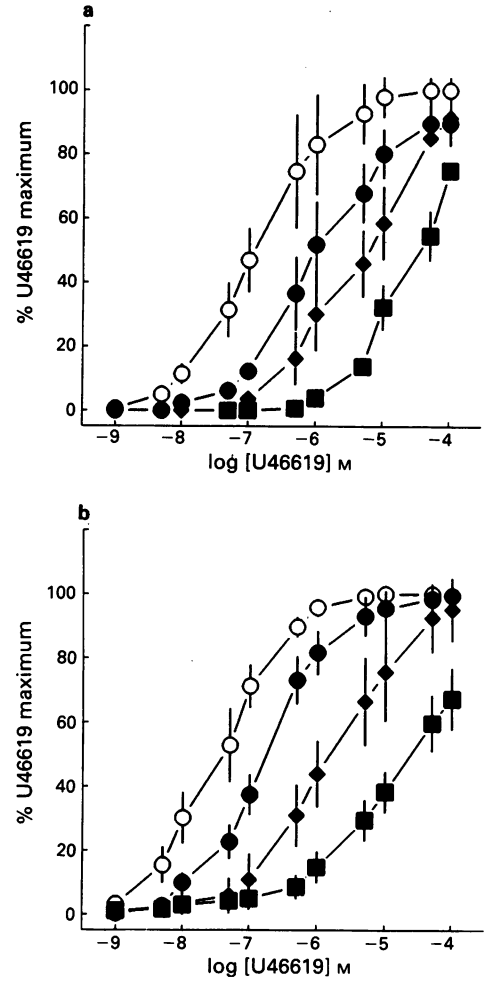


Figure 3 The effect of BAY u3405 on U46619-induced contractions of (a) human lung strip and (b) human bronchial muscle. U46619: control (○); BAY u3405 10^{-8} M (●), 10^{-7} M (◆) and 10^{-6} M (■): mean from 4 preparations; vertical lines show s.e.mean.

on guinea-pig lung strip, BAY u3405 was found to behave as a competitive antagonist (Figure 4).

Other prostanoids also contract airway smooth muscle via activation of TXA_2 receptors, generally with a maximal response markedly less than that of U46619 (McKenniff *et al.*, 1988; 1989). Contractile responses, to these prostanoids, of human and ferret airway smooth muscle were essentially abolished in the presence of 10^{-6} M BAY u3405 (Table 2). Only with the guinea-pig tissues was it possible to show competitive antagonism consistent with the Schild equation. BAY u3405 competitively antagonized contractions of guinea-pig trachea induced by PGD_2 (Figure 2) or its metabolite 9α, 11β-PGF_2. Similarly, on guinea-pig lung strip it acted as a competitive antagonist of contractions induced by PGD_2 (Figure 4) and abolished, the smaller, contractions induced by $PGF_{2\alpha}$ and 9α, 11β-PGF_2 (Table 2).

BAY u3405 and GR32191 were compared on three preparations against U46619 at an antagonist concentration of 10^{-7} M. On guinea-pig trachea both compounds gave a mean pK_B of 8.8 (data not shown). On both human bronchial muscle and rat lung strip a smaller shift in the U46619 concentration-response curve was observed in the presence of GR32191 than BAY u3405 (Figure 5). In these experiments the mean pK_B for BAY u3405 was 8.4 on both preparations whereas GR32191 gave pK_B values of 6.9 on rat lung strip and 7.7 on human bronchial muscle.

Table 1 The potency of BAY u3405 against U46619-induced contractions of respiratory smooth muscle

Tissue	pA_2	*Slope*	n
Human bronchial muscle	8.8 ± 0.3	1.15 ± 0.38	4
Human lung strip	8.9 ± 0.3	0.88 ± 0.13	4
Ferret tracheal ring	8.1 ± 0.3	1.09 ± 0.19	4
Guinea-pig tracheal ring	8.7 ± 0.2	0.78 ± 0.27	6
Guinea-pig lung strip	8.1 ± 0.3	1.09 ± 0.40	6
Rat lung strip	8.6 ± 0.1	0.70 ± 0.34	6

Data are shown as the mean ± 95% confidence limits from *n* preparations.

Selectivity

Non-prostanoids BAY u3405 (10^{-6} M) had no effect on the guinea-pig trachea against any of the following non-prostanoid agonists: carbachol, histamine, 5-hydroxytryptamine, LTC_4, LTD_4 or isoprenaline, nor against LTD_4 on human lung strip or carbachol on rat lung strip. Even 10^{-5} M BAY u3405 had no effect against histamine or LTD_4 on guinea-pig trachea (this concentration was not tested against other agonists).

Prostanoids The lack of affinity of BAY u3405 for other prostanoid receptor types was demonstrated by the absence of an effect of BAY u3405 (10^{-6} M) at the following prostanoid receptor types:— DP (PGD_2 and BW245C on rabbit jugular vein); EP_1 (16,16-dimethyl-PGE_2 and $PGF_{2\alpha}$ on guinea-pig

Table 2 The potency of BAY u3405 against prostanoid-induced contractions of respiratory smooth muscle

Tissue	*Agonist*	pK_B	pA_2	n
Guinea-pig trachea	PGD_2		8.6 ± 0.2[a]	8
	$9\alpha,11\beta$-PGF_2		9.1 ± 0.3[a]	4
	$PGF_{2\alpha}$	<5		4
	16,16-dimethyl-PGE_2	<5		4
Guinea-pig lung strip	PGD_2	8.9 ± 0.8[b]		6
	$PGF_{2\alpha}$	>8[c]		4
	I-BOP		7.8 ± 0.1[a]	4
Human lung strip	16,16-dimethyl-PGE_2	>8[d]		4
	PGD_2	>8[d]		4
	$PGF_{2\alpha}$	>8[d]		4
Human bronchial muscle	16,16-dimethyl-PGE_2	>8[d]		3
	PGD_2	>8[d]		1
	$PGF_{2\alpha}$	>8[d]		1
Ferret tracheal ring	16,16-dimethyl-PGE_2	>8[c]		4

[a] From Schild analysis, mean ± 95% confidence limits. The slopes were not significantly different from unity.
[b] In the presence of 10^{-8} M BAY u3405. Geometric mean ± s.e.mean.
[c] No contractile response observed in the presence of 10^{-7} M BAY u3405.
[d] No contractile response observed in the presence of 10^{-6} M BAY u3405.
For abbreviations, see text.

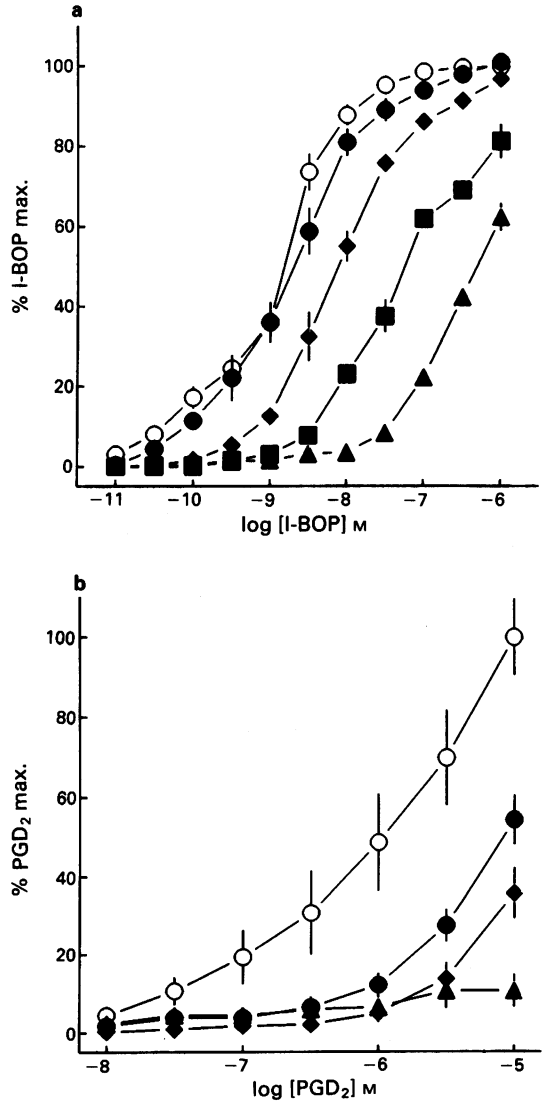


Figure 4 The effects of BAY u3405 on contractions of guinea-pig lung strip induced by (a) I-BOP: control (○); BAY u3405 10^{-8} M (●), 10^{-7} M (◆), 10^{-6} M (■) and 10^{-5} M (▲). Points are the mean from 4 preparations. (b) PGD_2: control (○); BAY u3405 10^{-8} M (●); 3×10^{-8} M (◆) and 3×10^{-7} M (▲). Points are the mean from 6 preparations. Vertical lines show s.e.mean in both (a) and (b).

trachea); EP_2 (butaprost on guinea-pig trachea); FP ($PGF_{2\alpha}$ and fluprostenol on rat colon); IP (PGI_2 on rabbit jugular vein).

Stereoselectivity Since BAY u3405 is a chiral antagonist we also evaluated the ability of its enantiomer BAY u3406 to

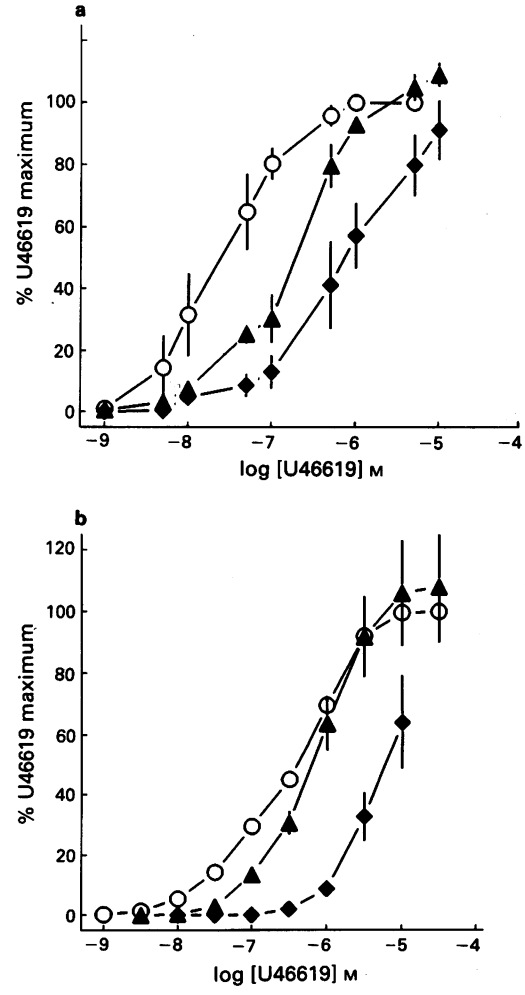


Figure 5 Comparison of the effect of 10^{-7} M BAY u3405 (◆) and 10^{-7} M GR32191 (▲) against U46619 control (○) on (a) human bronchial muscle and (b) rat lung strip. Mean from 4 preparations; s.e.mean shown by vertical lines.

Table 3 The potency of BAY u3405 against U46619-induced contractions of airway smooth muscle

Tissue	pK_B	n
Guinea-pig trachea	7.1 ± 0.2[a]	6
Human lung strip	6.6, 7.2[b]	2
Human bronchial muscle	6.7, 7.3[b]	2

[a] Geometric mean ± s.e.mean in the presence of 10^{-6} M BAY u3406.
[b] Observed values.

antagonize U46619-induced contractions of a number of preparations (Table 3). In each case this compound only produced a significant shift in the U46619 concentration-response curve at the highest concentration tested (10^{-6} M) from which pK_B values were calculated.

Discussion

We have shown BAY u3405 to be a potent TXA_2 receptor antagonist on airway smooth muscle preparations from a range of species including man. On no occasion was any inherent contractile activity observed with BAY u3405 on any of the tissues studied. The high potency of this drug on human airways suggests that it will be an ideal tool to elucidate the role of the TXA_2 receptor in airway diseases such as asthma.

BAY u3405 is a competitive antagonist of TXA_2 receptors. On all of the tissues studied it produced a parallel concentration-related rightward shift of the U46619 concentration-response curve with no suppression of the maximal response. The antagonism was surmountable and slopes of the Schild plots did not differ significantly from unity.

BAY u3405 was a selective TXA_2 antagonist. It had no effect on contractile responses induced by non-prostanoids at concentrations which were approximately three orders of magnitude greater than those shown to be effective against U46619. Similarly, when evaluated against selective agonists for each receptor, on a selection of tissues reported to possess a homogeneous or predominant population of each prostanoid receptor, BAY u3405 was inactive as an antagonist. We and others have previously found that on some airway preparations PGD_2, its metabolite 9α, 11β-PGF_2, and 16,16- dimethyl-PGE_2 induce contractile responses through TXA_2 receptors (McKenniff *et al.*, 1988; Featherstone *et al.*, 1990). It was not surprising therefore to find that BAY u3405 was a potent antagonist of these prostanoids on tissues where they were purported to activate TXA_2 receptors. On other tissues, such as the guinea-pig trachea where $PGF_{2\alpha}$ and 16,16-dimethyl-PGE_2 induce contractions via EP_1 receptors, BAY u3405 was inactive against such effects (McKenniff *et al.*, 1988).

Evidence from a number of studies points to the existence of TXA_2 receptor subtypes. However, from the available evidence it is uncertain how many subtypes exist. Mais *et al.* (1988) found a difference in rank orders of potency with a series of azapinane TXA_2 antagonists evaluated on both human platelets and canine saphenous vein. In an extensive study using both platelets and smooth muscle from three species Tymkewycz *et al.* (1991) concluded that variations in antagonist affinity could only be explained by the existence of TXA_2 receptor subtypes.

We have recently described differences in antagonist affinity on rat and guinea-pig lung strips as further evidence for TXA_2 receptor heterogeneity (Cuthert *et al.*, 1991). In the present studies it would appear that BAY u3405 displayed little selectivity between such subtypes. Human bronchial muscle, human lung strip and rat lung strip are probably the best airway preparations to demonstrate this point. Although most TXA_2 antagonists are effective on human lung strip they have markedly reduced potencies on the rat lung strip and human bronchial muscle (Cuthbert *et al.*, 1991, and unpublished observations). In contrast, however, BAY u3405 had comparable activity on all three tissues. We investigated this point further by comparing BAY u3405 with GR32191 on both human bronchial muscle and rat lung strips under identical conditions to those described in the present BAY u3405 studies (Figure 5). The potency of GR32191 on both these tissues was markedly less than that reported by Lumley *et al.*, (1989) on a range of TXA_2 receptor systems. Our results, on both rat lung and guinea-pig trachea were comparable to those reported. In contrast BAY u3405 had comparable activity on both tissues similar to that on human lung strip and a wide range of non-airway tissues. Thus although distinct TXA_2 receptor subtypes may exist, as suggested by the results with GR32191, BAY u3405 displays little, or no, subtype selectivity.

On some tissues, however, BAY u3405 did have a lower potency than expected against TXA_2-mimetics but not PGD_2 or $PGF_{2\alpha}$. On the guinea-pig lung strip where TXA_2 receptors are reported to be predominant, this drug was approximately one order of magnitude weaker against U46619 or another TXA_2-mimetic (I-BOP) than normally observed. Whilst this might be explained by the presence of a heterogeneous population of TXA_2 receptors neither we (McKenniff *et al.*, 1988) nor others (Coleman *et al.*, 1990) have observed, using a range of TXA_2 antagonists against U46619 concentration-response curves, Schild plots with slopes significantly different from unity or obtained pA_2 values on this tissue that differ markedly from those on the guinea-pig trachea. Even against I-BOP, where BAY u3405 was employed over a 1000 fold concentration range, there was no indication that the slope of the Schild plot deviates from unity (slope 0.96 ± 0.14). However, it is possible to observe linear Schild plots in heterogeneous systems (Kenakin, 1987).

The U46619 concentration-response curve on guinea-pig lung is shallow (spanning four orders of magnitude) possibly suggestive of the presence of two TXA_2 receptor types. If two receptor types are present then it would be anticipated that an antagonist might give different pA_2 values against different agonists. The low potency and efficacy of PGD_2 (Figure 4) make it difficult to obtain Schild plots. Only in the presence of 10^{-8} M BAY u3405 did the PGD_2 concentration-response curve reach its own EC_{50} level. This resulted in a large variation in observed pK_Bs. Thus any difference in the observed affinities of BAY u3405, against the three agonists employed, is probably apparent rather than real. To ascertain whether this preparation contains two subtypes would require evaluation of an antagonist against a wider range of agonists.

It has been shown that substantial quantities of both TXA_2 and PGD_2 are produced in the lung upon allergen challenge in asthmatics (Wenzel *et al.*, 1989). However it remains unresolved at present whether TXA_2 receptors play a significant role in airway diseases. As yet GR32191 is the only TXA_2 antagonist reported to have been evaluated against both PGD_2 and allergen challenge in man (Beasley *et al.*, 1989). Its activity against both challenges was modest and has not resolved this problem. While this result may be due to factors other than receptor affinity it is tempting to speculate that the limited profile of TXA_2 antagonism of GR32191 on airway preparations, as shown in this paper diminishes its value as a drug tool to clarify this area. In contrast BAY u3405 has a good profile at all TXA_2 subtypes especially in human tissue, suggesting that it is the ideal drug tool for investigating TXA_2 receptors in airway disease.

In summary, BAY u3405 is a potent, competitive and selective TXA_2 receptor antagonist on a range of airway smooth muscle including human. Such activity coupled with good oral potency and duration of action in the guinea-pig (Francis *et al.*, 1991) and tolerance in man (Weber *et al.*, 1990) suggest that BAY u3405 is the most promising drug tool for clinical investigation of the role of TXA_2 and prostanoids (e.g. PGD_2) which act through TXA_2 receptors in airway disease, most notably asthma.

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Inhibitory effects of BAY u3405 on prostanoid-induced contractions in human isolated bronchial and pulmonary arterial muscle preparations

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1 The thromboxane-mimetic, U46619, was a more potent contractile agonist than prostaglandin D_2 (PGD_2), $PGF_{2\alpha}$ or histamine in human isolated bronchial and pulmonary arterial muscle preparations.

2 Human isolated proximal bronchial muscles were less sensitive to contractile agents than distal bronchial preparations. However, the former tissues developed a greater contractile force (E_{max}) when compared with results obtained in the latter tissues.

3 BAY u3405 attenuated the contractions induced by U46619, $PGF_{2\alpha}$ and PGD_2 in both human isolated bronchial and pulmonary arterial muscle preparations.

4 BAY u3405 did not alter histamine concentration-effect curves nor the relaxation induced by Butaprost (TR4979) in human isolated bronchial muscle preparations. In addition BAY u3405 did not modify the relaxation induced by PGI_2 in isolated pulmonary arterial muscle preparations.

5 The contractions induced by different prostaglandins were blocked by BAY u3405, suggesting a common functional site for these agents found both on human bronchial and pulmonary arterial muscles.

Keywords: Human bronchus; human pulmonary arteries; prostanoids; histamine; thromboxane A_2 antagonist; BAY u3405

Introduction

One major interest in the development of compounds with specific blocking activity for thromboxane A_2 (TxA_2), apart from their possible therapeutic value, is their use in the identification and classification of those sites associated with the function of this mediator. Recently, a number of workers (McKenniff *et al.*, 1988; Armour *et al.*, 1989; Lumley *et al.*, 1989) have shown that TxA_2 functional sites exist in airway muscles of several species and prostanoid contractile agents generally produce their effects via this TxA_2-receptor (McKenniff *et al.*, 1988; Coleman & Sheldrick, 1989). However, the classification of prostanoid receptors has been impeded by the known paradoxical effects of these agents on tissues derived from various parts of the lung and the lack of potent selective antagonists.

The information available concerning the potent bronchoconstrictor activity of TxA_2 has been derived from studies which utilized stable synthetic analogues, such as U46619 (Spannhake *et al.*, 1978). The potency of U46619 has been reported to vary in human isolated airway preparations with large airways (Featherstone *et al.*, 1988) being more sensitive than smaller ones (McKenniff *et al.*, 1988; Armour *et al.*, 1989). However, some of these results are based on protocols in which the tissues have been continuously exposed to a variety of drug combinations such as atropine, indomethacin, phentolamine and/or mepyramine (Featherstone *et al.*, 1988; McKenniff *et al.*, 1988) before the effects of the TxA_2-mimetic (U46619) and TxA_2 antagonists were examined. A comparison of data from one paper with results obtained in another is difficult since alterations in tissue responsiveness and sensitivity due to the various drug pretreatments cannot be excluded. The purpose of the present study was to determine the inhibitory action of BAY u3405 on contractions induced by the TxA_2-mimetic (U46619), different prostanoids and histamine in human isolated bronchial muscle preparations of different size. In addition, similar experiments were performed on human isolated pulmonary arterial muscle preparations. All protocols were undertaken in tissues where no prior drug treatments were performed.

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Methods

Isolated preparations

Human lung tissue was obtained from patients who had undergone surgery for lung carcinoma. After the resection of a lung or a lobe, parts of the bronchus and human pulmonary artery were dissected free from parenchymal lung tissue and placed in Tyrode solution at 4°C for 12 h. Bronchial preparations were divided into two groups based on internal diameter (mm) and wet weight (mg). The proximal bronchial preparations were 7.12 ± 0.35 mm and weighed 235.74 ± 21.34 mg (51 preparations (p) from 19 lung samples (n)) while the distal bronchial tissues were 2.25 ± 0.17 mm and weighed 21.13 ± 2.44 mg ($n = 17$; $p = 42$). In this study the human pulmonary arterial muscle preparations were taken from the same region as the proximal bronchial and no attempt was made to study the regional differences of these tissues. Human pulmonary arterial preparations were 4.13 ± 0.57 mm (internal diameter) and 23.06 ± 5.44 mg (wet weight; $n = 14$; $p = 47$). All preparations were set up as rings and suspended in 10 ml organ baths containing Tyrode solution (concentrations mM): NaCl 139.2, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, $NaHCO_3$ 11.9, NaH_2PO_4 0.4 and glucose 5.5; pH 7.4; gassed with 5% CO_2 in 95% O_2 and maintained at 37°C. The preparations were set up under initial loads (2–3 g) proportional to their size. These loads ensured that responses to contractile agonists were maximal. Isometric force displacement transducers (Narco F-60) and physiographs (Linseis) were used to record the changes in force. The tissues were allowed to equilibrate for 90 min; during this period the bath fluid was exchanged every 10 min with fresh Tyrode solution.

Protocols

After the equilibration period, the preparations were contracted initially with a maximally effective concentration of histamine (50 μM). When the response reached a plateau, the bath fluid was exchanged at 10 min intervals until the preparations returned passively to their initial resting tone. A second contraction was induced with histamine (50 μM) and the tissues were again washed with Tyrode solution. After

these two contractions, when the basal tone was re-established, three different protocols were followed. In the first protocol pulmonary arterial, distal and proximal bronchial preparations were incubated for 30 min with different concentrations of the antagonist (BAY u3405: 1 nM, 10 nM or 100 nM) and subsequently a contractile agonist was added to the bath fluid at 5 min intervals in a cumulative fashion, beginning with the lowest concentration. In the second protocol, BAY u3405 cumulative concentration-effect curves were performed on the distal bronchial preparations. In order to determine the maximal relaxation of these preparations, after the last concentration of BAY u3405, isoprenaline (1 μM) was added to the bath. In the third protocol proximal bronchial preparations and pulmonary arterial muscles were incubated (30 min) with BAY u3405 (0.1 μM) and contracted with histamine (50 μM). When the contraction reached a plateau, proximal bronchial preparations were challenged with Butaprost (TR 4979) in a cumulative fashion and arterial preparations were stimulated with prostacyclin (PGI_2). In all protocols preparations were incubated for 30 min with Tyrode solution and served as controls.

Data analysis

Changes in force were measured from isometric recordings and expressed in grams (g). The maximal response induced with an agonist (E_{max} values) and the concentration of agonist yielding 50% of maximal response (EC_{50} values), were interpolated from the individual concentration-effect curves. These latter values were transformed into pD_2 values, that is, negative logarithms of EC_{50} values. The apparent dissociation constant (Furchgott, 1972) was calculated for the agonist–antagonist interaction according to the equation: $K_B = [B]/(CR - 1)$ where [B] is the concentration of the antagonist and CR (concentration ratio) is the ratio of equieffective concentrations of agonist in the presence and absence of antagonist. The K_B value was transformed into pK_B value, that is, negative logarithm of the K_B value. Concentration-effect curves in the presence of BAY u3405 were expressed as a percentage of the second histamine (50 μM) contraction. All results are expressed as mean ± s.e.mean. Statistical analysis was performed by use of Student's t test for paired or unpaired variables. A probability value (P) of less than 0.05 was considered to be statistically significant.

Drugs

The drugs and their sources were: histamine dihydrochloride, isoprenaline hydrochloride, $PGF_{2\alpha}$, PGI_2, PGD_2 and U46619 (9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$) (Sigma Chemical Co., St. Louis, MO, U.S.A.). Butaprost (TR4979) and the antagonist BAY u3405 (3(**R**)-[[4-fluorophenyl) sulphonyl] amino]-1,2,3,4-tetrahydro-9H carbazole-9-propenoic acid) (Bayer U.K. Ltd, Research Dept., Stoke Poges, England).

Histamine was dissolved in Tyrode solution, PGD_2, $PGF_{2\alpha}$, Butaprost (TR 4979) and U46619 were dissolved in ethanol 100%. PGI_2 was dissolved in Tyrode solution with pH adjusted to 8.5. BAY u3405 was dissolved in NaOH 1 M, pH was adjusted to 7.4 with HCl. Subsequent dilutions of each drug were made in Tyrode solution (pH 7.4).

Results

Human isolated bronchial muscle preparations

The results of various contractile agonists in proximal and distal human isolated bronchial muscle preparations are presented in (Figure 1). Distal preparations were more sensitive and less responsive to the three agonists (Table 1). In addition, bronchial muscle preparations were more sensitive to U46619 than either PGD_2 or histamine, an effect that was independent of tissue size.

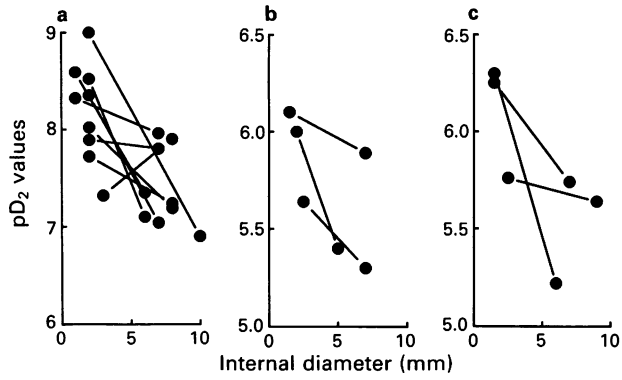


Figure 1 The effect of contractile agonists on human isolated proximal and distal bronchial preparations from the same lung samples. The results presented in the panels: (a) U46619; (b) prostaglandin D_2 and (c) histamine; are the agonist pD_2 values which were interpolated from individual concentration-effect curves.

BAY u3405 (30 min; 0.1 μM) relaxed the basal tone of human isolated proximal bronchial preparations. This effect was approximately 15% of the second histamine (50 μM)-induced contraction. The relaxation in paired control tissues was 0.7% ($n = 8$ and $p = 15$ for both treated and control tissues). In addition, concentration-effect curves with BAY u3405 were performed on basal tone of human isolated distal bronchial preparations (Figure 2).

BAY u3405 was a potent inhibitor of the U46619-induced contraction in proximal and distal human isolated bronchial muscle preparations. The data shown in Figure 3a and Table 2 indicate that BAY u3405 (0.1 μM) caused a significant change in sensitivity to U46619, pK_B values were 8.99 ± 0.06 and 9.45 ± 0.33 for proximal and distal preparations respectively. These unpaired pK_B values were not significantly different. In contrast, BAY u3405 did not significantly modify the (E_{max}) values to U46619 of any preparations, whether expressed in grams (g) or as a percentage of the second histamine (50 μM)-induced contraction: 124 ± 15% (control) vs 160 ± 20% (BAY u3405) in proximal preparations and 123 ± 23% (control vs 150 ± 22% (BAY u3405) in distal preparations.

Lower concentrations of BAY u3405 caused concentration-dependent shifts of the TxA_2-mimetic concentration-effect curve. After incubation of (none, 1 nM, 10 nM or 100 nM) with BAY u3405, U46619 pD_2 values for proximal bronchial prep-

Table 1 The response and sensitivity of human isolated bronchial muscle preparations to various contractile agonists

Bronchial preparations	n/p	*Histamine response* (g)*	*Agonist* E_{max} (g)*	*Agonist* pD_2 *value**
			U46619	
Proximal	9/17	2.30 ± 0.40	3.72 ± 0.65	7.39 ± 0.13
Distal	9/18	1.71 ± 0.31	1.81 ± 0.28	8.19 ± 0.17
			PGD_2	
Proximal	3/5	2.46 ± 0.84	1.46 ± 0.15	5.53 ± 0.18
Distal	3/4	0.90 ± 0.20	0.61 ± 0.12	5.91 ± 0.14
			Histamine	
Proximal	3/3	3.00 ± 0.88	4.03 ± 0.74	5.53 ± 0.16
Distal	3/3	0.94 ± 0.10	1.07 ± 0.11	6.10 ± 0.17

Histamine response was induced with 50 μM. E_{max} indicates the maximal contraction produced by the agonist expressed in grams (g). Values are means ± s.e.mean, n indicates the number of lung samples and p represents the number of preparations used. * Values significantly different when results from proximal and distal preparations were compared ($P < 0.05$).

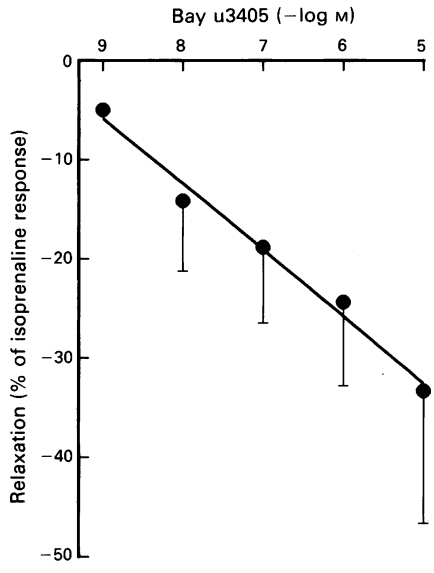


Figure 2 BAY u3405 concentration-effect curves on basal tone of human isolated distal bronchial muscle preparations ($n = 4$; p = 8). Relaxations were expressed as percentage of the maximal relaxation induced with isoprenaline (1 μM; -0.87 ± 0.35 g) at the end of each experiment. Values are means with s.e.mean shown by vertical bars.

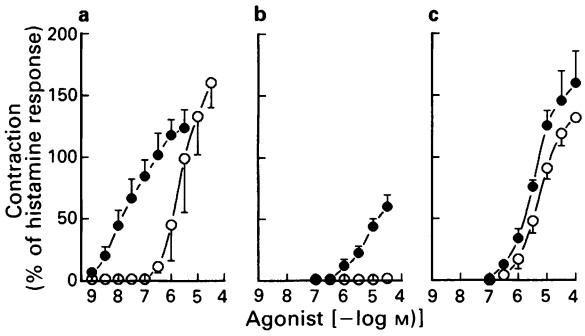


Figure 3 The effects of BAY u3405 on different agonists concentration-effect curves in human isolated proximal bronchial muscle preparations. Data are derived from curves performed after a 30 min incubation in Tyrode solution (●) or Tyrode solution containing BAY u3405 (0.1 μM; ○). Cumulative concentration-effect curves were performed with (a) U46619 ($n = 4$; p = 8), (b) prostaglandin D_2 ($n = 3$; p = 10), (c) histamine ($n = 3$; p = 6). Values are means with s.e.mean shown by vertical bars.

Table 2 The effects of BAY u3405 on the U46619-induced response in human isolated distal bronchial muscle preparations

		Histamine response	*U46619*	
	n/p	(g)	E_{max} (g)	*pD_2 value**
None	4/5	0.97 ± 0.20	1.28 ± 0.47	8.06 ± 0.28
BAY u3405	4/4	0.79 ± 0.12	1.17 ± 0.19	5.61 ± 0.08

Histamine response was induced with 50 μM prior to incubation. Tissues were exposed to Tyrode solution (none) or to Tyrode solution containing BAY u3405 (0.1 μM; 30 min). E_{max} indicates the maximal contraction produced by the agonist as force in grams (g). Values are means ± s.e.mean, *n* indicates the number of lung samples and p the number of preparations used. * Values significantly different when results from control and treated tissues were compared ($P < 0.05$).

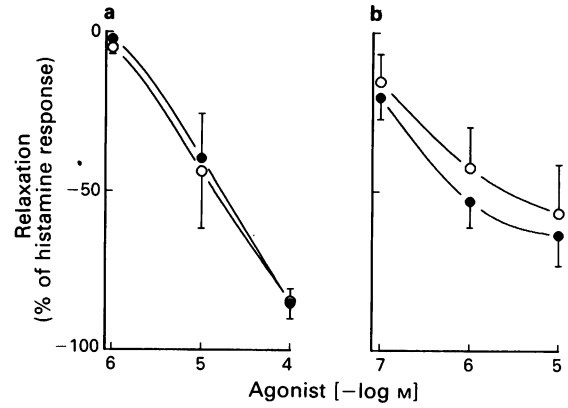


Figure 4 Effects of BAY u3405 on Butaprost (TR4979)-induced relaxation in human isolated bronchial muscle preparations (a; $n = 3$; p = 8) and on prostacyclin (PGI_2)-induced relaxation in human isolated pulmonary arterial muscle preparations (b; $n = 4$; p = 10). Concentration-effect curves to the relaxant agonists in the absence (●) and in the presence (○) of BAY u3405 (0.1 μM) were produced on histamine (50 μM)-contracted preparations. The contractions induced with histamine were: (a) control, 4.08 ± 0.28 g; BAY u3405, 3.82 ± 0.70 g and (b) control, 2.62 ± 0.46 g; BAY u3405, 2.72 ± 0.41 g. Values are means with s.e.mean shown by vertical bars.

arations ($n = 1$; p = 4) were: 7.77; 7.97; 6.28 and 5.75 respectively.

The inhibitory activity of BAY u3405 was also observed against PGD_2-induced contractions (Figure 3b) as well as $PGF_{2\alpha}$ data obtained in one lung sample (data not shown) but not against those produced by histamine (Figure 3c).

The relaxant effect of Butaprost (TR4979), an analogue of PGE_1 on histamine-contracted human proximal bronchial preparations was unaffected by BAY u3405 (Figure 4a).

Human isolated pulmonary arterial muscle preparations

Human isolated pulmonary arterial muscle preparations were more sensitive to U46619 than PGD_2 or $PGF_{2\alpha}$ (Figure 5 and

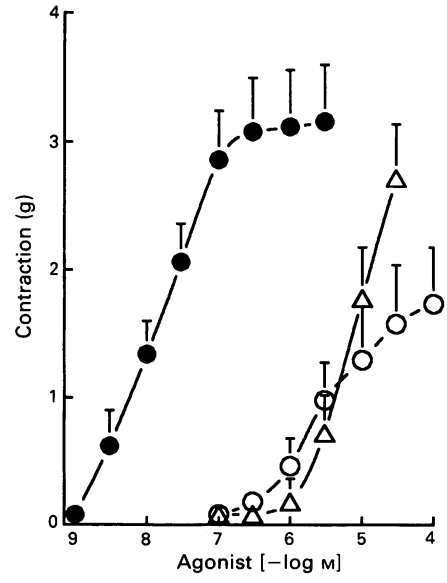


Figure 5 Agonist concentration-effect curves generated in human isolated pulmonary arterial muscle preparations by U46619 (●; $n = 5$; $p = 10$), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, △; $n = 3$; p = 3) and PGD_2 (○; $n = 5$; p = 9) using the cumulative dosing method. Values are means with s.e.mean shown by vertical bars and contraction is presented as force in grams (g).

Table 3 The effects of various contractile agonists on human isolated pulmonary arterial muscle preparations

Agonist	n/p	Histamine response (g)	Agonist E_{max} (g)	Agonist pD_2 value
U46619	5/10	1.67 ± 0.45	3.14 ± 0.43	7.96 ± 0.21
PGD_2	5/9	2.06 ± 0.70	1.74 ± 0.41	5.37 ± 0.19
$PGF_{2\alpha}$	3/3	2.72 ± 0.31	2.72 ± 0.42	5.25 ± 0.18
Endothelin	4/5	1.83 ± 0.31	2.89 ± 1.30	9.82 ± 0.07

Histamine response was induced with 50 μM. The maximal response (E_{max}) is expressed in grams (g). Values are means ± s.e.mean, n indicates the number of lung samples and p represents the number of preparations used.

Table 3). A comparison of the results derived from the vascular preparations with those obtained in bronchial tissues indicates that the sensitivities of these tissues to each of the contractile agents were similar when results for each contractile agonist were compared. BAY u3405 significantly shifted the concentration-effect curve to U46619 in human isolated pulmonary arterial muscle preparations (Figure 6a). The pK_B value was 9.25 ± 0.23. However, as in the case of the human isolated bronchial muscle preparations the contractile effects of $PGF_{2\alpha}$ were also blocked by BAY u3405 (Figure 6b). In contrast, BAY u3405 was without effect on endothelin concentration-effect curves (Figure 6c) in human isolated pulmonary arterial preparations.

The relaxant effect of prostacyclin (PGI_2) on histamine-contracted human pulmonary arterial preparations was not altered subsequent to a 30 min incubation with BAY u3405 (0.1 μM; Figure 4b). Similar data were also obtained with iloprost in one lung sample (data not shown).

Discussion

Airway preparations derived from different levels of the lung in a variety of animal species including man (Persson & Ekman, 1976; Drazen *et al.*, 1979; Brink *et al.*, 1980a; Raffestin *et al.*, 1985) exhibit differences in reactivity and sensitivity to specific contractile agonists. The data presented in this paper on the contractile effects of the TxA_2-mimetic and PGD_2 further support and extend this notion since these results demonstrate a greater contractile response in human isolated proximal airway muscle preparations than in distal airways. A difference which may be explained in part by the greater muscle mass found in the proximal airways. However, the agonist sensitivity is greater in the distal preparations

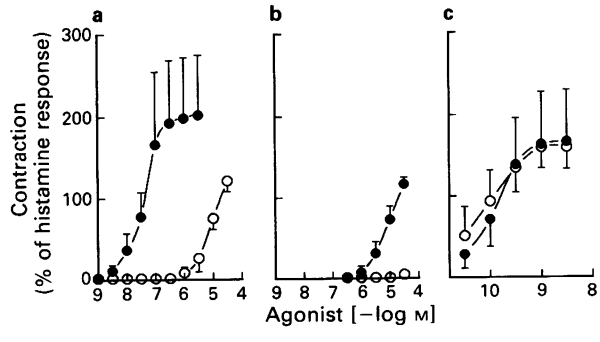


Figure 6 The effects of BAY u3405 on different agonists concentration-effect curves performed in human isolated pulmonary arterial muscle preparations: (a) U46619 curves (control, ●; n = 4, p = 7 and treated, ○; 0.1 μM, n = 4, p = 6), (b) prostaglandin $F_{2\alpha}$ curves (control, ● and treated, ○; 0.1 μM, n = 3, p = 3, respectively); (c) endothelin curves (control, ● and treated, ○; 0.1 μM, n = 4, p = 5, respectively). Values are means with s.e.mean shown by vertical bars.

when results for each agent were compared with the data obtained in the proximal tissues. This latter observation may indicate that the receptors in the distal bronchial preparations have a greater affinity for the agonist or are more directly coupled to the membrane and cellular events which lead to the contraction of the tissues. Another possible explanation is that the distal preparations are homogeneous that is, these airways lack non-muscle tissue mass such as, cartilage and therefore the diffusion barrier for the agonist is decreased when compared with proximal airways. Whether these regional differences are due to pharmaco-chemical coupling in muscle and/or binding characteristics of receptors (Roth, 1979) requires further investigation.

All bronchial preparations were more sensitive to U46619 than the other contractile agents used ($PGF_{2\alpha}$, PGD_2 and histamine). The U46619 pD_2 values reported by Armour and co-workers (1989) were similar to those observed for distal human isolated bronchial muscle preparations (this paper). In addition, McKenniff *et al.* (1988) showed similar results for the potency of U46619 in human isolated bronchial muscle preparations which had been pretreated with a number of receptor antagonists prior to the production of the TxA_2-mimetic concentration-effect curves. Together these data would suggest that the endogenous release of local mediators (acetylcholine, histamine, catecholamine and prostanoids) do not alter the sensitivity of the preparations to the TxA_2-mimetic. However, the region from which the tissue is derived may be a factor for consideration in agonist potency.

The relaxations obtained on basal tone with BAY u3405 suggest that prostaglandins may be involved in the maintenance of resting tone in human isolated bronchial muscle preparations. These results appear different from data obtained in similar preparations which have been treated with cyclo-oxygenase inhibitors. Initial studies have shown that indomethacin did not affect basal tone nor the histamine-induced contractions in human bronchial tissues (Brink *et al.*, 1980b). Although indomethacin inhibited the histamine-stimulated release of prostaglandins, the cyclo-oxygenase activity was not completely abolished (Haye-Legrand *et al.*, 1986). Thus prostaglandins which contract and relax the muscle preparations are continuously produced *in vitro*, albeit in smaller quantities after indomethacin treatment. The data (this paper) suggest that only when the site associated with the contractile effect of these mediators is blocked can the relaxations be observed. However, further experiments with BAY u3405 need to be performed to preclude the possibility that relaxations may be induced via some other mechanism of action in this tissue.

The identification of prostanoid sites has been undertaken using a variety of agonists by establishing rank order potencies in different tissues or cells and has led to a number of propositions concerning the classification of these sites (Gardiner & Collier, 1980; Kennedy *et al.*, 1982; Jones *et al.*, 1982; Coleman & Kennedy, 1985; McKenniff *et al.*, 1988). Since the development of compounds with potential TxA_2 blocking activity (Jones *et al.*, 1982; Brittain *et al.*, 1985; Lumley *et al.*, 1989) the notion of a single site namely, the TP receptor (Kennedy *et al.*, 1982; Coleman & Sheldrick, 1989) through which a number of prostanoids may be acting has received considerable support. Since BAY u3405 did not alter histamine contractions in bronchial preparations nor endothelin curves in arterial tissues, these data suggest that there is no antagonism other than at the site associated with prostanoid contraction. BAY u3405 is equipotent at the TP site in both airway and pulmonary arterial muscle preparations. The contractile prostaglandins PGD_2 and $PGF_{2\alpha}$ were markedly inhibited by this compound in both types of smooth muscle preparations while the relaxation induced by PGI_2 in human pulmonary arterial preparations or by Butaprost in human proximal bronchial preparations were unaltered. These data are in agreement with previously published results (McKenniff *et al.*, 1988; Armour *et al.*, 1989; Lumley *et al.*, 1989) and indicate that those sites associated with prostanoid contraction or

relaxation in smooth muscle preparations from the human lung may be distinct as has already been suggested for guinea-pig airway preparations (Gardiner & Collier, 1980). The results presented here also demonstrate that BAY u3405 is a potent contractile prostanoid antagonist on human isolated bronchial and pulmonary arterial muscle preparations and therefore may be a useful tool and/or therapeutic agent for understanding the role of these prostanoids in airways disease.

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BAY u3405 an antagonist of thromboxane A_2- and prostaglandin D_2-induced bronchoconstriction in the guinea-pig

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1 The novel thromboxane (TX) antagonist, BAY u3405, has been evaluated against bronchoconstriction induced by the TXA_2 mimetic U-46619, prostaglandin D_2 (PGD_2), 5-hydroxytryptamine (5-HT), leukotriene D_4 (LTD_4) and histamine in the guinea-pig *in vivo* by use of a modification of the model described by Konzett & Rössler.

2 When given intravenously (i.v.) at 30 or 100 $\mu g\,kg^{-1}$, U-46619 caused 80% maximal bronchoconstriction in most animals. In contrast, PGD_2 caused a smaller 40%–50% maximal bronchoconstriction at the highest dose tested (300 $\mu g\,kg^{-1}$, i.v.).

3 BAY u3405, given intravenously, orally (p.o.) or by aerosol antagonized U-46619-induced bronchoconstriction in a dose-related manner. The approximate ID_{50} values were 600 $\mu g\,kg^{-1}$, i.v., 1.7 $mg\,kg^{-1}$ p.o. and 0.1% w/v 20 breaths by aerosol.

4 BAY u3405 had similar inhibitory activities against U-46619-induced bronchoconstriction and hypertension suggesting that it had no preferential activity on the airways.

5 When given intravenously BAY u3405 antagonized the bronchoconstrictor effect of intravenous PGD_2 with ID_{50} values between 30–100 $\mu g\,kg^{-1}$.

6 The action of BAY u3405 (10 $mg\,kg^{-1}$, p.o.) was long lasting, causing significant inhibition of U-46619-induced bronchoconstriction 7 h after dosing.

7 At 1 $mg\,kg^{-1}$, i.v., a dose that abolished the response to U-46619 and PGD_2, BAY u3405 had no effect on histamine-, 5-HT- or LTD_4-induced bronchoconstriction.

8 BAY u3405 potently and selectively antagonized U-46619- or PGD_2-induced bronchoconstriction in the Konzett-Rössler model of guinea-pig lung function. It should therefore prove to be a useful tool for defining the role of TXA_2 and PGD_2 in airway diseases such as asthma.

Keywords: BAY u3405; guinea-pig lung; U-46619; PGD_2 broncoconstriction; blood pressure; thromboxane antagonist

Introduction

Asthma is a disease characterized by a number of distinct symptoms including acute bronchospasm and an increased sensitivity to inhaled spasmogens; 'bronchial hyperresponsiveness'. Although the profile of symptoms is well defined, there is much speculation about the mediators which cause them.

Since the discovery of cyclo-oxygenase products of arachidonic acid, weight has been added to the theory that they are implicated as mediators in the pathogenesis of asthma. This is based on the following three sets of findings: (a) the profile of mediators released from the lung, (b) the biological activity of those mediators and (c) the blockade of action of those mediators by antagonists and inhibitors.

Increased levels of metabolites of thromboxane A_2 (TXA_2) have been measured in the plasma and urine following antigen provocation in asthmatics (Green *et al.*, 1974; Shepherd *et al.*, 1985; Sladek *et al.*, 1990) and increased prostaglandin D_2 (PGD_2) and TXA_2 levels have been observed in bronchoalveolar lung fluid obtained following antigen challenge (Murray *et al.*, 1986; Wenzel *et al.*, 1989; Liu *et al.*, 1990). Challenge of sensitized human lung tissue *in vitro*, with antigen, causes the release of large amounts of PGD_2 with smaller quantities of TXA_2 (Steel *et al.*, 1979; Schulman *et al.*, 1981; 1982). The exact site of release of these mediators in the lung remains unclear, although mast cells are known to release PGD_2 in large quantities and macrophages and platelets release abundant amounts of TXA_2.

The contractile activity of TXA_2 and PGD_2 on airway tissues is well documented (Coleman *et al.*, 1980; Black *et al.*, 1986; McKenniff *et al.*, 1988; Giles & Leff, 1988). The metabolite of PGD_2 (9α, 11β-PGF_2) also has potent contractile activity on human and guinea-pig airways *in vitro* (Featherstone *et al.*, 1990) and by inhalation in man (Beasley *et al.*, 1987). In asthmatics, inhaled PGD_2 is 10–30 times more potent than histamine and causes bronchoconstriction and coughing at approximately a 100 fold lower concentration than in normal subjects exemplifying the 'bronchial hyperresponsiveness' characteristic of this disease (Hardy *et al.*, 1984).

In recent years many groups have become interested in airways hyperresponsiveness and a number of animal models have been established to investigate this phenomenon. By use of data generated in these models, it has been suggested that TXA_2 may be responsible for airways hyperresponsiveness following a variety of stimuli. These include ozone (Aizawa *et al.*, 1985), allergen exposure (Chung *et al.*, 1985), leukotriene B_4 (LTB_4, O'Byrne *et al.*, 1985), or PAF (Chung *et al.*, 1986a) in dogs. Such effects have been reversed with the thromboxane synthase inhibitor OKY-046 (Chung *et al.*, 1986b). OKY-046 has also been shown to improve bronchial hyperresponsiveness in asthmatics (Fujimura *et al.*, 1986). Letts & McFarlane (1988) have demonstrated that exposure of monkeys to the TXA_2-mimetic, U-44069, conferred a 10 fold increase in sensitivity to inhaled acetylcholine. This effect was reversed by treatment with the TXA_2 receptor antagonist L-655, 240. Brunelli *et al.* (1987) have suggested that PGD_2 induces hyperreactivity to acetylcholine in the guinea-pig *in vivo*. Although thromboxane receptor-mediated events appear to be involved in this phenomenon the mechanism for underlying airways hyperresponsiveness is poorly understood.

It is apparent that a potent and selective TXA_2 receptor antagonist would be a useful tool to determine the role of these mediators in asthma. BAY u3405 has been shown to be such an antagonist on a wide range of airway preparations *in vitro* (McKenniff *et al.*, 1991). In this paper we describe the results of a series of studies which demonstrate that BAY u3405 when given orally, intravenously or by aerosol is a

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potent, long acting and selective antagonist of U-46619- or PGD_2-induced bronchoconstriction in the anaesthetized guinea-pig.

Methods

Lung volume recording

Male Dunkin Hartley guinea-pigs (300–450 g) were anaesthetized with sodium pentobarbitone 120 mg kg^{-1}, i.p. sufficient to induce anaesthesia and abolish natural respiration. The trachea was cannulated and the cannula connected to a Harvard small animal pump ventilating at 60 breaths per min. Lung volume was measured by the method of Konzett & Rössler (1940) as modified by Gardiner (1971). An external jugular vein was cannulated for drug administration. Arterial blood pressure was measured in some experiments by means of a carotid artery cannula attached to a Bell and Howell pressure transducer and recorded on a Grass Polygraph recorder. At the end of each experiment the maximal lung volume (designated 100%), was established by clamping the trachea, thus allowing the full stroke volume to pass into the overflow chamber. A decrease in lung volume was taken to indicate bronchoconstriction and the size of each response was expressed as a percentage of maximum overflow.

In experiments where the test compound was administered by aerosol, an aerosol generator (Devilbiss 'Pulmosonic') was introduced into the system as described by Lees & Payne (1985).

Experimental design

For each study, 24 animals were assigned to four treatment groups in a randomized fashion. Each animal received vehicle or treatment at a designated time before the start of U-46619 or PGD_2 administration. Twelve animals were tested on each day and where studies lasted two days the treatments were evenly divided between the two days. Where treatments were given orally animals were fasted overnight.

Protocols

Intrinsic activity A number of TXA_2 antagonists have been reported to have intrinsic bronchoconstrictor and hypertensive activity so BAY u3405 was tested in this model for these actions. BAY u3405 was administered intravenously over the dose range 0.01–10 mg kg^{-1} using a half log dosing regimen with 5 min between each dose. A vehicle-treated group received the vehicle at 5 min intervals corresponding to the time of drug administration.

Antagonism of U-46619 or prostaglandin D_2 Bronchoconstriction was induced by the intravenous administration of U-46619 or PGD_2. Each agonist was administered sequentially in half log increments with maximal recovery allowed between each dose. For U-46619 the dose range was 0.1–100 μg kg^{-1} and for PGD_2 10–300 μg kg^{-1}. BAY u3405 was given intravenously, orally or by aerosol for 20 breaths; 20 min, 60 min or 5 min (respectively) before the start of the U-46619 administration. It was given i.v. 20 min before the start of the PGD_2 administration.

Selectivity Investigations of selectivity were made with the agonists, histamine, 5-hydroxytryptamine (5-HT) and leukotriene D_4 (LTD_4). Histamine and 5-HT were administered over the dose range 1–10 μg kg^{-1}, i.v., with maximal recovery allowed between each dose. LTD_4 was administered over the dose-range 0.1–10 μg kg^{-1}, i.v. in the presence of indomethacin (10 mg kg^{-1}) and sotalol (1 mg kg^{-1}) given i.v. BAY u3405 (1 mg kg^{-1}) was given i.v. 20 min before the start of histamine, 5-HT or LTD_4 administration.

Statistical analysis of data

Using computerised regression analysis based on Tallarida & Murray (1981), the ED_{50} for U-46619 was determined for each animal in the control and treatment groups. The ED_{50} was designated the dose which caused 50% maximal bronchoconstriction. The dose of BAY u3405 calculated to reduce this response by 50% was designated the ID_{50}. Comparisons of ED_{50} values obtained in selectivity studies were made by Student's t test.

For analysis of the inhibitory activity of BAY u3405 against the agonist, one-way analysis of variance was used at each level of U-46619 or PGD_2 for each parameter. If the overall treatment effect was statistically significant ($P \leq 0.05$), then the control group was compared with each treated group by Student's t tests with a pooled estimate of variance from the ANOVA. Where the treatment effect was not significant, no further comparisons were made.

Materials

BAY u3405 (3(**R**)-[[(4-flurophenyl)sulphonyl]amino-1,2,3,4-tetrahydro-9H-carbazole-9-propanoic acid]) was supplied by Dr U. Rosentreter (Institute of Chemistry, Bayer AG, Wuppertal, Germany). It was dissolved by addition of 1 M NaOH followed by saline. The pH was adjusted to neutrality by the addition of 1 M HCl. The final volume was made up with physiological saline (0.9% w/v).

Drugs were obtained from the following suppliers: PGD_2, U-46619 (11α,9α-epoxymethano-prostaglandin $F_2\alpha$) indomethacin, histamine acid phosphate and 5-hydroxytryptamine (Sigma, Poole, Dorset); LTD_4 (Dr T.S. Abram, Bayer U.K.). All salts used were of AnalaR grade. Sotalol was a gift from Bristol-Myers.

Results

Intrinsic activity

BAY u3405 (0.01–10 mg kg^{-1}, i.v.) caused no bronchoconstriction, nor did it cause any hypertension over this dose-range (Figure 1a and b).

In the vehicle-treated group an increase in overflow volume was recorded over the observation period. BAY u3405 significantly reduced this increase in overflow volume at a dose of 300 μg kg^{-1} and above. The reversal observed with the 1 mg kg^{-1} dose was not statistically significant at the 95% level ($P = 0.061$).

Over the same observation period, BAY u3405 caused no hypertension. Large variation in responses to BAY u3405 or vehicle administration was observed with animals yielding a mild increase or decrease in mean blood pressure with each injection. The mean blood pressure tended to increase over the observation period but two animals exhibited a transient hypotension when BAY u3405 was injected at 10 mg kg^{-1}. Due to the variability of response in the vehicle and treatment groups there was no statistically significant difference between them ($P > 0.05$).

BAY u3405 – antagonism of U-46619

Inhibitory potency

Intravenous U-46619 caused a dose-related increase in lung overflow volume and an increase in mean systemic blood pressure. The administration of BAY u3405 caused a dose-related reduction in these responses (Figure 2a and b). At 1 mg kg^{-1} BAY u3405 caused a statistically significant inhibi-

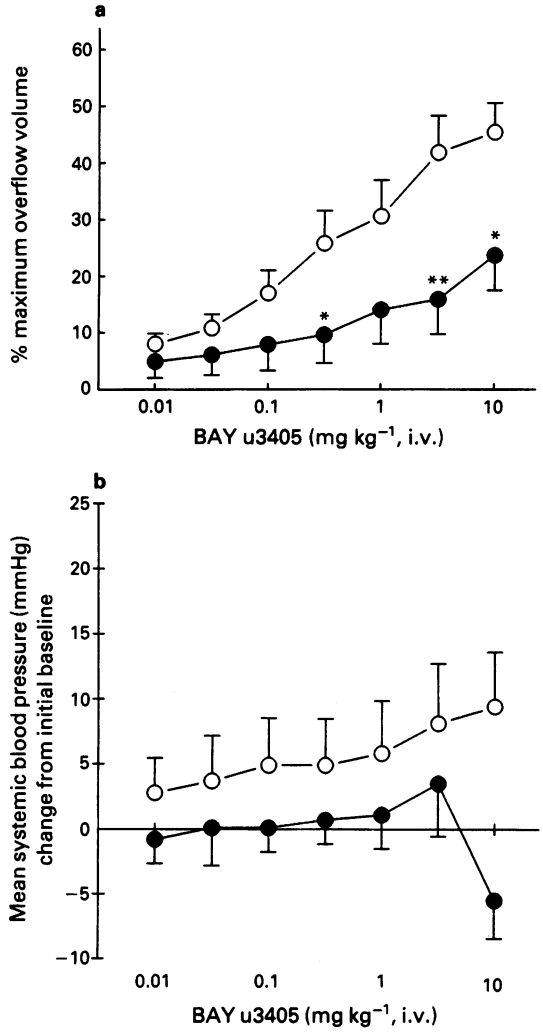


Figure 1 Effect of intravenous BAY u3405 on (a) lung overflow volume or (b) mean systemic blood pressure. Animals were given either vehicle (○) or BAY u3405 (●) cumulatively at 5 min intervals. BAY u3405 was given over the dose-range 0.01–10 mg kg^{-1} and vehicle was given at the corresponding time points. Each point represents the mean (s.e.mean shown by vertical bars) of $n = 6$. Asterisks denote statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$.

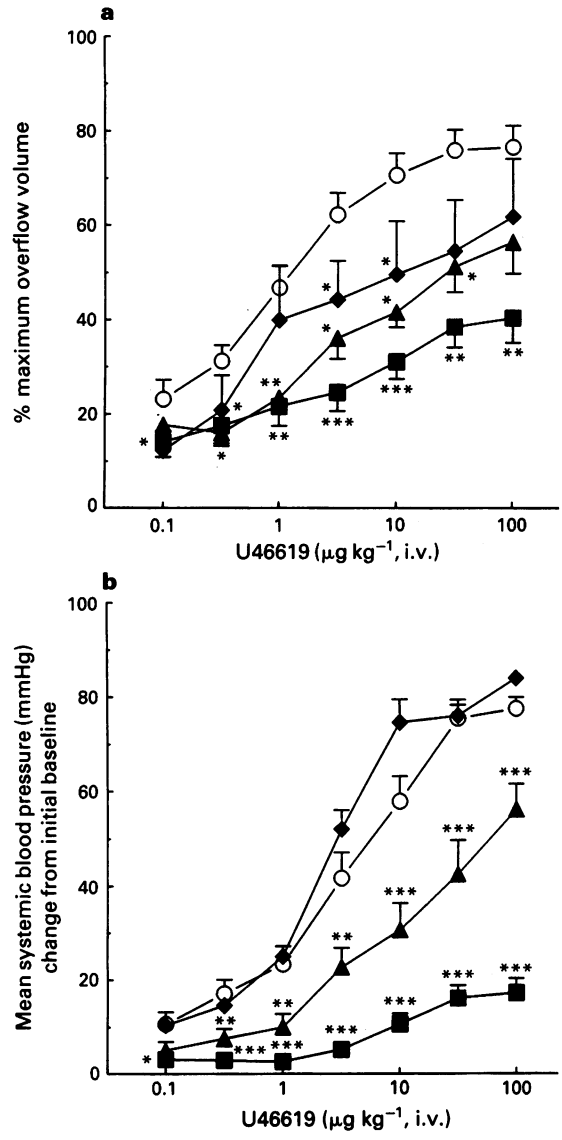


Figure 2 Effect of intravenous BAY u3405 on U-46619-induced changes in (a) lung overflow volume or (b) increase in mean systemic blood pressure. Animals were given either vehicle (○) or BAY u3405 (◆) 10 μg kg^{-1}, (▲) 100 μg kg^{-1} or (■) 1 mg kg^{-1} i.v. 20 min before the start of the U-46619. Each point represents the mean (s.e.mean shown by vertical bars) of $n = 6$. Asterisks denote statistical significance: $^{*}P > 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

tion of the bronchoconstrictor and hypertensive effects of U-46619, at all doses of the agonist tested. When given at 100 μg kg^{-1}, a significant inhibition of the bronchoconstriction was observed against doses of U-46619 up to 30 μg kg^{-1} i.v. and of the hypertensive effects at all doses of U-46619 given. The approximate ID_{50} for the effect on the airways was 600 μg kg^{-1}.

At 3 μg kg^{-1} U-46619 caused an approximate doubling of mean systemic blood pressure which was reduced by 50% by 100 μg kg^{-1} BAY u3405. Conversely the lower dose of 10 μg kg^{-1} had no statistically significant effect on the hypertension ($P > 0.05$), whilst exerting some inhibition of the airway response to 3 or 10 μg kg^{-1} U-46619 ($P < 0.05$).

Oral When given via the oral route BAY u3405 also caused a dose-related reduction in the bronchconstrictor response to U-46619 (Figure 3a). Blood pressure was not measured routinely on this occasion but it was recorded in a single experiment where BAY u3405 was given at 10 mg kg^{-1} p.o. 60 min before the start of the U-46619 dose–response curve (Figure 3b). BAY u3405, 10 mg kg^{-1}, p.o., caused a marked, and statistically significant reduction in the bronchoconstriction induced by all doses of U-46619 up to 100 μg kg^{-1}, and a reversal of the hypotension, abolishing the effect of doses up to 10 μg kg^{-1} U-46619, and attenuating those to 30 or 100 μg kg^{-1}. The lower dose of 1 mg kg^{-1} caused a small reduction in the bronchoconstrictor response to U-46619 which was only statistically significant against 10 μg kg^{-1} U-46619 ($P < 0.05$); 100 μg kg^{-1} had no statistically significant effect. The approximate ID_{50} against the bronchoconstrictor effect was 1.7 mg kg^{-1}.

Aerosol BAY u3405 1% w/v for 20 breaths, caused a profound and statistically significant reduction in the bronchoconstrictor response to U-46619, with very little response apparent even at the highest dose of 100 μg kg^{-1}. A 10 fold reduction in the nebuliser concentration of BAY u3405 to 0.1% w/v caused less inhibition, but the response to U-46619 was still attenuated by approximately 50% and was statistically significant. A further reduction to 0.03% w/v caused little inhibition of the response to U-46619 (Figure 4a). When given via this route BAY u3405 had little effect on the hypertensive response to U-46619. Only 1% w/v BAY u3405 caused any statistically significant reduction in the change in mean systemic blood pressure, although at 0.1% w/v a small but

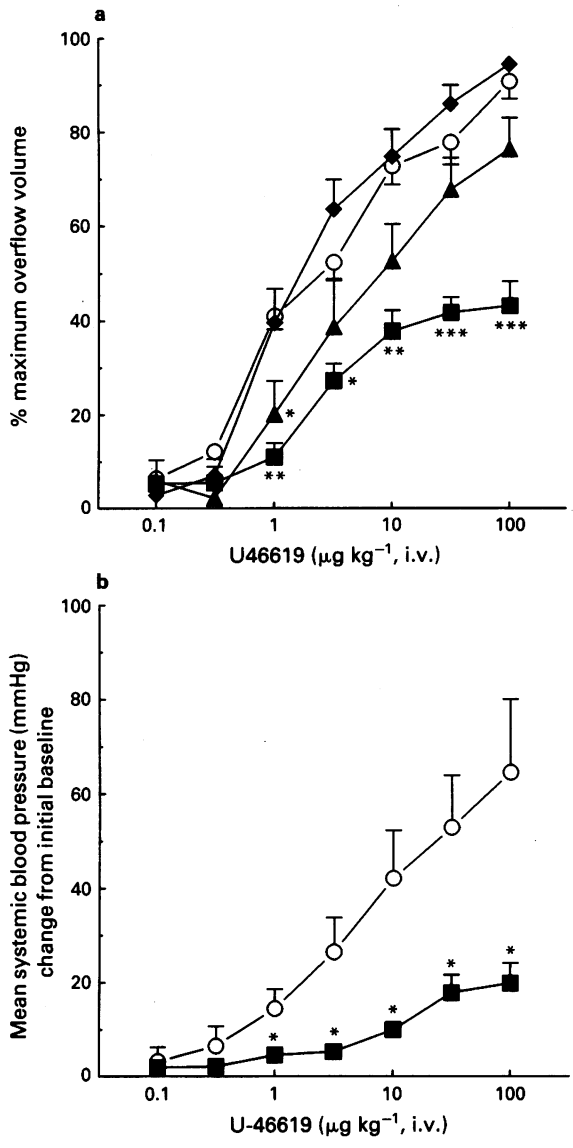


Figure 3 Effect of oral BAY u3405 on U-46619-induced changes in (a) lung overflow volume or (b) increases in mean systemic blood pressure. Animals were given either vehicle (○) or BAY u3405 (◆) 100 $\mu g\,kg^{-1}$, (▲) 1 $mg\,kg^{-1}$ or (■) 10 $mg\,kg^{-1}$ p.o. 60 min before the start of U-46619. Each point represents the mean (s.e.mean shown by vertical bars) of $n = 6$. Asterisks denote statistical significance: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

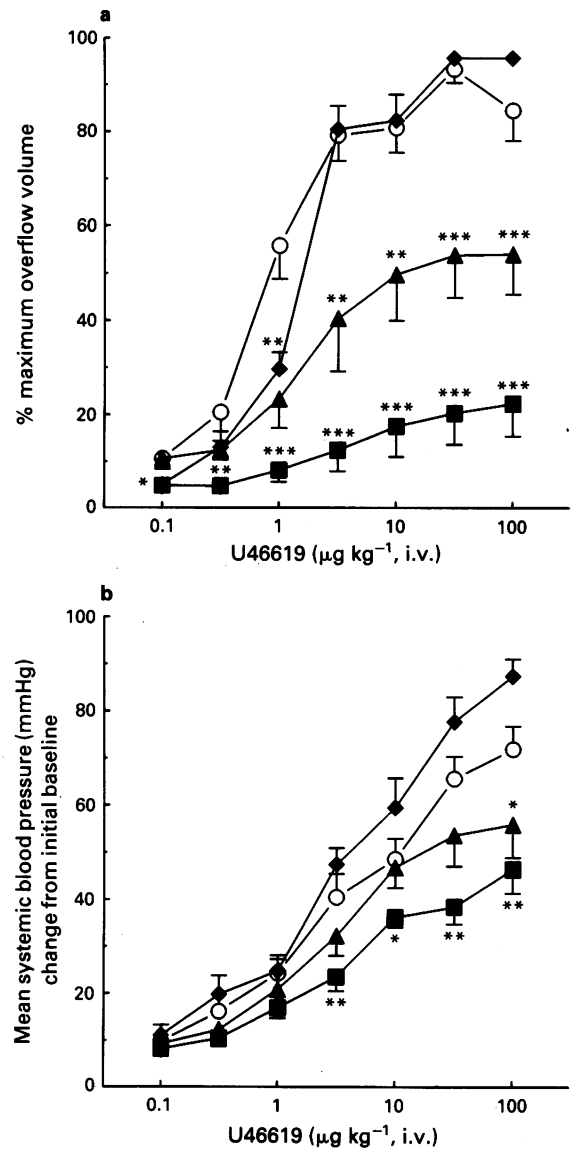


Figure 4 Effect of aerosol BAY u3405 on U-46619-induced changes in (a) lung overflow volume or (b) increases in mean systemic blood pressure. Animals were given either vehicle (○) or BAY u3405 (◆) 0.03% w/v, (▲) 0.1% w/v or (■) 1% w/v by aerosol 5 min before the start of U-46619. Each point represents the mean (s.e.mean shown by vertical bars) of $n = 6$. Asterisks denote statistical significance: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

significant reversal of the response to the highest dose of U-46619 was observed (Figure 4b).

Duration of action

BAY u3405 was given at a dose of 10 $mg\,kg^{-1}$, p.o., 1, 4 or 6 h before U-46619. One hour after treatment, a statistically significant reduction in the response to all doses of U-46619 was observed. The inhibition appeared to be reduced 4 h after dosing, but remained statistically significant. Little change in inhibition was seen 6 h after dosing and the responses remained significantly reduced (Figure 5).

BAY u3405-antagonism of prostaglandin D_2

When administered i.v., PGD_2 caused a dose-related bronchoconstriction. The response was biphasic with a rapid initial constriction followed by a partial recovery then a smaller, slower response. The peak height of the initial response was recorded and used in data analysis. The dose of 300 $\mu g\,kg^{-1}$ PGD_2 caused a maximal bronchoconstriction which was approximately 50% of the total lung volume.

BAY u3405 over the dose range 30–300 $\mu g\,kg^{-1}$ i.v. caused a dose-related reduction in the response to PGD_2 (Figure 6). At 30 $\mu g\,kg^{-1}$ i.v., BAY u3405 had little inhibitory effect but appeared to enhance the response to low doses of PGD_2;

Table 1 Effect of BAY u3405 (i.v.) on bronchoconstriction induced by non-prostanoid agonists

Bronchoconstrictor (i.v.)	ED_{50} ($\mu g\,kg^{-1}$) *Control*	*BAY u3405*	P *value*
Histamine	7.01 ± 0.95	7.17 ± 0.61	NS
5-Hydroxytryptamine	4.97 ± 0.62	4.76 ± 0.96	NS
Leukotriene D_4	1.57 ± 0.34	1.05 ± 0.36	NS

Each value denotes the mean (±s.e.mean) of 6–8 determinations for each agonist with or without prior treatment with BAY u3405 1 $mg\,kg^{-1}$ (i.v.). NS signifies no statistically significant difference between the BAY u3405-treated group and the corresponding control ($P > 0.05$) Student's t test.

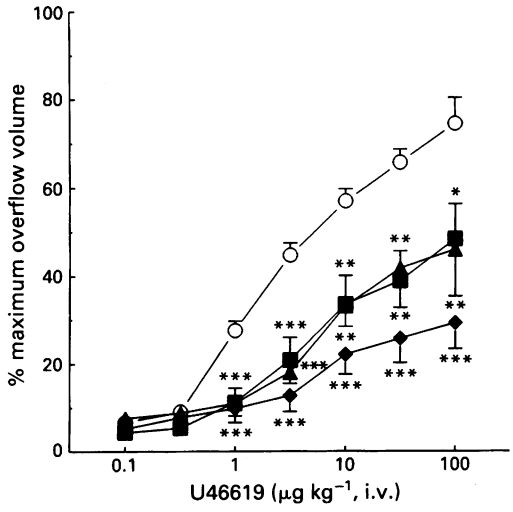


Figure 5 Duration of action of BAY u3405 10 mg kg^{-1} p.o. against U-46619-induced changes in lung overflow volume. Animals were given vehicle (○) or BAY u3405 1 h, (◆) 4 h (▲) or 6 h (■) before the start of U-46619. Each point represents the mean (s.e.mean shown by vertical bars) of $n = 6$. Asterisks denote statistical significance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

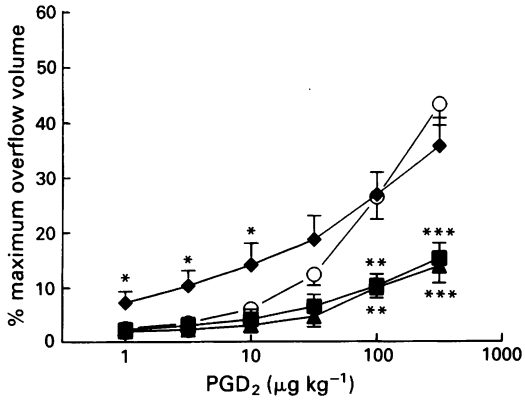


Figure 6 Effect of intravenous BAY u3405 on prostaglandin D_2 (PGD_2)-induced changes in lung overflow volume. Animals were given either vehicle (○) or BAY u3405 (◆) 30 μg kg^{-1}, (▲) 100 μg kg^{-1} or (■) 300 μg kg^{-1} i.v. 20 min before the start of the PGD_2. Each point represents the mean (s.e.mean shown by vertical bars) of $n = 6$. Asterisks denote statistical significance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

100 μg kg^{-1} reduced the response to PGD_2 at all doses. The higher dose of BAY u3405 300 μg kg^{-1} had no greater effect. However, only the reduction in the response to 100 and 300 μg kg^{-1} PGD_2 was statistically significant. From these data the approximate ID_{50} for BAY u3405 against PGD_2-induced bronchoconstriction appears to lie between 30 and 100 μg kg^{-1} i.v.

Selectivity studies

A 1 mg kg^{-1}, i.v. dose of BAY u3405, which abolished the response to U-46619 and PGD_2, was tested against histamine-, LTD_4- and 5-HT-induced bronchoconstriction. It had no effect against any of these agonists (Table 1).

Discussion

In the studies described above we have demonstrated that BAY u3405 is a potent and selective antagonist of both U-46619 and PGD_2-induced bronchoconstriction in the anaesthetized guinea-pig. Unlike some TXA_2 antagonists (AH23848, Humphrey *et al.*, 1986 and BM13505, Greenham, unpublished observations), BAY u3405 does not itself cause hypertension or bronchoconstriction in this model.

Over the observation period in the intrinsic activity studies, it became clear that ventilation of the lungs alone caused a steady decrease in lung volume (Figure 1). In the BAY u3405-treated group this trend was not apparent, suggesting that the compound reversed this effect. In 1973 Palmer *et al.* reported that mechanical stimulation of guinea-pig lungs led to the release of 'Rabbit aorta constricting substance' which was later isolated and termed thromboxane A_2. It is probable that with the Konzett-Rössler preparation, the mechanical ventilation of the lungs causes the release of TXA_2 which contributes to the progressive decrease in lung volume. This effect was reversed by the thromboxane antagonist, BAY u3405. It is unclear which mechanism underlies this decrease in lung volume, be it bronchoconstriction, fluid or mucus accumulation or oedema, but it is clear that this effect is reversed by BAY u3405.

BAY u3405 not only has effects against putative endogenously released TXA_2, but also against the administered agonists U-46619 and PGD_2. Given by a number of routes, BAY u3405 antagonizes the bronchoconstriction and hypotensive effects of U-46619.

Comparison of the ID_{50} doses of BAY u3405 for inhibition of U-46619-induced bronchoconstriction obtained after i.v. or oral dosing, demonstrated a difference of approximately 3 fold. This small difference suggests good bio-availability after oral dosing for this compound in the guinea-pig, as has been reported in man (Weber *et al.*, unpublished observations).

Although BAY u3405 appears to have a half life of less than 1 h in man, it exhibits inhibitory activity, as assessed by *ex vivo* inhibition of U-46619-induced platelet aggregation, up to 10 h after dosing (Weber *et al.*, unpublished observation). In the guinea-pig, maximal inhibition of the bronchoconstrictor response was seen 1 h after oral dosing, but after 6 h significant inhibition was still apparent. Since each U-46619 dose-response curve took approximately 60 min to complete, the responses to the higher doses were evaluated an additional hour after the initial dosing with BAY u3405. Thus BAY u3405 had good antagonist activity some 7 h after dosing.

BAY u3405 not only antagonized the bronchoconstrictor effects of U-46619 but also reversed the hypertension induced by this mediator. High doses of U-46619 caused an approximate trebling of the mean systemic blood pressure which was initially low in this model due to the action of the barbiturate anaesthetic. The approximate ID_{50} values of BAY u3405 against U-46619-induced bronchoconstriction and hypertension were of the same order, suggesting that the drug had no preferential activity on the respiratory or cardiovascular system. When given by aerosol, BAY u3405 had potent activity against the bronchoconstriction caused by U-46619 but had a reduced effect against its hypertensive action. This difference in potency reflects the high local concentration of BAY u3405 in the lungs obtained via this route; only the highest aerosol concentration (1% w/v) had any observable effect on the blood pressure.

When given intravenously, PGD_2 was a weaker bronchoconstrictor than U-46619 in this model. This is in agreement with its reported weak activity in the airways *in vitro* (Coleman *et al.*, 1980; Featherstone *et al.*, 1990) and *in vivo* (Hamid-Bloomfield *et al.*, 1989). In our studies PGD_2 appears 500–1000 times less potent at causing bronchoconstriction than U-46619, a difference in the same range as that reported by Coleman *et al.* (1981) and Featherstone *et al.* (1990). Hamid-Bloomfield *et al.* (1990) reported very little bronchoconstrictor activity of PGD_2 in the guinea-pig using the Dixon and Brodie model. The Konzett-Rössler model works on a slightly different principle from the Dixon and Brodie model: (changes in volume at a constant pressure as opposed to changes in pressure at a fixed volume) and this may explain

the slightly greater responses which we observed. BAY u3405 had potent activity against PGD_2 in this model, inhibiting the response with an ID_{50} some 6 to 20 fold lower than that required to reverse the U-46619-induced effects. It is not clear why this should occur, but it is possible that the weaker agonism of PGD_2 at the TXA_2 receptor is more easily reversed than that of the higher affinity agonist U-46619.

It is clear that BAY u3405 was very effective against the bronchoconstriction induced by both U-46619 and PGD_2, strengthening suggestions from *in vitro* studies that both mediators activate the TXA_2 receptor. This does not however reflect a non-specific effect of BAY u3405 as it had no effect on histamine-, 5-HT- or LTD_4-induced bronchoconstriction.

Recent reports suggest that the TXA_2 antagonist GR 32191 had only a small effect against antigen challenge in asthmatics (Beasley *et al.*, 1989). However, at the same dose it also had variable activity against PGD_2-induced bronchoconstriction suggesting that it may have been an inappropriate drug tool to investigate the role of TXA_2 or PGD_2 in antigen-induced asthma. Although the limited activity of GR 32191 may simply reflect low potency, it might alternatively reflect limited activity against all TXA_2 receptor subtypes. McKenniff *et al.* (1988, 1991) have suggested that subtypes of TXA_2 receptors exist in airway smooth muscle. They have demonstrated that some TXA_2 antagonists, including GR 32191, were not equipotent at all such subtypes. It is possible that high antagonist potency at all TXA_2 subtypes is essential for a drug to clarify the role of TXA_2 and PGD_2 in asthma. It is probable that BAY u3405 is such a drug as it has been shown to have good activity at all TXA_2 receptor subtypes (McKenniff *et al.*, 1991).

In summary, we have shown that BAY u3405 is a potent, long acting and selective TXA_2 antagonist administered by a number of routes in the guinea-pig. It may therefore by the most appropriate drug to probe the role of TXA_2 and PGD_2 in airway diseases such as asthma.

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The change in the threshold for short-term desensitization in isolated smooth muscle cells showing an all-or-none response to acetylcholine

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1 Isolated smooth muscle cells from guinea-pig taenia caecum were prepared by collagenase digestion. The cells showed an all-or-none response to acetylcholine (ACh) under our experimental conditions.

2 Desensitized cells showed an all-or-none response but required a higher concentration of ACh for induction of contraction, indicating that the desensitization was due to a change in the threshold concentration.

3 In [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) binding to the desensitized cells, K_D and B_{max} were not significantly different from those estimated in the control cells. The competitive inhibition curve for specific binding of [^{3}H]-QNB by ACh in the desensitized cells was in agreement with that of control cells.

4 The ACh-stimulated increase of the $^{45}Ca^{2+}$ influx was very rapid and correlated well with the contraction of the cells. The concentrations of ACh inducing the maximal $^{45}Ca^{2+}$ influx were increased by desensitization.

5 These results indicated that although the binding of ACh to the receptor was not changed by desensitization, the threshold concentration of ACh for their contraction was raised by desensitization, and the $^{45}Ca^{2+}$ influx accompanying the contraction was shifted to the side of high concentration of ACh.

6 These results suggest that the development of short-term desensitization is due to an uncoupling of the receptor from the mechanism for initiation of the contraction.

Keywords: Desensitization; smooth muscle cells; taenia caecum; acetylcholine; Ca^{2+} influx; muscarinic receptor

Introduction

Desensitization of the muscarinic response seems to occur in two phases, short-term desensitization within minutes (Mita & Uchida, 1987; Mita *et al.*, 1988; Hishinuma & Uchida, 1988; Horio *et al.*, 1990; Leurs *et al.*, 1990) and long-term desensitization within hours (Higuchi *et al.*, 1982). In general, a decrease in muscarinic acetylcholine (ACh) receptors was observed in long-term desensitization because degradation of these receptors through clustering and endocytosis are accelerated.

Short-term desensitization of smooth muscle to acetylcholine (ACh) was first observed by Cantoni & Eastman (1946). Short-term desensitization as an inductory phase of long-term desensitization has been studied (Triggle, 1980; Siegel *et al.*, 1984), but its mechanism is still unknown, compared with that of long-term desensitization. Previously we found that isolated smooth muscle cells from guinea-pig taenia caecum showed short-term desensitization to ACh when incubated with 10^{-4} M ACh for 10 min (Mita & Uchida, 1987). Single smooth muscle cells isolated by our mild procedure show an all-or-none response to ACh. These desensitized cells also showed an all-or-none response but required a higher concentration of ACh for induction of contraction: i.e. the magnitude of their response was not changed appreciably ('All') but the threshold concentration of ACh for this contraction was increased ('None'). The change in the threshold for excitability resulting in change in sensitivity is a general character of excitable cells. This short-term desensitization of isolated smooth muscle cells was also induced in the absence of external Ca^{2+} (Mita *et al.*, 1988). It was suggested that short-term desensitization was induced before massive influx of Ca^{2+}.

Isolated smooth muscle cells have been used to simplify analyses of the mechanism of muscle contraction (Bagby *et al.*, 1971; Fay & Singer, 1977; Bitar *et al.*, 1979; Momose *et al.*, 1981; Hasegawa *et al.*, 1987; Koike *et al.*, 1990). Isolated smooth muscle cells have little extracellular space and are therefore very useful for monitoring transmembrane ion movements (Momose *et al.*, 1983; Scheid & Fay, 1984; Bitar *et al.*, 1986) and for studying drug-receptor interactions (Seidel & Johnson, 1983; Takayanagi *et al.*, 1986; Nakazawa *et al.*, 1987) because their use avoids the complexities of intact tissue preparations. Previously we also reported that in isolated smooth muscle cells showing an all-or-none response to ACh, the sensitivity of the ACh receptor of smooth muscle cells was the same as that of whole tissue and ACh bound to its receptor in a concentration-dependent manner (Mita & Uchida, 1988). Moreover, the contraction of smooth muscle cells in response to ACh depended on the influx of extracellular Ca^{2+}, and the ACh-stimulated increase of the Ca^{2+} influx was very rapid and correlated well with the contraction of the cells. We suggested that ACh binds to its receptor in a concentration-dependent manner, and that when the binding reached a certain threshold, a rapid influx of Ca^{2+} occurs and the cells show an all-or-none response. The concentration of ACh that allows the maximal $^{45}Ca^{2+}$ influx corresponded to this threshold level for the rapid Ca^{2+} influx in non-desensitized cells.

We have now studied the binding of a radiolabelled cholinolytic agent to muscarinic ACh receptors on isolated smooth muscle cells and directly monitored the ACh-stimulated influx of $^{45}Ca^{2+}$ into the cells after desensitization in relation to the all-or-none contractile response.

Methods

Isolation of cells

A suspension of isolated smooth muscle cells from guinea-pig taenia caecum was prepared by the method described previously (Mita & Uchida, 1987), which is a modification of the

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procedure of Momose *et al.* (1981). Briefly, strips of taenia caecum were minced and incubated with 0.1% collagenase in 2.0 ml of Ca^{2+}-free solution containing 0.2% trypsin inhibitor and 1.0% bovine serum albumin at 37°C for 80 min with gentle stirring. The suspension of cells was centrifuged at 120 *g*. The precipitate was suspended in 4 ml of Ca^{2+}-free solution and cells dispersed by pipetting the suspension several times with a wide-bore pipette. The suspension of single cells was filtered through a nylon mesh to remove undigested material. Then the cells were harvested, resuspended in Ca^{2+}-free solution and used for experiments within 180 min. The cell viability, measured by trypan blue exclusion, was more than 90%. The medium was HEPES-Tyrode solution composed of (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, glucose 5.6 and HEPES 4.2, pH 7.4 at 37°C (normal solution). The Ca^{2+}-free solution had the same composition except that $CaCl_2$ was omitted.

[^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) binding assay

[^{3}H]-QNB binding was measured by the procedure described previously (Mita & Uchida, 1988). Single cells were incubated with various concentrations of [^{3}H]-QNB in the reaction medium containing (mM): NaCl 137, $CaCl_2$ 1.8 and HEPES 4.2 (pH 7.4) at 30°C for 10 min. The incubation mixture was then filtered through a Whatman GF/C glass fibre filter and the filters were washed twice with ice-cold reaction medium. Specific binding was calculated as the difference between the total binding and the binding in the presence of 10^{-5} M atropine. The radioactivity remaining on the filter was counted in Triton/toluene-based scintillation fluid in a liquid scintillation counter (Packard TRI-CARB 4530). For determination of the concentrations of ACh that caused 50% inhibition of [^{3}H]-QNB binding (IC_{50}), the competitive antagonism between [^{3}H]-QNB (0.4 nM) and the drug was assessed. Protein was determined by the method of Lowry *et al.* (1951).

Measurement of $^{45}Ca^{2+}$ influx

$^{45}Ca^{2+}$ influx was measured by the procedure described previously (Mita & Uchida, 1988). The cells were suspended in normal HEPES-Tyrode solution (normal solution) containing $^{45}Ca^{2+}$ (1.5 μCi) at 30°C for 5 min. Various concentrations of ACh were then added and after exactly 3 s, ice-cold Ca^{2+}-free solution containing EGTA at a final concentration of 10 mM was added to remove extracellular Ca^{2+}. After 1 min, this suspension was passed through a glass fibre filter (Whatman GF/C), and the filter was promptly washed 3 times with ice-cold Ca^{2+}-free solution. The filter was dried and the radioactivity was counted in a liquid scintillation counter in a toluene based scintillator.

Measurement of contraction of single cells

Contraction of isolated smooth muscle cells was measured by the method of Mita & Uchida (1987). The cell suspension was placed on a silicon-coated glass slide with a cover slip, and the cells were perfused continuously with normal solution at 30°C. Then the perfusion fluid was changed successively to solutions containing increasing concentrations of ACh. The perfusion fluid was introduced from one side and was blotted with filter paper from the other side of the slide at a rate of about 0.7 ml min^{-1}. The silicon-coated glass slide was placed on a temperature-controlled stage (30°C) of a phase contrast microscope. The contraction of a cell was observed on video tape, and the size of the cell was measured in photographs.

Desensitizing treatment

In the [^{3}H]-QNB binding assay and the measurement of $^{45}Ca^{2+}$ influx, desensitization was induced by incubating the cell suspension with 10^{-4} M ACh for 10 min in Ca^{2+}-free solution. The cell suspension was then washed twice with Ca^{2+}-free solution by centrifugation at 120 *g*. The precipitate was suspended in each medium of the experiments. [^{3}H]-QNB binding and $^{45}Ca^{2+}$ influx were determined within 20 min after the end of the incubation. In the measurement of contraction of the cells, desensitization was induced by the procedure described previously (Mita *et al.*, 1988). After responses in normal solution (first contraction) had been recorded, the cells were washed with Ca^{2+}-free solution and then incubated with 10^{-4} M ACh for 10 min in Ca^{2+}-free solution. Then they were washed with Ca^{2+}-free solution and their response was determined in normal solution 10 min after the end of incubation (second contraction).

Drugs used

Collagenase (Type I) and soybean trypsin inhibitor (Type II-S) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bovine serum albumin (Fraction V) was obtained from Nakarai Chemicals (Kyoto, Japan). Acetylcholine (Ovisot) was from Daiichi Seiyaku Co. (Tokyo, Japan). 4-(2-Hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) and ethyleneglycol-*bis*-(β-aminoethylether)-N,N'-tetraacetic acid (EGTA) were from Dojindo Laboratories (Kumamoto, Japan). [^{3}H]-QNB and $^{45}CaCl_2$ were from New England Nuclear (Boston, MA, U.S.A.). All other chemicals were of reagent grade.

Results

A Scatchard plot of the specific binding of [^{3}H]-QNB to isolated smooth muscle cells gave a straight line and the mean values for the dissociation constant (K_D) and the maximum binding sites (B_{max}) were 0.18 ± 0.03 nM and 1.11 ± 0.10 pmol mg^{-1} protein (111.2 ± 16.9 fmol/10^4 cells). On the other hand, in [^{3}H]-QNB binding to the desensitized cells, K_D and B_{max} were 0.26 ± 0.05 nM and 1.19 ± 0.14 pmol mg^{-1} protein (166.0 ± 24.9 fmol/10^4 cells) (Table 1). These values were not significantly different from those estimated in the control cells. The Hill coefficient (nH) for [^{3}H]-QNB binding to the desensitized cells was calculated to be 0.99 ± 0.02 and was not significantly different from that of the control cells (0.94 ± 0.05).

Table 1 Characteristics of [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) binding to control cells and desensitized cells

	K_D (nM)	B_{max} (pmol mg^{-1} protein)	r
Control cells (n = 3)	0.18 ± 0.03	1.11 ± 0.10 (111.2 ± 16.9 fmol/10^4 cells)	0.938 ± 0.032
Desensitized cells (n = 4)	0.26 ± 0.05	1.19 ± 0.14 (166.0 ± 24.9 fmol/10^4 cells)	0.928 ± 0.023

Each value is presented as a mean ± s.e. n: number of experiments. K_D: the dissociation constant; B_{max}: the maximum binding sites; r: relative coefficient.

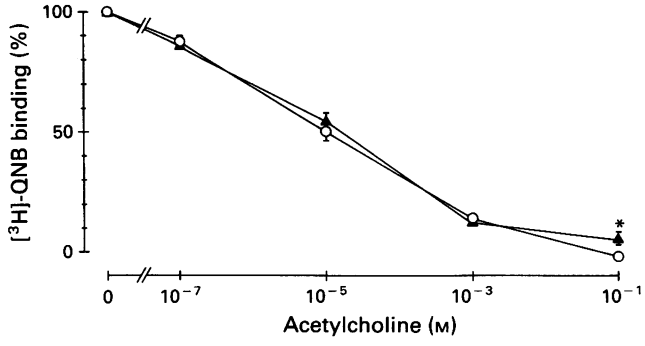


Figure 1 Inhibition of [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) binding to control cells and desensitized cells by acetylcholine. Points and bars are means and s.e. for 4 experiments and are percentages of the specific binding of [^{3}H]-QNB (0.4 nM) in the absence of ACh. (○) Control cells; (▲) desensitized cells. *Significantly different from the value for control cells ($P < 0.05$) by Student's t test for unpaired data.

Figure 1 shows the competitive inhibition curves of specific binding of [^{3}H]-QNB by ACh in the control cells and the desensitized cells. The specific binding of [^{3}H]-QNB in both the control and the desensitized cells was competitively inhibited by ACh in a concentration-dependent manner. The competitive inhibition curve in the desensitized cells was in agreement with that of control cells. Their 50% inhibition concentrations (IC_{50} values) were $1.21 \times 10^{-5} \pm 0.21 \times 10^{-5}$ M (control cells) and $1.00 \times 10^{-5} \pm 0.09 \times 10^{-5}$ M (desensitized cells), respectively, and were not significantly different from each other. In the control cells and the desensitized cells, ACh had nH of 0.42 ± 0.03 and 0.41 ± 0.02, respectively. Thus, neither the affinity nor the number of muscarinic ACh receptors was changed by short-term desensitization.

Figure 2 shows the change in the threshold concentration of ACh that induced an all-or-none response in the desensitized

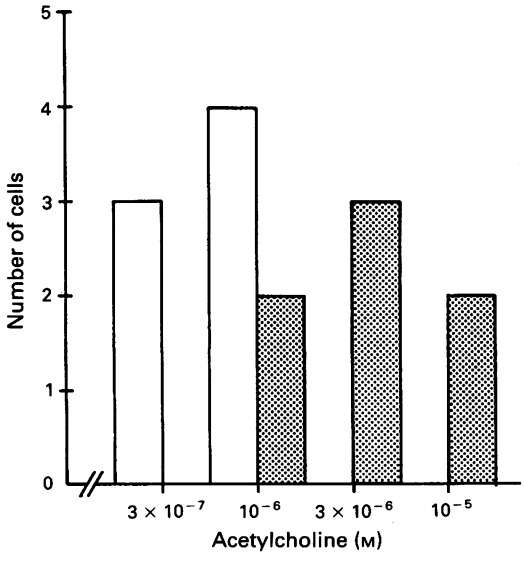


Figure 2 The change in the threshold concentrations of acetylcholine (ACh) that induced an all-or-none response in the desensitized smooth muscle cells. After the first contraction, desensitization was induced by incubating the cells with 10^{-4} M ACh for 10 min. The cell was then washed with HEPES-Tyrode solution and its response was examined 10 min after the end of incubation. The abscissa scale indicates the threshold concentration of ACh for an all-or-none response of each cell. The ordinate scale represents the number of cells showing an induced all-or-none response to ACh. Open columns indicate the first contraction and stippled columns indicate the second contraction of a desensitized cell in response to ACh.

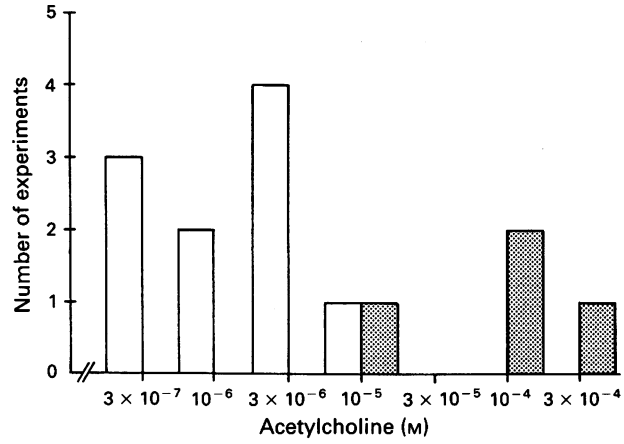


Figure 3 The change in the concentration of acetylcholine (ACh) that induced maximal $^{45}Ca^{2+}$ influx into the desensitized smooth muscle cells. The abscissa scale indicates the concentration of ACh that induced maximal $^{45}Ca^{2+}$ influx. The ordinate scale indicates the number of experiments. Open columns indicate the $^{45}Ca^{2+}$ influx into control cells and stippled columns indicate that into desensitized cells.

cells. Although the change in the threshold concentrations of ACh was different in each cell sample, these desensitized cells required a higher concentration of ACh to induce a contraction. The $^{45}Ca^{2+}$ influx stimulated by ACh was prompt and was correlated with the contraction of the cells (Mita & Uchida, 1988). Furthermore, in the desensitized cells, the concentration of ACh inducing the maximal $^{45}Ca^{2+}$ influx was higher than in control cells (Figure 3).

Discussion

We previously showed that isolated smooth muscle cells from guinea-pig taenia caecum showed an all-or-none response to ACh and that desensitized cells also showed an all-or-none response but required a higher concentration of ACh for induction of the contraction (Mita & Uchida, 1987). Incubation of the whole tissue with 10^{-4} M ACh for 10 min also caused desensitization (Mita & Uchida, 1987). This desensitization was accompanied by reduction of the contractile response at intermediate concentrations of ACh. The mode of desensitization of isolated smooth muscle cells determined from the average response of the isolated cells was almost the same as that of whole muscle. Hence, it was concluded that the desensitization occurred in each cell irrespective of its tissue organization and that the desensitization was due to an increase in the threshold for contraction by ACh of each cell.

In [^{3}H]-QNB binding to the desensitized cells, K_D and B_{max} values were not significantly different from those estimated in the control cells (Table 1). Moreover, the competitive inhibition curve for specific binding of [^{3}H]-QNB by ACh in the desensitized cells was in agreement with that of control cells. These results indicate that neither the affinity nor the total number of muscarinic ACh receptors of individual smooth muscle cells was changed by short-term desensitization. As a result, the binding properties of both the agonist and the antagonist to the muscarinic ACh receptor were not changed by short-term desensitization. On the other hand, Kagiya *et al.* (1986) found that carbachol-induced short-term desensitization of the muscarinic receptor of ventricular muscle was caused by reduction in the affinity of the receptor for the agonist without reduction in its number or affinity for the antagonist. The inhibition curves of [^{3}H]-QNB binding by carbachol was found to fit the three-site model, superhigh(SH), high(H) and low(L) affinity sites. This curve was shifted to the right in the desensitized membrane. In desensitized ventricular

muscle membrane, the populations of SH and H sites were decreased significantly and the populations of L sites were increased. These results indicated the possibility of the interconversion of the H site to the L site in carbachol-induced desensitization. Our data indicated that the nH of the Hill plot for ACh (0.42 ± 0.03) was significantly less than unity. This result suggests that ACh exhibits binding characteristics consistent with the presence of several binding sites. In fact, Shimada & Uchida (1988) reported that the displacement curve of [^{3}H]-QNB binding by carbachol shifted to the right after desensitizing treatment with carbachol for more than 30 min, but that K_D and B_{max} of [^{3}H]-QNB were not changed by desensitization. These results indicated the possibility of the interconversion of the H site to the L site on desensitizing treatment with carbachol for more than 30 min. However, our results showed that after desensitizing treatment with ACh for 10 min, the displacement curve by ACh did not shift to the right. Ten minutes was enough to cause desensitization of muscarinic ACh receptors in our experimental conditions, and further exposure to the agonist may have resulted in decreased receptor affinity. The results in ventricular muscle differ from our results, and our data show that ACh treatment leads to a decrease of neither the affinity nor the number of receptors. These differences probably come from differences in the preparations used in which a different mechanism of desensitization may be operating.

Recently phosphorylation of receptors, including α_1(Leeb-Lundberg *et al.*, 1985) and β-adrenoceptors (Sibley & Lefkowitz, 1985) and nicotinic (Huganir *et al.*, 1986) and muscarinic cholinoceptors (Kwatra & Hosey, 1986; Ho *et al.*, 1987), has been thought to be involved in desensitization. Ho *et al.*, (1987) found that phosphorylation of muscarinic ACh receptors was accompanied with a loss in specific binding to [^{3}H]-QNB. On the other hand, Kwatra & Hosey (1986) reported that phosphorylation of muscarinic ACh receptors was not accompanied by a reduction in muscarinic ACh receptor protein but a decrease in affinity of the receptor for the agonist. They suggested that receptor phosphorylation might increase as a result of receptor desensitization. Therefore, there is a possibility that a phosphorylated receptor has diminished ability to interact with the guanine nucleotide regulatory protein (Benovic *et al.*, 1986), but our results showed that ACh treatment led to a decrease of neither the affinity nor the number of receptors. After desensitization, the ability of the muscarinic receptors to stimulate phosphatidylinositol turnover was not reduced (Hishinuma & Uchida, 1989). Therefore, phosphorylation of receptors is not involved in short-term desensitization to ACh in taenia caecum.

Ca^{2+} is known to trigger the contraction of smooth muscle. Membrane excitation results in the entry of Ca^{2+} from extracellular space and/or mobilization from internal stores in the myoplasm of muscle cells. This free Ca^{2+} activates the contractile elements. In particular, contractions of visceral smooth muscle in response to muscarinic receptor stimulation are known to be highly dependent on the influx of extracellular Ca^{2+} (Bolton *et al.*, 1981). It is suggested that the action potential in smooth muscle, especially taenia caecum, is due to the influx of Ca^{2+} (Kuriyama & Tomita, 1970). We showed previously that the contraction of isolated smooth muscle cells from taenia caecum in response to ACh was inhibited concentration-dependently by nicardipine, a calcium antagonist, and was complete at 3×10^{-7} M nicardipine (Mita & Uchida, 1988). We suggested that the contraction of cells to ACh depends upon the influx of extracellular Ca^{2+} through Ca channels.

Since the tissue has a large extracellular space, it is difficult to monitor $^{45}Ca^{2+}$ influx exactly. Therefore, $^{45}Ca^{2+}$ influx into taenia caecum in response to ACh or carbachol in about 15 min has been measured (Nasu & Urakawa, 1973). As both tissues and cells contract in a few seconds in response to ACh, we measured the $^{45}Ca^{2+}$ influx stimulated by ACh in a short time. Moreover, recovery from short-term desensitization is rapid. In the whole tissue, recovery from the desensitization may occur during the measurement of $^{45}Ca^{2+}$ influx to ACh. We monitored Ca^{2+} influx into the cells directly by use of $^{45}Ca^{2+}$. The $^{45}Ca^{2+}$ influx induced by 3×10^{-7}–3×10^{-6} M ACh was prompt and rapid and was correlated with the contraction of the cells (Figure 7 in Mita & Uchida, 1988). In the desensitized cells, the $^{45}Ca^{2+}$ influx was induced in the same manner as that of the control cells. The concentration of ACh inducing the maximal $^{45}Ca^{2+}$ influx was increased to a higher concentration of 10^{-5}–3×10^{-4} M ACh. This result indicates that the threshold concentration of ACh for Ca^{2+} influx into the cells was raised in the same manner as the increase in the threshold concentration for their contraction by short-term desensitization. These results suggested that after desensitization with ACh, a step in the signal transduction pathway of the Ca^{2+}-mobilizing receptor is modulated, and that the development of desensitization was due to an uncoupling of the receptor with a mechanism for development of the threshold membrane potential.

El-Fakahany & Richelson (1980) showed previously that short-term desensitization of histamine or carbachol-mediated responses in mouse neuroblastoma cells involved inactivation of Ca channels. In these desensitized cells, neither the number of receptors nor the affinity of antagonist ligands was changed (Taylor *et al.*, 1979), but they suggested that this desensitization involved inactivation of calcium entry. The mechanisms that lead to inactivation of Ca channels are still unknown. It was suggested that they were as follows. One of the possible mechanisms for closure of the Ca channels upon desensitization is phosphorylation of membrane fragments by protein kinase. In fact, phosphorylation of proteins bound to synaptosomal membranes decreased calcium uptake through the membrane (Weller & Morgan, 1977).

Another suggested mechanism is the involvement of phosphatidylinositol turnover. Increased turnover of this compound occurs in response to stimulation of the muscarinic receptor which controls cell surface permeability to Ca^{2+} (Michell, 1975). In taenia caecum, phosphatidylinositol turnover was stimulated by carbachol and inositol phosphates were produced (Hishinuma & Uchida, 1989). Further, Watson *et al.* (1988) recently provided evidence that the release of intracellular Ca^{2+}, presumably the action of inositol 1,4,5-trisphosphate (IP_3), was also involved in the opening of the voltage-operated Ca channels in guinea-pig ileum. Moreover, inositol 1,3,4,5-tetrakisphosphate (IP_4), produced by the phosphorylation of IP_3, may be involved in the opening of Ca channels in the cell surface membrane (Irvine & Moor, 1986). Irvine (1987) proposed that IP_4 induces the influx of extracellular Ca^{2+} due to refilling of Ca^{2+} into Ca stores from which Ca^{2+} was released by IP_3. However, desensitization did not result in reduction of phosphatidylinositol turnover elicited by carbachol (Hishinuma & Uchida, 1989). Hishinuma & Uchida (1988) reported that the contractile response in Ca^{2+}-free solution to carbachol was reduced by short-term desensitization with carbachol. They suggested that carbachol-induced desensitization is due to insufficient release of Ca^{2+} from intracellular Ca stores. These findings suggest that Ca^{2+}-release from Ca stores may be involved in triggering the influx of extracellular Ca^{2+} and that decrease in Ca^{2+} release by desensitization may change the influx of extracellular Ca^{2+}. In addition, short-term desensitization in guinea-pig taenia caecum with carbachol was heterologous desensitization (Hishinuma & Uchida, 1988), because the contraction to histamine was decreased by carbachol-induced desensitization. Bolton *et al.* (1981) found that histamine and carbachol might open the same Ca channel in longitudinal smooth muscle of guinea-pig ileum. Therefore, these findings suggest that short-term desensitization with ACh may involve inactivation of Ca channels and this is probably because of the reduction of Ca^{2+} release from intracellular Ca stores.

In conclusion, in this study we have shown that neither the affinity nor the number of muscarinic acetylcholine receptors were changed by short-term desensitization, but that the threshold concentration of ACh for Ca^{2+} influx into the cells

was raised by short-term desensitization. These results suggest that after desensitization with ACh in smooth muscle cells, a step in the signal transduction pathway of Ca^{2+}-mobilization is modulated and that the development of desensitization was due to interference with the coupling between the receptor and the mechanisms to open the Ca channels which lead to rapid influx of Ca^{2+}. We are now studying the mechanism for the threshold phenomena in the contraction of isolated smooth muscle cells to ACh.

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Response of rabbit ear artery to endothelin-1 during cooling

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1 The effects of cooling on the response of rabbit central ear artery to endothelin-1 and the role of the endothelium in these effects were studied in 2 mm long cylindrical arterial segments.

2 Concentration-response curves for endothelin-1 (10^{-10}–3×10^{-7} M) were recorded isometrically in arteries with and without endothelium at 37°C and during cooling (24°C). To analyze further the endothelial mechanisms of the response to endothelin-1 during cooling, the effects of this peptide in the presence of N^G-nitro-L-arginine methyl ester (L-NAME) (10^{-4} M) or meclofenamate (10^{-5} M) were also determined.

3 In every condition tested, endothelin-1 produced a marked, concentration-dependent arterial contraction. Sensitivity of intact arteries to this peptide was consistently lower at 24°C than at 37°C. At 37°C there were comparable responses of arteries with and without endothelium, but at 24°C arteries without endothelium showed a higher sensitivity than intact arteries to endothelin-1.

4 L-NAME (10^{-4} M) increased the maximal contraction at 37°C, and both the sensitivity and maximal contraction at 24°C of intact arteries to endothelin-1. Meclofenamate (10^{-5} M) did not affect the arterial response to endothelin-1.

5 Sensitivity of arteries with and without endothelium to nitroprusside (10^{-9}–10^{-3} M) was significantly decreased during cooling, and endothelium removal did not affect the relaxation to this nitrovasodilator.

6 These results suggest that cooling decreases sensitivity of cutaneous arteries (ear artery) to endothelin-1 probably by increasing the availability of endothelial nitric oxide.

Keywords: Endothelium, skin arteries, temperature, nitric oxide; endothelin-1; rabbit ear artery, cooling; N^G-nitro-L-arginine methyl ester (L-NAME)

Introduction

It is widely accepted that the endothelium plays a major role in the regulation of vascular tone by releasing relaxing and contracting factors (Furchgott & Vanhoutte, 1989). As vascular relaxants, the endothelium can produce prostacyclin by the metabolic pathway of cyclo-oxygenase (Moncada, 1982), and nitric oxide the precursor of which is L-arginine (Palmer *et al.*, 1987). The endothelium can also produce the vasconstrictor endothelin, a 21-amino acid peptide (Yanagisawa *et al.*, 1988a) of which three distinct isopeptides have been described (ET-1, ET-2 and ET-3) (Inoue *et al.*, 1989). Endothelin-1 causes a potent constrictor effect in several vascular beds *in vivo* and *in vitro* (Hughes *et al.*, 1988; Yanagisawa *et al.*, 1988b; Eglen *et al.*, 1989; Gardiner *et al.*, 1989; 1990; Hinojosa-Laborde *et al.*, 1989; Kasuya *et al.*, 1989; García *et al.*, 1991).

It appears that temperature affects cutaneous vascular responses (Vanhoutte, 1980) and knowledge of the mechanisms involved in the effects of cooling on vascular reactivity could be of relevance to the understanding of the pathophysiology of cold-induced vasospasm in humans. Since it has been shown that healthy subjects have increases in endothelin-1 plasma levels during cooling and that this feature is more marked in patients with Raynaud's phenomenon (Zamora *et al.*, 1990), the present study was performed to analyze the effects of moderate cooling on the response of the rabbit central ear artery to endothelin-1, analyzing also the role of the endothelium in these effects. The action of endothelin-1 was recorded isometrically in arterial segments at normal temperature (37°C) and after cooling (24°C). The central ear artery was selected because this vessel is easily accessible and widely used as a model of cutaneous blood vessels (Roberts & Zygmunt, 1984).

Methods

Twenty two male New Zealand white rabbits, weighing 2–2.5 kg, were killed by i.p. injection of sodium pentobarbitone (Sigma, 100 mg kg^{-1}). Central ear arteries were dissected free and cut into cylindrical segments 2 mm in length. Each segment was prepared for isometric tension recording in a 6 ml organ bath containing modified Krebs-Henseleit solution with the following composition (mM): NaCl 115, KCl 4.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11.1. The solution was equilibrated with 95% oxygen and 5% carbon dioxide to give a pH of 7.3–7.4. Two, fine, stainless steel pins, 150 μm in diameter, were introduced through the lumen of the vascular segment. One pin was fixed to the organ bath wall while the other was connected to a strain gauge for isometric recording, thus permitting the application of passive tension in a plane perpendicular to the long axis of the vascular cylinder. The recording system included a Universal Transducing Cell UC3 (Statham Instruments, Inc.), a Statham Microscale Accessory UL5 (Statham Instruments, Inc.) and a Beckman Type RS Recorder (model R-411, Beckman Instruments, Inc.). A resting tension of 0.5 g was applied to the tissue and the segments were allowed to equilibrate for 60–90 min before the experiments began.

Response of arterial segments to endothelin-1 (10^{-10}–3×10^{-7} M) was determined in a cumulative manner under the following conditions: (a) arteries with (intact) and without endothelium at 37°C and 24°C: (b) arteries with endothelium, treated with the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M) (Moore *et al.*, 1990) at 37°C and 24°C, and (c) arteries with endothelium treated with the cyclo-oxygenase inhibitor meclofenamate (10^{-5} M) at 24°C.

Removal of the endothelium was accomplished by gently rubbing the lumen of the vessel with a roughened steel rod. The presence of the endothelium and the adequacy of endothelium removal was functionally tested in precontracted arteries by recording the effects of acetylcholine (10^{-6} M), and morphologically at the end of the experiments by silver staining.

L-NAME and meclofenamate, dissolved in distilled water, were added to the organ bath 20–30 min before beginning the endothelin-1 concentration-response curve and were used to analyze the effects of endothelium-derived nitric oxide and prostanoids, respectively, on the vascular effects to endothelin-1 during cooling.

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The effects of nitroprusside (10^{-9}–10^{-3} M) on arteries with and without endothelium precontracted with endothelin-1 were also obtained in a cumulative manner at 37°C and at 24°C.

Responses to potassium chloride (50 mM) were also recorded in arteries with and without endothelium at 37°C and 24°C.

Concentrations of endothelin-1 and nitroprusside causing 50% of the maximal response (EC_{50}) were calculated from each individual concentration-response curve. The geometric mean of EC_{50} and its 95% confidence interval were calculated for each group of experiments and the logarithms of the EC_{50} in the different experimental conditions were compared statistically.

Data analysis

The data, expressed as means ± s.e.mean, were evaluated by Student's t test; $P < 0.05$ was considered to be statistically significant.

Drugs and peptides

Materials used were endothelin-1 (human, porcine) (Peninsula Laboratories Europe, Ltd.), L-NAME (N^G-nitro-L-arginine methyl ester, Sigma) and sodium nitroprusside (Sigma). Sodium meclofenamate was a gift from Parke Davis.

Results

Endothelin-1 produced a marked, concentration-dependent contraction of the arterial segments in all cases. The response of arteries with endothelium differed at 37°C and 24°C (Figure 1a). In terms of the EC_{50} values, sensitivity of intact arteries at 24°C ($EC_{50} = 9.9 \times 10^{-9}$ M, 95% confidence interval 8.0×10^{-9}–1.2×10^{-8}) was 1.7 times lower ($P < 0.01$) than at 37°C ($EC_{50} = 5.9 \times 10^{-9}$ M, 4.5×10^{-9}–7.8×10^{-9}); maximal contraction was similar at both temperatures. Arteries without endothelium showed a response (sensitivity and maximal contraction) that was similar at 37°C and 24°C.

At 37°C (Figure 1b) sensitivity of vessels to endothelin-1, in terms of EC_{50} values, was similar in arteries with endothelium and without endothelium ($EC_{50} = 5.0 \times 10^{-9}$ M, 4.0×10^{-9}–6.3×10^{-9}). However, at 24°C (Figure 1c) arteries without endothelium ($EC_{50} = 5.0 \times 10^{-9}$ M, 4.1×10^{-9}–6.0×10^{-9}) were about 2 times ($P < 0.001$) more sensitive than arteries with endothelium. Endothelium removal did not significantly affect the maximal contraction to endothelin-1 at either temperature.

In intact arteries, treatment with L-NAME (10^{-4} M) significantly increased the maximal contraction (3.98 ± 0.32 vs. 3.24 ± 0.15 mg) without affecting the sensitivity to endothelin-1 at 37°C (4 animals), whereas this treatment significantly increased both the sensitivity ($P < 0.05$) and maximal contraction ($P < 0.001$) to this peptide during cooling (5 animals) (Table 1).

Treatment with meclofenamate (10^{-5} M) did not significantly affect the response of intact arteries to endothelin-1 during cooling. At 24°C the EC_{50} values and maximal contraction were comparable ($P > 0.05$) in arteries treated and not treated with this cyclo-oxygenase inhibitor (Table 1).

At 37°C and 24°C arteries precontracted with endothelin-1 ($10^{-8} - 3 \times 10^{-8}$ M) (tone ranged from 1650 to 2340 mg) showed a concentration-dependent relaxation to nitroprusside ($10^{-9} - 10^{-3}$ M) and the maximal relaxation in both conditions was about 96–100% of the initial tone. However, the sensitivity of intact arteries during cooling (5 animals) ($EC_{50} = 1 \times 10^{-6}$ M, 5.2×10^{-7}–2×10^{-6}) was about 14 times lower than at 37°C (5 animals) ($EC_{50} = 7.4 \times 10^{-8}$ M, 1.6×10^{-8}–3.4×10^{-7}) ($P < 0.003$). Removal of the endothelium did not significantly modify the response of arteries to nitroprusside at 37°C (4 animals) and 24°C (4 animals), and the sensitivity of the denuded arteries was also lower (about 12 times) at 24°C than at 37°C ($P < 0.001$).

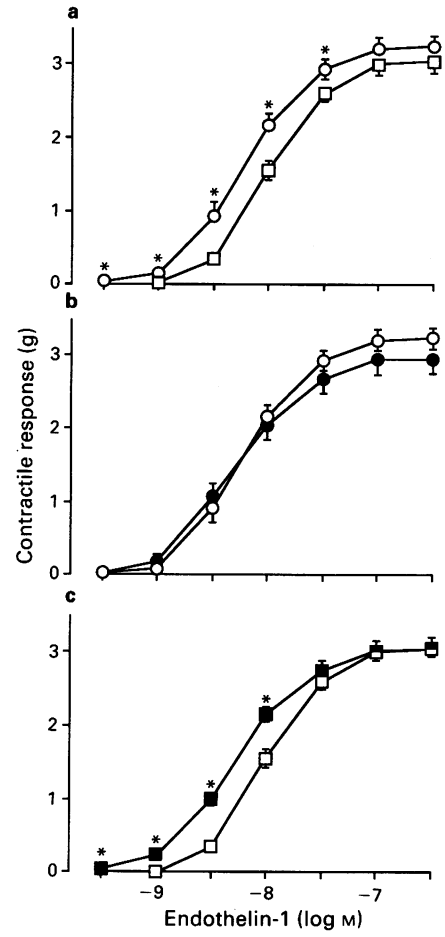


Figure 1 Contractile response of rabbit central ear artery to endothelin-1. (a) Responses obtained in intact arteries at 37°C (○) and 24°C (□) (15 animals); (b) responses obtained in arteries with (○) and without (●) endothelium at 37°C (9 animals); (c) responses obtained in arteries with (□) and without (■) endothelium at 24°C (16 animals). Values are means with s.e.mean shown by vertical bars. *Statistically significant differences ($P < 0.01$).

Potassium chloride (50 mM) produced a contractile response in intact arteries that was lower during cooling (0.68 ± 0.06 g) than at 37°C (1.97 ± 0.18 g) ($P < 0.0001$). After removing the endothelium the arterial contraction to potassium chloride at 37°C and 24°C tended to be higher, but not significantly ($P > 0.05$), than in the intact arteries.

Table 1 Effects of endothelin-1 on rabbit central ear artery with endothelium obtained during cooling (24°C) in the absence (control) and in the presence of N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M) or meclofenamate (10^{-5} M)

	EC_{50} (M) (95% confidence interval)	*Maximal contraction* (g)
Control	1.1×10^{-8}	3.07 ± 0.11
($n = 7$)	($8.1 \times 10^{-9} - 1.5 \times 10^{-8}$)	
L-NAME	7.0×10^{-9}*	3.78 ± 0.19**
($n = 5$)	($5.9 \times 10^{-9} - 8.3 \times 10^{-9}$)	
Meclofenamate	9.4×10^{-9}	3.08 ± 0.25
($n = 6$)	($6.4 \times 10^{-9} - 1.4 \times 10^{-8}$)	

*, ** Statistically significant (*$P < 0.05$; **$P < 0.01$) compared to control.
Maximal contraction values are means ± s.e.mean.
n = number of animals.

Discussion

The present results show that endothelin-1 induces a marked contraction of the isolated ear artery of rabbits, and that the sensitivity to this peptide is within the range found in other blood vessels of different species (Eglen *et al.*, 1989; Miller *et al.*, 1989; Martin de Aguilera *et al.*, 1990; García *et al.*, 1991). At 37°C, removal of the endothelium did not affect the endothelin-1-induced contraction in the ear artery as occurs in other vascular beds (Kasuya *et al.*, 1989; Marsden *et al.*, 1989; García *et al.*, 1991). However, in the presence of L-NAME we observed an augmented contractility of intact arteries to endothelin-1 thus suggesting that this peptide is able to increase the release of nitric oxide from the endothelium; this would oppose the contraction of the ear artery to endothelin-1 at a normal temperature.

In the rat mesenteric arterial bed it has been observed that both treatment with L-NAME and chemical destruction of the endothelium increased the contractility to endothelin-1, suggesting that the endothelium modulates the vasoconstrictor activity of this peptide (Douglas & Hiley, 1990). The reason for the difference observed in our study between arteries without endothelium and treated with L-NAME at 37°C is not apparent. A reduced capacity of rubbed arteries to contract may not be the cause, since the contraction of these arteries to potassium chloride tended to be higher than that of intact arteries. It is probable, however, that mechanical destruction of the endothelium and treatment with L-NAME cannot be compared directly and inhibition of nitric oxide synthesis may be a more appropriate intervention for evaluating some of the effects of endothelial function on vascular reactivity.

A major finding of our study is that cooling inhibits the response of ear artery of rabbits to endothelin-1 by altering the function of the endothelium. As compared with the results obtained at 37°C, the data obtained at 24°C show that sensitivity of intact arteries to endothelin-1 decreased during cooling. This inhibitory effect of cooling was of relatively small magnitude but it was present in every experiment.

Cooling did not seem to affect the response of arterial smooth muscle to endothelin-1, since there was no significant difference between the response of endothelium-denuded arteries at 24°C and 37°C. However, at 24°C the concentration-response curve of the arteries without endothelium was shifted to the left in relation to that of intact arteries. This suggests that the endothelium decreases the sensitivity of ear artery to endothelin-1 during cooling. From the experiments with L-NAME and meclofenamate it is suggested that nitric oxide, but not prostanoids, is involved in the lower sensitivity of ear artery to endothelin-1 during cooling. L-NAME, an inhibitor of nitric oxide synthesis in the endothelium (Moore *et al.*, 1990), was able to increase reactivity of the intact arteries to endothelin-1 during cooling.

The relaxation of the ear artery to nitroprusside, a compound that relaxes vascular smooth muscle in a similar way to nitric oxide (Ignarro & Kadowitz, 1985), was independent of the endothelium and was decreased during cooling. Evidence that the endothelium modulates the effect of nitrovasodilators is controversial and there are findings showing potentiation (Shirasaki & Su 1985; Gardiner *et al.*, 1991; Moncada *et al.*, 1991), attenuation (White *et al.*, 1986), and no effect (Furchgott *et al.*, 1981; Rapoport & Murad, 1983). These contrasting results may be related to differences in vascular beds, species and experimental approaches used. The decreased relaxation to nitroprusside observed in intact arteries during cooling could indicate that increased availability of endogenous nitric oxide down-regulates sensitivity to exogenous nitric oxide (Moncada *et al.*, 1991). However, as removal of the endothelium did not alter the vasorelaxation to nitroprusside at 37°C or 24°C, and the relaxation of the endothelium-denuded arteries to this compound is, as for intact arteries, decreased during cooling, it can be assumed that the relaxation of smooth musculature of ear artery to nitric oxide released from the endothelium in response to endothelin-1 during cooling is also decreased. Therefore, the decreased reactivity of rabbit ear artery to endothelin-1 at 24°C could be due to an increased availability of endothelial nitric oxide counteracting the arterial contraction to this peptide during cooling. This increased availability could be related to an augmented release and/or an augmented biological half-life of endogenous nitric oxide in that circumstance.

In the rat jugular vein it has been shown that cooling decreases the relaxation and increases the contraction induced by 5-hydroxytryptamine by endothelial mechanisms (Bodelsson *et al.*, 1989). In that study the authors suggested that cooling decreases the effectiveness of 5-hydroxytryptamine in stimulating the release of EDRF from the endothelium which, in addition to reducing the endothelium-mediated relaxation, contributed to the augmented contraction of the rat jugular vein to this amine during cooling. The discrepancy between this study and ours may be related to the different way in which cooling affects the venous and arterial endothelium. The relaxation to cholinergic stimulation in the rat jugular vein is not altered (Bodelsson *et al.*, 1989) whereas in the rat aorta it is augmented (Karaki & Nagase, 1987) during cooling. Also the effects of cooling on the response of the vascular endothelium may depend on the activation mechanisms.

In conclusion, the present results suggest that cooling decreases sensitivity of ear artery, a cutaneous artery, to endothelin-1 probably by increasing the availability of endothelial nitric oxide. If the features observed *in vitro* are also present *in vivo*, endothelin-1 might play a role in the regulation of cutaneous circulation. In the pathological conditions in which a lesion of the vascular endothelium is present, endothelin-1 may contribute to development of cutaneous vasospasm such as Raynaud's phenomenon.

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Muscarinic receptor subtypes coupled to generation of different second messengers in isolated tracheal smooth muscle cells

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1 Activation of muscarinic receptor subtypes leads to contraction, an increase in the accumulation of inositol phosphates (IPs) and a decrease in adenosine 3':5'-cyclic monophosphate (cyclic AMP) synthesis in tracheal smooth muscle. The concentrations of carbachol that produced a half-maximal effect (EC_{50}) in inhibition of cyclic AMP generation, stimulation of IPs formation and contraction were 15 nM, 2.0 μM and 0.17 μM, respectively.

2 Pirenzepine, a selective M_1 antagonist, displayed a low affinity for antagonizing cyclic AMP inhibition, IPs formation and contraction induced by carbachol (pK_B = 6.8, 7.0, and 7.1, respectively).

3 Methoctramine, a cardioselective M_2 antagonist, blocked cyclic AMP inhibition with a high affinity (pK_B = 7.5), while it antagonized IPs formation and contraction with a low affinity (pK_B = 6.2 and 6.1, respectively).

4 4-Diphenylacetoxy-N-methylpiperidine (4-DAMP), a selective smooth muscle M_3 antagonist, possessed a high affinity in blocking IPs formation (pK_B = 8.8) and contraction (pK_B = 9.2) as well as a low affinity for antagonism of cyclic AMP inhibition (pK_B = 8.1).

5 In conclusion, we have demonstrated that M_2 and M_3 receptor subtypes are coupled to different effector systems in tracheal smooth muscle. An M_1 receptor subtype is not involved in the generation of the second messengers examined. Inhibition of cyclic AMP formation may be coupled to the M_2 receptor subtype. The accumulation of IPs and presumably IP-induced Ca^{2+} release may function as the transducing mechanism for cholinergic contraction of tracheal smooth muscle through the activation of M_3 receptors.

Keywords: Smooth muscle; cyclic AMP; inositol phosphates; muscarinic receptor subtypes; contraction

Introduction

Contractions of the tracheal smooth muscle are mediated in part through muscarinic receptors. One of factors that may contribute to the diversity of physiological responses is the presence of multiple subtypes of the muscarinic receptors. By the use of pirenzepine, these receptors have been subdivided into M_1 and M_2 subtypes (Hammer *et al.*, 1980). Recently, experimental evidence has been obtained for the subclassification of muscarinic receptors into M_1, M_2 and M_3, based on studies with selective antagonists (Nathanson, 1987; Doods *et al.*, 1987). Pirenzepine has high affinity for M_1 receptors (Hammer *et al.*, 1980), AF-DX 116 and methoctramine for M_2 receptors (Giachetti *et al.*, 1986; Melchiorre *et al.*, 1987; Micheletti *et al.*, 1987; Giraldo *et al.*, 1988) and 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP) as well as HHSiD for M_3 receptors (Barlow *et al.*, 1976; Mutschler & Lambrecht, 1984). Furthermore, genetic studies have indicated that M_1, M_2, and M_3 subtypes are encoded by separate m1, m2, and m3 genes (Bonner, 1989; Buckley *et al.*, 1989). In addition, distinct m4 and m5 genes have been detected (Buckley *et al.*, 1989). That different receptor subtypes are coupled to various effector systems has been substantiated by the recent demonstration that the expressed m1, m3, and m5 subtypes are preferentially coupled to hydrolysis of phosphoinositides, whereas the m2 and m4 subtypes are coupled to attenuation of adenylate cyclase (Ashkenazi *et al.*, 1987; Bonner *et al.*, 1988; Shapiro *et al.*, 1988; Peralta *et al.*, 1988; Bonner, 1989). Expression of these receptor genes in cell lines has made it possible to explore the pharmacological properties of each distinct structure, leading to a direct correlation among genes, protein structure and pharmacological properties. Whether a single receptor subtype couples to a single effector system or each muscarinic receptor subtype can interact with multiple effectors remains unclear.

Muscarinic receptors are also known to interact via a G protein regulated process, with multiple effector systems leading to inhibition of adenylate cyclase, increased phosphoinositide breakdown, arachidonate release, and modulation of potassium channels (Nathanson, 1987). In the tracheal smooth muscle, activation of muscarinic receptors leads to phosphoinositide breakdown and contraction of the smooth muscle (Grandordy *et al.*, 1986; Roffel *et al.*, 1990) as well as inhibition of adenylate cyclase (Sankary *et al.*, 1988). Also, M_2 and M_3 receptor subtypes have been characterized in tracheal smooth muscle (Roffel *et al.*, 1988; Yang, 1991). Therefore, the question as to how the receptor subtypes regulate the function of tracheal smooth muscle through the generation of different second messenger systems requires study.

The purpose of the present study was to determine the correlation between muscarinic receptors mediating adenylate cyclase inhibition and receptors mediating the generation of IPs and contraction. The pharmacological investigation performed by use of several muscarinic antagonists and carbochol revealed that these two second messenger responses involved distinct muscarinic receptor subtypes in tracheal smooth muscle. The data suggest that both effectors may play roles in the overall physiological function of tracheal smooth muscle, but phosphoinositide breakdown is the transducing mechanism of muscarinic stimulation for smooth muscle contraction.

Methods

Animals

Mongrel dogs, 20–30 kg, purchased from a local supplier, were used throughout this study. The dogs were fed with standard laboratory chow and tap water *ad libitum* and were housed indoors in the animal facilities under automatically controlled temperature and light cycle. Dogs of either sex were anaesthetized with pentobarbitone (30 mg kg^{-1}, intravenously) and

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the lungs were ventilated mechanically via an orotracheal tube. The trachea was removed from the animal.

Preparation of tracheal smooth muscle

The trachea, about 20 cm long from the larynx to bifurcation, was quickly removed and transferred into oxygenated (95% O_2 and 5% CO_2; pH 7.2) Krebs Henseleit solution (KHS; Farley & Miles, 1977). The tracheal smooth muscle was maintained in KHS throughout the experiment. The trachea was opened by cutting the cartilage rings opposite the muscle. The muscle was cleaned in two steps. The initial step in each dissection involved removal of the epithelium and submucosal tissue with forceps. The connective tissue on the serosal surface was carefully cleaned under a microscope and cartilage up to the point of insertion of the muscle deep into the cartilage rings, then the trachea was cut into separate individual rings. Each of the tracheal rings was suspended in KHS in a muscle chamber and continuously bubbled with 95% O_2 and 5% CO_2 at 37°C. The tracheal rings were hooked to Gould (BG-25 gm) force displacement transducers with fine silk thread. Isometric muscle tension was recorded with a Gould 3000 recorder (Cleveland, Ohio, U.S.A.). A base line tension of 0.5 g was applied in preliminary experiments and found to be adequate for maximum force generation. The muscles were allowed to stabilize in the muscle chambers for 1 h with two or three changes of the KHS during this period. A minimum of six muscles from different animals was used to generate the dose-response curves.

Isolation of tracheal smooth muscle cells

For studying the effects of activation of muscarinic receptors on the phosphoinositide breakdown and adenosine 3':5'-cyclic monophosphate (cyclic AMP) generation, enzymatically isolated tracheal smooth muscle cells (TSMCs) were used in this study. The smooth muscle was dissected out, minced and transferred to the dissociation medium containing 0.2% collagenase I, 0.01% DNase I, and 0.01% elastase IV as well as antibiotics in physiological solution. The physiological solution used contained (mM): NaCl 137, KCl 5, $CaCl_2$ 1.1, $NaHCO_3$ 20, NaH_2PO_4 1, glucose 11 and HEPES 25 (pH 7.4). The tissue pieces were then gently agitated at 37°C in a rotator for 1 h. The released cells were collected and the remaining tissue was digested in the same fresh enzyme solution for an additional 1 h at 37°C. All the released cells were collected and washed with KHS. The cell number was counted and diluted with KHS to 10^6 cells ml^{-1}.

In order to characterize the isolated TSMCs and to exclude the possibility of epithelial cells and fibroblast contamination, the TSMCs were identified by an indirect immunofluorescence method (Gown *et al.*, 1985). Over 95% of the cells isolated in this study were smooth muscle cells.

Accumulation of inositol phosphates

The effects of muscarinic agents on the hydrolysis of phosphoinositide were assessed by monitoring the accumulation of ^{3}H-labelled inositol phosphates as described by Berridge *et al.* (1983). Isolated TSMCs were incubated with 10 μCi ml^{-1} of *myo*-[2-^{3}H]-inositol at 37°C for 2 h. TSMCs were washed 3 times with and incubated in Krebs Henseleit buffer (pH 7.4) containing (mM): NaCl 117, KCl 4.7, $MgSO_4$ 1.1, KH_2PO_4 1.2, $NaHCO_3$ 20, $CaCl_2$ 2.4, glucose 1, HEPES 20 and LiCl 10, at 37°C for 10 min. After 10 min, carbachol was added at the indicated concentration and incubation was continued for a further 30 min. When an antagonist was used, it was added 10 min before the addition of the agonist. Reactions were terminated by adding 5% perchloric acid followed by sonication and centrifugation at 3000 *g* for 15 min.

The perchloric acid-soluble supernatants were extracted 4 times with ether, neutralized with KOH and applied to a column of the anion exchange resin (AG1-X8, formate form, 100–200 mesh). The resin was washed successively with 5 ml of water and 5 ml of 60 mM ammonium formate/5 mM sodium tetraborate to eliminate free [^{3}H]-*myo*-inositol and glycerophosphoinositol, respectively. A total IPs fraction was then eluted with 10 ml of 1 M ammonium formate/0.1 M formic acid. The amount of [^{3}H]-inositol phosphates were determined by scintillation counting of 0.5 ml samples.

Cyclic AMP assay

TSMCs were incubated with 3-isobutyl-1-methylxanthine (IBMX) at a final concentration of 0.5 mM at 37°C for 10 min and then exposed to 1 μM isoprenaline alone or combined with the specific muscarinic agonist carbachol used at the indicated concentrations and continuously incubated at 37°C for 10 min. Muscarinic antagonists, when used, were added at the indicated concentrations 10 min before the addition of carbachol and/or isoprenaline. Reactions were stopped by boiling the cells for 3 min and followed by centrifugation at 10,000 *g* for 20 min at 4°C. Cyclic AMP was assessed by Amersham cyclic AMP assay kit. Cyclic AMP levels were expressed as pmol/10^6 cells. Inhibition of cyclic AMP accumulation mediated by carbachol was evaluated as the difference between cyclic AMP due to isoprenaline and cyclic AMP obtained in the simultaneous presence of isoprenaline and carbachol. Alternatively, inhibition was expressed as the percentage of the inhibitory response to a maximally effective concentration of carbachol.

Data analysis

The EC_{50} values of carbachol for inhibition of cyclic AMP formation, IPs accumulation and contraction were estimated by Graph Pad programme (Graph Pad, San Diego, California, U.S.A.), sharing the same estimate of the maximum response between the control data and the data measured in the presence of muscarinic antagonists. When several concentrations of antagonists were used, the dissociation constants (K_B) of muscarinic antagonists were estimated by measurement of their abilities to antagonize carbachol-mediated inhibition of cyclic AMP formation, stimulation of phosphoinositide hydrolysis and contraction of the tracheal smooth muscle. The K_B values were calculated as described by Furchgott (1972).

All the data are expressed as the means ± standard error (s.e.) of at least three experiments with statistical comparisons based on a two-tailed Student's *t* test. The level of significance was chosen at the $P < 0.05$ level. The Graph Pad programme was used to generate graphical data and in nonlinear regression analyses.

Drugs

Myo-[2-^{3}H]-inositol (18 Ci $mmol^{-1}$) and [^{3}H]-cyclic AMP assay kit were obtained from Amersham (Buckinghamshire). 4-DAMP and methoctramine were purchased from RBI (Boston, MA, U.S.A.). Pirenzepine was a gift from Dr K. Noll at Dr Karl Thomae, GmbH. AG1-X8 was ordered from Bio-Rad (Richmond, CA, U.S.A.). Other enzymes and chemicals were purchased from Sigma (St Louis, MO, U.S.A.).

Results

Effects of muscarinic antagonists on muscarinic receptor-mediated inhibition of cyclic AMP generation

The data obtained in Figure 1, show the high affinity interaction of carbachol with the inhibitory pathway of adenylate cyclase system in TSMCs. Carbachol inhibited isoprenaline-stimulated cyclic AMP formation in a dose-dependent manner with maximal inhibition at 1 μM carbachol. The mean values ± s.e. of cyclic AMP levels from carbachol inhibition

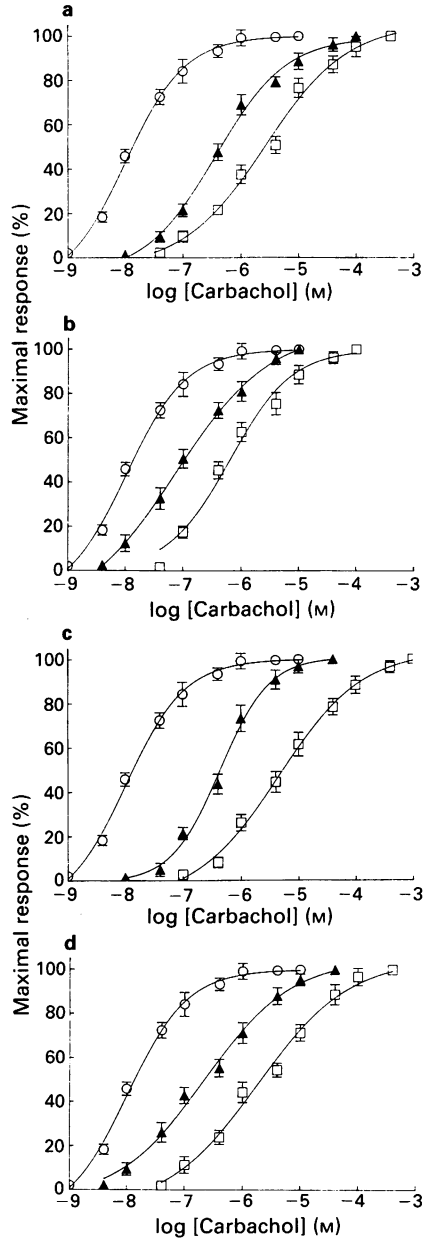


Figure 1 Competitive antagonism of carbachol-mediated inhibition of isoprenaline-stimulated cyclic AMP formation by (a) atropine, (b) pirenzepine, (c) methoctramine, and 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP) in tracheal smooth muscle cells. Each point represents the mean of three experiments determined in triplicate; s.e. shown by vertical bars. Basal levels of cyclic AMP, in the absence of isoprenaline, were 110 ± 20 and 95 ± 15 pmol/10^6 cells in the absence and the presence of carbachol, respectively. The amount of cyclic AMP inhibited by carbachol was evaluated as the amount of cyclic AMP stimulated by isoprenaline (1 μM) alone (190 ± 30 pmol/10^6 cells) minus the amount of cyclic AMP generated by the simultaneous presence of isoprenaline and carbachol (1 μM). Results are expressed as the percentage of response to a maximally effective concentration of carbachol (maximal inhibition = 95 ± 20 pmol/10^6 cells). The dose-response curves were constructed in the presence of various concentrations of carbachol in the absence (○) and in the presence of various antagonists: (a) atropine, (▲) 10 nM and (□) 100 nM; (b) pirenzepine, (▲) 1 μM and (□) 10 μM; (c) methoctramine, (▲), 1 μM and (□) 10 μM; (d) 4-DAMP, (▲) 0.1 μM and (□) 1 μM.

experiments in the absence of antagonists were as follows: maximal level produced by 1 μM isoprenaline was 190 ± 30 pmol/10^6 cells; minimal level remaining after the maximal inhibition by 100 μM carbachol was 95 ± 15 pmol/10^6 cells; and basal level, 110 ± 20 pmol/10^6 cells. EC_{50} for the inhibitory effect of carbachol on the accumulation of cyclic AMP stimulated by isoprenaline was 15 ± 3 nM. The abilities of muscarinic antagonists, atropine (10 and 100 nM), pirenzepine (1 and 10 μM), methoctramine (1 and 10 μM) and 4-DAMP (0.1 and 1 μM) to antagonize carbachol-mediated inhibition of cyclic AMP accumulation were investigated in TSMCs (Figure 1). In general, these antagonists caused parallel rightward shifts in the concentration-effect curves of carbachol. The dissociation constants (K_B) of each antagonist were calculated from dose-ratios by the method of Furchgott (1972). Two concentrations of each antagonist were used in these experiments; consequently, two K_B values were calculated for each antagonist. Table 1 lists the K_B values of the muscarinic antagonists. There was no significant difference between these two values ($P > 0.05$). The rank order of potency for preventing the inhibition of cyclic AMP accumulation was atropine > 4-DAMP > methoctramine > pirenzepine. The pK_B values of pirenzepine, methoctramine and 4-DAMP for antagonizing cyclic AMP inhibition were 6.7, 7.5 and 8.2 (Table 1), respectively, which corresponded to the high affinity for methoctramine and low affinity for pirenzepine and 4-DAMP in this response (see review Hulme *et al.*, 1990).

Table 1 The dissociation constants (K_B) for muscarinic antagonists to antagonize the carbachol-mediated inhibition of adenylate cyclase activity

Antagonist	EC_{50} (μM)	*Dose-ratio*	pK_B	n
Atropine				
(10 nM)	0.39 ± 0.11	26	9.4 ± 0.2	3
(100 nM)	2.0 ± 0.7	130	9.1 ± 0.1	3
Pirenzepine				
(1 μM)	0.11 ± 0.03	7	6.8 ± 0.2	3
(10 μM)	0.63 ± 0.14	43	6.6 ± 0.3	3
Methoctramine				
(1 μM)	0.44 ± 0.13	30	7.5 ± 0.2	3
(10 μM)	56 ± 1.4	380	7.6 ± 0.2	3
4-DAMP				
(0.1 μM)	0.22 ± 0.09	15	8.1 ± 0.1	3
(1 μM)	2.2 ± 0.7	150	8.2 ± 0.2	3

Values [mean ± s.e. for number (n) of experiments] were calculated from the dose-response curves for carbachol as shown in Figure 1. EC_{50} for the inhibitory effect of carbachol on the isoprenaline-stimulated cyclic AMP formation was 15 ± 3 nM.
4-DAMP = 4-diphenylacetoxy-*N*-methylpiperidine.

Effects of muscarinic antagonists on muscarinic receptor-mediated stimulation of inositol phosphates accumulation

Carbachol caused a concentration-dependent accumulation of IPs in TSMCs, with a maximal effect being a 2.5 fold increase over the basal levels (8000 d.p.m./10^6 cells per 0.5 ml). The EC_{50} value for the carbachol-stimulated IPs accumulation was approximately 2.0 ± 0.8 μM (Figure 2). The concentration-effect relationship of carbachol was shifted to the right in a nearly parallel fashion, without a reduction in maximal response, upon the addition of atropine (10 and 100 nM), pirenzepine (1 and 10 μM), methoctramine (10 and 100 μM), and 4-DAMP (0.1 and 1 μM) (see Figure 2). The dissociation constants (K_B) were calculated from dose-ratios by the method developed by Furchgott (1972). There was no significant difference between these two K_B values for each antagonist (Table 2). The order of potency of the antagonists in antagonizing the carbachol-stimulated IPs accumulation was atropine > 4-DAMP > pirenzepine > methoctramine. The pK_B values of pirenzepine, methoctramine and 4-DAMP for antagonizing the carbachol-mediated IPs accumulation were 7.0, 6.2, and 8.8 (Table 2), respectively, which were in good agreement with the low affinity for pirenzepine and methoctramine and high affinity for 4-DAMP in this response (see review Hulme *et al.*, 1990).

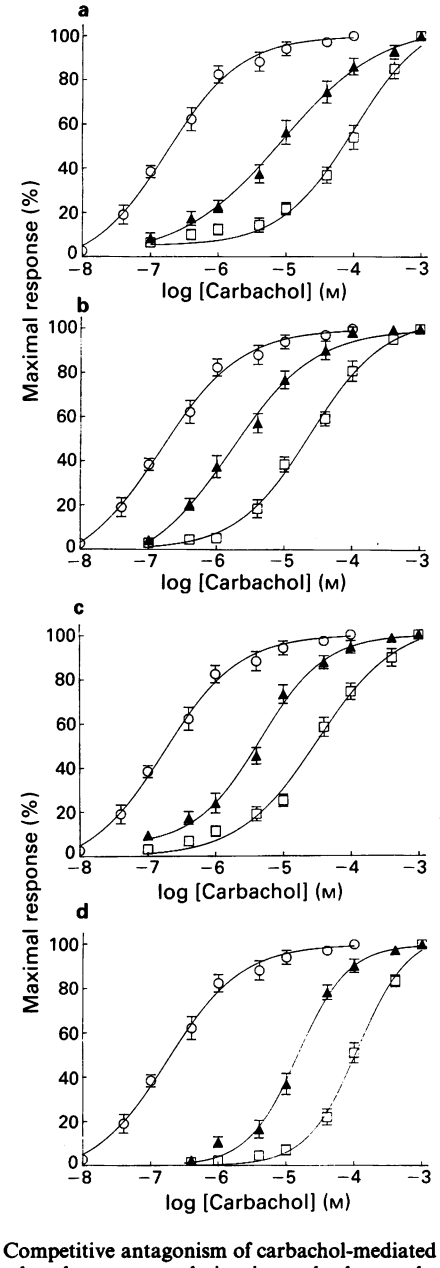


Figure 2 Competitive antagonism of carbachol-mediated stimulation of inositol phosphates accumulation in tracheal smooth muscle cells by (a) atropine, (b) pirenzepine, (c) methoctramine, and (d) 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP). Each point represents the mean of three experiments determined in triplicate; s.e. shown by vertical bars. Total [^{3}H]-inositol phosphates (IPs) were measured as d.p.m./10^6 cells. Results for the measurement of IPs production are expressed as the percentage of the IPs accumulation (19300 ± 1200 d.p.m./10^6 cells) stimulated by a maximal concentration of carbachol in the absence of antagonists. Carbachol-stimulated IPs accumulation was measured in the absence (○) and presence of atropine (▲, 10 nM and □, 100 nM), pirenzepine (▲, 1 μM and □, 10 μM), methoctramine (▲, 10 μM and □, 100 μM), and 4-DAMP (▲, 0.1 μM and △, 1 μM).

Effects of muscarinic antagonists on muscarinic receptor-mediated contractile responses

Carbachol caused a dose-dependent contraction of dog tracheal smooth muscle and the geometric mean EC_{50} for carbachol was 0.17 ± 0.10 μM (Figure 3). When the muscarinic antagonists, atropine (10 and 100 nM), pirenzepine (1 and 10 μM), methoctramine (10 and 100 μM), and 4-DAMP (10 and 100 nM), were examined for their ability to antagonize the carbachol-mediated contractile response, the concentration-effect relationship of carbachol was shifted to the right in a parallel fashion, without a change in the maximal response

Table 2 The dissociation constants (K_B) for muscarinic antagonists antagonizing the carbachol-mediated accumulation of inositol phosphates

Antagonist	EC_{50} (μM)	*Dose-ratio*	pK_B	n
Atropine				
(10 nM)	70 ± 15	34	9.5 ± 0.3	3
(100 nM)	600 ± 160	290	9.5 ± 0.3	3
Pirenzepine				
(1 μM)	23 ± 9	11	7.0 ± 0.2	3
(10 μM)	210 ± 80	100	7.0 ± 0.2	3
Methoctramine				
(10 μM)	38 ± 13	18	6.2 ± 0.2	3
(100 μM)	275 ± 110	130	6.1 ± 0.3	3
4-DAMP				
(0.1 μM)	150 ± 70	72	8.9 ± 0.2	3
(1 μM)	990 ± 170	480	8.7 ± 0.3	3

Values [mean ± s.e. for number (*n*) of experiments] were calculated from the dose-response curves for carbachol as shown in Figure 2. EC_{50} of carbachol-stimulated inositol phosphates accumulation was 2.0 ± 0.8 μM. 4-DAMP = 4-diphenylacetoxy-*N*-methylpiperidine.

(Figure 3). The dissociation constants (K_B) of muscarinic antagonists were calculated from dose-ratios by the method of Furchgott (1972). Table 3 lists the K_B values for muscarinic antagonists used in these experiments. Two K_B values were calculated for each antagonist, since two concentrations of each antagonist were used in this study. There was no significant difference between these two K_B values ($P > 0.05$). The rank order of potency of muscarinic antagonists in antagonizing the carbachol-mediated contraction was atropine > 4-DAMP > pirenzepine > methoctramine. As in the IPs accumulation, the pK_B values of pirenzepine, methoctramine and 4-DAMP were 7.1, 6.1 and 9.3 (Table 3), respectively, which were consistent with the low affinity for pirenzepine and methoctramine and high affinity for 4-DAMP (see review (Hulme *et al.*, 1990).

Discussion

The activation of muscarinic receptor subtypes coupled to the generation of different second messengers was investigated in tracheal smooth muscle. It was found that (1) activation of muscarinic receptors in tracheal smooth muscle leads to accumulation of IPs, inhibition of cyclic AMP formation, and generation of the contractile response; (2) an M_1 subtype is not involved in the observed muscarinic-mediated second messenger responses; (3) inhibition of adenylate cyclase seems to be coupled to M_2 subtype distinct from the M_3 subtype coupled

Table 3 The dissociation constants (K_B) for muscarinic antagonists antagonizing the carbachol-mediated contraction

Antagonist	EC_{50} (μM)	*Dose-ratio*	pK_B	n
Atropine				
(10 nM)	5.8 ± 1.4	35	9.5 ± 0.2	8
(100 nM)	110 ± 50	650	9.8 ± 0.3	8
Pirenzepine				
(1 μM)	2.1 ± 0.8	13	7.1 ± 0.2	10
(10 μM)	21 ± 5	130	7.1 ± 0.1	10
Methoctramine				
(10 μM)	1.7 ± 0.5	10	6.0 ± 0.2	8
(100 μM)	24 ± 8	150	6.2 ± 0.2	8
4-DAMP				
(0.1 μM)	30 ± 0.9	19	9.2 ± 0.2	12
(1 μM)	36 ± 7	220	9.3 ± 0.1	12

Values [mean ± s.e. for number (*n*) of experiments] were calculated from the dose-response curves for carbachol as shown in Figure 3. EC_{50} of carbachol-mediated contraction of tracheal smooth muscle was 0.17 ± 0.10 μM.
4-DAMP = 4-diphenylacetoxy-N-methylpiperidine.

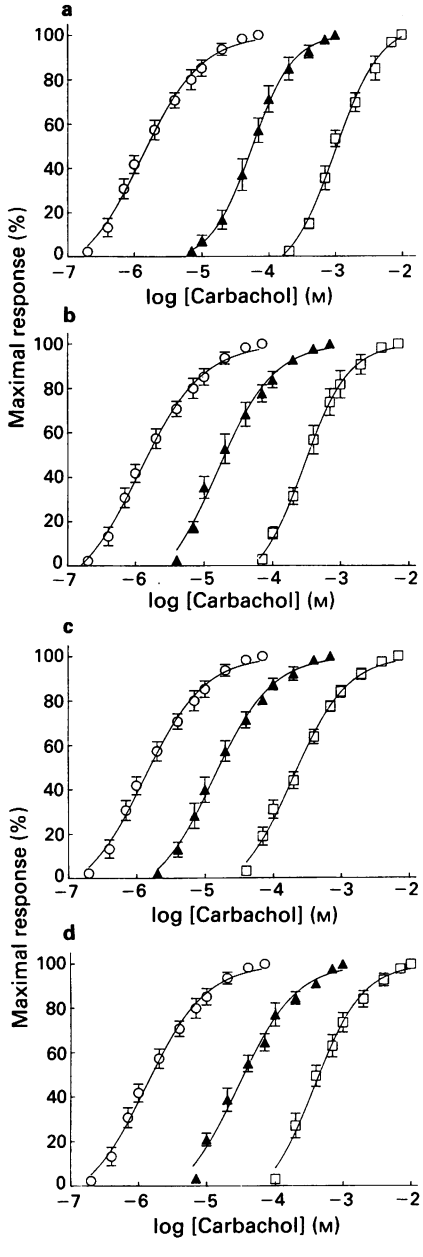


Figure 3 Competitive antagonism of carbachol-mediated contraction in tracheal smooth muscle by (a) atropine, (b) pirenzepine, (c) methoctramine, and (d) 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP). Each point represents the mean ± s.e. of at least eight experiments; s.e. shown by vertical bars. Curves of the contraction induced by carbachol (5 min exposure) were generated and expressed as the percentage of the response to a maximal effect of carbachol. Carbachol-mediated smooth muscle contraction was measured in the absence (○) and presence of atropine (▲, 10 nM and □, 100 nM), pirenzepine (▲, 1 μM and □, 10 μM), methoctramine (▲, 10 μM and □, 100 μM), and 4-DAMP (▲, 0.1 μM and □, 1 μM).

to phospholipase C activation; and (4) the contractile response was triggered mainly by the M_3 subtype via the IPs pathway.

The discrepancy in the dose-response relationships for the effect of carbachol on the metabolism of cyclic AMP and phosphoinositide in tracheal smooth muscle is consistent with those reported by others using different tissue and cell preparations (Brown & Brown, 1984). Our findings may reflect the presence of muscarinic receptor subtypes (M_2 and M_3) in the intact TSMCs, each coupled selectively to a single effector system. The presence of spare receptors is one of the explanations which may be used to rationalize the low and high affinity agonist interactions (Harden *et al.*, 1986). The differences in the properties of the two second messenger responses could then lie at the level of receptor-effector coupling: the partial receptor occupancy would trigger a maximal inhibition of adenylate cyclase whereas a total receptor occupancy should be required for the generation of IPs.

As an alternative approach to define receptor subtypes, the determination of antagonist affinities can provide more accurate information than the use of agonist relative potencies (Birdsall *et al.*, 1978). From the experiments, the effects of discriminating antagonists have been analysed at the level of functional and biochemical responses. Atropine was non-selective in antagonizing the different muscarinic responses, displaying K_i values in the nanomolar range corresponded to pK_B values. Furthermore, pirenzepine could not differentially antagonize the different muscarinic effects. The pK_B values of pirenzepine for inhibiting the generation of IPs, tension and for reversing cyclic AMP attenuation (7.0, 7.1, and 6.7, respectively) were of low affinity (Hammer *et al.*, 1980; Hulme *et al.*, 1990). This suggests that the M_1 receptor subtype does not contribute to these two biochemical responses. This was confirmed by the competitive inhibition of [^{3}H]-N-methylscopolamine binding which excluded the presence of M_1 subtype in the TSMCs (Roffel *et al.*, 1988; Yang, 1991).

The results obtained with methoctramine and 4-DAMP, differentiated between the receptors mediating the IPs and cyclic AMP responses. The muscarinic receptors possessing high affinity for methoctramine ($pK_B = 7.6$) and low affinity for 4-DAMP ($pK_B = 8.1$) on carbachol-mediated attenuation of the cyclic AMP response can be considered as the M_2 subtype (Giachetti *et al.*, 1986; Melchiorre *et al.*, 1987; Micheletti *et al.*, 1987). The receptor with low affinity for methoctramine ($pK_B = 6.1$) and a high affinity for 4-DAMP ($pK_B = 8.8$) can be classified as the M_3 subtype which is involved in carbachol-mediated generation of IPs and consequently contraction (Roffel *et al.*, 1990; Hulme *et al.*, 1990).

The pK_B values for inhibiting the carbachol-mediated contraction are similar to those for antagonizing carbachol-induced accumulation of IPs (Tables 2 and 3). This is in agreement with reports by Baron *et al.* (1984), which showed that muscarinic contraction is associated with the generation of inositol trisphosphate (Berridge *et al.*, 1984; Roffel *et al.*, 1990) which mobilizes Ca^{2+} from intracellular stores in the smooth muscle cells (Hashimoto *et al.*, 1985). Contraction of tracheal smooth muscle by muscarinic agonists is not dependent on extracellular Ca^{2+} (Farley & Miles, 1977). Therefore, the demonstration that muscarinic agonists stimulate the accumulation of IPs in tracheal smooth muscle concurs with the assumption that phosphoinositide breakdown is the transducing mechanism for muscarinic contraction through the release of Ca^{2+} from its internal stores. The contribution of the M_2 subtype, via the cyclic AMP system, to the modulation of the contractile or another response in smooth muscle remains an unsolved problem.

In addition, our data can be considered as indirect evidence for the presence of both M_2 and M_3 receptor subtypes in tracheal smooth muscle which is supported by radioligand binding studies in this tissue (Roffel *et al.*, 1988; Yang, 1991). Furthermore, our results are in agreement with those of a recent paper published whilst this article was in review by Candell *et al.* (1990) on rat longitudinal smooth muscle of ileum, which comes to a similar conclusion.

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The actions of propofol on inhibitory amino acid receptors of bovine adrenomedullary chromaffin cells and rodent central neurones

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1 The interaction of the intravenous general anaesthetic propofol (2,6-diisopropylphenol) with the $GABA_A$ receptor has been investigated in voltage-clamped bovine chromaffin cells and rat cortical neurones in cell culture. Additionally, the effects of propofol on the glycine and $GABA_A$ receptors of murine spinal neurones were determined.

2 Propofol (1.7–16.8 μM) reversibly and dose-dependently potentiated the amplitude of membrane currents elicited by GABA (100 μM) applied locally to bovine chromaffin cells. Intracellular application of propofol (16.8 μM) was ineffective. In rat cortical neurones and murine spinal neurones, extracellular application of 8.4 μM and 1.7–16.8 μM propofol respectively produced a potentiation of GABA-evoked currents qualitatively similar to that seen in the bovine chromaffin cell.

3 The potentiation by propofol (1.7 μM) was not associated with a change in the reversal potential of the GABA-evoked whole cell current. On outside-out membrane patches isolated from bovine chromaffin cells, propofol (1.7 μM) had little or no effect on the GABA single channel conductances, but greatly increased the probability of the GABA-gated channel being in the conducting state.

4 The potentiation of GABA-evoked whole cell currents by propofol (1.7 μM) was not influenced by the benzodiazepine antagonist flumazenil (0.3 μM). A concentration of propofol (1.7 μM) that substantially potentiated GABA currents had little effect on currents induced by the activation of the $GABA_A$ receptor by pentobarbitone (1 mM).

5 Bath application of propofol (8.4–252 μM), to bovine chromaffin cells voltage clamped at −60 mV, induced an inward current associated with an increase in membrane current noise on all cells sensitive to GABA. Intracellular application of propofol (16.8 μM) was ineffective in this respect. Local application of propofol (600 μM) induced whole cell currents with a reversal potential dependent upon the Cl^- gradient across the cell membrane.

6 On outside-out membrane patches formed from bovine chromaffin cells, propofol (30 μM) induced single channels with mean chord conductances of 29 and 12 pS. The frequency of propofol channels was greatly reduced by coapplication of 1 μM bicuculline. Under identical ionic conditions, GABA (1 μM) activated single channels with mean chord conductances of 33, 16 and 10 pS.

7 Bath applied propofol (0.84–16.8 μM) dose-dependently potentiated strychnine-sensitive currents evoked by glycine (100 μM) in murine spinal neurones.

8 The relevance of the present results to the general anaesthetic action of propofol is discussed.

Keywords: Propofol; $GABA_A$ receptors; glycine receptors; patch clamp; general anaesthetic; bovine adrenomedullary chromaffin cells; mouse spinal neurones; rat cortical neurones

Introduction

The mechanism(s) by which intravenous general anaesthetics produce their depressant effects is unclear. A variety of central neurotransmitter receptors and ion channels have been implicated as possible targets and it is conceivable that these drugs do not produce their effects by a unitary molecular mechanism of action. However, a number of studies have demonstrated that a variety of structurally diverse anaesthetics share a common property, namely the ability to enhance the action of γ-aminobutyric acid (GABA) on $GABA_A$ receptors at clinically relevant doses (Keane & Biziere, 1987). Such drugs include the anaesthetic barbiturates (e.g. Macdonald *et al.*, 1986; Owen *et al.*, 1986; Olsen *et al.*, 1986; Olsen, 1988), steroidal anaesthetics (Harrison & Simmonds, 1984; Barker *et al.*, 1987; Harrison *et al.*, 1987; Cottrell *et al.*, 1987; Callachan *et al.*, 1987; Lambert *et al.*, 1987; Lambert & Peters, 1989), chlormethiazole (Gent & Wacey, 1983; Simmonds & Turner, 1987; Hales & Lambert, 1988a), propanidid (Kirkness & Turner, 1986; Peters *et al.*, 1989) and etomidate (Olsen *et al.*, 1986; Robertson, 1989).

Propofol, the active component of which is 2,6-diisopropylphenol, is an intravenous general anaesthetic (Glen, 1980) recently introduced into clinical practice in an emulsion formulation (Coates *et al.*, 1985; de Grood *et al.*, 1985; MacKenzie & Grant, 1985; Langley & Heel, 1988). In cat spinal cord, propofol enhances synaptic inhibition, suggesting an action of the drug on $GABA_A$ receptors (Lodge & Anis, 1984). Consistent with this proposal, propofol potentiated GABA-induced depolarizations in slices of rat olfactory cortex (Collins, 1988). In view of the increasing use of propofol as an intravenous anaesthetic, we have investigated its actions on $GABA_A$ and glycine receptors in an attempt to understand better its mechanism of action. A preliminary account of a part of this work has appeared in abstract form (Hales & Lambert, 1988b; Hales *et al.*, 1990).

Methods

Dissociation and culture of bovine chromaffin cells

Bovine adrenomedullary chromaffin cells were isolated and cultured by the method of Fenwick *et al.*, 1982 with minor

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modifications (Cottrell *et al.*, 1987; Peters *et al.*, 1989) and used in electrophysiological experiments 1–7 days after plating.

Dissociation and culture of rat cortical neurones

Seven embryos (E18) were dissected from Sprague-Dawley rats which had been killed by cervical dislocation. The embryos were decapitated and their brains removed. The cerebral cortex was teased away from both hemispheres of the brain with fine-dissecting forceps and was isolated in Hank's balanced salt solution at 17–21°C. Hippocampal tissue and the meninges were removed from both cortical sections and the remainder of the cortex was chopped into fragments. The procedure from then on was similar to that described for the culture of neurones from the rat visual cortex by Heuttner & Baughman (1986), with minor modifications (Halliwell *et al.*, 1989). Cortical neurones were plated directly onto thirty 35 mm diameter 'Primaria' (Falcon) culture dishes and used for electrophysiological recording 10 to 30 days after plating. The proliferation of non-neuronal cells was prevented, as they approached confluency, by the inclusion of cytosine arabinoside (10 μM) in the growth medium for 48 h. Cells were incubated at 37°C in an atmosphere of 95% air, 5% CO_2 at 100% relative humidity, in growth medium comprising Minimal Essential Medium supplemented with 5% (v/v) foetal calf serum, 5% horse serum, streptomycin and penicillin (50 iu ml^{-1}). The final concentrations of glutamine and glucose in the medium were adjusted to 2 and 20 mM respectively.

Dissociation and culture of murine spinal neurones

Embryonic spinal neurones were dissociated and maintained in culture by a method similar to that of Barker *et al.* (1982). Briefly, the spinal cords of 13–14 day murine embryos were dissected out and dissociated in Hank's balanced salt solution supplemented with 0.25% trypsin at 37°C in 90% air, 10% CO_2 for 30 min. Partially dissociated tissue was transferred to a 10 ml centrifuge tube containing incubation medium consisting of Dulbecco's Modified Eagles Medium (with pyruvate), supplemented with $NaHCO_3$ (1.5 mg ml^{-1}), 10% v/v heat inactivated foetal calf serum, 10% v/v heat inactivated horse serum, penicillin/streptomycin (50 iu ml^{-1}) and glutamine (2 mM). The osmolarity of the incubation medium was adjusted to 330 mOsmol. The tissue was gently triturated with a flame polished Pasteur pipette. This process was repeated, with pipettes flame polished to reduce their aperture, until complete dissociation was achieved. The cell suspension was removed from the top of the medium during trituration and added to a centrifuge tube containing fresh incubation medium.

Cells were plated on to 35 mm diameter tissue culture dishes at a density of 1×10^6 cells/plate and incubated in 1.5 ml of incubation medium and a humid atmosphere of 90% air, 10% CO_2. After 5 days, the incubation medium was replaced by a similar medium supplemented with $NaHCO_3$ (1.5 mg ml^{-1}) and glucose (5 mg ml^{-1}). Initially this medium also contained the mitotic inhibitors 2-deoxy-5-fluoridine (15 μg ml^{-1}) and uridine (35 μg ml^{-1}). After two days the medium was replaced by one lacking the mitotic inhibitors, but otherwise containing the same constituents. The medium was renewed every third day and cells were used for electrophysiological recording between weeks 6 and 12.

Electrical recordings

'Whole cell' and 'outside-out' patch recordings of agonist-activated currents were made by use of a List Electronics L/M EPC-7 converter headstage and amplifier. Currents were low-pass filtered (Bessel characteristic) at the cut off frequencies indicated in the figure legends and recorded with an FM tape recorder (Racal Store 4DS) on magnetic tape (Ampex). Cells were continuously superfused (3–5 ml min^{-1}) with a recording saline containing (in mM): NaCl 140, KCl 2.8, $MgCl_2$ 2.0, $CaCl_2$ 1.0, HEPES-NaOH 10 (pH 7.2). For chromaffin cell experiments, the pipette solution used to dialyse the cell interior contained (in mM): CsCl 140, $MgCl_2$ 2.0, $CaCl_2$ 0.1, EGTA 1.1 and HEPES-NaOH 10 (pH 7.2). In some recordings, the internal chloride ion concentration was reduced to 30 mM, by the partial replacement of CsCl with Na glutamate. Liquid junction potentials arising at the tip of the patch pipette were corrected for by use of the method of Fenwick *et al.* (1982). For experiments on rodent neurones, the pipette saline comprised (in mM): KCl 140, $MgCl_2$ 2, $CaCl_2$ 0.1, EGTA 1.1 and HEPES-KOH 10 (pH 7.2).

For the intracellular application of propofol, the anaesthetic was diluted into the pipette saline at the desired concentration from a stock solution of 10^{-2} M made up in ethanol. The diffusional exchange rate between the patch pipette solution and the cell is dependent upon the molecular weight of the drug, the cell-pipette access resistance (3.2 ± 0.3 MΩ, range 1.9–4.4 MΩ, $n = 6$), and the cell volume, which can be estimated from the cell capacitance (7.6 ± 0.3 pF, range 6.4–8.6 pF, $n = 6$) assuming a specific membrane capacitance of 1 μF cm^2 (Pusch and Neher, 1988). Under the conditions of this study, the time constant for the diffusional exchange of the drug between the recording pipette solution and the cell interior was calculated to be approximately 16 s. Drugs were applied either locally, by pressure ejection (General Valve Picospritzer II) from modified patch pipettes (at a pressure of 1.4×10^5 Pa, a frequency of 0.05 Hz and a duration of 10–30 ms, unless otherwise stated), or added to the superfusion medium. All experiments took place at 17–21°C.

Data analysis

Whole cell currents, elicited by locally applied agonists, were either replayed onto a pen recorder (Lectromed multitrace 2) and measured manually, or analysed by a computer programme (Dempster, 1988) run on a PDP 11-73 minicomputer. In the latter method, whole cell currents were digitized into 512 points at 100 Hz by a Cambridge Electronic Design 502 analogue to digital converter, inspected on a display screen and edited. Unless stated otherwise, four suitable records were then averaged, analysed, and for illustration plotted on a Hewlett Packard 7470A plotter.

Single channel currents recorded from outside-out membrane patches were low pass filtered (Bessel characteristic, cut-off frequency 1 kHz) and stored on magnetic tape. Data (30–120 s duration) were replayed into the computer (PDP 11-73) via a Cambridge Electronic Design 502 analogue to digital converter sampling at 10 kHz and stored in digital form. The digitized signal was then viewed on an Electronic Visual 8060 oscilloscope as adjacent records of 512 samples. The display could be expanded to allow a closer inspection of the signal. Single channel amplitudes were determined by two methods.

(1) A semi-automated procedure was used in which the digitized signal was visually inspected record by record on the display screen. The mean current for a channel opening was determined by marking the beginning and end using a keyboard operated display cursor then allowing the computer programme to average the sample points between the cursors. Care was taken to ensure points during the opening and closing transitions were excluded and channels with a duration greater than 400 μs only were used. Currents were measured relative to the zero current baseline level.

(2) An automated procedure in which a threshold level was set during a quiescent section of the recording as close to the baseline as possible. The programme was allowed to sweep through the digital recording, using the threshold to determine the location of each channel opening and closure. The mean channel current for each opening was determined by averaging the continuous series of sample points between each channel opening and closure. The first and last 2 sample points were always excluded to prevent the measurements being biased towards smaller values by the opening and

closing transitions. This had the effect of totally excluding all openings with a duration less than 400 μs.

Little difference was found in the current amplitudes determined by the two methods. The lists of channel mean currents obtained by methods (1) and (2) were compiled into current amplitude histograms. Estimates of the amplitude of each observed channel conductance state were obtained by simultaneous fitting of two Gaussian curves to the distribution, according to the equation:

$$Y_{bin} = \Sigma_{g=1}^{2} \frac{a_g w}{\sigma_g \sqrt{2\pi}} \exp\left(\frac{-(I_{bin} - I_g)^2}{2\sigma_g^2}\right)$$

where Y_{bin} is the contents of the amplitude histogram bin with mid-point current I_{bin}, I_g is the channel mean current amplitude, σ_g the standard deviation, and a_g the area underneath the Gaussian curve g, and w the histogram bin width. An iterative non-linear curve fitting routine (Brown & Dennis, 1972) was used to find the best fitting set of values for the parameters I_g, σ_g and a_g for each Gaussian.

The probability (P_{open}) of at least one channel being in a conducting state was determined for 30–120 s segments of data using the equation:

$$P_{open} = T_{open}/(T_{open} + T_{closed})$$

where T_{open} and T_{closed} are the total open and closed times obtained as the sums of the channel open and closed times, determined by the threshold method described in (2) above.

All quantitative results are expressed as the arithmetic mean ± s.e.mean.

Drugs used

Drugs used in the study were: glycine, γ-aminobutyric acid (GABA), (+)-bicuculline, sodium pentobarbitone and strychnine (all Sigma), 2,6-diisopropylphenol (Aldrich), Intralipid (an aqueous solution of soya bean oil, phospholipid and glycerol; (Kabi Vitrum)), Diprivan (10 mg 2,6-diisopropylphenol, 10 ml of Intralipid vehicle); (ICI)), flumazenil and diazepam (Roche).

Stock solutions of 2,6-diisopropylphenol (Aldrich) and diazepam were prepared in ethanol. The final concentration of ethanol in the electrophysiological experiments did not exceed 0.1% (v/v) a concentration which does not influence GABA currents of bovine chromaffin cells (Cottrell *et al.*, 1987).

Results

Potentiation of GABA-evoked currents by propofol

Propofol in the 'Intralipid' formulation (8.4 μM), applied via the superfusion medium, potentiated the amplitude of submaximal whole-cell currents evoked by locally applied GABA (100 μM) on bovine chromaffin cells to 858 ± 210% (mean ± s.e.mean; n = 8) of control (Figure 1a). This concentration of propofol produced a similar potentiation of GABA-evoked currents recorded from rat cortical neurones (Figure 1b). To confirm that under our experimental conditions the GABA potentiating actions of propofol are not influenced by the Intralipid vehicle, we performed experiments with propofol made up in ethanol (see Methods). Previous experiments have demonstrated that ethanol has no effect on GABA-evoked responses recorded from chromaffin cells (Cottrell *et al.*, 1987). This formulation of propofol (8.4 μM) potentiated GABA-evoked whole cell currents of bovine chromaffin cells (Figure 1c) to 931 ± 65% (n = 5) of control. The effect of propofol in either formulation was concentration-dependent (Figure 1d) over the range investigated (1.7–16.8 μM) and readily reversed on washout. Inspection of Figure 1d suggests that propofol is more potent than pentobarbitone in potentiating GABA. In the majority of the following experiments propofol in the ethanol formulation was used.

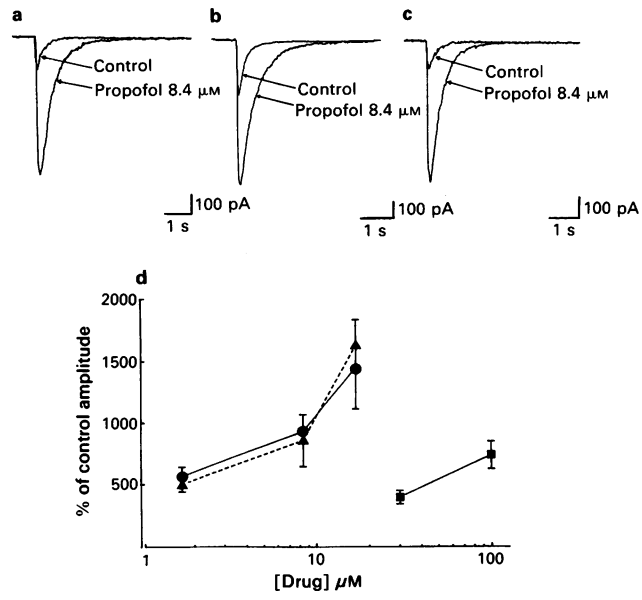


Figure 1 Potentiation of γ-aminobutyric acid (GABA)-evoked whole-cell currents by propofol. Propofol (8.4 μM in the Intralipid formulation) produces a potentiation of whole cell currents activated by GABA (100 μM) in (a) a bovine chromaffin cell and (b) a rat cortical neurone. (c) Propofol (8.4 μM in the ethanol formulation) produces a similar potentiation of the GABA-evoked whole cell current of bovine chromaffin cells to that seen in (a). In all cases cells were voltage-clamped at −60 mV and currents were low pass filtered at 500 Hz. All whole cell currents illustrated are the computer generated average of four responses. (d) Illustrates graphically the dose-dependency of the potentiating effect of propofol in Intralipid (▲) and ethanol (●) determined from bovine chromaffin cells. Pentobarbitone (■) appeared less potent than propofol in potentiating GABA-evoked currents. Data are plotted as a percentage of the control response amplitude against the concentration (μM) of propofol or pentobarbitone (on a logarithmic scale) in the bathing medium. Data points are the mean of at least four experiments obtained from different cells and the vertical bars indicate the standard error of the mean.

The molecular mechanism of the propofol-induced potentiation

Propofol could potentiate GABA-evoked responses by one of several mechanisms, including an inhibition of GABA uptake by the chromaffin cell (Kataoka *et al.*, 1984), a change in the driving force upon chloride ions which mediate the GABA$_A$ response (Cottrell *et al.*, 1985; Peters *et al.*, 1989), an increase in the conductance of GABA-activated single channels, or an increase in the probability of the GABA-channel being in a conducting state. The GABA uptake blocker, nipecotic acid (1 mM), has no effect on GABA-evoked whole-cell currents in bovine chromaffin cells (Cottrell *et al.*, 1987). Therefore, an action of propofol to inhibit the active uptake system for GABA is unlikely to account for the potentiation. The potentiation of GABA-evoked whole-cell currents evoked by propofol (1.7 μM), occurred without any significant effect on the reversal potential of the response (Figure 2), excluding a change in the driving force on chloride ions by the anaesthetic contributing to the potentiation.

Figure 3 depicts single channel currents induced by 1 μM GABA bath applied to an outside-out membrane patch. The determination of the current amplitudes (see Methods) and the calculation of the chord conductances (Bormann *et al.*, 1987) from four membrane patches (Table 1) confirms previous reports that the GABA channel of bovine chromaffin cells exhibits more than one conductance state (Bormann & Clapham, 1985). The addition of propofol (1.7 μM) to the bath had little effect on the GABA single channel amplitudes and chord conductances although, in one case (Cell No. 2 – Table 1), propofol appeared to increase the smaller GABA chord conductance from 15.3 to 20.8 pS. Further experiments are

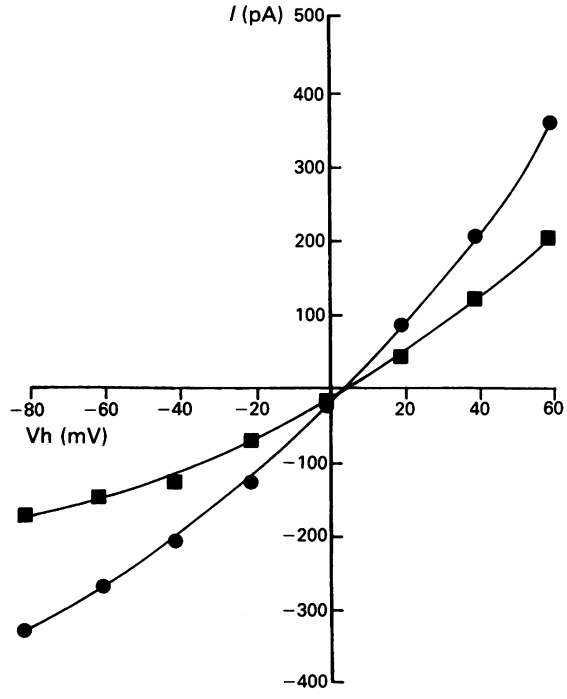


Figure 2 Propofol does not change the reversal potential of γ-aminobutyric acid (GABA)-evoked whole-cell currents. The relationship between holding potential and the amplitude of the GABA-evoked current (100 μM, applied locally by pressure ejection) is plotted in the absence (■) and presence (●) of propofol (1.7 μM). The curves, fitted to the data points by eye, yield reversal potentials of +3.5 mV and +3.0 mV for GABA-evoked responses in the absence and presence of propofol (1.7 μM), respectively. The data were obtained from the same chromaffin cell.

required to establish the importance of this observation. It is evident from inspection of Figure 3, that propofol produces a dramatic effect on the kinetics of the GABA single channel currents. The presence of multiple conductance states has thus far precluded a detailed assessment of the effects of propofol on single GABA-activated ion channels. However, an estimate of the propofol effect was determined by calculating the total time that at least one GABA-activated channel was open during a continuous 30–120 s segment of data. The probability of at least one channel being open, estimated in this manner, was increased in all cases by propofol (1.7 μM; $n = 4$) although clearly there was a large variability between membrane patches for this effect (range: 157–1214% of control) see Table 1.

The action of propofol is not reversed by the benzodiazepine antagonist, flumazenil

The benzodiazepines, diazepam and flunitrazepam, potentiate GABA-evoked whole cell currents in bovine chromaffin cells, an effect which is reversed by flumazenil (Cottrell *et al.*, 1985; 1987; Peters *et al.*, 1989). In confirmation of these studies, 0.3 μM flumazenil reversed the potentiation of the GABA-evoked current induced by 1 μM diazepam (Figure 4a). In contrast, the GABA potentiating action of 1.7 μM propofol (Figure 4b) was insensitive to this concentration of flumazenil ($n = 4$). These results suggest that the potentiation induced by propofol is not via a direct interaction with the benzodiazepine recognition site of the $GABA_A$ receptor.

The action of propofol on pentobarbitone-induced currents

Pentobarbitone activates the $GABA_A$ receptor of bovine chromaffin cells (Peters *et al.*, 1988). Propofol (1.7 μM) produced

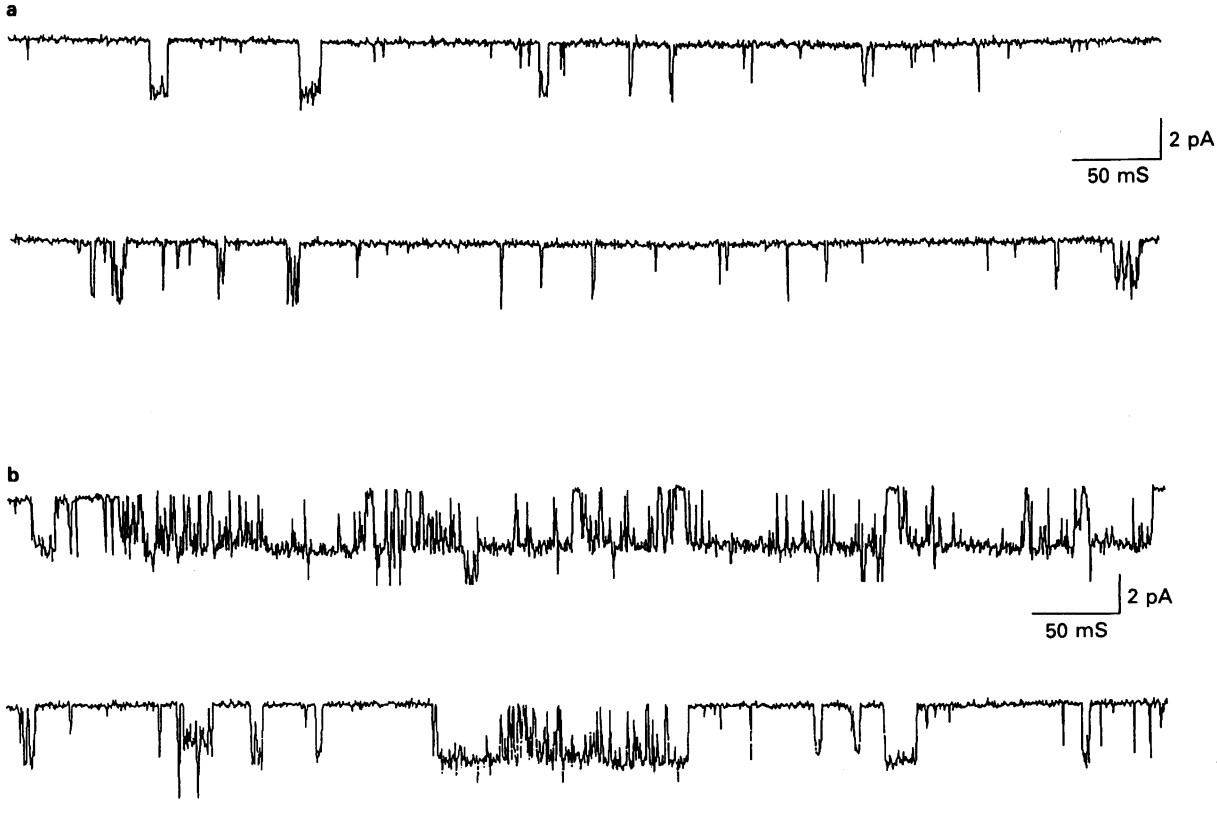


Figure 3 Modulation of γ-aminobutyric acid (GABA)-activated single channel activity by propofol. Illustrated are GABA (1 μM)-activated channels recorded from an outside-out membrane patch excised from a bovine chromaffin cell in the absence (a) and presence (b) of propofol (1.7 μM). For this membrane patch, propofol (1.7 μM) increased the probability of at least one GABA channel being in a conducting state (see Methods) from 0.054 to 0.229 (i.e. a 424% increase, determined from a 60 s segment of data before and after propofol). Visual inspection of the figure suggests that propofol (1.7 μM) has little effect on the GABA single channel amplitudes (see Table 1).

Table 1 The action of propofol (1.7 μM) on γ-aminobutyric acid (GABA)-activated single channels recorded from outside-out membrane patches excised from bovine chromaffin cells

	GABA (1 μM)			*GABA* (1μM) + *propofol* (1.7 μM)			
Membrane patch No.	*Chord conductances* (pS)		*Open probability*	*Chord conductances* (pS)		*Open probability*	*Open probability as a % of control*
1	30.4	14.5	0.054	30.3	14.4	0.229	424
2	31.6	15.3	0.00695	30.3	20.8	0.0844	1214
3	31.5	13.6	0.0205	32.4	14	0.0322	157
4	30.4	13.3	0.0094	29.1	12.6	0.0539	574
$\bar{x} \pm$ s.e.mean	31.0 ± 0.4	14.2 ± 0.5		30.5 ± 0.7	15.5 ± 2.1		

The mean chord conductances (pS) of GABA channels calculated assuming a reversal potential of 0 mV (Bormann *et al.*, 1987). The open probabilities of channels activated by GABA (1 μM), under control conditions and in the presence of propofol (1.7 μM) are also given. The right hand column lists open probabilities for GABA-evoked channels in the presence of propofol (1.7 μM) as a percentage of control GABA-evoked channel open probabilities.

only a modest potentiation of pentobarbitone (1 mM)-induced currents to 187 ± 16% of control ($n = 8$). In contrast, this dose of propofol caused a large potentiation of GABA (100 μM)-induced currents to 564 ± 78% of control ($n = 4$). A similar result was obtained from cells to which the local application of GABA (100 μM) and pentobarbitone (1 mM) was alternated and the inward currents induced by the two 'agonists' approximately matched for amplitude (Figure 4c).

Electrophysiological (Callachan *et al.*, 1987; Cottrell *et al.*, 1987; Lambert *et al.*, 1987) and radioligand binding experiments (Gee *et al.*, 1988; Peters *et al.*, 1988; Kirkness, 1989; Turner *et al.*, 1989) suggest that anaesthetic steroids and barbiturates bind to different sites on the $GABA_A$ receptors to potentiate the action of GABA. In confirmation of previous results (Peters *et al.*, 1988) the anaesthetic steroid 5β-pregnan-3α-ol-20-one (0.5 μM) produced a marked potentiation of both GABA currents (856 ± 194% of control, $n = 10$) and pentobarbitone currents (906 ± 121% of control, $n = 16$). A similar qualitative result was obtained from cells alternately challenged with 100 μM GABA and 1 mM pentobarbitone (Figure 4d).

Propofol activates the $GABA_A$ receptor

Bath application of propofol (8.4–252 μM), to a bovine chromaffin cell voltage-clamped at −60 mV, induced an inward current associated with an increase in membrane noise (Figure 5a). The threshold concentration for this effect is <8.4 μM (Figure 5a,b). At high concentrations (252 μM) the peak inward current produced was less than that obtained with the lower concentration of 84 μM and the current waned during the continuous application of propofol (Figure 5a,b). The direct action was more conveniently studied in experiments where propofol (600 μM) was locally applied to cells by pressure ejection. Propofol currents induced in this way had a reversal potential of 3.4 ± 0.2 mV ($n = 4$, see Figure 5c,d). Under these ionic conditions (i.e. an approximately symmetrical distribution of Cl^- across the cell membrane: $[Cl^-]_o = 148$ mM; $[Cl^-]_i = 144$ mM) GABA induces currents which reverse in sign at 2.9 ± 1.3 mV ($n = 5$). Reduction of the internal chloride concentration to 30.2 mM changed the reversal potential of the propofol and GABA-induced currents to −36.4 ± 1.8 mV ($n = 4$) and −34.7 ± 1.4 mV ($n = 5$), respectively. The reversal potential of the propofol-induced current under the two internal chloride concentrations studied are similar to those predicted by the Nernst equation for Cl^- and suggest that propofol, like GABA, selectively increases the chloride conductance of the cell membrane. In the present study, GABA activated channels with multiple chord conductances of approximately 33, 16 and 10 pS (Figure 6, Table 2). These values are similar to the slope conductances of 31, 18 and 11 pS reported in a previous study (Bormann & Clapham,

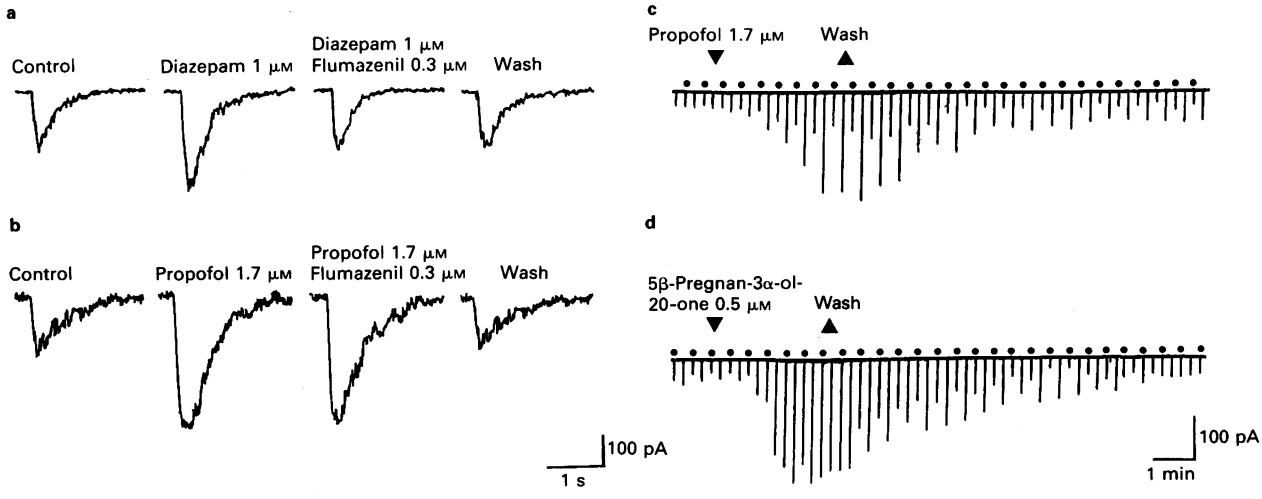


Figure 4 The potentiation of γ-aminobutyric acid (GABA)- and pentobarbitone-evoked currents by propofol. (a,b) Influence of flumazenil (0.3 μM) upon the potentiation of GABA-evoked whole cell currents (100 μM, applied locally by pressure ejection) by (a) diazepam (1 μM) and (b) by propofol (1.7 μM). Data obtained from the same chromaffin cell. (c,d) Traces illustrating currents evoked alternately by (●) pentobarbitone (1 mM; locally applied by pressure ejection 1.4×10^5 Pa for 30 ms at a frequency of 0.03 Hz) and by (no symbol) GABA (100 μM; locally applied by pressure ejection 1.4×10^5 Pa for 20 ms) and their modulation by bath applied (c) propofol (1.7 μM), and (d) 5β-pregnan-3α-ol-20-one (0.5 μM), lower panel. Note that propofol greatly potentiated the GABA-evoked currents but produced only a modest increase of the pentobarbitone-evoked current. In contrast the steroidal anaesthetic 5β-pregnan-3α-ol-20-one (0.5 μM) produced a substantial enhancement of both GABA- and pentobarbitone-activated currents. All traces illustrated in this figure were recorded from bovine chromaffin cells, voltage-clamped at −60 mV and low pass filtered at 500 Hz.

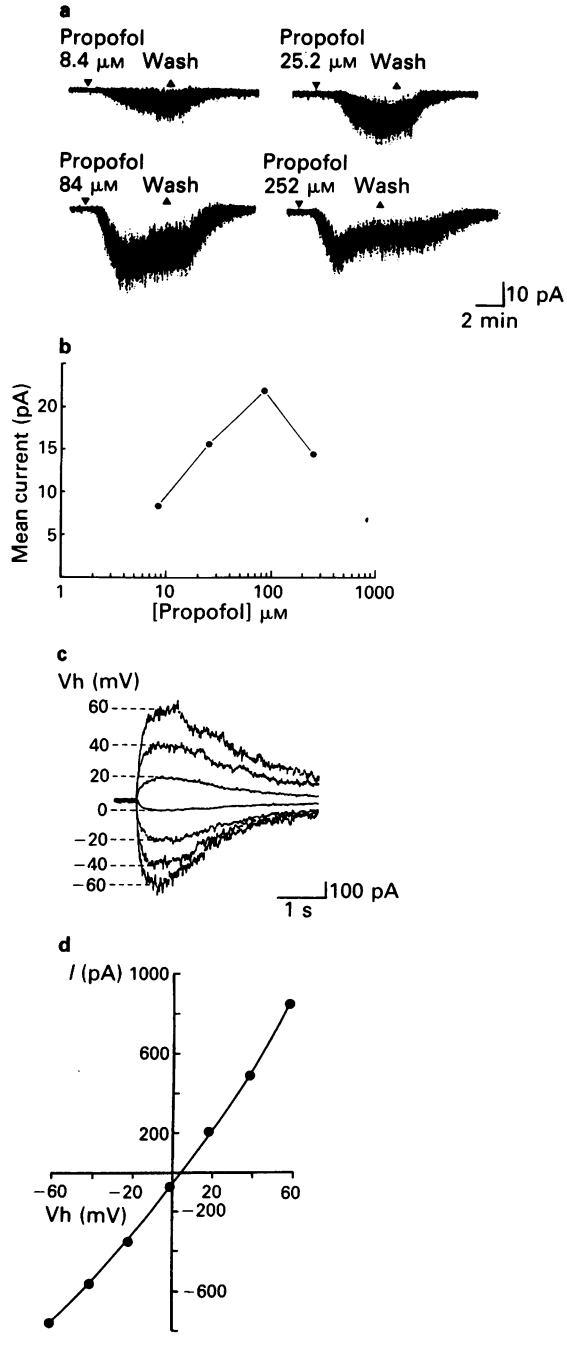


Figure 5 A direct agonist action of propofol. (a) Bath application of propofol (8.4–84 μM) to a bovine chromaffin cell voltage clamped at −60 mV induced a dose-dependent inward current associated with a large increase in membrane current noise. At higher concentrations of propofol (252 μM) this effect was reduced. The mean propofol-induced currents were determined during the plateau phase of the drug effect using a computer programme (Dempster, 1988; Peters *et al.*, 1988). Data were low pass filtered at 1 kHz. (b) A graph of the amplitude of propofol-induced currents (shown in (a)) is plotted against the concentration of propofol (on a logarithmic scale) in the medium. From this plot it is clear that the threshold for activation occurs with doses of propofol <8.4 μM. (c) Traces illustrating the currents evoked by locally applied propofol (600 μM pressure applied at 1.4×10^5 pA for 50 ms) at holding potentials (Vh) ranging from −60 to +60 mV. (d) The relationship between holding potential and response amplitude is shown graphically, and indicates that the propofol-induced current has a reversal potential of +3 mV (determined by interpolation) in this example. The data in (d) were corrected for the liquid junction potential and currents in (c) were low pass filtered at 500 Hz.

1985). A large (45 pS) conductance state of the $GABA_A$ channel (Bormann & Clapham, 1985) was not present, although we have occasionally observed a channel of this conductance on a small proportion of membrane patches (Hales, Lambert & Peters, unpublished observations). Propofol (30 μM) activated single channels on previously quiescent membrane patches (Figure 6). Most membrane patches exhibited two main conductance states with values of approximately 29 and 12 pS (see Table 2). The frequency of propofol-induced single channels was greatly reduced by co-application of 1 μM bicuculline, a $GABA_A$ receptor antagonist (Figure 6).

Propofol modulation of $GABA_A$ receptors exhibits membrane asymmetry

Propofol is highly membrane soluble (James & Glen, 1980) and hence may produce its effects on $GABA_A$ receptors indirectly via an interaction with the surrounding lipid membrane. In an attempt to define further the site of action of this anaesthetic the potentiating effect of bath applied propofol (1.7 μM) on GABA-evoked currents was compared in control cells and in cells dialysed intracellularly for 15–60 min with 16.8 μM propofol (see Methods). The potentiation of GABA currents by 1.7 μM propofol was similar for control cells (564 ± 78% of

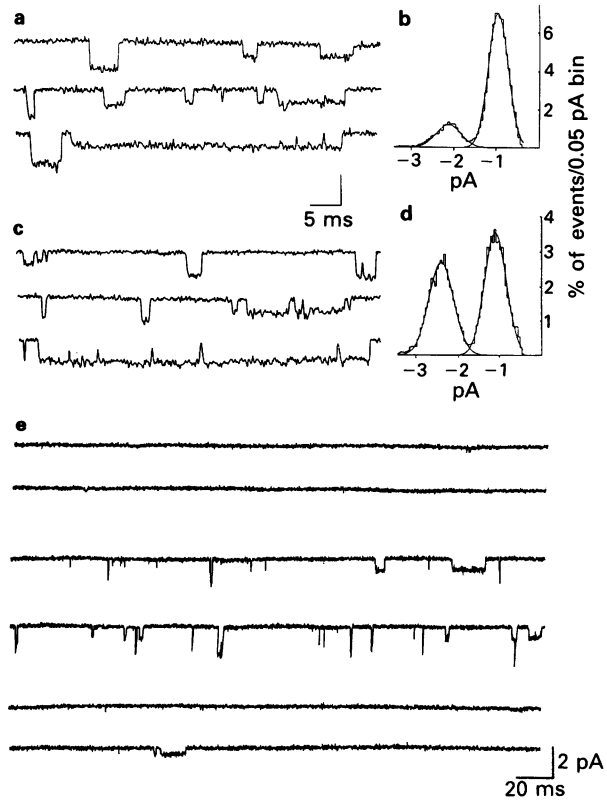


Figure 6 Propofol activates single channel currents. Bath application of propofol (30 μM) to the superfusate of an outside-out membrane patch activated single channels depicted in (a). The distribution of channel amplitudes determined from a continuous 30 s recording is known in (b). The mean channel amplitudes determined by simultaneously fitting two Gaussian curves to this distribution were −2.14 and −0.95 pA. Bath application of γ-aminobutyric acid (GABA) (1 μM) to a different outside-out membrane patch activated single channels depicted in (c). The distribution of channel amplitudes determined from a continuous 30 s recording is shown in (d). The mean channel current amplitudes determined by simultaneously fitting two Gaussian curves to this distribution were −2.43 and −1.06 pA. The top two traces in (e) depict a control recording from an outside-out membrane patch. Bath application of propofol (30 μM) to this patch induced the appearance of single channel currents (middle two traces) which were reduced in frequency by coapplication with the $GABA_A$ receptor antagonist bicuculline (1 μM) (bottom two traces). All records illustrated in this figure were low pass filtered at 1 kHz and recorded at a holding potential of −80 mV from outside-out membrane patches of bovine chromaffin cells. The vertical calibration bar is 2 pA for (a) and 3 pA for (c).

Table 2 The single channel conductances activated by γ-aminobutyric acid (GABA) (1 μM) or propofol (30 μM) recorded from outside-out membrane patches, excised from bovine chromaffin cells

A				B		
Membrane patch No.	*GABA (1 μM) single channel chord conductances* (pS)			*Membrane patch No.*	*Propofol (30 μM) single channel chord conductances* (pS)	
1	31.6	15.3	—	9	32.5	—
2	30.4	14.5	—	10	25.1	10.9
3	31.5	13.6	—	11	28.9	11.8
4	35.3	16.9	—	12	28.8	12.2
5	30.4	13.3	—			
6	31.9	—	—			
7	33	18.5	9.3			
8	36.1	16.6	10.2			
$\bar{x} \pm$ s.e.mean	32.5 ± 0.8	15.5 ± 0.8	9.8		28.8 ± 1.7	11.6 ± 0.5

The chord conductances (pS) of single channels activated (A) by GABA (1 μM) and (B) propofol (30 μM) assuming a reversal potential of 0 mV (Bormann *et al.*, 1987). Membrane patches were voltage clamped at either −80 or −100 mV.

control; $n = 4$) and cells 'dialysed' intracellularly with 16.8 μM propofol (550 ± 150% of control, $n = 6$; see Figure 7).

In addition to potentiating GABA-evoked currents, propofol directly activates chloride conducting channels (Figures 5 and 6). In cells 'dialysed' intracellularly for 15–60 min with propofol (16.8 μM) a low level of baseline noise was observed with no obvious single channel events (Figure 7). Subsequent bath application of 16.8 μM propofol to such cells, induced an inward current associated with an increase of membrane noise (Figure 7).

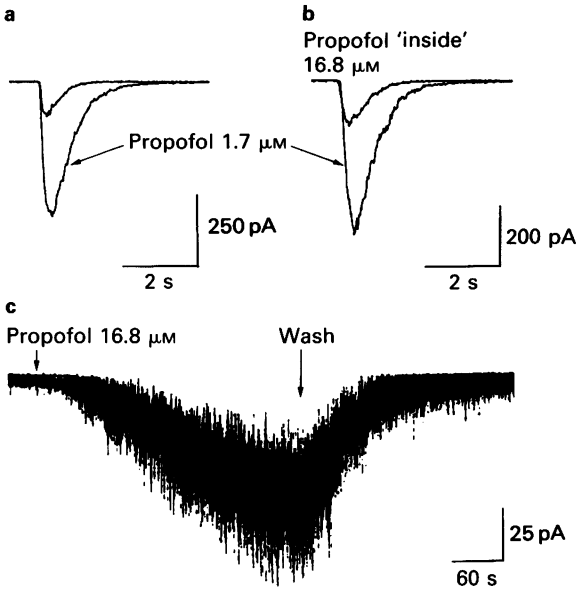


Figure 7 The potentiation of γ-aminobutyric acid (GABA)-evoked currents and activation of the $GABA_A$ receptor by propofol exhibits membrane asymmetry. (a and b) Illustrated are GABA (100 μM)-evoked currents before and after the bath application of 1.7 μM propofol. The traces illustrated in (b) are taken from a cell 'dialysed' for 15 min with a recording electrode containing 16.8 μM propofol. Note that the bath application of 1.7 μM propofol is equally active in both control (a) and test (b) conditions. All traces illustrated in (a) and (b) represent the computer generated average of four responses to (100 μM) GABA applied locally to a chromaffin cell voltage-clamped at −60 mV. (c) A trace obtained from a chromaffin cell 15 min after establishing the whole cell recording mode with a pipette containing 16.8 μM propofol. Visual inspection of the membrane current record at this time indicates low baseline noise with no obvious single channel events. The subsequent bath application of 16.8 μM propofol to this cell induced an inward current, associated with an increase in membrane noise which reversed upon washout. The current was recorded at a holding potential of −60 mV and low-pass filtered at 500 Hz.

Potentiation of glycine-evoked currents by propofol

Embryonic spinal neurones were used to study the effects of propofol on the glycine receptor. Local pressure application of glycine (100 μM) to voltage-clamped neurones, elicited inward currents in all cells tested ($n = 38$). These currents were reversibly abolished by bath-applied strychnine (100 nM; $n = 5$). In contrast, GABA (100 μM) activated currents in spinal neurones were insensitive to strychnine (100 nM; $n = 3$). The GABA currents were observed to run down with time after obtaining the whole-cell configuration ($n = 5$). Experiments investigating the pharmacology of the GABA response in spinal neurones were postponed for 10–20 min, sufficient time for GABA-activated currents to become consistent in amplitude. The phenomenon of GABA-evoked current run-down was not quantified in this study but has previously been documented and is believed to be caused by the loss of ATP from the intracellular milieu due to dialysis by the recording electrode (Stelzer *et al.*, 1988; Song & Huang, 1990). In contrast to currents activated by GABA, those evoked by glycine did not run down during the course of the experiments ($n = 38$). In many cases, an increase in the amplitude of glycine activated currents was observed after acquiring the whole-cell configuration ($n = 15$). Although this phenomenon was not quantified, the time course was similar to that of the run-down of GABA-evoked responses. There is recent evidence implicating the guanosine 5′ triphosphate (GTP) binding protein G_s and adenosine 3′:5′-cyclic monophosphate (cyclic AMP) in the intracellular regulation of the glycine receptor, but not the $GABA_A$ receptor (Song & Huang, 1990).

Currents activated by GABA (100 μM) were reversibly and dose-dependently potentiated by bath-applied propofol (1.7–16.8 μM; Figure 8a). In addition, propofol (8.4–16.8 μM) dose-dependently potentiated strychnine sensitive currents activated by glycine (100 μM; Figure 8b,c). This is not a property of all intravenous anaesthetics since pentobarbitone (10 and 100 μM) had no effect on glycine-evoked currents (Figure 8c).

Discussion

The results described here clearly demonstrate that the phenolic anaesthetic propofol, in either formulation (Intralipid or ethanol), can potentiate the actions of GABA on $GABA_A$ receptors. Propofol is approximately 30 fold more potent than pentobarbitone in this respect. What is the molecular mechanism underlying this effect? The results of experiments on outside-out membrane patches clearly demonstrated that propofol had no significant effect on GABA single channel conductances but greatly increased the probability of a single channel being in the conducting state. The multiple conductance states of the GABA channel have thus far precluded a

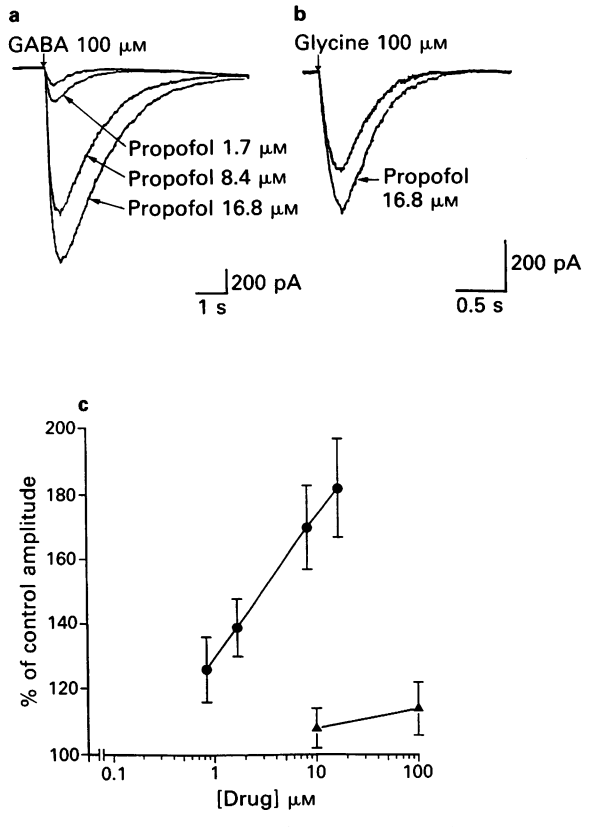


Figure 8 Potentiation by propofol of currents evoked by glycine and γ-aminobutyric acid (GABA) in spinal neurones. The dose-dependent potentiation by propofol (1.7–16.8 μM), of GABA (100 μM)-evoked whole-cell currents recorded from a mouse spinal neurone, is illustrated in (a). The potentiation by propofol (16.8 μM) of a glycine (100 μM)-activated current, recorded from the same cell, is shown in (b). Cells were voltage-clamped at −60 mV and currents were low-pass filtered at 500 Hz. The illustrated currents are computer generated averages of four responses. (c) Illustrates graphically the dose-dependent potentiation of glycine-activated currents by propofol (●) and the lack of effect of pentobarbitone in this respect (▲). Data are plotted as a percentage of the control response amplitude against the concentration of propofol or pentobarbitone in the bathing medium on a logarithmic scale. Data points are the mean of at least four experiments obtained from different cells and vertical bars indicate the standard error of the mean.

formal kinetic analysis of the actions of the anaesthetic on GABA channels. However, visual inspection of records such as those of Figure 3 clearly suggests that propofol prolongs the GABA channel burst duration in a manner similar to that produced by pentobarbitone (Twyman *et al.*, 1989; Macdonald *et al.*, 1990).

A number of drugs of diverse chemical structure are now known to act as positive allosteric modulators of the $GABA_A$ receptor (Keane & Biziere, 1987; Lambert *et al.*, 1991). However, with the possible exception of the benzodiazepines, little is known about their site of action. Indeed, as many of them are highly lipid soluble, it is possible that they modulate the $GABA_A$ receptor indirectly by perturbation of the surrounding membrane (Lambert *et al.*, 1990). Propofol is unlikely to act in this way as it was inactive on $GABA_A$ receptors when applied intracellularly. If propofol has a direct action then where on the $GABA_A$ receptor protein does it bind? It is now established that the $GABA_A$ receptor is a multisubunit protein comprised of several polypeptides, which may themselves exist in multiple forms (Lüddens & Wisden, 1991). The coexpression of various combinations of these polypeptides has defined the subunit requirements for modulation of the $GABA_A$ receptor by benzodiazepines, barbiturates and anaesthetic steroids. Benzodiazepines appear to require at least three subunits (α, β, γ_2) for functional activity, although the benzodiazepine pharmacology of the complex is influenced by the α subunit type (Lüddens & Wisden, 1991; Pritchett & Seeburg, 1991). In contrast, anaesthetic steroids and barbiturates do not require the γ_2 subunit for activity (Lüddens & Wisden, 1991). In the present study the benzodiazepine antagonist, flumazenil, had no influence on the GABA potentiating action of propofol and hence it is unlikely that the anaesthetic interacts directly with the benzodiazepine binding site. In support of this suggestion, in preliminary experiments propofol potentiated GABA-evoked currents recorded from Chinese hamster ovary cells transfected with only α_1 and β_1 subunits (Hill-Venning & Lambert, unpublished observations). These cells are insensitive to benzodiazepines (Moss *et al.*, 1990).

Electrophysiological and radioligand binding experiments suggest that barbiturates and steroids bind to distinct sites to produce their action on $GABA_A$ receptor channel kinetics (Cottrell *et al.*, 1987; Gee *et al.*, 1988; Peters *et al.*, 1988; Kirkness, 1989; Lambert & Peters, 1989; Turner *et al.*, 1989). In support of this proposal, the steroid 5β-pregnan-3α-ol-20-one was equieffective in potentiating GABA- and pentobarbitone-induced currents. In contrast, propofol produced only a modest potentiation of pentobarbitone-induced currents, but a large potentiation of GABA-induced currents recorded from the same cell. The results of radioligand binding experiments are consistent with propofol binding to a site distinct from that occupied by barbiturates or steroids (Concas *et al.*, 1990; 1991b). Studies investigating the specific binding of [^{3}H]-propofol to rat cortical membranes should further characterize the propofol binding site (Concas *et al.*, 1991a).

In the absence of GABA, propofol induced whole cell currents with a reversal potential dependent upon the chloride gradient across the cell membrane. Propofol induced single channel currents on previously quiescent, isolated, outside out membrane patches, deliberately positioned at least 5 mm away from the nearest cell. Such channel events are therefore unlikely to be produced by a potentiation of background GABA (Cottrell *et al.*, 1987). The chord conductance states of the propofol-induced channels were similar to two of the single channel conductance states activated by GABA and were blocked by the $GABA_A$ receptor antagonist, bicuculline. Collectively, these results are consistent with propofol in some way activating the $GABA_A$ receptor-chloride channel complex. Similar observations have been made for anaesthetic steroids (Barker *et al.*, 1987; Callachan *et al.*, 1987; Cottrell *et al.*, 1987; Lambert *et al.*, 1987), the hypnotic and anticonvulsant chlor-methiazole (Hales & Lambert, 1988a) the intravenous general anaesthetic propanidid (Peters & Lambert, unpublished observations), and pentobarbitone (e.g. Owen *et al.*, 1986; Peters *et al.*, 1988; Robertson, 1989). It seems unlikely that such chemically diverse structures directly activate the $GABA_A$ receptor complex by binding to the GABA recognition site and their mechanism of activation remains unknown.

Propofol, at similar concentrations to those effective at the $GABA_A$ receptor of rodent neurones and bovine chromaffin cells, potentiated glycine-activated currents recorded from mouse spinal neurones. In contrast to the actions of propofol on the $GABA_A$ receptor, the modest propofol-induced potentiation of glycine-evoked currents was not mimicked by pentobarbitone. Potentiation of glycine-activated currents by chlormethiazole, but not by alphaxalone, has also been observed (Hales *et al.*, 1990; Harrison & Simmonds, 1984; Lambert *et al.*, 1991). The primary structure of the $GABA_A$ receptor and the glycine receptor exhibit a high degree of amino acid sequence homology (Barnard *et al.*, 1987). Hence, the possibility exists that propofol binds to similar, or equivalent amino acids on each of these receptors, to produce a common effect, an enhanced agonist-induced chloride flux through their associated ion channels. However, an alternative possibility that the potentiation is secondary to an action of propofol on glycine uptake mechanisms has not been tested.

What is the relevance of the actions of propofol on inhibi-

tory amino acid receptors to its behavioural effects? The number of general anaesthetics of different molecular structure which have been reported both to potentiate GABA-mediated neurotransmission and to activate the $GABA_A$ receptor (Scholfield, 1980; Gage & Robertson, 1985; Keane & Biziere, 1987; Olsen, 1988; Nakahiro *et al.*, 1989) suggests that such mechanisms may be involved in general anaesthesia for some agents. However, a variety of ion channels and central neurotransmitter receptors have been implicated as potential target sites (Olsen, 1988), including a block of voltage-activated calcium channels (Macdonald *et al.*, 1986; Owen *et al.*, 1986), various potassium channels (Krnjevic, 1986) and antagonism of glutamate receptors (Richards & Smaje, 1976; Macdonald & Barker, 1979; Teichberg *et al.*, 1984; Simmonds & Horne, 1987; Lambert *et al.*, 1990; 1991). Hence, further work on the selectivity of action of propofol is required before the relative importance of $GABA_A$ receptors can be assessed, Even less clear is the importance of glycine receptors in anaesthesia. The discovery of selective positive allosteric modulators of this receptor is required to clarify their role.

In conclusion, the present study clearly demonstrates that the general anaesthetic, propofol, acts as a positive allosteric modulator of both $GABA_A$ and glycine receptors. These actions may well contribute to the central depressant actions of propofol.

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Furosemide inhibits the centrally-mediated pressor response to clonidine in conscious, normotensive rats

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1 The effect of furosemide on the pressor response induced by intracerebroventricular (i.c.v.) injection of clonidine was investigated in freely moving, normotensive rats with chronically implanted arterial catheters.

2 When injected i.c.v., clonidine at doses of 5 and 10 μg produced a dose-dependent pressor response and a decrease in heart rate. No depressor response was induced by clonidine.

3 Systemic (i.v.) pretreatment with furosemide (2–10 mg kg^{-1}) increased urine volume and dose-dependently inhibited the pressor response to i.c.v. clonidine (10 μg), and a long-lasting depressor response to clonidine was observed. However, furosemide treatment did not alter the bradycardia produced in response to clonidine.

4 The systemic treatment with furosemide (10 mg kg^{-1}, i.v.) had no effect on the pressor response to i.v. noradrenaline.

5 These results suggest that reduction of body fluid volume inhibits the centrally-mediated pressor response to clonidine and leads to the hypotensive effect. We also suggest that combined treatment with a diuretic increases the hypotensive efficacy of clonidine.

Keywords: Furosemide; clonidine; central pressor effect; body fluid volume; conscious rat

Introduction

We have reported that clonidine, an antihypertensive drug, increases blood pressure when injected into the cerebroventricles of conscious, normotensive rats, but not of pentobarbitone, anaesthetized rats (Kawasaki & Takasaki, 1986; Kawasaki *et al.*, 1986; Kawasaki & Takasaki, 1987a). Since central pretreatment with yohimbine, an α_2-adrenoceptor antagonist, but not prazosin, a selective α_1-adrenoceptor antagonist, abolished the pressor response to i.c.v. clonidine, we proposed that the response is mediated by central α_2-adrenoceptors (Kawasaki & Takasaki, 1986). However, the precise mechanism underlying the pressor response to clonidine remains unknown.

In the clinical treatment of hypertension, combining clonidine with a diuretic has been shown to lead to a greater hypotensive effect than when either drug is given alone (Rosenman, 1975; Hui *et al.*, 1986). Thus, the hypotensive effect of clonidine is probably affected by changes in extracellular fluid volume. This gives rise to the speculation that reducing body fluid volumes may suppress the centrally-mediated hypertensive response to clonidine and subsequently the hypotensive effect may predominate. In fact, we have observed that i.c.v. injection of clonidine produced no pressor response in the conscious rat after water deprivation for 48 h, and instead caused a long-lasting depressor response (Kawasaki & Takasaki, 1987b). Therefore, in the present study, we investigated further the influence of body fluid volumes on the centrally-mediated pressor response to clonidine, using a diuretic, furosemide, in freely moving, normotensive rats.

Methods

Experimental animals

Male Wistar rats weighing 300–350 g were used in this study. The animals were given food and water *ad libitum*. They were housed in the Experimental Animal Centre of Miyazaki Medical College at a controlled ambient temperature of 22°C with 50 ± 10% relative humidity and a 12 h light/dark cycle (light on 07 h 30 min). After surgery for chronic catheter implantation, the animals were transferred to individual cages.

Surgical procedures

Under pentobarbitone-anaesthesia (50 mg kg^{-1}, i.p.), stainless-steel guide cannulae (0.7 mm outer diameter) were implanted bilaterally into the lateral cerebroventricle as described previously (Kawasaki & Takasaki, 1986). A stainless-steel bipolar electrode was also placed above the frontal cortex to measure EEG activity. The animals were allowed to recover for 10 days before the surgery for chronic catheter implantation.

For the direct recording of blood pressure, the animals with the chronic guide cannulae and electrodes were anaesthetized with ether. Polyethylene catheters (PE 10 and PE 20) were implanted into the abdominal aorta via the left femoral artery and into the inferior vena cava via the left femoral vein as described previously (Kawasaki *et al.*, 1979; Kawasaki & Takasaki, 1986). The remainder of each catheter was passed beneath the skin to emerge on the back of the neck. Both catheters were prefilled with sterile heparinized 0.9% saline (500 iu ml^{-1}) and were flushed every 2 days. A recovery period of 1 week after surgery was allowed before starting the experiment.

Recording

On the day of the experiment, the arterial catheter was attached to polyethylene tubing, which was connected to a pressure transducer (P23ID, Statham). The arterial blood pressure (pulsatile and mean), heart rate (HR) triggered by arterial pulses, and EEG activity were measured simultaneously (while behaviour was observed) and were recorded on a polygraph (RM-6000, Nihon Kohden). Both mean blood pressure (MBP) and HR were digitized by use of a minicomputer (ATAC 450, Nihon Kohden) and printed out at 1 min intervals.

Experimental protocols

For i.c.v. injection of the drugs, a stainless-steel injection cannula (0.35 mm outer diameter) was lowered into the lateral cerebroventricle through the guide cannula. The injection cannula was attached to an extension polyethylene tube (PE 20), which was connected to a Hamilton microsyringe (25 μl).

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The venous catheter was connected to a piece of polyethylene tubing approximately 60 cm long (PE 20, containing 0.2 ml).

When the animal was completely relaxed and a drowsy EEG pattern (i.e., high voltage and slow waves) was recorded for over 5 min, the drugs were injected. Clonidine or the equivalent volume of 0.9% saline was injected i.c.v. with a microinjector in a volume of 5 μl through the right guide cannula. There was no significant change in MBP and HR after i.c.v. injection of saline.

Furosemide (2, 5 and 10 mg kg^{-1}) or 0.9% saline (1 ml kg^{-1}) was injected i.v. through the extension tubing 60 min before i.c.v. injection of clonidine. Noradrenaline was injected i.v. with stepwise increases in the dose every 10–20 min. After i.v. injection, the tubing was flushed for 60 s with 0.3 ml of sterile saline.

In some rats, urine volume was measured for 60 min after i.v. furosemide (2, 5 and 10 mg kg^{-1}) or 0.9% saline (1 ml kg^{-1}).

Each animal received only one dose of clonidine and one pretreatment at 1 week intervals on each occasion. At the end of each experiment the catheters were sealed with stoppers and the animals were returned to their home cages.

After completion of the experiment the animals were anaesthetized with large doses of pentobarbitone. To determine the site of injection cannula placement, 5 μl of dye was injected and the brain was removed. Correct guide cannula placement was indicated by the diffusion of dye in the ventricle.

Statistical analysis

All values are expressed as mean ± s.e.mean. Statistical analysis was performed using the unpaired t test and one way ANOVA. A P value less than 0.05 was considered statistically significant.

Drugs

The following drugs were used: clonidine HCl (Nippon C.H. Boerigner Sohn, Kawanishi, Japan), furosemide (Lasix inj., Hoechst Japan, Tokyo, Japan), (−)-noradrenaline HCl (Sigma Chemical Co., St. Louis, MO, U.S.A.). All drugs were dissolved in sterile 0.9% saline.

Results

Pressor response to i.c.v. clonidine

When injected i.c.v., clonidine at doses of 5 and 10 μg produced a dose-dependent and long-lasting rise in blood pressure concomitant with a decrease in heart rate as shown in Figures 1, 2 and 3. No depressor response to clonidine was observed (Figures 1 and 2). Clonidine injection (5 and 10 μg) caused a marked EEG drowsy pattern (Figure 1) and the animal was sedated for more than 60 min.

Effect of furosemide treatment

As shown in Figures 1, 2 and 3, pretreatment with 10 mg kg^{-1} i.v. furosemide abolished the pressor response to i.c.v. clonidine (5 and 10 μg), and there was a marked depressor response after clonidine injection. Furosemide treatment did not affect the clonidine-induced decrease in heart rate and EEG drowsy pattern.

Furosemide at doses of 2–10 mg kg^{-1} increased urine volume in a dose-dependent manner, as shown in Figure 4a. After i.v. furosemide, the basal MBP and HR before i.c.v. injection of clonidine were 95.3 ± 2.1 mmHg and 324.8 ± 16.4 beats min^{-1} (1 ml kg^{-1} saline control), 98.8 ± 3.4 mmHg and 317.8 ± 8.5 beats min^{-1} (2 mg kg^{-1} furosemide), 96.3 ± 1.4 mmHg and 305.2 ± 6.0 beats min^{-1} (5 mg kg^{-1} furosemide) and 104.3 ± 1.5 mmHg and 359.0 ± 17.5 beats min^{-1} (10 mg kg^{-1} furosemide), respectively. Pretreatment with furosemide dose-dependently inhibited the clonidine (10 μg)-induced pressor response, and a depressor response to clonidine appeared. However, this treatment did not affect the clonidine-induced decrease in HR.

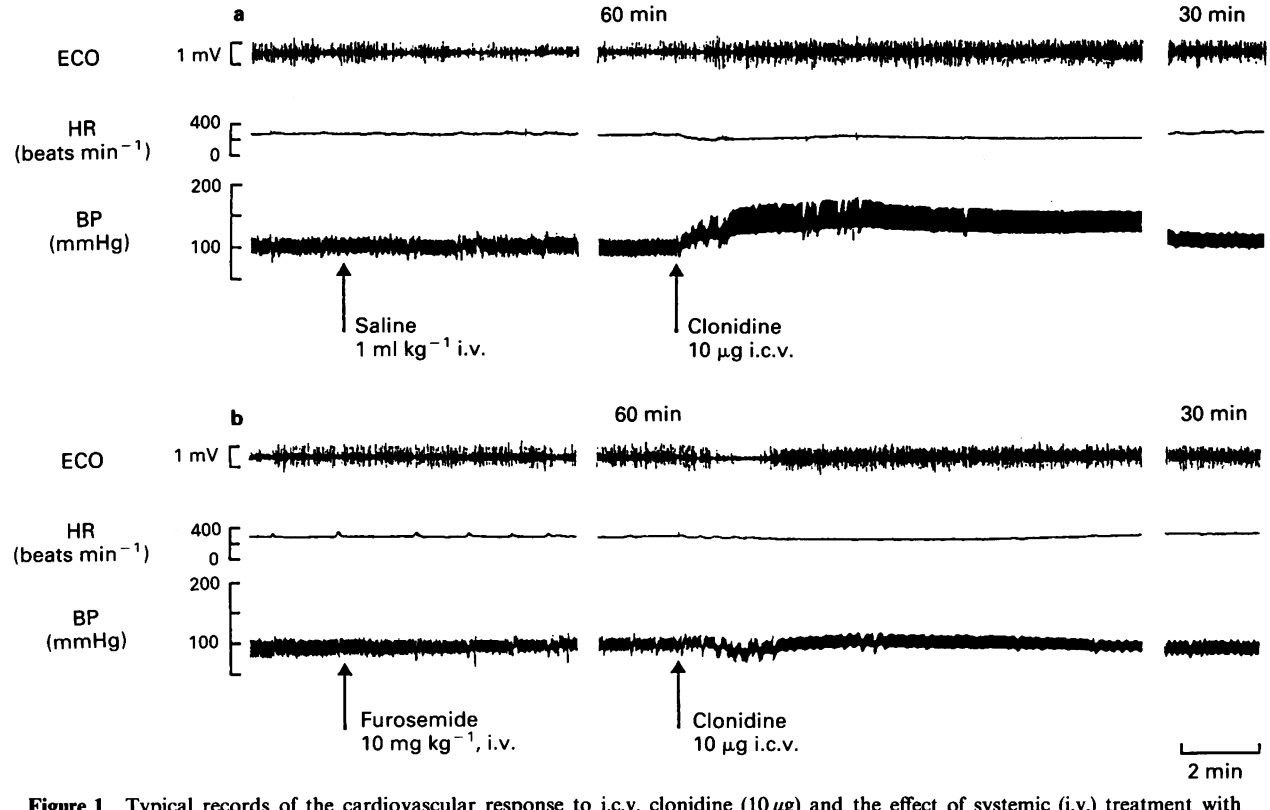


Figure 1 Typical records of the cardiovascular response to i.c.v. clonidine (10 μg) and the effect of systemic (i.v.) treatment with furosemide (10 mg kg^{-1}) in the conscious, normotensive rat. (a) Saline treatment (1 ml kg^{-1}, i.v.); (b) furosemide treatment, which was carried out 1 week after saline treatment in the same animal. ECO, electrocorticogram. HR, heart rate. BP, blood pressure.

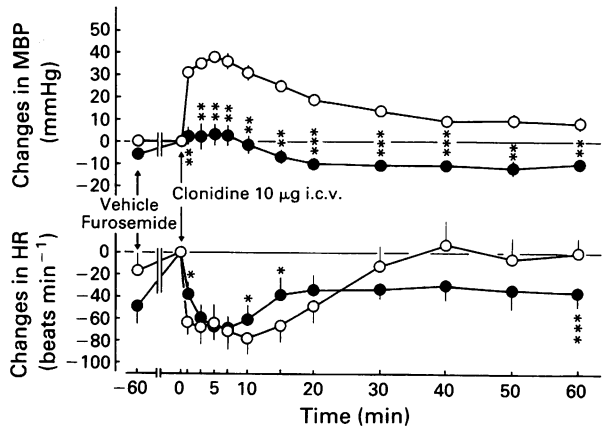


Figure 2 Changes in mean blood pressure (MBP) and heart rate (HR) following i.c.v. clonidine (10 μg) in vehicle (saline 1 ml kg^{-1}, i.v., ○)-and furosemide (10 mg kg^{-1}, i.v., ●)-treated rats. $n = 6$ for both groups. The rats were treated 60 min before clonidine injection. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with the vehicle (saline) control.

Effects of pressor response to i.v. noradrenaline

As shown in Figure 5, systemic treatment with 10 mg kg^{-1} i.v. furosemide did not affect the dose-response curve for pressor responses induced by i.v. injected noradrenaline (0.1–1.0 μg kg^{-1}).

Discussion

The present study shows that i.c.v. clonidine produces a dose-dependent and long-lasting pressor response in conscious rats, and does not lower blood pressure. This finding is in accord with previous reports that i.c.v. clonidine produces a hypertensive response only in conscious rats (Kawasaki & Takasaki, 1986; Kawasaki *et al.*, 1986; Kawaski & Takasaki, 1987a).

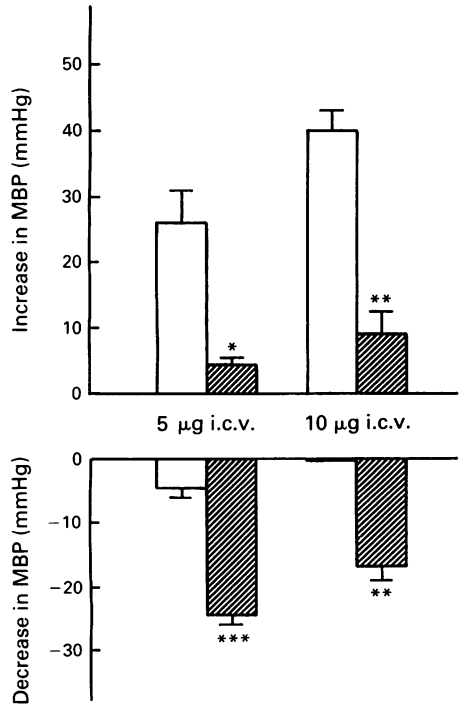


Figure 3 Effect of systemic treatment with furosemide (10 mg kg^{-1}, i.v., hatched columns) on the pressor and depressor responses induced by i.c.v. injected clonidine (5 and 10 μg) in conscious, normotensive rats. $n = 6$ in treated and control groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with the vehicle (saline, open columns)

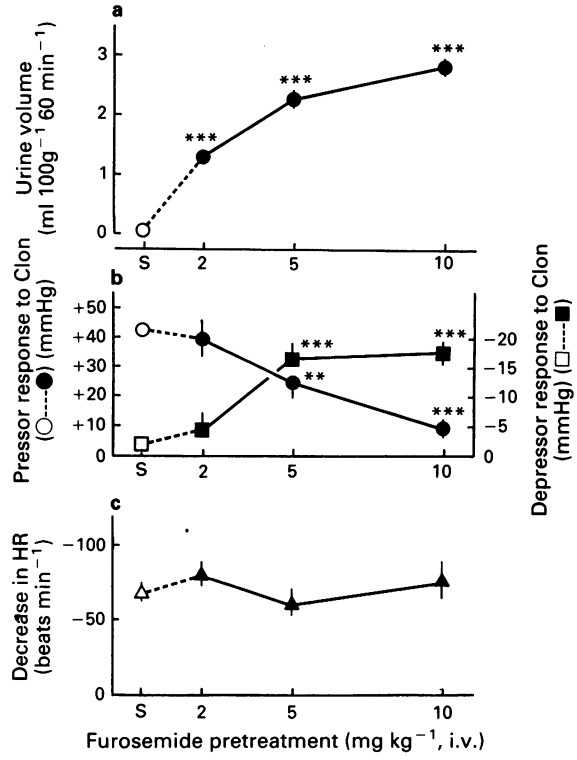


Figure 4 Effect of systemic treatment with furosemide (2–10 mg kg^{-1}, i.v.) on the urine volume (a) and the pressor response (b, circles), depressor response (b, squares) and decrease in heart rate (c) induced by i.c.v. clonidine (Clon, 10 μg) in conscious normotensive rats. The abscissa scale indicates dose of furosemide; S being saline (1 ml kg^{-1}, i.v.) treatment. The rats were treated 60 min before clonidine injection. ** $P < 0.01$; *** $P < 0.001$, compared with the saline control.

The pressor response to clonidine is abolished by pentobarbitone anaesthesia (Kawasaki & Takasaki, 1986; 1987a), which has no effect on α_2-adrenoceptors (Armstrong *et al.*, 1982), and by central (i.c.v.) pretreatment with the α_2-adrenoceptor antagonist, yohimbine (Kawasaki & Takasaki, 1986). Therefore, we propose that the pressor response to i.c.v. injected clonidine is of central origin and is mediated by central α_2-adrenoceptors. However, it has recently been proposed that the hypotensive effect of clonidine is mediated not only by

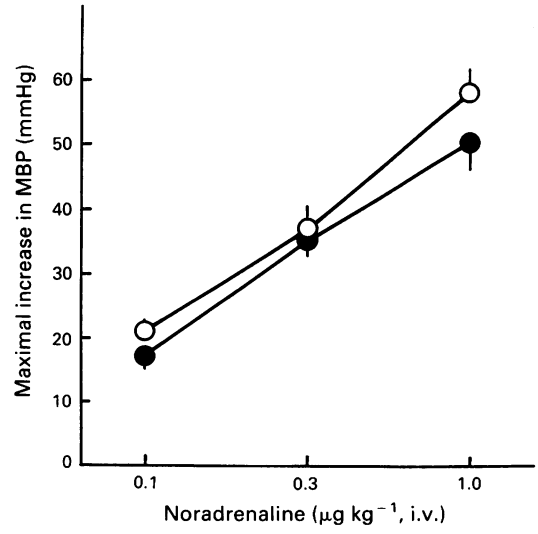


Figure 5 Effect of systemic treatment with furosemide (10 mg kg^{-1}, i.v., ●) and vehicle (saline 1 ml kg^{-1}, i.v., ○) on the pressor response induced by i.v. noradrenaline (0.1–1.0 μg kg^{-1}) in conscious normotensive rats. $n = 6$ for both groups. The rats were treated 60 min before noradrenaline injection.

α_2-adrenoceptors but also by imidazoline-preferring receptors (Bousquet *et al.*, 1986). Since yohimbine binds selectively to α_2-adrenoceptors but not to imidazoline-preferring receptors (Lehmann *et al.*, 1989), central α_2-adrenoceptors are mainly responsible for the pressor response to i.c.v. clonidine.

In conscious rats treated by furosemide, i.c.v. injected clonidine produced little pressor response and instead induced a marked depressor response. The inhibitory effect of furosemide on the pressor response was dose-dependent and was correlated with increased urine volume, indicating that the inhibition is associated with loss of body fluid volumes. These results are consistent with our previous report that the clonidine-induced pressor response was abolished by water deprivation for 48 h (Kawasaki & Takasaki, 1987b). The present finding that clonidine produces a marked depressor response in rats treated with furosemide is also consistent with the previous observation of a depressor response to clonidine in water-deprived rats. Collectively, the results suggest that clonidine has both hypertensive and hypotensive effects, which are mediated by central α_2-adrenoceptors. It seems likely that the pressor component of clonidine masks the hypotensive effect and that reduced body fluid volumes affects only the pressor component.

Changes in sodium ion concentration *in vitro* (Woodcock & Murley, 1982) and sodium balance *in vivo* (Diop *et al.*, 1984) have been shown to alter the binding properties of central and peripheral α_2-adrenoceptor agonists. Therefore, it is possible that the inhibition of the clonidine-induced pressor response after furosemide treatment might be due to alterations in the binding activities of α_2-adrenoceptors. These changes in binding activity would result from electrolyte alterations, which are well known to be caused by furosemide. However, this is unlikely because furosemide did not inhibit either the hypotensive effect or the drowsy EEG pattern induced by clonidine, which are mediated by central α_2-adrenoceptors (Florio *et al.*, 1975; Kobinger, 1978; Kostowski *et al.*, 1984). Furthermore, the present results show that furosemide treatment in conscious rats does not affect the pressor response to i.v. noradrenaline, a mixed α_1- and α_2-adrenoceptor agonist. Thus, it seems likely that electrolyte alterations by furosemide at doses used in this study have little effect on the binding activities of central and peripheral α_2-adrenoceptors.

In the present study we used high doses of clonidine, i.e. 6–13 times the usual maximum single dose used in man. This raises the possibility that i.c.v. clonidine might leak into the circulation to cause vasoconstriction via peripheral α_2-adrenoceptors. Furthermore, the reduction in body fluid volumes caused by furosemide might lead to a decrease in intravascular volume, to the extent that the α-adrenoceptor-mediated vasoconstriction is unable to overcome the volume depletion and maintain an elevated pressure. However, this is not the case in the present study. The present finding that the pressor response to i.v. injected noradrenaline was not altered by furosemide indicates that blood vessels treated with furosemide at doses used here still have the ability to constrict and maintain blood pressure. This also supports the idea that the clonidine-induced hypertensive response is central in origin.

Decreased extracellular fluid volume induced by water deprivation has been shown to lead to loss of brain arginine-vasopressin (AVP) (Epstein *et al.*, 1983). The present and previous findings that the pressor response to i.c.v. injected clonidine is inhibited by reduction of body fluid volumes raises the possibility that endogenous brain AVP may play a role in the clonidine-induced pressor response. This is supported by the preliminary result that central pretreatment with an AVP V_1-receptor antagonist markedly inhibits the pressor response to i.c.v. clonidine in conscious rats (Nakamura *et al.*, 1990). In addition, central administration of AVP has been shown to increase blood pressure in conscious rats (Janiak *et al.*, 1989). On the other hand, it is well known that reduction of body fluid volume activates the renin-angiotensin system. The increased angiotensin II level might affect the pressor response to i.c.v. clonidine. Further studies are needed to clarify the actual mechanism.

In conclusion, the present study suggests that reduction of body fluid volume inhibits the centrally-mediated pressor response to clonidine and leads to the clonidine-induced hypotensive response in conscious rats. We also suggest that combined treatment with a diuretic increases the hypotensive efficacy of clonidine.

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Nitric oxide from vascular smooth muscle cells: regulation of platelet reactivity and smooth muscle cell guanylate cyclase

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1 Incubation of smooth muscle cells (SMC) from bovine aorta for 3 min with human washed platelets treated with indomethacin (10 μM) promoted a cell number-related inhibition of platelet aggregation induced by thrombin (40 mu ml^{-1}). This inhibition was not attributable to products of the cyclo-oxygenase pathway for the SMC were also treated with indomethacin (10 μM).

2 The inhibitory activity of the SMC on platelet aggregation was enhanced by incubating the SMC with *E. coli* lipopolysaccharide (LPS, 0.5 μg ml^{-1}) for a period of 9 to 24 h. This effect was attenuated when cycloheximide (10 μg ml^{-1}) was incubated together with LPS. Cycloheximide did not prevent the inhibitory activity of the non-treated cells.

3 The inhibition of platelet aggregation obtained with non-treated or LPS-treated SMC was potentiated by superoxide dismutase (SOD, 60 u ml^{-1}) and ablated by oxyhaemoglobin (OxyHb, 10 μM). Preincubation of the SMC with N^G-monomethyl-L-arginine (L-NMMA, 30–300 μM) for 60 min prevented their anti-aggregatory activity. This effect was reversed by concurrent incubation with L-arginine (L-Arg, 100 μM) but not with D-arginine (D-Arg, 100 μM).

4 Exposure of the non-treated SMC (5×10^5 cells) to stirring (1000 r.p.m., 37°C) for 10 min led to a significant increase in their levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) but not adenosine 3':5'-cyclic monophosphate (cyclic AMP). L-NMMA (300 μM) attenuated the increase in cyclic GMP induced by stirring but did not affect the basal levels of cyclic GMP in the cells. The inhibitory activity of L-NMMA was reversed by co-incubation with L-Arg (100 μM) but not D-Arg (100 μM). L-Arg alone had no effect on the levels of cyclic GMP. In the absence of stirring, a 10 min stimulation of the non-treated SMC with glyceryl trinitrate (GTN, 200 μM) or atrial natriuretic factor (ANF, 10^{-7} M) led to an increase in the levels of cyclic GMP but not cyclic AMP. The increase in cyclic GMP promoted by GTN or ANF was not affected by L-NMMA. The levels of cyclic GMP were higher in the LPS (0.5 μg ml^{-1}, 18 h)-treated cells (5×10^{-5}) and stirring was more effective in increasing the levels of cyclic GMP in these cells.

5 These findings support the idea that non-treated or LPS-treated cultured SMC can produce an NO-like factor. Production by the latter requires protein synthesis as evidenced by blockade with cycloheximide. This NO-like factor may play a role in the auto-regulation of smooth muscle cell reactivity through a cyclic GMP-dependent mechanism.

Keywords: Nitric oxide; platelets; smooth muscle cells; guanylate cyclase; cyclic GMP; N^G-monomethyl-L-arginine; EDRF

Introduction

Endothelium-derived relaxing factor (EDRF), which is released from the vascular endothelium in response to a number of stimuli, induces vasodilatation (Furchgott & Zawadzki, 1980), inhibits platelet aggregation (Azuma *et al.*, 1986; Radomski *et al.*, 1987a) and platelet adhesion (Radomski *et al.*, 1987b; Sneddon & Vane, 1988) through stimulation of soluble guanylate cyclase. Nitric oxide (NO) accounts for the biological activity of EDRF (Palmer *et al.*, 1987; Ignarro *et al.*, 1987). Although there is still debate as to whether EDRF is NO or a closely related substance such as nitrosothiol (Myers *et al.*, 1990), the inhibition of EDRF formation by analogues of L-arginine (L-Arg) such as N^G-monomethyl-L-arginine (L-NMMA, Hibbs *et al.*, 1987), helps to substantiate L-Arg as the precursor for EDRF/NO (Palmer *et al.*, 1988). We shall refer to EDRF as NO in this paper. NO is released by macrophages and endothelial cells following stimulation with LPS and various cytokines (Marletta *et al.*, 1988; Salvemini *et al.*, 1990a) and participates in the cardiovascular events associated with the *in vivo* administration of these agents (Thiemermann & Vane, 1990; Kilbourn *et al.*, 1990). However, the main cell type involved in these responses is not known.

Bovine pulmonary arteries denuded of endothelium release a factor with the same pharmacological and chemical profile as NO (Wood *et al.*, 1990). In addition, vascular reactivity is diminished during endotoxin administration, possibly as a result of increased NO formation (Julou-Schaeffer *et al.*, 1990).

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Here we demonstrate the release of an NO-like factor from smooth muscle cells (SMC) cultured from bovine aortae in the absence or presence of *E. coli* lipopolysaccharide (LPS) using platelet aggregation and guanosine 3':5'-cyclic monophosphate (cyclic GMP) accumulation as a bioassay (Salvemini *et al.*, 1989). Furthermore, we have compared the release of an NO-like factor from SMC with or without LPS with that from endothelial cells, macrophages or monocytes.

Methods

Preparation of washed platelets

Human washed platelets were prepared as described by Radomski & Moncada (1983). Indomethacin (10 μM) was added to the final platelet suspension to prevent the formation of cyclo-oxygenase products. The platelet count was adjusted to approximately $1.5–2 \times 10^8$ ml^{-1}.

Preparation of endothelial cells, macrophages or monocytes

Bovine aortic endothelial cells (EC), mouse macrophage cell line J774 or human monocytic cell line U937 were prepared as described earlier (Salvemini *et al.*, 1989; 1990a,b). Indomethacin (10 μM) was added to the final suspensions of ECs (9×10^6 cells ml^{-1}), J774 (1×10^8 cells ml^{-1}) or U937 cells (1×10^8 cells ml^{-1}). In some experiments, LPS (0.5 μg ml^{-1}) was added directly to the EC and J774 cells in culture for 9 to 24 h. Maximum anti-platelet activity of EC and J774 cells was reached after 18 h incubation with LPS.

Preparation of smooth muscle cells

Smooth muscle cells were explanted from bovine aortae and grown to confluence in T75 tissue culture flasks. On the day of the experiment, the SMC were treated briefly (10–20 s) with 0.05% (w/v) trypsin and spun at 1000 r.p.m. for 5 min to form a pellet. The pellet containing the SMC was then washed twice with warm calcium-free Krebs buffer and the final SMC count then adjusted to 11×10^6 cells ml^{-1}. They were characterized as smooth muscle cells by the presence of smooth muscle specific alpha actin identified with a Sigma kit. Indomethacin (10 μM) was always added to the final platelet or SMC suspension to prevent the formation of cyclo-oxygenase products. In some experiments, LPS (0.5 $\mu g\,ml^{-1}$) was added directly to the SMC in culture for periods of 9 to 24 h. Cycloheximide (10 $\mu g\,ml^{-1}$) was added to SMC incubated with LPS for 18 h. An 18 h incubation time with LPS was selected for comparing the increase in anti-platelet action with that of EC and J774 cells. The SMC preparation was more than 95% viable as assessed by the uptake of trypan blue. Incubation of the SMC with L-NMMA (300 μM) for 60 min or with LPS (0.5 $\mu g\,ml^{-1}$) in the absence or presence of cycloheximide (10 $\mu g\,ml^{-1}$) for 18 h did not alter the cell viability.

Bioassays of NO

Platelet aggregation A suspension of washed platelets was incubated at 37°C for 4 min in a Payton dual channel aggregometer (Born & Cross, 1963) with continuous stirring at 1000 r.p.m. and then stimulated with thrombin (40 mu ml^{-1}) to give a submaximal aggregation (80–90%). The decrease in optical density was recorded for 5 min. In other experiments, SMC were added to the platelet suspension 3 min before stimulation with a submaximal concentration of thrombin. Cell numbers added were in the range of 0.1–3 $\times 10^5$ cells. Supernatants from suspensions of SMC (3×10^5 cells) obtained by centrifugation at 1000 r.p.m. for 1 min were also tested at this stage. When required, SOD (60 u ml^{-1}) or OxyHb (10 μM) was added to the platelet suspension immediately before the addition of the SMC. L-NMMA (30–300 μM) either alone or in the presence of L-Arg (100 μM) or D-Arg (100 μM) was pre-incubated with smooth muscle cells for 60 min before the cells were added to the platelet suspension. Results are expressed as % inhibition of platelet aggregation as described previously (Salvemini *et al.*, 1989). When oxyhaemoglobin was used, the calibrations were performed in the presence of this agent to compensate for possible changes in light transmission. Similar protocols were used for testing the anti-platelet activity of the other cell types.

Measurement of cyclic nucleotides Cyclic GMP levels were measured by radioimmunoassay (Steiner *et al.*, 1972) following prior acetylation of the samples with acetic anhydride (Harper & Brooker, 1975). A suspension of non-treated or LPS (0.5 $\mu g\,ml^{-1}$ for 18 h)-treated SMC (5×10^5 cells) in the presence of IBMX (0.1 mM) to inhibit phosphodiesterase activity was diluted in Krebs buffer and preincubated in an aggregometer for 10 min with or without stirring (37°C, 1000 r.p.m.). When required, GTN (200 μM) or ANF (10^{-7} M) was added to the non-treated SMC for 10 min without stirring. Ice cold trichloroacetic acid (TCA, final concentration 5% w/v) was then added, the samples stored at −20°C, and the cyclic nucleotides extracted from TCA with 0.5 M tri-n-octylamine dissolved in 1,1,2-trichloro-trifluoroethane. The addition of [^{3}H]-cyclic GMP or [^{3}H]-cyclic AMP to SMC showed a recovery of >98%; therefore the results have not been corrected for recovery. All determinations were performed in duplicate.

Uptake of thrombin by smooth muscle cells

A suspension of SMC (3×10^5 cells) in Krebs buffer was incubated for 3 min with thrombin and then centrifuged at 10,000 *g* for approximately 2 min. Platelet aggregatory activity of the supernatant was compared with the aggregatory activity of thrombin at the same dilution as that added to the suspension of smooth muscle cells.

Determination of endotoxin levels

Endotoxin levels in the distilled water, Krebs buffer and culture medium were determined by use of the Limulus Amebocyte Lysate (E-Toxate, Sigma).

Statistics

Results are expressed as mean ± s.e.mean for (*n*) experiments and each experiment was performed with blood obtained from a different donor. Student's unpaired *t* test was used to determine the significant difference between means, and a *P* value of <0.05 was taken as significant.

Materials

The composition of the modified (Sneddon & Vane, 1988) Krebs bicarbonate buffer was (mM): NaCl 137, KCl 2.7, $NaHCO_3$ 11.9, NaH_2PO_4 0.3, $MgSO_4$ 0.8, glucose 5.6 and $CaCl_2$ 1. The cell culture medium consisted of Dulbecco's Modified Eagle's Medium with the addition of 10% new born calf serum, L-glutamine 4 mM, penicillin 100 iu ml^{-1}, streptomycin 100 $\mu g\,ml^{-1}$, gentamycin 0.1 mg ml^{-1}, insulin 5 $\mu g\,ml^{-1}$, transferrin 5 $\mu g\,ml^{-1}$ and sodium selenite 5 ng ml^{-1}. Human thrombin, *E. coli* lipopolysaccharide (serotype 0127:B8), haemoglobin (from bovine blood), superoxide dismutase (from bovine erythrocytes), indomethacin, L-arginine (free base), D-arginine (free base), cycloheximide, E-Toxate (Limulus Amebocyte Lysate). E-Toxate (Limulus Amebocyte Lysate, No. 210), adenosine deaminase, sodium nitrite, atrial natriuretic factor and kits for alpha smooth muscle actin (procedure number S1H 903) were obtained from Sigma (Poole, Dorset). L-Glutamine, penicillin, streptomycin, gentamycin, insulin, transferrin and sodium selenite were obtained from Flow Laboratories (Rickmansworth, Herts). New born calf serum was obtained from Gibco (Paisley). Tri-n-octylamine and 3'-isobutyl-1-methylxanthine (IBMX) were obtained from Aldrich. Stock solutions of IBMX were prepared in 0.1 N NaOH and then diluted in Krebs buffer as required. Kits for radioimmunoassay of adenosine 3':5'-cyclic monophosphate (cyclic AMP) were purchased from Amersham as was [^{125}I]-cyclic GMP. N^G-monomethyl-L-arginine was obtained from Ultrafine Chemicals Ltd. Oxhyaemoglobin was prepared by reduction of bovine haemoglobin with sodium hydrosulphite as described previously (Salvemini *et al.*, 1989). Glyceryl trinitrate (Nitronal) was obtained from Lipha Pharmaceuticals Ltd (West Drayton, Middlesex). All the other reagents were obtained from BDH. Prostacyclin was a gift from the Wellcome Research Laboratories (Beckenham, Kent). The cyclic GMP-specific antibodies were kindly provided by Dr K. Schror (Institute of Pharmacology, University of Dusseldorf, Germany).

Results

Inhibition of thrombin-induced platelet aggregation by bovine aortic smooth muscle cells

Thrombin (40 mu ml^{-1}) produced a submaximal aggregation of washed platelets within 3 min. The anti-platelet activity of the SMC was maximal following a 3 min incubation with platelets. Thus, all experiments were performed at this time period of incubation. Addition of SMC for 3 min before thrombin inhibited the aggregation according to the number of cells added (cell number required to induce 50% inhibition $IC_{50} = 0.8 \pm 0.03 \times 10^5$, $n = 8$; Table 1). Hence, SMC in the aggregometer released a factor that inhibited platelet aggregation. Stirring the cell suspensions may contribute to the release of this factor as originally shown with endothelial cells (Stamler *et al.*, 1989). The inhibition was not due to thrombin

Table 1 Inhibition by different numbers of bovine aortic smooth muscle cells (SMC) of platelet aggregation induced by thrombin (40 mu ml^{-1})

	% *inhibition of platelet aggregation*			
Number of	*Time of incubation with LPS* (h)			
SMC ($\times 10^5$)	0	9	18	24
0.125	2 ± 1	7 ± 1	13 ± 1†	20 ± 2§
0.25	6 ± 2	14 ± 1*	25 ± 2†	40 ± 2§
0.5	30 ± 6	44 ± 2*	64 ± 8†	83 ± 3
1	66 ± 8	72 ± 1	96 ± 4†	100
2	100	100	100	

Incubation of SMC for a period of 3 min with platelets resulted in inhibition of platelet aggregation according to the cell number. This inhibition was potentiated by pretreatment of the SMC with LPS (0.5 μg ml^{-1}) for 9 to 24 h. * $P < 0.005$ when compared to the values obtained in the absence of LPS, † $P < 0.05$ when compared to the effects obtained at 9 h and § $P < 0.05$ when compared to the values obtained at 18 h by the ANOVA test followed by a least significance procedure. Results are expressed as % inhibition of platelet aggregation ± s.e.mean for $n = 8$ experiments.

uptake by SMC, for supernatants from suspensions of SMC that had been incubated with thrombin produced the same degree of aggregation as that obtained with thrombin alone (% aggregation with thrombin alone at 40 mu ml^{-1} was 85 ± 2% and with thrombin incubated for 3 min with 3×10^5 cells 87 ± 3% aggregation, $n = 4$). Incubation of the SMC with adenosine deaminase (60 μg ml^{-1}) for 30 min did not modify their anti-platelet activity ($n = 3$, not shown).

Characterization of the platelet inhibitory factor derived from smooth muscle cells

The virtually complete inhibition of platelet aggregation with 2×10^5 SMC was not seen when platelets were incubated with the supernatant from 2×10^5 cells taken 5 min before addition to the platelet suspension ($n = 4$). In addition, neither the supernatant nor the suspension of 2×10^5 cells that were sonicated for approximately 1 min inhibited thrombin-induced platelet aggregation (not shown). The inhibitory activity of 0.25×10^5 cells was enhanced by SOD (60 u ml^{-1} from 6 ± 2% inhibition to 71 ± 4% inhibition, $n = 4$, $P < 0.0005$) and the inhibitory activity of 2×10^5 cells was attenuated by OxyHb (10 μM from 100% inhibition to 20 ± 5% inhibition, $n = 4$, $P < 0.0005$). Preincubation for 60 min of 2×10^5 SMC with L-NMMA (30–300 μM) reduced their ability to inhibit thrombin-stimulated platelet aggregation (Table 2A). L-Arginine (100 μM) but not D-arginine (100 μM) when co-incubated with L-NMMA counteracted the effects of L-NMMA (Table 2A). A preincubation period of 60 min with L-NMMA was necessary in order to reverse fully the inhibitory action of the SMC. Thus when SMC (2×10^5) were incubated with L-NMMA for only 30 min a partial reversal of their inhibitory effects was observed (from 100% inhibition to 55 ± 4% inhibition, $n = 4$).

Incubation with E. coli *lipopolysaccharide enhances the platelet-inhibitory activity of smooth muscle cells*

Preincubation of SMC with LPS (0.5 μg ml^{-1}) for periods of 9 to 24 h enhanced their ability to inhibit thrombin-induced platelet aggregation (Table 1). The IC_{50} before LPS was $0.8 \pm 0.03 \times 10^5$ and after 18 h of LPS treatment was $0.3 \pm 0.02 \times 10^5$ ($n = 8$, $P < 0.0005$). Thus, LPS induced a 2.6 fold potentiation of the inhibitory effects of SMC on platelet aggregation. LPS (20 μg ml^{-1}) when incubated with the SMC for 3 min failed to enhance their anti-aggregatory activity (not shown). The inhibitory activity of 0.25×10^5 cells was potentiated by SOD (60 u ml^{-1} from 25 ± 2% inhibition to 71 ± 3% inhibition, $n = 4$, $P < 0.0005$) and that of 1×10^5 cells was reversed by OxyHb (10 μM, from 96 ± 4% inhibition to 16 ± 4% inhibition, $n = 4$, $P < 0.0005$). Preincubation for 60 min of 1×10^5 cells with L-NMMA (30–300 μM) reduced their ability to inhibit platelet aggregation (Table 2B). Co-incubation with L-arginine (100 μM) but not D-arginine (100 μM) counteracted the effects of L-NMMA (Table 2B).

Effects of cycloheximide on the platelet-inhibitory activity of smooth muscle cells

The potentiation of the platelet inhibitory activity of SMC (1×10^5 cells) by LPS (0.5 μg ml^{-1} for 18 h) was ablated when cycloheximide was incubated together with LPS for 18 h (from 96 ± 4% inhibition to 51 ± 4% inhibition, $n = 4$, $P < 0.0005$). Cycloheximide (10 μg ml^{-1}) when incubated with the SMC for 18 h in the absence of LPS did not reverse the inhibitory activity of the cells (1×10^5) (from 66 ± 8% to 70 ± 4% inhibition, $n = 4$, $P < 0.35$).

Relative potency of LPS treatment for 18 h in potentiating the anti-platelet activity of SMC, EC, J774 or U937

The platelet inhibitory activity of the SMC following a 3 min contact with platelets was 2.6 fold greater in cells incubated for 18 h with LPS (0.5 μg ml^{-1}; Table 1). The IC_{50} obtained for EC ($n = 5$) or J774 cells ($n = 4$) in the absence of LPS was $1.75 \pm 0.02 \times 10^5$ and $13 \pm 0.4 \times 10^5$. The IC_{50}s obtained after an 18 h pre-treatment of the EC ($n = 5$) or J774 cells ($n = 4$) with LPS (0.5 μg ml^{-1}) were $0.42 \pm 0.3 \times 10^5$ and

Table 2 The platelet inhibitory of (**A**) non-treated bovine aortic smooth muscle cells (SMC) and (**B**) *E. coli* lipopolysaccharide (LPS, 0.5 μg ml^{-1}, for 18 h)-treated SMC and its reversal by N^G-monomethyl-L-arginine (L-NMMA, 30–300 μM)

	% *inhibition of platelet aggregation*		
A			
L-NMMA (μM)	*L-NMMA alone*	*L-NMMA + L-Arg*	*L-NMMA + D-Arg*
0	100	100	100
30	60 ± 3*	100	55 ± 4
100	35 ± 2*	95 ± 2†	33 ± 3
300	21 ± 2*	73 ± 9†	28 ± 4
B			
L-NMMA (μM)	*L-NMMA alone*	*L-NMMA + L-Arg*	*L-NMMA + D-Arg*
0	96 ± 4	96 ± 4	96 ± 4
30	55 ± 3*	98 ± 2†	57 ± 2
100	38 ± 2*	90 ± 2†	35 ± 4
300	18 ± 1*	70 ± 2†	20 ± 2

The inhibitory activity of (**A**) non-treated SMC (2×10^5) or (**B**) LPS-treated SMC (1×10^5) cells was reversed in a concentration-dependent manner by L-NMMA (30–300 μM). L-Arginine (L-Arg, 100 μM) but not D-arginine (D-Arg, 100 μM) when coincubated with L-NMMA, reversed the effects of L-NMMA in non-treated (**A**) and LPS-treated SMC (**B**). * $P < 0.0005$ when compared to the value obtained in the absence of L-NMMA and † $P < 0.0005$ when compared to the value obtained with L-NMMA alone. Results are expressed as % inhibition of platelet aggregation ± s.e.mean for $n = 4$ experiments.

$3.95 \pm 0.2 \times 10^5$. Thus, LPS induced a 4.4 fold for EC and 3.3 fold for J774 cells magnification of the inhibitory effects on platelet aggregation (Figure 1a and b). The U937 did not respond to LPS under these conditions ($n = 3$, data not shown).

Cyclic GMP concentrations in non-treated smooth muscle cells after stirring

When SMC (5×10^5) in the presence of IBMX (0.1 mM) were stirred for 10 min, a significant increase in the levels of cyclic GMP was observed ($n = 4$, $P < 0.01$; Figure 2). There were no changes in the levels of cyclic AMP (2.2 ± 0.5 pmol/5×10^5 cells without stirring and 1.6 ± 0.2 pmol/5×10^5 cells after 10 min stirring, $n = 4$, $P < 0.15$). The increase in cyclic GMP observed after 10 min stirring was prevented by coincubation with L-NMMA (300 μM, $n = 4$, $P < 0.01$). The inhibitory activity of L-NMMA was reversed by co-incubation with L-Arg (100 μM, $n = 4$, $P < 0.01$) but not by D-Arg (100 μM, $n = 4$, $P < 0.3$; Figure 2). L-NMMA (300 μM) did not reduce the basal levels of cyclic GMP in cells that had not been exposed to stirring ($n = 4$, $P < 0.25$). In the presence of stirring, L-Arg (100 μM) did not further increase the levels of cyclic GMP in these cells (from 132 ± 13 fmol/5×10^5 cells to 127 ± 16 fmol/5×10^5 cells, $n = 4$, $P < 0.4$).

Increase of the concentrations of cyclic GMP in smooth muscle cells by glyceryl trinitrate or atrial natriuretic factor

The basal levels of cyclic GMP in non-treated SMC (5×10^5) with IBMX (0.1 mM) obtained in the absence of stirring were increased by a 10 min stimulation with glyceryl trinitrate (GTN, 200 μM) or atrial natriuretic factor (ANF, 10^{-7} M) ($n = 4$, $P < 0.025$ and $P < 0.0005$ respectively) (Figure 3). This increase was not abolished when the cells (5×10^5) had been pretreated with L-NMMA (300 μM) for a period of 60 min ($n = 4$, $P < 0.475$ and $n = 4$, $P < 0.35$) (Figure 3). Neither GTN nor ANF at the concentrations tested increased the levels of cyclic AMP in these cells (from 1.6 ± 0.2 pmol/5×10^5 cells to 1.4 ± 0.2 or 1.3 ± 0.1 pmol/5×10^5 cells respectively, $n = 4$, $P < 0.2$ in both cases).

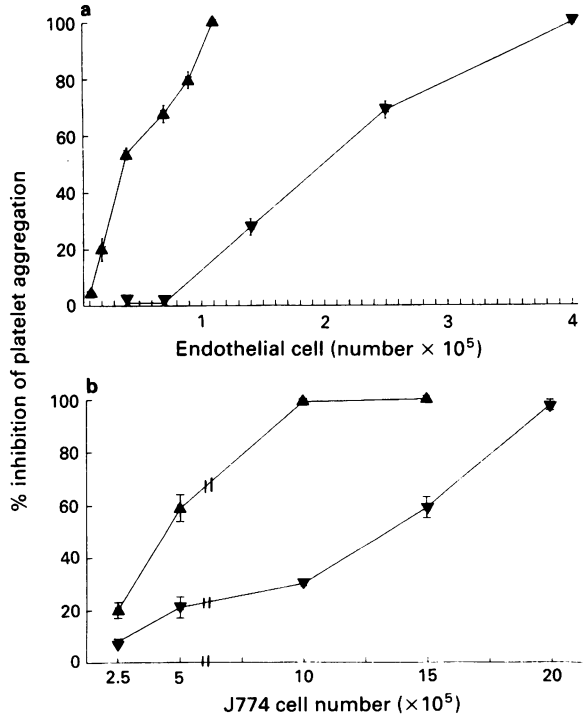


Figure 1 The relative potency of (a) endothelial cells (EC, $n = 5$) or (b) J774 cells ($n = 4$) an inhibiting thrombin (40 mu ml^{-1})-induced platelet aggregation with (▲) or without (▼) an 18 h pre-treatment with *E. coli* lipopolysaccharide (LPS, 0.5 μg ml^{-1}). The inhibitory activity of the EC and that of the J774 were enhanced by LPS treatment. Results are expressed as % inhibition of platelet aggregation. Vertical bars represent the s.e.mean of (n) experiments.

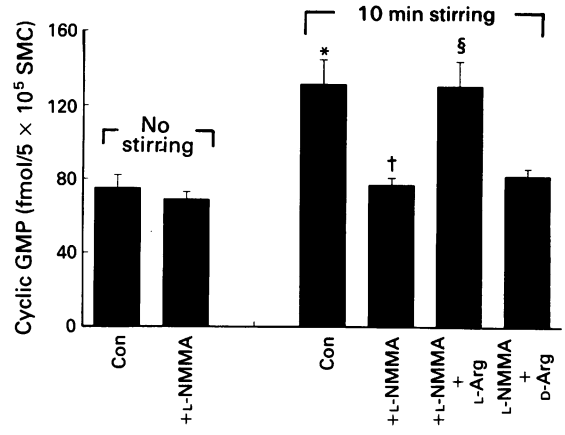


Figure 2 The effects of stirring on cyclic GMP levels in bovine aortic smooth muscle cells (SMC). Stirring the SMC (5×10^5 cells) for 10 min led to a significant increase in the levels of cyclic GMP. This rise was reduced by N^G-monomethyl-L-arginine (L-NMMA, 300 μM). The effect of L-NMMA was reversed by L-arginine (L-Arg, 100 μM) but not by D-arginine (D-Arg, 100 μM). L-NMMA (300 μM) did not reduce the basal levels of cyclic GMP in the absence of stirring. * $P < 0.01$ when compared to the value obtained in the absence of stirring, † $P < 0.01$ when compared to the value obtained in the presence of stirring and § $P < 0.01$ when compared to the value obtained in the presence of L-NMMA. Results are expressed as cyclic GMP fmol/5×10^5 cells. Vertical bars represent the s.e.mean of 4 experiments.

Cyclic GMP concentrations in LPS-treated smooth muscle cells after stirring

The basal levels of cyclic GMP in the LPS-treated (0.5 μg ml^{-1} for 18 h) SMC (5×10^5) in the presence of IBMX (0.1 mM) were higher than in the non-treated SMC (75 ± 7 fmol in the non-treated SMC and 99 ± 4 fmol cyclic GMP/5×10^5 in the LPS-treated SMC, $n = 4$, $P < 0.025$). The levels of cyclic AMP in the LPS-treated cells were similar to those in the non-treated cells ($n = 4$, $P < 0.25$). Stirring the LPS-treated cells for 10 min led to a marked increase in the levels of cyclic GMP (from 99 ± 4 to 345 ± 30 fmol cyclic GMP/5×10^5 SMC, $n = 4$, $P < 0.005$) but not cyclic AMP (from 2 ± 0.1 to 2.2 ± 0.5 pmol cyclic AMP/5×10^5 SMC, $n = 4$, $P < 0.25$). The increase in cyclic GMP observed after a

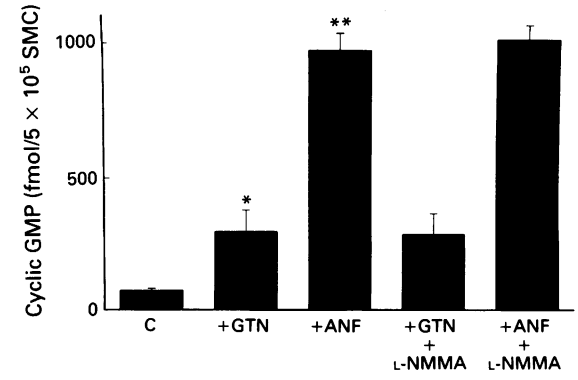


Figure 3 The effects of glyceryl trinitrate (GTN) or atrial natriuretic factor (ANF) on cyclic GMP levels in bovine aortic smooth muscle cells (SMC). Stimulation of SMC (5×10^5 cells) for 10 min in the absence of stirring with GTN (200 μM) or ANF (10^{-7} M) caused a significant increase in the levels of cyclic GMP. This increase was not reversed by N^G-monomethyl-L-arginine (L-NMMA, 300 μM). Results are expressed as cyclic GMP fmol/5×10^5 cells. Vertical bars represent the s.e.mean of 4 experiments. * $P < 0.25$ and ** $P < 0.0005$ when compared to basal cyclic GMP levels.

10 min stirring was prevented by co-incubation with L-NMMA (300 μM, from 345 ± 30 to 104 ± 6 fmol cyclic GMP/5 $\times 10^5$ SMC, $n = 4$, $P < 0.005$).

Determination of endotoxin levels

Levels of LPS in the distilled water, Krebs buffer and culture medium were below the detection limit of the assay (0.03 EU ml^{-1}).

Discussion

Cultured bovine aortic smooth muscle cells treated with indomethacin release a factor that inhibits thrombin-induced platelet aggregation. This factor has the same pharmacological profile as NO. Thus, the anti-aggregating activity of this NO-like factor was unstable, was potentiated by SOD and prevented by OxyHb or by L-NMMA. Furthermore, the effects of L-NMMA were reversed by co-incubation with L-Arg but not with D-Arg. These results, therefore, strongly suggest that the inhibitory factor released from SMC is NO and that, as in endothelial and other cells the precursor is L-arginine. The release of the NO-like factor from SMC could have been the result of cell contact activation favoured by the stirring mechanism that takes place during the aggregation experiments. Evidence that stirring may play a role in such a release is supported by the findings that in the absence of stirring, the levels of cyclic GMP in SMC were not affected by L-NMMA. On the other hand, stirring the SMC suspension for 10 min increases the levels of cyclic GMP and this increase was blocked by L-NMMA. The effects of L-NMMA were reversed by co-incubation with L-Arg but not D-Arg. Such a finding is consistent with the release of NO-like activity after smooth muscle cell stimulation and supports previous observations showing an increase in cyclic GMP when NO-generating cells are stimulated (Boulanger *et al.*, 1990).

Although both GTN and ANF increased the levels of cyclic GMP in the SMC, such an increase was not reduced by L-NMMA. These results confirm previous studies performed on other cell types (Salvemini *et al.*, 1990c; Boulanger *et al.*, 1990) and demonstrate the presence in these cells of the pathway responsible for the conversion of GTN to form NO and of the presence of the particulate guanylate cyclase. Furthermore, they show that the effects of L-NMMA are specific to the L-Arg-NO pathway.

Incubation of SMC with adenosine deaminase did not modify their anti-platelet action suggesting that adenosine does not play a role in their anti-platelet effects. There is no evidence in the literature that LPS can enhance the release of adenosine from SMC; here we have demonstrated that LPS enhances the anti-platelet action of SMC through a mechanism involving enhanced production of NO as evidenced by total reversal with L-NMMA and OxyHb. Adenosine inhibits platelets through stimulation of the adenylate cyclase and increase in the levels of cyclic AMP (Gerrard, 1988). No changes in the levels of cyclic AMP were observed in our cells. Thus it is unlikely that a synergism between NO and another possible anti-platelet mediator is released from the SMC takes place.

Endothelial cells (Salvemini *et al.*, 1990a), J774 cells (Salvemini *et al.*, 1990b), human neutrophils (PMNs, Salvemini *et al.*, 1989) but not U937 cells (Salvemini *et al.*, 1989) when co-incubated with platelets release an inhibitory factor with the pharmacological properties identical to those of NO. The cell counts ($\times 10^5$) needed to cause 50% inhibition of aggregation were 1.75 ± 0.02 for EC, 13 ± 0.4 for J774 cells and 19 ± 0.2 for PMNs. In this study, the IC_{50} for SMC under these conditions was $1.2 \pm 0.02 \times 10^5$ cells. Thus, under these experimental conditions SMC and EC are more potent than J774 and PMN cells in inhibiting platelet aggregation. In the present study we have also demonstrated that the anti-platelet action of SMC was enhanced in a time-dependent manner by LPS through a mechanism involving increased production of NO-like activity. Furthermore when compared to the non-treated SMC, LPS-treated SMC responded to stirring with a much greater elevation in cyclic GMP levels. This increase in cyclic GMP was attenuated by co-incubation with L-NMMA and can therefore be taken as representing an enhanced production of NO-like activity after stimulation with LPS.

Hence, our work supports that of others who conclude that SMC can make a NO-like factor following stimulation with LPS or cytokines. Thus, L-NMMA blocks the enhanced constrictor response in LPS-treated endothelium-denuded rat aortic strips (Julou-Schaeffer *et al.*, 1990) and the increase in cyclic GMP in rabbit smooth muscle cells stimulated with various cytokines (Busse & Mulsch, 1990). In the absence of LPS, the SMC are the most potent and PMNs the least potent in inhibiting platelet aggregation. On the other hand, following 18 h of incubation with LPS the EC and J774 cells became more potent than the SMC. It seems likely, therefore, that after LPS stimulation, expression of the enyzme responsible for the synthesis of NO-like activity and the degree to which it can be induced differs from cell type to cell type. It must, however, be kept in mind that such differences may be related to species variation. For instance, human neutrophils release NO (Wright *et al.*, 1989) but guinea-pig neutrophils do not (Rimele *et al.*, 1990).

Two distinct enzymes are responsible for the biosynthesis of NO. One is constitutive, calcium-calmodulin and NADPH-dependent and the other is induced by LPS and various cytokines, is calcium-independent and requires NADPH and tetrahydrobiopterin (Knowles *et al.*, 1989; Busse & Mulsch, 1990; Radomski *et al.*, 1990). Endothelial cells possess both the constitutive and inducible form of the NO-synthase (Radomski *et al.*, 1990). The NO-synthase expressed in rabbit SMC in response to tumour necrosis factor and interferon is of the inducible type (Busse & Musch, 1990) and is similar to that found in macrophages (Kwon *et al.*, 1989; Stuehr *et al.*, 1990). Indeed, Rees *et al.* (1990) have clearly demonstrated that Krebs buffer contaminated with endotoxin promoted the expression of the inducible form of the NO-synthase in rat aortic smooth muscle and that as a result of this, loss of vascular tone was observed. No direct evidence is at present available in the literature to demonstrate that SMC contain the constitutive form of the NO-synthase. We cannot exclude the possibility that during the period of culturing, our SMC may have expressed the NO-synthase. Thus, the nature of the NO-synthase in our cultured SMC in the absence of LPS is at present not known since studies on the enzyme activity have not been performed. However, what is clear from our experiments is that cycloheximide when incubated with the non LPS-treated SMC did not reverse their anti-platelet action.

We have recently demonstrated that in the presence of LPS the increased release of NO-like activity from EC is immediate (Salvemini *et al.*, 1990a) while that in the macrophage needs hours indicating enzyme induction (Salvemini *et al.*, 1990b). In the present study, we have found that in contrast to the EC but in accordance with the macrophages, LPS does not cause an immediate release of NO-like activity from SMC but rather requires prolonged incubation periods. This effect requires protein synthesis since the actions of LPS were abrogated by the protein synthesis inhibitor cycloheximide. Although it has been demonstrated that NO participates in the initial fall in blood pressure associated with the *in vivo* administration of LPS (Thiemermann & Vane, 1990) and that its overproduction under these conditions may be responsible for the reduced vascular responsiveness to vasoconstrictor agents (Julou-Schaffer *et al.*, 1990), the main cell types involved in these responses is not known. From our previous work, and from the results presented in this paper, it is likely that NO released mainly from EC is involved in the early stages of endotoxin shock whereas NO released from EC, macrophages and smooth muscle cells may be involved in the changes associated with the later phases. In addition, the fact that the SMC can be stimulated by LPS to produce more

NO-like activity is of considerable interest in the pathophysiological implication of these cells in disease states such as atherosclerosis. For example, it is known, that NO is cytotoxic (Hibbs *et al.*, 1988), and depending upon the dose, possesses either anti-proliferative or proliferative effects on various cells (O'Connor *et al.*, 1990). Through negative feedback, an excess of NO after LPS stimulation may act on the guanylate cyclase in the EC to reduce their own release of NO. This, in turn, may lead to a less thromboresistant surface allowing the deposition and activation of platelets or other blood cells.

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Contraction of vascular smooth muscle induced by phorbol 12,13 dibutyrate in human and rat pulmonary arteries

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1 The effect of phorbol 12,13 dibutyrate (PDB) on vascular tone was studied in both human and rat isolated pulmonary arterial strips (HPA and RPA, respectively).

2 PDB (1 nM to 2 μM) produced slowly developing, sustained and concentration-dependent contractions in HPA (mean EC_{50} = 3.5 nM, n = 5) and RPA (mean EC_{50} = 120 nM, n = 5). The maximal response was 185.6 ± 25% and 207 ± 27.5% (n = 5) of that induced by K^+-rich (80 mM) solution, and 223 ± 34.5% and 176.5 ± 38.6% of the noradrenaline (10 μM)-induced contraction in HPA and RPA, respectively.

3 PDB-induced contractions were not altered either by the presence of atropine (10 μM), propranolol (5 μM), phentolamine (5 μM) or tetrodotoxin (10 μM) in the bathing solution, or by the removal of endothelium from pulmonary arteries.

4 In HPA, the amplitude of PDB-induced contractions was significantly reduced by removal of external calcium ions, addition of verapamil (10 μM) or trifluoperazine (TFP, 5 μM) and significantly increased by Bay K 8644 (0.5 μM). In contrast, in RPA, calcium-free solution and verapamil had only a moderate effect on the maximal PDB-induced contraction (approximately 20% reduction), whereas Bay K 8644 and TFP had no significant effect. In both HPA and RPA, PDB-contractions in calcium-free solutions were not modified by ryanodine (25 μM) or by 8-(N,N diethylamino)octyl-3,4,5, trimethoxybenzoate hydrochloride (TMB-8, 50 μM).

5 PDB-induced contractions were inhibited by protein kinase C (PKC) antagonists. The maximal response was decreased by 60 ± 10.5% and 35 ± 11.5% (n = 5) by 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H_7, 50 μM), 70.5 ± 12.2% and 56 ± 18% (n = 5) by phloretin (100 μM) and 80.7 ± 8.4% and 71 ± 14% (n = 5) by staurosporine (25 nM) in HPA and RPA, respectively.

6 Long term treatment (15–20 h) of arterial strips with phorbol esters (phorbol 12,13 didecanoate, or PDB) abolished the contractile response to subsequent addition of PDB.

7 These results show that PDB is a potent vasoconstrictor agent in human and rat pulmonary arteries. Unlike the rat, part of the PDB response depends on calcium influx in human preparations. PDB action appears mainly mediated by the activation of protein kinase C. PKC could thus play a major role in the control of vascular pulmonary reactivity.

Keywords: Smooth muscle; human tissue; pulmonary arteries; contraction; phorbol ester; protein kinase C

Introduction

Smooth muscle of large arteries (e.g. aorta, pulmonary artery) is able to develop and maintain maximal contraction in response to hormone and neuromediator stimulation. It has been demonstrated that the onset of the contraction is linked to an increase in cytosolic calcium concentration ($[Ca^{2+}]_i$) and subsequent formation of a calcium-calmodulin-myosin light chain kinase (MLCK) complex leading to the phosphorylation of myosin light chains (MLC) (Adelstein & Hataway, 1979; Murphy *et al.*, 1983). In contrast, the mechanism responsible for the maintenance of tone which occurs together with a decrease in both $[Ca^{2+}]_i$ and phosphorylation of MLC (Kamm & Stull, 1985; Himpens *et al.*, 1988; Jiang & Morgan, 1989), remains unclear. Among the different hypotheses that have been proposed to explain this maintained component of tension, the one which ascribes a major role to the activation of protein kinase C (PKC) has received considerable attention (Chatterjee & Tejada, 1986; Rasmussen *et al.*, 1987; Jiang & Morgan, 1987). PKC is present in high concentrations in vascular smooth muscle (Kuo *et al.*, 1980; Kariya & Takai, 1987), and specifically activated by diacylglycerol (DAG) under physiological conditions. PKC can also be directly activated by tumour promoting agents, the so-called phorbol esters (Nishizuka, 1984; Abdel-Latif, 1986).

In a variety of arteries from the systemic circulation, phorbol esters induce slowly-developing and sustained contractions, the calcium requirement of which varies from one vessel to another (Spedding, 1987; Sawamura *et al.*, 1987; Chiu *et al.*, 1988; Jiang & Morgan, 1989). In contrast, the effect of phorbol esters in the pulmonary circulation has been less extensively studied. Information about the involvement of protein kinase C in the control of pulmonary vascular tone especially in human tissue could be relevant to a better understanding of pathophysiological processes such as hypoxic vasoconstriction or pulmonary hypertension.

We therefore designed the present study to investigate the effect of phorbol 12,13 dibutyrate (PDB), a potent PKC activator (Castagna *et al.*, 1982), on vascular tone in both human and rat isolated pulmonary arteries. The sensitivity of PDB-induced contractile responses to calcium ions, calmodulin antagonists and PKC inhibitors was studied in the two types of preparations. A brief account of some of these results has been published previously (Savineau *et al.*, 1991).

Methods

Tissue preparations

Experiments were performed on small strips (200–300 μm in diameter, 8–10 mm in length) isolated from human lobar pulmonary artery (HPA) and the rat main pulmonary artery (RPA). Human preparations were dissected from lungs obtained at thoracotomy from patients aged from 52 to 66 years undergoing resection for lung carcinoma. From a macroscopically tumour-free part of the specimen, arteries

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(3–4 mm in internal diameter) were dissected out free of surrounding parenchyma, then transferred to physiological solution and opened along the longitudinal axis. Animal preparations were obtained from male Wistar rats aged from 15–18 weeks, after intraperitoneal injection of 40 mg sodium pentobarbitone and intracardiac administration of 100 iu heparin. After removal of connective tissue, the pulmonary artery was opened and strips (2–3 for each artery) were cut transversely.

Mechanical recording

Isometric contraction was measured in an experimental chamber (Savineau *et al.*, 1988) by means of a highly sensitive force transducer (Akers 801, Horten, Norway).

At the beginning of the experiments, K^+-rich (80 mM) solution or noradrenaline (10 μM) was repeatedly applied in order to obtain at least three contractions similar in both amplitude and kinetics before the addition of phorbol ester. Concentration-response curves to PDB were then constructed by adding cumulative concentrations of PDB (from 1 to 25 nM in HPA, and from 25 nM to 2 μM in RPA). A concentration increment was made once the maximal contractile effect of the preceding concentration had been recorded (usually 10–12 min). Generally two or three cumulative concentration-response curves (CCRC) were constructed in the same preparation. It was verified in control experiments that three or even four identical CCRC to PDB could be obtained from the same arterial strip. The response to each concentration of PDB alone or of PDB in the presence of another tested substance was expressed as a percentage of the maximal response obtained in the first CCRC.

Experiments were also performed on endothelium-free vascular preparations. Endothelium-free arteries were prepared by gently rubbing the luminal surface with a stainless steel wire. Strips dissected from these arteries were precontracted by noradrenaline (1 μM) and then exposed to acetylcholine (ACh, 10 μM): a percentage of ACh-induced relaxation, less than 10%, was accepted as evidence of endothelium removal. CCRCs to PDB were compared between intact and endothelium-free arteries. In this case, the response to an individual concentration of PDB was expressed as a percentage of the contraction evoked by 80 mM K^+.

Solutions

Physiological solutions had the following composition: (a) reference solution (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11. The solution was aerated with 5% CO_2 in O_2. (b) Calcium-free solution was prepared by omitting $CaCl_2$ and adding EGTA (0.2 mM). (c) K^+-rich (80 mM) solution was prepared by substituting an equimolar amount of KCl for NaCl.

Chemicals

Chemicals used in the present study were: acetylcholine chloride, atropine, ethyleneglycol-*bis*(β-amino-ethylether)N,N,N′,N′-tetraacetic acid (EGTA), 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H7), 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8), noradrenaline, phentolamine, phloretin, propranol, phorbol 12,13 dibutyrate, phorbol 12,13 didecanoate (PDD), 4 α-phorbol 12,13 didecanoate (4αPDD), tetrodotoxin, trifluoperazine (TFP), verapamil, N-(6-aminohexyl)-5-chloro-1-naphthalene-sulphonamide (W7) all from Sigma (La Verpillère, France); methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4(2-trifluoromethylphenyl)-pyridine-5-carboxylate (Bay K 8644) from Bayer (Leverkusen, Germany); staurosporine from Fluka (Mulhouse, France); ryanodine from Reserach Biomedical Inc. (Wayland, MA, U.S.A.).

Bay K 8644, phorbol esters, phloretin and staurosporine were dissolved in dimethyl sulphoxide (DMSO). The maximum concentration of DMSO was <0.1% and had no effect on the contractility of pulmonary arteries.

Analysis of results

The amplitude of contraction is expressed as mean ± s.d. for n, number of experiments. Significance was assessed by a paired Student's t-test. A difference between means was considered significant when $P < 0.05$. Values for EC_{50} (concentration producing 50% of the maximal response) are given as geometric means with 95% confidence limits (CL).

Results

Contractile responses induced by phorbol 12,13 dibutyrate in pulmonary arteries

Figure 1 shows a typical example of the effect of cumulatively applied concentrations of PDB on the tone of human and rat isolated pulmonary arteries. PDB induced a slowly developing and sustained contraction (the mean time for the maximal effect for each concentration was: 6 ± 1.2 min and 8.2 ± 2.4 min, $n = 10$, in HPA and RPA, respectively). The geometric mean EC_{50} was 3.5 nM, $n = 5$, (95% CL: 2.7 nM and 4.8 nM) in HPA, and 120 nM, $n = 5$, (95% CL: 80 nM and 220 nM) in RPA. The maximal response was 185.6 ± 25% and 207 ± 27.5% ($n = 5$) of the K^+-rich (80 mM) solution-evoked contraction, and 223 ± 34.5% and 176.5 ± 38.6% ($n = 5$) of the noradrenaline (10 μM)-evoked contraction in HPA and RPA, respectively. Removal of PDB after recording the maximal response was followed by a slow relaxation of tissues which returned to their basal tone in 46 ± 3.7 min and 36.7 ± 3.2 min ($n = 5$) in HPA and RPA, respectively (not shown).

CCRC to PDB was not significantly modified by the presence in the bathing solution of any of the following substances: atropine (10 μM), phentolamine (5 μM), propranolol (5 μM), tetrodotoxin (10 μM) ($n = 6$ in HPA and RPA). Removal of endothelium produced no significant change of the PDB-induced contractions in either HPA or RPA ($P > 0.05$, $n = 5$) (not shown). The inactive phorbol 4α12,13 didecanoate (1 nM to 5 μM) had no contractile effect in HPA ($n = 3$) or in RPA ($n = 4$).

Calcium-dependence of phorbol 12,13 dibutyrate-induced contractions

Effect of calcium-free solutions Removal of external calcium ions significantly reduced the amplitude of PDB-responses in

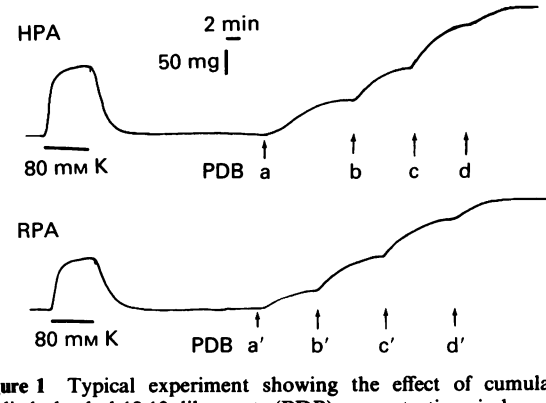


Figure 1 Typical experiment showing the effect of cumulatively-applied phorbol 12,13 dibutyrate (PDB) concentrations in human pulmonary artery (HPA) and rat pulmonary artery (RPA). PDB concentrations were: 2.5 (a), 5 (b), 10 (c) and 25 nM (d) in HPA; 50 (a'), 100 nM (b'); 0.5 (c') and 2 μM (d') in RPA. The maximum PDB-induced contraction was 190 and 200% of that induced by the potassium-rich (80 mM K^+) solution in HPA and RPA, respectively.

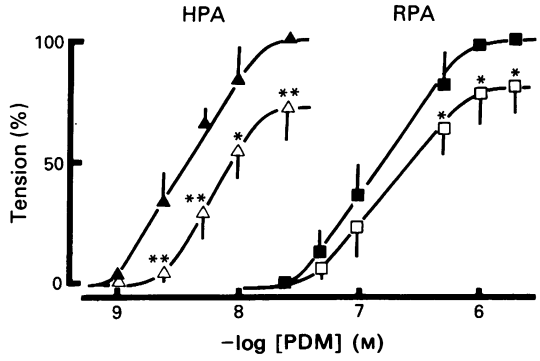


Figure 2 Concentration-response curves for phorbol 12,13 dibutyrate (PDB) in the presence (▲, ■) and in the absence (△, □) of external calcium ions, in human (HPA, ▲, △) and rat (RPA, ■, □) pulmonary arteries, respectively. The calcium-free solution contained 0.2 mM EGTA. Abscissa scale: −log concentration (M) of PDB. Ordinate scale: tension as a percentage of the maximal response induced by PDB—25 nM in HPA, or 2 μM in RPA. Data points are mean, $n = 5$. Vertical lines show s.d. $*P < 0.05$ and $**P < 0.01$ indicate a response in the absence of external Ca-ions which is significantly different from the corresponding response in the control experiment.

HPA, especially those evoked by low concentrations (1–5 nM) of PDB, (contractions evoked by 2.5 and 25 nM PDB were reduced by 85.5 ± 17.8% and 28 ± 7.7%, $n = 5$, respectively). In RPA, this effect was less important (the maximal response was reduced by only 17.6 ± 11%, $n = 5$) (Figure 2). In another set of experiments, CCRC were performed in calcium-free solution containing either ryanodine (25 μM) or TMB-8 (50 μM). No significant difference was observed between the CCRC obtained in the presence and in the absence of ryanodine ($n = 3$) and TMB-8 ($n = 4$) in both types of arteries.

Effect of drugs acting at the voltage-dependent calcium channel We tested the effect of a calcium-channel agonist, Bay K 8644, and of a calcium-channel antagonist, verapamil, on PDB-induced contractions. Bay K 8644 (0.5 μM) added 10 min before the beginning of the CCRC had *per se* no effect on the vascular tone in both types of arteries, but increased the amplitude of PDB-induced contractions. This increase was significant only in HPA. Addition of verapamil (10 μM) 20 min before PDB significantly reduced the contractile responses in HPA (70 ± 8.4% and 25 ± 12.5% reduction for 2.5 and 25 nM PDB, respectively), but had only a slight effect on the maximal responses in RPA (Figure 3).

Effect of calmodulin-antagonists

Pretreatment of pulmonary arterial strips for 15 min with trifluoperazine (TFP, 5 μM) significantly decreased PDB-induced contractions in HPA (especially those evoked by low PDB concentrations, 1–5 nM). This effect of TFP was not significant in RPA (Figure 4). Similar results were obtained in the presence of W7 (10 μM) ($n = 3$ in HPA and RPA, not shown).

Effects of protein kinase C inhibitors

In the subsequent series of experiments, we tested the effect of three substances commonly accepted as PKC-inhibitors: H7, phloretin and staurosporine. Each substance was added to the bathing solution for 15 min before the beginning of the CCRC. H7 (50 μM), phloretin (100 μM) and staurosporine (25 nM) inhibited in a potent manner PDB contractile responses (Figures 5, 6, 7) in the two types of arteries. In HPA, the maximal response was decreased by 60 ± 10.5%, 70.5 ± 12.2% and 80.5 ± 8.4% ($n = 5$) for H7, phloretin and staurosporine, respectively. Similarly in RPA the maximal response was

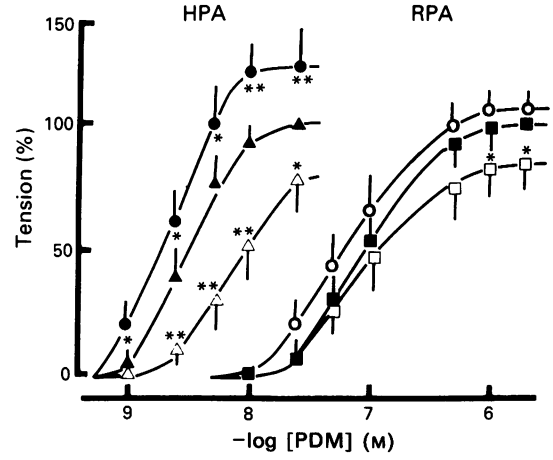


Figure 3 Concentration-response curves for phorbol 12,13 dibutyrate (PDB) alone (▲, ■), in the presence of either 0.1 μM Bay K 8644 (●, ○) or 10 μM verapamil (△, □) in human (HPA) and rat (RPA) pulmonary arteries, respectively. Abscissa sacle: −log concentration (M) of PDB. Ordinate scale: tension as a percentage of the maximal response induced by PDB—25 nM in HPA, or 2 μM in RPA. Data points are mean, $n = 5$. Vertical lines show s.d. $*P < 0.05$ and $**P < 0.01$ indicate a response in the presence of Bay K 8644 or verapamil which is significantly different from the corresponding response in the control experiment.

decreased by 35 ± 11.5%, 56 ± 18% and 71 ± 14% ($n = 5$) for H7, phloretin and staurosporine, respectively.

Effect of long-term treatment of preparation with phorbol esters

After recording K^+-rich (80 mM)-induced contraction and constructing CCRC to PDB, pulmonary arterial strips were incubated for 15–20 h in the presence of phorbol 12,13 didecanoate or PDB itself at a concentration of 10 nM in HPA and 0.5 μM in RPA. This treatment was followed by a 2 h washing period with the reference solution. K^+-rich solution and PDB were then successively applied again. Potassium-induced contraction was reduced only by 21 ± 7.2% ($n = 4$; $P < 0.05$) and 17 ± 5.6% ($n = 4$, $P > 0.05$) in HPA and RPA, respectively. In contrast, contractile responses to PDB stimulation completely disappeared in HPA ($n = 4$). In RPA, 2 μM of PDB produced only a small response, the amplitude of which was 15.6 ± 6.4% ($n = 4$, $P < 0.01$) of the maximal control response.

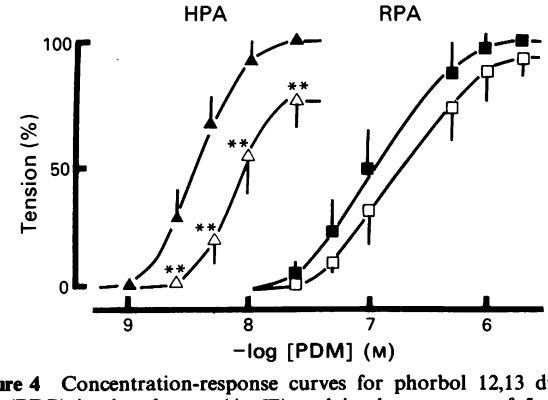


Figure 4 Concentration-response curves for phorbol 12,13 dibutyrate (PDB) in the absence (▲, ■) and in the presence of 5 μM trifluoperazine (TFP, △, □), in human (HPA) and rat (RPA) pulmonary arteries, respectively. Abscissa scale: −log concentration (M) of PDB. Ordinate scale: tension as a percentage of the maximal response induced by PDB—25 nM in HPA, or 2 μM in RPA. Data points are mean, $n = 5$. Vertical lines show s.d. $**P < 0.01$ indicate a response in the presence of TFP which is significantly different from the corresponding response in the control experiment.

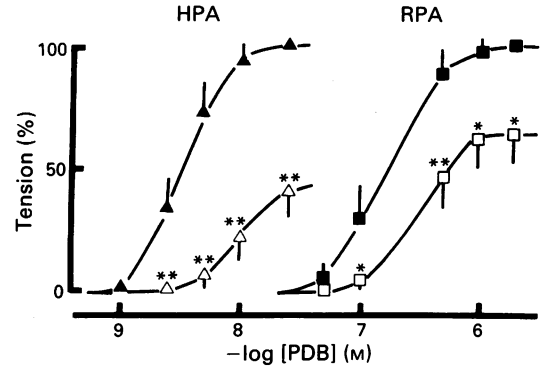


Figure 5 Concentration-response curves for phorbol 12,13 dibutyrate (PDB) in the absence (▲, ■) and in the presence of 50 μM 1-(5-isoquindinesulphonyl)-2-methylpiperazine (H7) (△, □) in human (HPA) and rat (RPA) pulmonary arteries, respectively. Abscissa scale: −log concentration (M) of PDB. Ordinate scale: tension as a percentage of the maximal response induced by PDB—25 nM in HPA, or 2 μM in RPA. Data points are mean, $n = 5$. Vertical lines show s.d. * $P < 0.05$ and ** $P < 0.01$ indicate a response in the presence of H7 which is significantly different from the corresponding response in the control experiment.

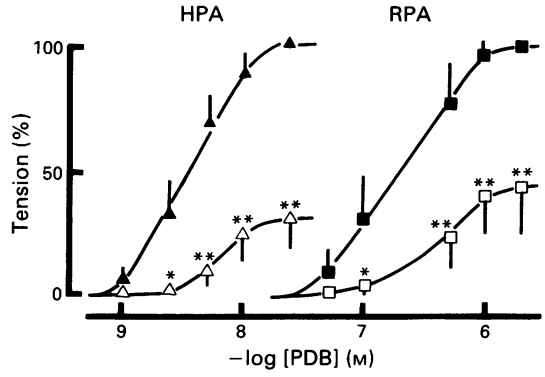


Figure 6 Concentration-response curves for phorbol 12,13 dibutyrate (PDB) in the absence (▲, ■) and in the presence of 100 μM of phloretin (△, □) in human (HPA) and rat (RPA) pulmonary arteries, respectively. Abscissa scale: −log concentration (M) of PDB. Ordinate scale: tension as a percentage of the maximal response induced by PDB—25 nM in HPA, or 2 μM in RPA. Data points are mean, $n = 5$. Vertical bars show s.d. * $P < 0.05$ and ** $P < 0.01$ indicate a response in the presence of phloretin which is significantly different from the corresponding response in the control experiment.

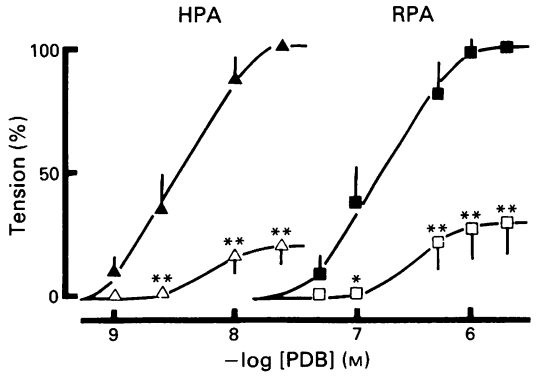


Figure 7 Concentration-response curves for phorbol 12,13 dibutyrate (PDB) in the absence (▲, ■) and in the presence of 25 nM staurosporine (△, □) in human (HPA) and rat (RPA) pulmonary arteries, respectively. Abscissa scale: −log concentration (M) of PDB. Ordinate scale: tension as a percentage of the maximal response induced by PDB—25 nM in HPA, or 2 μM in RPA. Data points are mean, $n = 5$. Vertical lines show s.d. * $P < 0.05$ and ** $P < 0.01$ indicate a response in the presence of staurosporine which is significantly different from the corresponding response in the control experiment.

Discussion

The present work has revealed that PDB is a potent constrictor agent in mammalian pulmonary arteries. Its efficacy is approximately twice that of potassium-rich solution or noradrenaline. Moreover, for the first time, it is shown that human pulmonary vascular tissue is very sensitive to phorbol esters, the potency of which is in the nanomolar range.

In both human and rat pulmonary arteries, PDB produced sustained contractions which were characterized as follows. First, the time course of the contraction was slow as in other vascular beds (Forder *et al.*, 1985; Spedding, 1987; Salaices *et al.*, 1990). Second, the contraction was not altered by the presence of adrenoceptor or cholinoceptor antagonists or by removal of endothelium. Third, the contraction was reduced in a potent manner by inhibitors of protein kinase C activity such as H7, phloretin and staurosporine (Gschwendt *et al.*, 1984; Hidaka *et al.*, 1984; Tamaoki *et al.*, 1986). The rank order of efficacy of these inhibitors was staurosporine > phloretin > H7, this order being similar to that observed in the rat aorta (Auguet *et al.*, 1989). Fourth, the contraction disappeared after prolonged treatment of tissues with PKC activators. Such a treatment has been shown to induce a 'down-regulation' of PKC activity in vascular smooth muscle cells (Pfeilschifter *et al.*, 1989). It has been suggested that this 'down-regulation' could be related to a persistent state of membrane translocation of PKC leading to its hydrolysis by proteases (Wolf *et al.*, 1985; Secrest *et al.*, 1991). Moreover, the inactive 4 phorbol 12,13 didecanoate (Castagna *et al.*, 1982) had no effect on the tone of these arteries. These results together with the facts that: (1) PDB directly binds to and activates PKC (Kikkawa *et al.*, 1983); (2) PKC is present in arterial smooth muscle (Kuo *et al.*, 1980; Kariya & Takai, 1987) strongly suggest that the PDB-effect in pulmonary arteries is mainly mediated by activation of smooth muscle cell PKC. Taking into account the kinetics of PDB-induced responses (Figure 1), it thus appears that activation of PKC could be an effective pathway in the maintenance of vascular tone during agonist stimulation in pulmonary arteries.

The mechanism by which activation of PKC leads to smooth muscle contraction is not fully elucidated and different cellular sites have been proposed (Abdel-Latif, 1986; Rasmussen *et al.*, 1987). One of them is the voltage-dependent calcium channel. Although PDB increases calcium channel current in some enzymatically dispersed vascular cells (Fish *et al.*, 1988; Loirand *et al.*, 1990), conflicting results have been reported regarding the external calcium-dependence of contractions induced by phorbol esters in blood vessels. In this respect, our results on the effect of calcium-free solutions (Figure 2), verapamil and Bay K 8644 (Figure 3), show a clear species difference: PDB-induced contraction was not dependent on calcium influx in RPA, whereas it exhibited a significant external calcium-dependence in HPA, especially at low concentrations. Since Bay K 8644 alone had no effect on pulmonary arterial tone, we suggest that in HPA, PDB depolarizes smooth muscle cells producing an influx of calcium ions through voltage-dependent channels. This calcium entry could then be responsible for part of the PDB response via a pathway involving the activation of MLCK by a Ca-calmodulin complex. This hypothesis is supported by the results of the experiments with TFP (Figure 4) and W7, two calmodulin-antagonists (Levin & Weiss, 1977; Asano *et al.*, 1982). The component of the tension independent of calcium influx in HPA as well as that more important in amplitude in RPA could be due to the action of PKC on other sites in the vascular smooth muscle cell. These sites include sarcoplasmic reticulum (SR), myosin light chains (MLC) and myofilaments. It has been shown that phorbol esters act on the calcium-release mechanism of SR in cardiac muscle (Rogers *et al.*, 1990). It is unlikely, however that such an action could have occurred in our experiments for the following reasons. PDB contractions in calcium-free solution were (i) unaffected by the presence of ryanodine or TMB8, both substances known to

alter the Ca release from smooth muscle SR (Chiou & Malagodfi, 1975; Hwang & Van Breemen, 1987); (ii) not significantly different from those obtained in the presence of TFP in calcium-containing solution (compare Figures 2 and 4). These results suggest that the component of tension independent of calcium influx was not related to a variation in $[Ca^{2+}]_i$. Phorbol esters could also directly phosphorylate MLC on sites different from those phosphorylated by MLCK (Ikebe *et al.*, 1985; Singer, 1990). Lastly, phorbol esters could act at the site of myofilaments either by direct stimulation (Chatterjee & Tajeda, 1986) or by increasing their calcium sensitivity (Nishimura *et al.*, 1990). Additional experiments with skinned fibres as well as biochemical measurements are required to determine precisely the mechanism of action of PKC in pulmonary smooth muscle cells.

In conclusion, the present work has shown that phorbol 12,13 dibutyrate is one of the most potent constrictor agents in pulmonary arteries. In human tissue, a part of this effect is dependent on calcium influx. Activation of PKC appears to be a primary step in the action of PDB. Our findings suggest that PKC activity could be an important factor in pulmonary vascular responsiveness.

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Differentiation by hydroquinone of relaxations induced by exogenous and endogenous nitrates in non-vascular smooth muscle: role of superoxide anions

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1 The influence of hydroquinone on relaxations induced by nitric oxide (NO), nitrovasodilator drugs, and non-adrenergic, non-cholinergic (NANC) field stimulation has been investigated in three tissues in which endogenous nitrates have been implicated in the NANC response; the mechanism of action of hydroquinone was also studied.

2 In mouse anococcygeus, hydroquinone (10–100 μM) produced a concentration-dependent inhibition of relaxations induced by 15 μM NO. Hydroquinone, 100 μM, which reduced responses to NO by 85%, had no effect on relaxations induced by NANC field stimulation (10 Hz; 20 s trains), hydroxylamine (10 μM), sodium nitroprusside (1 μM) or sodium azide (20 μM).

3 In guinea-pig trachea, 100 μM hydroquinone reduced relaxations to 150 μM NO by 75%, but had no effect on those to NANC stimulation (10 Hz; 30 s trains) or sodium azide (5 μM).

4 In rat gastric fundus, 100 μM hydroquinone reduced relaxations to 1 μM NO by 85%, but had no effect on those to NANC stimulation (0.5 Hz; 15 s trains) or sodium azide (2 μM).

5 Superoxide dismutase (SOD; 50 u ml^{-1}) had no effect on relaxations of the mouse anococcygeus in response to 15 μM NO or 10 Hz NANC stimulation. Further, the inhibition of responses to NO by hydroquinone was unaffected in the presence of SOD.

6 Hydroquinone (10–100 μM) failed to generate superoxide anions, as detected by a chemiluminescent assay. However, 100 μM hydroquinone, like SOD (50 u ml^{-1}), produced almost complete inhibition of superoxide anion chemiluminescence induced by xanthine (500 μM): xanthine oxidase (0.07 u ml^{-1}).

7 It is concluded that, in our system, hydroquinone inhibits NO by acting as a free radical scavenger rather than by generating superoxide anions. The ability of hydroquinone to block relaxations to NO, but not NANC stimulation, may suggest that the endogenous nitrate substance released by these NANC nerves may not be free NO, but may be an NO-containing, or NO-generating, molecule.

Keywords: Guinea-pig trachea; hydroquinone; mouse anococcygeus; nitric oxide; nitrovasodilators; non-adrenergic, non-cholinergic; rat gastric fundus; superoxide anions; superoxide dismutase

Introduction

Certain non-adrenergic, non-cholinergic (NANC) relaxations may be mediated by the release of an endogenous nitrate neurotransmitter, generated via a system similar to that responsible for the production of endothelium-derived relaxing factor (EDRF). Thus, drugs known to inhibit EDRF synthesis, such as L-N^G-monomethyl-arginine (Moncada *et al.*, 1989) and L-N^G-nitro-arginine (L-NOARG; Moore *et al.*, 1990), reduce NANC relaxations in a variety of tissues, including the anococcygeus (Gillespie *et al.*, 1989; Ramagopal & Leighton, 1989; Gibson *et al.*, 1990; Hobbs & Gibson, 1990), trachea (Tucker *et al.*, 1990; Li & Rand, 1991), blood vessels (Toda & Okamura, 1990), gastrointestinal tract (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1990; Li & Rand, 1990), and urogenital tract (Gillespie, 1990; Ignarro *et al.*, 1990a; Andersson *et al.*, 1991; Holmquist *et al.*, 1991). In addition, L-arginine:nitric oxide (NO) synthase, the enzyme involved in the generation of EDRF, has been detected by immunocytochemistry in nerve terminals in both the central and peripheral nervous systems (Bredt *et al.*, 1990). The biological activity of EDRF has been attributed mainly to NO (Moncada *et al.*, 1988), which therefore has been considered the most likely candidate for the NANC transmitter. However, some differences between the vascular and NANC endogenous nitrate systems have been reported (Gillespie & Sheng, 1989; Gibson *et al.*, 1990; Toda & Okamura, 1990). If the two systems are the same, then drugs known to influence EDRF/NO function should show parallel effects on the endogenous nitrate NANC system; indeed, such evidence would be necessary to establish NO as a neurotransmitter. Hydroquinone has been used frequently as an inhibitor of EDRF/NO relaxations of vascular smooth muscle (Furchgott, 1984) where it might act either as a free radical scavenger (Griffith *et al.*, 1984) or as a generator of superoxide anions (Moncada *et al.*, 1986). However, its effects on NANC responses have been less widely studied and results have been varied; Gillespie & Sheng (1990) found that hydroquinone produced a small reduction of NANC relaxations of the rat anococcygeus but had no effect on the bovine retractor penis. The object of the present study was to investigate in more detail the interaction between hydroquinone and NANC nerves by determining its effects on three other tissues possessing an endogenous nitrate NANC system: the mouse anococcygeus (Gibson *et al.*, 1990), guinea-pig trachea (Tucker *et al.*, 1990; Li & Rand, 1991), and the rat gastric fundus (Li & Rand, 1990). In addition, an attempt was made to determine whether the effect of hydroquinone resulted from production of superoxide anions. Some of the work has been presented to the British Pharmacological Society (Gibson & Mirzazadeh, 1989a; Hobbs *et al.*, 1991).

Methods

Mouse anococcygeus

Male mice (LACA; 25–35 g) were killed by stunning and exsanguination. The paired anococcygeus muscles were dissected, joined by the ventral bar, and set up in series in 2 ml

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glass organ baths containing Krebs bicarbonate buffer (mM: NaCl 118.1, KCl 4.7, $MgSO_4$ 1.0, KH_2PO_4 1.0, $CaCl_2$ 2.5, $NaHCO_3$ 25.0 and glucose 11.1) which was maintained at 37°C and gassed continously with 95% O_2:5% CO_2. A resting tension of 200–400 mg was placed on the tissues and changes in tension recorded with a Grass FTO3 force-displacement transducer attached to a Graphtec pen-recorder (WR 3101). Muscles were allowed to equilibrate for 45 min before beginning the experiment. Field stimulation was applied via two parallel platinum electrodes running down either side of the tissue; these were attached to a Grass S48 stimulator (0.5 ms pulse width; 70 V). To observe relaxations to field stimulation in the anococcygeus it is necessary to raise muscle tone and negate contractions due to release of noradrenaline from sympathetic nerves. Tone was raised with 50 μM carbachol in all cases; sympathetic responses were prevented by including 1 μM phentolamine in the Krebs solution and by pre-incubating each muscle with 30 μM guanethidine for 10 min during the 45 min equilibration period. Field stimulation (10 Hz; 10 s trains every 100 s), under these conditions, produced NANC relaxations which were reduced by 95% in the presence of 50 μM L-NOARG (Gibson *et al.*, 1990).

To record relaxations to drugs, tone was first raised with 50 μM carbachol. When carbachol-induced tone had reached a plateau (usually within 3 min), the relaxant drug was added to the organ bath. The response was calculated as the peak % reduction of carbachol-induced tone occurring within 4 min of addition of the relaxant drug; if no peak occurred within 4 min, the response at that time was used. Both carbachol and the relaxant were then washed out of the organ bath and the muscle allowed to rest for 20 min before tone was raised again.

Guinea-pig trachea

Male guinea-pigs (Dunkin-Hartley; 600–900 g) were stunned, exsanguinated and the trachea excised. Surrounding connective tissue was removed and tranverse strips consisting of two adjacent cartilage rings were set up for the recording of isometric tension responses and application of field stimulation as described above (resting tension 0.5 g). All experiments were conducted in the presence of atropine (1 μM), propranolol (1 μM), indomethacin (2 μM) and α-chymotrypsin (2 u ml^{-1}). Muscle tone was raised with the thromboxane-mimetic U46619 (50 nM). Again, relaxations were calculated as the % reduction of tone occurring within 4 min of application of relaxant drug or field stimulation.

Rat gastric fundus

Male rats (Wistar; 250–350 g) were stunned, exsanguinated and their stomachs removed. Longitudinal muscle strips approximately 10 mm long by 2 mm wide were prepared from the fundus by cutting parallel to the greater curvature and set up for the measurement of isometric tension responses and application of field stimulation as described above (resting tension 0.5 g). All experiments were conducted in the presence of atropine (2 μM) and guanethidine (5 μM). Muscle tone was raised with U46619 (100 nM). Relaxations were calculated as % reduction of tone, occurring within 4 min of application of relaxant drug or field stimulation.

In all three of the above preparations, hydroquinone (or SOD) was added to the bath 10 min before the relaxing stimulus. Only one concentration of relaxant, or one train of field stimulation, was applied on each occasion that tone was raised with carbachol or U46619.

Rabbit aorta

Male rabbits (New Zealand White; 2.5–3.5 kg) were killed by overdose of pentobarbitone (60 mg kg^{-1}, i.v.). The thoracic aorta was dissected out and cleared of connective tissue. Intact rings, 3–4 mm in diameter, were set up for the recording of isometric tension responses as described above (resting tension 2 g). Rings were pre-contracted with phenylephrine (500 nM). Methacholine (0.1–10 μM) was administered cumulatively to elicit concentration-dependent relaxations.

Chemiluminescent studies

Superoxide anions were detected by chemiluminescence (Cherry *et al.*, 1990). Two ml Krebs solution containing 250 μM lucigenin was added to clear polystyrene cuvettes. These were positioned in an LKB-Wallac 1250 luminometer in which they were maintained at 37°C and gassed continuously with 95% O_2:5% CO_2. The chemiluminescent signal (measured in mV) was monitored continuously by digital read-out, and in analogue form, by an attached pen-recorder. After a 15 min equilibration period, increasing concentrations of hydroquinone were added to the cuvette via an auto-injector (LKB 1250-104). In some experiments, a pair of anococcygeus muscles from a single mouse were minced and added to the cuvette prior to the hydroquinone. A new cuvette, and tissue where appropriate, was used for each new concentration of hydroquinone. The ability of the system to detect superoxide anions was determined by use of xanthine:xanthine oxidase. Here 0.07 u ml^{-1} xanthine oxidase was included with 250 μM lucigenin in 2 ml Krebs solution in the cuvette; increasing concentrations of xanthine were then added via the auto-injector. In certain experiments superoxide dismutase (SOD; 50 u ml^{-1}) or hydroquinone (100 μM) was added to the cuvette 10 min before the xanthine.

Statistics

Results are expressed as mean ± s.e.mean. Statistical analysis was by Student's *t* test (unpaired); a probability (*P*) value of <0.05 was taken to indicate statistical significance.

Drugs

All drugs used in this study were dissolved in distilled water, except xanthine oxidase, which was in 2.3 M $(NH_4)_2SO_4$ buffer, and indomethacin which was in 0.5% Na_2CO_3. NO solutions and concentrations were prepared and calculated as described previously (Gibson & Mirzazadeh, 1989b).

Drugs used were: atropine sulphate (Sigma); α-chymotrypsin (Type I-S; Sigma); carbachol (BDH); guanethidine sulphate (Ciba); hydroquinone (Sigma); hydroxylamine hydrochloride (Sigma); indomethacin (Sigma); lucigenin (*bis*-methylacridinium nitrate; Sigma); methacholine chloride (Sigma); nitric oxide (99%; BDH); pentobarbitone sodium (Sigma); phentolamine mesylate (Ciba); phenylephrine hydrochloride (Sigma); propranolol hydrochloride (ICI); sodium azide (Sigma); sodium nitroprusside (Sigma); U46619 (9,11-dideoxy methanolepoxy-9α, 11α-prostaglandin $F_{2\alpha}$) (Sigma); xanthine (Sigma); xanthine oxidase (Sigma).

Results

The effect of hydroquinone on nitrovasodilator- and NANC-induced relaxations

Mouse anococcygeus As described previously (Gibson & Mirzazadeh, 1989b; Gibson *et al.*, 1990), carbachol-induced tone of the mouse anococcygeus showed graded relaxations in the presence of NO (3–30 μM), hydroxylamine (5–200 μM), sodium nitroprusside (SNP; 0.01–10 μM) and sodium azide (1–100 μM). Concentrations of each drug which reduced tone by between 30–80% were chosen to study the effect of hydroquinone (see control columns in Figure 2). To establish an effective concentration of hydroquinone it was first tested against NO. Relaxations induced by 15 μM NO were reduced, in a concentration-dependent manner, by hydroquinone (10–100 μM; Figure 1); the inhibitory effect of each concentration of hydroquinone was easily reversed by washout. Hydro-

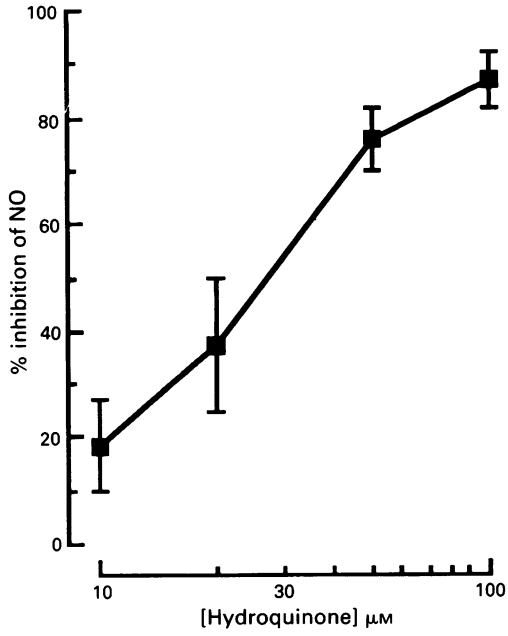


Figure 1 Concentration-response curve for hydroquinone causing inhibition of nitric oxide (NO; 15 μM)-induced relaxations of the mouse anococcygeus muscle. Tone was raised with 50 μM carbachol; hydroquinone was added 10 min prior to the NO. Each point represents the mean from at least 6 individual muscle preparations; s.e.mean shown by vertical bars.

quinone, 100 μM, which reduced responses to NO by about 85%, had no effect on relaxations induced by hydroxylamine (10 μM), SNP (1 μM) or sodium azide (20 μM; Figure 2). Field stimulation (10 Hz; 20 s trains) caused reproducible NANC relaxations which have previously been shown to be blocked by L-NOARG (Gibson *et al.*, 1990); these relaxations were, however, unchanged in the presence of 100 μM hydroquinone (Figure 2). Although not shown, we also studied the effect of hydroquinone with 20 s trains at 1, 2 and 5 Hz ($n = 2$ in each case) but, again, no change in response was observed.

Guinea-pig trachea In these experiments, in addition to propranolol (1 μM), indomethacin (2 μM) and α-chymotrypsin

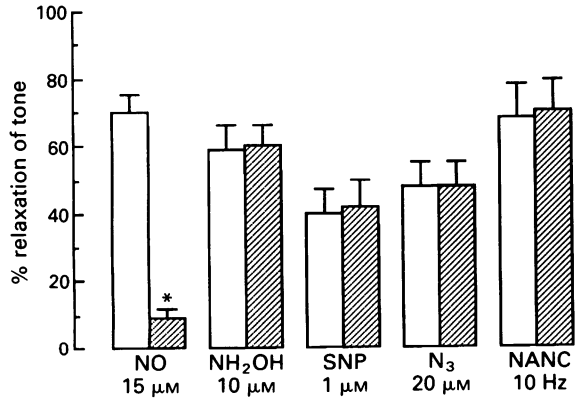


Figure 2 Histogram showing the effect of hydroquinone (100 μM) on relaxations of the mouse anococcygeus in response to nitric oxide (NO), hydroxylamine (NH_2OH), sodium nitroprusside (SNP), sodium azide (N_3) and non-adrenergic, non-cholinergic field stimulation (NANC). Muscle tone was raised with 50 μM carbachol; hydroquinone was in contact with the tissue for 10 min before application of the relaxing stimulus. Open columns, control; hatched columns, in the presence of 100 μM hydroquinone. Each column represents the mean from at least 6 individual muscle preparations; s.e.mean shown by vertical bars. * $P < 0.05$, significant reduction of response. Hydroquinone reduced responses to NO but not to NANC stimulation or other nitrovasodilators.

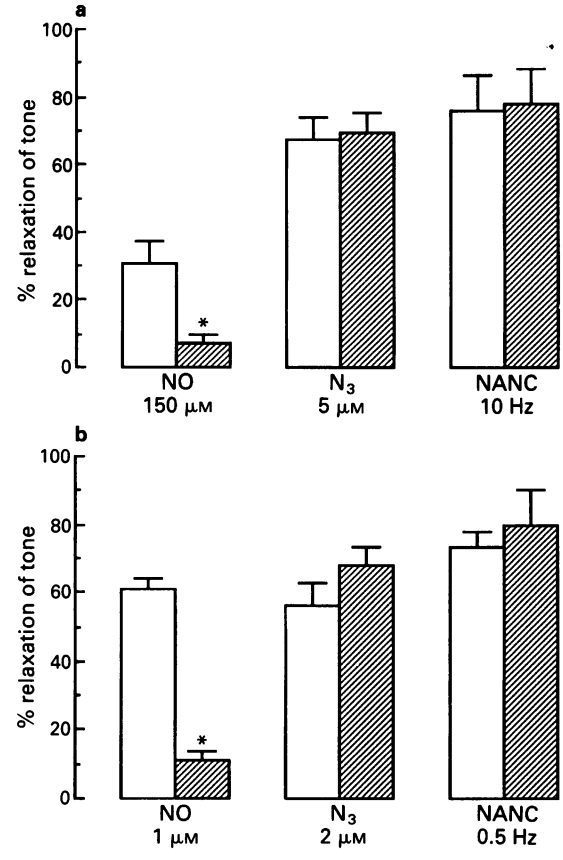


Figure 3 Histograms showing the effect of hydroquinone (100 μM) on relaxations of the guinea-pig trachea (a) and rat gastric fundus (b) in response to nitric oxide (NO), sodium azide (N_3) and non-adrenergic, non-cholinergic field stimulation (NANC). Muscle tone was raised with U46619 (50 nM in trachea; 100 nM in fundus); hydroquinone was in contact with tissue for 10 min before application of the relaxing stimulus. Open columns, control; hatched columns, in the presence of 100 μM hydroquinone. Each column represents the mean of at least 6 individual muscle preparations; s.e.mean shown by vertical bars. * $P < 0.05$, significant reduction of response. Hydroquinone reduced responses to NO in both tissues but not those to NANC stimulation or sodium azide.

(2 u ml^{-1}), atropine (1 μM) was included in the Krebs solution to prevent any contractile effects due to released acetylcholine. For this reason, we could not use carbachol to raise tone as in our original experiments (Tucker *et al.*, 1990). However, field stimulation (10 Hz; 30 s trains) produced clear NANC relaxations of U46619-induced tone, which were inhibited by 90% in the presence of 100 μM L-NOARG (Hobbs *et al.*, 1991). U46619-induced tone was also reduced by NO (3–300 μM) and sodium azide (0.5–100 μM). Hydroquinone (100 μM) reduced responses to NO (150 μM) by 75% (Figure 3a), but had no effect on those to sodium azide (5 μM) or NANC field stimulation.

Rat gastric fundus U46619 (100 nM)-induced tone in rat gastric fundus was reduced by NANC field stimulation (0.5 Hz; 15 s trains); these relaxations were reduced by 50% in the presence of 50 μM L-NOARG, confirming the involvement of an endogenous nitrate (Li & Rand, 1990). NO (1–20 μM) and sodium azide (0.5–10 μM) also reduced U46619-induced tone. Hydroquinone (100 μM) reduced relaxations to 1 μM NO by 85% (Figure 3b), but had no effect on those to sodium azide (2 μM) or NANC field stimulation.

Investigation of the mechanism of action of hydroquinone

Hydroquinone on methacholine-induced relaxations of rabbit aorta To ensure that hydroquinone was capable of inhibiting

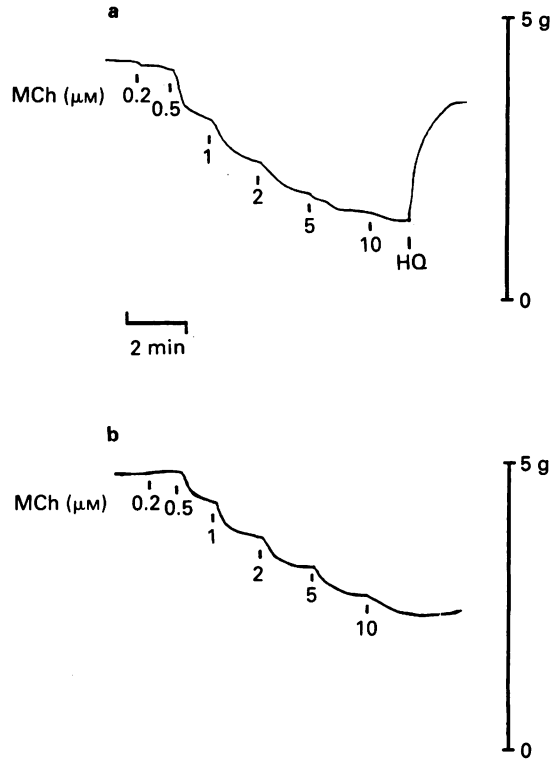


Figure 4 Relaxation of a rabbit aortic ring in response to cumulative additions of methacholine (MCh, a and b) and its reversal by hydroquinone (HQ; 100 μM; a). Muscle tone was raised with 500 nM phenylephrine. The trace is representative of similar results obtained in 10 out of 10 tissues.

endogenously-released nitrate in our system, we investigated its effect on rabbit aortic rings. In rings pre-contracted with phenylephrine (500 nM), methacholine (0.1–10 μM) caused concentration-related relaxations; these were abolished by gently rubbing the luminal surface with cotton wool and were therefore deemed to be due to the release of EDRF. Methacholine (10 μM) reduced tone by approximately 60%; hydroquinone (100 μM) produced a rapid reversal of this relaxation (Figure 4).

The effect of prior field stimulation on the inhibitory effect of hydroquinone It was possible that the passage of current through the Krebs solution might lead to breakdown of hydroquinone. However, the inhibitory effect of 100 μM hydroquinone on NO (15 μM)-induced relaxations of the mouse anococcygeus was still observed after prior exposure to a 10 s train of field stimulation (10 Hz; Figure 5).

The role of superoxide anions The involvement of superoxide anions in the inhibitory effects of hydroquinone was investigated in two series of experiments. In the first, relaxations were obtained in the presence and absence of SOD (50 u ml^{-1}). By itself, SOD had no effect on relaxations induced by 15 μM NO (control relaxation = 54 ± 5%; with SOD = 54 ± 9%; $n = 40$; $P > 0.05$) or 10 Hz NANC stimulation (control relaxation = 55 ± 9%; with SOD = 54 ± 9%; $n = 6$; $P > 0.05$). In addition, the inhibitory effect of 100 μM hydroquinone on relaxations induced by NO was unchanged in the presence of SOD (control inhibition = 91 ± 6%; with SOD = 96 ± 4%; $n = 6$; $P > 0.05$).

In the second series of experiments, superoxide anion generation was assessed by chemiluminescence. In the presence of xanthine oxidase (0.07 u ml^{-1}) and lucigenin (250 μM), the addition of xanthine (10–500 μM) caused a concentration-related generation of superoxide anions, detected by an increase in the chemiluminescent signal; the time course of the effect of 100 μM xanthine and the peak increase produced by

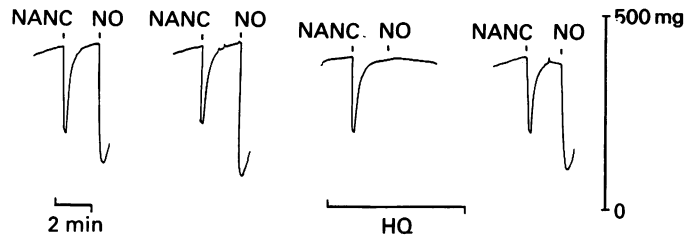


Figure 5 The effect of hydroquinone (HQ; 100 μM) on relaxations of a mouse anococcygeus muscle induced by non-adrenergic, non-cholinergic field stimulation (NANC; 10 Hz; 10 s train) and nitric oxide (NO; 15 μM). Muscle tone was raised by carbachol (50 μM) which was washed out of the bath after each dose of NO and the muscle allowed to rest for 20 min before tone was raised again. Hydroquinone was added 10 min before NANC stimulation. Hydroquinone reduced responses to NO, but not NANC stimulation, even after prior exposure to field stimulation. The trace is representative of similar results obtained in 5 out of 5 tissues.

each concentration of xanthine are shown in Figure 6. SOD (50 u ml^{-1}) produced almost complete inhibition of superoxide anion chemiluminescence (Figure 7). Hydroquinone (10–100 μM) produced no generation of superoxide anions; however, 100 μM hydroquinone greatly reduced the chemiluminescence generated by 500 μM xanthine (Figure 7). Essentially, similar results were obtained when the above experiments were repeated with the inclusion of minced anococcygeus tissue in the cuvette, in which xanthine (500 μM):xanthine oxidase chemiluminescence was reduced by

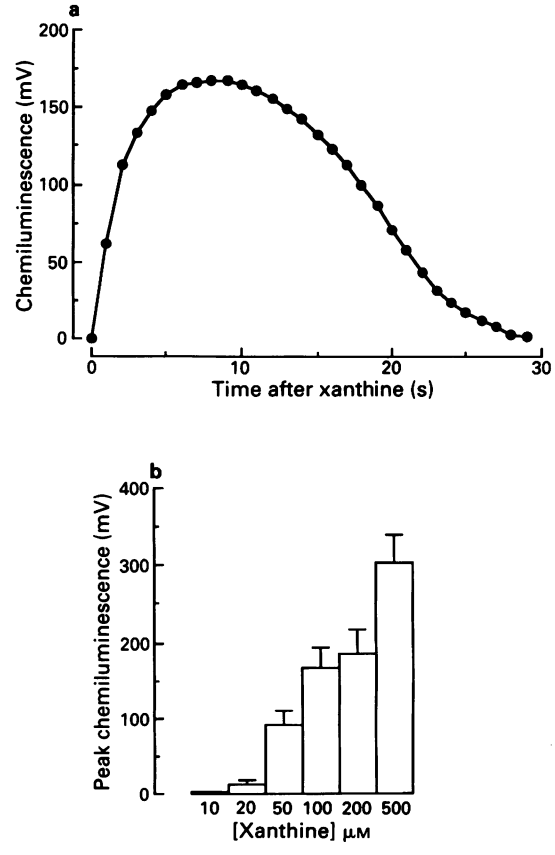


Figure 6 Time-course (a) and peak increase (b) of the chemiluminescent signal generated by xanthine:xanthine oxidase. A constant concentration of xanthine oxidase (0.07 u ml^{-1}) was used; in (a) 100 μM xanthine and in (b) increasing concentrations of xanthine were added to induce the production of superoxide anions. Each point represents the mean of at least 6 experiments; s.e.mean shown by vertical bars in (b) and omitted in (a) for clarity.

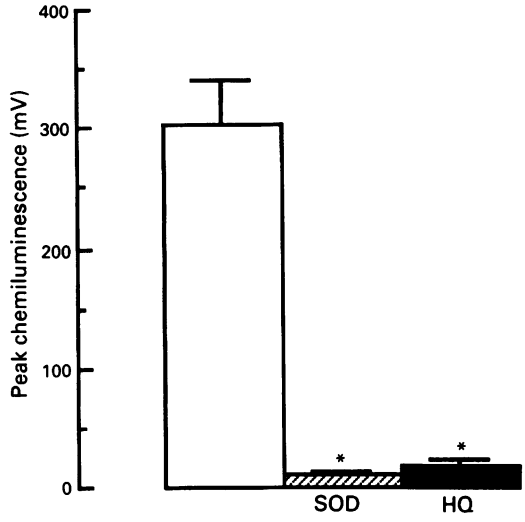


Figure 7 Histogram showing the effect of superoxide dismutase (SOD; 50 u ml^{-1}) and hydroquinone (HQ; 100 μM) on the peak chemiluminescent signal generated by xanthine (500 μM):xanthine oxidase (0.07 u ml^{-1}). SOD and HQ were added 10 min before xanthine. Each column represents the mean of at least 6 individual observations; s.e.mean shown by vertical bars. * $P < 0.05$, significant reduction of chemiluminscent signal.

93 ± 2% ($n = 5$) in the presence of SOD (50 u ml^{-1}) and by 96 ± 1% ($n = 5$) in the presence of hydroquinone (100 μM).

Discussion

NO-synthase inhibitors have been shown to block NANC relaxations in the mouse anococcygeus (Gibson *et al.*, 1990), guinea-pig trachea (Tucker *et al.*, 1990; Hobbs *et al.*, 1991; Li & Rand, 1991) and rat gastric fundus (Li & Rand, 1990; Boeckxstaens *et al.*, 1991); the inhibition is stereoselective and is reversed by L-, but not D-, arginine. Further, in the anococcygeus the relaxations to both NANC stimulation and nitrovasodilators are selectively reduced by the guanylate cyclase inhibitor N-methylhydroxylamine (Gibson & Mirzazadeh, 1989b). Such observations have led to the proposal that, in each tissue, endogenous nitrates contribute to the NANC response. Since the system is similar to that which generates EDRF/NO (Moncada *et al.*, 1988), and since NO-synthase has been detected in nerves (Bredt *et al.*, 1990), NO has been considered as the putative NANC transmitter. If NO is the actual transmitter substance released by the NANC nerves it would be expected that drugs which influence the actions of NO would exert a similar effect on the NANC response. However, in the present study, hydroquinone produced marked inhibition of relaxations to NO in all three tissues but had no effect on those to NANC stimulation. Hydroquinone also failed to influence NANC relaxations in the bovine retractor penis (Gillespie & Sheng, 1990), but produced a small inhibitory effect in the rat anococcygeus; why the rat anococcygeus should differ from the other tissues in its response to hydroquinone is not known. It is unlikely that lack of penetration of hydroquinone into the tissue can account for its differentiation between exogenous and endogenous nitrate in the present study because it produced the expected reversal of EDRF/NO-induced relaxations of rabbit aorta (Furchgott, 1984). Similarly, breakdown of hydroquinone by the current passing through the Krebs solution during field stimulation cannot be an explanation, since the activity of hydroquinone persisted after prior exposure to field stimulation. Consequently, the observed lack of parallelism may suggest that free NO is not liberated by the NANC nerves. While there is now strong evidence that an endogenous nitrate system contributes to NANC relaxations in several tissues (see Introduction), the precise nature of the substance released by the nerves remains to be established. Gillespie & Sheng (1989) found that NO and inhibitory factor from bovine retractor penis (the putative NANC transmitter) differed in their ability to penetrate red blood cells and suggested that the NANC nerves might release a NO-containing, or NO-generating, molecule rather than NO itself. Our findings would support this proposal since hydroquinone not only differentiated between NO and NANC stimulation, but also between NO and other nitrovasodilators. In this respect, hydroquinone appears to be more useful than haemoglobin as a probe to investigate possible roles for NO, since haemoglobin is known to bind and inactivate not just NO but also a wide range of nitrovasodilators (Colter & Quastel, 1950; Mittal *et al.*, 1978; Martin *et al.*, 1985; Laurence & Bennett, 1987). Bult *et al.* (1990) reported the release of NO from dog ileocolonic junction following NANC nerve stimulation and used this to support the view that NO is the NANC transmitter. However, as pointed out by Ignarro *et al.* (1990b), the detected NO could equally be coming from other sources having been generated or liberated by the actual transmitter. Even in the case of EDRF there have been recent reports that some nitroso compounds more closely mimic the properties of EDRF than does NO (Graser *et al.*, 1990; Myers *et al.*, 1990; Rubanyi *et al.*, 1990), and it may be that a similar substance is liberated from the NANC nerves. Several physiologically-relevant S-nitrosothiols have recently been shown to relax smooth muscle (Kowaluk & Fung, 1990), although interpretation of results is complicated by their differing abilities to liberate free NO in solution. Nevertheless, these and other S-nitrosothiols are clearly of interest in terms of the NANC transmitter and require closer investigation; so too does hydroxylamine, which has been proposed as a possible intermediate in the generation of NO from L-arginine (DeMaster *et al.*, 1989) and which, like NANC stimulation, was resistant to hydroquinone. Of course, the possibility that free NO is the transmitter, and that there is some other reason for the differential effect of hydroquinone, cannot be dismissed; until such a reason is found the precise nature of the NANC transmitter remains in doubt.

It has been suggested that hydroquinone inhibits EDRF/NO relaxations of vascular smooth muscle by acting either as a free radical scavenger (Griffith *et al.*, 1984) or as a generator of superoxide anions (Moncada *et al.*, 1986). Our observations with SOD and with the chemiluminescent assay suggest that superoxide anion generation cannot explain the inhibitory effect of hydroquinone on NO in the present study. If hydroquinone was acting as a superoxide anion generator its inhibitory effects on NO should have been blunted by SOD. However, the inhibitory potency of hydroquinone was unchanged in the presence of SOD; indeed, superoxide anions do not seem to play an important role in our system since SOD had no effect on relaxations induced by NO or NANC stimulation. This was confirmed by the chemiluminescent studies in which no superoxide anion chemiluminescence was present under control conditions and, further, no superoxide anion generation was induced by hydroquinone. The assay was capable of detecting superoxide anions since concentration-related increases in chemiluminescence were obtained with xanthine:xanthine oxidase, an effect prevented by SOD. However, while hydroquinone did not generate superoxide anions it acted like SOD, and reduced the chemiluminescent response to xanthine:xanthine oxidase, which would be consistent with it acting as a free-radical scavenger. The lack of effect of SOD on the NANC relaxation is similar to the observations of Toda & Okamura (1990) on cerebral arteries; again, these authors suggested the substance released by the NANC nerves may be an NO-related compound that is resistant to superoxide anions, rather than NO itself.

In conclusion, hydroquinone blocks relaxations induced by NO, but not NANC nerve stimulation or other nitrovasodilator drugs, in three tissues in which NANC relaxations involve an endogenous nitrate. Free radical scavenging, rather than superoxide anion generation, appears to be the mecha-

nism of action of hydroquinone. In the absence of other satisfactory explanations, the differential effect of hydroquinone on NO- and NANC-induced relaxations may suggest that the transmitter released by the NANC nerves is not free NO, but may be an NO-containing, or NO-generating, substance (Gillespie & Sheng, 1989).

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Dicentrine, a natural vascular α_1-adrenoceptor antagonist, isolated from *Lindera megaphylla*

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1 The pharmacological activity of dicentrine, isolated from *Lindera megaphylla*, was determind in rat isolated thoracic aorta, guinea-pig isolated trachea and human platelet-rich plasma.

2 Dicentrine was found to be a potent α_1-adrenoceptor blocking agent in rat thoracic aorta as revealed by its competitive antagonism of noradrenaline- ($pA_2 = 8.19 \pm 0.09$) or phenylephrine ($pA_2 = 9.01 \pm 0.10$)-induced vasoconstriction. These effects still persisted in denuded aorta. It was less potent than prazosin ($pA_2 = 10.60 \pm 0.10$), but was more potent than phentolamine ($pA_2 = 7.53 \pm 0.10$) or yohimbine ($pA_2 = 6.20 \pm 0.05$).

3 Inositol monophosphate formation induced by noradrenaline (3 μM) in rat thoracic aorta was suppressed by dicentrine (3–10 μM) and prazosin (3 μM).

4 A high concentration of dicentrine (30 μM) did not affect the aortic contraction induced by the thromboxane receptor agonist U-46619 (1 μM), angiotensin II (1 μM), high potassium (60 mM) or carbachol (3 μM).

5 Contraction of guinea-pig trachea caused by histamine or carbachol was slightly inhibited by dicentrine (30 μM), while β-adrenoceptor relaxation to isoprenaline in trachea was not affected.

6 Aggregation in human platelet-rich plasma induced by adrenaline (10 μM) was blocked by yohimbine (5 μM). A high concentration of dicentrine (>30 μM) caused slight inhibition of aggregation, the release reaction and thromboxane formation. Complete blockade was obtained with 150 μM dicentrine.

7 It is concluded that dicentrine is a potent, selective α_1-adrenoceptor antagonist in vascular smooth muscle.

Keywords: α_1-Adrenoceptor antagonist; dicentrine; prazosin; rat thoracic aorta; vascular smooth muscle; *Lindera megaphylla*

Introduction

Medicinal plants have been used as traditional remedies in oriental countries over hundreds of years. In a large scale screening test, we have found many biologically active compounds isolated from plant sources. Some of them inhibited the contraction of aortic smooth muscles. For example, magnolol (isolated from *Magnolia officinalis*) causes vasorelaxation of rat aorta by releasing endothelium-derived relaxing factor (EDRF) (Teng *et al.*, 1990a); two vasorelaxants, denudatin B and fargesone B (isolated from *Magnolia fargesii*) block the voltage-dependent calcium channel (Yu *et al.*, 1990; Teng *et al.*, 1990b); norathyriol (isolated from Gentianaceae spp.) is also a calcium-channel blocker (Ko *et al.*, 1991).

Recently, we found dicentrine, an aporphine derivative (Figure 1) isolated from *Lindera megaphylla*, possessed antiplatelet activity (Chen *et al.*, 1991). It inhibited platelet aggregation and release reaction through the suppression of thromboxane formation and elevation of adenosine 3':5'-cyclic monophosphate (Yu *et al.*, 1991). In the present study, we show that dicentrine has a strong vasorelaxant action when tested against noradrenaline-induced contraction of rat thoracic aorta and have investigated the mechanism of this activity.

Methods

Rat aortic contraction

Wistar rats of either sex weighing 250 to 300 g were killed by a blow to the head. The thoracic aorta was isolated and excess fat and connective tissue were removed. Vessels were cut into rings of about 5 mm in length and mounted in organ baths containing 5 ml Krebs solution of the following composition (mM): NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.7, $CaCl_2$ 1.9 and $NaHCO_3$ 25.0. The tissue bath solution was maintained at 37°C and gassed with 95% O_2 plus 5% CO_2. Two stainless steel hooks were inserted into the aortic lumen, one was fixed while the other was connected to a transducer. Aortae were equilibrated in the medium for 90 min with three changes of Krebs solution and maintained under an optimal tension of 1 g before specific experimental protocols were initiated. Contractions were recorded isometrically via a force-displacement transducer connected to a Grass polygraph. In some experiments, the endothelium was removed by rubbing with a cotton ball and the absence of acetylcholine-induced relaxation was taken as an indicator that vessels were denuded successfully. Aortae were allowed to

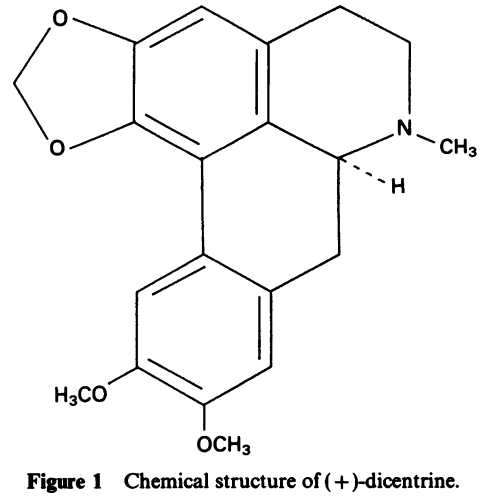


Figure 1 Chemical structure of (+)-dicentrine.

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preincubate for 15 min with dicentrine before generating the cumulative concentration-response curve with each agonist for 15–30 min at 3 min intervals. Results are expressed as percentage of the maximal control response for each agonist.

Guinea-pig tracheal contraction

Tracheae from guinea-pigs were dissected out, transferred to a dish containing Krebs solution and cut transversely between the segments of cartilage. Several of these, usually about 5, were tied together so as to form a chain, which was then mounted in Krebs solution at 37°C, gassed with 95% O_2 plus 5% CO_2. One end of the chain was attached to a fixed pin in the bath and the other to a force-displacement transducer connected to a Grass polygraph. Resting tension on each tissue was set at 1 g. Tracheae were allowed to equilibrate for at least 1 h and washed periodically. Cumulative concentration-response curves were obtained by application of various concentrations of spasmogens for 15–18 min at 3 min intervals. Responses were found to be reproducible with this procedure. Tracheal rings were preincubated with dicentrine (30 μM) for 15 min, then various concentrations of spasmogens were added for 15–18 min at 3 min intervals. Results are expressed as percentage of the maximal control response for each agonist.

Measurement of [^{3}H]-inositol monophosphate

The same procedure as described by Hirata *et al.* (1990) was used. Briefly, rat thoracic aortae were exposed to Krebs solution containing 10 μCi ml^{-1} of [^{3}H]-myo-inositol for 3 h and gassed with 95% O_2 plus 5% CO_2 mixture. Tissues were then transferred to tubes containing fresh Krebs solution with dimethylsulphoxide (DMSO) or dicentrine and incubated for 15 min. Saline or noradrenaline (3 μM) was added and the tubes incubated for another 15 min. LiCl (10 mM) was added 5 min before noradrenaline to inhibit metabolism of inositol monophosphates (Berridge *et al.*, 1982). Aortae were then frozen in liquid nitrogen and homogenized in 1.3 ml of 10% trichloroacetic acid. After centrifugation, 1 ml of supernatant was collected and trichloroacetic acid was removed by washing with 4 × 3 vol of ether. The inositol monophosphate in the aqueous phase was analyzed by application of the sample to a column of 1 ml Dowex-1 ion-exchange resin according to the method of Neylon & Summers (1987). The pellets of the tissues were resuspended in 1.0 N NaOH and assayed for protein according to the method of Lowry *et al.* (1951).

Human platelet aggregation

Platelet-rich plasma (PRP) was obtained from blood collected from healthy individuals with sodium citrate (3.8%, 1:14) added as anticoagulant, and centrifuged for 10 min at 90 *g*. Three min before the addition of the aggregation inducer, PRP was preincubated with dicentrine (15–150 μM), indomethacin or yohimbine and stirred at 1200 r.p.m. The absorbance of PRP was taken as 0% aggregation and the absorbance of platelet-poor plasma as 100% aggregation. Adenosine 5'-triphosphate (ATP) released from platelets was detected by the bioluminescence method (DeLuca & McElory, 1978). ATP at a known concentration was used to calibrate the intensity of bioluminescence. Both the aggregation and release of ATP were simultaneously measured by a Lumi-aggregometer (Chrono-Log Co. U.S.A.) connected to a dual channel recorder. In order to eliminate the effect of the solvent on aggregation, the final concentration of DMSO was fixed at 0.5%.

Data analysis

In each experiment, agonist dose-response curves in the presence of dicentrine were related to the control dose-response curve, of which the maximum response was taken as 100%. In most experiments, three or four concentrations of dicentrine were tested and the slopes of the resulting Schild plots were used to assess competitive antagonism. The pA_2 values were calculated for each concentration of dicentrine according to: $pA_2 = -\log([\text{antagonist}]/[\text{dose-ratio} - 1])$ (Mackay, 1978).

The experimental results are expressed as the mean ± s.e.mean and accompanied by the number of observations. Statistical significance was assessed by Student's *t* test and *P* values less than 0.05 were considered significant.

Drugs

(+)-Dicentrine (Figure 1) was prepared according to the method described previously by us (Chen *et al.*, 1991). The following drugs were used: noradrenaline, yohimbine, prazosin, phentolamine, U-46619 (9,11-dideoxy methanoepoxy-9α, 11α-prostaglandin $F_{2\alpha}$), angiotensin II, carbachol, *myo*-inositol, trichloroacetic acid and Dowex-1 (100–200 mesh: × 8 chloride) were obtained from Sigma Chemical Co.; phenylephrine was from Denmarks Apotekerforening and *myo*-[2-^{3}H] inositol was purchased from Amersham. If drugs were dissolved in DMSO, the final concentration of DMSO in the bathing solution did not exceed 0.1% and had no effect on the muscle contraction.

Results

α_1-Adrenoceptor antagonism in rat aorta

In rat thoracic aorta, noradrenaline (3 μM) caused a phasic contraction and then a tonic contraction (1.7 ± 0.1 g) maintained for at least 30 min. Pretreatment of aorta with dicentrine (1–10 μM) for 15 min inhibited this contraction in a concentration-dependent manner (Figure 2a). Phenylephrine (100 μM) gave a similar tracing to noradrenaline but with a lesser tension (1.4 ± 0.1 g). Dicentrine (1–3 μM) also showed potent inhibition, while yohimbine (3 μM) did not affect significantly phenylephrine-induced contraction (Figure 2b).

Dicentrine was then evaluated against a concentration-response curve to noradrenaline. Dicentrine (0.3–10 μM) produced a parallel, rightward shift of the curve consistent with competitive blockade (Figure 3a). This concentration-related shift by dicentrine was also observed, without significant diminution, in rat thoracic aorta in which the endothelium had been removed (Figure 3b). The maximal contraction induced by noradrenaline was a little higher than that obtained by phenylephrine, and maximal tension was increased in denuded aortae when the contraction was induced by either noradrenaline or phenylephrine (Table 1). The EC_{50} of noradrenaline

Table 1 Comparison of the responses of rat aorta to α_1-adrenoceptor agonists in the presence and absence of endothelium

Agonist	*Endothelium*	n[a]	E_{max} (g)[b]	EC_{50} (× 10^{-7} M)	*Relative potency*[c]
Noradrenaline	present	32	1.70 ± 0.12	2.70 ± 0.50	0.60
	absent	32	2.09 ± 0.13	1.80 ± 0.13	1.00
Phenylephrine	present	51	1.45 ± 0.15	7.00 ± 0.10	0.26
	absent	51	1.73 ± 0.14	4.01 ± 0.14	0.45

[a] Number of experiments; [b] maximum developed tension (means ± s.e.mean); [c] relative potency calculated with reference to the EC_{50} of noradrenaline (endothelium absent).

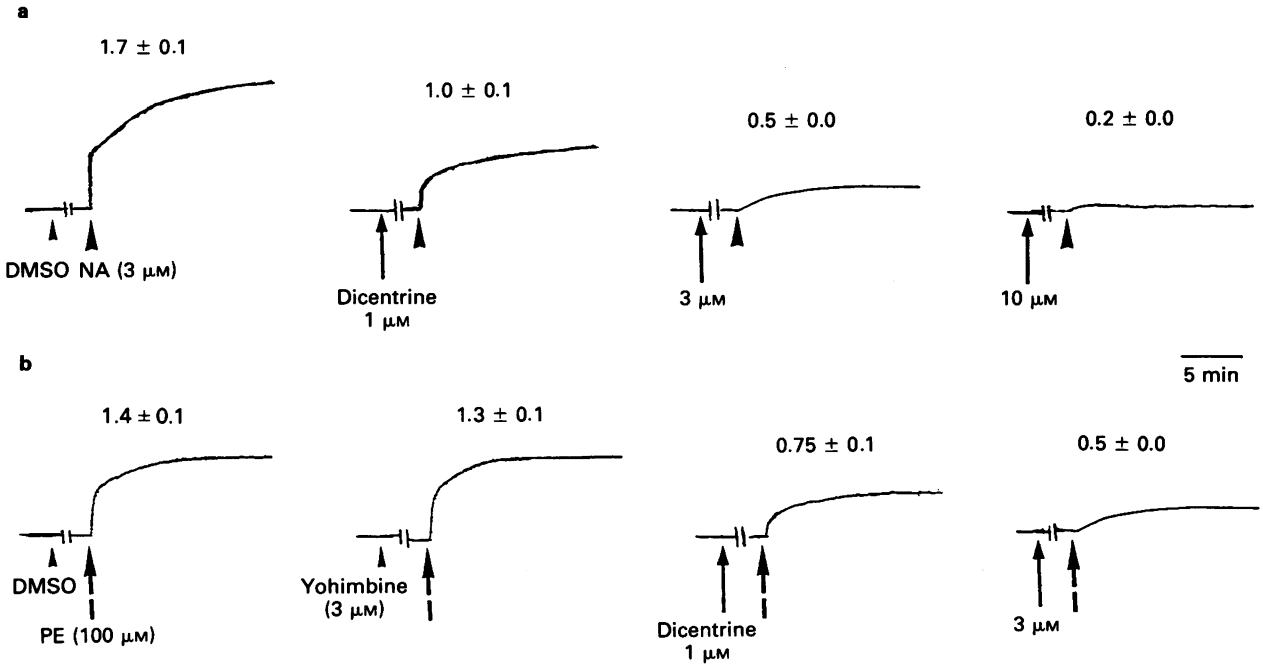


Figure 2 Typical tracings of dicentrine inhibition on the contraction of rat thoracic aorta induced by noradrenaline and phenylephrine. Dicentrine (1, 3, 10 μM), yohimbine (3 μM) or dimethylsulphoxide (DMSO) (0.1%, control) was incubated with aorta for 15 min, then noradrenaline (NA, 3 μM, a) or phenylephrine (PE, 100 μM, b) was added to trigger the contraction. Data (means ± s.e.mean) shown above the tracings are the contractile force (g) developed by aorta at 15 min after the addition of agonists.

was lower than that of phenylephrine, and was also lower in denuded aortae (Table 1).

Concentration-response curve analysis of phenylephrine-induced contraction of rat aortae was performed for the α_1-adrenoceptor antagonism by dicentrine in comparison with prazosin, yohimbine and phentolamine (Figure 4). The pA_2 of dicentrine against noradrenaline and phenylephrine was 8.19 ± 0.09 (slope 0.85–1.20) and 9.01 ± 0.10 (slope 0.95–1.32), respectively. Dicentrine was less potent than the α_1-adrenoceptor blocker prazosin ($pA_2 = 10.60 \pm 0.10$) against phenylephrine but was much more potent than phentolamine ($pA_2 = 7.53 \pm 0.10$) or yohimbine ($pA_2 = 6.20 \pm 0.05$), which is known as an α_2-adrenoceptor blocking agent. In all cases the Schild slopes were not significantly different from 1.0.

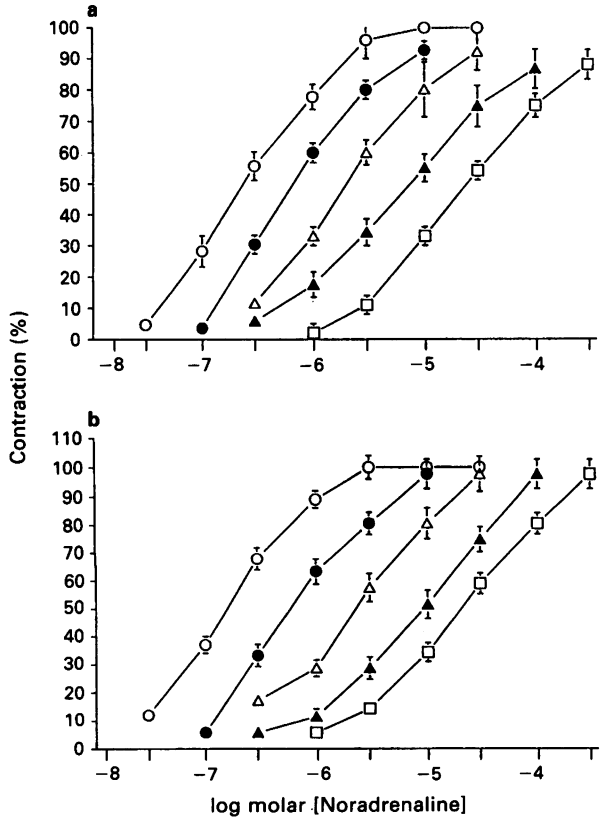


Figure 3 Cumulative concentration-response curves to noradrenaline in rat thoracic aorta with intact (a) or without (b) endothelium. Dimethylsulphoxide (0.1%, control) (○) or dicentrine, 0.3 μM (●), 1.0 μM (△), 3.0 μM (▲) and 10 μM (□) were preincubated with aorta for 15 min. Each point represents the mean and vertical bars show s.e. mean ($n = 6$).

To see if signal transduction after α_1-adrenoceptor activation was blocked by dicentrine, rat thoracic aortae were labelled with [^{3}H]-*myo*-inositol. The accumulation of inositol monophosphate in rat aortae was increased in the presence of noradrenaline (3 μM). This increase was significantly suppressed by prazosin (3 μM) or dicentrine (3 and 10 μM) (Table 2).

A high concentration of dicentrine (30 μM) was used to test its antagonism against contraction of rat thoracic aorta caused by vasoactive agents other than adrenoceptor agonists. Tension of aorta was developed by U-46619 (1 μM), angiotensin II (1 μM), KCl (60 mM) or carbachol (3 μM). Dicentrine (30 μM) did not suppress the increase in tension by these vasoactive agents (not shown).

Antagonism against histamine, carbachol and isoprenaline in guinea-pig trachea

In guinea-pig trachea, histamine and carbachol caused contraction of tracheal smooth muscle. Dicentrine (30 μM) produced a slight depression of both concentration-response curves (Figure 5a,b). Dicentrine (30 μM) had no effect on the isoprenaline-induced relaxation of guinea-pig trachea precontracted by carbachol (1 μM) (Figure 5c).

Antiaggregation in human platelet-rich plasma

Adrenaline (10 μM) can induce biphasic aggregation in human platelet-rich plasma. The primary aggregation is mediated through the activation of α_2-adrenoceptors (Hsu *et al.*, 1979), the secondary aggregation is accompanied by the release reac-

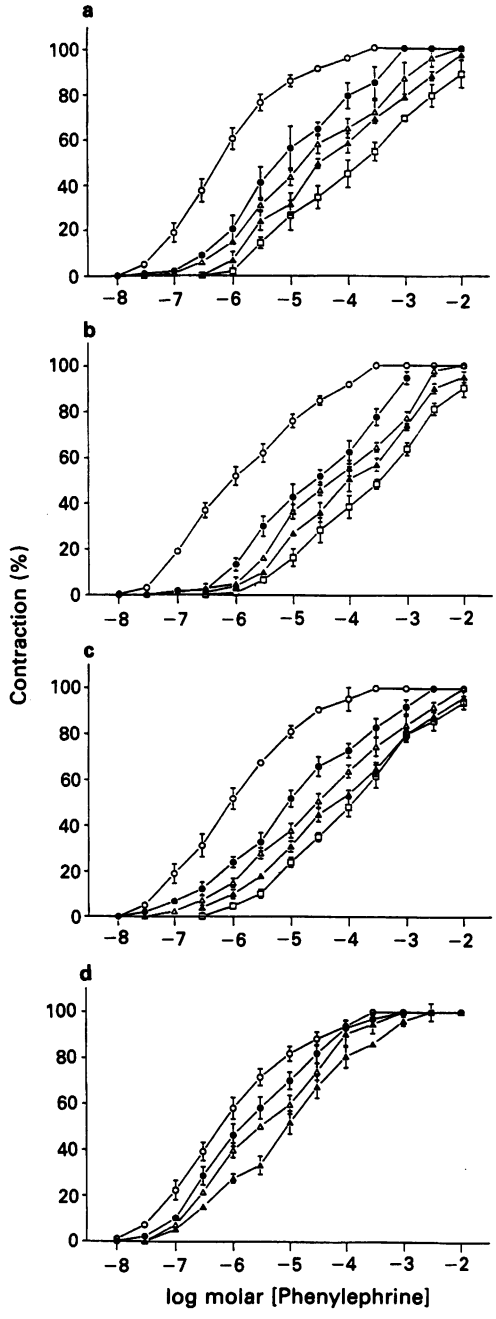


Figure 4 Antagonism of the concentration-response curves to phenylephrine by 15 min pretreatment of denuded aorta with (a) prazosin (●, 0.3 nM; △, 1 nM; ▲, 3 nM; □, 10 nM), (b) dicentrine, (c) phentolamine (●, 0.3 μM; △, 1 μM; ▲, 3 μM; □, 10 μM) and (d) yohimbine (●, 1 μM; △, 3 μM; ▲, 10 μM) or dimethylsulphoxide (0.1%, control) (○). Each point represents the mean and vertical bars show s.e.mean ($n = 6$).

tion and also formation of thromboxanes. As shown in Figure 6, the α_2-adrenoceptor blocker yohimbine (5 μM) (Motulsky *et al.*, 1980) blocked completely the aggregation and ATP release. Dicentrine caused partial inhibition of the adrenaline-induced response of human platelet-rich plasma only at the high concentration of 30 μM; complete blockade was obtained with 150 μM of dicentrine.

Discussion

The present experiments have demonstrated that dicentrine, an antiplatelet agent isolated from *Lindera megaphylla*, depressed the contractile responses of rat aorta to the adrenoceptor agonist, noradrenaline, or the more selective

Table 2 Effects of prazosin and dicentrine on the accumulation of inositol monophosphate in rat aortae by noradrenaline

Treatment	*Inositol monophosphate* (c.p.m. mg^{-1} protein)
Resting	1651 ± 284
Noradrenaline (3 μM)	2463 ± 23
Prazosin (3 μM)	1801 ± 263*
Dicentrine (3 μM)	1984 ± 180*
Dicentrine (10 μM)	1667 ± 241***

Rat aortic segments were preincubated with prazosin (3 μM), dicentrine (3, 10 μM) or dimethylsulphoxide (DMSO, 0.1%, control) for 15 min, followed by the stimulation with noradrenaline (3 μM) for 15 min. Data are represented as total inositol monophosphates accumulated (c.p.m. mg^{-1} protein) and expressed as means ± s.e.mean. * $P < 0.05$; *** $P < 0.001$ compared with the control value.

α_1-adrenoceptor stimulant, phenylephrine. It acts as a selective α_1-adrenoceptor antagonist without affecting the contraction of rat aorta caused by the thromboxane receptor agonist (U-46619), angiotensin II, the muscarinic receptor agonist

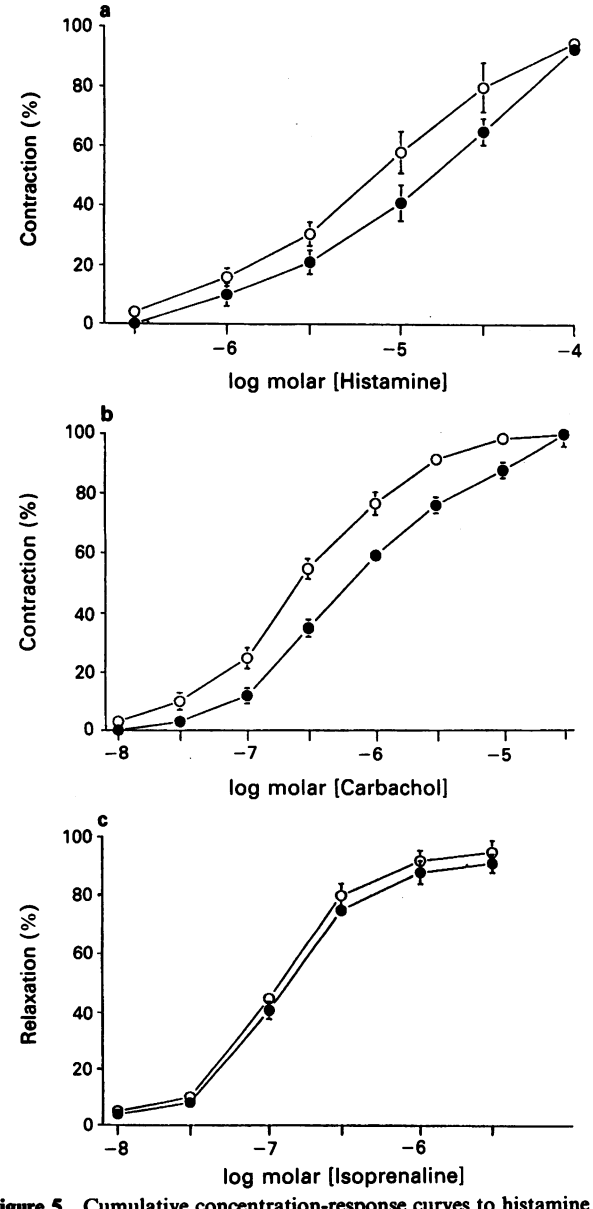


Figure 5 Cumulative concentration-response curves to histamine (a), carbachol (b) and isoprenaline (c) in the absence (○) or presence (●) of dicentrine (30 μM) in guinea-pig trachea. Each point represents the mean and vertical bars show s.e.mean ($n = 6$).

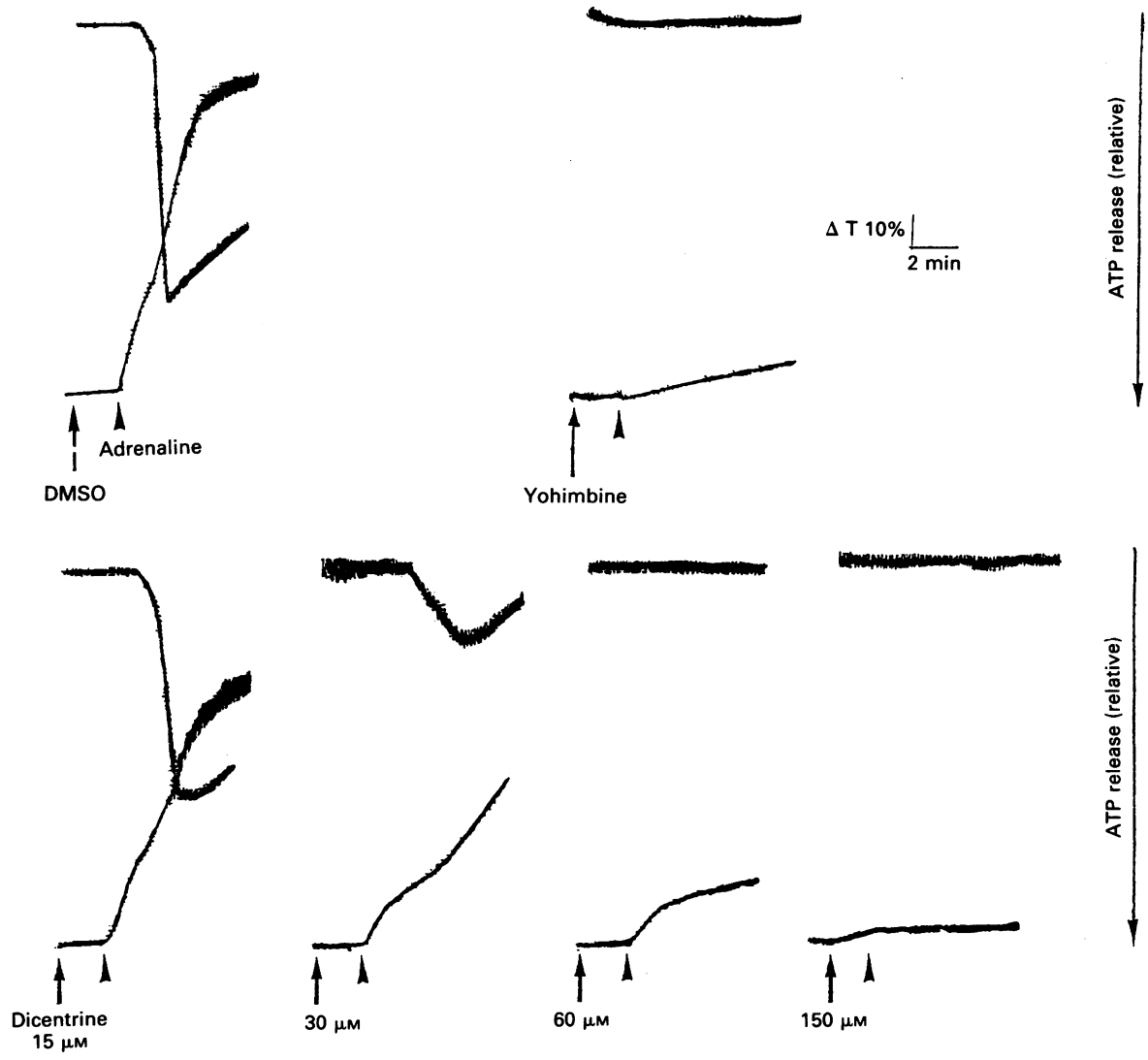


Figure 6 Effect of dicentrine on platelet aggregation and ATP release from platelet-rich plasma induced by adrenaline. Platelet-rich plasma was preincubated with yohimbine (5 μM), dicentrine of various concentrations or dimethylsulphoxide (DMSO, 0.5%) for 3 min, then adrenaline (10 μM) was added to trigger the aggregation (upward tracings) and ATP release (downward tracings).

(carbachol) or high-potassium depolarization. It also had no apparent effects on β-adrenoceptors, histamine and muscarinic receptors in guinea-pig trachea. Comparison of the α-adrenoceptor antagonism of dicentrine with prazosin (α_1-subtype) and yohimbine (α_2-subtype) in rat aorta and human platelets clearly showed that dicentrine is a selective α_1-adrenoceptor blocker with a pA_2 of 8.19 and 9.01 for competitive antagonism of noradrenaline and phenylephrine, respectively. It is about 40 times less potent than prazosin, but 1000 times more potent than yohimbine and 40 times more potent than phentolamine in phenylephrine-induced contraction of rat aorta.

It is increasingly clear that α_1-adrenoceptors are not pharmacologically homogeneous. Evidence from radioligand binding studies, functional studies of smooth muscle contractile responses, and studies of receptor-mediated second messenger accumulation shows substantial differences in the affinities of antagonists for α_1-adrenoceptors in different tissues and may explain the difference in the K_D values for dicentrine against noradrenaline and phenylephrine in rat aorta.

Vascular endothelium is a very important part of blood vessels. It responds to a variety of neurochemical and physical stimuli to release endothelium-dependent relaxing factor (EDRF) and prostacyclin (PGI_2) (Jaffe, 1985; Vanhoutte *et al.*, 1986). Endothelium modulates the vasoconstrictor responses to many agonists, and EDRF is mainly responsible for these effects (Egleme *et al.*, 1984; Palmer *et al.*, 1987). The relaxant action of dicentrine persisted in denuded aorta. Thus, the vasorelaxation caused by dicentrine was independent of the endothelium and was not mediated by either EDRF or PGI_2. Analysis of the concentration-response curves of dicentrine revealed that competitive antagonism against noradrenaline occurred in aorta in the presence or absence of endothelium. It has been reported that prazosin acts as a non-competitive antagonist against noradrenaline or phenylephrine in the presence of endothelium and as a competitive antagonist in the absence of endothelium (Alosachie & Godfraind, 1986; 1988). Thus vascular endothelium modifies the antagonist action of prazosin but not that of dicentrine. On the other hand, endothelium also modulates the contractile response to α-adrenoceptor agonists. The EC_{50} of noradrenaline or phenylephrine was lower in denuded aorta (Table 1). Similar observations were also reported by Alosachie & Godfraind (1988). These authors suggested that this effect of endothelium was related to a change in agonist efficacy and receptor reserve.

In conclusion, dicentrine is a novel vascular α_1-adrenoceptor blocking agent isolated from a plant source, it also shows antiplatelet action at high concentrations.

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Delineation of three pharmacological subtypes of α_2-adrenoceptor in the rat kidney

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1 Simultaneous computer modelling of plain and ARC 239- and guanoxabenz-masked [^{3}H]-RX821002 saturation curves, plain ARC 239 and guanoxabenz competition curves as well as ARC 239-masked guanoxabenz competition curves revealed that the drugs bound to three α_2-adrenoceptor subtypes in the rat kidney with grossly differing selectivities. These α_2-adrenoceptor subtypes were termed α_{2A}, α_{2B1} and α_{2B2}. The order of affinities for [^{3}H]-RX821002 for the adrenoceptor sites was $\alpha_{2A} > \alpha_{2B1} > \alpha_{2B2}$, the K_ds being 0.62 ± 0.05, 2.52 ± 0.11 and 6.74 ± 1.21 nM, respectively. The order of affinities for ARC 239 was $\alpha_{2B1} > \alpha_{2B2} \gg \alpha_{2A}$ with K_ds 4.78 ± 1.04, 28.8 ± 4.1 and 1460 ± 270 nM, respectively. For guanoxabenz the order of affinities was $\alpha_{2A} > \alpha_{2B1} \gg \alpha_{2B2}$ with K_ds 99.7 ± 15.1, 508 ± 135 and 25,400 ± 2400 nM, respectively.

2 Binding constants for 14 compounds for the three rat kidney α_2-adrenoceptor subtypes were determined by the simultaneous computer modelling of plain and ARC 239- and guanoxabenz-masked drug competition curves, plain ARC 239 and guanoxabenz competition curves as well as ARC 239-masked guanoxabenz competition curves. Of the 14 compounds tested, oxymetazoline and guanfacine were found to bind with low affinities to both of the α_{2B1}- and α_{2B2}-adrenoceptors but with high affinity to the α_{2A}-adrenoceptor. Prazosin instead bound with high affinities to the α_{2B1}- and α_{2B2}-adrenoceptors but with low affinity to the α_{2A}-adrenoceptor. By contrast, guanoxabenz and ARC 239 delineated clearly between all the three α_2-adrenoceptor subtypes. Notably the affinities of guanoxabenz for α_{2B1}- and α_{2B2}-adrenoceptors differed 72 fold and for α_{2A}- and α_{2B2}-adrenoceptors 380 fold. The selectivities of a number of other drugs were less marked but their K_ds were consistent with all three sites being α_2-adrenoceptors.

3 (−)-Adrenaline and (−)-noradrenaline showed dissimilar order of affinities for the three α_2-adrenoceptors. For (−)-adrenaline the order of affinities was $\alpha_{2B1} \geqslant \alpha_{2A} > \alpha_{2B2}$ and for (−)-noradrenaline $\alpha_{2B2} \geqslant \alpha_{2B1} > \alpha_{2A}$. All three α_2-adrenoceptors showed the expected stereoselective binding for adrenaline enantiomers, the (+)-form being 7–10 fold less potent than the (−)-form.

4 [^{3}H]-yohimbine was also used as radioligand. The data with this ligand were fully compatible with the [^{3}H]-RX821002 data. However, [^{3}H]-yohimbine appeared to label only α_{2B1}- and α_{2B2}-adrenoceptors presumably because it had too low an affinity for α_{2A}-adrenoceptors.

5 We conclude that three pharmacological subtypes of α_2-adrenoceptors are labelled by [^{3}H]-RX821002 in the rat kidney. Guanoxabenz and ARC 239 may be used in competition studies to delineate between these three α_2-adrenoceptor subtypes. Moreoever, we here present a method allowing the determination of binding constants for an arbitrary drug to the three α_2-adrenoceptor subtypes.

Keywords: α_2-adrenoceptor subtypes; rat kidney; receptor classification; [^{3}H]-RX821002 ligand binding; computer modelling

Introduction

Recent advances in molecular biology indicate that the effects of adrenaline and noradrenaline released from the adrenal glands and the sympathetic nerve terminals are mediated by multiple subtypes of structurally similar receptors. Using pharmacological criteria these receptors were originally divided into two classes called α and β (Ahlquist, 1948). Subsequent studies then indicated that these receptors consisted of at least four classes for which the names α_1 and α_2 (see Berthelsen & Pettinger, 1977; Wikberg, 1978) and β_1 and β_2 (Lands *et al.*, 1967) were used. More recently the molecular cloning of the genes for adrenoceptors have indicated that there exist at least two α_1-, three α_2- and three distinct β-adrenoceptor genes in mammals which code for receptors with differing pharmacological properties (Dixon *et al.*, 1986; Firelle *et al.*, 1987; Kobilka *et al.*, 1987a,b; Cotecchia *et al.*, 1988; Regan *et al.*, 1988; Emorine *et al.*, 1989; Schwinn *et al.*, 1990; Lomasney *et al.*, 1990; Chalberg *et al.*, 1990; Zeng *et al.*, 1990; Voigt *et al.*, 1991). However, although radioligand binding techniques and other functional methods have indicated the presence of multiple receptors of the α_2-type (Maura *et al.*, 1985; Bylund, 1985; 1988; Adams *et al.*, 1986; Ruffolo *et al.*, 1987; Michel *et al.*, 1989a; Uhlén *et al.*, 1990) it has not yet been possible to merge the molecular biological data with the functional into a unifying hypothesis as to the number of distinct α_2-adrenoceptor subtypes (Lomasney *et al.*, 1990). Some reasons have been the lack of suitable drugs to delineate between the receptors and the presence of species differences in the structure and ligand binding properties of the receptors, making the comparison of results from different studies difficult. In a previous study we reported that the α_2-adrenoceptor antagonist ligand [^{3}H]-RX821002 was useful to label α_{2A}- and α_{2B}-adrenoceptors in the rat kidney (Uhlén & Wikberg, 1991a). In the present study we show that radioligand binding using [^{3}H]-RX821002 in conjunction with combinations of suitable competing drugs as well as simultaneous computer analysis of multiple saturation and competition curves can further subdivide the α_{2B}-adrenoceptors in the rat kidney into two pharmacological subtypes of α_2-adrenoceptors termed α_{2B1} and α_{2B2}.

Methods

Membrane preparation

Male Sprague-Dawley rats weighing 200–300 g were decapitated and their kidneys rapidly excised and homogenized in ice-cold 50 mM Tris-HCl, 5 mM EDTA, 0.1 mM PMSF (phenyl methyl sulphonyl fluoride), 10 μg ml^{-1} soybean trypsin inhibitor and 200 μg ml^{-1} bacitracin, pH 7.5, with a motordriven

teflon glass homogenizer. The homogenates were spun at 500 *g* and the supernatants collected and spun at 38,000 *g* for 30 min. The pellets were then washed once with the same buffer and then once again with a similar buffer containing a lower concentration of EDTA (1.5 mM). The final pellets were diluted to protein concentrations of about 2.4 mg protein ml^{-1} with 1.5 mM EDTA, 50 mM Tris-HCl pH 7.5. Aliquots of the membranes were frozen and stored at 80°C until used. Protein was determined according to Lowry *et al.* (1951).

Binding studies

Radioligand binding was performed by incubation of about 240 μg of the membranes in 150 μl of 1 mM EDTA, 100 μM Gpp(NH)p (guanyl-5'-yl-imido-diphosphate), 140 mM NaCl, 33 mM Tris-HCl, pH 7.5 with [^{3}H]-RX821002 or [^{3}H]-yohimbine and drugs, as indicated, for 1 h at 25°C. Termination of the assays was performed by filtering and washing on Whatman GF/C fibre glass filterpaper with 20 ml ice-cold 50 mM Tris-HCl, pH 7.5, using a cell harvester (Brandel, Gaithersburg, MD, U.S.A.). The filters were then counted in a Beckman LS 1801 β-counter each with 3.5 ml of Ready Protein scintillation cocktail (Beckman Instruments). The counting efficiency was determined by use of an external standard and the Beckman Instruments H-number method. Assays were performed in duplicates.

Statistical methods

Computer modelling of radioligand binding data was performed essentially as described (De Lean *et al.*, 1982; Bergström & Wikberg, 1986) by using programmes coded by us suitable for the Macintosh and IBM-PC computers. In brief the experimental data were fitted into the equation:

$$B_i = \sum_{b=1}^{n} \left(K_{ib} F_i R_b \Big/ \left(1 + \sum_{a=1}^{m} K_{ab} F_a \right) \right) + N_i F_i \qquad (1)$$

which assumes that ligands bound reversibly to independent sites according to the law of mass action by using Marquardts algorithm for non-linear regression analysis (Marquardt, 1963). In the formula, B_i is the concentration of ligand 'i' bound to the membranes, K_{ib} and K_{ab} the affinity constants for ligand 'i' and 'a' for receptor 'b', respectively, F_i and F_a the free concentrations of ligands 'i' and 'a', respectively, R_b the concentration of receptor site 'b', and N_i the constant ratio of bound/free (i.e. 'non-specific binding') for ligand 'i'. The free concentrations of ligands were estimated numerically from their total concentrations, as described by De Lean *et al.* (1982). Weighting of residuals were performed as described (De Lean *et al.*, 1982). In the calculations all of the curves of a separate experiment were simultaneously fitted by setting the appropriate values for 'n' (number of sites) and 'm' (number of ligands) and arranging the data fed to the computer algorithm appropriately. Moreover, for each set of data several separate fits were performed assuming that one or several sites were present. An F-test comparing the sums of squares for the different models (see De Lean *et al.*, 1982) was then used to judge which model approximated the data the best. A significant improvement of a regression model was assumed when $P < 0.05$. In the following this procedure to select the best model will be referred to as computer modelling. Binding constants of competing drugs are given as dissociation constants (K_ds) and were calculated from the reciprocal of their affinity constants calculated according to eq. (1).

Isotopes, drugs and chemicals

[^{3}H]-RX821002 [1,4-(6,7(n)-^{3}H]benzo-dioxan-2-methoxy-2-yl)-2-imidazoline; 51 Ci $mmol^{-1}$) and [^{3}H]-yohimbine (89 Ci $mmol^{-1}$) was from Amersham; (−)-adrenaline, chlorpromazine, corynanthine, dopamine, (−)-noradrenaline, prazosin and yohimbine were from Sigma Chemical Co.; (+)-adrenaline was from Sterling-Winthrop Res. Inst., Rensselaer, N.Y., U.S.A.; ARC 239 2-(2,4-(O-methoxyphenyl)-piperazin-1-yl)-ethyl-4,4-dimethyl-1,3-(2H,4H)-isoquinolindione) from Thomae, Biberach, Germany; BDF 8933 (4-fluoro-2-(imidzoline-2-ylamino)-isoindoline) from Beiersdorf AG, Hamburg, Germany; guanoxabenz from Roussel, Romainville, France; oxymetazoline from Draco, Lund, Sweden; rauwolscine from Carl Roth, Karlsruhe, Germany; rilmenidine from Servier, Gidy, France; WB 4101 from Research Biochemicals Inc., Natick, MA, U.S.A.; guanfacine was a kind gift from Dr. Claes Post, Astra, Södertälje, Sweden. All other chemicals were purchased from Merck or Sigma and were of analytical quality.

Results

Delineation of three putative α_2-adrenoceptor binding sites in rat kidney by use of [^{3}H]-RX821002 radioligand binding

In these experiments saturation curves of [^{3}H]-RX821002 were obtained by adding various concentrations of the tritiated ligand in the absence and presence of fixed concentrations of ARC 239 (0.2 μM), guanoxabenz (6 μM) and BDF 8933 (1 μM) to rat kidney membranes. In the same experiment full competition curves were also obtained by adding various concentrations of ARC 239 or guanoxabenz in the presence of a fixed concentration of [^{3}H]-RX821002 (~2 nM). Moreover, a competition curve for guanoxabenz was also obtained for which fixed concentrations of ARC 239 (0.2 μM) and [^{3}H]-RX821002 (~2 nM) has been included, as exemplified in Figure 1 and summarized for six separate experiments in Table 1. All data of each experiment were subjected to simultaneous computer modelling assuming that ligands bound reversibly to independent sites. For all six separate experiments this analysis showed that fitting the data into a model that assumed that ligands bound to three sites resulted in highly significant ($P \ll 0.001$ for all six tests) reductions in the sums of squares, as compared to fitting the data to a model that assumed that two sites were present. Since models that assumed four sites to be present gave no further reductions in the sums of squares it was assumed that a three site model approximated the data the best. The three sites were termed α_{2A}, α_{2B1} and α_{2B2} (Table 1). As seen from the table, [^{3}H]-RX821002 bound with non-uniform affinity to the three sites, the order of affinities being $\alpha_{2A} > \alpha_{2B1} > \alpha_{2B2}$. The order of affinities for ARC 239 was instead $\alpha_{2B1} > \alpha_{2B2} \gg \alpha_{2A}$ and for guanoxabenz $\alpha_{2A} > \alpha_{2B1} \gg \alpha_{2B2}$. These differences in affinities are clearly reflected in the curves shown in Figure 1. Thus, the ARC 239 competition curve was strongly biphasic, the upper part corresponding to competition at α_{2B1}- and α_{2B2}-sites and the lower to competition at α_{2A}-sites (Figure 1c). For guanoxabenz the plain competition curve was only weakly resolved into visually distinguishable components. However, when 0.2 μM of ARC 239 was added the guanoxabenz curve became clearly biphasic (Figure 1c). The 0.2 μM concentration of ARC 239 blocked the α_{2B1}-sites almost completely whereas almost all of the α_{2A}- and part of the α_{2B2}-sites remained. Since the α_{2B1}-site had an affinity for guanoxabenz which fell between α_{2A}- and α_{2B2}-affinities for guanoxabenz, the blockade of the α_{2B1}-sites would cause the competition curve of guanoxabenz to become more biphasic. Moreover, support for three sites is obtained in the saturation studies. As seen from the Scatchard transformation (Figure 1a) the curve representing total specific binding was only slightly curved. When 0.2 μM of ARC 239 was present, the Scatchard plot became a sharply bent curve. This is because 0.2 μM ARC 239 almost totally blocked the α_{2B1}-sites while sparing almost all of the α_{2A}- and part of the α_{2B2}-sites. Since the [^{3}H]-RX821002 affinity for the α_{2B1}-site fell between its affinities for α_{2A}- and α_{2B2}-sites the blockade of the α_{2B1}-sites would lead to the increased curvature of the transform. In contrast, the saturation isotherm of [^{3}H]-

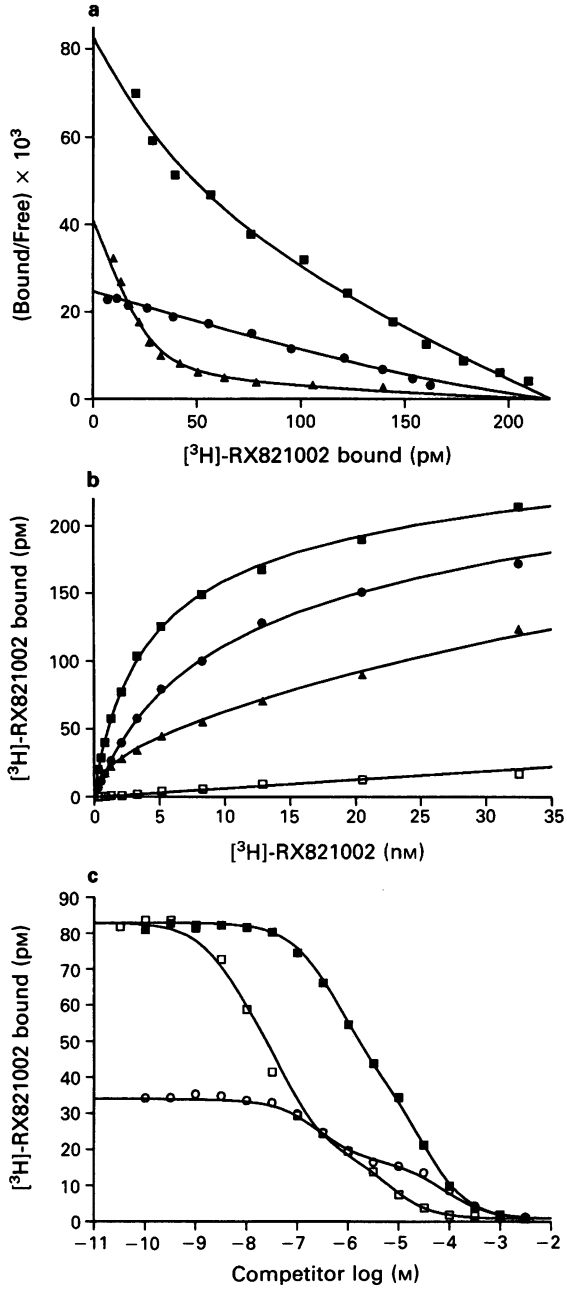


Figure 1 (a–c) Saturation and competition experiments, performed on the same day, and using the same batch of rat kidney membranes and [^{3}H]-RX821002 as labelled ligand. Shown in (b) are saturation curves for total [^{3}H]-RX821002 binding (■), binding of [^{3}H]-RX821002 in the presence of 6 μM guanoxabenz (●), in the presence of 0.2 μM ARC 239 (▲) and in the presence of 1 μM BDF 8933 (□). (BDF 8933 was used to define non-specific binding). Shown in (c) are competition curves for guanoxabenz (■), ARC 239 (□) and of guanoxabenz in the presence of a fixed concentration of ARC 239 (0.2 μM) (○). The lines represent the computer drawn fits from the simultaneous fitting of the data in (b) and (c) assuming that ligands bound to three independent sites. In (a) is shown the Scatchard transformation of the data shown in (b); (■) represent the total specific binding; (●) the specific binding obtained in the presence of 6 μM guanoxabenz, and (▲) the specific binding obtained in the presence of 0.2 μM ARC 239. The figure represents one experiment, with assays in duplicate, out of six experiments showing essentially the same results.

RX821002 obtained in the presence of 6 μM guanoxabenz generated an almost straight Scatchard plot (Figure 1a). This is because 6 μM guanoxabenz almost completely blocked both the α_{2A}- and α_{2B1}-sites while sparing the α_{2B2}-site to which [^{3}H]-RX821002 binds.

The computer modelling showed that the proportions of sites were, for α_{2A} 10%, α_{2B1} 25% and for α_{2B2} 65%. These relative amounts of sites corresponded to receptor densities of 12, 29 and 76 fmol mg^{-1} protein, respectively (see Table 1B); the total number of α_2-adrenoceptor sites being 117 ± 1 fmol mg^{-1} protein (mean ± s.e.mean; n = 6).

Determination of drug binding constants for α_{2A}-, α_{2B1}- and α_{2B2}-adrenoceptors in rat kidney

In order to test the binding of further drugs to the three binding sites for [^{3}H]-RX821002 we developed an assay for the determination of drug K_ds for the three sites. For each test of a drug, full competition curves were obtained for the tested drug either alone or in the presence of either 2 μM guanoxabenz or 0.1 μM ARC 239. In addition, for each test, full competition curves were obtained for guanoxabenz and ARC 239 as well as a competition curve for guanoxabenz in the presence of 0.1 μM ARC 239, as exemplified in Figures 2 and 3 for the tests of oxymetazoline and prazosin. (In these tests the ARC 239 concentration was reduced from 0.2 μM to 0.1 μM because 0.1 μM ARC 239 is sufficient to block completely the α_{2B1}-sites while more of the α_{2B2}-sites will become uncovered. In these conditions the ARC 239 masked curve will give more information on drug affinities for both α_{2A}- and α_{2B2}-sites. Similarly, reduction of guanoxabenz from 6 to 2 μM was to incorporate more information concerning α_{2B2}-sites in this curve.) All data were then subjected to simultaneous computer modelling and the results from the tests of 14 compounds are summarized in Table 2. For all tests the computer modelling indicated that a three site model was representing the data best. As can be seen from Figure 2c the plain prazosin competition curve was slightly tailed. In the presence of 2 μM guanoxabenz the prazosin competition curve was shifted to the left. This is because guanoxabenz will block α_{2A}- (as well as α_{2B1}-sites), thus eliminating the low affinity component (e.g. the α_{2A}-receptors) for prazosin while sparing the α_{2B2}-sites (e.g. one of the high affinity sites for prazosin). When 0.1 μM ARC 239 was present most of the α_{2B1}- and part of the α_{2B2}-adrenoceptors were blocked. Therefore the prazosin competition curves aligned more towards the low affinity part of the plain prazosin competition curve (i.e. the α_{2A}-site).

For oxymetazoline the plain competition curve was slightly distorted at the low concentration range (Figure 3c). In the presence of 2 μM guanoxabenz the competition curve aligned to the low affinity component of the plain curve because oxymetazoline had low affinity for the remaining α_{2B2}-receptors. However, in the presence of 0.1 μM ARC 239 the competition curve was clearly bisphasic; the high affinity component representing competition at unblocked α_{2A}-receptors and the low affinity component competition at the remaining α_{2B2}-receptors.

As can be seen from Table 2 all three binding sites showed stereoselectivity for adrenaline, the (−)-form of adrenaline being 7–10 fold more potent than the (+)-form, as expected for an adrenoceptor. Moreover, yohimbine showed higher affinity than prazosin for the binding sites, indicating their α_2-adrenoceptor nature. As seen from Table 2, guanfacine, like oxymetazoline, showed much higher affinity for the α_{2A}-site than for the α_{2B1}- and α_{2B2}-sites. Interestingly, the affinity of (−)-noradrenaline was highest for the α_{2B2}-site, the order of affinities being $\alpha_{2B2} \geq \alpha_{2B1} > \alpha_{2A}$. These results are in marked contrast with those for (−)-adrenaline where the order of potency was $\alpha_{2B1} \geq \alpha_{2A} > \alpha_{2B2}$. The affinities of dopamine for all the three sites were lower than those for either (−)-noradrenaline or (−)-adrenaline. Essentially the same K_ds for guanoxabenz and ARC 239 were obtained in these tests (Table 2) as those estimated above (Table 1).

[^{3}H]-yohimbine binding in rat kidney

In order to obtain data for comparison we performed tests using [^{3}H]-yohimbine as a radioligand in the rat kidney.

Table 1 Results from computer modelling six separate experiments of the type shown in Figure 1

A

Receptor	α_{2A}	α_{2B1}	α_{2B2}	*Selectivity ratios*		
Drug	K_d (nM)	K_d (nM)	K_d (nM)	I	II	III
[^{3}H]-RX821002	0.62 ± 0.05	2.52 ± 0.11	6.74 ± 1.21	4.1	2.7	11
ARC 239	1460 ± 270	4.78 ± 1.04	28.8 ± 4.1	0.0033	6.0	0.020
Guanoxabenz	99.7 ± 15.1	508 ± 135	25,400 ± 2400	5.1	50	250

B

Receptor	α_{2A}	α_{2B1}	α_{2B}
Receptor densities (fmol mg^{-1})	11.8 ± 1.1	29.4 ± 2.5	75.7 ± 2.8
Proportion of sites (%)	10.2 ± 0.9	25.1 ± 2.1	64.7 ± 2.4

Shown in **A** are the K_ds ± s.e.mean obtained for [^{3}H]-RX821002, ARC 239 and guanoxabenz for the α_{2A}-, α_{2B1}- and α_{2B2}-sites; also the selectivity ratios for the drugs are as follows: I = $K_d\alpha_{2B1}/K_d\alpha_{2A}$; II = $K_d\alpha_{2B2}/K_d\alpha_{2B1}$ and III = $K_d\alpha_{2B2}/K_d\alpha_{2A}$. The computer analysis showed that three site fits gave highly significant ($P \ll 0.001$ for all cases) reductions in the sums of squares as compared to two site fits whereas four site fits resulted in only marginal and insignificant reductions in the sums of squares as compared to three site fits. Shown in **B** are the receptor densities and proportion of sites of the α_{2A}-, α_{2B1}- and α_{2B2}-sites obtained from the computer modelling calculations.

Saturation studies (with 1 μM BDF 8933 to define non-specific binding) indicated that [^{3}H]-yohimbine labelled sites with similar affinities since two or three site fits did not improve the regression model over one site fits in the computer modelling calculations (data not shown). The apparent K_d of [^{3}H]-yohimbine obtained was 18.2 ± 2.2 nM and the receptor density 116 ± 4 fmol mg^{-1} protein (n = 2). (The reason that two or three site models did not improve the regressions was

Table 2 Dissociation constants (K_ds in nM) of drugs for α_{2A}-, α_{2B1}- and α_{2B2}-adrenoceptors in rat kidney membranes obtained by computer modelling competition experiments of the type shown in Figures 2 and 3

Drug	n	α_{2A} K_d (nM)	α_{2B1} K_d (nM)	α_{2B2} K_d (nM)	*Selectivity ratios* I	II	III
(−)-Adrenaline	4	3180 ± 390	1270 ± 480	12,800 ± 1100	0.40	10	4.0
		(12.2 ± 1.5)	(27.0 ± 2.0)	(60.7 ± 2.9)			
(+)-Adrenaline	4	31,000 ± 3500	8460 ± 4730	86,000 ± 13,200	0.27	10	2.8
		(12.1 ± 1.5)	(28.5 ± 3.8)	(59.4 ± 4.0)			
(−)-Noradrenaline	3	14,300 ± 1400	4910 ± 1100	2860 ± 920	0.34	0.58	0.20
		(9.53 ± 0.19)	(43.2 ± 1.0)	(47.3 ± 1.1)			
Dopamine	3	36,100 ± 5200	79,200 ± 6500	46,700 ± 6900	2.2	0.59	1.3
		(9.68 ± 0.21)	(38.8 ± 1.0)	(51.4 ± 0.8)			
BDF 8933	3	0.395 ± 0.071	0.882 ± 0.089	1.70 ± 0.06	2.2	1.9	4.3
		(10.4 ± 0.1)	(41.7 ± 0.4)	(47.9 ± 0.4)			
Oxymetazoline	4	14.8 ± 0.8	1240 ± 290	1460 ± 430	84	1.2	99
		(10.3 ± 1.6)	(28.8 ± 1.6)	(60.9 ± 3.2)			
Guanfacine	3	24.5 ± 2.60	2780 ± 660	2930 ± 1100	110	1.1	120
		(12.3 ± 1.2)	(36.0 ± 3.7)	(51.8 ± 4.2)			
Yohimbine	3	70.5 ± 2.1	6.81 ± 3.02	20.9 ± 3.1	0.097	3.1	0.30
		(8.50 ± 1.10)	(25.9 ± 7.8)	(65.6 ± 8.9)			
Guanoxabenz	23	72.6 ± 9.3	384 ± 49	27,500 ± 1500	5.3	72	380
		(10.0 ± 0.3)	(32.0 ± 1.3)	(57.9 ± 1.5)			
Rauwolscine	3	98.7 ± 11.6	12.4 ± 3.8	29.9 ± 1.5	0.13	2.4	0.30
		(8.97 ± 0.88)	(29.2 ± 5.2)	(61.8 ± 6.1)			
WB4101	3	322 ± 4	33.0 ± 3.8	79.7 ± 11.2	0.10	2.4	0.25
		(8.13 ± 1.02)	(28.4 ± 5.5)	(63.5 ± 6.4)			
Prazosin	3	1210 ± 220	35.6 ± 16.5	72.7 ± 5.0	0.029	2.0	0.060
		(7.85 ± 0.15)	(19.9 ± 2.8)	(72.2 ± 3.0)			
Rilmenidine	3	2170 ± 220	1420 ± 430	3200 ± 200	0.65	2.3	1.5
		(8.83 ± 0.85)	(30.1 ± 6.2)	(61.1 ± 7.0)			
ARC 239	23	2690 ± 180	6.14 ± 0.42	38.3 ± 2.8	0.0023	6.2	0.014
		(10.0 ± 0.3)	(32.0 ± 1.3)	(57.9 ± 1.5)			
Chlorpromazine	4	4040 ± 1070	448 ± 67	1220 ± 290	0.11	2.7	0.30
		(10.1 ± 0.7)	(35.8 ± 4.9)	(54.1 ± 5.1)			
Corynanthine	2	10,000 ± 600	498 ± 311	1320 ± 550	0.050	2.7	0.13
		(9.34 ± 0.45)	(37.8 ± 6.2)	(52.8 ± 6.2)			

For each drug tested a total of 6 competition curves were obtained and used simultaneously for the computer calculations. The data were simultaneously fitted to eq. 1 (see Methods) using non-linear regression analysis. In the calculations the affinities of [^{3}H]-RX821002 were set constant and assumed to be the reciprocals of the K_ds determined in Table 1. The computer analysis showed for all cases that three site fits gave significant ($P \ll 0.001$) reductions in the sums of squares as compared to two site fits, whereas four site fits resulted in only marginal and insignificant reductions in the sums of squares as compared to the three site fits. The analysis also showed that the proportion of sites was close to that given in Table 1. A value given in parentheses, below a K_d-value, represents percentage sites obtained from the tests of one drug using the 6 curve computer modelled approach. The table shows the mean ± s.e.mean. n = number of experiments. Shown also are the selectivity ratios for the drugs, as follows: I = $K_d\alpha_{2B1}/K_d\alpha_{2A}$; II = $K_d\alpha_{2B2}/K_d\alpha_{2B1}$ and III = $K_d\alpha_{2B2}/K_d\alpha_{2A}$.

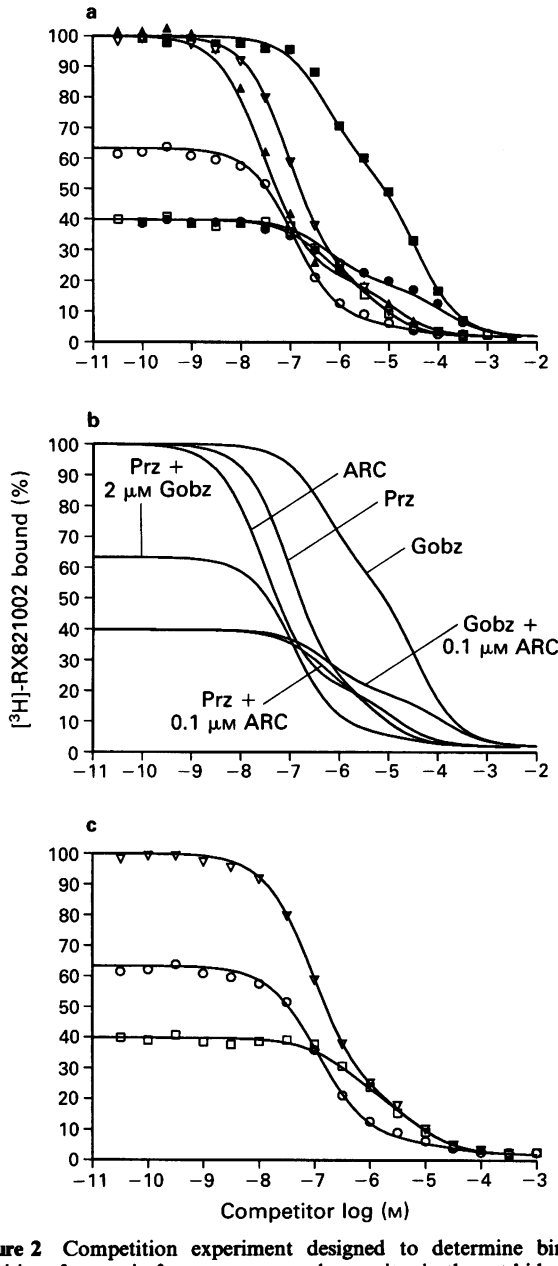


Figure 2 Competition experiment designed to determine binding affinities of prazosin for α_{2A}-, α_{2B1}- and α_{2B2}-sites in the rat kidney. In this test a fixed concentration of [^{3}H]-RX821002 (~2 nM) was used and plain competition curves of guanoxabenz (Gobz, ■), prazosin (Prz; ▽) and ARC 239 (ARC; ▲), as well as competition curves of prazosin in the presence of 2 μM guanoxabenz (○), prazosin in the presence of 0.1 μM ARC 239 (□) and guanoxabenz in the presence of 0.1 μM ARC 239 (●). All data were subjected to simultaneous computer modelling assuming that ligands bound to three independent sites. Shown in (a) are the experimental data and the computer drawn fit. In (b) is shown only the computer drawn fit in order to visualize better the separate curves. In (c) is shown (for the same reason) only the fit and the data for the prazosin competition curves. As can be seen the model represent an accurate approximation of the experimental data.

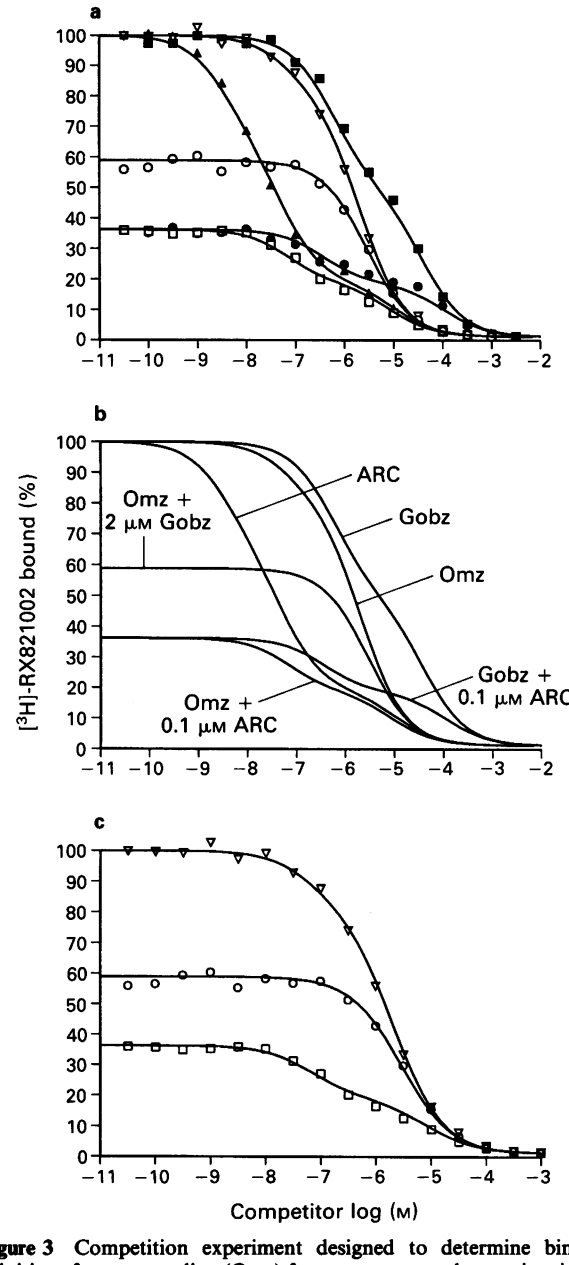


Figure 3 Competition experiment designed to determine binding affinities of oxymetazoline (Omz) for α_{2A}-, α_{2B1}- and α_{2B2}-sites in the rat kidney. The experiment was performed in an identical fashion to the one described in the legend to Figure 2, except that prazosin was exchanged for oxymetazoline; see Figure 2 for further details and abbreviations.

presumably because yohimbine showed too small selectivity for any of the three α_2-receptor subtypes; cf. Table 2).

Competition experiments with 8 nM [^{3}H]-yohimbine are shown in Figure 4. As can be seen from the figure, the competition curves of ARC 239 and guanfacine were steep and uniphasic; ARC 239 showing high and guanfacine low affinity. By contrast, guanoxabenz gave clearly bisphasic competition curves. Computer modelling showed that the ARC 239 and guanfacine competition curves modelled best into one site fits

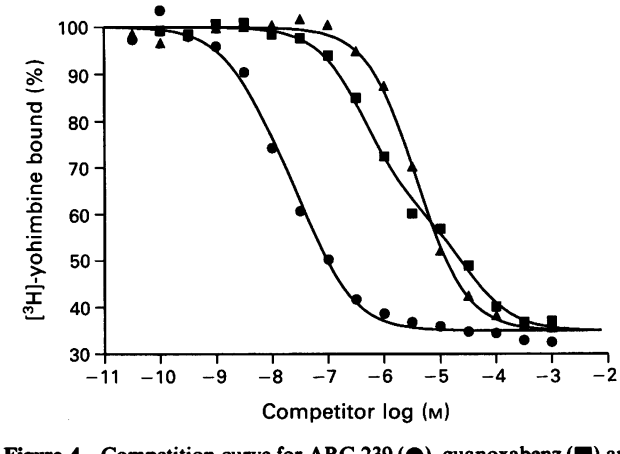


Figure 4 Competition curve for ARC 239 (●), guanoxabenz (■) and guanfacine (▲) in the rat kidney with 8 nM [^{3}H]-yohimbine used as radioligand.

with apparent K_ds of 12.2 ± 1.2 and 2090 ± 150 nM, respectively. The guanoxabenz curves modelled best into two site fits ($P < 0.001$ compared to a one site model; no significant further improvement with a three site model) with apparent K_ds of 232 ± 32 and 38,100 ± 15,100 nM ($n = 2$). (See discussion why three sites were not revealed with [^{3}H]-yohimbine). The high non-specific binding of [^{3}H]-yohimbine (more than 30%) made a more detailed analysis of [^{3}H]-yohimbine binding difficult.

Discussion

The ability to obtain accurate estimates of the dissociation constants of drugs for the three rat kidney α_2-adrenoceptor subtypes is due to the simultaneous analysis of several competition curves for each assay of a drug, as shown in Figures 2 and 3, Thus, in the presence of 0.1 μM ARC 239, the competition curve of the tested compound will represent the displacement of [^{3}H]-RX821002 from the α_{2A}- and part of α_{2B2}-receptors, whereas in the presence of 2 μM guanoxabenz it will represent displacement almost only from the α_{2B2}-receptors. The plain competition curve will of course represent competition from all three sites. Thus, these curves will contain data to determine dissociation constants of the test compound for the three α_2-adrenoceptor sites. Moreover, since competition curves of guanoxabenz, ARC 239 and of guanoxabenz in the presence of 0.1 μM ARC 239 are included in each assay, accurate information becomes available to determine the proportions of the three receptor sites. This will in turn increase the accuracy in the determination of the K_ds of the test compounds.

The validity of the multicurve approach is also evident from the experiments depicted in Figure 1, which were designed for determination of the K_ds of [^{3}H]-RX821002 for the three α_2-adrenoceptor sites. Thus, the saturation curve obtained in the presence of 6 μM guanoxabenz will contain information concerning [^{3}H]-RX821002 binding mainly to α_{2B2}-sites, whereas the one obtained in the presence of 0.2 μM ARC 239 contains information of binding mainly to α_{2A}- and α_{2B2}-sites. The unmasked saturation curve will of course contain information on [^{3}H]-RX821002 binding for all three sites. Moreoever, inclusion of competition curves for guanoxabenz and ARC 239 as well as of guanoxabenz in the presence of 0.1 μM ARC 239 will add enough information to determine the K_ds of guanoxabenz and ARC 239 for the three sites in analogy with what was said in the previous paragraph. In addition, the inclusion of 1 μM BDF 8933 in one of the saturation curves will give accurate information about non-specific binding.

In this context we would again like to emphasize that the drug K_ds are determined simultaneously from the information present in all data for each particular experiment (i.e. all of the 6–7 curves obtained in the two types of experiments shown in Figures 1–3). The ability to extract drug K_ds for three different binding sites from the complex information is due to the application of non-linear regression analysis to formulae derived from the law of mass action. We have verified the ability of the computer modelling procedure to determine the correct K_ds from the complex information by using simulated data utilizing the Monte Carlo approach (data not given; see De Lean *et al.* (1982) and Uhlén & Wikberg (1991a) for examples of the use of this approach). Moreover, our multicurve approach renders the problem of determining dissociation constants for three different sites into a mathematically well conditioned problem, something which was evident from rapid convergence in the computations. (Such well conditioning was not possible with a single curve approach.) The reliability of our approach is also evident from the overall low errors in the estimates of drug K_ds given in Table 1 and 2. Apart from this, it was helpful that the order of affinities of [^{3}H]-RX821002 for the α_2-receptor subtypes was $\alpha_{2A} > \alpha_{2B1} > \alpha_{2B2}$, whereas the order of receptor densities in the kidney was reversed: $\alpha_{2A} < \alpha_{2B1} < \alpha_{2B2}$. This situation resulted in a signal of similar magnitude from each receptor subtype, when 2 nM [^{3}H]-RX821002 was used in competition studies.

The [^{3}H]-yohimbine experiments of the present study give full support to the conclusions obtained from the experiments with [^{3}H]-RX821002. Thus, the number of total α_2-adrenoceptor sites labelled by [^{3}H]-yohimbine and [^{3}H]-RX821002 was the same (116 versus 117 fmol mg^{-1} protein, respectively) indicating the validity of the methods. In the competition studies using [^{3}H]-yohimbine it appeared that only α_{2B}-adrenoceptors are being labelled due to the apparent uniform high and low affinity of ARC 239 and guanfacine, respectively. It is also evident from the biphasic guanoxabenz curve that these α_{2B}-adrenoceptors consist of two subtypes (e.g. the α_{2B1}- and α_{2B2}-types). Moreover, the estimates of the K_ds of ARC 239, guanfacine and guanoxabenz with [^{3}H]-yohimbine were very similar to those obtained with [^{3}H]-RX821002. The reason that [^{3}H]-yohimbine failed to reveal the α_{2A}-adrenoceptor site is probably due to the low affinity of yohimbine for α_{2A}-adrenoceptors (see Table 2), as well as their low density in the kidney (Table 1). This will render the signal from α_{2A}-adrenoceptors insignificant compared to the signal obtained from the α_{2B}-adrenoceptor subtypes. These findings are consistent with data in the literature suggesting that [^{3}H]-yohimbine (or [^{3}H]-rauwolscine) labels only α_{2B}-adrenoceptors in the rat kidney (Cheung *et al.*, 1986; Michel *et al.*, 1989a).

The relations of our α_2-adrenoceptors to those for which the gene structures have been determined (Kobilka *et al.*, 1987a; Regan *et al.*, 1988; Lomasney *et al.*, 1990; Chalberg *et al.*, 1990; Zeng *et al.*, 1990; Voigt *et al.*, 1991) are not entirely clear. Our two α_{2B}-adrenoceptors show similar pharmacology to the expressed rat α_2-RNG and rat α_2-pA2d receptors (Zeng *et al.*, 1990; Voigt *et al.*, 1991) due to their similar high affinities for yohimbine and prazosin and low affinities for oxymetazoline, but available data does not allow definite classification of either α_{2B1} or α_{2B2} as being identical to the α_2-RNG or α_2-pA2d receptors. (α_2-pA2d has also been referred to as 'rat α_2-C4'; Voigt *et al.*, 1991.) Our α_{2A}-receptor shows similar pharmacology to the human α_2-C10 receptor (Lomasney *et al.*, 1990) due to their similar high and low affinities for oxymetazoline and prazosin, respectively. However, yohimbine, rauwolscine and WB4101 show comparatively low affinities for our α_{2A}-receptor compared to the human α_2-C10 receptor (Kobilka *et al.*, 1987a; Lomasney *et al.*, 1990) possibly due to species differences in α_{2A}-receptor types (see also Michel *et al.*, 1989a; Brown *et al.*, 1990; Harrison *et al.*, 1991).

Our two α_{2B}-receptors show pharmacology more similar to the human α_2-C2 and α_2-C4 receptors than to the α_2-C10 receptor, but species differences may make comparisons of binding data of less value. (The rat α_2-RNG amino acid sequence is 95, 73 and 71% homologous to the human α_2-C2, α_2-C4 and α_2-C10 genes, respectively, when comparison is made starting from the tyrosine residue just before the 1st transmembranal segment and excluding the 3rd intracellular loop. The corresponding data for rat α_2-pA2d are 72, 97 and 71%, respectively.) Very limited binding data are available for the α_2-adrenoceptor cloned from rat brain (cA2-47 clone (Chalberg *et al.*, 1990); 95, 71 and 71% homologous to α_2-C10, α_2-C4 and α_2-C2, respectively) which makes it difficult to link this receptor with any of our present ones. Moreover, α_2-cA2-47 and α_2-C10 probes hybridized to different restriction fragments in the rat genome (Chalberg *et al.*, 1990) possibly indicating that α_2-cA2-47 may represent yet another α_2-receptor subtype. Guanoxabenz, and ARC 239, in conjunction with [^{3}H]-RX821002 binding and use of expressed receptor proteins may prove useful in linking structure to pharmacology for α_2-adrenoceptors.

The drug K_ds for the rat kidney α_2-adrenoceptor subtypes determined in the present study do not represent agonist binding to high affinity forms of α_2-adrenoceptors since

several previous studies have indicated that the use of NaCl, Gpp(NH)p and EDTA eliminates the agonist high affinity binding sites of α_2-adrenoceptors in various tissues (Michel *et al.*, 1980; Snavely & Insel, 1982; Uhlén & Wikberg, 1991b). Moreoever, as the effects of Gpp(NH)p and EDTA are mediated via *G*-proteins and the effect of Na^+ is mediated by interaction with a specific aspartate residue (Horstman *et al.*, 1991) which appears to be conserved among all G-protein coupled receptors, including the α_2-adrenoceptors, it is conceivable that the inclusion of NaCl, Gpp(NH)p and EDTA will eliminate the agonist high affinity conformation for all of our three rat kidney α_2-adrenoceptor subtypes. Moreoever, the computer modelling indicated that a three sites model best described the experimental data, irrespective of whether agonists or antagonists were used in the tests.

The binding data of our present study clearly indicate the α_2-adrenoceptor natures of the three receptors studied. This is due to their expected stereoselective binding properties for (+)- and (−)-adrenaline and the expected affinities for classical α_1- and α_2-adrenoceptor blockers such as yohimbine, rauwolscine, corynanthine and prazosin. Moreover, since dopamine showed much lower affinities than either (−)-adrenaline or (−)-noradrenaline the sites cannot be classified as being dopamine receptors. In accordance with the original definition of α_{2A}- and α_{2B}-adrenoceptors (Bylund, 1985) prazosin showed low affinity for the α_{2A}-site and high for the α_{2B1}- and α_{2B2}-sites, whereas oxymetazoline showed high affinity for the α_{2A}-site but low for the α_{2B1}- and α_{2B2}-sites. Although the affinities of prazosin were comparatively high for the α_{2B1}- and α_{2B2}-sites its affinities for these sites are still more than 100 fold lower than its reported (Michel *et al.*, 1989b) affinities for α_1-adrenoceptors. Moreover, the three sites labelled by [^{3}H]-RX821002 do not represent imidazoline binding sites ('I-receptors') since these invariably show low non-stereoselective affinities for catecholamines as well as negligible affinity for RX821002 itself (Wikberg, 1989; Wikberg & Uhlén, 1990; Langin *et al.*, 1990; Wikberg *et al.*, 1991).

Previous studies have, in addition to α_{2A}- and α_{2B}-subtypes, described α_{2C}- and α_{2D}-subtypes of α_2-adrenoceptors (for review see Bylund *et al.*, 1991). The α_{2C}-type was present in the oppossum and data for this receptor (Bylund *et al.*, 1991) clearly show that it does not correspond to any of the rat kidney $\alpha_2$adrenoceptor subtypes identified in the present study. This is mainly due to the fact that the α_{2C}-type show about equally high affinity for both prazosin and oxymetazoline whereas this is not the case for any of the rat kidney types. It has been claimed that the α_{2D}-type is present in the rat submaxillary gland (Michel *et al.*, 1989a; Bylund *et al.*, 1991). It is conceivable that our kidney α_{2A}-adrenoceptor is closely similar to this α_2-adrenoceptor due to excellent correlations of our α_{2A} K_ds to the ones reported for α_{2D}s (cf. our results to Table 1 in Bylund *et al.*, 1991). The main reason that the α_{2D}-receptor was placed in a group of its own seems to have been that this receptor shows fairly low affinity for yohimbine, rauwolscine and WB4101 compared to human (as well as rabbit) α_{2A}-receptors. However, recent literature indicates that the rat α_{2D}-adrenoceptor is probably coded for by a rat analogue to the α_2-C10 gene (e.g. the human α_{2A}-receptor gene) (see Harrison *et al.*, 1991 and Lorenz *et al.*, 1991). Thus the presence of species differences in α_{2A}-adrenoceptors may explain the difference in yohimbine, rauwolscine and WB 4101 affinities.

Previous binding studies in the rat have identified α_{2B}-adrenoceptors in the brain and neonatal lungs. It is not possible to classify the brain and neonatal lung α_{2B}-adrenoceptors into either α_{2B1}- or α_{2B2}-types since none of the previous studies included guanoxabenz, which at present is the only known drug which shows major selectivity for the two types of α_{2B}-adrenoceptors. Thus, classification of rat brain and neonatal lung α_{2B}-adrenoceptors into α_{2B1}-, α_{2B2}- or even yet another α_{2B}-subtype of the α_2-adrenoceptor family must await further studies.

The present study provides evidence that guanfacine is selective for α_{2A}-adrenoceptors as compared to either α_{2B1}- or α_{2B2}-adrenoceptors. As can be seen from Table 2 the selectivity of guanfacine is about 100 fold which is comparable to the selectivity for oxymetazoline which is the previously most well-characterized α_{2A}-selective compound (Bylund, 1985).

It was an interesting finding that, whereas (−)-adrenaline showed highest affinities for α_{2A}- and α_{2B1}-adrenoceptors, (−)-noradrenaline showed the highest affinity for the α_{2B2}-adrenoceptor. It is tempting to speculate that these differences in affinities are due to different physiological roles for the α_2-adrenoceptor subtypes. However, as detailed above, it is important to realize that our approach will determine the affinities of drugs only to the agonist low affinity conformation of the α_2-adrenoceptors. Thus, no information on the ability of the agonists to activate the receptors is achieved. Therefore the physiological roles for α_2-adrenoceptor subtypes remain a subject for future studies.

In conclusion the data of the present study show that three pharmacological subtypes of α_2-adrenoceptors are present in the rat kidney. The best drugs to distinguish between these receptors are guanoxabenz, which shows an order of potency for the receptors $\alpha_{2A} > \alpha_{2B1} \gg \alpha_{2B2}$ and ARC 239, which shows an order of potency $\alpha_{2B1} > \alpha_{2B2} \gg \alpha_{2A}$.

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Salmeterol, a novel, long-acting β_2-adrenoceptor agonist: characterization of pharmacological activity *in vitro* and *in vivo*

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1 Salmeterol, a novel, long-acting β-adrenoceptor agonist, has been compared with isoprenaline and salbutamol for activity *in vitro* on a range of β-adrenoceptor containing preparations from laboratory animals and man, and *in vivo* for bronchodilator activity in the conscious guinea-pig.

2 Salmeterol, like isoprenaline and salbutamol, relaxed preparations of both guinea-pig trachea (contracted by prostaglandin $(PG)F_{2\alpha}$ or electrical stimulation) and human bronchus (contracted by $PGF_{2\alpha}$) in a concentration-related fashion. Salmeterol was of similar potency to isoprenaline and more potent than salbutamol on both airway preparations.

3 Relaxant responses of superfused guinea-pig trachea and human bronchus to isoprenaline and salbutamol declined rapidly when the agonists were washed from the tissues, with complete recovery within 10 min, whereas responses to salmeterol were more persistent. In electrically-stimulated guinea-pig trachea preparations, inhibition by salmeterol persisted for periods of up to 12 h, despite continuous superfusion with agonist-free medium. However, these persistent responses were rapidly and fully reversed by the β-adrenoceptor blocking drug, propranolol (0.1 μM). In further studies, on guinea-pig trachea, propranolol caused concentration-related parallel, rightward shifts of salmeterol concentration-effect curves, yielding a pA_2 of 9.0. The slope of the Schild plot was 1.02.

4 Another β-adrenoceptor blocking drug, sotalol (10 μM), also fully and rapidly reversed established submaximal responses to salmeterol in superfused guinea-pig trachea. However, after administration of sotalol was stopped, the antagonism waned, and salmeterol responses were reasserted without the addition of further agonist.

5 In the β_1-adrenoceptor containing preparation, rat left atria, isoprenaline exhibited potent, concentration-related, positive inotropic activity, whereas salbutamol and salmeterol were at least 2000–5000 fold less potent, and appeared to be partial agonists. At a concentration of 72 μM, salmeterol exhibited weak antagonism of isoprenaline-induced increases in atrial force of contraction. This antagonism was less marked than that caused by salbutamol (42 μM).

6 On the guinea-pig isolated gastric fundus strip, a putative β_3-adrenoceptor containing preparation, salbutamol and salmeterol had only modest agonist activity, being 20–30 fold less potent than isoprenaline and the selective β_3-adrenoceptor agonist, BRL 35135.

7 In conscious guinea-pigs, inhaled salmeterol and salbutamol were approximately equipotent in causing dose-related bronchodilatation. Whereas the duration of action of salbutamol at its threshold-effective dose was less than 90 min, the responses to a similarly effective dose of salmeterol were well-maintained for at least 6 h.

8 Salmeterol is therefore a potent and selective β_2-adrenoceptor agonist with a remarkably long duration of action in isolated superfused airways smooth muscle. It also causes persistent bronchodilatation *in vivo*, in the guinea-pig, when administered by the inhaled route.

Keywords: β-Adrenoceptor agonists; salmeterol; guinea-pig trachea; human bronchus; duration of action; conscious guinea-pig; bronchodilatation

Introduction

In the treatment of bronchial asthma, inhaled β_2-adrenoceptor agonists, such as salbutamol, produce effective bronchodilatation with few associated side-effects (McFadden, 1980; Brittain *et al.*, 1981; Clarke, 1982; Tattersfield, 1982; Svedmyr & Lofdahl, 1987). The major disadvantage of the currently available drugs is in their relatively short duration of action of 4–6 h (Leifer & Wittig, 1975; Svedmyr, 1985a,b). A bronchodilator with a substantially longer duration of action would not only have the advantage of less frequent dosing, but may afford protection against nocturnal asthma, which is currently inadequately controlled. The availability of such a compound could therefore be an important therapeutic advance. Whilst orally-administered, sustained-release preparations, pro-drugs and drugs with long plasma half-lives may offer some benefit, they lack the functional selectivity afforded by the inhaled route of administration. We have therefore attempted to design a molecule with a long duration of action at β_2-adrenoceptors and, which, as a consequence, would result in a long-lasting bronchodilatation after administration by inhalation.

We now describe some results comparing potency, duration of action and selectivity of salmeterol (4-hydroxy-α[[[6-[4-phenylbutyl)oxy]hexyl]amino]methyl] - 1,3 - benzenedimeth - anol), a novel long-acting β_2-adrenoceptor agonist (Bradshaw *et al.*, 1987), with the β-adrenoceptor agonists, isoprenaline and salbutamol (Figure 1) both on a range of β-adrenoceptor containing preparations *in vitro*, and in conscious guinea-pigs *in vivo*.

Preliminary accounts of this work have been communicated to the British Pharmacological Society (Ball *et al.*, 1987a,b).

Methods

Tissue preparations

Tracheae and stomach fundi were obtained from male guinea-pigs (Dunkin-Hartley, 350–500 g), and atria from male rats (A

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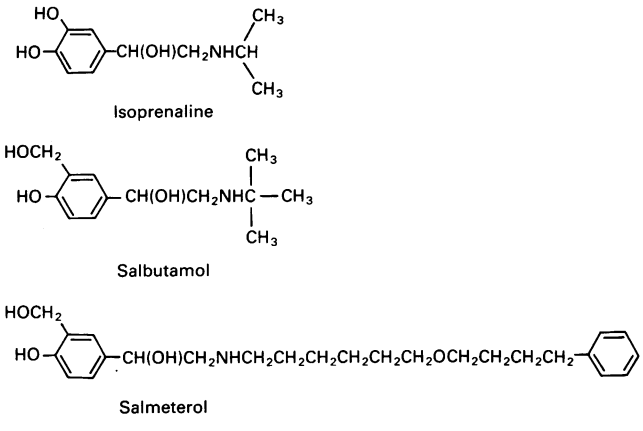


Figure 1 Chemical structures of isoprenaline, salbutamol and salmeterol.

& H strain, 200–300 g). All animals were killed by a blow to the head and subsequent exsanguination. Human bronchi were dissected from macroscopically normal lung parenchyma, obtained from patients undergoing surgical removal of carcinomas of the lung and bronchi. Tracheal and bronchial preparations were cut into rings which were opened by cutting the cartilage opposite the smooth muscle (Coburn & Tomita, 1973). A cotton thread was tied to one cartilage for attachment to a strain gauge, and a cotton loop to the other end for anchoring the tissue in the superfusion chamber or organ bath.

Left atrial preparations were prepared as described by Blinks & Koch-Weser (1961). A thread was sewn to the uppermost portion of the atrium, and the tissue was then impaled on a 2-pin electrode and placed in a 2 ml organ bath.

The gastric fundus was dissected away from the rest of the stomach, the mucosal layer removed, and strips prepared as previously described (Coleman, 1987). One end of each preparation was attached to a strain gauge with cotton thread and the other anchored in the organ bath by means of a cotton loop.

Tissue superfusion

Superfusion apparatus The superfusion apparatus employed in these experiments has been previously described (Coleman *et al.*, 1986; Coleman & Nials, 1989). Preparations were mounted under a resting tension of 1 g, and superfused at a rate of 2 ml min^{-1} with oxygenated (5% CO_2 in O_2) modified Krebs solution maintained at 37°C. For experiments involving electrical stimulation, bipolar platinum electrodes positioned parallel with, and in close proximity to, the superfused tissue were used.

Determination of agonist activity on guinea-pig trachea and human bronchus In order to measure relaxant activity on guinea-pig trachea and human bronchus, smooth muscle tone was elevated by the inclusion of $PGF_{2\alpha}$ (2.8 μM) in the Krebs solution. Alternatively, in the case of guinea-pig trachea, phasic contractile responses were induced by electrical field-stimulation with 10 s trains of square wave pulses of 5 Hz frequency, 0.1 ms duration and just maximal voltage every 2 min. These electrically induced responses were highly reproducible over periods of at least 8–10 h of stimulation. For experiments involving electrical stimulation, preparations were taken from guinea-pigs pretreated intraperitoneally with 6-hydroxydopamine (6-OHDA, 200 mg kg^{-1}), in order to eliminate any adrenergic component in the electrically-induced response (Coleman & Nials, 1986; 1989).

For each preparation, constant responses to the standard agonist, isoprenaline, were first obtained either by repeating cumulative bolus dose-effect curves or by infusing increasing concentrations in sequential manner, until sensitivity was constant. A 30 min equilibration period was then allowed before a concentration-effect curve to a test compound was started. The magnitude of each response was measured and calculated as a percentage of the maximum isoprenaline response obtained in the final control curve.

Potency values for the β-adrenoceptor agonists were expressed in both absolute terms (concentration required to induce 50% of the maximum response to each agonist, EC_{50}) and relative to isoprenaline, as an equipotent concentration (EPC, i.e. EC_{50} for the test agonist/EC_{50} for isoprenaline). An EPC less than unity indicates greater potency than isoprenaline, and an EPC greater than unity, a lower potency than isoprenaline.

Determination of the rates of onset and offset of action Onset time (Ot_{50}) is defined as the time from administration of an EC_{50} concentration of an agonist to attainment of 50% maximal response and recovery (Rt_{50}) is defined as the time from stopping administration of the test agonist to attainment of 50% recovery from an EC_{50} concentration. Ot_{50} and Rt_{50} values were determined by interpolation from a plot of % response against time to attainment of 50% of each response (for Ot_{50}), or of 50% recovery from the response for (Rt_{50}) (Coleman & Nials, 1989).

Determination of agonist and antagonist activity in rat left atria

Atrial preparations were placed in 2 ml jacketed organ baths containing oxygenated (5% CO_2 in O_2) Krebs solution maintained at 32°C. A resting tension of 1 g was applied. Electrical stimuli of 1 Hz frequency, 1 ms duration and 5 volts amplitude were delivered by a square wave stimulator. Agonist potencies were determined in the same manner as on guinea-pig trachea and human bronchus. Responses were measured as increase in force, and expressed as a % of the maximum isoprenaline response. If the maximum increase in force achieved by the test compound was less than 50% of the preceding isoprenaline control, then it was tested for antagonist activity by repeating the isoprenaline concentration-effect curve in its presence. The isoprenaline concentration ratio was defined as:-

$$\frac{EC_{50} \text{ for isoprenaline in the presence of the test compound}}{EC_{50} \text{ for isoprenaline in the absence of the test compound}}$$

Determination of agonist activity in guinea-pig fundus

Guinea-pig fundic strip preparations were mounted in 2 ml jacketed organ baths and immersed in oxygenated (5% CO_2 in O_2) Krebs solution containing indomethacin (2.8 μM), atropine (0.4 μM) and phenoxybenzamine (1 μM), and maintained at 37°C. A tension of 1 g was applied to the tissue. Smooth muscle tone was elevated by addition of a submaximally-effective concentration of $PGF_{2\alpha}$ (2.8 μM) to the organ bath.

Agonist potencies were determined as for the other preparations, agonist potency being expressed as equipotent concentration ratio (EPC, isoprenaline = 1).

Conscious guinea-pigs

Bronchodilator activity was assessed in conscious guinea-pigs by the method described by Ball *et al.* (1987b). Briefly, guinea-pigs were placed in perspex, whole-body plethysmographs, pressure changes being recorded via a side-arm attached to a UP2 low pressure transducer. Each plethysmograph was connected via a head chamber to an aerosol expansion chamber. A gated port allowed the guinea-pig to breathe the atmosphere from the expansion chamber, but when this was closed, two other ports opened, one to a supply of 5% CO_2 in O_2, and the other to waste. The synchronous opening and closing of the three ports was achieved with a sliding valve. Respiratory rate was continuously computed from the cyclic pressure changes in each plethysmograph. With the animals breathing the O_2/CO_2 mixture, an aerosol generated from a

9 mM histamine solution was driven into the expansion chamber for 15–30 s by use of a Devilbiss 645 nebulizer. The port to the expansion chamber was then opened and the animals exposed to the aerosolised histamine. This caused a mild bronchoconstrictor response, seen as a small fall in plethysmograph pressure and an increase in respiratory rate. The animals were exposed to histamine for 4 min or until the respiratory rate had increased by 40%, whichever occurred first, at which point they were permitted to breathe the O_2/CO_2 mixture again. During histamine exposure, a purpose-built computer measured and recorded the area under the respiratory rate curves as an index of bronchoconstriction. In most animals, repeated histamine challenges yielded reproducible bronchoconstrictor responses. Each was then treated with a β-adrenoceptor agonist or vehicle, by either the inhaled or oral route, and then re-challenged with aerosolized histamine 30, 90, 180 and 360 min later. Inhalation of β-adrenoceptor agonists was achieved by attaching each plethysmograph to a head/expansion chamber and a Devilbiss nebulizer. Guinea-pigs were exposed to aerosolized β-adrenoceptor agonists for 60 s.

Drugs used

In the present study, the following compounds were used: ascorbic acid (BDH Chemicals, U.K.), atropine sulphate (Sigma, U.S.A.) BRL35135 (Glaxo Group Research, U.K.), 5-hydroxytryptamine creatinine sulphate (5-HT, Sigma, U.S.A.), histamine acid phosphate (BDH Chemicals, U.K.), indomethacin (Sigma, U.S.A.), (−)-isoprenaline sulphate (Sigma, U.S.A.), prostaglandin $F_{2\alpha}$ tromethamine salt (Lutalyse, Upjohn, U.S.A.), propranolol hydrochloride (Sigma, U.S.A.), salbutamol (Glaxo Group Research, U.K.), salmeterol (Glaxo Group Research, U.K.) and sotalol hydrochloride (Glaxo Group Research, U.K.).

BRL35135, isoprenaline, salbutamol and salmeterol were each dissolved in 2 drops of glacial acetic acid and diluted to stock concentration with phosphate buffer (pH 7.0). Dilutions of stock concentrations were made with 0.9% saline. All solutions of β-adrenoceptor agonists contained ascorbic acid (11 μM).

Indomethacin was dissolved in 1% sodium bicarbonate in 0.9% saline. Atropine, 5-hydroxytryptamine, histamine, propranolol and sotalol were dissolved in distilled water. Prostaglandin $F_{2\alpha}$ was supplied in aqueous solution containing 5 mg ml^{-1} free acid. All dilutions were made in 0.9% saline.

The Krebs solution used had the following composition (gl^{-1}): NaCl 6.92, $NaHCO_3$ 2.1, KCl 0.35, $MgSO_4 \cdot 7H_2O$ 0.15, KH_2PO_4 0.16, glucose 2.0 and $CaCl_2 \cdot 6H_2O$ 0.28.

Results

Guinea-pig trachea

Prostaglandin $F_{2\alpha}$-contracted preparation Each of the β-adrenoceptor agonists caused concentration-related relaxation of $PGF_{2\alpha}$-contracted preparations of superfused guinea-pig trachea (Figure 2, Table 1). Salmeterol and isoprenaline (EC_{50} = 3.2 nM) were equipotent, salbutamol being approximately 2 fold less potent. All three agonists caused virtually complete inhibition of $PGF_{2\alpha}$-induced tone at the highest concentrations used. However, there were differences in their rates of onset and offset of action (see Table 1). Thus, while the onset of action of both isoprenaline and salbutamol was rapid (Ot_{50} = 1.2 and 1.8 min respectively), that for salmeterol was substantially longer (Ot_{50} = 19.5 min). Similarly, both isoprenaline and salbutamol were short-acting (Rt_{50} = 2.1 min and 3.5 min respectively), whereas little or no recovery was observed from responses to salmeterol for up to 45 min, the maximum time for which measurements could reliably be made.

Electrically-stimulated preparation All three β-adrenoceptor agonists caused concentration-related inhibition of electrically-induced contractile responses of the guinea-pig superfused trachea (Table 2). Salmeterol was the most potent, being approximately 2 fold more potent than isoprenaline (EC_{50} = 15.4 nM) and approximately 8 fold more potent than salbutamol. No attempt was made in these experiments to achieve a maximal inhibitory response with either salbutamol or salmeterol (see Methods). The onset and offset of action times observed broadly reflected those on the $PGF_{2\alpha}$-contracted preparation (see Table 2), with isoprenaline and salbutamol being rapid both in onset and offset of action, and salmeterol having a slow onset of action and showing virtually no recovery even after periods in excess of 12 h,

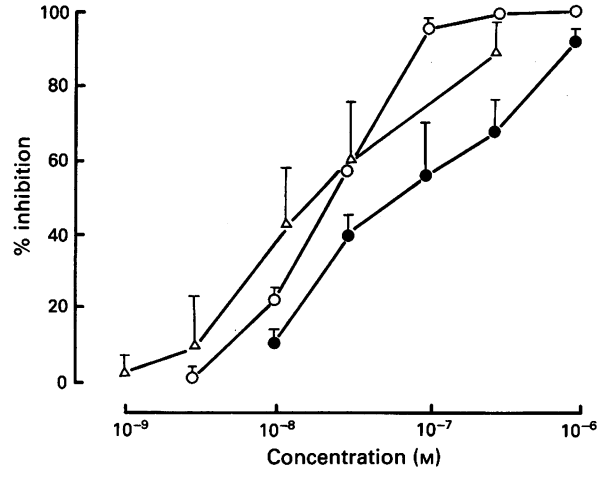


Figure 2 Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-contracted, superfused guinea-pig tracheal strip: mean relaxant concentration-effect curves for isoprenaline (O, n = 12), salbutamol (●, n = 7) and salmeterol (Δ, n = 5). Each point is the mean of n determinations, and vertical bars represent s.e.mean.

Table 1 Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-contracted superfused guinea-pig tracheal strip: potency and times for onset and recovery for isoprenaline, salbutamol and salmeterol

Agonist	*Equipotent*[1] *concentration* (Isoprenaline = 1)	*Onset*[2] *time* (Ot_{50}, min)	*Recovery*[2] *time* (Rt_{50}, min)	n
Isoprenaline	1	1.2	2.1	20
	EC_{50} = 3.2 (2.5–3.9) nM	(1.0–1.4)	(1.8–2.6)	
Salbutamol	2.0	1.8	3.5	7
	(1.2–3.3)	(1.3–2.5)	(2.5–4.7)	
Salmeterol	0.9	19.5	>12.4–>46	12
	(0.6–1.2)	(8.4–30.6)		

[1] Potency data are geometric means of n observations with (95% CL).
[2] Onset and recovery data are arithmetric means of n observations with (95% CL) or expressed as a range.

Table 2 Electrically-stimulated guinea-pig trachea: potency and times for onset and recovery for isoprenaline, salbutamol and salmeterol

Agonist	*Equipotent*[1] *concentration* (Isoprenaline = 1)	*Onset*[2] *time* (Ot_{50}, min)	*Recovery*[2] *time* (Rt_{50}, min)	n
Isoprenaline	1 EC_{50} = 15.4 (12.4–19.1) nM	> 1 < 3	> 2 < 4	25
Salbutamol	3.0 (1.5–6.5)	3.2 (2.0–5.4)	10.7 (7.8–14.7)	8
Salmeterol	0.4 (0.2–0.7)	29.1 (23.8–35.7)	> 420– > 770	14

[1] Potency data are geometric means of *n* observations with (95% CL).
[2] Onset and recovery data are arithmetic means of *n* observations with (95% CL) or expressed as a range.

despite continuous superfusion of the tissue (Figure 3). The persistent inhibitory effects of salmeterol were, however, reversed (Figure 3) by the β-adrenoceptor blocking drug propranolol (0.1 μM).

Human bronchus

Isoprenaline, salbutamol and salmeterol all caused concentration-related relaxations of the $PGF_{2\alpha}$-contracted superfused human bronchial strip preparation (Figure 4). The mean EC_{50} (95% confidence limits) for isoprenaline was 6.0 (3.0–9.1, *n* = 9) nM. Salmeterol was of similar potency to isoprenaline (EPC = 0.7 (0.4–1.2, *n* = 6)), while salbutamol was approximately 10 fold less potent (EPC = 10.9 (9.8–12.1, *n* = 6)). The maximum degree of relaxation induced by salmeterol was however, consistently less (70%) than that induced by isoprenaline (Figure 4), which fully relaxed the induced tone. In contrast, despite its relatively lower potency, salbutamol appeared to be a full agonist with respect to isoprenaline. Both isoprenaline (Rt_{50} = 1.1 (0.8–1.6, *n* = 9) min) and salbutamol (Rt_{50} = 5.5 (2.8–10.8, *n* = 6) min) were short-acting on the human bronchus preparation when compared with salmeterol, the effects of which persisted with no obvious recovery

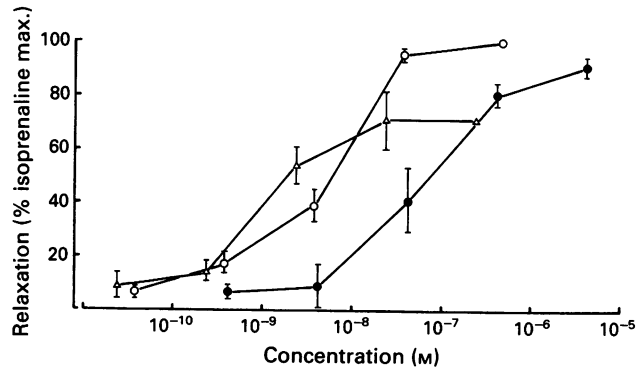


Figure 4 Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-contracted superfused human bronchial strip. Mean relaxant concentration-effect curves to isoprenaline (○, *n* = 9), salbutamol (●, *n* = 6) and salmeterol (△, *n* = 6). Each point is the mean of *n* determinations, and vertical bars represent s.e.mean.

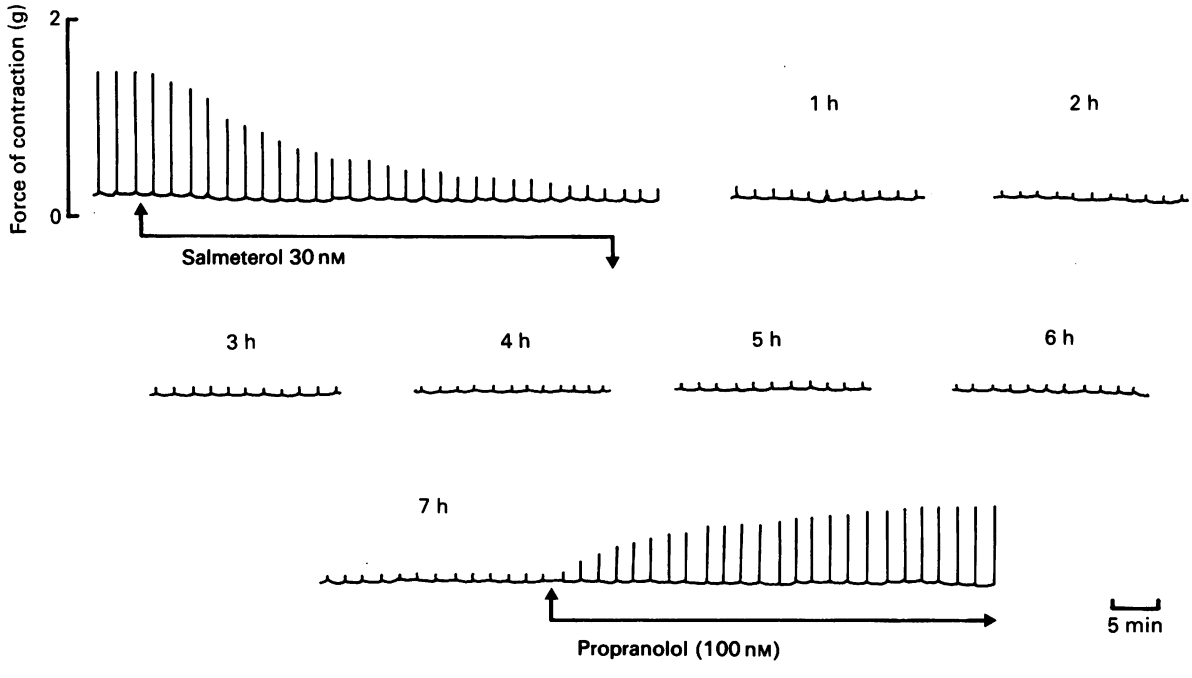


Figure 3 Electrically-stimulated, superfused, guinea-pig tracheal strip: typical inhibitory response to salmeterol, illustrating lack of recovery from inhibition despite 7 h continuous superfusion in drug-free Krebs solution, but rapid reversal by propranolol.

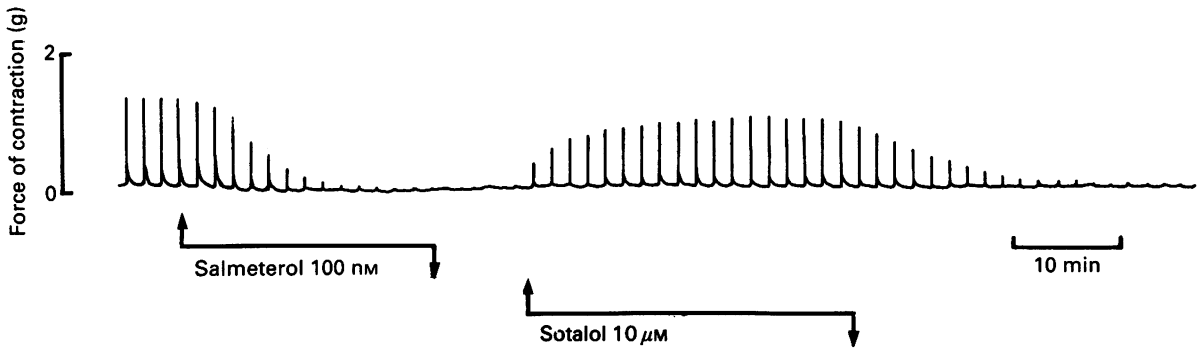


Figure 5 Superfused, electrically-stimulated guinea-pig trachea: representative experiment illustrating the effects of administration and subsequent washout of sotalol on the inhibitory responses persisting after washout of salmeterol. Note the reassertion of the salmeterol response without further agonist administration following washout of the sotalol.

for periods in excess of 50 min, despite continuous superfusion of the tissue.

Antagonist potency of propranolol against salmeterol on guinea-pig trachea

The interaction between propranolol and salmeterol was determined on $PGF_{2\alpha}$-contracted guinea-pig tracheal preparations. Propranolol (0.1, 0.3 and 1.0 μM) caused concentration-related, parallel, rightward shifts of salmeterol concentration-effect curves, with no evidence for a reduction in the maximal response. A pA_2 value for propranolol (mean; 95% confidence limits) of 8.97 (8.12–9.81, $n = 11$), the plot having a slope of −1.02 (0.57–1.47) was determined by the method of Arunlakshana & Schild (1959). There was, however, a marked degree of variability observed as indicated by the wide confidence limits on both pA_2 and slope.

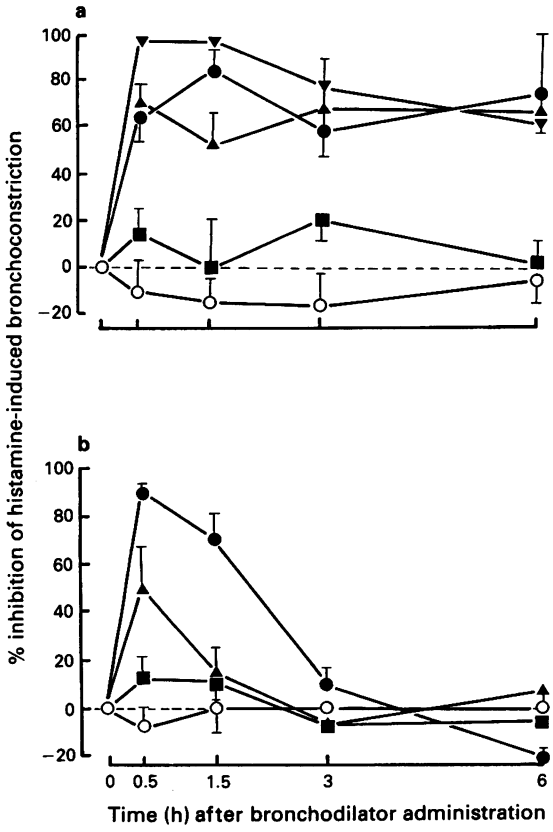


Figure 6 Conscious guinea-pig: time course of inhibition of histamine-induced increases in respiratory rate following aerosol administration of vehicle (○) or (a) salmeterol, nebulizer concentrations 0.012 mM (■), 0.12 (▲), 1.2 (●) and 12 mM (▼) or (b) salbutamol 0.02 mM (■), 0.2 mM (▲) and 2.0 mM (●). Each point is the mean of 6 determinations. Vertical bars represent s.e.means.

Interaction of sotalol with salmeterol

On both $PGF_{2\alpha}$-contracted and electrically-stimulated guinea-pig trachea preparations, infusions of the β-adrenoceptor blocking drug, sotalol (10 μM), caused a rapid and complete reversal of the responses persisting after administration and subsequent washout of salmeterol in concentrations (0.7–1.2 μM) sufficient to cause 75–90% maximal relaxation. When the sotalol infusion was halted, the salmeterol-induced relaxation was gradually re-established over a period of 40–60 min, despite the fact that no further salmeterol was administered (Figure 5).

In 3 experiments, administration of sotalol, and its subsequent withdrawal was repeated up to 4 times, with re-establishment of the original salmeterol-induced response on each occasion. In all of these experiments, concurrent control preparations demonstrated both the reproducibility of electrically-induced contractile responses in the absence of salmeterol and sotalol treatment over the time course of the experiment, and the maintenance of the established salmeterol-induced inhibition over the same time course.

Rat atria

Isoprenaline (0.4–500 nM) induced concentration-related increases in the force of contraction on rat atria, with a mean EC_{50} value of 33 (15–71, $n = 8$) nM. The mean maximal increase in force of atrial contraction was 0.51 (0.39–0.66, $n = 8$) g. In contrast, salbutamol and salmeterol caused increases in the force of contraction only at concentrations of 1 μM and above, causing no more than 38% of the isoprenaline maximum response. When concentration-effect curves to isoprenaline were constructed in the presence of salmeterol (72 μM), a 3.5 (1.7–7.4, $n = 5$) fold, rightward displacement of the isoprenaline concentration-effect curves was observed. In contrast, the effects of salbutamol (42 μM) were more marked, producing a 68 (22–208, $n = 6$) fold rightward shift of curves to isoprenaline, indicating that the compound has some degree of affinity for β_1-adrenoceptors.

In a subsequent series of experiments, similar results were obtained with guinea-pig left atria.

Guinea-pig fundus

Isoprenaline (0.001–30 μM), salbutamol (0.01–300 μM), salmeterol (0.001–300 μM) and the selective 'β_3-adrenoceptor agonist', BRL 35135 (0.001–30 μM), all caused concentration-related relaxation of $PGF_{2\alpha}$-contracted guinea-pig fundic strip preparations. None of the agonists was potent, isoprenaline having an EC_{50} of 120 (90–170, $n = 48$) nM, and BRL 35135 was of similar potency, with an EPC of 1.3 (0.9–1.8, $n = 5$). Both salbutamol and salmeterol were less potent, with EPC values of 37 (35–56, $n = 5$) and 23 (4–127, $n = 4$) respectively.

The rates of relaxation of guinea-pig fundus in response to isoprenaline and salbutamol were rapid, with equilibrium being reached within 3–6 min. Salmeterol and BRL35135 caused much slower relaxations, with equilibrium being attained only after 15–30 min. No attempt was made to quantify the rate of offset of action of any of the agonists on guinea-pig fundus.

Conscious guinea-pig

Aerosol administration Inhaled nebulized solutions of salmeterol (0.012–12 mM, $n = 6$) and salbutamol (0.02–2 mM, $n = 6$) caused dose-related protection against histamine-induced bronchoconstriction in the conscious guinea-pig (Figure 6), achieving almost complete inhibition at the highest dose tested. Over the effective dose-range, there were clear differences in the durations of action of the two compounds. Thus, while threshold-effective doses (i.e. the lowest dose required to cause at least a 50% inhibition) of salbutamol (nebulizer concentration, 0.2 mM) had a duration of action of less than 1.5 h, an equi-effective dose of salmeterol (nebulizer concentration, 0.12 mM) caused an inhibition of bronchoconstriction which was well-maintained for at least 6 h.

Even when the nebulizer concentration of salbutamol was increased 10 fold, the resulting inhibition of histamine-induced bronchoconstriction lasted for less than 3 h.

Discussion

In the *in vitro* pharmacological studies, salmeterol, like isoprenaline and salbutamol, was potent in relaxing both guinea-pig and human airways smooth muscle. Its effects on guinea-pig trachea were competitively antagonized by propranolol, indicating that these effects were mediated by β-adrenoceptors. Like salbutamol, salmeterol was shown to be only a weak partial agonist on the β_1-adrenoceptor-containing preparations, rat and guinea-pig left atria. It also possessed only modest agonist activity on the putative β_3-adrenoceptor (Zaagsma & Nahorski, 1990)-containing preparation, guinea-pig fundus (Coleman *et al.*, 1987), where it was approximately 20 fold less potent than either isoprenaline or the selective β_3-adrenoceptor agonist, BRL35135 (Wilson *et al.*, 1984). Although salmeterol does exhibit some relaxant activity on guinea-pig fundus, it is important to appreciate that this preparation is not sensitive to the relaxant effects of any of the β-adrenoceptor agonists, effective concentrations of salmeterol being in the micromolar range. In view of the high concentrations of salmeterol required on guinea-pig fundus and the lack of availability of a definitive β_3-adrenoceptor blocking drug, it is by no means certain that the activity of salmeterol on this preparation is mediated by β_3-adrenoceptors. Thus, while we can say that it is at least 20 fold weaker than isoprenaline at these receptors, it may in fact be weaker still. What is clear, however, is that salmeterol appears to possess a high degree of selectivity for β_2-adrenoceptors.

In isolated airways smooth muscle, salmeterol has a slow onset of action, the responses in many experiments often taking periods in excess of an hour to achieve equilibrium. This contrasts with the relatively rapid onset observed with the other β-adrenoceptor agonists included in this study. The most outstanding feature of salmeterol, however, is its long duration of action. Thus, in superfused preparations of both guinea-pig trachea and human bronchus, which demonstrated rapid and full recovery from the relaxant effects of isoprenaline and salbutamol, responses to salmeterol were well-maintained, despite continuous superfusion with drug-free medium. Indeed it was demonstrated in guinea-pig trachea that such responses were maintained for several hours. Interestingly, these responses to salmeterol were fully and rapidly reversed by infusions of the β-adrenoceptor antagonists propranolol and sotalol.

Furthermore, in guinea-pig trachea preparations in which salmeterol responses had been inhibited by sotalol, after stopping the sotalol infusion, the salmeterol-induced responses were gradually fully reasserted. The rapid and complete reversal of the sustained agonist effects of salmeterol by the β-adrenoceptor blocking drugs suggests that the long duration of its action does not result from irreversible binding to the β_2-adrenoceptors. However, the fact that on washout of the β-adrenoceptor blocking drug, sotalol, the salmeterol effect is reasserted, suggests that the drug must persist in the tissue in such a manner that it can freely associate with and dissociate from the β-adrenoceptors. The demonstration that this process can be repeated up to four times in succession with no loss in the salmeterol response, gives further weight to this suggestion. It is interesting however, that on guinea-pig trachea at least, there appears to be a simple competitive interaction between salmeterol and propranolol which is perhaps surprising if indeed the agonist is anchored in the vicinity of the β-adrenoceptors. We have previously suggested that the mechanism by which salmeterol exhibits its long duration of action involves binding to a specific exo-site in the cell membrane adjacent to the β-adrenoceptor (Bradshaw *et al.*, 1987), and may result in part from the lipid solubility of the compound. Indeed, the rank order of both onset and offset action for the three agonists included in this study correspond with the rank order of the lipophilicities of the molecules. Clearly, data obtained from only three compounds are insufficient to be able to draw any firm conclusions and further studies are required to establish the true relationship between onset and duration of action and the differential lipid solubility of β-adrenoceptor agonists.

Salmeterol is not only a potent, long-acting β_2-adrenoceptor agonist on airways smooth muscle preparations *in vitro*, but the present study shows that it is also a long-acting bronchodilator *in vivo* in conscious guinea-pigs. Thus, by aerosol administration, salmeterol caused dose-related inhibition of the increases in respiratory rate resulting from histamine-induced bronchoconstriction, being of a similar order of potency to salbutamol. However, as predicted from its *in vitro* activity, it exhibits a substantially longer duration of action than salbutamol. For example, at doses causing a 50% or greater inhibition of histamine-induced bronchoconstrictor responses, the effects of salmeterol persisted for at least 6 h with little or no decline in activity. In contrast, similarly effective doses of salbutamol exhibited only relatively transient activity, with no residual bronchodilatation being apparent 90 min after inhalation of a dose causing 70% inhibition of the histamine response. Even after administration of a dose of salbutamol 10 fold greater, the duration of bronchodilatation was still less than 3 h.

In clinical studies, salmeterol has also been shown to exhibit long-lasting bronchodilator activity after aerosol administration to both normal and asthmatic subjects (Maconochie *et al.*, 1987; Ullman & Svedmyr, 1988). Thus, the guinea-pig appears to resemble man, where again salmeterol is about ten fold more potent than salbutamol (Ullman & Svedmyr, 1988). Similarly, in terms of absolute duration of action, inhaled salbutamol has a well-established 4 to 6 h action in man (Leifer & Wittig, 1975), whereas preliminary data with salmeterol suggests a 12 h duration of action

(Ullman & Svedmyr, 1988). On this basis, therefore, it appears that the conscious guinea-pig is of some predictive value, in terms of relative potencies and durations of action of inhaled β_2-adrenoceptor bronchodilators in man.

In conclusion, salmeterol is a potent and selective β_2-adrenoceptor agonist with a long duration of action in relaxing airways smooth muscle *in vitro*. It also behaves as a long-acting bronchodilator *in vivo*, after administration by the inhaled route to guinea-pigs, where it is substantially longer acting than salbutamol. This profile of activity parallels that previously reported in man (Maconochie *et al.*, 1987; Ullman & Svedmyr, 1988). Salmeterol may therefore represent an important advance in the treatment and management of bronchial asthma.

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Salmeterol: a potent and long-acting inhibitor of inflammatory mediator release from human lung

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1 The effects of salmeterol, a novel long-acting β_2-adrenoceptor agonist, have been investigated on antigen-induced mediator release from passively sensitized fragments of human lung *in vitro*.

2 Salmeterol was a potent inhibitor of the release of histamine ($-\log IC_{50} = 8.54$), leukotriene C_4 (LTC_4)/LTD_4 ($-\log IC_{50} = 9.07$) and prostaglandin D_2 ($-\log IC_{50} = 8.81$). It was slightly less potent (1–3 fold) than isoprenaline, but significantly more potent (10–35 fold) than salbutamol.

3 Propranolol competitively antagonized the inhibitory effects of salmeterol on histamine release ($pA_2 = 8.41$) and LTC_4/LTD_4 release, ($pA_2 = 8.40$) indicating an action via β-adrenoceptors.

4 The inhibitory effects of isoprenaline (20 nM) and salbutamol (200 nM) were removed after washing the lung tissue for 2 h and 4 h respectively. In contrast, the inhibitory effects of salmeterol (40 nM) were much longer-lasting, and were still evident after 20 h.

5 Salmeterol therefore exhibits potent and persistent inhibition of anaphylactic mediator release from human lung. This anti-inflammatory effect may be important for the therapeutic potential of salmeterol in the treatment of bronchial asthma.

Keywords: β-Adrenoceptor agonists; salmeterol; human lung fragments; mast cell; anaphylactic mediator release

Introduction

The release of inflammatory and/or spasmogenic mediators from cells in the lung contributes towards airway obstruction in bronchial asthma (Holgate, 1988). Histamine, leukotrienes and prostanoids released from tissue mast cells can cause bronchoconstriction, increase vascular permeability, stimulate the accumulation of inflammatory cells and contribute to hypersecretion of mucus (Lewis & Austen 1981; Wassermann, 1983). β_2-Adrenoceptor agonists such as salbutamol, as well as being potent bronchodilators, have been shown to inhibit the release of inflammatory and spasmogenic mediators from human lung tissue *in vitro* (Butchers *et al.*, 1980), and to inhibit the rise in levels of circulating mediators after antigen challenge of asthmatic subjects *in vivo* (Howarth *et al.*, 1985). These effects are believed to be mediated by β_2-adrenoceptors on lung mast cells (Butchers *et al.*, 1980). However, prior treatment of asthmatics with β-adrenoceptor agonists, such as salbutamol, fails to attenuate the late asthmatic response after allergen challenge (Cockroft & Murdock, 1987). Moreover, the characteristic hyper-responsiveness of asthmatic airways to bronchoconstrictor agents is not reduced by acute or long-term treatment with either salbutamol or terbutaline (Cockcroft & Murdock, 1987; Kraan *et al.*, 1985). As it is considered that inflammation of the airways is responsible for both the late response and increased airway responsiveness, it is generally accepted that β-adrenoceptor agonists have little or no demonstrable anti-inflammatory activity in clinical asthma. One explanation for this apparent anomaly is that the duration of action of the β-adrenoceptor agonists currently used is insufficiently long to protect against continual or intermittent exposure to allergen.

Salmeterol (Bradshaw *et al.*, 1987) is a potent β_2-adrenoceptor agonist with a long duration of action on airways smooth muscle *in vitro* (Ball *et al.*, 1987a), and as a bronchodilator *in vivo*, both in animals (Ball *et al.*, 1987b) and in man (Ullman & Svedmyr, 1988). In the present paper, we describe the effect of salmeterol as an inhibitor of antigen-induced mediator release from human lung fragments. In these experiments, the effects of salmeterol have been compared with those of the non-selective β-adrenoceptor agonist, isoprenaline, and the selective β_2-adrenoceptor agonist, salbutamol.

A preliminary account of this work has been communicated to the British Pharmacological Society (Butchers *et al.*, 1987).

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Methods

Passive sensitization of human lung tissue

The preparation and sensitization of human lung tissue was carried out as described previously (Butchers *et al.*, 1979). Briefly, samples of macroscopically normal human lung tissue were cut into fragments (HLF), weighing approximately 10 mg (wet weight) each, and washed free of blood in bicarbonate-buffered, Tyrode solution, pH 7.4. The HLF were sensitized by incubation at 20°C overnight in serum from an allergic donor, and then washed free of serum with Tyrode solution.

Each experiment was performed on tissue from a separate donor and within each experiment, treatments were carried out in triplicate.

Determination of β-agonist potency

Sensitized HLF were placed in 5 ml plastic tubes containing Tyrode solution (3 fragments in 500 μl) and incubated at 37°C. After 10 min, 10 μl of the β-agonist or vehicle was added, followed 30 min later by 50 μl of specific antigen (Bencard, U.K.) or Tyrode solution. After a further 20 min, the fluid from each tube (diffusate) was aspirated and stored at −70°C before assay for histamine, leukotriene C_4 (LTC_4)/LTD_4 and prostaglandin D_2 (PGD_2). The remaining HLF were stored at −70°C, and then lysed with boiling distilled water and assayed for unreleased histamine. To determine the time of onset of inhibitory activity, the β-agonist or vehicle was added at different times before challenge with antigen or Tyrode solution.

Antagonist studies

In experiments with the β-adrenoceptor antagonist, propranolol, sensitized HLF were preincubated with propranolol (0.01–1 μM) for 20 min, before addition of salmeterol. The

tissue was then incubated for a further 10 min before challenge with antigen or Tyrode solution. Concentration-ratios for salmeterol were calculated at the half maximum inhibition values achieved by salmeterol alone, and pA_2 values for each experiment were determined according to the method of Arunlakshana & Schild (1959).

Duration of action

To compare the durations of action of β-agonists, approximately 100 sensitized HLF were incubated for 20 min at 37°C with each test compound or vehicle. β-Agonists were tested at a single, equipotent concentration, derived from previous experiments, sufficient to cause just maximal inhibition of histamine release (Butchers *et al.*, 1980; 1987). The concentrations selected were: salmeterol (40 nM), salbutamol (200 nM) and isoprenaline (20 nM). At time 0, some HLF were transferred to tubes containing 500 μl of the same concentration of β-agonist or vehicle, and challenged with antigen. The remaining HLF were washed and incubated in a large volume (250 ml) of Tyrode solution at 37°C. At intervals thereafter, the washing fluid was replaced with fresh Tyrode solution at 37°C, and aliquots of HLF were removed and challenged with antigen or buffer as before.

In a separate series of experiments, HLF were preincubated with salmeterol (40 nM) or vehicle for 20 min at 37°C, washed for 8 h in Tyrode solution, and then incubated for a further 90 min in the presence of propranolol (1 μM) or vehicle before antigen challenge.

Assay of mediators

Histamine was assayed either on the guinea-pig isolated, superfused ileum (Butchers *et al.*, 1980) in the presence of the leukotriene receptor and β-adrenoceptor antagonists, FPL55712 (0.4 μM) and propranolol (0.7 μM), or by radioimmunoassay (Serotec, U.K.). LTC_4/LTD_4 was assayed by radioimmunoassay (Amersham, UK). PGD_2 was only measured in studies to determine agonist potency and was assayed by radioimmunoassay (Amersham, U.K.).

Calculation of results

The release of histamine was expressed as a percentage of the total histamine content for the tissue in each tube. The release of leukotrienes and prostaglandin was corrected for variations in fragment size by dividing values by the total histamine content. Values were then corrected for spontaneous release, and inhibition expressed as a percentage relative to vehicle-treated controls. Mean inhibition values within an experiment were calculated, and individual IC_{50} values was determined.

Statistical analysis

Values are expressed as mean ± s.e.mean. The statistical significance of differences was analysed by Student's unpaired *t* test and *P* values of less than 0.05 were considered to be significant.

Drugs

(−)-Isoprenaline hydrochloride (Sigma, U.K.) and (±)-salbutamol (Glaxo Group Research, U.K.) were dissolved in 20 μg ml^{-1} ascorbic acid in water and used immediately. Salmeterol (Glaxo Group Research, U.K.) was dissolved in ascorbic acid in water with the addition of acetic acid (final concentration 25 mM). 7-[3-(4-Acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylic acid (FPL55712, synthesized at Glaxo Group Research, U.K.) and (±)-propranolol (Sigma U.K.) were dissolved in distilled water and used immediately.

Results

Determination of β-agonist potency

Salmeterol (0.2–100 nM), salbutamol (0.8–500 nM) and isoprenaline (0.2–100 nM) each caused concentration-dependent inhibition of the release of histamine, LTC_4/LTD_4 and PGD_2 from HLF (Figure 1). Salmeterol appeared to be slightly less potent (1–3 fold) than isoprenaline, although only with inhibition of LTC_4/LTD_4 release was the difference statistically significant (Table 1). In contrast, salmeterol was significantly more potent (10–35 fold) than salbutamol with regard to inhi-

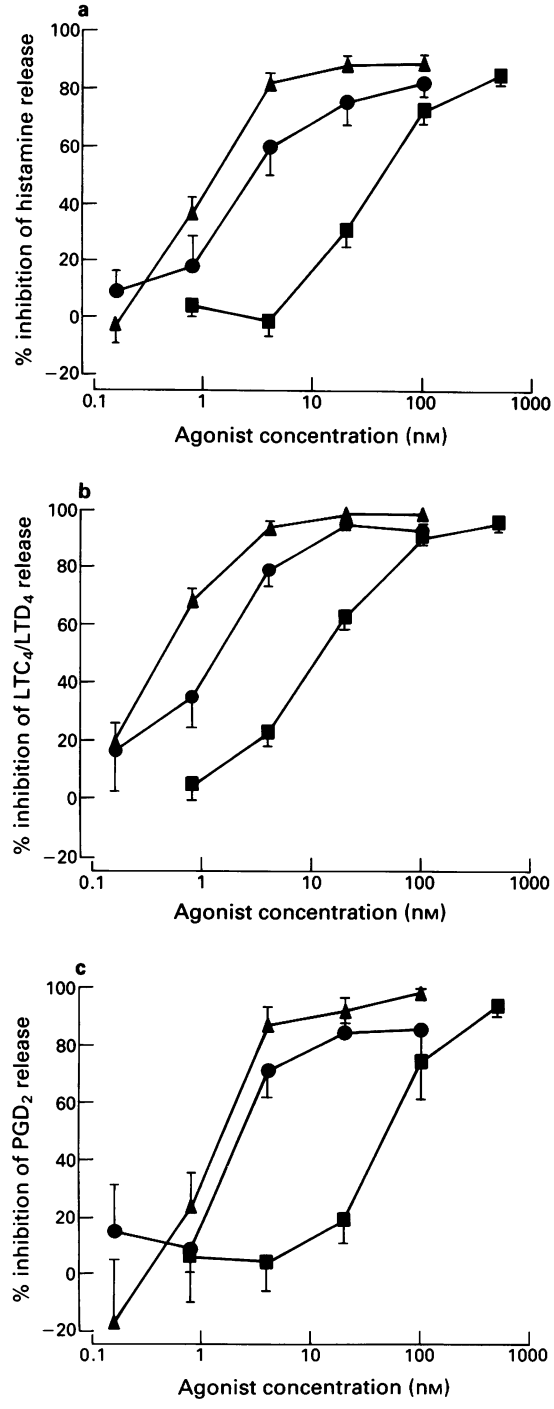


Figure 1 Inhibition of antigen-induced release of (a) histamine, (b) leukotriene C_4 (LTC_4)/LTD_4 and (c) prostaglandin D_2 (PGD_2) from human lung by salmeterol (●), isoprenaline (▲) and salbutamol (■). Human lung fragments were incubated with agonist for 30 min before antigen challenge. Mean curves from 11–24 (histamine and LTC_4/LTD_4) or 4–6 (PGD_2) separate experiments. Bars indicate s.e.mean.

Table 1 Inhibition of antigen-induced mediator release from human lung by β-adrenoceptor agonists

	$-log\ IC_{50}$					
Agonist	*Histamine*	n	LTC_4/LTD_4	n	PGD_2	n
Salmeterol	8.54 ± 0.22	11	9.07 ± 0.18	11	8.81 ± 0.12	4
Isoprenaline	8.95 ± 0.07	24	9.38 ± 0.06*	24	8.86 ± 0.11	6
Salbutamol	7.45 ± 0.09**	24	7.90 ± 0.07**	24	7.29 ± 0.13**	6

Lung fragments were incubated with agonist for 30 min before antigen challenge. Values are mean ± s.e.mean of *n* separate experiments. * $P < 0.05$; ** $P < 0.02$ compared with salmeterol.

bition of all three mediators. The maximum inhibition obtained with salmeterol was >80% in each case, and was not significantly different from the maxima obtained with salbutamol or isoprenaline ($P > 0.05$; Figure 1).

The onset of action of salmeterol was rapid, in that there was no significant difference in potency when salmeterol was incubated with HLF for 1, 10, 20 or 30 min before antigen challenge (Table 2).

Table 2 Inhibition of antigen-induced histamine release from human lung by β-adrenoceptor agonists: time of onset of inhibition

	$-log\ IC_{50}$					
Time (min)*	*Salmeterol*	n	*Isoprenaline*	n	*Salbutamol*	n
1	8.66	2	NT		NT	
10	8.44 ± 0.08	21	8.94 ± 0.12	12	7.40 ± 0.21	7
20	8.28 ± 0.16	7	8.77 ± 0.11	7	7.33 ± 0.12	7
30	8.54 ± 0.22	11	8.95 ± 0.07	24	7.45 ± 0.09	24

* Time of incubation with β-agonist before challenge. IC_{50} values are mean ± s.e.mean of *n* separate experiments. There was no significant difference ($P > 0.05$) between potency values at different time points for each agonist. NT = not tested.

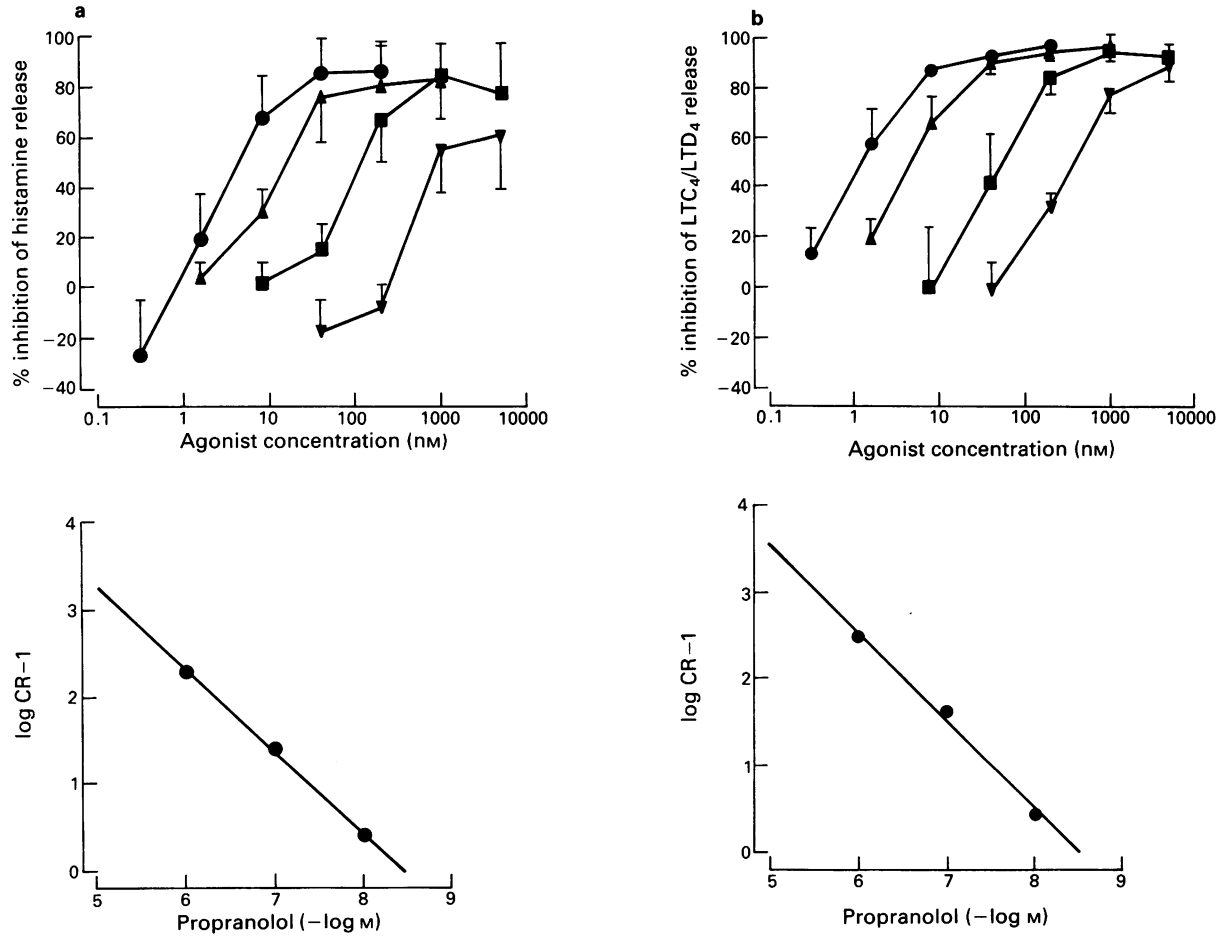


Figure 2 Inhibition of antigen-induced release of (a) histamine and (b) leukotriene C_4 (LTC_4)/LTD_4 from human lung by salmeterol: antagonism by propranolol. Human lung fragments were incubated with antagonist for 20 min before the addition of agonist and then for 10 min before antigen challenge. Upper panels: concentration-effect curves to salmeterol in the absence (●) and presence of propranolol at 0.01 μM (▲), 0.1 μM (■) and 1.0 μM (▼). Mean curves from 3 separate experiments. Bars indicate s.e.mean. Lower panels: Schild analysis of antagonism using concentration ratios (CR) calculated at half maximum inhibition of salmeterol alone. Mean pA_2 for histamine release (± s.e.mean) = 8.41 ± 0.11, slope = 0.99 ± 0.02 and for LTC_4/LTD_4 release = 8.40 ± 0.04, slope = 1.02 ± 0.03.

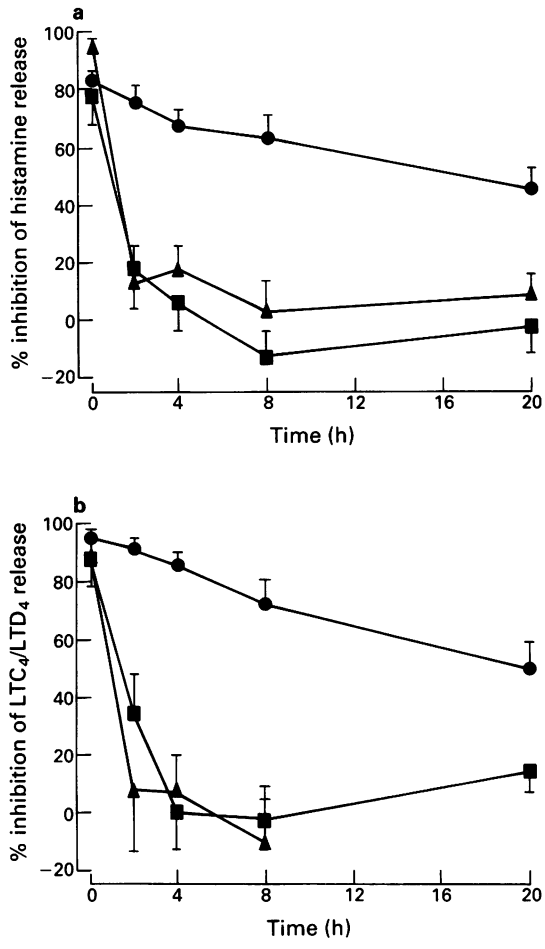


Figure 3 Duration of action of approximately equipotent concentrations of salmeterol, 40 nM (●), isoprenaline 20 nM (▲) and salbutamol, 200 nM (■) as inhibitors of antigen-induced release of (a) histamine and (b) leukotriene C_4 ($LTC)_4/LTD_4$ from human lung. Human lung fragments were incubated with agonist for 20 min before addition of antigen (zero time point), or washing and subsequent antigen challenge at the times indicated. Mean curves from 4–12 separate experiments. Bars indicate s.e.mean.

Antagonist studies

Propranolol (0.01–1.0 μM) antagonized the inhibitory effects of salmeterol in a competitive manner (Figure 2), giving mean pA_2 values of 8.41 ± 0.11 and 8.40 ± 0.04 against inhibition of histamine and LTC_4/LTD_4 release respectively. The slopes obtained from Arunlakshana & Schild plots were not significantly different from unity. Although the highest concentration of propranolol tested (1.0 μM) appeared to reduce the maximum inhibition of histamine release obtainable with salmeterol, this was not statistically significant ($P > 0.05$ compared to control maximum), and was not observed in experiments where leukotriene release was measured.

Duration of action

The inhibitory effects of isoprenaline (20 nM) and salbutamol (200 nM) on the release of histamine and LTC_4/LTD_4 were poorly maintained when HLF were washed (Figure 3), with complete loss of inhibitory activity ($P > 0.05$) within 2 h (isoprenaline) and 4 h (salbutamol). In contrast, the inhibitory effect of an approximately equipotent concentration of salmeterol (40 nM) persisted, despite repeated washing, with significant ($P < 0.005$) inhibition of the release of both mediators still being observed after 20 h. In a separate study, the residual effects of salmeterol (40 nM) on histamine (55.5 ± 11.1% inhibition) and LTC_4/LTD_4 (53.5 ± 18.0% inhibition) release after 8 h of washing, were completely reversed by propranolol (1 μM), the resulting levels of inhibition being −1.3 ± 16.4% and −11.3 ± 11.3% respectively ($n = 4$).

Discussion

Salmeterol is a β_2-adrenoceptor agonist with a long duration of action on airways smooth muscle *in vitro* (Ball *et al.*, 1987a) and as a bronchodilator *in vivo* (Ball *et al.*, 1987b). Clinical investigations with salmeterol in asthmatic patients have demonstrated improved airways function for up to 12 h, after a single inhaled dose of 50 μg (Ullman & Svedmyr, 1988).

The results presented here indicate that salmeterol also has a long duration of action as an inhibitor of antigen-induced release of both preformed (histamine) and newly generated (LTC_4/LTD_4) mediators from human lung tissues *in vitro*, and that it is significantly longer-acting than either salbutamol or isoprenaline. In a separate study, we have also shown salmeterol to have a longer duration of action than formoterol (Nials *et al.*, 1990). The mechanism by which salmeterol maintains its inhibitory activity is under investigation. It has been postulated that the agonist is retained in the vicinity of the β-adrenoceptor at a membrane 'exosite' (Bradshaw *et al.*, 1987; Nials & Coleman, 1988).

The potency of salmeterol in the present study is similar to that previously obtained on isolated airways smooth muscle from guinea-pig and man (Johnson *et al.*, 1989). However, the observation that the onset of action of salmeterol is rapid (< 1 min) in the present study contrasts with its slower onset of action on airways smooth muscle (Ball *et al.*, 1991), where submaximally effective concentrations require periods in excess of 30 min to achieve equilibrium effect. The reason for this apparent discrepancy is not clear, but it may be a reflection of different diffusion rates of salmeterol to the appropriate β-adrenoceptors in the two preparations.

Pretreatment of the tissue with propranolol, antagonized the inhibitory effects of salmeterol with characteristics indicative of simple competitive antagonism. The pA_2 values obtained for propranolol fell within the accepted range for β-adrenoceptors (Butchers *et al.*, 1980). In addition, the residual inhibitory effects of salmeterol 8 h after washing the tissue, were fully reversed by propranolol in experiments where a longer equilibration time with the antagonist was used. The effect of propranolol with shorter equilibration times has yet to be evaluated. Clearly, the inhibition of mediator release by salmeterol is achieved by an action on β-adrenoceptors, presumably on the lung mast cell.

Although we have studied the effect of salmeterol on only three of the proinflammatory substances known to originate from lung mast cells, it is probable that the release of other granular constituents, such as the potent mucus secretagogue, chymase (Sommerhoff *et al.*, 1989), and other newly-formed products are also inhibited.

In addition to their effects on mast cells, β-adrenoceptor agonists have the capacity to inhibit increases in vascular permeability, a processs that plays an important role in airway mucosal inflammation (Persson *et al.*, 1982; Svensjo & Grega, 1986). Whelan & Johnson (1990) have recently shown that the inhibitory effects of salmeterol on histamine-induced plasma protein extravasation in guinea-pig lung are significantly longer lasting than those of salbutamol. The same authors have also shown that aerosol administration of salmeterol to guinea-pigs has inhibitory effects on granulocyte accumulation in the lung (Whelan & Johnson, 1991).

Treatment of asthma with existing β-adrenoceptor agonists is largely symptomatic, and the compounds currently available have relatively short durations of action. As a result, the effect of these agonists may not be manifest at critical periods of the inflammatory response, so that they have little effect on airway inflammation. If, however, inhibitory effects of salmeterol on both anaphylactic mediator release and vascular permeability are sustained *in vivo*, then the drug should have

significant anti-inflammatory effects in man. Twentyman *et al.* (1990), showed that pretreatment of asthmatics with a therapeutic dose of salmeterol prevents the late asthmatic response and, perhaps more importantly, attenuates the increase in airway hyperresponsiveness following allergen challenge. Although these findings are encouraging, other studies are required before concluding that salmeterol has clinically important anti-inflammatory effects.

In conclusion, this study shows salmeterol to be a potent inhibitor of anaphylactic mediator release from human lung tissue, with a long duration of action comparable with that on airways smooth muscle. If salmeterol exhibits such persistent anti-inflammatory activity *in vivo* in man, these combined properties may be of great importance in the treatment and management of bronchial asthma.

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Histamine-induced inositol phosphate accumulation in HeLa cells: lithium sensitivity

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1 In the presence of 10 mM Li^+ the histamine-stimulated accumulation of [^{3}H]-inositol monophosphates ([^{3}H]-IP_1) in HeLa cells prelabelled with [^{3}H]-inositol increased over 10–20 min to a plateau level, which was normally maintained up to 60 min. Levels of [^{3}H]-inositol bis- and trisphosphates ([^{3}H]-IP_2 and [^{3}H]-IP_3) initially increased rapidly but declined to near basal levels by 20 min.

2 The same pattern of histamine-induced [^{3}H]-IP_1 accumulation was observed in cells in which [^{3}H]-inositol was present 30 min before and during the incubation with histamine. Concentration-response curves for histamine measured in the presence of 10 mM Li^+ were closely similar in cells prelabelled for 24 h with [^{3}H]-inositol and in cells exposed to [^{3}H]-inositol for only 30 min before addition of histamine, without removing the [^{3}H]-inositol. The EC_{50} for histamine was 1.6 ± 0.2 μM.

3 [^{3}H]-IP_1 accumulation induced by a sub-maximal concentration of histamine, 1 μM, also reached a plateau, but at a lower level than with 1 mM histamine.

4 Addition of 10 mM NaF at the plateau phase of [^{3}H]-IP_1 accumulation induced by 1 mM histamine resulted in a further increase in the level of [^{3}H]-IP_1. The level of [^{3}H]-IP_1 in the presence of histamine + NaF was 1.4 ± 0.2 fold of that of the sum of the responses to histamine and NaF acting alone (basal levels subtracted).

5 Addition of 1 μM mepyramine at the plateau phase of [^{3}H]-IP_1 accumulation induced by 1 mM histamine in the presence of 10 mM Li^+ resulted in a decline in the level of [^{3}H]-IP_1, implying that the metabolism of [^{3}H]-IP_1 is not completely blocked by 10 mM Li^+ in HeLa cells.

6 Omission of the 15 min preincubation period with 10 mM Li^+ before stimulation with 100 μM histamine for 15 min resulted in an approximate halving of the level of [^{3}H]-IP_1, without any significant change in basal accumulation. Periods of preincubation with 10 mM Li^+ longer than 15 min did not produce any further increase in the level of [^{3}H]-IP_1 induced by histamine.

7 Basal and histamine-induced levels of [^{3}H]-IP_1 increased as the concentration of Li^+ was increased from 0 to 60 mM, but the effect on the histamine-induced response was greater than on the basal level. Increasing the concentration of Li^+ from 0 to 60 mM had only a small and mostly statistically insignificant effects on the levels of [^{3}H]-IP_2 and [^{3}H]-IP_3. The EC_{50} for histamine-induced [^{3}H]-IP_1 accumulation in the presence of 30 mM Li^+ was 2.4 ± 0.4 μM.

8 The results indicate that IP_1 metabolism in HeLa cells is much less sensitive to Li^+ than in mammalian brain. The plateau phase of histamine-induced [^{3}H]-IP_1 accumulation in the presence of 10 mM Li^+ thus represents a steady-state level. On a simple model the plateau level will be proportional to the rate of [^{3}H]-IP_1 formation at that time. The lower the concentration of Li^+ present, the better the approximation will be. The information obtained is thus not the same as when [^{3}H]-IP_1 metabolism is completely blocked, in which case [^{3}H]-IP_1 accumulated can be used to calculate a mean rate of formation over the whole of the incubation period.

Keywords: Histamine; HeLa cells; inositol phosphates; lithium; fluoride

Introduction

HeLa cells, originally derived from a human uterine carcinoma, are potentially a useful model system in which to study histamine H_1-receptor mechanisms in intact cells. The cells are relatively easy to grow in culture and histamine has been shown to stimulate phosphoinositide hydrolysis (Tilly *et al.*, 1990a,b; Bristow & Young, 1991; Raymond *et al.*, 1991), which appears to be the primary response to H_1-receptor activation in all mammalian tissues (reviewed by Hill, 1990). Preliminary measurements with [^{3}H]-mepyramine indicate that HeLa cell membranes contain a reasonably high density of H_1-receptors (Bristow & Young, 1991), which would make the cells particularly suitable for binding studies with a new quaternary radioligand, [^{3}H]-(+)-N-methyl-4-methyldiphenhydramine (Treherne & Young, 1988), designed to detect changes in the numbers and properties of plasma membrane H_1-receptors under various conditions, such as desensitization. However, the regulation of H_1-receptors in HeLa cells may differ in some respects from those in other cell lines. Pre-exposure to histamine has been reported not to cause desensitization of H_1-receptors in HeLa cells, as indicated by the failure of histamine pretreatment to decrease histamine-induced [^{3}H]-IP_1 accumulation (Tilly *et al.*, 1990a). In contrast, in the BC3H-1 smooth muscle cell line (Brown *et al.*, 1986), in the 1321N1 human astrocytoma cell line (Nakahata & Harden, 1987) and in cultured rat cerebellar granule cells (Dillon-Carter & Chuang, 1989) there is evidence for desensitization of cellular responses to histamine H_1-receptor activation and that it may involve changes at the receptor level. We have therefore set out to characterize histamine H_1-receptor-mediated phosphoinositide hydrolysis in HeLa cells in more detail. We show here that assessment of histamine-induced [^{3}H]-inositol phosphate accumulation in these cells over extended periods of incubation is complicated by the relative insensitivity of monophosphatase activity to inhibition by lithium ions.

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Methods

Cell culture

HeLa cells were grown to near confluence in Dulbecco's modified Eagle's medium (DMEM, Gibco), containing 10% (v/v) bovine calf serum (Gibco) and supplemented with penicillin (50 u ml^{-1}) and streptomycin (50 mg ml^{-1}) (Flow Laboratories), in flasks at 37°C in a CO_2-incubator (5% CO_2).

Labelling of cells with [^{3}H]-inositol and incubation with histamine

Two main protocols were used for labelling cellular inositol phospholipids with [^{3}H]-inositol. In most experiments cells were cultured in an [^{3}H]-inositol-containing medium for the last day of growth and free extracellular [^{3}H]-inositol washed out before stimulation with agonist. In the second protocol, cells were incubated for a short period (30 min) with [^{3}H]-inositol before addition of the agonist and further incubation in the continued presence of [^{3}H]-inositol.

(a) The culture medium was removed and the cells washed in approximately 10 ml inositol-free DMEM before addition of inositol-free DMEM containing 10% dialysed calf serum, 10 μM *myo*-inositol and 5 μCi ml^{-1} [^{3}H]-inositol (0.32 μM). The cells were then incubated for 20–24 h at 37°C in a CO_2-incubator (5% CO_2 atmosphere). The [^{3}H]-inositol labelled cells were washed three times with approximately 15 ml phosphate buffered saline (in mM: NaCl 137, KCl 2.7, Na_2HPO_4 8.1 and KH_2PO_4 1.5) and once in phosphate buffered saline containing 0.6 mM ethylenediaminetetraacetic acid (EDTA) before dissociation with 10 ml trypsin/EDTA (500–750 BAEE units ml^{-1}/0.6 mM, Sigma). After centrifugation at 220 *g* for 5 min, the cells were resuspended in Krebs-Henseleit buffer (in mM: NaCl 116, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5, glucose 11, O_2:CO_2 95:5% gassed). Cells (50 μl, approximately 3 × 10^5 cells) were added to 190 μl Krebs-Henseleit solution containing LiCl (5–60 mM), where appropriate, and incubated for 15 min at 37°C, before addition of 10 μl histamine (final concentration 0.1–1000 μM). Antagonists, where present at the beginning of an incubation with histamine, were added 15 min before the agonist. After each addition the tubes were gassed with O_2:CO_2 (95:5%, v/v) and the incubation continued at 37°C in a shaking water bath for 15 s–60 min.

(b) HeLa cells were recovered from the culture flasks by treatment with trypsin/EDTA and centrifugation as above. The cells were resuspended in Krebs-Henseleit medium (1–2 ml per 150 cm^2 flask) and incubated at 37°C for 20 min. Aliquots of the cell suspension (50 μl) were then added to 190 μl Krebs-Henseleit medium, containing 1 μCi [^{3}H]-inositol (0.32 μM) and 10 mM LiCl and incubated for 30 min at 37°C before addition of 10 μl histamine and further incubation as above.

In a limited number of experiments a combination of the above protocols was adopted, in that cells were labelled for 20–24 h with [^{3}H]-inositol, but the same concentration of [^{3}H]-inositol was present during incubation with the agonist.

Measurement of [^{3}H]-inositol phosphate accumulation

Incubations were terminated by addition of 250 μl of an ice-cold solution of 10% perchloric acid, containing 1 mM EDTA and 1 mg ml^{-1} phytic acid, and allowed to stand on ice for 15 min. Inositol phosphates were extracted by the trioctylamine-freon method (Sharpes & McCarl, 1982; Downes *et al.*, 1986). Trioctylamine/1,1,2-trichlorotrifluoroethane (1:1, v/v) (0.4 ml) was added to the sample, the solution mixed thoroughly and then centrifuged at 7000 *g* for 3 min to separate the phases. A sample (0.38 ml) of the upper phase was transferred to an insert vial, 3 ml 100 mM HEPES, pH 7.5, added and the mixture applied to an AG 1-X8 (formate form, 100–200 mesh; Biorad) anion-exchange column.

[^{3}H]-inositol and [^{3}H]-glycerophosphoinositol were eluted with 10 ml water and 10 ml 60 mM ammonium formate/5 mM sodium tetraborate, respectively, and [^{3}H]-inositol monophosphates ([^{3}H]-IP$_1$) then eluted with 10 ml 200 mM ammonium formate/100 mM formic acid. Higher phosphates were eluted by sequential addition of 10 ml 400 mM ammonium formate/100 mM formic acid (for [^{3}H]-inositol bisphosphates, [^{3}H]-IP$_2$), 10 ml 800 mM ammonium formate/100 mM formic acid (for [^{3}H]-inositol trisphosphates, [^{3}H]-IP$_3$) and 10 ml 1 M ammonium formate/100 mM formic acid (for [^{3}H]-inositol tetrakisphosphates, [^{3}H]-IP$_4$). Quicksafe A (10 ml, Zinnser) was added to each fraction and tritium determined by liquid scintillation counting. Within an experiment 3–4 replicate determinations were made of each incubation condition.

Analysis of data

Concentration-response data for histamine-induced [^{3}H]-IP$_1$ accumulation (after subtraction of basal accumulation) were fitted to a Hill equation (logistic equation) using the Harwell Library non-linear regression programme VB01A as implemented on the Cambridge IBM 3084. The actual equation fitted was:

$$[^3H]\text{-}IP_1 \text{ accumulated} = Resp_{max} \times C^n/(C^n + EC_{50}^n)$$

where C is the histamine concentration, EC_{50} is the concentration giving half-maximal response, and $Resp_{max}$ is the maximum response. Each point was weighted according to the reciprocal of the variance associated with it.

Drugs

[^{3}H]-*myo*-inositol, 15–18 Ci mmol^{-1}, was purchased from New England Nuclear and histamine dihydrochloride and mepyramine maleate from Sigma.

Results

Time-course of histamine-stimulated [^{3}H]-inositol phosphate accumulation in 10 mM Li$^+$

The time-course of the accumulation of [^{3}H]-IP$_1$, [^{3}H]-IP$_2$ and [^{3}H]-IP$_3$ induced by 1 mM histamine in the presence of 10 mM Li$^+$ in HeLa cells prelabelled for 20 h with [^{3}H]-inositol is shown in Figure 1. Appreciable amounts of [^{3}H]-IP$_2$ and [^{3}H]-IP$_3$ were present at early times after histamine addition (2–10 min), but thereafter the accumulation in the presence of histamine was only modestly greater than basal levels (Figures 1b and 1c). In contrast, histamine-stimulated [^{3}H]-IP$_1$ increased to a plateau which was maintained over the period of the experiment (Figure 1a). Since the amounts of [^{3}H]-IP$_2$ and [^{3}H]-IP$_3$ after 15 min incubation with histamine are a small proportion of the total, measurements made on [^{3}H]-IP$_1$, rather than on total [^{3}H]-inositol phosphates, have the advantage of significantly reducing basal levels (mean basal level of [^{3}H]-IP$_1$ 6069 ± 514 d.p.m., 21 measurements, cf. 10,430 ± 594 d.p.m. for the combined basal levels of [^{3}H]-IP$_1$, [^{3}H]-IP$_2$ and [^{3}H]-IP$_3$ in the same experiments), without introducing any appreciable error in the determination of histamine-induced total [^{3}H]-inositol phosphate formation, thereby increasing the ratio of histamine-induced to basal accumulation (2.1 ± 0.2, 16 measurements with 1 mM histamine).

[^{3}H]-IP$_1$ formation stimulated by 100 μM histamine was inhibited by 83 ± 7% (5 measurements) by 1 μM mepyramine, an H_1-receptor antagonist (theoretical inhibition 81%, assuming a K_d of 3.8 nM for mepyramine, Bristow & Young, 1991, and a K_d of 1.6 μM for histamine, see below). Cimetidine, an H_2-receptor antagonist, had no significant effect on the

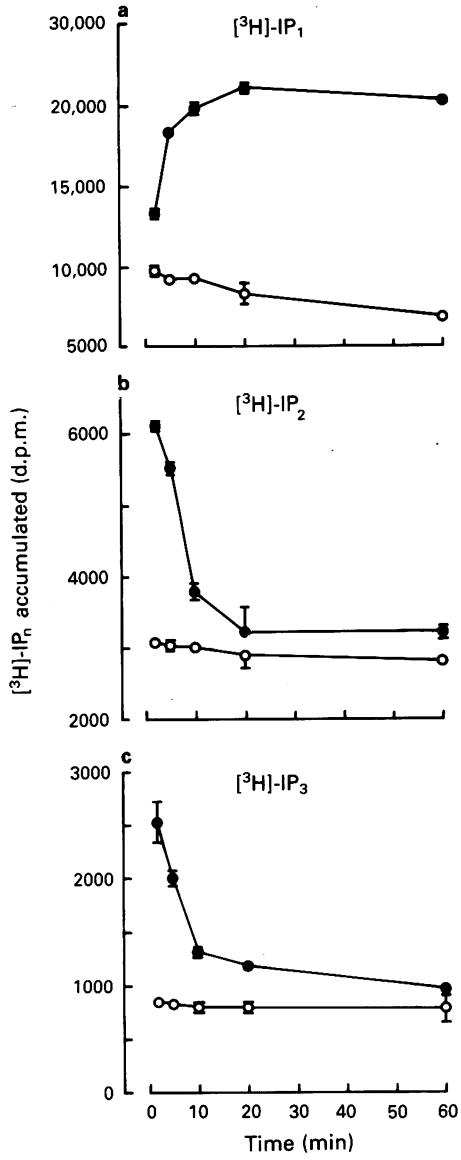


Figure 1 Time-course of histamine-induced [^{3}H]-inositol phosphate accumulation. HeLa cells, prelabelled for 20–24 h with [^{3}H]-inositol, were incubated in the presence of 10 mM Li^+ in the presence or absence of 1 mM histamine and (a) [^{3}H]-IP_1, (b) [^{3}H]-IP_2 and (c) [^{3}H]-IP_3 measured as described under Methods. Points are the means from triplicate determinations within a single experiment; s.e.mean shown by vertical bars. Where no error bars are shown the error was within the size of the symbol. The whole experiment was repeated a further 3 times with qualitatively similar results. Note that the ordinates of the graphs in (a) and (b) do not start at zero. (○), No histamine (basal accumulation); (●), 1 mM histamine.

response to 100 μM histamine (2 ± 2% inhibition, 3 measurements).

In many experiments, but not in all, histamine (100 μM or 1 mM) also induced the accumulation of labelled material in the [^{3}H]-IP_4 fraction after 60 min incubation. This stimulated accumulation was apparent even in experiments in which the histamine-stimulated levels of [^{3}H]-IP_2 and [^{3}H]-IP_3 at 60 min were negligible. The basal level of tritium in the [^{3}H]-IP_4 fraction was usually high (mean 3428 ± 378 d.p.m., 19 measurements), with the result that the stimulation by histamine expressed as fold over basal was modest (1.2 ± 0.1, 14 measurements). The identity of the presumed [^{3}H]-IP_4 isomer(s) present in resting and stimulated cells remains unknown.

The most apparent feature of the time-course of histamine-stimulated [^{3}H]-IP_1 accumulation was that a plateau phase was reached. There are several possible factors which could be responsible, in whole or in part, for the failure of histamine to produce a sustained increase in [^{3}H]-IP_1 accumulation. One is that the amount of available phosphatidylinositol-4,5-bisphosphate ([^{3}H]-PIP_2) could be limiting in [^{3}H]-inositol-prelabelled cells. However, a similar time-course with 1 mM histamine to that shown in Figure 1 was observed in experiments in which [^{3}H]-inositol was added 30 min before the agonist and maintained in the medium during the incubation period, although the absolute magnitude of the response was less (data not shown). The lack of influence of the labelling conditions on the pattern of the response to histamine was also indicated by the close similarity in the position and shape of concentration-response curves to histamine measured in cells prelabelled with [^{3}H]-inositol for 20–24 h and in cells prelabelled for 30 min (Figure 2). The curves approximated closely to hyperbolae (best-fit value of the Hill coefficient to the combined data 1.07 ± 0.14) and the EC_{50}, 1.6 ± 0.2 μM, was in accord with the EC_{50} of approximately 1 μM reported by Tilly *et al.* (1990a). A similar time-course of 1 mM histamine-induced [^{3}H]-IP_1 accumulation to that shown in Figure 1a was also observed in one experiment in which cells were both prelabelled for 20 h and the [^{3}H]-inositol concentration maintained at the same level during the incubation with histamine. All of these data suggest that the plateau phase of histamine-induced [^{3}H]-IP_1 accumulation is not a consequence of a limitation in the amount of [^{3}H]-PIP_2 available to the H_1-receptor/G-protein-coupled phosphoinositidase C.

This conclusion is strengthened by time-course measurements with sub-maximal concentrations of histamine. [^{3}H]-IP_1 accumulation induced by 1 μM and 1 mM histamine in the presence of 10 mM Li^+ is shown in Figure 3. A plateau phase was again reached by 15 min with 1 μM histamine, but at a lower level than with 1 mM histamine. Amounts of [^{3}H]-IP_2 in the presence of 1 μM histamine did not differ significantly from basal levels at any time. There was a small peak of [^{3}H]-IP_3

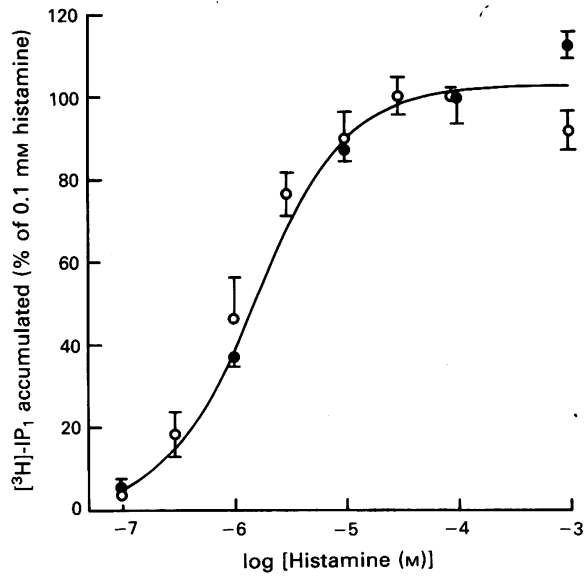


Figure 2 Concentration-response curve for histamine-induced [^{3}H]-inositol monophosphate ([^{3}H]-IP_1) accumulation in the presence of 10 mM Li^+. Cells prelabelled for 20–24 h with [^{3}H]-inositol (●) or cells exposed to [^{3}H]-inositol for 30 min before addition of histamine, but without removal of extracellular [^{3}H]-inositol (○), were incubated with various concentrations of histamine for 60 min in the presence of 10 mM Li^+ (added 15 or 30 min, respectively, before histamine). To allow for differences in the magnitude of the response between experiments, all values are expressed as a percentage of the response to 100 μM histamine. Points are the combined values (weighted means ± approximate s.e.mean) from 3 (prelabelled 20–24 h) or 4 (prelabelled 30 min) experiments. The line drawn is the best-fit curve obtained from non-linear regression analysis as described under Methods.

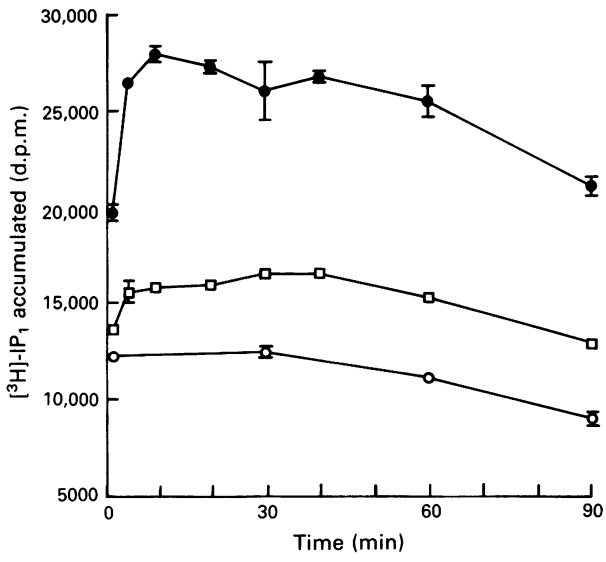


Figure 3 Time-course of [^{3}H]-inositol monophosphate ([^{3}H]-IP_1) accumulation induced by 1 μM and 1 mM histamine in the presence of 10 mM Li^+. [^{3}H]-IP_1 was measured as described under Methods in the presence of 10 mM Li^+. Points are the means from triplicate determinations within a single experiment; s.e.mean shown by vertical bars. Where no error bars are shown the error was within the size of the symbol. The whole experiment was repeated a further 3 times (twice with 100 μM histamine instead of 1 mM). Note that the ordinate scale does not start at zero. (○), No histamine (basal accumulation); (●) 1 mM histamine; (□), 1 μM histamine.

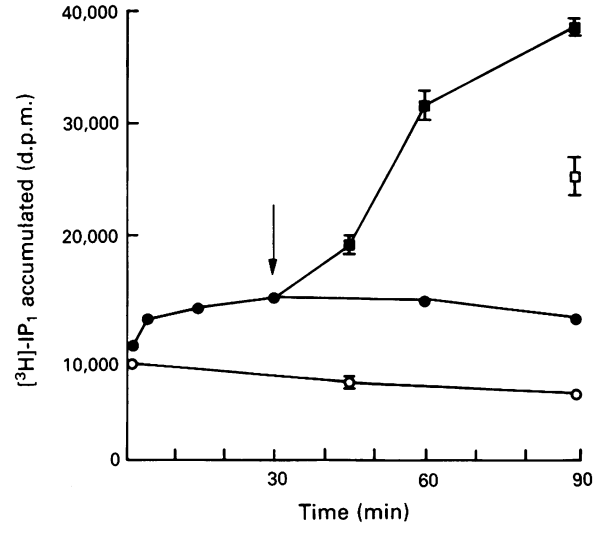


Figure 4 Time-course of [^{3}H]-inositol monophosphate ([^{3}H]-IP_1) accumulation induced by histamine in the presence and absence of NaF. Accumulation of [^{3}H]-IP_1 in the presence of 10 mM Li^+ was measured as described under Methods. NaF (10 mM) was added at 30 min (arrow) in the presence or absence of 1 mM histamine. Points are the mean from triplicate determinations in a single experiment; s.e.mean shown by vertical bars. Where no error bars are shown the error was within the size of the symbol. The experiment was repeated with qualitatively similar results. (○) No histamine, no NaF (basal accumulation); (□) 10 mM NaF; (●) 1 mM histamine; (■) 1 mM histamine + 10 mM NaF.

at early times with 1 μM histamine, but as with [^{3}H]-IP_3 induced by 1 mM histamine (Figure 1c) the level had fallen to near basal by 20 min. The lower plateau level of [^{3}H]-IP_1 in the presence of 1 μM histamine provides further evidence against a limitation in the size of the [^{3}H]-PIP_2 pool available to histamine. It is also notable that the rates at which the plateau is reached with 1 μM and 1 mM histamine are similar.

Not every experiment with histamine in the presence of 10 mM Li^+ showed a maintained plateau phase. In one experiment with 1 mM histamine on [^{3}H]-inositol prelabelled cells with [^{3}H]-inositol still present, [^{3}H]-IP_1 accumulation reached a peak at *circa* 10 min and then declined, so that after 90 min incubation the stimulated level did not differ significantly from basal. The basal level also declined over 90 min, but to a lesser extent than the histamine-stimulated response. A similar decline in [^{3}H]-IP_1 levels after *circa* 20 min was also observed in one experiment each with 1 and 5 μM histamine. Some indication of a decline with time in basal and 1 mM histamine-induced levels of [^{3}H]-IP_1 after 60 min is also apparent in Figure 3.

Effect of fluoride ions on the response to histamine

Evidence that the plateau phase in histamine-stimulated [^{3}H]-IP_1 accumulation (Figure 1a) is not due to a loss of responsiveness at the post-receptor level was obtained by measuring the effect of fluoride ions, which stimulate G-proteins directly by acting in the form of the complex anion AlF_4^- (Bigay *et al.*, 1985, and references therein). HeLa cells prelabelled with [^{3}H]-inositol were stimulated with 1 mM histamine for 30 min in the presence of 10 mM Li^+ in order to reach the plateau level of [^{3}H]-IP_1 accumulation and 10 mM NaF then added. The effect of the added F^- was to cause a large increase in [^{3}H]-IP_1 levels (Figure 4). In the absence of F^- the level of [^{3}H]-IP_1 induced by histamine was maintained for a further 60 min (Figure 4). In 3 experiments in which the effect of 10 mM NaF added at 30 min was measured 60 min later, the response to histamine (100 μM or 1 mM) + NaF (10 mM) was greater than the sum of the responses to histamine and NaF acting alone (ratio 1.4 ± 0.2, 3 experiments, basal accumulations subtracted throughout). This additivity (or, possibly, synergism) argues strongly against a loss of response at the G-protein or post-G-protein level in histamine-stimulated cells.

Effect of mepyramine addition during the plateau phase

An explanation for the plateau phase in the response to histamine in the presence of 10 mM Li^+ which does not require any severe limitation in some aspect of histamine receptor function is that the plateau is in fact a steady-state level representing a balance between [^{3}H]-IP_1 formation and breakdown. This implies that 10 mM Li^+ could not be causing a complete block of [^{3}H]-IP_1 breakdown. Direct evidence for the steady-state proposition was provided by experiments in which HeLa cells were first incubated with histamine for 30 min in order to achieve the plateau level of [^{3}H]-IP_1 and then treated with 1 μM mepyramine to inhibit further histamine-stimulated [^{3}H]-IP_1 formation. The addition of mepyramine resulted in a fall in the level of [^{3}H]-IP_1 (Figure 5), indicating that the H_1-receptor is still functional 30 min after addition of 1 mM histamine and that [^{3}H]-IP_1 metabolism has not been blocked completely by 10 mM Li^+.

Effect of the length of the preincubation with 10 mM Li^+

To investigate whether the inability of 10 mM Li^+ to prevent [^{3}H]-IP_1 breakdown was due to a slow penetration of Li^+ into HeLa cells, measurements were made of the effect of varying the preincubation period with 10 mM Li^+ between 0 and 60 min on the levels of [^{3}H]-IP_1 determined after a further 15 min incubation in the presence or absence of histamine (Figure 6). Basal levels of [^{3}H]-IP_1 were not significantly affected by changing the preincubation period from 0 to 15 min, but the level of histamine-induced [^{3}H]-IP_1 was approximately doubled (level with no preincubation with 10 mM Li^+ 51 ± 3% of that with 15 min preincubation, mean ± s.e.mean from 3 experiments). Increasing the time of preincubation with Li^+ beyond 15 min had no further effect

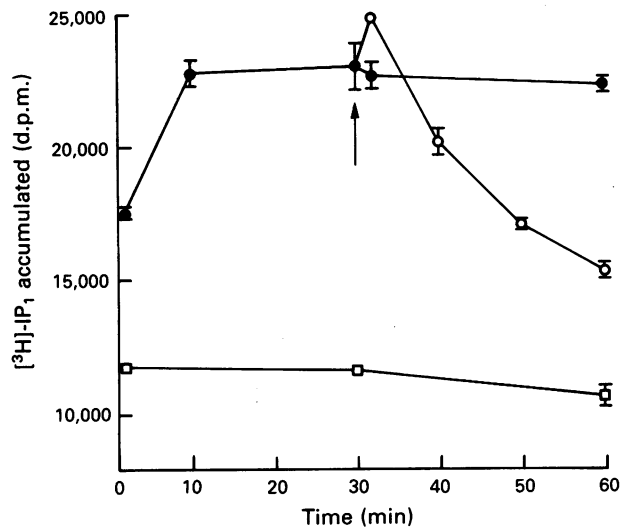


Figure 5 Effect of the addition of mepyramine at the plateau phase of histamine-induced [^{3}H]-inositol monophosphate ([^{3}H]-IP_1) accumulation in the presence of 10 mM Li^+. Mepyramine (1 μM) was added 30 min after 1 mM histamine (at arrow). Values are means from triplicate determinations from a single experiment; s.e.mean shown by vertical bars. Where no error bars are shown the error was within the size of the symbol. The experiment was repeated, in whole or in part, twice with similar results. Note that the ordinate scale does not start at zero. (□) No histamine (basal); (●) 1 mM histamine; (○) 1 mM histamine + 1 μM mepyramine.

on the level of [^{3}H]-IP_1 in the presence of histamine (Figure 6).

Basal levels of [^{3}H]-IP_2 and [^{3}H]-IP_3 were not significantly altered by increasing the time of preincubation with 10 mM Li^+ from 0 to 60 min. The change in the level of these phosphates after 15 min incubation with 100 μM histamine was small or statistically insignificant and plotting the data as [^{3}H]-IP_1 + [^{3}H]-IP_2 + [^{3}H]-IP_3 did not change the pattern of the response from that shown in Figure 6.

The marked increase in histamine-stimulated [^{3}H]-IP_1 accumulation argues strongly that Li^+ can penetrate HeLa cells. The lack of any further increase with longer preincubation suggests that the inability of Li^+ to cause a complete block of [^{3}H]-IP_1 breakdown is due to a lower sensitivity of the monophosphatases in HeLa cells than those in mammalian brain.

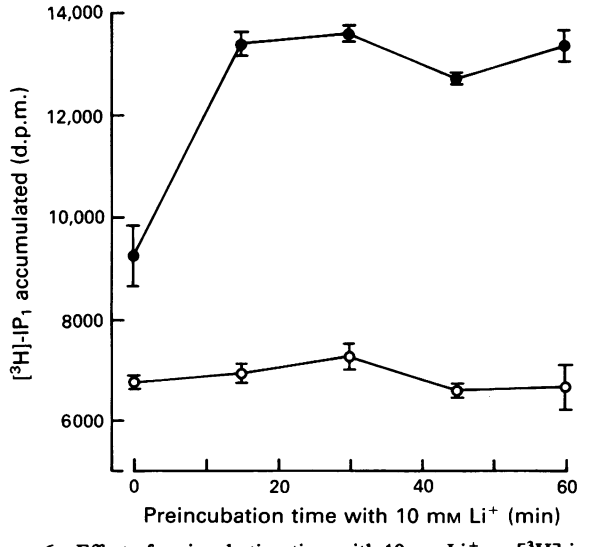


Figure 6 Effect of preincubation time with 10 mM Li^+ on [^{3}H]-inositol monophosphate ([^{3}H]-IP_1) accumulation. Cells were preincubated with 10 mM Li^+ for 0–60 min before addition of 100 μM histamine and further incubation for 15 min. Points are the means of triplicate determinations from a single experiment; s.e.mean shown by vertical bars. The experiment was repeated twice with 100 μM and once with 1 mM histamine with qualitatively similar results. Note that the ordinate scale does not start at zero. (○) No histamine (basal accumulation); (●) 100 μM histamine.

Concentration-dependence of Li^+ potentiation of [^{3}H]-inositol monophosphate accumulation

The effect of increasing the concentration of Li^+ from 0 to 60 mM on the basal and histamine-induced [^{3}H]-IP_1 accumulation is shown in Figure 7. Preincubation with Li^+ was for 15 min and stimulation with 1 mM histamine for 60 min. Both basal and histamine-stimulated [^{3}H]-IP_1 levels increased with increasing concentrations of Li^+, but even with 30 mM Li^+ the maximal effect was not obtained. However, Li^+ had a much greater effect on the histamine-induced level than on the basal level (Figure 7). With no Li^+ present the basal level of [^{3}H]-IP_1 was 57 ± 2% of that with 30 mM Li^+, whereas in the

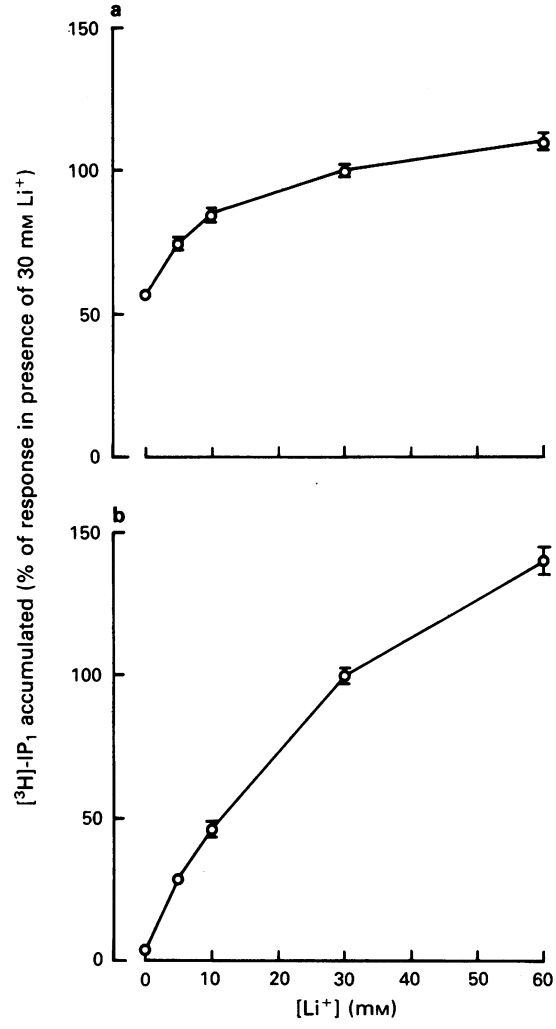


Figure 7 Concentration-effect curve for Li^+ on (a) basal and (b) histamine-induced [^{3}H]-inositol monophosphate ([^{3}H]-IP_1) accumulation. HeLa cells were preincubated with 0–60 mM Li^+ for 15 min before addition of 1 mM histamine and incubation for 60 min. Points are the combined values (weighted mean with approximate s.e.mean shown by vertical bars) from 3 experiments. To allow for differences in the absolute magnitude of basal and histamine-stimulated [^{3}H]-IP_1 accumulation between experiments, levels of [^{3}H]-IP_1 are expressed as a percentage of the level in the presence of 30 mM Li^+. Where no error bars are shown the error was within the size of the symbol. Mean values of [^{3}H]-IP_1 accumulation with 30 mM Li^+: basal, 4927 ± 272 d.p.m.; histamine-induced, 10,763 ± 396 d.p.m. Note that the scales of the two graphs are the same.

absence of Li^+ the histamine-induced accumulation was only $4 \pm 1\%$ of that with 30 mM Li^+.

Increasing the concentration of Li^+ had only a small and, mostly, statistically insignificant effect on the basal and histamine-stimulated levels of [^{3}H]-IP_2 and [^{3}H]-IP_3. As a consequence the concentration-effect curve for the effect of Li^+ on histamine-stimulated accumulation of [^{3}H]-IP_1 + [^{3}H]-IP_2 + [^{3}H]-IP_3 was practically identical to that for [^{3}H]-IP_1.

In the presence of 30 mM Li^+, a concentration well up the concentration-effect curve (Figure 7), the nature of the time-course of [^{3}H]-IP_1 accumulation induced by 100 μM or 1 mM histamine varied somewhat between experiments. In 3 experiments the level of [^{3}H]-IP_1 increased rapidly for 10–20 min and then continued with a slow linear increase with time, but in 3 other experiments the level of [^{3}H]-IP_1 reached a plateau which was then maintained. This variability may reflect differences in the extent of inhibition of monophosphatase activity between experiments. Consistent with this, in one experiment in which [^{3}H]-IP_1 continued to increase with time, addition of 1 μM mepyramine at 30 min resulted in a maintained level of [^{3}H]-IP_1 for the next 30 min. However, in two other experiments in which the level of [^{3}H]-IP_1 induced by 100 μM histamine came to a plateau, addition of 1 μM mepyramine caused a slow fall in the level of [^{3}H]-IP_1.

The concentration-response curve for histamine-induced accumulation of [^{3}H]-IP_1 in the presence of 30 mM Li^+, measured after 60 min incubation with histamine, had an EC_{50} of 2.4 ± 0.4 μM (best-fit value to the combined data from 3–8 determinations), similar to the value, 1.6 ± 0.2 μM determined in the presence of 10 mM Li^+. However, the Hill coefficient, 0.77 ± 0.07, was numerically less than that, 1.07 ± 0.14, in the presence of 10 mM Li^+.

Discussion

The results presented here show that in the presence of 10 mM Li^+, the concentration used in the original studies of histamine-induced phosphoinositide metabolism in HeLa cells (Tilly *et al.*, 1990a,b; Bristow & Young, 1991), the plateau phase in histamine-induced [^{3}H]-IP_1 accumulation is a steady-state balance between formation and metabolism. The monophosphatase enzyme(s) responsible for IP_1 metabolism in HeLa cells are thus much less sensitive to blockade by Li^+ than the monophosphatase of mammalian brain. Li^+ is an uncompetitive inhibitor of the enzyme from bovine brain, as originally demonstrated by Hallcher & Sherman (1980), and the extent of the inhibition is dependent on the nature of the substrate and on its concentration. Thus the K_i for Li^+, determined on a purified preparation from bovine brain, was 1.0 mM against inositol 1-phosphate and 0.26 mM against inositol 4-phosphate (Gee *et al.*, 1988a). The uncompetitive nature of the inhibitory action of Li^+ complicates comparisons with assays in other tissues under different experimental conditions, particularly since at very low substrate concentrations Li^+ can appear to be ineffective as an inhibitor (see Shears, 1989). However, under the normal assay conditions employed in brain slices, maximal effects of Li^+ are obtained with 5–10 mM Li^+ (see e.g. Brown *et al.*, 1984, for rat cerebral cortical slices), although there is one report of higher concentrations being required in rat hippocampal slices (Lenox *et al.*, 1988). In contrast, there are now several studies in transformed cell lines which indicate that the metabolism of IP_1 in these cells is much less sensitive to Li^+. In clonal NCB-20 neurohybridoma cells the concentration of Li^+ required for half-maximal potentiation of muscarinic agonist-induced [^{3}H]-IP_1 accumulation was 17 mM, whereas in rat cerebral cortical slices, examined in the same study, the EC_{50} was 1.8 mM (Chuang, 1986). A similar decreased sensitivity to Li^+ has been reported in C6 glioma (Ananth *et al.*, 1987) and N1E-115 neuroblastoma (Thompson & Fisher, 1990) cells. HeLa cells can clearly be added to this list.

The increase in the level of [^{3}H]-IP_1 in HeLa cells preincubated with 10 mM Li^+ for 15 min before addition of histamine for 15 min, and the lack of effect of further increasing the preincubation period (Figure 6) strongly suggests that the low sensitivity to Li^+ is not a consequence of a slow entry into the cells. It is notable that in the same experiments preincubation with 10 mM Li^+ had no effect on the basal level of [^{3}H]-IP_1. A similar, probably related, effect was observed in the concentration-effect experiments with Li^+ (Figure 7), in which an increase from 0 to 30 mM Li^+ had much less effect on the basal [^{3}H]-IP_1 level than on the histamine-induced response. An understanding of this difference will require a knowledge of the identity of the radiolabelled compounds in the [^{3}H]-IP_1 fraction in control and stimulated cells. Neither inositol 2-phosphate nor inositol 1:2 cyclic phosphate are substrates for the monophosphatase enzyme in mammalian brain (Shears, 1989, but see Attwood *et al.*, 1988), but whether they are present to any appreciable extent in HeLa cells prelabelled for 20–24 h with [^{3}H]-inositol remains to be established.

In brain slices increasing the concentration of Li^+ also leads to inhibition of the enzyme inositol polyphosphate 1-phosphatase, which removes the 1-phosphate from inositol 1,4-bisphosphate and inositol 1,3,4-trisphosphate. The inhibition by Li^+ is again uncompetitive (Shears, 1989) and the K_is for Li^+ in bovine brain against these two substrates are 9.63 and 0.46 mM, respectively (Gee *et al.*, 1988b). However, we have not observed any significant change in the histamine-induced levels of either [^{3}H]-IP_2 or [^{3}H]-IP_3 in HeLa cells, even at 60 mM Li^+. It therefore seems likely that the inositol polyphosphate 1-phosphatase of HeLa cells is also much less sensitive to Li^+ than the enzyme in brain. The lack of any appreciable potentiation by Li^+ of the levels of [^{3}H]-IP_2 or [^{3}H]-IP_3 in stimulated cells over incubation times of 15 min or more further underlines the gain in sensitivity in the assay, without the introduction of any appreciable error, in measuring only [^{3}H]-IP_1. Where we have made direct comparison by analysing both histamine-induced [^{3}H]-IP_1 accumulation and the combined accumulation of [^{3}H]-IP_1, [^{3}H]-IP_2 and [^{3}H]-IP_3, the results were essentially the same. Whether the [^{3}H]-IP_4 fraction should be included in the measurement of total [^{3}H]-inositol phosphates after extended periods of incubation is debatable, since the nature and origin of the histamine-stimulated product in this fraction is unknown and could conceivably be derived from the breakdown of higher polyphosphates.

The use of Li^+ in the assay of agonist-induced phosphoinositide metabolism was originally devised as a method of amplifying the response over extended periods of incubation (Berridge *et al.*, 1982) and the concentration of Li^+ used in most studies is chosen to produce a complete inhibition of IP_1 breakdown. The [^{3}H]-IP_1 accumulated divided by the incubation time with agonist is then a measure of the *average* activity of phosphoinositidase C (PIP_2-selective phospholipase C). This is not the case when the level measured is a steady-state level, as in HeLa cells. However, steady-state levels may still provide useful, but different, information. In the simplest notional case if the concentration of IP_1 (labelled and unlabelled) is much less than the K_m of the monophosphatase (assuming a single enzyme), then the rate of breakdown of IP_1 will approximate to $(V_{max}/K_m) \times [IP_1]$ (setting the substrate concentration $S < K_m$ in the Michaelis-Menten equation). The change in the agonist-induced concentration of $[IP_1]$ with time will be described by the differential equation:

$$d([IP_1])/dt = F - k \times [IP_1]$$

where $k = V_{max}/K_m$ and F is the rate of formation of IP_1 expressed as $mol\,l^{-1}$ and will be a function of receptor occupancy by the agonist. Integrating and setting $[IP_1] = 0$ at $t = 0$

$$[IP_1] = [IP_1]_{ss} \times \{1 - \exp(-k \times t)\}$$

where $[IP_1]_{ss}$ is the steady-state concentration of IP_1. Thus under these conditions the rise of $[IP_1]$ to the plateau would be exponential, with a rate constant of k, i.e. the rate at which the plateau is approached depends only on the rate constant for breakdown. This is the well-known 'plateau principle' of the pharmacokinetics of infusions (Goldstein *et al.*, 1968). At the steady-state $F = k \times [IP_1]_{ss}$. Thus, under these conditions the level of [^{3}H]-IP_1 at the plateau will be proportional to the rate of formation of [^{3}H]-IP_1 at that time, making the further assumption that in cells prelabelled with [^{3}H]-inositol for 20–24 h there is no marked change in the specific activity with time. However, in the presence of Li^+ the situation becomes more complex, since the rate of breakdown of IP_1, v, becomes

$$v = V_{max} \times [IP_1]/\{K_m + [IP_1](1 + [Li^+]/K_i)\}$$

making no assumptions about the relative values of $[IP_1]$ and K_m. However, if $[IP_1] \ll K_m$, then

$$v = V_{max} \times [IP_1]/(K_m + [IP_1] \times [Li^+]/K_i)$$

and a simple proportionality between the plateau level of IP_1 and the rate of formation of IP_1 will depend on $[Li^+]/K_i$ being small.

In the absence of a knowledge of the K_ms of the [^{3}H]-IP_1 isomers present in histamine-stimulated HeLa cells and the corresponding K_is for Li^+, together with a degree of uncertainty of how good an approximation [^{3}H]-IP_1 is to total $[IP_1]$, it is not possible to calculate how directly related changes in the plateau level of [^{3}H]-IP_1 are to changes in the rate of formation of [^{3}H]-IP_1. However, it is clear that the lower the concentration of Li^+ the more direct the proportionality will be. At the lowest levels of Li^+ the determination of the very low levels of agonist-induced [^{3}H]-IP_1 will be subject to considerable experimental error. Thus, 10 mM Li^+ could represent a useful compromise in experiments designed to detect changes in rates of phosphoinositide hydrolysis from changes in the steady-state level. Little experimental evidence can be advanced at present to support the suitability of 10 mM Li^+ for this purpose, but it may be noted that the rates at which the plateau was approached with 1 μM and 1 mM histamine in the experiment depicted in Figure 3 look similar. This is consistent with the simple model in which the rate constant for the rise in the concentration of [^{3}H]-IP_1 is determined solely by the rate constant for breakdown.

The fact that the plateau level in the presence of 10 mM Li^+ is a steady-state level means that some caution has to be exercised in interpreting experiments designed to measure changes in receptor function with time, such as agonist-induced desensitization. If desensitization leads to a decrease in the rate of agonist-stimulated [^{3}H]-IP_1 formation, such as observed in turkey erythrocytes pre-exposed to ADPβS (Martin & Harden, 1989), rather than a complete inhibition, then if desensitization occurs within say 10 min it might be missed in measurements made under steady-state conditions, in which the level at the plateau is proportional to the rate of formation at that point. Thus the ingenious experimental design of Tilly *et al.* (1990a) in which HeLa cells were exposed to 100 μM histamine for different periods of time before addition of 10 mM Li^+ to initiate [^{3}H]-IP_1 accumulation, on the implicit assumption that [^{3}H]-IP_1 had been blocked, may not be so clear-cut evidence of the absence of H_1-receptor desensitization as it might seem. This might go some way to explaining the apparent discrepancy with our own preliminary measurements on the effect of pre-exposing HeLa cells to histamine before the addition of 10 mM Li^+ and subsequent rechallenge with histamine, in which the level of the plateau reached was not changed by histamine pretreatment, but the rate at which the plateau was approached was clearly slowed (Bristow & Young, 1991). However, since the plateau level of [^{3}H]-IP_1 in the presence of 10 mM Li^+ depends on both the rate of formation and the rate of metabolism, there is clearly scope for considerable complexity.

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Direct effects of adenylyl 5'-(β,γ-methylene)diphosphonate, a stable ATP analogue, on relaxant P_1-purinoceptors in smooth muscle

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1 Previous results obtained with the rat colon muscularis mucosae, which contracts in response to adenosine and adenosine 5'-triphosphate (ATP), had suggested that adenylyl 5'-(β,γ-methylene)diphosphonate (AMPPCP), a stable ATP analogue, acted on P_1-purinoceptors rather than, as expected, on P_2-purinoceptors. This possibility has been examined in two tissues in which adenosine and ATP both cause relaxation, the guinea-pig taenia caeci and the rat duodenum.

2 ATP, 2-methylthio-ATP (2-MeSATP), AMPPCP, adenosine 5'-(α,β-methylene)triphosphonate (AMPCPP) and adenosine each relaxed the taenia caeci and the duodenum, and the order of potency of the nucleotides in each tissue was 2-MeSATP > ATP > AMPCPP > AMPPCP, indicating that these effects were mediated by P_{2Y}-purinoceptors.

3 The P_1 antagonist 8-(*p*-sulphophenyl)theophylline (8-SPT) (100 μM) did not affect the responses to ATP, 2-MeSATP or AMPCPP in either tissue, but inhibited the responses of adenosine and of AMPPCP in both tissues. In the duodenum a lower concentration of 8-SPT caused a parallel shift to the right of the concentration-response curve to adenosine and to AMPPCP but to different extents, with AMPPCP being inhibited more powerfully than adenosine. A dose-ratio of around 5 was observed for adenosine and AMPPCP at concentrations of 8-SPT of 20 μM and 2 μM respectively, but Schild analysis resulted in plots with slopes greater than unity. In the taenia caeci, however, 8-SPT inhibited adenosine more powerfully than AMPPCP, and a range of concentrations (10–20 μM) only caused a two fold shift in the concentration-response curve for AMPPCP, although the concentration-response curve to adenosine was shifted in a concentration-dependent manner and Schild analysis gave a pA_2 value of 5.13 with a slope of 0.90.

4 As has been shown in other tissues, including the guinea-pig taenia caeci, ATP (100 μM) was rapidly dephosphorylated by enzymes present in the rat duodenum, with less than 10% remaining after 20 min incubation, whereas AMPPCP (100 μM) was resistant to degradation, with greater than 90% remaining at the same time point.

5 AMPPCP therefore has pronounced but variable agonist actions on P_1-purinoceptors, and appears to act entirely via these receptors on the rat duodenum although in the guinea-pig taenia caeci this action is less important and it acts largely via P_{2Y}-purinoceptors. These P_1-purinoceptor effects of AMPPCP are direct and are not due to its degradation to adenosine.

Keywords: Purines; adenosine; ATP; smooth muscle; analogues of ATP; receptors

Introduction

Extracellular adenosine and adenosine 5'-triphosphate (ATP) are thought to cause their pharmacological effects by acting at two distinct receptor types called P_1- and P_2-purinoceptors respectively (Burnstock, 1978). Each of these receptors has been subdivided into at least two major subtypes, the P_1-purinoceptors into A_1 and A_2 (Williams, 1987) and the P_2-purinoceptors into P_{2X} and P_{2Y} (Burnstock & Kennedy, 1985) with additional P_2-purinoceptor subtypes existing on platelets (P_{2T}) and some other cell types such as mast cells (P_{2Z}) (Gordon, 1985). In smooth muscle, adenosine usually causes relaxation probably via A_2 receptors but contracts some tissues via A_1 receptors (Kenakin & Pike, 1987; Smith *et al.*, 1988), whereas ATP can cause either relaxation via P_{2Y} or contraction via P_{2X} depending on the tissue (White, 1988; Burnstock, 1989). Adenosine also presynaptically inhibits neurotransmitter release in many preparations, an effect which is generally thought to be mediated by A_1 receptors (Williams, 1987; Burnstock, 1989) although a separate subtype, A_3, has also been proposed to mediate this effect (Ribeiro & Sebastiao, 1986).

As well as acting directly on P_2-purinoceptors, ATP is rapidly dephosphorylated ultimately to adenosine by ecto-nucleotidases present on smooth muscle, and this breakdown can complicate studies of the actions of ATP and its analogues. For example, the resistance to dephosphorylation of ATP analogues correlates well with their potency in contracting the guinea-pig bladder via P_{2X}-purinoceptors, but not with their potency in relaxing the guinea-pig taenia caeci via P_{2Y}-purinoceptors, implying that degradation does greatly affect potency on some tissues (Welford *et al.*, 1986; 1987). Although no selective inhibitors of the breakdown of ATP are known (Hourani & Chown, 1989), analogues of ATP in which a bridging oxygen between the phosphate groups has been replaced by a methylene group, such as adenosine 5'-(α,β-methylene)triphosphonate (AMPCPP) and adenylyl 5'-(β,γ-methylene)diphosphonate (AMPPCP) are much more resistant to degradation (Moody & Burnstock, 1982; Welford *et al.*, 1986; 1987; Welford & Anderson, 1988; Bailey & Hourani, 1990). These compounds together with 2-substituted ATP analogues such as 2-methylthio-ATP (2-MeSATP), which is broken down as fast as ATP (Welford *et al.*, 1986; 1987), are used to classify P_2-purinoceptors, the order of potency on P_{2X} receptors being AMPCPP, AMPPCP ≫ ATP = 2-MeSATP and on P_{2Y} receptors being 2-MeSATP > ATP > AMPCPP, AMPPCP (Burnstock & Kennedy, 1985). Although the rapid degradation of ATP and of 2-MeSATP complicates the use of these agonists in classifying receptors, P_{2X} and P_{2Y} receptors do possess different structure-activity relationships even for non-degradable analogues (Cusack *et al.*, 1987) and selective

agonists for these receptors have been found (Hourani et al., 1985; 1986; 1988).

In some tissues ATP has effects which appear to be mediated by P_1 receptors rather than P_2 receptors, although it is not clear whether ATP is acting directly or via adenosine formed by dephosphorylation (Dahlen & Hedqvist, 1980; Collis & Pettinger, 1982; Moody & Burnstock, 1982; Moody et al., 1984; Wiklund et al., 1985; Shinozuka et al., 1988). The finding that the more resistant analogue, AMPPCP, also has actions that appear to be mediated by P_1-purinoceptors has been taken by some authors as evidence that ATP acts directly on P_1 receptors (Dahlen & Hedqvist, 1980; Collis & Pettinger, 1982; Wiklund et al., 1985) although Shinozuka et al. (1988) suggested that these receptors, since they respond to adenosine and to nucleotides, represent a unique class which they called P_3. Moody & Burnstock (1982) attributed the P_1 effects of AMPPCP to its breakdown to adenosine, which was, however, much slower than that of ATP. They found no P_1 effect of AMPCPP which they believed to be due to a greater resistance to degradation of this analogue although after 10 min it had been broken down to the same extent as AMPPCP.

We have recently demonstrated (Bailey & Hourani, 1990) that in the rat colon muscularis mucosae, AMPPCP acts via P_1 receptors as its effects are inhibited by the adenosine antagonist 8-(*p*-sulphophenyl)theophylline (8-SPT), whereas ATP, 2-MeSATP and AMPCPP act, as one would expect, on P_2 receptors and are not inhibited by 8-SPT. This action of AMPPCP is almost certainly not due to its degradation to adenosine, as AMPPCP was highly resistant to degradation in this tissue whereas ATP was more readily broken down but nevertheless had no effect via P_1-purinoceptors. It appears therefore that AMPPCP may have an unexpected direct P_1 agonist action which could explain some of the findings referred to above. However, the rat colon muscularis mucosae has rather unusual responses to purines, as it contracts rather than relaxes to adenosine, and also contracts to ATP via a P_{2Y} receptor rather than the expected P_{2X} receptor (Bailey & Hourani, 1990). In the present study we therefore investigated whether the responses of AMPPCP were also mediated by P_1-purinoceptors rather than by P_{2Y}-purinoceptors in two preparations which relax to adenosine and to ATP, the rat duodenum and the guinea-pig taenia caeci.

Methods

Isolated tissue studies

Male Wistar rats (200–250 g) were killed by cervical dislocation and the duodenum dissected out by cutting at the base of the pyloris and 1.5 cm from this point, mounted in 10 ml organ baths by threads tied around each end so that the lumen was closed and maintained in Krebs solution of the following composition (mM): NaCl 118, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11. Male albino guinea-pigs (250–450 g) were killed by cervical dislocation and the taenia caeci (10 mm lengths) dissected out, mounted in 3 ml organ baths and maintained in modified Krebs solution of the following composition (mM): NaCl 120, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 15.4 and glucose 11. For both preparations the Krebs solution was bubbled with 95% O_2:5% CO_2 and warmed to 35°C, and the tissues were equilibrated for at least 45 min before addition of drugs. A resting tension of 1 g was applied to the tissues and force was recorded isometrically with Grass FTO3 transducers and displayed on Grass 79D polygraphs. Concentration-response curves were obtained non-cumulatively, and the inhibitory responses were quantified by precontracting the tissues with carbachol (40 nM for the taenia caeci, 100 nM for the duodenum) before challenge with purines, and expressed as % inhibition of carbachol contraction. In the taenia caeci, the concentration of carbachol used induced a sustained contraction of 50–70% of the maximal response, whereas in the duodenum the concentration of carbachol used increased the spontaneous contractions of the tissue to around 50% of the maximum, and made them more regular (see Nicholls et al., 1990 for further details and representative traces for the duodenum). In each tissue, doses of carbachol were applied at approximately 10 min intervals, and no tachyphylaxis was observed to either carbachol or any of the purines used. After control concentration-response curves to purines had been obtained, the tissues were incubated for 30 min with 8-SPT and the concentration-response curves were repeated in the presence of this antagonist. Responses to agonists returned to the control levels after no more than 40 min washout.

Degradation studies

The rat duodenum was dissected out as above and incubated in gassed Krebs solution at 35°C for 3 h with the buffer being changed every 15 min. The tissues were then placed in aerated Krebs solution (2.5 ml) at 35°C, containing ATP or AMPPCP (100 μM), and 100 μl aliquots were taken at various times and frozen for later analysis by high performance liquid chromatography (h.p.l.c.). To check for leakage of enzymes, tissues which had been washed as above were incubated in Krebs solution (2.5 ml) alone for 45 min, the tissues were then removed, the purines added to the buffer which had contained the tissue and samples were taken for analysis at 0 and 20 min. Samples were analysed with a Waters h.p.l.c. with a Techsphere 5 μm ODS C_{18} column, eluted with 0.1 M KH_2PO_4/8 mM tetrabutylammonium hydrogen sulphate (pH 6.0) (Solvent A) and a 60:40 mixture of Solvent A and acetonitrile (pH 6.73) (Solvent B), using a non-linear gradient (0–2.5 min 0% B, 2.5–5 min 0–20% B, 5–10 min 20–40% B, 10–13 min 40–100% B, 13–18 min 100% B), at a flow rate of 1.3 ml min^{-1}. The purines were detected by u.v. absorbance at 259 nm, and quantified from the height of their absorbance peaks, which was linearly related to the concentration in the incubation mixture.

Materials

Adenosine, ATP, AMPPCP, AMPCPP, tetrabutylammonium hydrogen sulphate and carbachol were obtained from Sigma Chemical Co., U.K., 2-MeSATP and 8-SPT were obtained from Research Biochemicals Inc., and buffer salts and solvents were of analytical or h.p.l.c. grade and were obtained from BDH.

Results

Guinea-pig taenia caeci

ATP, adenosine, AMPPCP, AMPCPP and 2-MeSATP each relaxed the carbachol-contracted taenia caeci, and the order of potency was 2-MeSATP > ATP > AMPCPP > AMPPCP > adenosine (Figure 1). The responses to ATP, 2-MeSATP and AMPCPP were not inhibited by 8-SPT (100 μM) (Figure 1a), whereas those to adenosine (Figure 1b) and, to a lesser extent, those to AMPPCP were inhibited (Figure 1c).

The inhibition of adenosine by 8-SPT was concentration-dependent, with increasing shifts in the concentration-response curve as the concentration of antagonist was increased from 10–200 μM (Figure 2a). In contrast, the same shift, approximately 2 fold, was observed in the concentration-response curve to AMPPCP for concentrations of antagonist from 10–200 μM (Figure 2c). The Schild plot for 8-SPT as an inhibitor of the effects of adenosine, derived from the data in Figure 2a by calculating dose-ratios for 25% inhibition of the carbachol-induced contraction in the presence and absence of antagonist, was linear ($r = 0.993$) with a slope of 0.90 and gave a pA_2 value of 5.13 (Figure 2b).

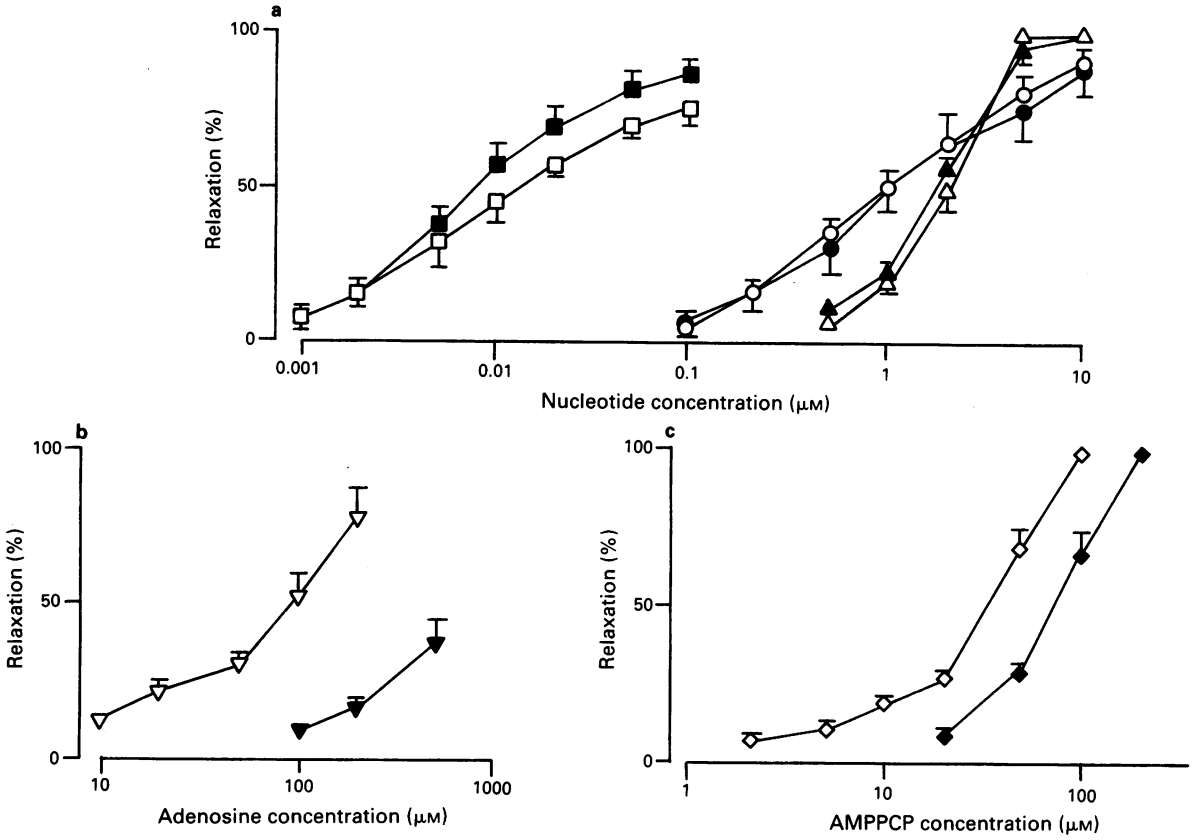


Figure 1 Effects of purines on guinea-pig taenia caeci in the absence (open symbols) or presence (closed symbols) of 8-SPT (100 μM): (a) ATP (○, ●); AMPCPP (△, ▲) and 2-MeSATP (□, ■); (b) adenosine (▽, ▼); (c) AMPPCP (◇, ◆). Each point is the mean of at least 6 determinations and the vertical bars show s.e.mean. Abbreviations as in text.

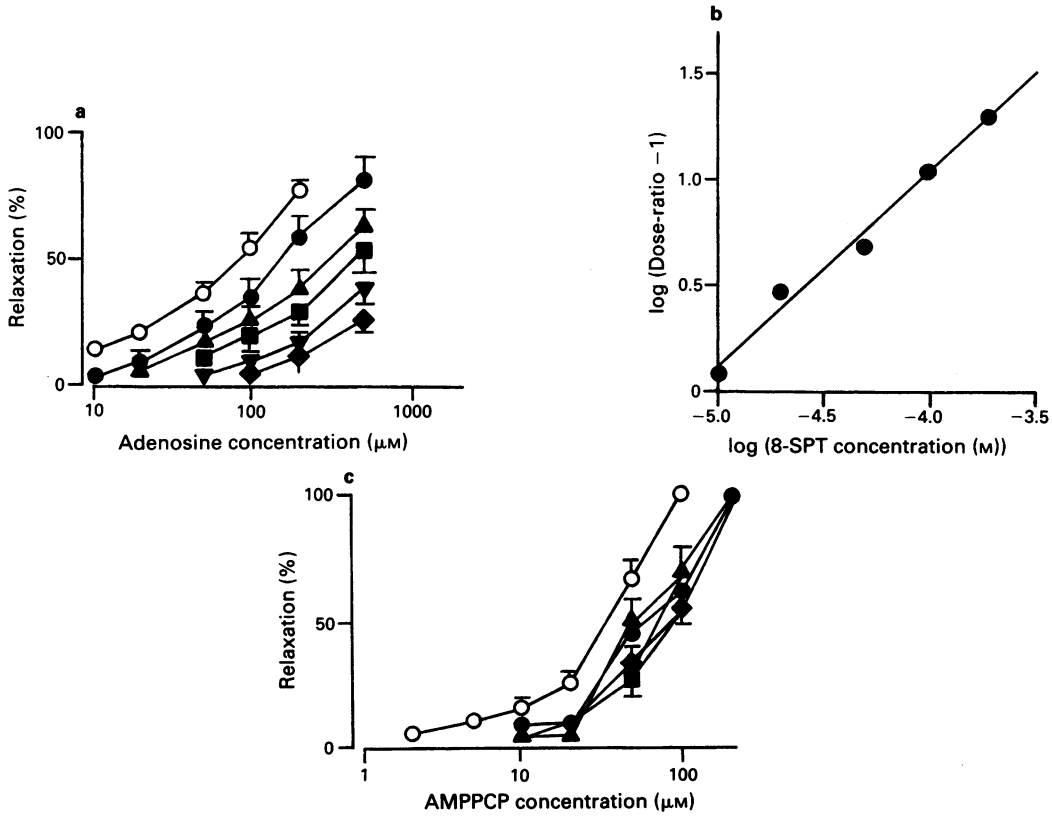


Figure 2 Relaxations of the guinea-pig taenia caeci induced by (a) adenosine or (c) AMPPCP in the absence (○) or presence of 8-SPT at 10 μM (●), 20 μM (▲), 50 μM (■), 100 μM (▼) or 200 μM (◆). Each point is the mean of at least 4 determinations and the vertical bars show s.e.mean. The Schild plot derived from the data in (a) is shown in (b). The line shown is that calculated by least squares linear regression analysis, has a slope of 0.90 and gives a pA_2 value of 5.13 taken from the intercept on the horizontal axis. Abbreviations as in text.

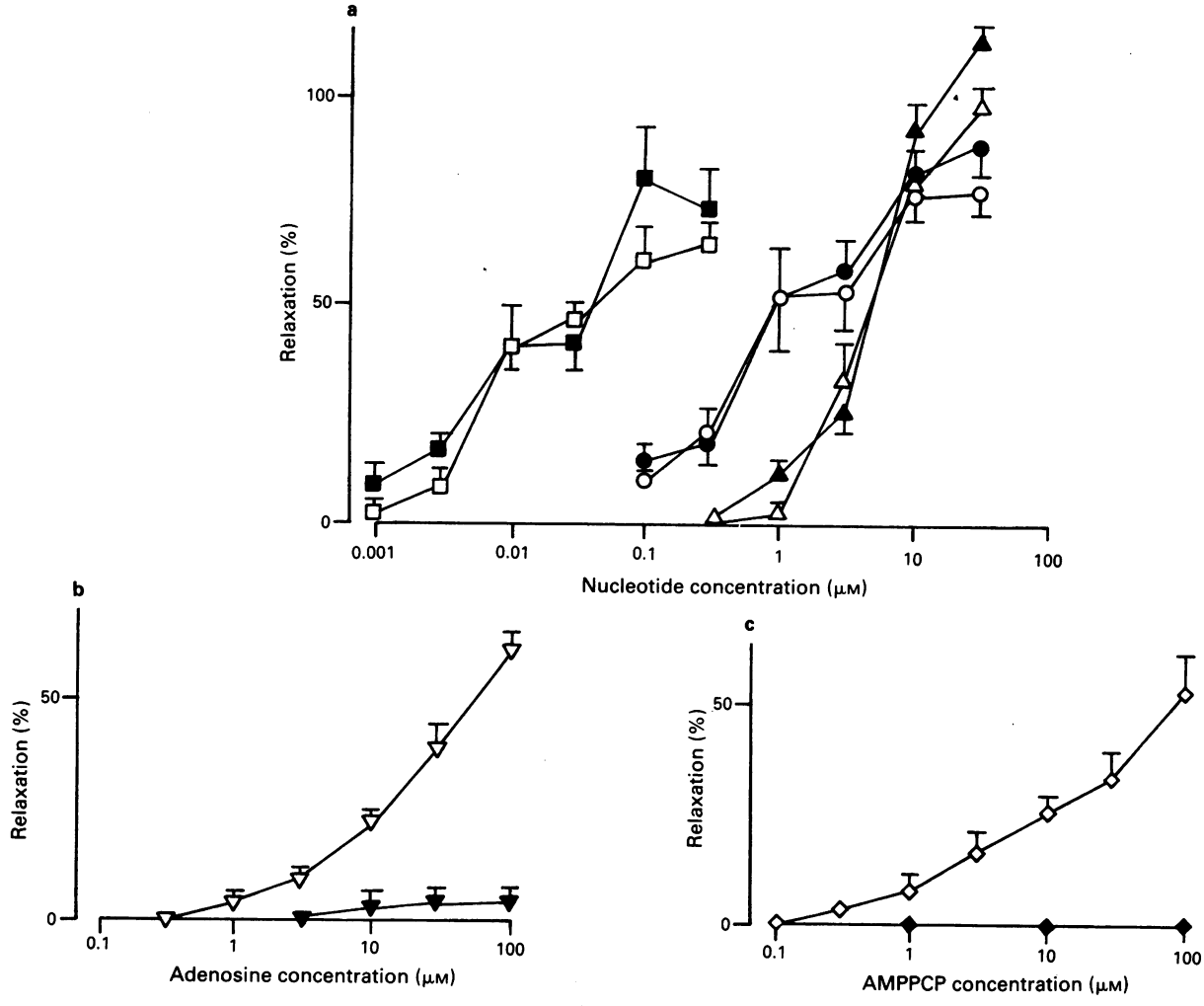


Figure 3 Effects of purines on rat duodenum in the absence (open symbols) or presence (closed symbols) of 8-SPT (100 μM): (a) ATP (○, ●); AMPCPP (△, ▲) and 2-MeSATP (□, ■); (b) adenosine (▽, ▼); (c) AMPPCP (◇, ◆). Each point is the mean of at least 5 determinations and the vertical bars show s.e.mean. Abbreviations as in text.

Rat duodenum

ATP, adenosine, AMPCPP, AMPPCP and 2-MeSATP all relaxed the carbachol-contracted rat duodenum, and the order of potency was 2-MeSATP > ATP > AMPCPP > AMPPCP = adenosine (Figure 3). The responses to ATP, 2-MeSATP and AMPCPP were not inhibited by 8-SPT (100 μM) (Figure 3a), whereas those to adenosine (Figure 3b) and AMPPCP (Figure 3c) were abolished.

Lower concentrations of 8-SPT shifted the concentration-response curves for adenosine and for AMPPCP to the right but to different extents, with a dose-ratio of around 5 being observed for adenosine and AMPPCP at concentrations of 8-SPT of 20 μM and 2 μM respectively (Figure 4). Schild analysis in this tissue using either agonist resulted in Schild plots with slopes greater than 1 (results not shown).

Degradation studies

ATP (100 μM) was rapidly degraded by the rat duodenum with only 9% remaining after 20 min, and was mainly converted to AMP (33%) and inosine (45%) during this time (Figure 5a). AMPPCP (100 μM) was resistant to degradation, with 92% remaining after 20 min incubation (Figure 5b). ATP (100 μM) added to buffer from which the tissue had been removed after incubation alone for 45 min was degraded with 29% remaining after 20 min, and the main degradation products were ADP (32%) and AMP (35%), with only 3% inosine being formed (Figure 5c). When AMPPCP was added to buffer from which the tissue had been removed, 97% remained after 20 min incubation (Figure 5d). No degradation of either purine was observed after incubation with buffer in which no tissue had been preincubated.

Discussion

These results show that AMPPCP acts directly via P_1-purinoceptors in two tissues which relax to ATP and adenosine, as well as in the rat colon muscularis mucosae which contracts to the purines (Bailey & Hourani, 1990). The extent to which the actions of AMPPCP are mediated by P_1-purinoceptors is not, however, the same in all tissues, as in the taenia caeci no more than a two fold shift in the dose-response curve could be achieved even with 200 μM 8-SPT, whereas in the rat duodenum, as in the rat colon, the dose-response curve to AMPPCP was shifted at least as much as that to adenosine, indicating that in these tissues AMPPCP acts entirely via P_1-purinoceptors. These actions of AMPPCP are unlikely to be due to adenosine formed as a result of degradation, as AMPPCP is known to be resistant to degradation in the guinea-pig taenia caeci (Welford *et al.*, 1986) and the rat colon (Bailey & Hourani, 1990) and was also degraded to a negligible extent by the rat duodenum (Figure 5). Also, the actions of ATP, which is degraded much faster than AMPPCP in these tissues, were not inhibited by 8-SPT, showing that the degradation products do not influence activity. Indeed, although AMP and ADP were formed, the main product of degradation of ATP was the inactive inosine, and no adenosine was detected (Figure 5a), confirming the work of Franco *et al.* (1988), who also found the activity of adenosine

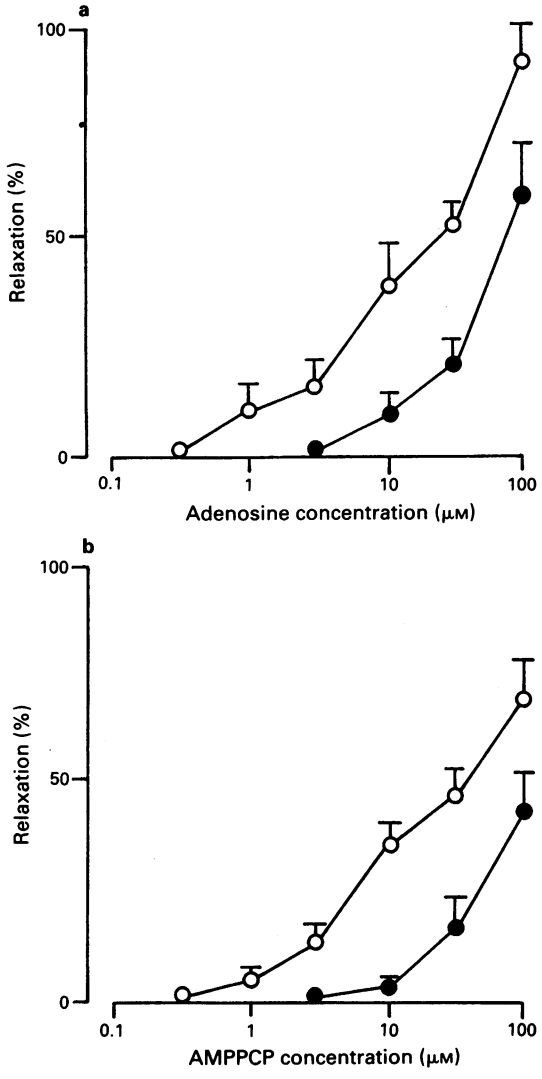


Figure 4 Relaxations of the rat duodenum induced by (a) adenosine or (b) AMPPCP in the absence (○) or presence (●) of 8-SPT (20 μM in (a), 2 μM in (b)). Each point is the mean of at least 8 determinations and the vertical bars show s.e.mean. Abbreviations as in text.

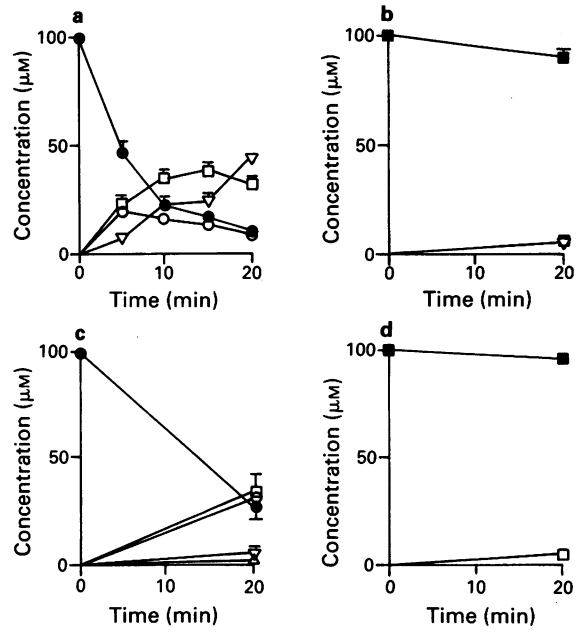


Figure 5 Degradation of ATP (100 μM) (●) (a and c) or of AMPPCP (100 μM) (■) (b and d) to ADP (○), AMP (□), adenosine (△) and inosine (▽) by enzymes present in the rat duodenum (a and b) or released into buffer from the rat duodenum (c and d). Each point is the mean of at least 4 determinations and the vertical bars show s.e.mean.

deaminase to be relatively high in the rat duodenum. Some leakage of enzymes was detected, as ATP was also degraded by buffer in which tissue had been incubated, although in this case the main degradation products were ADP and AMP, suggesting that 5'-nucleotidase and adenosine deaminase had remained bound to the tissue (Figure 5c).

The order of potency of the ATP analogues in both tissues confirms that the P_2-purinoceptor is of the P_{2Y} subtype, with 2-MeSATP being much more potent than ATP and AMPCPP being somewhat less potent, with none of these compounds having any effect on P_1-purinoceptors which could interfere with this analysis. Indeed, the concentration-response curves for these compounds on the two tissues looked almost identical, with the potencies on each tissue being very similar and the curve for AMPCPP being steeper than those for ATP or 2-MeSATP, possibly because of its resistance to degradation, and therefore crossing the top of the ATP curve. This supports the idea that the receptors in these tissues are the same, although agonist potencies and the shapes of concentration-response curves do not provide conclusive information on receptor subtypes, and definite proof must await the development of true subtype-selective, competitive ATP antagonists. The results described here show that AMPPCP is of little value for the definition of P_2-purinoceptor subtypes, as although on both tissues it is indeed less potent than ATP as expected for a P_{2Y}-purinoceptor (Burnstock & Kennedy, 1985), it has a significant but variable effect on P_1-purinoceptors. In the taenia caeci its true potency at P_{2Y}-purinoceptors is presumably that observed in the presence of 10–200 μM 8-SPT and is thus roughly half that observed in the absence of antagonist, whereas in the duodenum no P_{2Y} effect is detectable at the concentrations tested and AMPPCP appears to act entirely as a P_1 agonist in this tissue.

Whether the observed differences in the effects of AMPPCP between the tissues reflect qualitative differences between the P_{2Y} receptor populations in these tissues is hard to investigate with the pharmacological tools available, but there seems no compelling reason to propose P_{2Y} subtypes on this evidence. There are however some hints that the P_1-purinoceptors may be different, although the small differences observed in the inhibitory potency of 8-SPT are hard to interpret with certainty, particularly in the absence of convincing Schild plots in the duodenum and in view of reports that P_1 agonists and antagonists may in general have a rather higher affinity for rat tissues than for guinea-pig tissues (Collis *et al.*, 1989). Why the Schild plots in the rat duodenum gave slopes greater than unity is not clear, but problems were also encountered with the dose-dependence of the antagonist in the rat colon (Bailey & Hourani, unpublished observations), and this could imply that 8-SPT is not acting in a purely competitive fashion in these tissues. In the guinea-pig taenia caeci the Schild plot against adenosine was satisfactory and no problems were reported by Collis *et al.* (1987) on the guinea-pig atria and aorta, showing that in these tissues 8-SPT is acting competitively, and that again there may be differences between rat and guinea-pig tissues. 8-SPT did not cause dose-dependent shifts in the concentration-response curve for AMPPCP in the taenia caeci where it was acting as a competitive adenosine antagonist, showing that the P_1 action of AMPPCP is only a component of its overall effect in this tissue.

In conclusion, it is now clear that AMPPCP cannot simply be regarded as a P_2 agonist but also has a pronounced direct P_1 agonist action as well, and the extent of this varies depending on the tissue chosen. This direct P_1 effect complicates the use of AMPPCP as a stable ATP analogue, and may explain some earlier findings that AMPPCP (and ATP) acted like adenosine in some tissues (Dahlen & Hedqvist, 1980; Collis &

Pettinger, 1982; Moody & Burnstock, 1982; Moody et al., 1984; Wiklund et al., 1985; Shinozuka et al., 1988). ATP could well be acting in these cases via its breakdown product adenosine, although we have found no evidence for a direct or indirect P_1 effect, but it is likely that AMPPCP is acting directly on P_1-purinoceptors as in the results described here. The related stable analogue, AMPCPP, would appear to be a more suitable analogue to use in studies of P_2-purinoceptors as it lacks this anomalous P_1 effect, although it is not as selective as AMPPCP for P_{2X} receptors and has a higher potency on P_{2Y} receptors, being almost as potent as ATP itself in the taenia caeci and rat duodenum, and furthermore has a greater effect than ATP at high concentrations. The potent, specific P_{2X} agonist L-AMPPCP, the enantiomer of AMPPCP, is probably the best compound to use, as it has little or no P_{2Y} or P_1 effects in the guinea-pig taenia caeci, aorta or ileum (Hourani et al., 1985; 1986) or the rat colon muscularis mucosae (Bailey & Hourani, 1990). Our results with AMPPCP show that it is possible for an ATP analogue to act directly on P_1-purinoceptors however unlikely this may seem from the known structure-activity relationships, and emphasise the danger of making assumptions about the receptor selectivity of an agonist based solely on its structure.

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Sequential release of tumour necrosis factor, platelet activating factor and eicosanoids during endotoxin shock in anaesthetized pigs; protective effects of indomethacin

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1 The effects of indomethacin were investigated on haemodynamics, haematological and blood glucose values, and the release of tumour necrosis factor (TNF), platelet activating factor (PAF) and eicosanoids in anaesthetized pigs receiving 5 $\mu g\,kg^{-1}$ *E. coli* lipopolysaccharide (LPS) over 60 min into the superior mesenteric artery. The animals were observed for an additional period of 2 h after the termination of LPS infusion.

2 Eight of the 17 animals infused with LPS and not treated with indomethacin died within 30 min after the beginning of LPS infusion (non-survivors), while the other 9 survived the experimental period of 3 h though in a state of shock (survivors).

3 No alterations were observed in plasma concentrations of PAF and eicosanoids (thromboxane B_2 (TXB_2), 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) and leukotriene B_4 (LTB_4)) in non-survivors. However, a marked increase was detected in TNF release. A significant, though transient, increase in concentrations of PAF, TNF and eicosanoids occurred in the survivors. The peak in the concentrations of PAF and TXB_2 preceded the maximum in TNF values in survivors.

4 Another group of 7 LPS-infused pigs was treated with indomethacin (2 $mg\,kg^{-1}$, i.v. bolus 60 min before the start of LPS infusion, followed by a continuous infusion of 3 $mg\,kg^{-1}\,h^{-1}$). This treatment prevented death and shock despite the high concentrations of circulating TNF and PAF. Concentrations of cyclo-oxygenase enzyme products were reduced, whereas LTB_4 release was not affected. The effect of indomethacin on haemodynamic changes occurred earlier than on cyclo-oxygenase products.

5 In another group of 6 pigs indomethacin (2 $mg\,kg^{-1}$, i.v.) was given 20–25 min after the start of LPS infusion at which time mean arterial blood pressure (MABP) had decreased below 40 mmHg indicating imminent death. This indomethacin treatment immediately reversed the hypotension, restored the organ perfusion, delayed the haemoconcentration and thrombocytopenia and prevented death. However, TNF and PAF concentrations remained elevated. Concentrations of cyclo-oxygenase products studied were reduced by the end of the observation period, whereas LTB_4 production was unaffected.

6 The decrease in MABP induced by exogenous PAF was temporarily prevented by indomethacin.

7 These data indicate that the beneficial effect of indomethacin in LPS-induced septic shock is related to cyclo-oxygenase inhibition as well as to a direct vasoconstrictor property of the drug.

Keywords: Endotoxin; shock; indomethacin; tumour necrosis factor (TNF); PAF; eicosanoids

Introduction

Specific treatment for the cardiopulmonary derangements characteristic of septic shock is lacking, although circulatory shock resulting from bacterial sepsis continues to be a major medical problem (Zimmermann, 1990). Endotoxin, the lipopolysaccharide (LPS) moiety of the microbial cell wall, initiates synthesis of inflammatory mediators, such as eicosanoids, platelet activating factor (PAF) and tumour necrosis factor (TNF) (Morrison & Ryan, 1987; Beutler & Cerami, 1988). Although the opposing actions and interactions of these substances are complex, their net effect in initiating the shock state appears to be very significant (Morrison & Ryan, 1987). Non-steroidal anti-inflammatory drugs (NSAIDs), including indomethacin, represent a rational approach to break up the chain of mediators, because the clinical effectiveness of NSAIDs is thought to be due to the inhibition of cyclo-oxygenase enzyme (Vane, 1971). Indomethacin is effective in improving survival with concomitant decrease in cyclo-oxygenase enzyme products in endotoxin-induced shock in rats (Ball *et al.*, 1986). Furthermore, indomethacin is able to prevent the initial rapid drop in arterial blood pressure after endotoxin challenge in cats (Parratt & Sturgess, 1974; 1975). Additionally, indomethacin has been found to reduce the hypotensive and toxic effect of TNF (Kettelhut *et al.*, 1987; Goto *et al.*, 1989). Recently, BN 52021, a PAF receptor antagonist, has been claimed to protect against septic shock by attenuating the eicosanoid release (Fletcher *et al.*, 1990). These data, taken together, suggest an important relationship between PAF, TNF and eicosanoids and, what is more, seem to indicate that some of the effects of TNF and PAF may be mediated via the cyclo-oxygenase pathway. However, effective blockade of eicosanoid production by BW755C, an inhibitor of both cyclo-oxygenase and 5-lipoxygenase, failed to improve survival in endotoxaemia (McKechnie *et al.*, 1985). Though indomethacin, given i.v. during other kind of shock improved the haemodynamics (Mózes *et al.*, 1989), it is unclear whether the beneficial action was actually due to inhibition of cyclo-oxygenase enzyme or to some other pharmacological property of indomethacin.

The purpose of the present experiments was to determine the relation between the protective effect of indomethacin in

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septic shock and its effect on haemodynamics, haematological and blood glucose changes and the release of TNF, PAF and eicosanoids during septic shock.

Methods

Experimental set-up

After an overnight fast, young female Yorkshire pigs (body weight: 20–24 kg, age: 12–16 weeks) were initially anaesthetized with i.m. injections of ketamine (20 mg kg^{-1}) and midazolam (0.25 mg kg^{-1}) as well as atropine (0.05 mg kg^{-1}). The anaesthesia was maintained throughout the experiment with i.v. sodium pentobarbitone (20 mg kg^{-1} bolus followed by 20 mg kg^{-1} h^{-1} infusion). A tracheostomy was performed and the animals were ventilated with intermittent positive pressure (Bear 2E adult volume ventilator, Lameris, The Netherlands). Respiratory rate, tidal volume and oxygen to air ratio were adjusted to keep arterial blood gases within normal ranges: pH 7.35–7.45, $90 < Po_2$ mmHg < 150, $35 < Pco_2$, mmHg < 45. The animals' temperature, measured with a thermometer (Philips, HP 5311, Japan) attached to the liver, was maintained at around 38°C by use of an electric blanket.

Catheters were placed via femoral vessels into the aorta and pulmonary artery (Swan-Ganz 7 F catheter). For injection of radioactive microspheres a catheter (7 or 8 F) was inserted via the left carotid artery into the left ventricle. A midline laparotomy was performed and the superior mesenteric artery was exposed. After gently clearing the surrounding fat, nerves and connective tissues, a needle (0.5 mm external diameter) connected to a suitable polyethylene tube was inserted directly into the superior mesenteric artery for infusion of LPS or normal saline. Another catheter was inserted into the superior mesenteric vein through a branch vein for collecting blood samples and measurement of mean portal venous pressure (MPVP). The abdomen was then closed and a Ringer-lactate infusion at a rate of 4 ml kg^{-1} h^{-1} was started. The animals were allowed to remain undisturbed for 30 min to ensure haemodynamic stability. Mean arterial blood pressure (MABP), and MPVP were continuously monitored by electromanometers with Statham P23dB strain gauges (Hato Rey, PR). Cardiac output (CO) was determined intermittently by thermodilution (WTI Computer, Holland). The determinations were performed in triplicate and the results averaged. Systemic vascular resistance was calculated by dividing MABP by cardiac output and was reported in arbitrary units (u).

Regional blood flows

To measure organ perfusion five times during an experiment, microspheres (15 ± 1 μm labelled with ^{141}Ce, ^{113}Sn, ^{46}Sc, ^{103}Ru and ^{95}Nb; Dupont de Nemours, NEN Division, Dreieich, F.R.G.) were used as described elsewhere in detail (Saxena *et al.*, 1980). Briefly, approximately 1×10^6 microspheres, suspended in 1 ml of normal saline containing a drop of Tween 80, were injected into the left ventricular catheter over 10 s and the catheter flushed with an additional 3 ml of normal saline. A reference arterial blood sample, starting 10 s before the injection of microspheres and continuing for a total duration of 75 s, was withdrawn at a rate of 10.5 ml min^{-1} via the aortic catheter by use of a pump (B. Braun, Melsungen, FRG). At the end of the experiment, the animals were killed with an overdose of sodium pentobarbitone and heart, kidneys, liver and small intestine were dissected out, weighed and placed into vials. The radioactivity in the vials containing the tissues and blood samples was counted for 5–10 min in a gamma scintillation counter (Packard, Minaxi Autogamma 5000) using suitable windows for discrimination of the different isotopes used. The microsphere data were processed by a PDP-11/70 computer; a set of programmes were used that had been especially developed for the microsphere techniques (Saxena *et al.*, 1980).

Assay for serum levels of tumour necrosis factor

Blood was collected in sterile tubes (Costar, Cambridge, MA). Serum was separated by centrifugation and aliquots were stored at −20°C until assay. TNF activity was determined by measuring the cytostatic effect of TNFα on the murine transformed fibroblast cell line L929 (a gift from Dr W. Fiers, State University of Ghent, Belgium) (Hay & Cohen, 1989; Meager *et al.*, 1989). L929 cells were plated in a 96 well flat-bottom microtiter plate (NUNC, Roskilde, Denmark) at a density of 1×10^4 cells per well in 25 μl of RPMI 1640 culture medium. Medium (25 μl control), human recombinant TNF standard solutions (10, 100, 1000 u ml^{-1}) (Roche Research, Ghent, Belgium) or serum were added (final dilution 1 : 10) to quadruplicate wells. After incubation for 24 h in a humidified atmosphere (7.5% CO_2) at 37°C, 50 μl of a 10 μCi ml^{-1} [^{3}H]-thymidine (Amersham Laboratories, Amersham, England) solution was added. After 2 h of incubation, L929 cells were harvested on glass fibre filtermats (Shatron Inc., Sterling, VA, U.S.A.). The uptake of [^{3}H]-thymidine was measured by liquid scintillation spectroscopy. A standard curve of cytostasis by human recombinant TNF was obtained yielding progressive cytostasis ranging from 10–1000 u ml^{-1} of TNF. The bioactivity of TNF in experimental samples was determined in quadruplicate and compared against the standard curve.

Assay for blood levels of platelet activating factor

Blood (5 ml) was collected into polypropylene tubes containing 45 ml of ice-cold methanol, [^{3}H]-PAF (94000 d.p.m.; 80 Ci mmol^{-1}; radiochemical purity, 99.2%; Amersham, UK) was added and lipids were extracted as described by Filep *et al.*, (1989) and Mózes *et al.*, (1989). Briefly, samples were mixed by slow rotation for 60 min, and the methanolic extract was separated by centrifugation. The pellet was extracted once more with 5 vol of methanol. Supernatants were combined and chloroform and water were added to effect phase separation (methanol-chloroform-water 1 : 1 : 0.9 vol : vol : vol). The lower chloroform-rich phase contained all PAF activity. Chloroform was evaporated under a stream of nitrogen. The samples were redissolved in chloroform-methanol (1 : 1 vol : vol), spotted on silica gel plates (Merck, Darmstadt, FRG) and developed with methanol-chloroform-water (65 : 35 : 6 vol : vol : vol). The area corresponding to authenic PAF (R_F from 0.20 to 0.28) was scraped off and lipids were eluted with 2 ml of methanol. The clear solvent layer was carefully removed and considered to be PAF extract.

Bioassayable PAF activity was determined by platelet aggregation assay (Pinckard *et al.*, 1984) in which washed rabbit platelets were used (Ardlie *et al.*, 1970). Platelet aggregation was determined at 37°C in an aggregometer (Payton Aggregation Module, Payton Ltd., Scarborough, Ontario, Canada) with 200 μl of platelet suspension (200 G l^{-1}) containing 10 μM indomethacin and 0.4 u ml^{-1} apyrase (Sigma). A standard curve for platelet aggregation by authentic PAF was obtained, yielding progressive platelet aggregation ranging from 10^{-10} to 10^{-7} M PAF. PAF bioactivity of experimental samples (after extraction and purification) was determined in duplicate and compared against the standard curve. Values were corrected for individual recovery (ranging from 46 to 56%).

Assay for plasma levels of thromboxane B_2, 6-keto prostaglandin $F_{1\alpha}$, and leukotriene B_4

Blood samples for eicosanoid determinations were obtained from the superior mesenteric vein. The samples were collected into ice-cooled polypropylene tubes containing 20 μl of heparin (5000 u ml^{-1}, Thromboquine, Organon, Oss, The Netherlands) and 50 μl of indomethacin (0.1 mg ml^{-1} in 0.1 M phosphate buffer pH 8). After centrifugation plasma was decanted and stored at −20°C until extraction of eicosanoids, while the red blood cells were suspended in 0.9% saline and

were injected back after each blood collection. Details of the radioimmunoassay (RIA) for eicosanoid determinations have been described reported (Zijlstra & Vincent, 1984).

Determination of blood chemistry values

Plasma glucose concentrations were determined by Glucoquant kit (Boehringer, Mannheim, F.R.G.). Laboratory values of blood haemoglobin, haematocrit as well as leucocyte and platelet counts were measured by haematology analyzers (Sysmex CC-108 and Pl-100, Kobe, Japan, as appropriate).

Experimental protocols

Group I: Sham-operated Three animals were prepared as described above, except that instead of LPS, normal saline was infused into the superior mesenteric artery. Ten ml of blood was collected from the superior mesenteric vein for eicosanoid determination. Five ml of blood was taken from pulmonary artery for laboratory measurements.

Group II: LPS-induced shock Seventeen pigs were infused with $5\,\mu g\,kg^{-1}$ *Escherichia coli* LPS (O111, B4, Serva) into the superior mesenteric artery over a 60 min period and the animals were observed for an additional 120 min period. Blood was collected from the superior mesenteric vein 10 min before and 15, 30 and 60 min after starting LPS infusion and 60 and 120 min after LPS infusion. Radioactive microspheres were injected in 8 of the 17 pigs 2 min before each blood collection, except at 60 min after LPS infusion.

Group III: Indomethacin pretreated Seven pigs were pretreated with $2\,mg\,kg^{-1}$ i.v. of indomethacin 60 min before the start of LPS infusion followed by an infusion of $3\,mg\,kg^{-1}\,h^{-1}$ over the entire experimental period of 4 h. Blood was collected immediately before (−60 min), 15, 30, 60 min after starting indomethacin treatment (−45, −30, 0 min before and 15, 30 and 60 min after the start of LPS infusion and 60 and 120 min after the end of LPS infusion). Radioactive microspheres were injected in 3 of 7 pigs 2 min before each blood collection, except at −45, −30, 15 and 120 min.

Group IV: Indomethacin revived In 6 pigs just after the mean arterial blood pressure had decreased below 40 mmHg at 20–25 min after starting LPS infusion, $2\,mg\,kg^{-1}$ of indomethacin was given i.v. Blood was collected from the superior mesenteric vein and aorta at the same times as in group II. Radioactive microspheres were also injected, identical to group II in four animals.

Group V: PAF injections In 4 pigs $0.02\,\mu g\,kg^{-1}$ PAF (Sigma, Chemicals, Deisenhofen, F.R.G.) was injected into the superior mesenteric vein. Effectiveness of indomethacin treatment on mean arterial blood pressure was tested in four animals by administering PAF into the superiod mesenteric vein 60 min before and 5, 30 and 60 min after i.v. injection of indomethacin ($2\,mg\,kh^{-1}$).

Statistical analysis

All values are expressed as means ± s.e.mean. The data were evaluated by a non-parametric two-way analysis of variance (Friedman test) followed by the Wilcoxon-Wilcox' test to identify differences between measurements performed during the control period and at different times during and after LPS infusion. The Mann-Whitney U test was performed to evaluate the differences between the groups. A P value of 0.05 or less was considered statistically significant for all tests.

Results

Sham-operated animals

In sham-operated animals, systemic haemodynamic variables, organ blood flows, blood chemistry values and eicosanoid levels were stable during the experiment (Table 1).

Animal survival

A total number of 30 pigs received LPS infusion for 60 min (group II, III and IV). Group II consisted of 17 animals which received LPS only. Eight of these 17 animals died at or close to 30 min after the start of LPS infusion. The remaining 9 animals survived the experimental period of 3 h (i.e. 2 h after termination of the LPS infusion), though in a state of profound shock (survivors). None of the animals receiving indomethacin (group III, $n = 7$: indomethacin $2\,mg\,kg^{-1}$ 60 min before the start of LPS infusion and then $3\,mg\,kg^{-1}\,h^{-1}$; group IV, $n = 6$: indomethacin $2\,mg\,kg^{-1}$ 20–25 min after

Table 1 Systemic haemodynamic variables, organ blood flows, laboratory values and eicosanoid concentrations in sham-operated animals

Variables	*Control*	*60 min*	*120 min*	*180 min*
Systemic haemodynamics				
MABP (mmHg)	99 ± 8	116 ± 8	121 ± 8	121 ± 8
MPVP (mmHg)	6.0 ± 0.1	5.7 ± 0.9	5.0 ± 0.5	5.3 ± 0.6
CO ($l\,min^{-1}$)	2.7 ± 0.4	2.8 ± 0.1	2.2 ± 0.1	2.4 ± 0.3
SVR (u)	38 ± 5	42 ± 5	52 ± 4	51 ± 9
Regional haemodynamics ($ml\,min^{-1}\,100\,g^{-1}$)				
Heart	132 ± 44	115 ± 18	NM	100 ± 11
Kidneys	296 ± 22	280 ± 9	NM	291 ± 38
Liver	20 ± 7	18 ± 7	NM	27 ± 11
Small intestines	47 ± 1	46 ± 4	NM	42 ± 2
Laboratory values				
Hb ($mM\,l^{-1}$)	5.7 ± 0.4	6.0 ± 0.4	6.1 ± 0.5	6.3 ± 0.4
WBC count ($G\,l^{-1}$)	13.5 ± 2.3	20.0 ± 5.0	20.0 ± 5.0	20.0 ± 6.0
Platelet count ($G\,l^{-1}$)	350 ± 49	340 ± 48	330 ± 49	330 ± 52
Blood glucose ($mM\,l^{-1}$)	6.0 ± 0.2	6.3 ± 0.3	5.8 ± 0.2	5.8 ± 0.4
Eicosanoid concentration ($pg\,ml^{-1}$)				
TxB_2	180 ± 8	130 ± 14	120 ± 17	110 ± 37
6-ketoPGF$_{1\alpha}$	150 ± 10	160 ± 8	160 ± 10	150 ± 10
LTB_4	180 ± 27	170 ± 73	180 ± 19	160 ± 17

Values are means ± s.e.mean. TXB_2, thromboxane B_2; 6-keto $PGF_{1\alpha}$, 6-keto prostaglandin $F_{1\alpha}$; LTB_4, leukotriene B_4; Hb, haemoglobin; WBC, while blood cells, SVR, systemic vascular resistance (u).

starting LPS infusion) died during the observation period of 3 h.

Systemic haemodynamics (Figure 1)

In group II pigs not surviving the LPS infusion, MABP dropped to 28 ± 2 mmHg, MPVP increased to 12 ± 1 mmHg and CO decreased to 1.0 ± 0.1 l min^{-1}, shortly before death (30 min after the start of LPS infusion). In pigs surviving the LPS infusion, MABP gradually decreased to 55 ± 5 mmHg at the end of the 3 h observation period (2 h after the end of LPS infusion), MPVP first increased to peak values at 30 min before decreasing slightly but remaining higher than baseline values; CO first decreased rapidly (15–30 min after the start of LPS infusion) and then gradually to about 36% of the baseline value (Figure 1; a).

In group III treated with indomethacin before the start of LPS infusion, MABP increased immediately after indomethacin administration and then, at the start of LPS infusion, it gradually decreased but remained still higher than in indomethacin-untreated LPS survivors: 87 ± 6 mmHg (n = 7) vs. 55 ± 5 mmHg (n = 8), respectively, at the end of the 3 h observation period; $P < 0.05$. The early increase but not the late increase in MPVP levels as well as the early decrease but not the late decrease in CO levels were attenuated in this group (Figure 1; b).

In animals revived with indomethacin (group IV), MABP increased dramatically immediately after indomethacin and remained high up to the end of the observation period: 79 ± 8 mmHg (n = 6) vs. 55 ± 5 mmHg (n = 9) in the group surviving the LPS infusion; $P < 0.05$. MPVP increased shortly after the indomethacin administration in this group and then decreased to levels observed in the untreated LPS-infused survivors and in the indomethacin pretreated group. CO was transiently elevated and then decreased to the same level as in the untreated LPS-infused survivors: (1.0 ± 0.2 l min^{-1} vs. 1.0 ± 0.1 l min^{-1}) (Figure 1c). A typical record of the immediate reversing effect induced by i.v. administration of indomethacin on arterial blood pressure is shown in Figure 2.

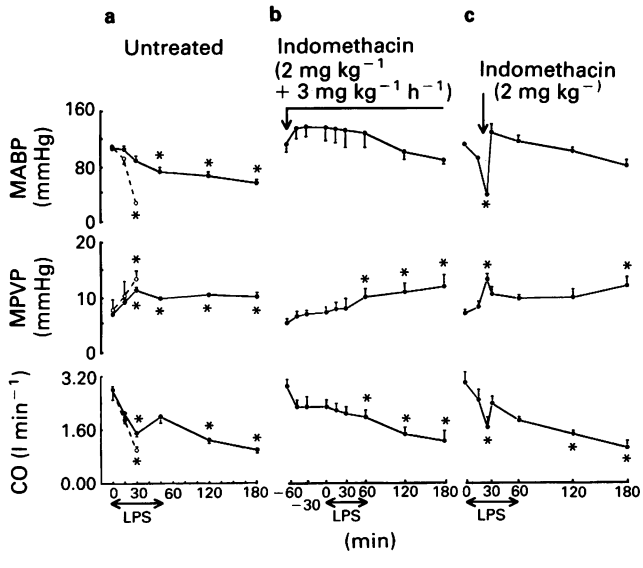


Figure 1 Effect of indomethacin treatments on systemic haemodynamics in pigs receiving endotoxin (LPS) infusion into the superior mesenteric artery. Arrows indicate the time for administration of either LPS or indomethacin, as appropriate. In the untreated group, survivors (●—●) and non-survivors (o----o) indicate, respectively, the animals that survived or died during the observation period of 3 h after the beginning of LPS infusion. Values are means with s.e.mean shown by vertical bars. Abbreviations; MABP, mean arterial blood pressure; MPVP, mean portal venous pressure; CO, cardiac output. *$P < 0.05$ value was obtained by 2-way analysis of variance (Friedman test) followed by Wilcoxon-Wilcox' test for multiple comparisons, control (t = 0 min) versus 15 min, control versus 30 min, etc.

Systemic vascular resistance (MABP/CO ratio) increased transiently in the untreated LPS-infused survivors from 15–30 min after the start of LPS infusion (from 43 ± 4 u to 66 ± 5 u and 68 ± 4 u at 15 and 30 min respectively), and then fell sharply to approximately the baseline value: 50 ± 5 u at 180 min. No changes in systemic vascular resistance were observed in LPS-infused non-surviving pigs: 38 ± 1 u at 30 min vs. 40 ± 3 u at baseline. Systemic vascular resistance increased slowly in the indomethacin pretreated group; at 30 min after the start of indomethacin administration the value was 64 ± 11 u vs. 37 ± 3 u at baseline. This increase was not affected by the LPS infusion and remained high up to the end of the observation period: 74 ± 23 u. Increase in the systemic vascular resistance was also observed in the animals revived with indomethacin.

Organ blood flows (Figure 3)

In LPS-infused non-survivors, LPS significantly decreased blood flow to all organs studied. The flow to the heart, kidneys and small intestine was reduced by about 40% and to the liver by more than 60% at 15 min after starting LPS infusion. In survivors, blood flow to the heart and kidneys was stable during LPS infusion, but by the end of the experiment (2 h after stopping LPS infusion) blood flow to the heart and kidneys was markedly decreased. Perfusion of the liver and small intestine was sharply reduced at 15 min after the start of LPS infusion. Subsequently the liver flow increased above the baseline levels and the flow to the small intestine returned to about the baseline levels at 30–60 min of LPS infusion. After stoppage of LPS infusion, perfusion of the liver and small intestine decreased again during the prolonged hypotension.

Indomethacin pretreatment in itself did not affect organ perfusion. The drug, however, attenuated the decreases in coronary and hepatic blood flow found at the end of the observation period. The decreases in the renal and small intestinal blood flow were not affected by indomethacin pretreatment.

The seriously reduced flow to the heart returned to baseline within 10 min in animals revived with indomethacin. Despite the gradual decline, the cardiac flow stabilized at a higher level than that observed in untreated survivors: 112 ± 21 ml min^{-1} 100 g^{-1} vs. 62 ± 7 ml min^{-1} 100 g^{-1}, $P < 0.05$. Similar changes were observed in the flow to the

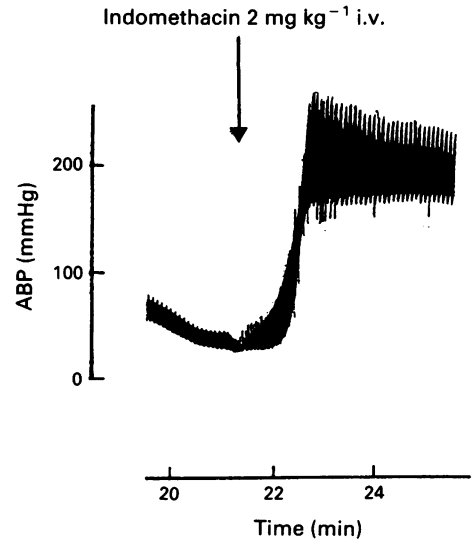


Figure 2 Effect of i.v. treatment with indomethacin on arterial blood pressure (ABP) in an anaesthetized pig during endotoxin shock. The arrow indicates the start of indomethacin administration. Time scale shows time after the start of LPS infusion.

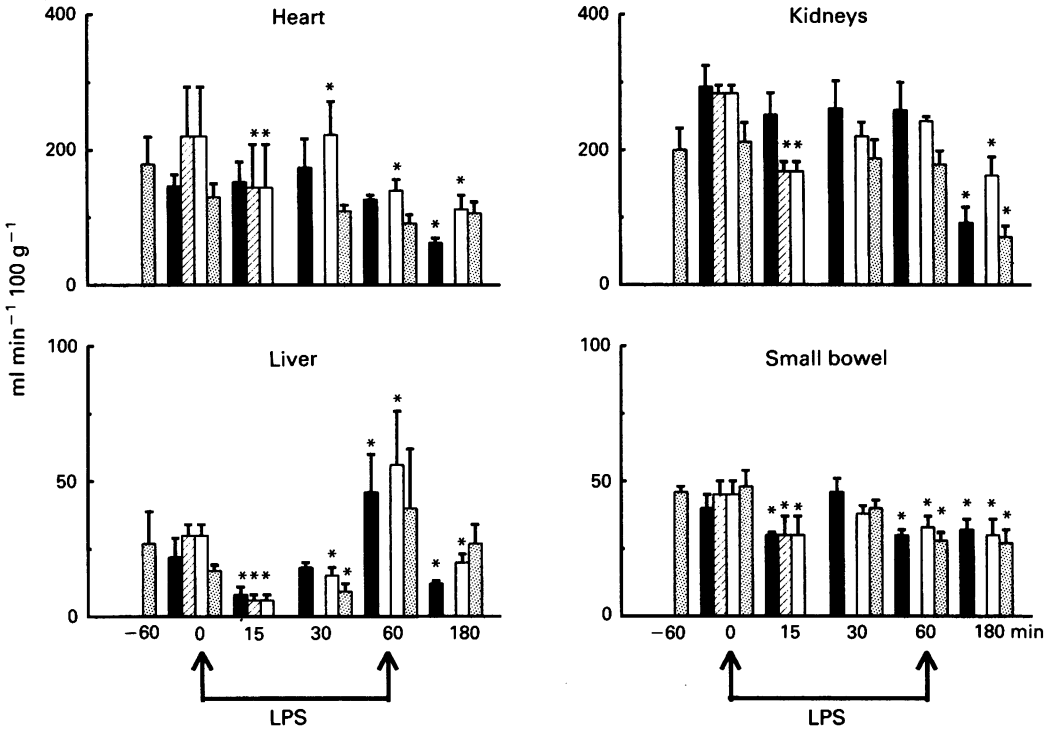


Figure 3 Effect of indomethacin treatment on organ blood flows in pigs receiving endotoxin (LPS) infusion into the superior mesenteric artery: solid columns, untreated survivors; hatched columns, untreated non-survivors; stippled columns, indomethacin ($2\,mg\,kg^{-1} + 3\,mg\,kg^{-1}\,h^{1}$) pretreated group; open columns, indomethacin ($2\,mg\,kg^{-1}$) revived group. $^{*}P < 0.05$ vs. control (t = 0 min).

kidneys and liver. The diminished flow to the small intestine was transiently elevated. This improvement did not last up to the end of the experiment.

Blood chemistry values (Figure 4)

LPS infusion was followed by a rapid increase in haemoglobin values, evident from 30 min after the start of LPS infusion. This haemoconcentration was apparent towards the end of the observation period in survivors and did not improve after the LPS infusion was stopped. The increase in haemoglobin concentration seemed attenuated in both the indomethacin-pretreated and indomethacin-revived groups.

Platelet counts dropped 30 min after LPS infusion was started in both survivors and non-survivors. There were no significant differences between groups at 30 min. After LPS infusion was stopped, platelet counts in the survivors remained significantly lower than at the baseline. The appearance of thrombocytopenia observed in endotoxic shock was not much affected by indomethacin.

In untreated LPS-infused non-survivors a clear hyperglycaemia developed, while in survivors a severe hypoglycaemia was observed. In the indomethacin pretreated group, hyperglycaemia failed to develop but hypoglycaemia was similar to that observed in endotoxic shock ($3.5 \pm 1.1\,mM\,l^{-1}$ vs. $2.8 \pm 0.4\,mM\,l^{-1}$). Hyperglycaemia decreased after revival with indomethacin, but hypoglycaemia was also not affected in this group.

Mediator release

In untreated LPS-infused non-survivors no changes were found in either eicosanoid ($n = 4$, data not shown) or PAF release ($n = 3$), but a marked increase was detected in serum TNF concentration ($n = 5$), which reached a maximum level at the time of death i.e., at 30 min after the start of LPS infusion (Figure 5). In contrast, blood concentration of TXB_2 in superior mesenteric vein increased markedly, but only for a short time, during LPS infusion in survivors ($n = 5$). Similarly a transient, but less marked increase was detected in 6-keto $PGF_{1\alpha}$ concentrations of superior mesenteric vein in survivors ($n = 5$). The increase in TXB_2 concentration started 30 min earlier than 6-keto $PGF_{1\alpha}$ elevation. In untreated survivors, the plasma concentration of LTB, also showed an increase in superior mesenteric vein ($n = 5$). LTB_4 production was elevated when the concentrations of TXB_2 and 6-keto $PGF_{1\alpha}$ had returned to baseline. In contrast to prostanoid release, the LTB_4 production seems to be continuous during the observation period. A significant, though transient increase was also measured in concentrations of PAF ($n = 2$) and TNF ($n = 4$) in survivors. The maximum release of PAF and thromboxane occurred at the same time and both preceded the peak in TNF concentration, which was lower in survivors than in non-survivors ($4000 \times 10^2\,u\,ml^{-1}$ $n = 5$, vs. $2000 \times 10^2\,u\,ml^{-1}$ $n = 4$) (Figure 5a).

Indomethacin pretreatment (Figure 5b)) did not modify the release of TNF ($n = 5$) and PAF ($n = 3$). Both concentrations increased to the same levels with a similar time course to that observed in animals that survived LPS infusion without treatment. Indomethacin infusion was followed by a significant decrease in plasma concentrations of TXB_2 and 6-keto $PGF_{1\alpha}$ evident from 30 min after the start of indomethacin treatment. This fall was apparent toward the end of the experiment ($n = 7$ each). However, LTB_4 production remained unaffected during indomethacin treatment ($n = 6$).

The markedly elevated TNF levels decreased in animals revived with indomethacin ($n = 4$). At the end of the experiment TNF concentrations were just detectable (Figure 6). In non-survivors a lack of PAF release was observed but after indomethacin injection PAF increased in the same manner as observed in LPS-infused survivors, but the peak occurred 30 min later ($n = 3$). The time course of PAF release in this group of animals was identical to that observed in untreated animals surviving LPS infusion as well as in the indomethacin pretreated group. At the end of the experiment the concentrations of TXB_2 and 6-keto $PGF_{1\alpha}$ were reduced significantly, however, LTB_4 production remained unchanged ($n = 4$, each).

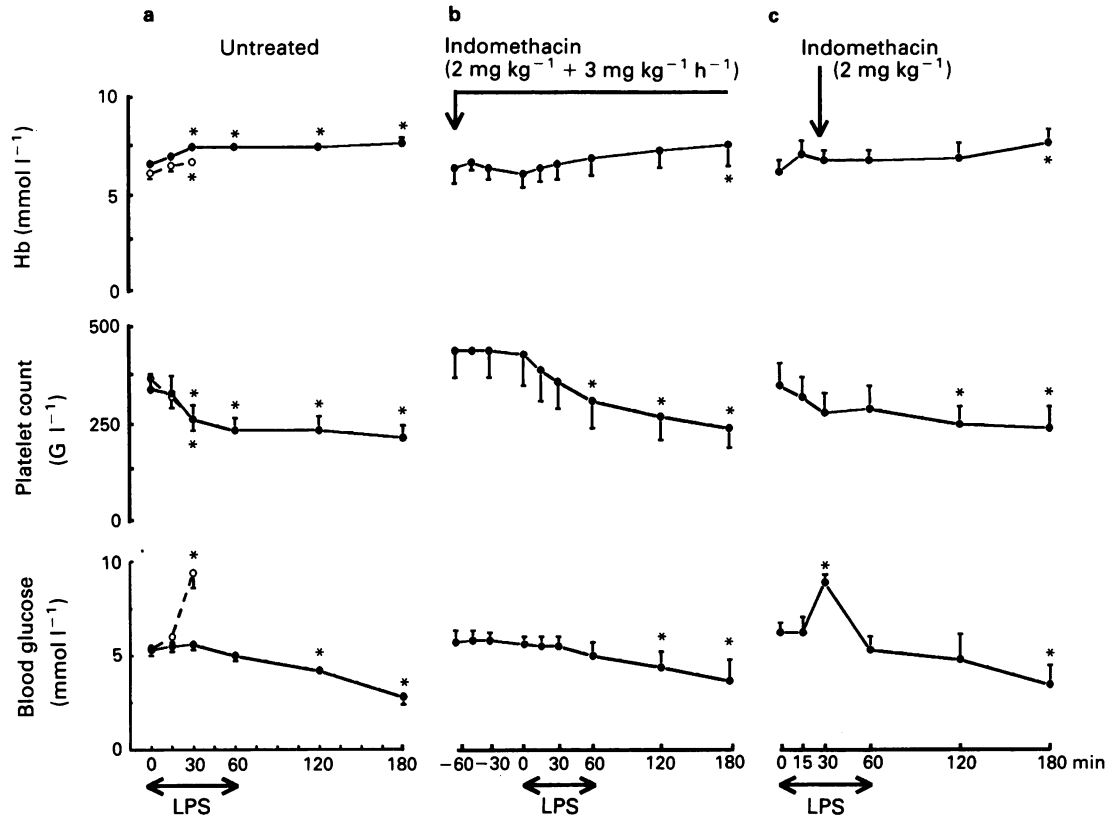


Figure 4 Effect of indomethacin treatments on haematological and blood sugar values in pigs receiving endotoxin (LPS) infusion into the superior mesenteric artery. In the untreated group, survivors (●—●) and non-survivors (o---o) indicate, respectively, the animals that survived or died during the observation period of 3 h after the beginning of LPS infusion. Hb, haemoglobin; $*P < 0.05$ vs. control (t = 0 min).

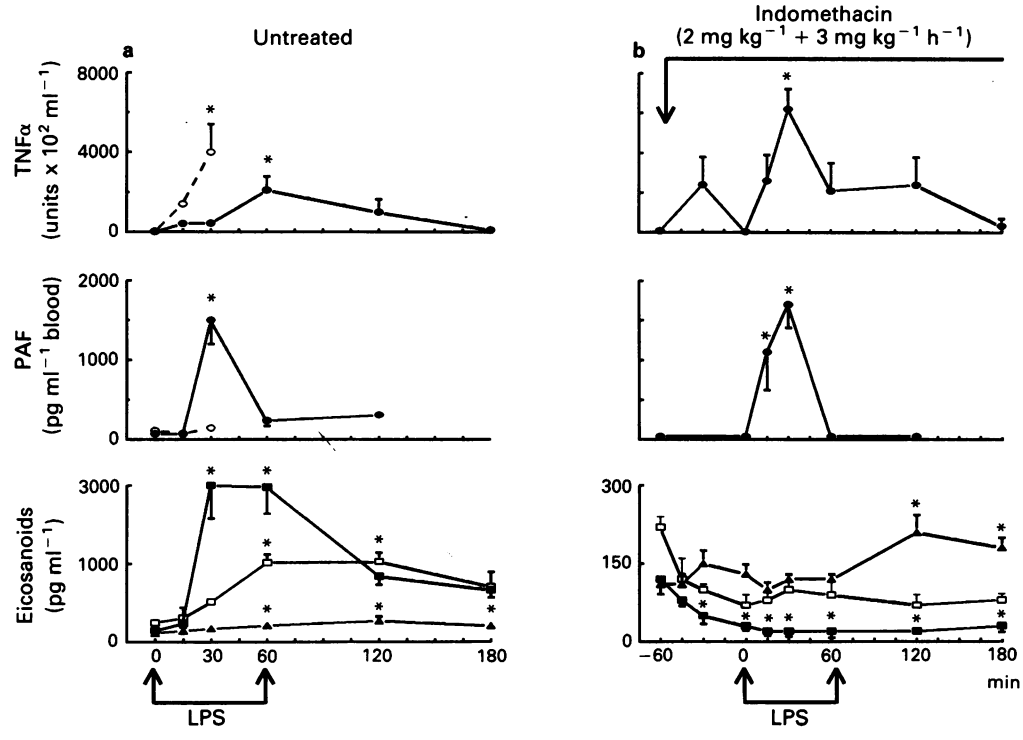


Figure 5 Effect of indomethacin pretreatment on blood levels of tumour necrosis factor (TNFα), platelet activating factor (PAF) and eicosanoids in pigs receiving endotoxin (LPS) infusion into the superior mesenteric artery. In the untreated group, suvivors (●—●) and non-survivors (o---o) indicate, respectively, the animals that survived or died during the observation period of 3 h after the beginning of LPS infusion. Bottom panel represents eicosanoid release in the survivors: thromboxane B_2(■); 6-keto prostaglandin $F_{1\alpha}$(□); leukotriene B_4 (▲). $*P < 0.05$ vs. control (t = 0 min).

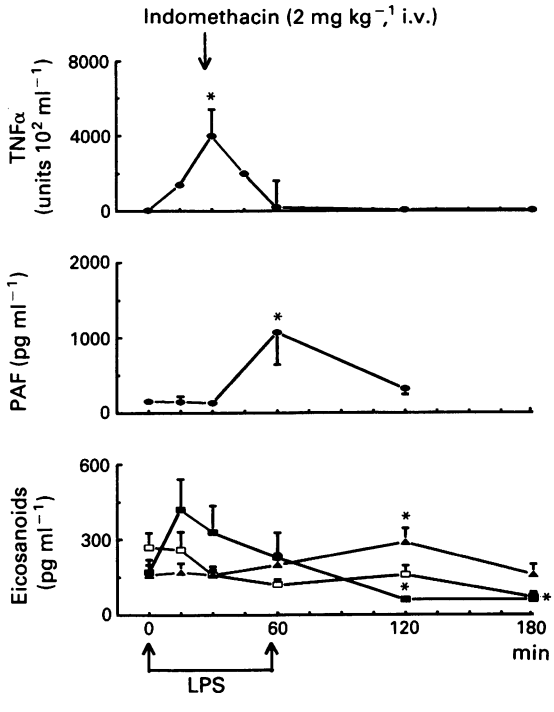


Figure 6 Effects of revival with indomethacin on blood levels of tumour necrosis factor (TNFα), platelet activating factor (PAF) and eicosanoids. Symbols as in Figure 5. *$P < 0.05$ vs. control (t = 0 min).

Effect of indomethacin on the hypotensive response to exogenous platelet activating factor

Administration of 0.02 μg kg^{-1} of PAF into the superior mesenteric vein reduced MABP by 74 ± 4% ($n = 4$) 60 min before and by 5 ± 5%, 13 ± 4% and 26 ± 1% at 5, 30 and 60 min, respectively, after 2 mg kg^{-1} of indomethacin (Figure 7, $P < 0.05$, $n = 4$ each). At 120 min after administration of indomethacin, MABP decreased again by 70 ± 5% after PAF injection. MABP increased after indomethacin injection from 95 ± 4 mmHg to 138 mmHg in 1 min. However, at 30 min after indomethacin injection MAPB had returned to the baseline (99 ± 5 mmHg) and remained at this level throughout the remaining period.

Discussion

The main findings in the present study are: (a) the non-survivors following LPS infusion showed marked haemodynamic alterations, no changes in release of PAF and eicosanoids but marked increase in TNF production; (b) the animals surviving LPS infusion remained in a severe shock state up to the end of the 3 hours observation period (2 hours after termination of LPS infusion); they presented a sequential release of PAF, TNF and eicosanoids but the increase in TNF was much less than in the non-surviving animals; (c) indomethacin administration, starting either before LPS (indomethacin pretreated group) or shortly before presumed death, improved circulatory derangements, reduced release of cyclo-oxygenase products without affecting leukotriene production; and (d) the release of TNF and PAF was not affected in the indomethacin pretreated group but the markedly elevated TNF levels decreased in animals revived with indomethacin.

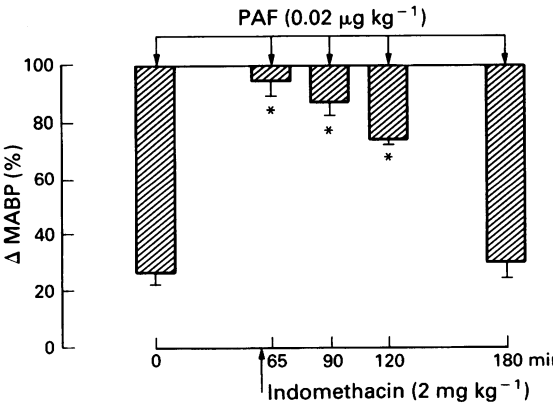


Figure 7 Effect of platelet activating factor (PAF; 0.02 μg kg^{-1}) injection into the superior mesenteric vein over 1 min on mean arterial blood pressure (MABP) in absence and presence of indomethacin. Arrows indicate the time of administration of either PAF or indomethacin as appropriate. *$P < 0.05$ vs. control ($t = 0$ min).

Endotoxic shock model

Infusion of a low amount of endotoxin is a recommended way to induce standardized shock which may more closely resemble clinical septic shock than bolus administration of LPS (Fink *et al.*, 1989; Naess *et al.*, 1989). In the present study with LPS infusion into the superior mesenteric artery, half of the animals died within 30 min of administration of endotoxin. The main changes observed in the pigs not surviving the LPS infusion were a marked decrease in blood pressure, rapid increase in portal pressure contributing to a reduced cardiac preload, reduction in CO without any compensatory increase in systemic vacular resistance, significant decrease in the blood flow to various organs, marked reduction in platelet counts, hyperglycaemia and marked increase in TNF release. These changes may have led to early death because of serious reduction of blood flow to essential organs and because of toxic properties of TNF. It seems that eicosanoids and PAF do not play an important role in causing death of the LPS-infused pigs because their levels remained unchanged in non-survivors. It might be that TNF, released in large quantities during rapid death, has a destructive effect on the cells able to synthesize PAF and eicosanoids.

The state of shock in the animals surviving the LPS infusion was manifested by systemic hypotension, elevated portal pressure, a transient increase in systemic vascular resistance, a damaged organ perfusion, haemoconcentration, leucocytopenia, thrombocytopenia, hypoglycaemia and a sequential release of eicosanoids. The survival following LPS infusion might be due to an increase in systemic vascular resistance which aids in maintenance of MABP, thus improving the supply of blood to essential organs. After exhausting this compensatory vasoconstriction the flow to organs markedly increased and, consequently, the systemic vacular resistance, cardiac output and arterial blood pressure were reduced. Moreover, the increase in TNF levels induced by LPS, was less in survivors than in non-survivors.

Indomethacin treatment

Indomethacin pretreatment prevented the circulatory collapse seen after LPS infusion despite the high concentration of TNF and PAF. The flow to heart and liver was preserved whereas renal and intestinal circulation diminished. Furthermore, an increase in portal pressure occurred reducing cardiac preload. This event may be responsible for the decreased cardiac output observed in the indomethacin pretreated group despite the preserved coronary circulation as demonstrated here. Haemoconcentration and thrombocytopenia were shifted to the end of the experiment after indomethacin pretreatment. Hypoglycaemia seen during endotoxic shock was not affected by indomethacin pretreatment but hyperglycaemia failed to develop.

Animals in group IV were treated with indomethacin (2 mg kg^{-1}, i.v.) at the time of the peak LPS-induced hypotensive effect, which, in the untreated animals, was followed by

death within 5–10 min. This indomethacin treatment was highly effective in preventing the imminent death and an immediate reversal of hypotension was observed with a concomitant improvement in cardiac output and organ blood flows. However, this beneficial effect on cardiac output and organ circulations was transient; by the end of the observation period both cardiac output and organ flows tended to decline again. Nevertheless, the flow to most of the organs studied was higher in animals revived with indomethacin than in untreated animals surviving LPS infusion. This may be due to the elevated systemic vascular resistance seen after indomethacin treatment. Importantly, renal blood flow was better preserved in the group revived with indomethacin than in the indomethacin pretreated group. The exact reason for this difference is not clear, but it may be due to the difference in the duration and dose of indomethacin treatment.

Indomethacin was effective in blocking cyclo-oxygenase as indicated by the lowered concentrations of TXB_2 and 6-keto $PGF_{1\alpha}$ in both types of indomethacin treatment. Since indomethacin effectively inhibited prostanoid production, it is reasonable to assume that its beneficial effects are due, at least in part, to the inhibition of prostanoid synthesis (Fletcher & Ramwell, 1977; Fletcher, 1982; Ball *et al.*, 1986; Lefer, 1989). However, the effect of indomethacin on blood pressure was rapid in onset, reaching its maximum within 60 s, implying that vasoconstriction seen after indomethacin administration may be independent of cyclo-oxygenase inhibition. Moreover, in the animals revived with indomethacin, no changes in prostanoid release were noticed before or immediately after indomethacin, yet the drug produced a marked vasoconstrictor response (see Figures 6 and 2, respectively). Treatment with indomethacin during mesenteric ischaemia/reperfusion-induced shock in dogs also restored the circulation in a similar way, namely, vasoconstriction occurred in 30 s while the decrease in prostanoid concentration occurred later on (Mózes *et al.*, 1989). Although indirect vasoconstriction via suppression of local prostacyclin generation in a particular vascular bed, associated with a slower change in the circulating 6-keto $PGF_{1\alpha}$, cannot be entirely excluded, this seems not very likely because both aspirin and ibuprofen, despite being potent cyclo-oxygenase inhibitors, are not capable of eliciting a rapid vasoconstrictor response (Fletcher & Ramwell, 1977; Balk *et al.*, 1988). The protective effect of ibuprofen against endotoxic shock in dogs develops with a delay of 45 min (Balk *et al.*, 1988) and the improvement in the survival during endotoxic shock in dogs by aspirin occurs without any attenuation of the haemodynamic effects of LPS (Fletcher & Ramwell, 1977).

Regardless of the precise mechanism of action, indomethacin has been shown to have a beneficial effect during different shock states in various animal species including endotoxic shock (Parratt & Sturgess, 1974; 1975; Fletcher & Ramwell, 1977; Fletcher, 1982; Mózes *et al.*, 1989). However, one should keep in mind, that this beneficial effect was transient in the present study. By the end of the experiment the decreased portal pressure increased again and the increased cardiac output improved diminishing organ blood flows in both types of indomethacin treatment. Despite these events the arterial blood pressure remained compensated probably due to elevated systemic vascular resistance. However, in these animals LPS infusion did cause hypoglycaemia, and only delayed the occurrence of thrombocytopenia and haemoconcentration. This indicates that mediators other than cyclo-oxygenase products may be involved in endotoxic shock. Indeed, the LTB_4 concentration remained elevated in animals revived with indomethacin reflecting an increased 5-lipoxygenase activity. Consequently, high concentrations of other lipoxygenase products (i.e. peptido-leukotrienes) can be expected. These peptido-leukotrienes (LTC_4 and LTD_4) are active on vascular smooth muscle and enhance leakiness (Lefer, 1989). Thus leukotrienes can contribute to the elevated portal pressure and haemoconcentration seen at the end of the experiment in the indomethacin-treated animals independently of the type of the treatment. Since leukotrienes have no effect on platelet aggregation (Lefer, 1989), the drop in platelet counts is probably due to the presence of increased release of PAF. Though indomethacin given i.v. markedly reduced the effect of PAF on blood pressure over more than 1 h, it failed to prevent the thrombocytopenia seen after LPS infusion. Since thrombocytopenia occurred later in indomethacin pretreated animals than in LPS infused animals, other mediators besides PAF may also be involved in this process, for example thromboxane.

Furthermore, it is worth mentioning that LTB_4 concentration in indomethacin-treated animals reached similar levels as measured in pigs treated with LPS alone. Thus a shift of arachidonic acid metabolism to the 5-lipoxygenase pathway in the presence of cyclo-oxygenase enzyme blockade was not observed in the present experiments.

In summary, indomethacin given either 60 min before or 20–25 min after starting LPS infusion (5–10 min before imminent death) attenuated or reversed most of the haemodynamic (hypotension, low cardiac output and reduced organ perfusion) and laboratory (haemoconcentration, thrombocytopenia and hyperglycaemia) changes in the early stage of endotoxic shock. Late changes, except hypoglycaemia, thrombocytopenia and portal hypertension, were less but significantly affected by indomethacin. The sequential release of different mediators during endotoxic shock indicates an important relationship between TNF, PAF and eicosanoids, though present results do not verify that effects of PAF and TNF are mediated via the cyclo-oxygenase pathway (Kettelhut *et al.*, 1986; Fletcher *et al.*, 1990). It should be noted that the effect of indomethacin on haemodynamics precedes the effective blockade of cyclo-oxygenase enzyme. Our data indicate that the protective effect of indomethacin in preventing septic shock death is probably due to the combined vasoconstrictor and cyclo-oxygenase blocking properties of the drug, which may be of value in rescuing patients in septic shock.

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Electrophysiological effects of amineptine on neurones of the rat substantia nigra pars compacta: evidence for an inhibition of the dopamine uptake system

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1 Intracellular recordings were made from substantia nigra pars compacta neurones of the rat maintained *in vitro* in order to study the effects of the tricyclic antidepressant drug, amineptine.

2 Amineptine hydrochloride (1–30 μM) decreased spontaneous firing and slightly hyperpolarized the membrane potential. In neurones voltage-clamped at −50 or −60 mV, amineptine produced an outward membrane current. These actions were concentration-dependent and were completely antagonized by (−)-sulpiride.

3 The amineptine-induced hyperpolarization was resistant to tetrodotoxin (1 μM) but it was abolished in 0 mM Ca^{2+} (plus 13 mM $MgCl_2$) solutions.

4 Amineptine (300 nM–30 μM) and cocaine (10–30 μM) increased the amplitude and duration of responses to exogenously applied dopamine. The effects of dopamine were potentiated about 5 fold by 10 μM amineptine; this potentiation persisted in calcium-free solutions.

5 Cocaine (10 μM) had no additional effect on the dopamine-induced responses in the presence of amineptine (30 μM). Amineptine (10 μM) produced no detectable effects in the presence of cocaine (30 μM).

6 It is concluded that amineptine acts as a dopamine uptake blocker in slices of rat substantia nigra.

Keywords: Amineptine; dopamine; cocaine; dopamine-uptake; antidepressant drugs; substantia nigra; intracellular recordings; brain slices

Introduction

The dopamine-containing neurones of the substantia nigra pars compacta are known to have autoreceptors for their own transmitter. Activation of these receptors by dopamine and dopamine agonists decreases firing rate (Aghajanian & Bunney, 1973; 1977; Pinnock, 1983) because of the hyperpolarization resulting from an increased potassium conductance (Lacey *et al.*, 1987; 1988). It has been recently reported that amphetamine, levodopa and cocaine, all indirectly-acting dopamine agonists (Fischman, 1987; Brannan *et al.*, 1989), have an inhibitory influence on the firing discharge of the dopaminergic cells by hyperpolarizing the membrane potential (Bunney *et al.*, 1973; Einhorn *et al.*, 1988; Mercuri *et al.*, 1989; Lacey *et al.*, 1990a; Mercuri *et al.*, 1990b; 1991). The predominant action of these drugs is to augment the extracellular brain content of dopamine by increasing the efflux (amphetamine, levodopa) or by blocking the uptake processes (cocaine). Amineptine is a tricyclic antidepressant drug that is commonly used in clinical medicine. It is devoid of anticholinergic effects and is suitable for the treatment of depression in elderly people (Marconi *et al.*, 1989). Although biochemical studies have shown that it may inhibit the uptake of dopamine in the brain (Samanin *et al.*, 1977: Bonnet *et al.*, 1987), there is no electrophysiological data concerning the effects of amineptine on neuronal dopamine responses. In the present study, we have tested the hypothesis that amineptine acts on the dopaminergic neurones in the substantia nigra pars compacta via blockade of dopamine uptake. This was investigated by measurement of the effects of amineptine on the responses to exogenously applied dopamine by use of intracellular recordings *in vitro*.

Methods

The methods used have been described previously (Lacey *et al.*, 1987; Mercuri *et al.*, 1990b). Albino Wistar rats (150–350 g) of either sex were killed by a heavy blow to the chest under ether anaesthesia: this severed major blood vessels. The brain was quickly removed and slices of the mesencephalon (300–350 μm thick) which contained the substantia nigra were cut with an Oxford vibratome. A single slice was then transferred to a recording chamber and continuously superfused at a rate of 2.5 ml min^{-1}, with a solution maintained at 35°C and gassed with 95% O_2/5% CO_2. The standard solution contained (mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.4, glucose 10 and $NaHCO_3$ 26. Intracellular recording electrodes were filled with either 2 M KCl or 2 M K-acetate and had a resistance of 30–120 MΩ. A single electrode voltage clamp amplifier was used (Axoclamp 2A) for both voltage and current recordings. Discontinuous single-electrode voltage clamp recordings were made at 2.5–3.5 kHz (30% duty cycle). The voltage signal at the head stage amplifier was continuously monitored on a separate oscilloscope (Mercuri *et al.*, 1989; 1990a). Membrane potentials and currents were displayed by use of a Gould RS 3400 or a Hewlett Packard 7402 A chart recorder. Drugs were applied by superfusion, substituting the control solution for one containing the drug at known concentration. Drug solutions entered the recording chamber no later than 20 s from changing solutions via a 3 way tap. Complete replacement of the medium in the chamber took 90 s. The following drugs were used: amineptine hydrochloride (Poli), dopamine hydrochloride (Sigma) and (−)-sulpiride (Ravizza). Numerical data are expressed as mean ± s.e.mean.

Results

Intracellular recordings were obtained from 46 'principal' dopaminergic neurones of the substantia nigra zona compacta (Lacey *et al.*, 1989). These cells had a mean resting potential of −54 ± 1.3 mV (n = 33) and a mean spontaneous firing rate of 2.5 ± 0.4 Hz (n = 25). Ten neurones were silent. The electrophysiological properties of the cells were similar to those already described for the dopaminergic neurones in the substantia nigra (Lacey *et al.*, 1987; 1988; 1989; 1990a; 1990b; Mercuri *et al.*, 1989; 1990a; 1990b; 1991). Dopamine (3–30 μM) was superfused on 34 cells, which all responded with a

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concentration-dependent reduction in firing activity and a hyperpolarization of the membrane, or an outward current (under voltage clamp) as previously described (Lacey *et al.*, 1987). The reduction in firing rate and the hyperpolarization of the somatic membrane were not always linearly related: a dopamine-induced hyperpolarization of distal dendrites may also account for the reduction in firing.

Actions of amineptine

Amineptine hydrochloride (1–30 μM) caused an inhibition of the spontaneous firing activity and a small hyperpolarization of the membrane. The action of the drug on the firing rate was studied on 20 neurones: these responded with a concentration-dependent decrease in rate (Figure 1). The average hyperpolarizations caused by 3 μM and 10 μM amineptine were 1.4 ± 0.4 mV ($n = 4$) and 2.6 ± 0.5 mV ($n = 8$) respectively (these cells were hyperpolarized by current injection just sufficient to prevent spontaneous firing activity). Although not studied in detail, there was an obvious parallel relationship between the cellular responses to dopamine and amineptine applications. Under voltage clamp, (at −50 or −60 mV, holding potential) an outward current was produced by amineptine (Figure 2b).

Four to seven minutes were typically required by amineptine to produce a steady action that lasted for the period of application of the drug. A complete recovery was not observed even after 130 min of washing. The inhibition of the firing and the hyperpolarization of the membrane were rapidly and fully antagonized by the selective D_2 receptor antagonist (−)-sulpiride (1 μM) (Figure 1b). (−)-Sulpiride had no effect on the membrane potential and firing activity of the cells when applied alone (Lacey *et al.*, 1987, Mercuri *et al.*, 1989).

The inclusion of tetrodotoxin (1 μM) in the superfusate did not block the cellular responses to amineptine (4 cells); in 3 neurones, however the hyperpolarizing responses was completely abolished when Ca^{2+} was removed from the superfusate (Figure 4).

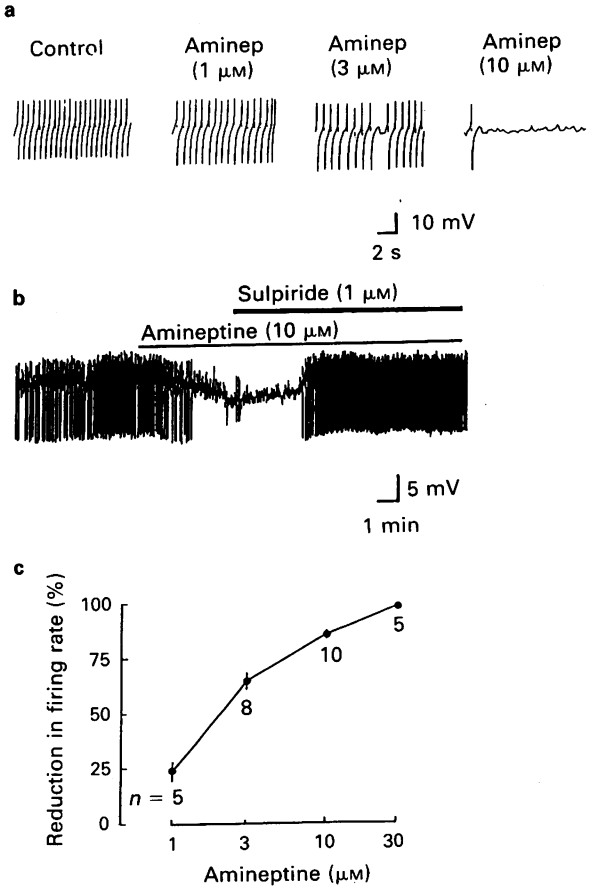


Figure 1 Amineptine-induced inhibition of the firing rate of dopaminergic cells is antagonized by (−)-sulpiride. (a) The spontaneous firing activity of a cell was reduced by increasing concentrations of amineptine (Aminep) applied cumulatively. In 10 μM amineptine the membrane potential was −58 mV. Note that the hyperpolarization of the membrane potential *per se* increases the spike afterhyperpolarization (Grace & Bunney, 1984; Lacey *et al.*, 1990b) in the substantia nigra pars compacta cells. Full amplitude of the action potentials in this and the following figures is not reproduced due to the low frequency response of the pen recorder. (b) Voltage trace shows that amineptine (10 μM) hyperpolarized a substantia nigra cell and blocked the spontaneous firing activity. (−)-Sulpiride (1 μM), applied at the period indicated by the bar, antagonized the hyperpolarizing effect of amineptine and restored spontaneous firing. The firing activity of this cell in sulpiride was similar to that observed before the application of amineptine. Sulpiride itself does not modify the firing rate of substantia nigra zona compacta cells (Lacey *et al.*, 1987, Mercuri *et al.*, 1989; 1990b). The cell membrane potential was −60 mV at the peak of the amineptine-induced hyperpolarization. (c) Dose-response curve of amineptine on the spontaneous firing rate of 20 neurones. Points show mean effects and vertical lines indicate s.e.mean for the numbers of experiments indicated.

Effects of amineptine on the responses induced by exogenous dopamine

It has previously been shown that dopamine cells are hyperpolarized by dopamine, acting on D_2 soma-dendritic autoreceptors (Lacey *et al.*, 1987). We found that the sensitivity of these cells to dopamine was consistently increased by aminep-

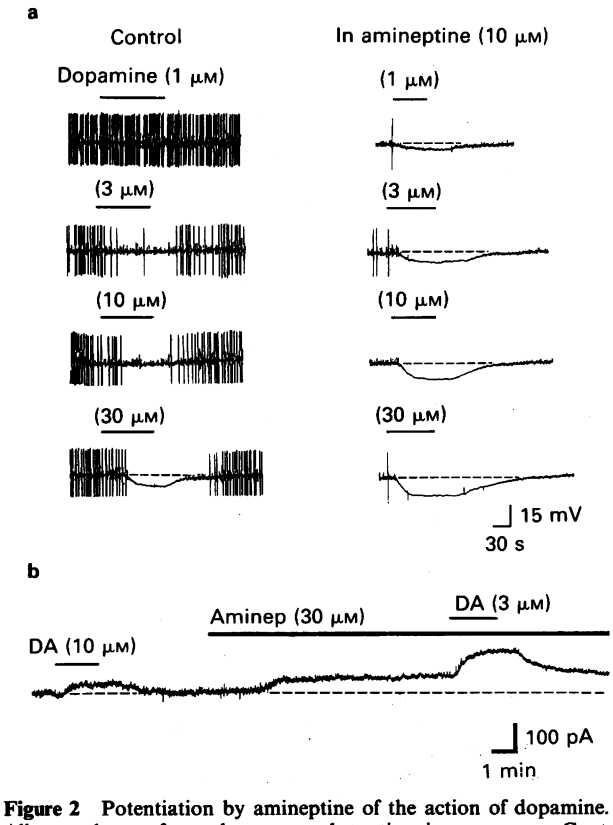


Figure 2 Potentiation by amineptine of the action of dopamine. (a) All records are from the same substantia nigra neurone. Control: increasing concentrations of dopamine, applied for the period indicated by the bars, hyperpolarized the membrane and inhibited the spontaneous firing activity. In amineptine (10 μM) the membrane hyperpolarizations produced by dopamine in the same cell were increased in amplitude and duration. Amineptine itself slightly hyperpolarized the cell and blocked the spontaneous firing. Dashed line indicates −60 mV. (b) Outward currents induced by dopamine (DA) under voltage clamp (different neurone; holding potential −56 mV) were enhanced in the presence of 30 μM amineptine (Aminep). Note the sustained outward current induced by this drug.

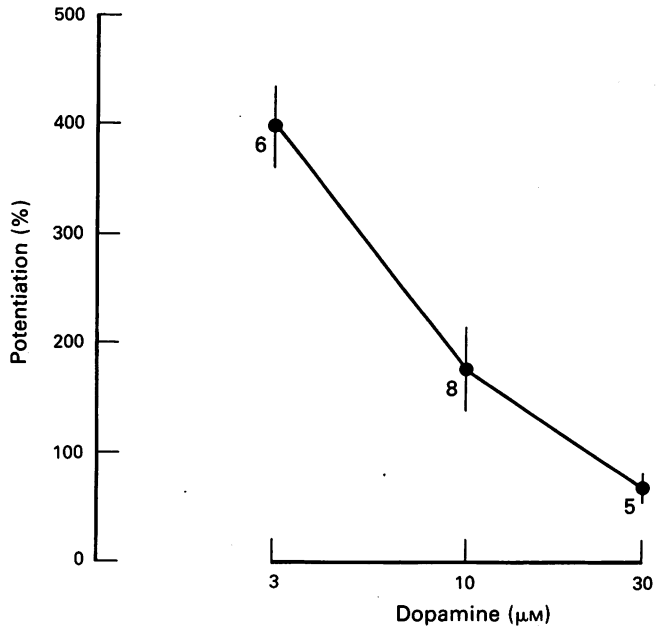


Figure 3 Pooled data showing percentage potentiation of the dopamine-induced membrane hyperpolarization by amineptine (10 μM) from all experiments performed in the substantia nigra. Ordinate scale is expressed as 100 × increment in dopamine-induced hyperpolarization caused by amineptine with respect to the hyperpolarization caused by dopamine alone. The number beside each point indicates the number of observations. Note the larger degree of potentiation caused by amineptine on the response produced by the lower concentrations of dopamine.

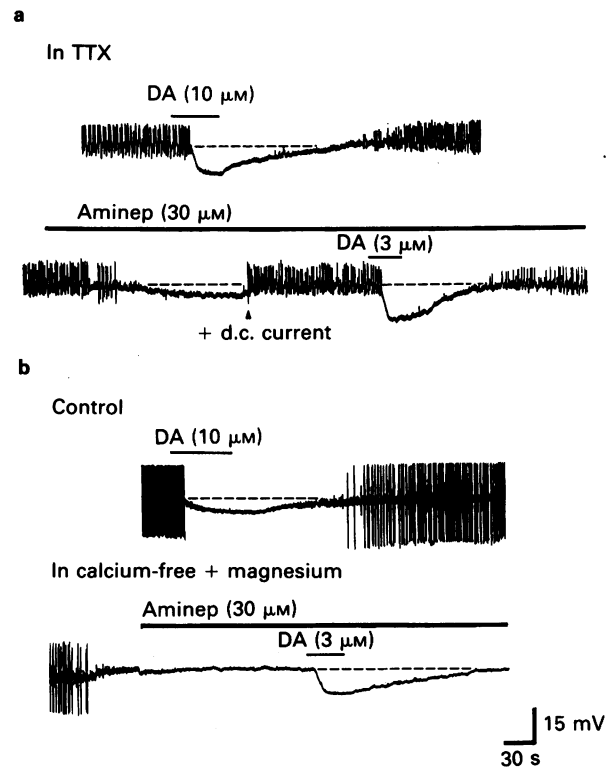


Figure 4 The effect of amineptine in tetrodotoxin (TTX) or calcium-free solutions. (a) Upper trace: dopamine (DA, 10 μM) hyperpolarized a substantia nigra cell in the presence of TTX (1 μM). Residual low amplitude spontaneous calcium-mediated depolarizations were present (Lacey *et al.*, 1987; 1990b; Mercuri *et al.*, 1989). Lower trace: Amineptine (Aminep) applied over the period indicated by the bar, blocked these spontaneous depolarizations and hyperpolarized the membrane. This cell was then repolarized to the resting level by positive d.c. current injection and dopamine (3 μM) reapplied to produce an enhanced hyperpolarization. Dashed lines indicate −58 mV membrane potential level. (b) Upper trace: dopamine (10 μM) hyperpolarized a different substantia nigra pars compacta cell and blocked the firing activity. Lower trace: in nominally calcium-free solution (plus 13 mM added magnesium) the spontaneous activity of the cell was eventually blocked. In this solution, amineptine did not modify the membrane potential but still clearly potentiated the hyperpolarizing response to applied dopamine (3 μM). Dashed line indicates −58 mV. Calibrations refer to the cell shown in (a) and (b).

tine. The dopamine-induced inhibition of the firing and membrane hyperpolarization (outward current) were increased in duration and in amplitude (Figure 2). The minimal effective concentration of amineptine was 300 nM while the maximum potentiating effect of the dopamine response was reached with 10–30 μM amineptine. The perfusion with amineptine (10 μM) caused a shift to the left of the dopamine dose-response curve by a factor of 5.5 ± 0.9 (n = 5), measured at the EC_{50} value. In Figure 3 the percentage potentiation of the hyperpolarization produced by increasing concentrations of dopamine in amineptine (10 μM) is plotted. It is clear that the membrane responses to low concentrations of dopamine were more effectively potentiated by amineptine. The potentiation of the dopamine responses by amineptine was also observed under conditions where the superfusate contained TTX (μM) (3 cells) or nominally 0 mM Ca^{2+} (plus 13 mM $MgCl_2$) (2 cells) (Figure 4).

Amineptine and cocaine effects on the same neurone

In four cells, cocaine (10–30 μM) inhibited the neuronal firing, hyperpolarized the membrane or produced an outward current (under voltage-clamp) and potentiated the responses to dopamine as previously described (Lacey *et al.*, 1990a) (Figure 5b).

The marked similarity between the effects of amineptine and cocaine suggested that they may act on these neurones by the same mechanism. Accordingly, in the presence of 30 μM amineptine, the potentiating effect of cocaine (10 μM) on the dopamine responses was reduced or blocked (Figure 5a). On the other hand, the enhanced membrane responses caused by dopamine in 30 μM cocaine were not further affected by the concomitant administration of amineptine on the same cells (4 neurones) (Figure 5b).

Discussion

Our intracellular study has shown that the tricyclic antidepressant drug amineptine has clear effects on the dopaminergic neurones of the substantia nigra; it reduces their firing activity, hyperpolarizes the cell membrane and increases the sensitivity of cells to exogenously-applied dopamine. Neurochemical studies have previously shown that amineptine increases the efflux of dopamine from brain synaptosomal preparations by blocking the dopamine uptake processes, with an EC_{50} of about 2 μM (Bonnet *et al.*, 1987). This concentration of drug produced detectable effects in our experiments. Thus, the depression of the firing discharge and the hyperpolarization of the membrane are probably due to an increased concentration of dopamine in the extracellular space within the slice, resulting from an amineptine-induced blockade of the dopamine uptake system. The subsequent activation of D_2 soma-dendritic receptors is supported by the antagonism obtained with sulpiride (a selective D_2 receptor antagonist) (Pinnock, 1984; Lacey *et al.*, 1987).

The potentiating effect on the membrane responses produced by added dopamine, is in keeping with the proposal that amineptine blocks active dopamine uptake sites in the brain (Bonnet *et al.*, 1987). Consequently, the diffusion of dopamine is limited and this catecholamine can function on dopamine receptors more effectively and for a longer period of time. Consistent with this interpretation is the similarity between the action of amineptine and cocaine (Lacey *et al.*, 1990a). No additional effects on the dopamine-induced

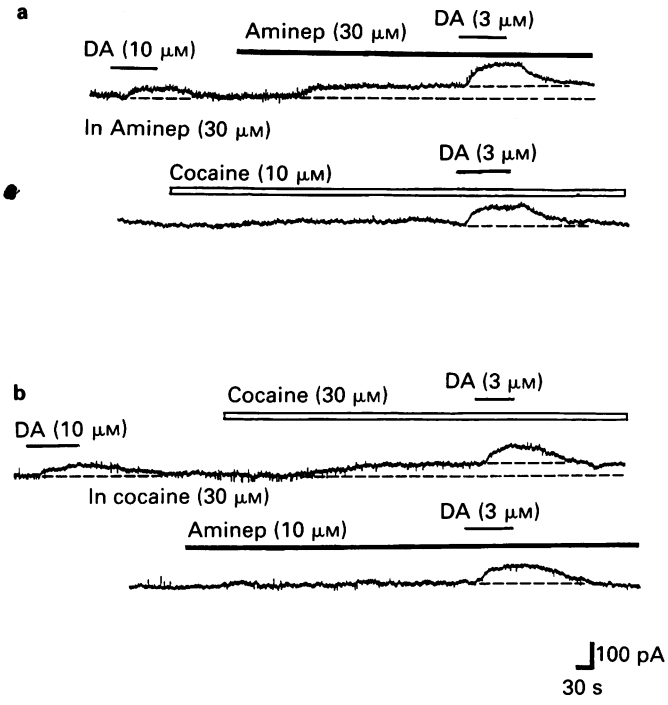


Figure 5 Outward currents induced by dopamine in the presence of amineptine and cocaine. (a) Upper trace: amineptine (Aminep) produced a sustained outward current and potentiated the response to exogenously applied dopamine (DA). Lower trace: no additional effect was observed on the membrane current and on the dopamine response when cocaine was superfused on the cell in the presence of amineptine. Same cell as in Figure 2b held at −56 mV. (b) Upper trace: cocaine also produced an outward current and potentiated the response produced by dopamine. Lower trace: in the presence of cocaine, amineptine was unable to induce clear effects on the membrane current and on the response to exogenously applied dopamine on the same cell. Holding potential = −60 mV.

hyperpolarization/outward current were observed when high concentrations of amineptine were applied in the presence of cocaine and *vice versa*. This phenomenon, known as non-additivity, indicates that amineptine acts on the uptake sites that are acted upon by cocaine. As reported for cocaine (Lacey *et al.*, 1990a) the action of amineptine was not affected by the presence of the fast Na^+ channel blocker, TTX. However, calcium-free solutions (with added magnesium) were able to block the effects of amineptine on the cellular membrane (hyperpolarization, outward current) but they did not prevent the potentiating effect on exogenously applied dopamine. This suggests that amineptine has no direct postsynaptic action and that the increased level of dopamine in the extracellular space is due to release from vesicular storage pools sensitive to the intracellular level of calcium. Influx of calcium ions in the cell is also critical for triggering dopamine release with L-DOPA while it is not necessary for the amphetamine-induced release of dopamine in the substantia nigra (Mercuri *et al.*, 1989; 1990b).

Thus, our experiments can differentiate between the electrophysiological effects of drugs that block the uptake (Lacey *et al.*, 1990a; Mercuri *et al.*, 1991) or induce the release of dopamine (Mercuri *et al.*, 1989; Mercuri *et al.*, 1990b) in this brain area.

Our data also support a previous neurochemical finding that amineptine is capable of blocking the dopamine uptake sites in the mammalian central nervous system (Bonnet *et al.*, 1987). This is in line with clinical results which have shown that amineptine has a potent antidepressant action, suggesting that this may result from an increase in brain levels of dopamine (Mocaer, 1983; Kemali, 1989). Because the actions of cocaine on the brain dopamine uptake system have been demonstrated to be essential to cocaine's reinforcing properties (Wise, 1984; Colpaert *et al.*, 1987; Ritz *et al.*, 1987) and the inhibition of dopamine uptake is the principal neuronal effect underlying the actions of amineptine, an increase in dopaminergic transmission by this dopamine uptake blocker might be a valid strategy for the treatment of cocaine abusers.

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The control of the contraction of myocytes from guinea-pig heart by the resting membrane potential

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1 The influence of different holding potentials (−120 to −70 mV) on the contraction of enzymatically dispersed myocytes from guinea-pig hearts was evaluated. Contractions were elicited by repetitive depolarizations to 0 mV at 0.5 Hz.

2 While ineffective at 140 and 5 mmol l^{-1} $[Na^+]_o$ and pipette Na^+, respectively, depolarization of the resting membrane with the holding potential increased myocyte shortening at reduced Na^+ gradients ($[Na^+]_o$ 70 or $[Na^+]_i$ 10–15 mmol l^{-1}). Elevated intracellular Na^+ after Na^+-pump inhibition with ouabain 1–10 μmol l^{-1} was similarly effective with regard to the inotropic response to different holding potentials.

3 At −70 mV holding potential, reduction of $[Na^+]_o$ from 140 to 70 mmol l^{-1} increased myocyte shortening and induced an inwardly directed component of the holding current which peaked at -44 ± 10 pA and declined thereafter in parallel with the inotropic effect. The relation of this inward current to $[Ca^{2+}]_i$ was confirmed by experiments at high Ca^{2+} buffer capacity where $[Na^+]_o$ reduction induced a Ni^{2+}-insensitive, outwardly directed component (36 ± 15 pA) of the holding current. The observed inward current is suggested to reflect the extrusion of $[Ca^{2+}]_i$ in exchange for $[Na^+]_o$ as a counter-regulatory mechanism which limits the increase of $[Ca^{2+}]_i$.

4 The interventions which increased the strength of the contraction also enhanced the transient tail current after repolarization, suggesting its close relation to $[Ca^{2+}]_i$. This finding confirmed the pattern found with cell shortening.

5 It is concluded that under certain conditions, voltage-dependent and Na^+-dependent Na^+–Ca^{2+} exchange during the interval between the contractions is relevant to the diastolic concentration of $[Ca^{2+}]_i$ which in turn determines the accumulation of Ca^{2+} in the sarcoplasmic reticulum and the magnitude of the subsequent contraction.

Keywords: Resting potential; sodium-calcium exchange; sodium; guinea-pig heart; myocytes

Introduction

While depolarization of the cell membrane with high K^+ concentrations leads to contracture (e.g., Niedergerke, 1956), moderate changes of K^+ concentration preserve the physiological contraction cycle. Negative inotropic effects (Reiter *et al.*, 1971), presumably related to Na^+-pump activation and alterations of the action potential, and transient increases of contraction (Kavaler *et al.*, 1972), however, indicate the diversity of K^+ actions. The reversal from a negative to a positive inotropic effect of K^+ after reduction of extracellular Na^+ in guinea-pig papillary muscle (Ebner & Siegl, 1986) and the role of K^+ in the inotropic response to intracellular Na^+ (Ebner *et al.*, 1986) could suggest a control of the contraction by the resting membrane potential via Na^+–Ca^{2+} exchange in a way which resembles the effect of high K^+ concentrations in frog atrial trabeculae on the Na^+ withdrawal contracture (Chapman & Tunstall, 1980) or the Na^+-dependence of the K^+-induced contracture (Chapman & Tunstall, 1981). The influence of high K^+ concentrations on Ca^{2+} fluxes (Busselen, 1982) or intracellular Ca^{2+} (Sheu *et al.*, 1986) is in line with the contracture experiments. However, the membrane potentials at those high, contracture-inducing K^+ concentrations which under physiological conditions only occur during the action potential and a possible role of voltage-dependent Ca^{2+} release from the sarcoplasmic reticulum (cf. Reiter, 1988) weaken the arguments in support of a common basis of the effects of different resting membrane potentials and of high K^+ concentrations. Moreover, some observations preclude a direct comparison of the effects of the resting membrane potential and of K^+. Firstly, an effect of K^+ different from its influence on the membrane potential cannot be excluded *a priori* (Cervetto *et al.*, 1989). Secondly, with respect to the resting membrane potentials of −100 to −70 mV associated with the K^+ effect on the regular contraction, reports on the electrical control of mechanical restitution seem to be at variance (Beeler & Reuter, 1970; Lipsius *et al.*, 1982). Potentials negative to −70 mV did not alter or only slightly altered the contraction after a priming event although the Na^+ withdrawal contracture of frog atrial trabeculae depended on the membrane potential (Chapman & Rodrigo, 1986). At presence, therefore, the question regarding a conceivable effect of the resting membrane potential on the regular contraction by interfering with the sarcoplasmic Ca^{2+} concentration and its precise mechanism seems to be unsettled.

Since the first description of Na^+–Ca^{2+} exchange (mammalian cardiac muscle; Reuter & Seitz, 1968; squid giant axons; Baker *et al.*, 1969; Blaustein & Hodgkin, 1969), the basic parameters of the exchange have been evaluated (for reviews see Eisner & Lederer, 1985; Sheu & Blaustein, 1986; Hilgemann, 1988; Wier, 1990), the features of which comprise: (1) the role of intra- and extracellular Na^+ and Ca^{2+} (Reuter & Seitz, 1968; Glitsch *et al.*, 1970; Miura & Kimura, 1989); (2) the stoichiometry of the exchange of 1 Ca^{2+} for n Na^+ ions (consistently $n > 2$; $n = 4$, Mullins & Brinley, 1975; $n = 3$, Reeves & Hale, 1984), and (3) the voltage-dependence (Ca^{2+} efflux from squid axon; Mullins & Brinley, 1975; $[Na^+]_i$-dependent accumulation of $[Ca^{2+}]_i$ in cardiac vesicles; Philipson & Nishimoto, 1980; Reeves & Sutko, 1980). These experimental details are condensed in equation 1 which couples the Na^+ and Ca^{2+} gradients to yield (Blaustein & Hodgkin, 1969; Mullins, 1976; Mullins, 1977) $2(V-E_{Ca}) = n(V - E_{Na})$ (1) and $[Ca^{2+}]_i = [Ca^{2+}]_o([Na^+]_i[Na^+]_o^{-1})^n \exp -[(2-n)(V\,F)(R\,T)^{-1}]$ (2); whereby V, E_{Ca}, and E_{Na} are membrane potential, and Ca^{2+} and Na^+ equilibrium potential, respectively; n is the number of Na^+ ions exchanged per

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Ca^{2+} ion; $[Ca^{2+}]_{i,o}$ and $[Na^+]_{i,o}$ are intra- and extracellular concentrations of the respective ions; F, R, T are Faraday number, gas constant, and absolute temperature, respectively.

With respect to the features of Na^+–Ca^{2+} exchange, depolarization of the cell membrane on excitation and changes of the resting membrane potential could both interfere with Na^+–Ca^{2+} exchange and alter the cytosolic concentration of Ca^{2+} ($[Ca^{2+}]_i$). Its accumulation in the sarcoplasmic reticulum in the interval between the beats could therefore depend on the membrane potential. Although the reports on K^+ and voltage effects appear to be conflicting, an influence of Na^+–Ca^{2+} exchange during diastole on the subsequent contraction may be overlooked when the effectiveness of a voltage change on $[Ca^{2+}]_i$ disappears with an inappropriate ratio of $[Na^+]_i$:$[Na^+]_o$; according to equation 2, depolarization of the membrane from -120 to -70 mV increases $[Ca^{2+}]_i$ from 0.6 to 3.9 nmol l^{-1} at a ratio of $[Na^+]_i$:$[Na^+]_o$ = 5:140, whereas this value rises from 16 to 105 nmol l^{-1} at 15:140; n was 3, $[Ca^{2+}]_o$ 1.2 mmol l^{-1}. Irrespective of the factors which in addition to Na^+–Ca^{2+} exchange modulate $[Ca^{2+}]_i$ and lead to actually higher concentrations than predicted by equation 2, the relation of voltage to $[Ca^{2+}]_i$, and consequently the relation of resting membrane potential to contraction, should vary with the ratio of $[Na^+]_i$:$[Na^+]_o$. Isolated myocytes were therefore studied under conditions of whole-cell voltage clamp with adequate control of the intracellular ionic milieu.

Methods

Cell preparation

Ventricular myocytes from the hearts of adult guinea-pigs (250–300 g) were isolated enzymatically. The procedure of Bendukidze *et al.* (1985) was modified as follows. The heart was retrogradely perfused at 36°C with a pressure of 6.8 kPa. The solutions were vigorously gassed with 5% CO_2 in O_2; pH was 7.0. Perfusion with 30–50 ml of a nominally Ca^{2+}-free solution at a flow rate of approximately 10 ml min^{-1} was followed by 30–40 ml of pronase solution. The ventricle was then minced with scissors, pronase solution containing 0.5 mmol l^{-1} $CaCl_2$ was added, and the tissue fragments were incubated for 5 min at 37°C. After filtration the myocytes were spun down at 37 *g*, 2–3 min, resuspended in modified Krebs-Henseleit solution and kept for use at room temperature.

On average, one third of the cells recovered were rod-shaped with clear cross-striation. Cell length was 107 ± 2 μm, diameter 20 ± 0.5 μm ($n = 101$) and estimated height 7.5 μm, yielding a cell surface of 6.24×10^3 μm^2. This value compares with 6.99×10^3 μm^2 calculated from membrane capacity (69.9 ± 3.4 pF, $n = 43$) assuming a specific capacity of 1 μF cm^{-2}. Determined with low-ohmic patch-clamp pipettes, the resting membrane potential was -93.3 ± 0.42 ($n = 99$), -74.0 ± 0.8 ($n = 42$), and -60.3 ± 1.49 ($n = 15$) mV at 2.4, 5.9, and 9.6 mmol l^{-1} K^+, respectively. In the voltage-clamp mode at 2.4 mmol l^{-1} K^+ the holding current was -658 ± 25 pA at -120 mV holding potential and 211 ± 17 pA at -70 mV in 20 cells.

Experimental set-up and procedure

The cell suspension was placed in a thermostated perfusion chamber (volume 0.5 ml) mounted on an inverse microscope (IM 35, Zeiss, Oberkochen, West Germany). After the cells had attached to the bottom, the bath was perfused at a flow rate of 2–3 ml min^{-1} with pre-warmed modified Krebs-Henseleit solution continuously gassed with 5% CO_2 in O_2. The temperature in the bath (35–36°C) was continuously monitored.

Voltage-clamp experiments were performed in the whole-cell clamp configuration (Hamil *et al.*, 1981). The pipettes were made from haematocrit glass in a two-step procedure. After fire-polished they were filled with pre-filtered solution (see below). D.c. resistance ranged from 1.5 for 4 MΩ. An Ag-AgCl electrode connected via an agar bridge (3% agar-agar in modified Krebs-Henseleit medium) to the bath solution served as a reference. The pipette was mounted on a micromanipulator (Narishige, Japan) and connected to a patch-clamp amplifier (List LM/EPC7) for control of voltage and monitoring current. After giga-seal formation cell capacity was compensated before the experiment. Series resistance was compensated until 'ringing' occurred (usually at 30–50% compensation). The signals were filtered with a 3 kHz, 3-pole Bessel filter.

Cell shortening was recorded simultaneously with a photometer attached to the microscope (MPM01K, Zeiss, Oberkochen, West Germany). A window was positioned in the light beam on the cell end or on the cell itself and the change of transmission, either an increase or decrease with cell shortening depending on the position of the window, was monitored. The contraction signal was filtered with a cut-off frequency of 100 Hz.

The voltage, current, and contraction signals were displayed on an oscilloscope and a pen recorder. Simultaneously, the signals were recorded and stored on diskette by a personal computer (IBM AT02) equipped with an AD board (Labmaster, Scientific Solutions, Solon, USA) at a sampling rate per channel of 1.33 and 1.1 kHz in the experiments with change of $[Na^+]_o$, respectively.

From a holding potential of -120 mV the cell was depolarized to 0 mV for 100 ms at a stimulus rate of 0.5 Hz. Following stabilization of the signals the holding potential was set to -70 mV and the cell was depolarized to 0 mV as before. While the voltage control with fast voltage-clamp steps may not have been ideal, this is unlikely to have influenced the principal results of the experiments. In some experiments perfusion was changed to a solution of different $[Na^+]_o$ at constant holding potential and otherwise identical experimental conditions. Exchange of solution in the dead space of the apparatus required approximately 1 min, but depending on the flow rate, $[Na^+]_o$ concentration in the vicinity of a cell could be changed within 15–30 s as monitored by the fast Na^+ inward current.

Data analysis and statistics

The active shortening was evaluated from the difference between baseline and peak shortening of the signals stored on diskette. Since contraction data could not be normalized sufficiently fast during the experiment in terms of μm shortening, the actual photometer output is given instead. It is representative for the shortening of an individual cell. Inwardly directed tail currents after repolarization from 0 to -70 or -120 mV were integrated numerically (Simpson rule) after subtraction of the holding current. Arithmetic means ± s.e.mean or the data of individual experiments are shown. Statistical significance was assessed with the two-tailed t test. Significance was assumed at $P < 0.05$.

Solutions

The concentrations are in mmol l^{-1} if not stated otherwise. pH was measured after gassing with 5% CO_2 in O_2 except the pipette solution. Modified Krebs-Henseleit solution: NaCl 115, $NaHCO_3$ 24.9, KCl 1.2, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.2, glucose 10, pH 7.4; at reduced $[Na^+]_o$ concentration 70 mmol l^{-1} NaCl was replaced by an equimolar amount of choline chloride; pipette solution: KCl 140, NaCl 5–15, $MgCl_2$ 2, Tris-ATP 3, HEPES 10, EGTA (if not stated otherwise) 0.02; adjusted to pH 7.2 with KOH; assuming a total of 3 μmol Ca^{2+} l^{-1} (Bendukidze *et al.*, 1985) on the basis of the log binding constants of Fabiato (1988) (H_1EGTA 9.353, H_2EGTA 8.794, Ca_1EGTA 10.593, Ca_2EGTA 5.33, Mg_1EGTA 5.21, Mg_2EGTA 3.37, Ca_1ATP 3.936, Ca_2ATP 1.735, Mg_1ATP 4.422, Mg_2ATP 2.841) non-complexed Ca^{2+}

is 57 nmol l^{-1} in the pipette solution at 35°C as calculated by solving a set of 10 non-linear equations (Newton-Raphson method) with Ca^{2+}, Mg^{2+}, and H^+ competing for EGTA and Ca^{2+} and Mg^{2+} for ATP according to

$$\left(I_{0,i} - \sum_{j=1}^{j=2}\sum_{k=1}^{k=n} I_{i,j}L_k\right) \times \left(L_{0,k} - \sum_{j=1}^{j=2}\sum_{i=1}^{i=m} I_{i,j}L_k\right) - K_{i,k,1}I_{i,1}L_k = 0$$

and

$$\left(I_{0,i} - \sum_{j=1}^{j=2}\sum_{k=1}^{k=n} I_{i,j}L_k\right)I_{i,1}L_k - K_{i,k,2}I_{i,2}L_k = 0;$$

where I_i, L_k, and $K_{i,k,1,2}$ are ion i ligand k, and binding constant K, respectively, and the subscript $_0$ denotes the total ion or ligand concentration; nominally Ca^{2+}-free solution: NaCl 100, $NaHCO_3$ 10, KCl 10, KH_2PO_4 1.2, $MgSO_4$ 5, glucose 20, taurine 50, pH 7.0; pronase solution: NaCl 80, $NaHCO_3$ 10, KCl 30, KH_2PO_4 1.2, $MgSO_4$ 5, glucose 20, taurine 50, $CaCl_2$ 0.04, pronase E 100 mg l^{-1}, essentially fat free bovine serum albumin 1 g l^{-1}, pH 7.0.

Materials

Pronase E from *Streptomyces griseus* 7.4 DMC-u mg^{-1}, taurine, Dowex G50WX8, and ouabain were obtained from Serva, Heidelberg, West Germany. Essentially fat free bovine serum albumin, EGTA (ethylene glycol *bis*-(β-aminoethylether)N,N,N',N'-tetraacetic acid), and HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid) were obtained from Sigma (München, West Germany). Tris-ATP was prepared from the disodium salt after passage over Dowex AG 50WX8 and adjustment with Tris(trishydroxymethyl-aminomethane) to pH 7.5. ATP, Tris and all other chemicals were purchased from Merck (Darmstadt).

Results

The effect of the holding potential (V_{hold}) on contraction depends on extracellular Na^+ ($[Na^+]_o$)

While the change of the holding potential from −120 to −70 mV did not alter the contraction elicited by repetitive depolarizations to 0 mV at 140 mmol l^{-1} $[Na^+]_o$ and 5 mmol l^{-1} pipette Na^+, depolarization with the same settings of the holding potential nearly doubled myocyte shortening when $[Na^+]_o$ was reduced to 70 mmol l^{-1} (Figure 1). The effect of depolarization to −70 mV was reversible on hyperpolarization to −120 mV, although some run down of contraction was usually found. When the holding potential was gradually changed to −120, −100 and −70 mV the shortening of myocytes followed in a stepwise manner. Relative to the value at −70 mV, V_{hold} the normalised shortening was 0.7 ± 0.05 ($n = 5$) at −100 mV and 0.58 ± 0.03 ($n = 13$) at −120 mV, 15 s after alteration of the holding potential.

The recordings of a single experiment illustrate the effect of depolarization of the membrane to less negative values of the holding potential (Figure 2). Figure 2a demonstrates the shortening of the cell subsequent to the voltage protocol in Figure 2c. The current traces in Figure 2b indicate the reversal of the holding current from the inward to the outward direction and some increase of the tail current after repolarization when the holding potential was set from −120 to −70 mV. When the membrane was clamped to 0 mV the flow of currents seemed to be hardly affected by the preceding V_{hold} although at higher resolution the inactivation phase of the initial inward current appeared to be accelerated and, in some cells, the subsequent current was slightly shifted to the inward direction.

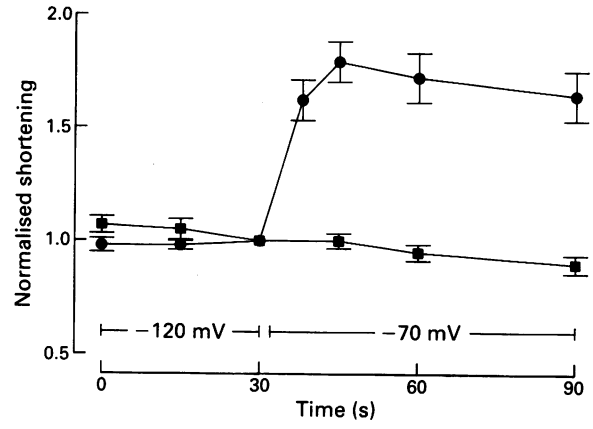


Figure 1 The effect of the holding potential on myocyte shortening depends on extracellular Na^+ ($[Na^+]_o$). The time courses of normalised shortening of 11 and 13 cells at 140 (■) and 70 (●) mmol l^{-1} $[Na^+]_o$ are shown when the holding potential is altered from −120 to −70 mV. The contractions were elicited by depolarization to 0 mV at a stimulus rate of 0.5 Hz. Na^+ concentration in the pipette was 5 mmol l^{-1}. Abscissa scale: time in s; ordinate scale normalised shortening, i.e., peak shortening relative to its value before V_{hold} was changed. Arithmetic means are presented with s.e.mean shown by vertical bars.

The influence of $[Na^+]_o$ on the holding current

The interference from several voltage-dependent processes precludes the isolation of a Na^+–Ca^{2+} exchange current in the preceding experiments with different holding potentials. The relevance of the exchange to the holding current was therefore tested with change of $[Na^+]_o$ at −70 mV holding potential in regularly depolarized cells. The individual recordings of clamp protocol, current, and myocyte shortening of

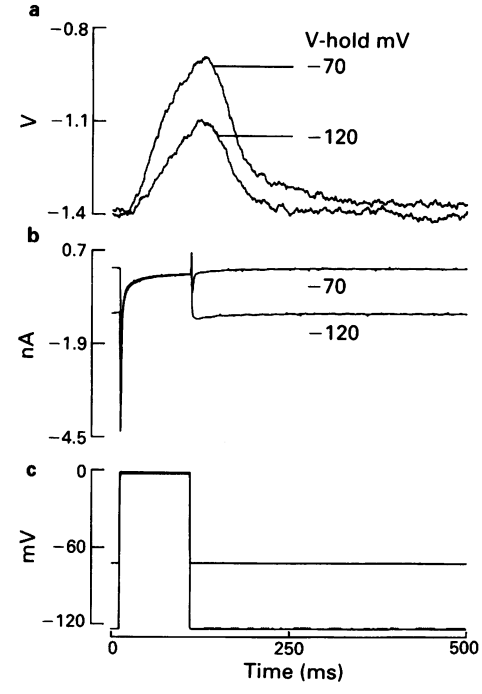


Figure 2 The influence of the holding potential on myocyte shortening and membrane currents at reduced $[Na^+]_o$. At 70 mmol l^{-1} $[Na^+]_o$, 5 mmol l^{-1} pipette Na^+, the effect of depolarization from a holding potential of −120 to −70 mV on the shortening (a) and membrane currents (b) is illustrated with data from a single myocyte. The digitized recordings were taken immediately before and 16 s after change of the holding potential. The myocyte was regularly stimulated at 0.5 Hz by depolarization to 0 mV; see (c) for clamp protocol. Ordinates: (a) photometer output in V, (b) membrane currents in nA; (c) clamp voltage in mV; common abscissae: time in ms.

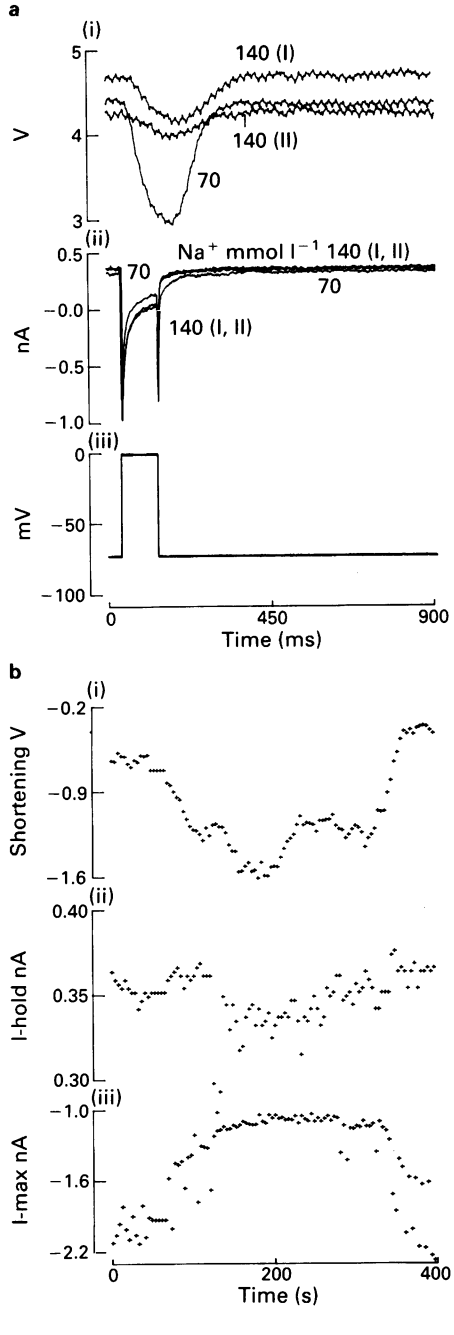


Figure 3 The influence of $[Na^+]_o$ reduction on the membrane currents and myocyte shortening at low intracellular Ca^{2+} buffer capacity: (a) individual recordings; (b) the time courses of some current parameters and of cell shortening. At low intracellular Ca^{2+} buffer capacity (20 μmol l^{-1} EGTA) superfusion was changed from 140 to 70 and back to 140 mmol l^{-1} $[Na^+]_o$; $Na^+_{pipette}$ was 5 mmol l^{-1}. Stimulation frequency was 0.5 Hz, temperature 35°C, V_{hold} −70 mV. (a) The digitized recordings of clamp potential (mV, (iii)), membrane current (nA, (ii)), and cell shortening (photometer output in V, (i) were taken before (140I), during (70), and after (140II) $[Na^+]_o$ reduction; for better resolution the maximal inward current was cut off; common abscissa scale: time in ms. (b) From recordings corresponding to (a) and of the same cell the maximal inward current to monitor $[Na^+]_o$ (averaged data from peak ± 1.8 ms, in nA, (iii)), the holding current (averaged data from 660 to 715 ms after repolarization, in nA, (ii)), and the cell shortening (peak-background value of photometer output, in V, (i) were evaluated and their time courses were followed during the change of $[Na^+]_o$; common abscissa scale: time in s. $[Na^+]_o$ was altered at $t = 0$.

Figure 3a before, during, and after superfusion with reduced $[Na^+]_o$ illustrate this effect at low $[Ca^{2+}]_i$ buffer capacity. The development of the positive inotropic effect of $[Na^+]_o$ reduction was paralleled by an outward shift of the current during depolarization while the holding current was slightly but reversibly shifted to the inward direction. For closer inspection the time courses of the maximal shortening and of the holding current were evaluated (Figure 3b). To monitor the actual Na^+ concentration in the vicinity of the cell we chose the maximal inward current flowing on depolarization as an indirect measure of $[Na^+]_o$, although the sampling rate of 1.1 kHz and the incomplete control of fast voltage changes in large cells preclude its precise evaluation. Nevertheless the change of $[Na^+]_o$ became clearly visible in its time course as exemplified in Figure 3b (lower panel). With the reduction of $[Na^+]_o$ the shortening of the myocyte increased and, beyond a maximum, approached a somewhat lower level of contractility (Figure 3b, upper panel). When the onset of Na^+ reduction became detectable in the shortening, the holding current (middle panel) was at first slightly and barely visibly shifted to the outward direction before an inward current of approximately 20 pA in this cell superimposed with the further development of the inotropic effect. This inward current faded to a variable extent in the different cells in parallel with shortening. After $[Na^+]_o$ had been reset to 140 mmol l^{-1}, the holding current returned to its previous value. In a total of 5 cells $[Na^+]_o$ reduction increased the shortening by 1.94 ± 0.33 and induced an inwardly directed component of the holding current of −44 ± 10 pA.

The relation between the inotropic effect of $[Na^+]_o$ reduction and an inwardly directed component of the holding current suggests a role of $[Ca^{2+}]_i$ in it. This idea was confirmed by clamping $[Ca^{2+}]_i$ with high buffer capacity of the pipette solution (EGTA 10 mmol l^{-1}). When under these conditions $[Na^+]_o$ was reduced from 140 to 70 mmol l^{-1} (Figure 4a) the membrane currents during depolarization to 0 mV were altered in a similar manner to that occurring in low Ca^{2+} buffer capacity, although the outward shift was particularly prominent in this cell. By contrast, the holding current was now differently affected. Reduction of $[Na^+]_o$ as monitored by the maximal inward current induced an outwardly directed component of the holding current which clearly dissociated from the overall outward drift (Figure 4b). The outward current related to $[Na^+]_o$ reduction was 36 ± 15 pA in 4 cells. To test its susceptibility to Ni^{2+}, the experiment with high Ca^{2+} buffer capacity was repeated while the cells were constantly superfused with 1 mmol l^{-1} Ni^{2+}, a concentration which substantially reduces the Na^+–Ca^{2+} current (Kimura *et al.*, 1987). Under otherwise identical conditions, $[Na^+]_o$ reduction induced an outward current of similar magnitude (58 ± 13 pA, $n = 3$). After prolonged superfusion with Ni^{2+}, however, this $[Na^+]_o$ effect increased progressively in repeated tests.

The effect of the holding potential (V_{hold}) on contraction depends on intracellular Na^+ ($[Na^+]_i$)

The role of $[Na^+]_i$ in the effect of different holding potentials was tested at 140 mmol l^{-1} $[Na^+]_o$ by raising Na^+ concentrations in the pipette or alternatively by inhibition of the Na^+ pump with ouabain. When, in the absence of ouabain, pipette Na^+ was 15 mmol l^{-1} a few minutes after the giga seal was formed, cell shortening increased reversibly with depolarization from V_{hold} = −120 to −70 mV (Figure 5). Qualitatively at least, the changes of contractile and current parameters correspond to the situation at reduced $[Na^+]_o$. The result shown in Figure 5 was confirmed in two other cells. In further experiments with 6 cells, pipette Na^+ was 10 mmol l^{-1}. Change of the holding potential from −120 to −70 mV increased the shortening relative to its value at −120 mV by 1.66 ± 0.25.

At low access resistance the pipette solution could entirely determine the intracellular milieu. The development of inotropic effects in myocytes in the whole-cell-clamp configuration after the addition of a cardioactive steroid to the superfusion medium (e.g., Berlin *et al.*, 1989), however, suggests that intracellular Na^+ is incompletely controlled in large cells. Super-

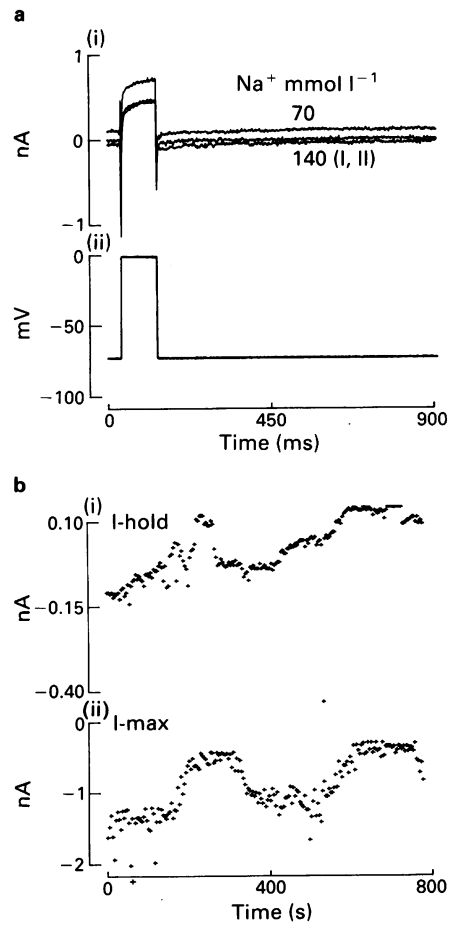


Figure 4 The influence of $[Na^+]_o$ reduction on membrane currents at high intracellular Ca^{2+} buffer capacity: (a) individual recordings; (b) the time courses of some parameters. At high intracellular Ca^{2+} buffer capacity (10 mmol l^{-1} EGTA) superfusion was changed twice from 140 to 70 and back to 140 mmol l^{-1} $[Na^+]_o$; $Na^+_{pipette}$ was 5 mmol l^{-1}. Stimulation frequency was 0.5 Hz, temperature 35°C, V_{hold} −70 mV. (a) The digitized recordings of clamp protocol (mV, (ii)) and membrane current (nA, (i)) were taken before (140I), during (70), and after (140II) the first $[Na^+]_o$ reduction; for better resolution the maximal inward current was cut off; common abscissa scale: time in ms. (b) From recordings corresponding to (a) and of the same cell the maximal inward current (in nA, (ii)) as an indirect measure of $[Na^+]_o$ concentration in the vicinity of the cell and averaged values of the holding current (from 660 to 715 ms after repolarization in nA, (i)) were evaluated and their time courses were followed during the change of $[Na^+]_o$; common abscissa scale: time in s. $[Na^+]_o$ was altered at t = 110 s.

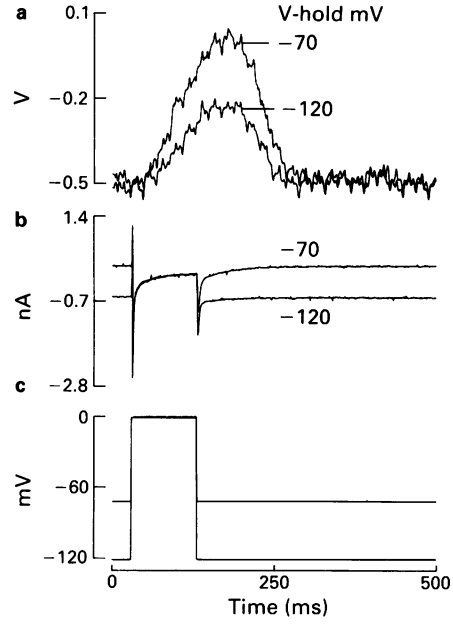


Figure 5 The influence of the holding potential on myocyte shortening and membrane currents at elevated intracellular Na^+. At 140 mmol l^{-1} $[Na^+]_o$, 15 mmol l^{-1} pipette Na^+, the effect of a change of the holding potential from −120 to −70 mV on the shortening (a) and membrane currents (b) of a single myocyte is shown. The digitized recordings were taken immediately before and 16 s after change of the holding potential. The myocyte was regularly stimulated by depolarization to 0 mV; see (c) for clamp protocol. Ordinates: (a) photometer output in V, (b) membrane currents in nA, (c) clamp voltage in mV; common abscissa scale: time in ms.

fusion of myocytes with ouabain therefore could also increase $[Na^+]_i$ in view of the effect of Na^+-pump inhibition in multicellular preparations and raise the inotropic effectiveness of different holding potentials. In resting guinea-pig papillary muscle under comparable conditions (K^+ 2.4, Ca^{2+} 1.2 mmol l^{-1}) and under the influence of 0.3 μmol l^{-1} ouabain $[Na^+]_i$ rose from 8 to 14, and to 20 mmol l^{-1} at 3.2 mmol l^{-1} Ca^{2+}, respectively (Ebner *et al.*, 1986). Corresponding to the situation in multicellular preparations in the isolated myocytes, too, a considerable inotropic effect developed at −120 mV V_{hold} (Figure 6a). Since in this experiment the window was positioned on the cell, light transmission reduced with contraction. In accordance with the development of the inotropic effect the currents both during excitation and diastole changed; alterations of $[Ca^{2+}]_i$-dependent K^+ conductivity (Siegelbaum & Tsien, 1980) and of the current generated by the Na^+ pump (Daut & Rüdel, 1981) could be relevant to this observation. After the maximum inotropic effect of ouabain had developed, contracture ensued with reduced active shortening; compare baseline and active shortening in upper panels of Figure 6a and b. When under these conditions the holding potential was changed from −120 to −70 mV, this resulted in 4 cells (as exemplified in Figure 6b), showing enhanced contracture (see the shift of the baseline in the upper panel), reduced active shortening, after-contractions associated with transient inward currents, shift of the current to the outward direction when the cell was clamped to 0 mV, and in large tail currents on repolarization. These effects were at least partially reversible on hyperpolarization (Figure 6b) with the exception of the current at 0 mV clamp potential. Before the signs of Ca^{2+} overload appeared and as long as contraction was regular, fractional shortening increased on depolarization by factors of 2.1 and 2 at 10 μmol l^{-1} ouabain in 2 cells and by 1.5 and 1.44 at 1 μmol l^{-1} ouabain in 2 additional cells (see Figure 6c).

The tail current on repolarization in relation to contraction

The inwardly directed tail current on repolarization has been shown to reflect the intracellular Ca^{2+} load (Fedida *et al.*, 1987; Shimoni & Giles, 1987; Mitchell *et al.*, 1987) subsequent to an activation of Na^+–Ca^{2+} exchange and possibly of non-selective cation channels (Colquhoun *et al.*, 1981). Accordingly, different magnitudes of the tail current and of its integral as a measure of total charge flow could be expected with the interventions of the preceding experiments. Increased pipette Na^+ and reduced $[Na^+]_o$ actually influenced the integral of charge similarly (Figure 7). Depolarization of the resting membrane to −70 mV augmented this effect. Ouabain also potentiated the flow of charge and, like the situation with different pipette Na^+, V_{hold} was a relevant factor with higher effectiveness under conditions of a reduced Na^+ gradient.

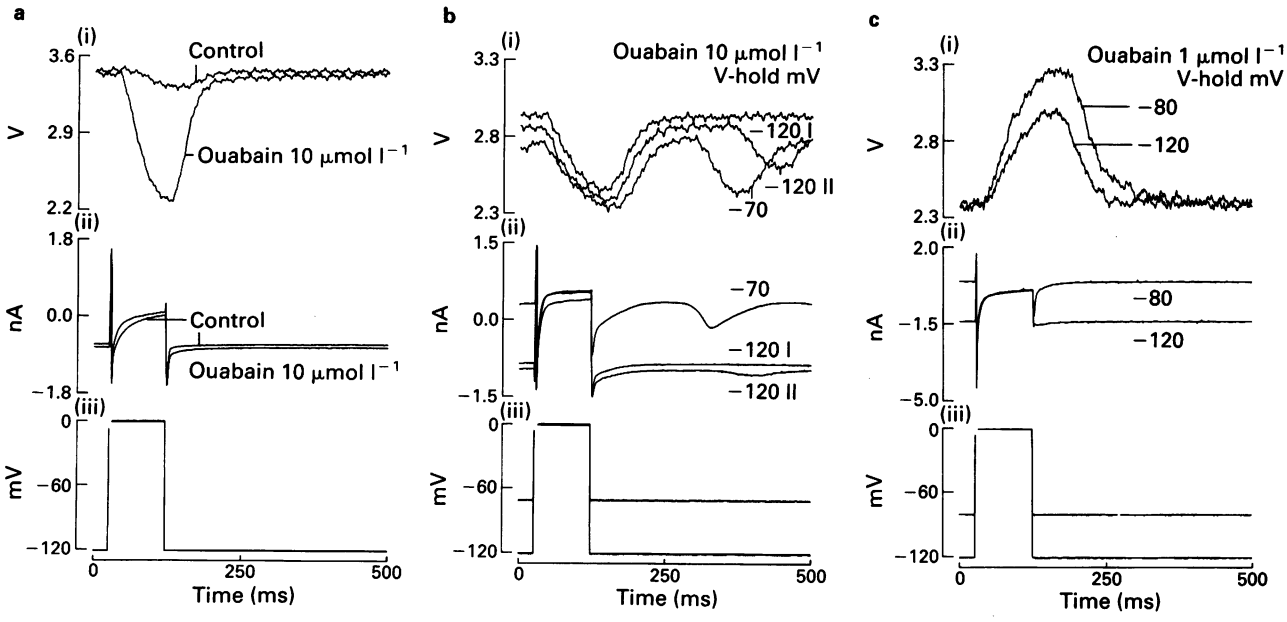


Figure 6 The influence of the holding potential on myocyte shortening and membrane currents in the presence of ouabain. The ouabain effect at constant holding potential (a); the effect of different holding potentials at 10 (b) and 1 (c) μmol l^{-1} ouabain. The shortening (upper panel) and membrane currents (middle panel) of a single myocyte subsequent to the voltage protocol of the lower panel are demonstrated with digitized data. In (a) at constant holding potential the signals were recorded before and 3 min after superfusion had been switched to 10 μmol l^{-1} ouabain; 3.5 min later the holding potential was changed from −120 to −70 and back to −120 mV after additional 1.5 min. The data of (b) were sampled immediately before (−120 I) and 80 s (−70) after the holding potential was altered while the traces (−120 II) were recorded 90 s after changing back to −120 mV. (a and b) are derived from the same cell. In a different myocyte (c) 11 min after superfusion with 1 μmol l^{-1} ouabain the holding potential was altered from −120 to −80 and back to −120 mV 1 min later. Immediately before and 80 s after resetting the holding potential from −80 to −120 mV the traces of (c) were recorded. The cells were regularly stimulated by depolarization to 0 mV at 0.5 Hz. $[Na^+]_o$ was 140, pipette Na^+ 5 mmol l^{-1}. Ordinates: (i) photometer output in V, (ii) membrane currents in nA, (iii) clamp voltage in mV; common abscissae: time in ms.

Although the tendency of an increased effectiveness of depolarization could be observed under all conditions of a reduced Na^+ gradient, this was statistically significant only at 70 mmol l^{-1} $[Na^+]_o$ ($P < 0.05$) and in the presence of ouabain ($P < 0.01$).

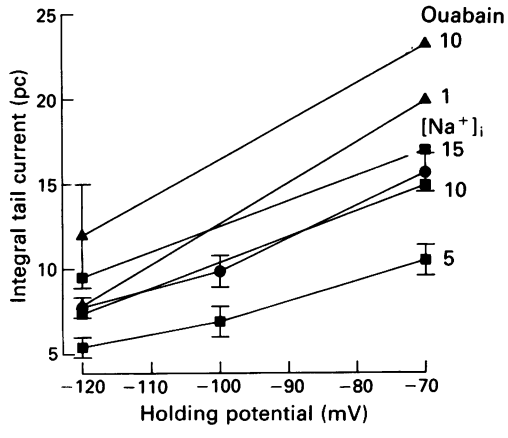


Figure 7 The charge carried with the tail current after repolarization. Influence of holding potential, intra- and extracellular Na^+, and ouabain. The tail current after repolarization from 0 to the respective V_{hold} of −120, −100, or −70 mV as indicated on the abscissa scale was integrated numerically to obtain the exchange of charge in pC (ordinate scale). At 140 mmol l^{-1} $[Na^+]_o$ (■) the number of cells was 18 (at −100 mV, $n = 7$), 6, and 3 at 5, 10, and 15 mmol l^{-1} pipette Na^+. At reduced $[Na^+]_o$ (70 mmol l^{-1}, pipette Na^+ 5 mmol l^{-1}; ●) the number of cells was 7 ($n = 5$ at −100 mV). In the experiments with ouabain (▲) two groups with 4 cells each were evaluated after the effect had stabilized. $[Na^+]_o$ was 140; pipette Na^+ 5 and 10 mmol l^{-1} in the experiments with 1 and 10 μmol l^{-1} ouabain, respectively. In the graph the Na^+ concentrations in the pipette ($[Na^+]_i$) are given in mmol l^{-1}, ouabain concentrations are in μmol l^{-1}. Arithmetic means and some s.e.mean (vertical bars) are shown).

Discussion

Depolarization of the resting membrane in the diastolic interval between the stimuli has been shown to interfere with the strength of the regular contraction in this paper, i.e., the resting membrane potential is a factor in the regulation of contraction. This effect of membrane potentials negative to −70 mV on contraction differs from the potential dependence of mechanical restitution (Beeler & Reuter, 1970; Lipsius *et al.*, 1982) where the relation of tension to membrane potential was S-shaped with saturation at potentials negative to −70 mV, while tension declined at more positive potentials. The relevance of the Na^+ gradient to our experiments, however, suggests that it plays a special role in this discrepancy. Moreover, decrease in a^i_{Na} (13.0 and 8.5 mmol l^{-1} at −110 and −80 mV, respectively, in sheep Purkinje fibres; January & Fozzard, 1984) with depolarization could contribute to the difference between the effects of the resting membrane potential in single cells and in multicellular preparations where $[Na^+]_i$ is not controlled. Decrease of $[Na^+]_i$ would evidently compensate for a positive inotropic effect of different origin.

Our results with regard to the contraction and the tail current as indirect measures of Ca^{2+} concentration in the cytosol ($[Ca^{2+}]_i$) indicate that depolarization increases the availability of Ca^{2+} in the cell. The role of the holding potential in contraction is supported by the increase of $[Ca^{2+}]_i$ found when, after a conditioning pulse to 60 mV, the holding potential was set to less negative values ($Na^+_{pipette}$ 15 mmol l^{-1}; Beuckelmann & Wier, 1989). Our experiments agree with the features of equation 2 which defines the effectiveness of a potential change on $[Ca^{2+}]_i$ relative to the Na^+ gradient. Therefore, and after considering the conceivable contribution of Na^+–Ca^{2+} exchange to the holding current (see below), Na^+–Ca^{2+} exchange may be inferred as the basis of the potential effect on contraction. Secondary to the effect of the holding potential on cytosolic Ca^{2+}, the accumulation of

Ca^{2+} in the sarcoplasmic reticulum must have been altered. The comparably slow process of mechanical restitution which indicates the filling of the sarcoplasmic reticulum with Ca^{2+} during diastole in periods of time between 0.5 and 1 s is consistent with the influence of the resting membrane potential in this process (Hoffman *et al.*, 1956; Edman & Johannson, 1976; Lipsius *et al.*, 1982). Relative to the role of Na^+–Ca^{2+} exchange, the activation of ion channels by depolarization from −120 to −70 mV appears to be irrelevant to the present findings since potentials step negative to −60 mV do not activate the Ca^{2+} channels of guinea-pig myocardial cells (Josephson *et al.*, 1984). Moreover, the driving force of passive Na^+ and Ca^{2+} influx would even be reduced with depolarization. The difference between the effects at −120 as compared with −100 mV V_{hold} on contraction (see Results) likewise indicates that ion channels did not measurably interfere with the effect of depolarization on contraction.

The relation to the concentration of $[Ca^{2+}]_i$ and its reversal potential determine the role of Na^+–Ca^{2+} exchange in cellular Ca^{2+} homeostasis. The low K_M value of 0.6 μmol l^{-1} $[Ca^{2+}]_i$ for $[Na^+]_o$–$[Ca^{2+}]_i$ exchange in guinea-pig ventricular cells as compared with 20.7 mmol l^{-1} $[Na^+]_i$ and 0.14 mmol l^{-1} $[Ca^{2+}]_o$ in the absence of external $[Na^+]_o$ for $[Na^+]_i$–$[Ca^{2+}]_o$ exchange (Miura & Kimura, 1989) and its high capacity as opposed to Ca^{2+}-ATPase (for review see Carafoli, 1988) support the relevance of the exchanger to Ca^{2+} extrusion at the $[Ca^+]_i$ concentration which prevails during diastole (in nmol l^{-1}): 272 (sheep ventricular muscle, Ca^{2+}-sensitive microelectrodes; Sheu & Fozzard, 1982) 100 and 137–177 (guinea-pig and rat myocytes, Quin-2; Powell *et al.*, 1984; Sheu *et al.*, 1986). In stimulated preparations diastolic $[Ca^{2+}]_i$ was 290 nmol l^{-1} in canine cardiac Purkinje fibres (aequorin; Wier & Hess, 1984) or 75 nmol l^{-1} in rat myocytes (Fura-2; Berlin *et al.*, 1989). In addition to the K_M value, the reversal potential of Na^+–Ca^{2+} exchange is essential to an assessment of its mode of action under special experimental conditions. The consistency of theoretical with observed reversal potentials of Ni^{2+}-sensitive currents in guinea-pig ventricular cells (Ehara *et al.*, 1989) validates the data of Figure 8 which were calculated from equation 1 assuming an exchange of 3 Na^+ for 1 Ca^{2+}. Its features are: increase of $[Na^+]_i$, or equivalent reduction of $[Na^+]_o$, and a shift of the reversal potential to negative potentials while $[Ca^{2+}]_i$ has the opposite effect; since low concentrations of $[Ca^{2+}]_i$ are especially effective, Na^+–Ca^+ exchange seems to be particularly sensitive to any change of its parameters during rest. Accordingly, at fixed membrane potential (see horizontal lines at −120 and −70 mV in Figure 8) and at 0.1–0.3 μmol l^{-1} $[Ca^{2+}]_i$ the driving force of Ca^{2+} extrusion ($V_m - E_r$) declines with increase of $[Na^+]_i$ and reduction of $[Na^+]_o$, respectively, or, dependent on membrane potential and $[Ca^{2+}]_i$, the mode of the exchanger even reverses. Depolarization from −120 to −70 mV reduces Ca^{2+} extrusion depending on the Na^+ gradient or leads to transient influx of Ca^{2+} at high $[Na^+]_i$ (low $[Na^+]_o$). Both mechanisms will increase $[Ca^{2+}]_i$ and shift the reversal potential to less negative values while establishing the new equilibrium. Since the driving force of the Ca^{2+} leak current also diminishes on depolarization, the potential effect on $[Ca^{2+}]_i$ via Na^+–Ca^{2+} exchange would be partially compensated for.

To consider the effects of $[Na^+]_o$ reduction with respect to Na^+–Ca^{2+} exchange it may be inferred that, as long as $[Ca^{2+}]_i$ is unaltered, the reduced driving force and competition by Na^+ (Reeves & Sutko, 1983) induce an outward current. Since the outward current of Figure 4 was insensitive to Ni^{2+}, participation of the exchanger is unlikely and its origin remains obscure. When on the other hand $[Ca^{2+}]_i$ increases and shifts the reversal potential towards positive potentials, a transiently reduced driving force subsequent to the reduction of $[Na^+]_o$ would be compensated for. Its magnitude could even increase in spite of the low Na^+ gradient depending on the extent of the increase of $[Ca^{2+}]_i$ (see Figure 8). A slowly developing inward current, related to the inotropic effect and susceptible to EGTA (Figures 3 and 4), which is consistent with this interpretation, was actually found. This inward current may therefore represent a counter-regulatory mechanism which limits the increase of $[Ca^{2+}]_i$. At first glance our finding appears to be incompatible with the transient inward current related to mechanical relaxation and induced by the rapid increase of $[Na^+]_o$ from 0 to 145 mmol l^{-1} in the presence of 10 mmol l^{-1} caffeine (−40 mV holding potential; Bridge *et al.*, 1990). However, different initial $[Ca^{2+}]_i$ concentrations resolve this apparent contradiction. The finding of Bridge *et al.* (1990) reflects the acute activation of Ca^{2+} extrusion by $[Na^+]_o$ from a level of high $[Ca^{2+}]_i$ where the reversal potential is less sensitive to any change of $[Ca^{2+}]_i$. In consequence, a higher driving force for Ca^{2+} extrusion is also less rapidly balanced by the $[Ca^{2+}]_i$ reduction. By contrast, diastolic $[Ca^{2+}]_i$ is low in our experiment. In view of the high sensitivity of the reversal potential to low $[Ca^{2+}]_i$ the activation of an outward current could be obscured and over-compensated by the inward current that is induced subsequent to an even small rise in diastolic $[Ca^{2+}]_i$. Our observation therefore supports the contribution of Na^+–Ca^{2+} exchange to the holding current whereas in view of a threshold concentration of 0.3 μmol l^{-1} $[Ca^{2+}]_i$ required for their activation (Ehara *et al.*, 1988) $[Ca^{2+}]_i$-activated non-selective cation channels do not seem to be involved.

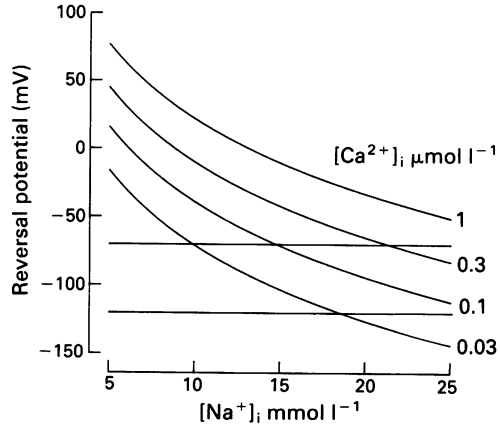


Figure 8 The reversal potential of Na^+–Ca^{2+} exchange in dependence on Na^+ and Ca^{2+} gradients. From equation 1 reversal potentials were calculated under variation of $[Na^+]_i$ and $[Ca^{2+}]_i$. Extracellular concentration of Ca^{2+} and Na^+ was 1.2 and 140 mmol l^{-1}, respectively; temperature was 35°C. The horizontal lines indicate the position of holding potentials (−70 and −120 mV). Changes of the same relative magnitude of intra- and extracellular Na^+ concentration are equivalent; e.g., increase of $[Na^+]_i$ from 5 to 10 mmol l^{-1} has the same effect as reduction of $[Na^+]_o$ from 140 to 70 mmol l^{-1}. Abscissa scale: intracellular Na^+ concentration in mmol l^{-1}, ordinate scale: reversal potential in mV.

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Action of heptaminol hydrochloride on contractile properties in frog isolated twitch muscle fibre

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1 Heptaminol stopped or delayed the progressive decline in tension which characterizes the phenomenon of fatigue in frog isolated twitch muscle fibre.

2 Heptaminol had no action on the sodium, potassium and calcium voltage-dependent ionic conductances.

3 The hypothesis of an action via an internal alkalinization was tested by comparison with the action of NH_4Cl. Both substances increased the tension.

4 The action of heptaminol was suppressed in sodium-free (TRIS) solution or in the presence of amiloride while the action of NH_4Cl was always observed.

5 These results could be explained by a stimulation of the Na/H antiport by heptaminol.

Keywords: Heptaminol; fatigue; excitation-contraction coupling; frog twitch muscle fibre.

Introduction

Heptaminol (6-amino-2-methyl-2-heptanol) hydrochloride (Heptamyl, Delalande) is primarily known as a potent cardiotonic agent (Loubatières, 1949). In animals as well as in man (Rigal *et al.*, 1963), early work showed that heptaminol may prevent fatigue in nervous and muscle system activities. Intraperitoneal injection of the drug in exhausted rat restored normal values of neuronal and muscular chronaxies (Chauchard & Mazoue, 1958). In dog heart, hypotensive and depressant effects of (+)-tubocurarine were minimized in the presence of the drug (Loubatières *et al.*, 1961). It has been reported (Coraboeuf & Boistel, 1953) that heptaminol increased action potential plateau, contraction amplitude and spontaneous activity frequency in nodal tissue; toxic effects of high CO_2 concentrations were reduced in its presence and a normal electrical activity was restored in exhausted cardiac tissues. Müller *et al.* (1964) observed similar potentiating actions on contraction in mammalian ventricular trabeculae; these authors mentioned that action potentials and Na/K pump activity were not modified.

The positive inotropic action of heptaminol is therefore well established; nevertheless the underlying cellular mechanisms remain unknown. Heptaminol could act at different steps involved in the process of excitation-contraction coupling or in the control of the function of the myofilaments. It has been shown on squid axon (Vassort *et al.*, 1986) that alcohol and amine groups can induce an increase in internal calcium and a decrease in internal protons. On the other hand it is well known that the calcium sensitivity of myofilaments and, in some way, the sarcoplasmic reticulum calcium release are increased during intracellular alkalinization (Fabiato & Fabiato, 1978). Heptaminol which has both an alcohol and an amine group could produce its positive inotropic effect by an internal alkalinization. Such an explanation has recently been envisaged by Berthiau *et al.* (1989) who suggested that the inotropic effect induced by heptaminol on ischaemic rat myocardium could be due to stimulation of the Na/H antiport.

In the present paper, experiments have been performed to investigate the effect of heptaminol on the electrical and mechanical responses of frog isolated twitch muscle fibre in conditions of repeated stimulation inducing fatigue. The main result of this study indicates that the progressive decline in tension which characterizes the phenomenon of fatigue during repeated stimulation was stopped or delayed in the presence of heptaminol.

An action of the drug on the regulation of the intracellular pH is discussed. Some of the results have been published in abstract form (Allard & Rougier, 1989a).

Methods

The experiments were performed at room temperature on intact isolated single fibres from the semitendinosus of the frog *Rana esculenta*.

Ionic currents, action potentials and isometric tension were recorded in the test gap (200 μm in length) of a double mannitol gap device as previously described in detail by Caille *et al.* (1978) and Potreau & Raymond (1980, 1982). The recording solutions used are shown in Table 1.

Tetrodotoxin (TTX) from Sigma, amiloride from Sigma and 5-(N-ethyl-N-isopropyl) amiloride) (EIPA) were added in some experiments. EIPA was a gift from Prof. M. Lazdunski. Heptaminol (Hept-a-myl) and tuamine were supplied by Laboratoires Delalande.

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Table 1 Composition of the recording solutions used (all concentrations mM)

Solution	*NaCl*	*LiCl*	*$CaCl_2$*	*KCl*	*$MgCl_2$*	*TRIS*	*TEA-CH_3SO_3*	*NH_4Cl*	*4-AP*
Control Ringer	110	/	1.8	2.5	2	10	/	/	/
Li Ringer	/	110	1.8	2.5	2	10	/	/	/
NH_4Cl Ringer	90	/	1.8	2.5	2	10	/	20	/
0 Na Ringer	/	/	1.8	2.5	2	120	/	/	/
0 Na NH_4Cl Ringer	/	/	1.8	2.5	2	100	/	20	/
TEA-CH_3SO_3 Ringer	/	/	1.8	2.5	2	10	122	/	3

TEA = tetraethylammonium; 4-AP = 4-aminopyridine.
In all solutions the pH was adjusted to 7.4.

At the beginning of each experiment the preparations were stimulated at a low rate to permit stabilization. Then different patterns of repeated stimulation were used to induce fatigue.

In current clamp experiments, the fibres were stimulated every minute by a 2 s tetanic train of depolarizing pulses (1 ms, 1.2 rheobase, 50 Hz).

In voltage clamp experiments, several patterns of stimulation were used; they are given in the results section. The holding potential was chosen so that the early sodium current reversed for a depolarization of 120 to 130 mV. In this condition the holding potential of the fibre could be assumed to have a value of −90 mV (see also Caille *et al.*, 1978).

Currents, potentials and tension were simultaneously chart recorded (Gould-Brush 220 or 2400), visualized on an oscilloscope (Textronix 565) and photographed (Camera Nihon-Kohden PC-3A). In some experiments, the records were digitized with a PCM converter (Sony 701) and stored on a video tape for further analysis.

Results

Action of heptaminol on mechanical tension in control Ringer solution

Figure 1a illustrates the contractile behaviour of a preparation under conditions of repeated stimulation in control Ringer solution. In this experiment, after an initial period of stabilization, the preparation was stimulated by voltage clamp depolarizing pulses (100 mV, 200 ms) at a frequency of 12 per min. The amplitude of tension decreased progressively during the course of the experiment. This decline which can be considered as an indication of the 'fatigue' of the preparation differed from fibre to fibre (Figure 1a and b) and varied between 20% to 40% during the first 10 min of the experiment with this pattern of stimulation.

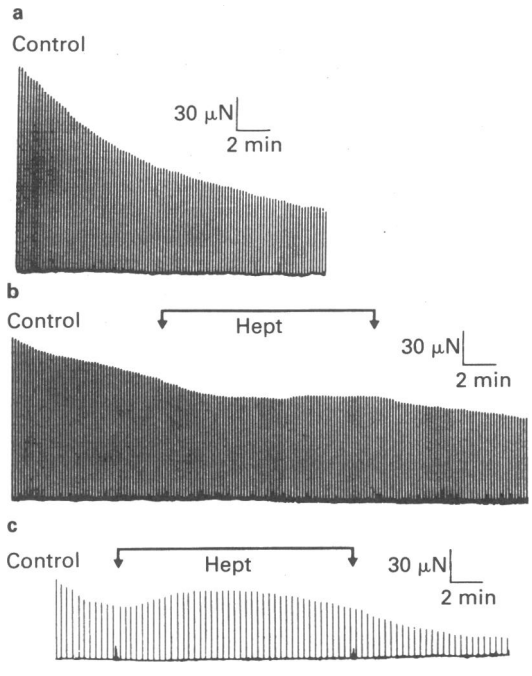


Figure 1 Action of heptaminol on tension during repeated stimulation in control solution. (a) Control experiment showing the progressive decline in tension which characterizes the phenomenon of fatigue. The preparation was stimulated by voltage clamp depolarizations (100 mV, 200 ms) at a frequency of 12 min^{-1}. (b) In this fibre, tension stopped declining during the addition of heptaminol (10^{-4} M). Same protocol of stimulation as in (a). (c) Note that in this fibre which developed a weak contraction, addition of heptaminol (10^{-4} M) increased the tension. The preparation was stimulated by voltage clamp depolarizations (100 mV, 1 s) at a frequency of 3 min^{-1}. (a), (b) and (c) were recorded from different fibres.

Addition of heptaminol (10^{-4} M) increased the tension or stopped its progressive decline (Figure 1b). When heptaminol was removed the tension decreased again. Similar results were obtained in 6 other fibres.

When heptaminol was added after a longer period of repeated stimulation which induced a decrease in tension greater than 50%, a marked increase in tension was always observed (Figure 1c, 4 experiments).

Similar observations were obtained on tetanic tension experiments performed in current clamp conditions (Figure 2). The preparation was stimulated at a tetanic rate as described in the methods section; it generated a train of action potentials (Figure 2a and b, upper row) and developed a tetanic contraction (Figure 2a and b, lower row). This tetanic tension was composed of two phases: an initial peak followed by a plateau. The amplitude and the time course of the plateau differed from fibre to fibre as illustrated in Figure 2a and b. Addition of heptaminol (10^{-4} M) induced a progressive increase in the plateau of the tetanic tension. The potentiating effect became maximal after 4 to 6 min. Action potentials were not appreciably modified, except for a slight decrease in spike amplitude; this was true for the envelope of the action potentials (Figure 2a and b) as well as for single corresponding records (not illustrated).

These results indicate that heptaminol is able to stop or to reverse the appearance of fatigue in an isolated fibre submitted to repeated stimulation.

In order to study the possible mechanism of action of heptaminol, experiments were first performed to test an eventual action on the ionic conductances which are implicated in the process of excitation-contraction coupling (see Caille *et al.*, 1985; Raymond, 1989 for reviews).

Does heptaminol act on ionic currents?

In Figure 3a the preparation was depolarized to $E = -30$ mV by 10 ms duration pulses at a frequency of 1 per second; it developed a large inward sodium current which was not sig-

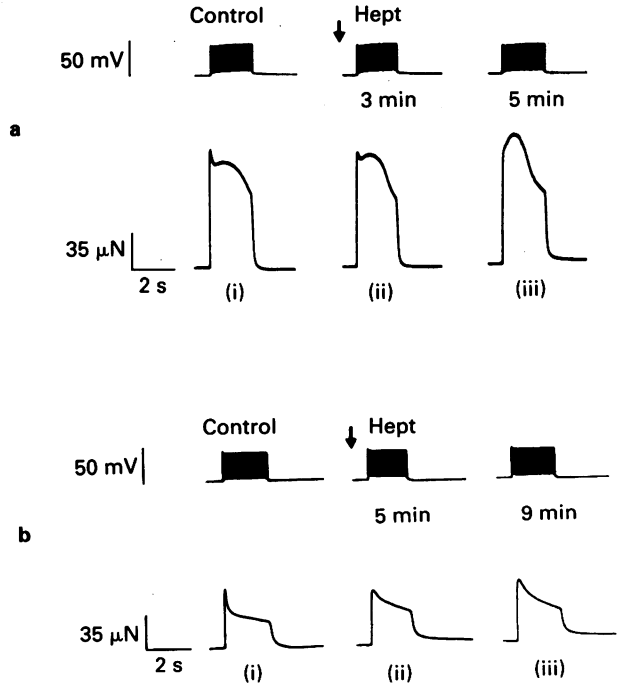


Figure 2 Action of heptaminol on action potentials and tetanic tension. Note that the time course of control tetanic contraction is different in two different fibres (a and b). When heptaminol (10^{-4} M) was added, the plateau of tetanic tension (lower row, (i), (ii) and (iii)) increased while the train of action potentials (upper row) was not modified. The preparations were stimulated by a 2 s train of depolarizing currents (1 ms, 1.2 rheobase, 50 Hz) at a frequency of 1 min^{-1}.

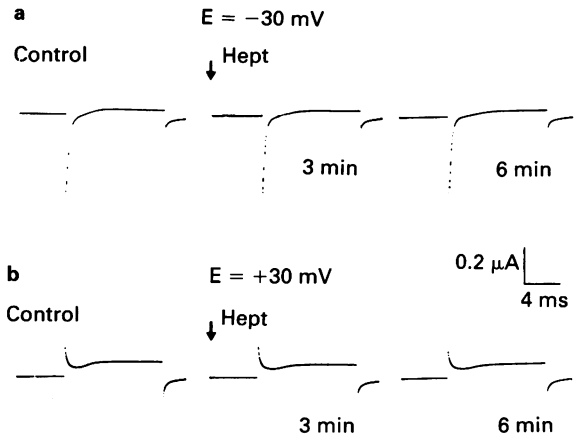


Figure 3 Action of heptaminol on sodium and potassium ionic current. (a) Action on sodium current. The sodium inward current which was activated for a depolarization to E = −30 mV was not modified by heptaminol (10^{-4} M). (b) Action on potassium current. The potassium delayed outward current which was activated for a depolarization to E = +30 mV was not modified by heptaminol (10^{-4} M). (a) and (b) were from the same fibre. The preparation was stimulated at a frequency of 1 s^{-1}.

nificantly modified after the addition of heptaminol (10^{-4} M). In Figure 3b the same fibre was depolarized to E = +30 mV; at this potential close to the sodium current reversal potential, the current which corresponded to the delayed outward potassium current was not modified by heptaminol (10^{-4} M). Similar results were obtained in 3 other fibres. Increasing the concentration of heptaminol (10^{-2} M) induced a decrease of both these membrane currents (not illustrated). This action is certainly due to the general anaesthetic properties of the alcohol group of the molecule.

The following experiment (Figure 4) was carried out to study the action of heptaminol on calcium current and contraction. In this experiment, performed in sodium-free TEA-CH_3SO_3 Ringer, the preparation was depolarized to E = −30 mV by pulses of 2.5 s duration at a frequency of 1 min^{-1}; it developed a slow calcium inward current and contraction (see also Jacquemond & Rougier, 1988). When heptaminol (10^{-4} M) was added neither calcium current nor contraction were modified. Similar results were obtained in 4 other fibres.

These results indicate that the potentiating effect of heptaminol on tension cannot be due to modifications of the ionic fluxes occurring during the first steps of the process of excitation-contraction coupling.

Does heptaminol act via an internal alkalinization?

Action of NH_4Cl It has been suggested that heptaminol exerts its positive inotropic effect by producing an internal alkalinization (Berthiau *et al.*, 1989). The influence of internal alkalinization on tension (see Fabiato & Fabiato, 1978) was tested in our experimental conditions by studying the action of NH_4Cl. It is well known that substituting NH_4Cl (20 mM) for NaCl induces a rapid alkalinization of 0.2, 0.3 pH unit (Aickin & Thomas, 1977; Roos & Boron, 1981; Putnam *et al.*, 1986).

All the following experiments (Figures 5–8) were performed under voltage clamp conditions in the presence of TTX (10^{-7} M).

In Figure 5 the preparation was stimulated by voltage clamp depolarizing pulses (100 mV, 200 ms) at a frequency of 12 min^{-1} inducing the phenomenon of fatigue. When NH_4Cl was introduced a marked and transient increase in tension developed. When returned to the control solution the tension decreased in two phases: an initial rapid phase which corresponded certainly to the sudden acidification due to NH_4Cl withdrawal (see Roos & Boron, 1981) followed by a slower one. Similar results were obtained in 5 other fibres.

These results indicate that an internal alkalinization is able to increase the progressively declining tension observed during fatigue. But if the mechanism of NH_4Cl alkalinization is well established (ROOS & BORON, 1981; LAZDUNSKI *et al.*, 1985 for a review), nothing is known concerning heptaminol; for example we have no evidence for a penetration of the molecule. In a series of experiments we found that tuamine, which corresponds to the amine group of heptaminol, decreased rather than increased tetanic tension during a train of repeated stimulation (not illustrated). Furthermore we observed that the presence of heptaminol (up to 10^{-2} M) in the 'internal' solution bathing cut fibres in the end pools of the double gap chamber did not modify the tension of the test gap fibre segment. So an intracellular alkalinization by a penetration of the molecule as observed with NH_4Cl seems improbable.

Action on the Na/H antiport The possibility of an action of heptaminol on the Na/H antiport as mentioned in the introduction (Berthiau *et al.*, 1989) was investigated.

This hypothesis was attractive for several reasons. A stimulation of the Na/H antiport (1) increases the intracellular pH, a situation which, as observed under the action of NH_4Cl, is able to increase tension; (2) promotes the entry of sodium ions whose intracellular concentration is important for the control of contraction in cardiac (Lazdunski *et al.*, 1985) and in skeletal muscle (Caille *et al.*, 1985). Two kinds of experiments were carried out to test this hypothesis.

In Figure 6a the preparation was stimulated with the same protocol as in Figure 5 but in a sodium-free solution (TRIS substitute). The addition of heptaminol had no effect on the regularly declining tension. Similar observations were made on 5 other fibres. This result confirms and explains the absence of action of heptaminol on tension described in Figure 4 where calcium current and tension were studied in a sodium-free solution.

When the preparation was stimulated in the presence of an inhibitor of the Na/H exchange, addition of heptaminol

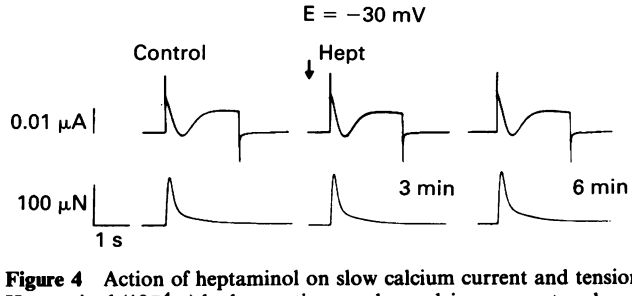


Figure 4 Action of heptaminol on slow calcium current and tension. Heptaminol (10^{-4} M) had no action on slow calcium current and contraction. The preparation was stimulated at a frequency of 1 min^{-1}.

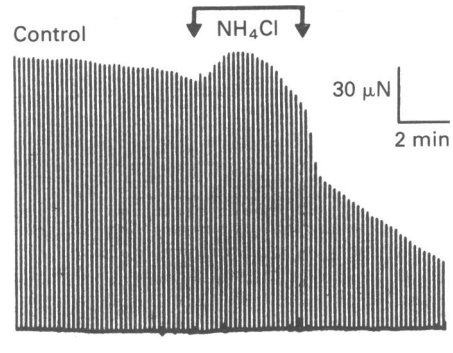


Figure 5 Action of NH_4Cl on tension during repeated stimulation in control solution (tetrodotoxin 10^{-7} M). The addition of NH_4Cl (20 mM) induced a marked increase in tension. Notice the sudden decrease in tension with NH_4Cl withdrawal. Same protocol of stimulation as in Figure 1a.

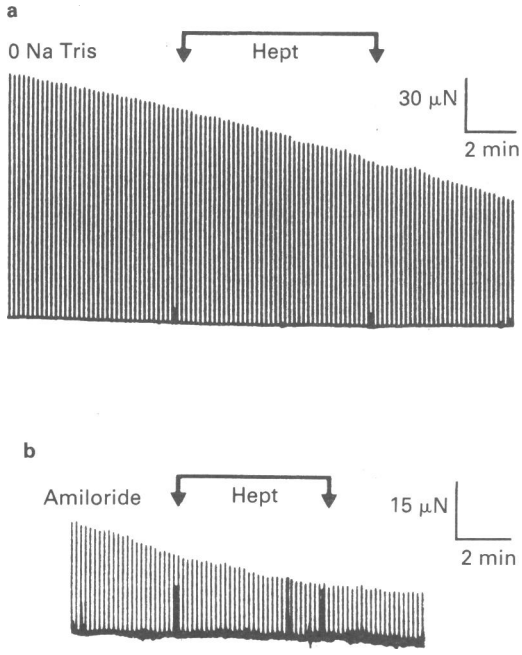


Figure 6 Action of heptaminol on tension during repeated stimulation in sodium-free or in amiloride-containing solution (tetrodotoxin 10^{-7} M). (a) In sodium-free solution (TRIS substitute) heptaminol (10^{-4} M) had no action on the declining tension. (b) In the presence of amiloride (10^{-3} M), heptaminol (10^{-4} M) had no action on tension. (a) and (b) were recorded from different fibres. Same protocol of stimulation as in Figure 1a.

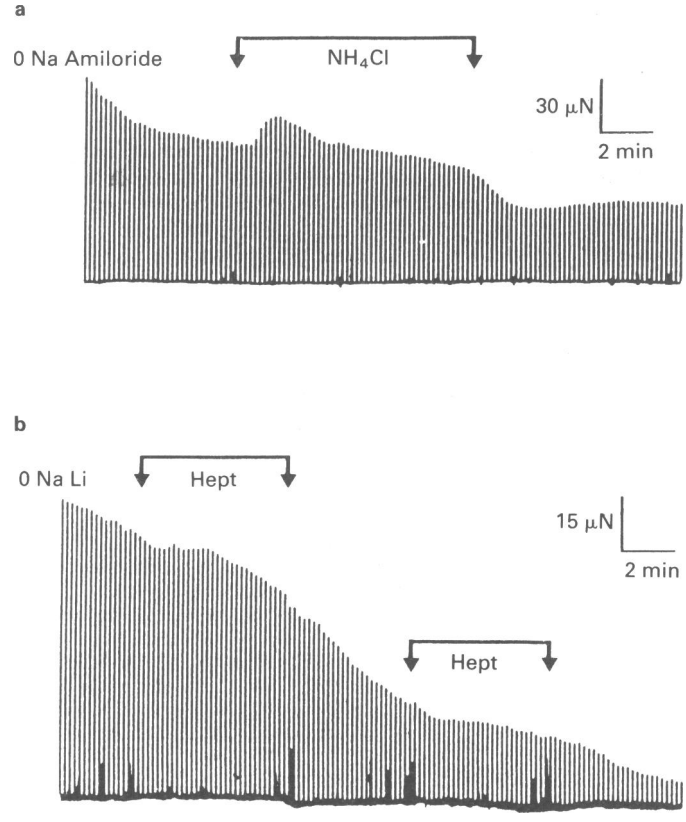


Figure 7 (a) Action of NH_4Cl on tension during repeated stimulation in sodium-free, amiloride-containing solution (tetrodotoxin (TTX) 10^{-7} M). In sodium-free (TRIS substitute) amiloride (10^{-3} M)-containing solution, addition of NH_4Cl (20 mM) induced a marked increase in tension. Same protocol of stimulation as in Figure 1(a). (b) Action of heptaminol on tension during repeated stimulation in lithium containing solution (TTX 10^{-7} M). Addition of heptaminol (10^{-4} M) delayed the decline in tension as observed in sodium-containing solution. Same protocol of stimulation as in Figure 1a.

(10^{-4} M) had no effect on tension; this was observed with amiloride (10^{-3} M) (Figure 6b) (4 preparations) and with EIPA (10^{-5} M) (not illustrated, 3 preparations).

It must be added that in our experimental conditions (performed in the presence of TTX) amiloride or EIPA had no action on the holding current and on the current during the depolarizing pulses. This conflicts with the recent work of Estrada & Sanchez (1991) who found a hyperpolarization as high as 12 mV in the presence of amiloride (10^{-3} M).

On the other hand the addition of NH_4Cl had always a potentiating action on tension in experiments performed in sodium-free amiloride (10^{-3} M) containing solution (Figure 7a, 3 preparations).

These results are in agreement with the hypothesis of a stimulation of the Na/H antiport by heptaminol. This conclusion is strengthened by the observation (3 preparations) that heptaminol was also active in sodium-free, lithium containing solution (Figure 7b), an experimental situation where the Na/H antiport is able to extrude H^+ (Aronson, 1985).

Discussion

Heptaminol has been shown to prevent fatigue of muscle and nervous activities (Chauchard & Mazoue, 1958; Rigal *et al.*, 1963). The cellular mechanisms underlying its action on skeletal muscle have been poorly studied. The results of our experiments show that heptaminol is able to prevent the decay or to increase the amplitude of tension during continuous repeated stimulation of single isolated twitch fibres.

Due to the presence of an alcohol group, heptaminol has general anaesthetic properties which can inhibit ionic conductances as shown by Haydon & Urban (1983) on squid giant axon; such an inhibition was observed at a concentration of 10^{-2} M. The absence of action of heptaminol at 10^{-4} M on the sodium, potassium and calcium currents is an indication that its effect on tension cannot be related to the general anaesthetic properties of the molecule.

An eventual action of heptaminol (10^{-4} M) on the intramembrane charge movements which are considered to play an important role in depolarization-contraction coupling (Schneider & Chandler, 1973) seems improbable. Indeed calcium channels as dihydropyridine receptors are considered to be the support of these charge movements (Rios & Brum, 1987); the fact that the calcium current is not modified by heptaminol is an indication that charge movements are certainly unaffected.

The absence of action of heptaminol during experiments with cut fibres is an indication that heptaminol does not act on some intracellular events implicated in the development of tension.

The possibility that heptaminol exerts its effect by producing an internal alkalinization was tested by comparison with a NH_4Cl-induced alkalinization. Both treatments had a positive inotropic effect but important differences were observed. The action of heptaminol was suppressed in experimental conditions where the Na/H exchange was inhibited, i.e. in sodium-free (TRIS substitute) and in amiloride containing solutions. The action of NH_4Cl was always observed in the same experimental situations. Furthermore, heptaminol exerted its action in sodium-free, lithium containing solution.

These results have several interesting meanings: (1) amiloride and its derivative EIPA cannot be considered in our experimental conditions as direct inhibitors of the sarcoplasmic reticulum calcium release as suggested by Nasri-Sebdani *et al.* (1989); (2) heptaminol is not able to produce directly an internal alkalinization as does NH_4Cl; (3) the fact that heptaminol exerts its action in lithium as well as in sodium solution indicates that the Na/Ca exchange (which is not working in lithium solution) cannot be implicated; on the other hand this strengthened considerably the implication of the Na/H exchange (which is working in lithium solution).

In conclusion the positive inotropic effect of heptaminol on skeletal muscle can be explained by an internal alkalinization due to a stimulation of the Na/H exchange system. This is in agreement with the results of Berthiau *et al.* (1989) on cardiac muscle. The mechanism(s) of heptaminol Na/H antiport stimulation remains to be studied; an alteration of the apparent affinity of the exchange for internal H^+ can be suggested as postulated by Moolenaar *et al.* (1983) for the action of growth factors. Whatever the mechanisms, these experimental results show that the Na/H exchanger plays an important role during the appearance of fatigue; any situation which stimulates this exchange can induce (1) an intracellular alkalinization which can increase tension through an action on contractile proteins (Fabiato & Fabiato, 1978), (2) an influx of sodium which can increase tension by an action on the sarcoplasmic reticulum calcium release (Caille *et al.*, 1978; Allard & Rougier, 1989b).

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Pharmacological analysis of 5-hydroxytryptamine effects on electrically stimulated human isolated urinary bladder

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1 5-Hydroxytryptamine (5-HT) is able to potentiate the contractions induced by electrical field stimulation of pieces of human isolated urinary bladder. On the basis of available selective 5-HT agonists and antagonists, we have further investigated the receptors involved and their site of action.

2 5-HT produced a concentration-dependent increase of the contractile response to electrical field stimulation from 0.1 nM to 1 μM. At higher concentrations (up to 100 μM) the effect decreased. These activities were mimicked by a variety of 5-HT agonists, for which the following rank order of potency was found: 5-HT > α-methyl 5-HT > 5-methoxytryptamine > 5-carboxamidotryptamine > 2-methyl 5-HT ⩾ GR 43175. In addition the gastro-prokinetics agents metoclopramide, cisapride and the 5-HT_3 antagonist ICS 205-930 behaved as 5-HT agonists, their EC_{50} values being 2.3, 0.3, and 0.5 (μM) respectively.

3 The 5-HT potentiating effect was resistant to antagonism by ondansetron (1 μM) and cyanopindolol (1 μM), selective 5-HT_3 and 5-$HT_{1A/1B}$ antagonists respectively. The 5-HT_2 antagonists ketanserin (1 μM), spiperone (1 μM) and methysergide (1 μM) also showed a weak inhibitory activity. Methiothepin (0.1–1 μM) antagonized only the inhibitory effect of 5-HT. Metoclopramide (0.1–1 μM), cisapride (0.01–0.1 μM) and ICS 205-930 (0.3–3 μM) all produced a rightward displacement of the 5-HT response curve with concomitant reduction of the maximum response. The pA_2 values calculated were 7.4, 8.5 and 7.0 respectively. The antagonism of metoclopramide was receptor specific and was not apparently related to interactions with dopaminergic activity since domperidone showed no antagonism of 5-HT, and metoclopramide, itself, did not antagonize the potentiating effect of prostaglandin $F_{2\alpha}$.

4 The receptor involved in the potentiating effect of 5-HT may be located prejunctionally because 5-HT did not potentiate responses to acetylcholine (ACh) or electrical field stimulation with the parameters of direct muscle excitation. Also, since the 5-HT potentiating effect was blocked by atropine, it may be attributed to a release of ACh.

5 This study suggests that in the human urinary bladder 5-HT causes two opposite effects on the contractile response to electrical field stimulation. A potentiating effect at low concentrations due to an interaction with an atypical receptor, different from the classical 5-HT_1, 5-HT_2 or 5-HT_3 subtypes and an inhibitory effect at greater concentrations probably due to an interaction with 5-HT_1-like receptors. The possibility that this atypical receptor possesses some characteristics of those found in other isolated preparations like guinea-pig ileum, rat oesophagus and mouse embryo colliculi neurones is discussed.

Keywords: Urinary-bladder; 5-HT; electrical-stimulation; ondansetron; methiothepin; ketanserin; methysergide; metoclopramide; cisapride; ICS 205-930.

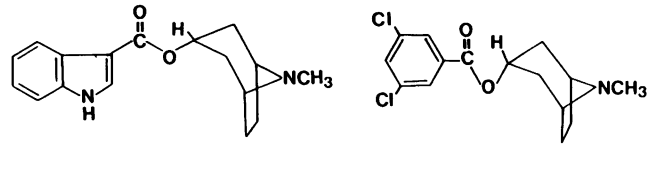
Introduction

5-Hydroxytryptamine (5-HT) has a variety of actions on many cell types and knowledge of the membrane receptors which mediate its cellular responses has progressed significantly in the last decade. The current view is that there are three main groups of functional 5-HT receptors, 5-HT_1-like, 5-HT_2 and 5-HT_3 (Bradley *et al.*, 1986). The latter two equate with Gaddum & Picarelli's 'D' and 'M' receptors respectively, first identified in guinea-pig ileum (Gaddum & Picarelli, 1957).

A range of potent and specific receptor blocking drugs is now available for either 5-HT_2, e.g. ketanserin, methysergide and spiperone (Leysen *et al.*, 1981; Humphrey *et al.*, 1982) or 5-HT_3 receptors e.g. ondansetron (Brittain *et al.*, 1987), ICS 205-930 (Richardson *et al.*, 1985) and MDL 72222 (Fozard, 1984), which allows their identification and characterization in both the brain and periphery (Figure 1). No good antagonists are available for 5-HT_1-like receptors which have largely been classified on the basis of data with agonists. These receptors are clearly heterogeneous comprising several subtypes, but all can be characterized by the high agonist potency of 5-carboxamidotryptamine (5-CT) and are blocked by methiothepin (Bradley *et al.*, 1986).

5-HT has been reported to potentiate the contraction induced by electrical field stimulation of isolated urinary bladder of mouse and man. In mouse bladder, the potentiating action of 5-HT has already been analysed and the activation of facilitatory 5-HT_{1B} and 5-HT_2 receptors, located on intrinsic nerves has been demonstrated (Holt *et al.*, 1986; Clean *et al.*, 1989). In human bladder the potentiating effect of 5-HT was observed at very low concentrations and was unaffected by methysergide and morphine (Hindmarsh *et al.*, 1977). However, a detailed pharmacological analysis of this effect in human bladder is still lacking.

Exploiting currently available selective 5-HT agonists and antagonists we have now examined further the potentiating



ICS 205-930 MDL 72222

Figure 1 Chemical structure of two 5-HT_3 antagonists, ICS 205-930 and MDL 72222.

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effect of 5-HT on electrically stimulated human bladder to characterize the receptors involved and to elucidate their site of action.

Methods

Preparation of human urinary bladder strips

Specimens from the anterior part of the dome of the urinary bladder were obtained from patients undergoing cystectomy *en bloc* because of bladder malignancy. Immediately after surgical removal, the serosal and mucosal layers were dissected away and muscular strips (approximately 10 mm long, 3 mm wide, 3 mm thick) were cut. Each bladder strip was mounted vertically between two platinum electrodes and placed in a 20 ml glass organ bath containing modified Krebs solution, aerated with 95% O_2 and 5% CO_2 and maintained at 37°C. The composition of this physiological solution was (mM): NaCl 120, KCl 4.7, $MgSO_4$ $7H_2O$ 0.6, KH_2PO_4 1.2, $NaHCO_3$ 2.5, $CaCl_2$ 2.0 and glucose 10. Preparations were initially placed under tension of 2 g and allowed to equilibrate for at least 1 h before an experiment was started. The tension was measured with a Grass Instrument FT03 isometric force displacement transducer, connected to a Linseis model 7025 polygraph. The contractile capacity of all preparations was tested by exposure to KCl 100 mM.

Characterization of the response to electrical stimulation

Bladder strips were electrically field stimulated with a Grass S88 stimulator. Trains of electrical pulses of 5 s in duration were delivered at intervals of 1 min, at pulse width of 0.1 ms and supramaximal output voltage (30–80 V). Pulse frequency was 20 Hz producing about half maximal contraction. These trains of electrical pulses produced reproducible contractile responses for at least 3 h. An analysis of the components of the twitch response was carried out by examination of the effect of tetrodotoxin (TTX) 0.3 μM and atropine (0.001–1 μM).

Measurement of the effects of 5-hydroxytryptamine agonists

Bladder strips were electrically stimulated at the parameters listed above. When the contractile responses were stabilized (usually within 30 min) a cumulative concentration-response curve (CRC) for 5-HT or 5-HT selective agonists was run at 0.5 $\log_{10}$ unit intervals (Van Rossum, 1963). Since the CRCs to 5-HT or 5-HT agonists were not reproducible (tachyphylaxis occurred) a single CRC was determined in each strip. In a typical experiment, from four to eight strips were obtained from a single bladder specimen. On each occasion, one of available strips was treated with 5-HT, so that the potentiating effect of 5-HT agonists could be examined in parallel with that of 5-HT. In some strips the potentiating effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), metoclopramide, cisapride and ICS 205-930 were also investigated.

Measurement of the effects of 5-hydroxytryptamine antagonists

Bladder strips were stimulated at the parameters listed above. When the contractile responses had stabilized, selective 5-HT antagonists were added to the bath and 2 h later a CRC to 5-HT was constructed. Comparisons of the effect of antagonists were made by comparing the CRC to 5-HT in test preparations and in matched controls. This protocol enabled the effect of antagonists on the twitch height *per se* to be examined. The effect of metoclopramide, cisapride, ICS 205-930, atropine and domperidone were also investigated following the same protocol. To minimize the binding of 5-HT antagonists to the glass, the organ baths were silanised with 5% dimethyldichlorosilane in toluene and rinsed in distilled water before use.

Effect of 5-hydroxytryptamine on direct electrical muscle excitation

Following a 30 min period of field stimulation at the parameters listed above, TTX (0.3 μM) was administered. When the twitch response was abolished, the pulse width was increased to 6 ms and once the response to stimulation had been stabilized, 5-HT was added cumulatively.

Effect of 5-hydroxytryptamine on contractile responses to acetylcholine

The effect of 5-HT on responses to a single submaximal concentration of acetylcholine (ACh) were also investigated. Initially, concentrations of ACh that produced equivalent increases in contractile tone to electrical stimulation (20 Hz for 5 s, every 60 s, 0.1 ms pulse width, V_{max}) were determined. Concentrations of 30–100 μM ACh were most commonly used and for each preparation this selected concentration was kept constant throughout the experiment. Once reproducible responses to ACh had been obtained, each preparation was electrically stimulated for 15 min and exposed to a single concentration of 5-HT. After maximum potentiation had been obtained the stimulator was switched off and ACh added immediately. Following a 30 min wash period this cycle was repeated, and the effect of different concentrations of 5-HT analyzed. One of each pair of preparations was exposed to vehicle to monitor spontaneous changes in responses to electrical stimulation and ACh.

Data analysis

Potentiating effects were transformed into a percentage of the contractile responses to 100 mM KCl. Data are expressed as means ± s.e.mean.

Where applicable, the analysis of CRC was performed with the logistic curve fitting programme ALLFIT (De Lean *et al.*, 1978). The logistic function used was:

$$Y = E_{max} \frac{[A]^n}{[A]^n + EC_{50}^n}$$

where Y is the observed response, [A] the concentration of agonist, E_{max} the maximal response to agonist, EC_{50} the location and n the slope. Ratios of concentration (CR) inducing 50% of maximal response to 5-HT, in the presence or absence of antagonists are expressed as means ± s.e.mean. pA_2 values were obtained by use of the Schild equation: $pA_2 = -\log[Ant] + \log(CR - 1)$ where [Ant] is the concentration of the antagonist used (Arunlakshana & Schild, 1959). The IC_{50} estimated for atropine represents the concentration of atropine yielding 50% inhibition. This value was obtained by regression analysis. The significance of differences between mean values was determined at $P < 0.05$ using a Student's t test.

Drugs used

The following compounds were used: atropine sulphate (Sigma), acetylcholine chloride (Merck), 5-hydroxytryptamine creatinine sulphate (Sigma), prostaglandin $F_{2\alpha}$ (Sigma), spiperone (RBI), domperidone (Janssen), metoclopramide hydrocloride (Lepetit), cisapride (Janssen), methysergide bimaleate (Sandoz).

The following compounds were gifts: ketanserin (Janssen), methiothepin maleate (Roche), cyanopindolol (Sandoz), ICS 205-930 (Sandoz) (Figure 1) and we acknowledge the generosity of these companies. GR 43175 (see Humphrey *et al.*, 1988) ondansetron, α-methyl 5-HT, 5-carboxamidotryptamine, 2-methyl 5-HT, 5-methoxytryptamine were synthesized by the Chemistry Research department at Glaxo Group Research.

All drugs were dissolved in Krebs medium, unless otherwise stated, to give a final concentration of 1–100 mM. Spiperone at a concentration of 10 mM was dissolved in dimethysulphoxide (DMSO) and diluted in distilled water; ketanserin at a concentration of 1 mM was dissolved in distilled water containing 1% v/v of lactic acid; domperidone at a concentration of 10 mM was dissolved in dimethylformamide, 50% v/v, and diluted in distilled water. Appropriate solvent controls were found to be without effect on 5-HT-induced potentiation (results not shown).

Results

Characteristics of the contractile response of the human isolated bladder to electrical field stimulation

Electrical field stimulation of the human isolated bladder (20 Hz for 5 s every 60 s, 0.1 ms pulse width, supramaximal voltage) produced reproducible contractile responses which were abolished by TTX (0.3 μM) and markedly inhibited by atropine (IC_{50} (with 95% C.L.) = 0.01 μM (0.005–0.03) $n = 3$, maximum inhibition approximately 90–95%). The small twitch (8.8 ± 2.0% of the original response), remaining after atropine, was insensitive to TTX.

Effects of 5-hydroxytryptamine and 5-HT agonists on contractile response of the human isolated bladder to electrical field stimulation

The cumulative addition of 5-HT produced a biphasic effect. At concentrations from 0.1 nM to 1 μM, 5-HT induced a concentration-dependent increase of the contractile response to electrical field stimulation. At greater concentrations (from 3 to 100 μM), 5-HT inhibited its own potentiating effect even though, at the highest concentration used, the level of contraction did not return to the control values (Figure 2).

The potentiating effect of 5-HT was mimicked by a variety of 5-HT agonists (Figure 3).

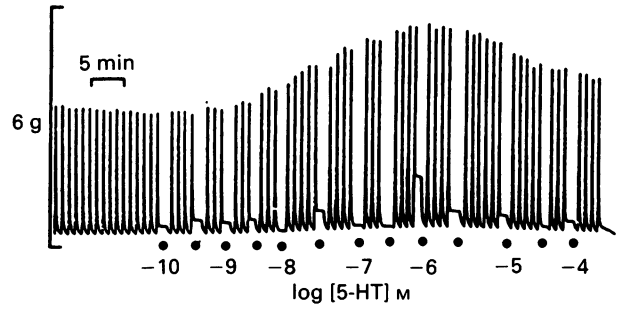


Figure 2 Experimental recording illustrating the potentiating effect of 5-hydroxytryptamine (5-HT) on the contractile response of the human bladder to neurogenic electrical field stimulation (20 Hz for 5 s, 0.1 ms pulse width, every 1 min, 60 V). Dots represent the points of 5-HT cumulative administration in 0.5 $\log_{10}$ unit intervals.

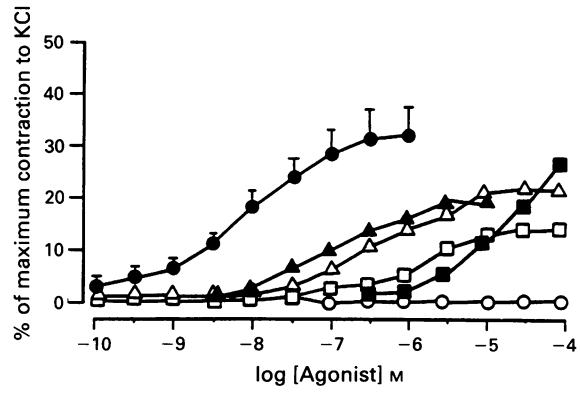


Figure 3 Potentiating effect of 5-hydroxytryptamine (5-HT) and of a variety of 5-HT agonists on the contractile response of the human isolated bladder to neurogenic electrical field stimulation. Symbols represent 5-HT (●); α-methyl-5-HT (▲); 5-methoxytryptamine (△); 2-methyl-5-HT (■); 5-carboxamidotryptamine (□) and GR 43175 (○). Values shown are the means from 12 experiments for 5-HT and 2–4 experiments for the 5-HT agonists. The s.e.mean is omitted for clarity.

The maximum responses (mean ± s.e.mean) produced by 5-CT (5-HT_1-like agonist), 5-methoxytryptamine (5-MeOTr, (5-HT_1-like agonist), α-methyl-5-HT (5-HT_2 agonist) and 2-methyl 5-HT (5-HT_3 agonist) were 55 ± 5.5, 65 ± 10.3, 65 ± 18 and 94 ± 14.5% respectively of the maximum response produced by 5-HT, GR 43175 (5-HT_1-like agonist) was completely ineffective. These results, together with the potency (as EC_{50}) and the equipotent molar concentration ratio (e.p.m.r.) of 5-HT and of 5-HT agonists are shown in Table 1.

The rank order of potency of these compounds was: 5-HT > α-Me5-HT > 5-MeOTr > 5-CT > 2-Me5-HT ≫ GR 43175.

Metoclopramide, cisapride and ICS 205-930 were all agonists and their EC_{50} (±s.e.mean) values (μM) were respectively: 2.3 (±0.7); 0.3 (±0.1); 0.5 (±0.2). The maximal responses to metoclopramide, cisapride and ICS 205-930 were approximately 70%, 45% and 15% of the 5-HT maximum (Figure 4). These compounds were also tested as antagonists (see below).

Effect of 5-hydroxytryptamine antagonists

The potentiating action of 5-HT was substantially resistant to antagonism by ondansetron (1 μM), a 5-HT_3 antagonist and cyanopindolol (1 μM), which has a high affinity at $5\text{-HT}_{1A}/5\text{-HT}_{1B}$ recognition sites in brain radioligand binding studies (data not shown). The potentiating effect of 5-HT was also resistant to methiothepin (0.1–1 μM), which conversely antagonized the inhibitory effect of 5-HT (Figure 5). At a low concentration (0.1 μM) methysergide had little effect on the potentiating action of 5-HT, whilst at a higher concentration (1 μM) it acted as an antagonist. However, this was largely

Table 1 Agonist potencies of 5-hydroxytryptamine (5-HT) and 5-HT agonists in the potentiation of responses to electrical stimulation of the human isolated urinary bladder

Agonist	n	EC_{50} (μM)	*e.p.m.r.* (5-HT = 1)	*% 5-HT maximum response*
5-Hydroxytryptamine	12	0.01 ± 0.002	1.0	100
5-Carboxamidotryptamine	4	2.6 ± 0.8	260	55.0 ± 15.0
α-Methyl 5-HT	3	0.3 ± 0.07	30	65.0 ± 18.0
2-Methyl 5-HT	3	30 ± 1.2	3000	100.0 ± 15.0
5-Methoxytryptamine	4	0.5 ± 0.08	50	65.0 ± 10.0
GR 43175	2	NSE up to 100 μM	—	—

Values are given ± s.e.mean. NSE = no significant effect.
EC_{50} values were determined as described in the methods.
e.p.m.r. = equipotent molar concentration ratio.

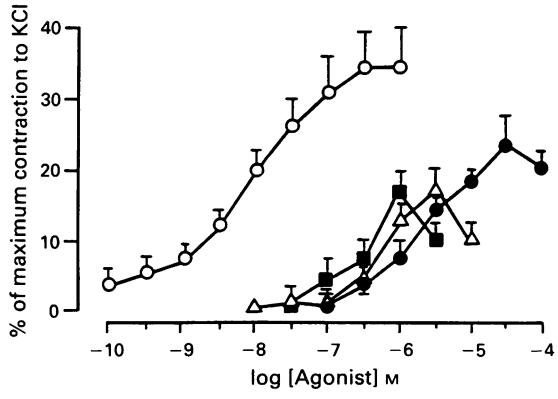


Figure 4 Potentiating effect of 5-hydroxytryptamine (5-HT) (○), metoclopramide (●), cisapride (■) and ICS 205-930 (△) on the contractile response of the human isolated bladder to neurogenic electrical field stimulation. Values are the mean of 12 experiments for 5-HT and 3 experiments for the other agonists, with s.e.mean shown by vertical bars.

associated with a significant depression of the maximum response to 5-HT ($P = 0.001$ versus control), there being little change in the threshold concentration of 5-HT (Figure 5). Only relatively high concentrations of 5-HT_2 antagonists, ketanserin (1 μM) and spiperone (1 μM), caused a displacement to the right of the CRC to 5-HT (approximately fourfold) yielding a pA_2 of 6.5 (Figure 6) without a significant depression of the maximum response ($P > 0.05$ versus control for both agonists). In none of these experiments did the 5-HT

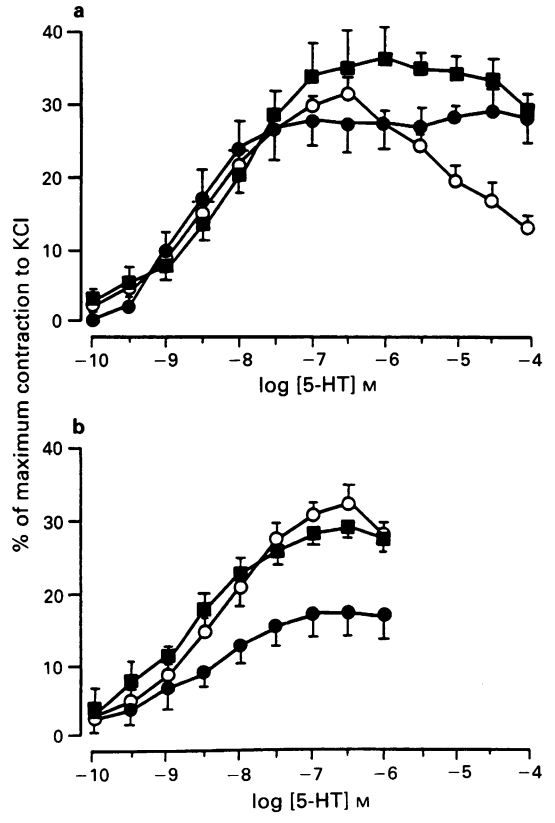


Figure 5 Concentration-effect curves to 5-hydroxytryptamine (5-HT) for the potentiation of responses to electrical stimulation of the human urinary bladder in the absence (○) and in the presence of methiothepin at 0.1 μM (●) and 1 μM (■) in (a) or methysergide at 0.1 μM (■) and 1 μM (●) in (b). Values are the mean of 10 experiments for 5-HT alone and 3 experiments for 5-HT in presence of antagonists, with s.e.mean shown by vertical bars.

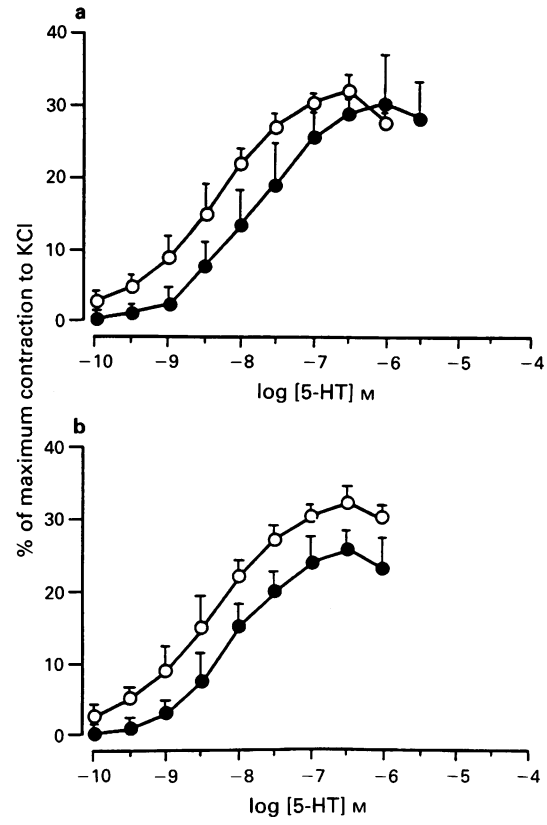


Figure 6 Concentration-effect curves to 5-hydroxytryptamine (5-HT) for the potentiation of responses to electrical stimulation of the human urinary bladder in the absence (○) and in the presence of 1 μM ketanserin (●) in (a) or 1 μM spiperone (●) in (b). Values are the mean of 10 experiments for 5-HT and 3 experiments for 5-HT in presence of antagonists, with s.e.mean shown by vertical bars.

receptor blocking drugs have any significant effect on the contractile response of the human isolated bladder to electrical field stimulation.

Effect of metoclopramide, cisapride and ICS 205-930

Metoclopramide (0.1–1 μM), cisapride (0.01–0.1 μM) and ICS 205-930 (0.3–3 μM) all produced rightward displacement of the CRC to 5-HT. These were accompanied by a concentration-related reduction in the maximum response (Figure 7). For metoclopramide and cisapride the reduction of the maximum response was statistically significant ($P < 0.02$ versus control) for all the concentrations used. Conversely the reduction of the maximum response induced by ICS 205-930 was significant ($P < 0.05$ versus control) only at concentrations of 1 and 3 μM. A calculation of the pA_2 using the ratio of concentrations inducing 50% of the effect in the absence or in the presence of antagonist was made, despite the depression of the maximum response. Thus for the concentrations of antagonists used the $pA_2 \pm$ s.e.mean was: 7.5 ± 0.03, 8.5 ± 0.03 and 7.0 ± 0.1 for metoclopramide, cisapride and ICS 205-930 respectively. When metoclopramide was tested in the presence of methiothepin (1 μM), which inhibits the inhibitory portion of the CRC to 5-HT, the rightward displacement was still accompanied by a significant reduction ($P = 0.02$ versus control) in the maximum response (Figure 7). The antagonistic activity of metoclopramide (1 μM) was specific in nature since it did not modify the potentiating effect of $PGF_{2\alpha}$ in the same tissue ($n = 3$, data not shown). Furthermore domperidone (1 μM) which has antidopamine properties like metoclopramide, did not produce any significant effect on the potentiation produced by 5-HT ($n = 3$, data not shown).

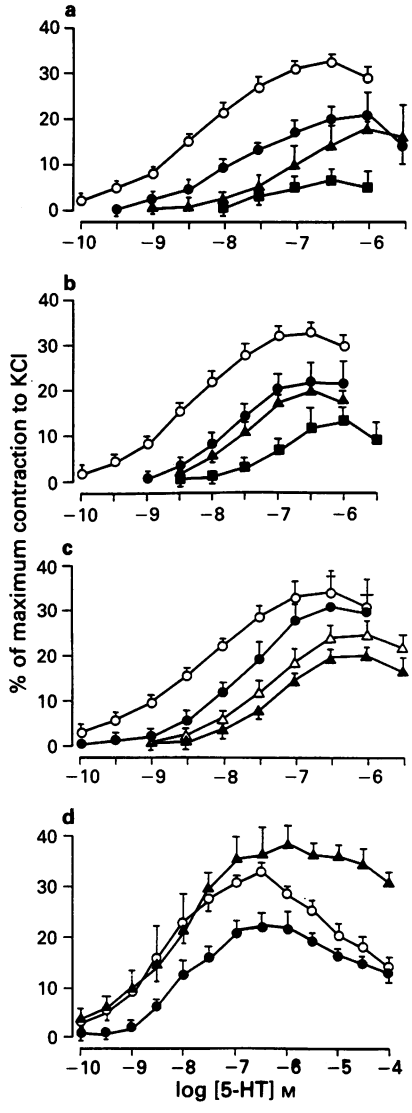


Figure 7 Antagonism by metoclopramide (a), cisapride (b), ICS 205-930 (c) and metoclopramide combined with methiothepin (d) of 5-hydroxytryptamine (5-HT)-induced potentiation of responses to electrical stimulation of the human urinary bladder. In (a) symbols represent control responses (○) or the presence of metoclopramide at 0.1 μM (●), 0.3 μM (▲) and 1 μM (■). In (b), symbols indicate control responses (○) or the presence of cisapride at 0.01 μM (●), 0.03 μM (▲) and 0.1 μM (■). In (c) symbols represent control responses (○) or the presence of ICS 205-930 at 0.3 μM (●), 1 μM (△) and 3 μM (▲). In (d) symbols indicate control responses (○), the presence of only methiothepin 1 μM (▲), the presence of 1 μM methiothepin and 0.1 μM metoclopramide (●). Values are the mean of 10 experiments for 5-HT alone and 3 experiments for 5-HT in the presence of antagonists. The s.e.mean is shown by vertical bars.

Effect of 5-hydroxytryptamine on direct electrical muscle excitation

Marked reproducible contractions of the human urinary bladder were obtained by direct excitation of the smooth muscle. In the presence of TTX, contraction could be induced at a pulse width of 6 ms (20 Hz, for 2 s, every min, 40 V). Cumulative addition of 5-HT up to 10 μM had no effect on responses to direct muscle excitation, but at concentrations of 10–100 μM responses were very slightly potentiated with a little increase in resting tone (n = 3, data not shown).

Effect of 5-hydroxytryptamine on contractile responses to acetylcholine

In experiments in which both myogenic and neurogenic stimulation was used, the location of the 5-HT site of action

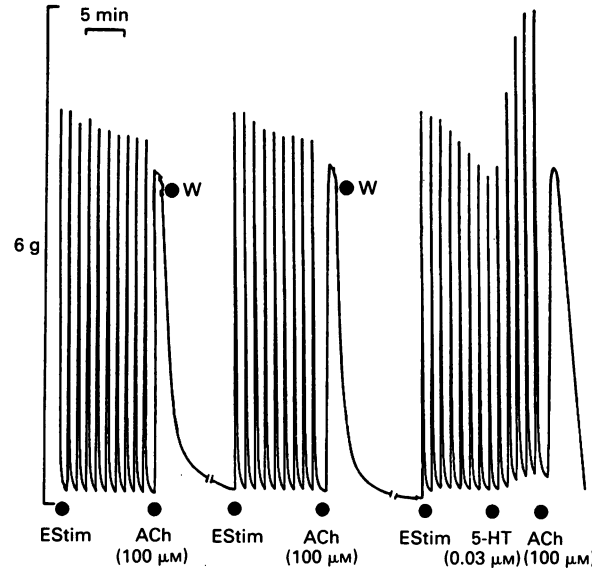


Figure 8 Experimental recording illustrating the effects of 0.03 μM 5-hydroxytryptamine (5-HT) on the contractile response of the human bladder to neurogenic electrical stimulation (20 Hz for 5 s, 0.1 ms pulse width, every 1 min, 50 V) or to exogenous 100 μM acetylcholine (ACh). Dots represent the points of agonist administration (5-HT or ACh) and the starting point for the electrical stimulation (EStim). W = wash.

was investigated. Myogenic contractions induced by concentrations of 30–100 μM ACh were submaximal and brought about similar contractile responses to those of nerve-mediated field stimulation (20 Hz for 5 s, every 60 s, 0.1 ms pulse width, V max). 5-HT, up to 1 μM, potentiated the electrical field stimulation, without modifying the response to ACh 100 μM (an example using 5-HT at 0.03 μM is shown in Figure 8).

Atropine (0.03 μM) attenuated by 49% the excitatory response to neuronal stimulation (n = 3). Furthermore it antagonized the CRC to 5-HT. This antagonism was not competitive, being largely associated with a depression of the maximum response to 5-HT (data not shown).

Discussion

In the present study, electrical stimulation of the human bladder induced reproducible contractile responses that were abolished by TTX and almost completely inhibited by atropine, thus supporting the suggestion that in man, unlike several other animal species (mouse, rat), the contractile responses to electrical stimulation of the detrusor are largely mediated by excitation of cholinergic neurones.

Neuronally induced contractions of the human bladder were potentiated in a concentration-related manner, by 5-HT from 0.1 nM to 1 μM confirming the previous findings of Hindmarsh *et al.* (1977). Our study has also revealed that at higher concentrations, 5-HT inhibited its own potentiating effect. Thus an activation of at least two receptors one excitatory, the other inhibitory would be conceivable. However there are two other possible explanations for the observed decreasing response at higher concentrations of 5-HT. Firstly, this may be a result of tachyphylaxis towards the end of the CRC. Secondly, 5-HT may be producing a direct contractile response of the preparation at the higher concentrations employed, thereby reducing the capacity for further contractions to field stimulation. However, both these hypotheses seem unlikely. In fact an antagonist (methiothepin) was found to antagonize the inhibitory effect of 5-HT without affecting the excitatory phase and on the other hand 5-HT, up to 100 μM did not modify the baseline tension (Figure 2). The inhibitory action of 5-HT was insensitive to 5-HT_2 and 5-HT_3 blockade with

ketanserin (Bradley et al., 1986) and ondansetron, but was prevented by methiothepin, a potent 5-HT_1-like and 5-HT_2 antagonist. Taken together these findings suggest that only a 5-HT_1-like receptor is involved. The tryptamine analogues α-methyl 5-HT, 5-CT, 5-methoxytryptamine and 2-methyl 5-HT mimicked the potentiating effect of 5-HT, thus implicating a specific 5-HT receptor in this response. Several results clearly indicate that this receptor does not belong to the 5-HT_1-like, 5-HT_2 and 5-HT_3 families. 5-CT, a potent specific 5-HT_1 agonist, was 260 fold less potent than 5-HT, whereas 5-CT is known to have a similar or an even higher potency than 5-HT at 5-HT_1-like receptors, (Humphrey, 1984) suggesting that such a receptor subtype is not involved. This suggestion is corroborated by the absence of an effect of the 5-HT_1-like receptor agonist, GR 43175 (Humphrey et al., 1988), and also the lack of antagonism by methiothepin and cyanopindolol, 5-HT_{1A} and 5-HT_{1B} antagonists. α-Methyl 5-HT, a potent 5-HT_2 antagonist, was 30 fold less potent than 5-HT, whereas, this tryptamine analogue is known to have a similar potency to 5-HT in isolated preparations containing 5-HT_2 receptors (Humphrey, 1984). The suggestion that 5-HT_2 receptors are not involved is corroborated by the weak antagonism of ketanserin and spiperone. In fact their apparent pA_2 values calculated in this study are at least 100 times less than the appropriate pA_2 values at 5-HT_2 receptors (Humphrey et al., 1982). The weak agonist potency of 2-methyl-5-HT, a potent specific 5-HT_3 agonist, (3000 fold less potent than 5-HT), and the lack of antagonism by ondansetron, a selective 5-HT_3 antagonist, clearly indicate that the potentiating action of 5-HT is unlikely to be due to an involvement of 5-HT_3 receptors.

At high concentrations metoclopramide, cisapride and ICS 205-930 show agonism. The order of potency is cisapride > ICS 205-930 > metoclopramide. At lower concentrations all three compounds can antagonize the 5-HT response. Because of the inhibitory effect of 5-HT it was not easy to test whether these compounds were competitive antagonists or not. Since methiothepin antagonized only the inhibitory part of the curve, experiments were designed in the presence of methiothepin with the aim of achieving a less complicated analysis of its type of antagonism of these compounds. From these experiments it was concluded that for metoclopramide at least part of the antagonism was non-competitive since a reduction of the maximal response was still apparent. This was not due to the interaction with dopamine receptors since domperidone was completely inactive in these experiments. However, the antagonism of metoclopramide was specific in nature since it did not modify the potentiating effect of $PGF_{2\alpha}$. It is possible to make an approximate estimate of the pA_2 value for each of these antagonists and these are 8.5 for cisapride, 7.4 for metoclopramide and 7.0 for ICS 205-930. In the way in which we obtained these numbers, they represent only a relative potency of the antagonists on the 5-HT-induced potentiating effect, without showing any evidence of competitiveness for the 5-HT receptor involved and thus comparison with data, reported by other authors, where the above antagonists are competitive would be difficult. However, it is possible that the receptor identified in this study is similar to the receptors identified by other workers in guinea-pig ileum (Sanger, 1987; Kay et al., 1989; Craig & Clarke, 1989), in rat oesophagus (Reeves et al., 1989) or in mouse embryo colliculi neurones (Dumuis et al., 1989). This receptor as well as those present in guinea-pig ileum (Craig & Clarke, 1990), rat oesophagus (Reeves et al., 1989) and mouse embryo colliculi neurones (Dumuis et al., 1989) is blocked in a competitive manner by micromolar concentrations of ICS 205-930 (pK_B 6.0–6.4), is resistant to 5-HT_3 antagonists having an indole group such as BRL 43694 (ileum and neurones) and ondansetron (neurones and oesophagus). Moreover this receptor, as well as those present in guinea-pig ileum (Sanger, 1987; Craig & Clarke, 1989) and mouse embryo colliculi neurones (Dumuis et al., 1989), is stimulated by high concentrations of benzamide compounds such as metoclopramide and cisapride. In both cases, cisapride was more potent than metoclopramide. The present receptor, as well as that present in rat oesophagus (Reeves et al., 1989), can also be antagonized by the same benzamide compounds such as metoclopramide and cisapride but at lower concentrations. As antagonists, unlike when acting as agonists, the potencies of the two benzamides were similar in the two preparations.

5-Methoxytryptamine is reported to be equipotent with 5-HT in mouse embryo colliculi neurones (Dumuis et al., 1988) and 8 times less potent than 5-HT in guinea-pig ileum (Craig & Clarke, 1990), whereas in the present study it is 50 times less potent. To explain these differences in activity, a different 'reserve' in the receptor-stimulus trasduction between the tissues could be postulated or in terms of a species difference. The same variation in sensitivity should be expected also with the other 5-HT selective agonists. However 2-methyl-5-HT, although it was only a weak partial agonist in rat oesophagus (Reeves et al., 1989) or ineffective in mouse embryo colliculi neurones (Dumuis et al., 1989) and guinea-pig ileum (Craig & Clark, 1990), was a weak, but full, agonist in our preparation. Therefore, only a variation in the amount of the effector-couplers for the same 5-HT receptor in different tissues, could be invoked to explain these findings.

A part of this study attempted to determine the specific location of this receptor and thus the site of the potentiating action of 5-HT in human bladder. Some lines of evidence suggest that this receptor is located prejunctionally and involves the release of ACh. Firstly, the contractile responses to neuronal stimulation were potentiated in a concentration-dependent manner by 5-HT. Yet, equivalent responses to ACh in the same preparation were largely unaffected. Secondly, 5-HT (up to 10 μM) did not alter responses to direct muscle excitation. The mechanism of action of 5-HT in potentiating responses to field stimulation may be attributed to the facilitation of the release of the excitatory transmitter ACh, since the potentiating effect of 5-HT was antagonized by atropine.

In conclusion, our study has demonstrated two opposite effects of 5-HT on the contractile responses to electrical field stimulation, a potentiation at lower doses of 5-HT and an inhibition at higher doses. Interestingly, the pharmacological characteristics of the receptor involved in the potentiating action exclude the possibility that it belongs to the known 5-HT_1, 5-HT_2 and 5-HT_3 receptor types. In several aspects the receptor resembles the atypical 5-HT receptor subtype reported in guinea-pig ileum, rat oesophagus and mouse embryo colliculi neurones. This is possibly the first finding of such an atypical 5-HT receptor in human tissue.

The authors wish to thank Mrs Bettelini Letizia for excellent technical assistance.

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Effects of flecainide on isolated vascular smooth muscles of rat

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1 The inhibitory effects of flecainide were studied on contractile responses in rat isolated aortae and caudal artery and on spontaneous mechanical activity in portal vein segments.

2 In rat isolated aorta flecainide, 10^{-6} M–5×10^{-4} M, inhibited in a dose-dependent manner the contractile responses induced by high K (80 mM) and noradrenaline (NA, 10^{-5} M). These inhibitory actions were observed when flecainide was added before or after the induced contractions and were similar in aortae with or without endothelium.

3 Contractile responses induced by addition of Ca to Ca-free high-K solution were also dose-dependently inhibited by flecainide ($IC_{50} = 2.5 \pm 0.3 \times 10^{-5}$ M). Moreover, flecainide inhibited the contractile responses elicited by NA in rings incubated in Ca-free solution.

4 Flecainide also inhibited the spontaneous mechanical activity in portal vein segments ($IC_{50} = 6.5 \pm 0.9 \times 10^{-5}$ M).

5 Flecainide, 10^{-4} M, inhibited ^{45}Ca uptake stimulated by high K or NA without altering ^{45}Ca uptake in resting aortae.

6 These results indicated that in rat isolated aortae, caudal arteries and portal veins, flecainide inhibited Ca entry through voltage-operated channels and NA-induced Ca uptake as well as Ca release from intracellular stores, thus decreasing the availability of intracellular free Ca required for vascular smooth muscle contraction.

Keywords: Flecainide; aorta; ^{45}Ca movements; rat tail artery; portal vein

Introduction

Flecainide acetate is a class Ic antiarrhythmic drug effective both in experimental and clinical ventricular and supraventricular tachyarrhythmias (Anderson *et al.*, 1981; Duff *et al.*, 1981; Campbell, 1983a; Hellestrand *et al.*, 1984; Holmes & Heel, 1985; Somberg & Tepper, 1986). In cardiac muscle fibres, flecainide exhibits both very slow onset kinetics and recovery from V_{max} block (Campbell & Vaughan Williams, 1983; Campbell, 1983a,b; Delpón *et al.*, 1991) due to its high affinity for the activated and inactivated states of the Na channels (Anno & Hondeghem, 1990).

In addition, there is some experimental and clinical evidence suggesting that flecainide may exhibit weak calcium channel blocking properties on cardiac tissues which can play some role in its antiarrhythmic effects. In fact, flecainide prolonged the PR interval of the ECG (Somberg & Tepper, 1986), depressed cardiac contractility both *in vitro* (Schulze & Knops, 1982) and *in vivo* (Serruys *et al.*, 1983; Josephson *et al.*, 1984) and inhibited the slow action potentials elicited by isoprenaline in ventricular muscle fibres partially depolarized with high K (Schulze & Knops, 1982). However, and even when the cardiac and haemodynamic effects of flecainide are well established (Josephson *et al.*, 1984; Holmes & Heel, 1985; Silke *et al.*, 1986), little is known about its direct action on peripheral blood vessels and the calcium antagonistic properties of flecainide. Therefore, the present study was undertaken to examine the effects of flecainide on contractile responses and ^{45}Ca uptake in rat isolated vessels.

Methods

Experimental procedure

Sprague-Dawley rats (either sex, 250–350 g) were killed by a blow on the head. Thoracic aorta, portal vein and central caudal artery were dissected and placed immediately in a modified Krebs-Henseleit solution (KS) of the following composition (mM): NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 1.8, KH_2PO_4 1.2 and glucose 11. After excess of fat and connective tissue were removed, the aortae and central caudal artery were cut into rings about 2–3 mm in length. Only the proximal part (0–2 cm) of the caudal artery was used in these experiments (Bao *et al.*, 1990). Aortic and caudal rings were mounted under a tension of 1 and 0.5 g respectively, by two parallel L-shaped stainless-steel holders (0.8 and 0.25 mm in diameter, respectively) inserted into the lumen. One holder was connected to a Grass FT03 force-displacement transducer while the other served as anchor (Tamargo & Tejerina, 1989; Bao *et al.*, 1990). Vein segments (2 mm in width and 15 mm in length) were mounted vertically under 1 g of tension in organ baths containing 20 ml of KS as previously described (Carrón *et al.*, 1991). The tissue bath was maintained at 34°C and bubbled with 95% O_2:5% CO_2 gas mixture. Contractile responses were recorded isometrically on a Grass polygraph (Tamargo & Tejerina, 1989). The presence of endothelium in the aorta was confirmed by the relaxant effect of acetylcholine 10^{-7} M–10^{-5} M against the contraction evoked by 10^{-7} M phenylephrine. In some arteries the endothelium was mechanically removed by gently rubbing the lumen. Each preparation was allowed to equilibrate for at least 90 min before experiments were started and during this period the incubation medium was changed every 20 min.

After equilibration the following experiments were performed: (1) In some experiments aortic and caudal rings were exposed to single submaximal concentrations of KCl (80 mM) and noradrenaline (NA, 10^{-5} M). An initial 15 min control contraction was obtained in each experiment with the appropriate stimulatory agent and rings were then rested for a minimum of 30 min. Control contractile responses for each agonist were obtained at the beginning of the experiment until two successive responses were almost identical in height. This was followed by exposure to flecainide for a 30 min period before the addition of KCl or NA and during the 25 min re-exposure to stimulatory agents. Only one agonist was used in each experiment. The results of these experiments are expressed as a percentage of the maximal control agonist-induced contractile responses. (2) To determine whether flecainide could relax an existing contraction, aortic rings were contracted by single submaximal concentrations of NA or

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KCl. When the contractile response to either agonist was maximum, flecainide was added in progressively increasing cumulative concentrations (10^{-6}–10^{-4} M) at 30 min intervals. The results were expressed as a percentage of the maximal control agonist-induced responses. (3) In additional experiments dose-response curves to $CaCl_2$ (0.5–5 mM) were obtained in aortic rings incubated in Ca-free KS containing 2 mM EGTA for 2 h and then in Ca-free high-K (80 mM) solution for 5 min before addition of the drugs (Carrón *et al.*, 1991). The results are expressed as a percentage of the maximal contractile response induced by 5 mM $CaCl_2$. (4) To study the effects of flecainide on a membrane store of Ca mobilized by NA, the experimental protocol employed was similar to that previously described (Ebeibge & Aloamaka, 1985; Carrón *et al.*, 1991). Aortic rings incubated in Ca-free KS were stimulated by successive additions of 10^{-5} M NA for 5 min ('Ca-washing'). When the contractile response to NA was suppressed the rings were exposed for 5 min to 2.7 mM Ca KS ('Ca-loading') and then again exposed to Ca-washing KS. The above protocol was repeated in the presence of flecainide, added at the end of the 'Ca-loading' period. Results are expressed as the percentage of the first contraction of the Ca-washing period in the presence of flecainide. Control experiments were performed with the same experimental four protocols in the absence of flecainide.

Rat isolated atria

Right and left atria were removed and mounted vertically under 1 g of tension in 20 ml organ baths containing Tyrode solution of the following composition (mM): NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 11.9, NaH_2PO_4 0.42, and glucose 5.5 bubbled with 95% O_2 and 5% CO_2 at 34°C. Right atria were allowed to beat spontaneously and left atria were electrically driven at a basal rate of 1 Hz through bipolar platinum electrodes with rectangular pulses (1 ms duration, twice threshold strength) delivered from a Grass SD-9 stimulator. Rate and amplitude of contractions were measured isometrically by a force-displacement transducer and recorded on a Grass polygraph (Pérez-Vizcaíno *et al.*, 1991). Resting tension was adjusted to 1 g and a 30 min equilibration period was allowed to elapse before control measurements were made.

Measurements of ^{45}Ca uptake

The ^{45}Ca uptake was estimated by measurement of the changes in specific activity of the calcium fraction resistant to displacement by lanthanum as previously described by Godfraind (1983) and Barrigón & Tamargo (1986). Aortic rings were equilibrated in KS of the following composition (mM): NaCl 122, KCl 5, $NaHCO_3$ 25, $CaCl_2$ 1.25, $MgSO_4$ 1.2 and glucose 11.5. This solution was adjusted to pH 7.4 and gassed with 95% O_2:5% CO_2. After equilibration, experimental rings were treated with flecainide for 30 min. Then both experimental and control rings were incubated for 5 min in KS containing ^{45}Ca (specific activity 1 μCi ml^{-1}; ICN Biomedicals, California) either with or without flecainide and then for another 2 min in the same solution with or without the addition of 80 mM KCl or 10^{-5} M NA. At this time rings were washed for 5 min in 500 ml of a lanthanum solution of the following composition (mM): NaCl 122, KCl 5.9, $MgCl_2$ 1.25, glucose 11, $LaCl_3$ 50 and Tris maleate 15 (pH 6.8) at 0°C. A high concentration of lanthanum has been shown to remove the extracellular ^{45}Ca, while low temperature inhibits transmembrane ^{45}Ca fluxes and thus preserves intracellular ^{45}Ca (Karaki & Weiss, 1979). After this,rings were removed, pressed with a roller and weighed. The rings were then placed in scintillation vials and 0.5 ml of Protosol (Dupont) added; the rings were digested overnight at 60°C. Radioactivity was assayed in a liquid scintillation counter (LKB Wallac 1211 Rack) as previously described (Barrigón & Tamargo, 1986).

Drugs

The following drugs were used: flecainide acetate (Laboratorios Dr. Esteve S.A.) and noradrenaline bitartrate (Sigma Chem. Co., London). Flecainide as a powder was initially dissolved in distilled deionized water and further dilutions were made in KS. Ascorbic acid (10^{-4} M) was added to each solution of NA, made up freshly every day.

Throughout the paper values are expressed as the mean ± s.e.mean and statistical analysis was performed with Student's t test. The differences between control and experimental values were considered significant when $P < 0.05$. Dose-response slopes were analyzed to obtain the concentration of flecainide producing a 50% inhibition of the maximal contractile response (IC_{50}) by linear regression analysis (method of least squares).

Results

Effects on resting tension and myogenic portal activity

At concentrations between 10^{-6} M and 5×10^{-4} M, flecainide had no effect on base-line tension in aortic and caudal rings. In 6 portal vein segments the control amplitude of spontaneous contractions was 465.9 ± 70.2 mg. At 10^{-5} M, flecainide decreased the rate but slightly increased the amplitude of contractions (by 17.9 ± 7.4%, $P > 0.05$). However, at higher concentrations flecainide inhibited in a concentration-dependent manner the amplitude of the contractions resulting in a complete suppression at 2×10^{-4} M ($IC_{50} = 6.5 \pm 0.9 \times 10^{-5}$ M). After a washout period of 40–60 min the effects on the frequency and amplitude of contractions were almost completely reversed.

Effect on contractions induced by KCl and noradrenaline in aorta and tail artery

The inhibitory effect of flecainide on contractile responses induced by KCl (80 mM) and NA (10^{-5} M) in rat aorta are shown in Figure 1a. Both agonists produced submaximal contractile responses of 687.6 ± 91.7 mg and 939.9 ± 191.0, respectively ($n = 8$), these values representing 94.1 ± 1.4% and 96.4 ± 3.4% of the maximal contraction evoked by KCl 100 mM and NA 10^{-4} M, respectively. At concentrations $\geq 10^{-5}$ M, flecainide produced a concentration-dependent inhibition of the contractile responses induced by both stimulatory agents, the IC_{50} being $3.5 \pm 0.8 \times 10^{-5}$ M

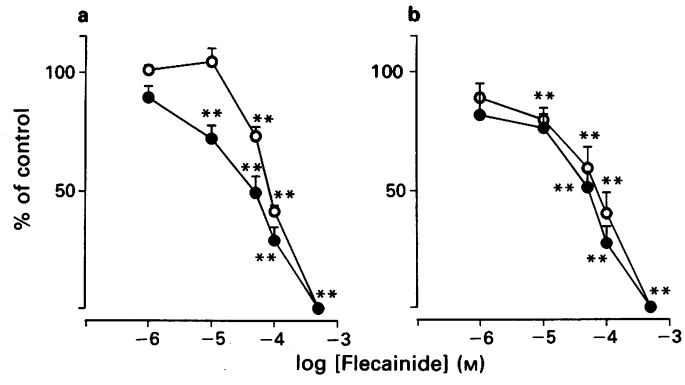


Figure 1 Effect of flecainide, 10^{-6} M–5×10^{-4} M, on the contractile responses induced by high K (●, 80 mM) and noradrenaline (○, 10^{-5} M) in rat aortae (a) and rat tail artery (b). Ordinate scale: relaxation (% of control values). Abscissa scale: flecainide concentration (M). Each point represents the mean ± s.e.mean of 8 experiments with s.e.mean shown by vertical bars. The asterisks denote significant differences (**$P < 0.01$) from control values.

and $8.4 \pm 0.5 \times 10^{-5}$ M, respectively, which indicated that flecainide was significantly more potent in inhibiting the contractile responses induced by high K than those induced by NA ($P < 0.01$). The inhibitory effects of flecainide were completely reversed by washing with drug-free KS. Similar results were obtained in aortic rings after removal of the endothelium.

Cumulative increases in the concentration of flecainide (10^{-6} M–10^{-4} M) in aortic rings previously contracted with 80 mM K and NA 10^{-5} M resulted in a concentration-dependent relaxation, but again flecainide was more potent against the contraction elicited by high K than by NA. Thus, the IC_{50} to relax the KCl-induced contraction was $8.6 \pm 3.1 \times 10^{-6}$ M, whereas at 10^{-4} M flecainide relaxed aortic rings previously contracted with NA by only $39.1 \pm 5.7\%$ of its maximum. The relaxant effects of flecainide were not altered after removal of the endothelium.

The contractile responses elicited by high K (80 mM) and NA 10^{-5} M in the rat tail artery were 379.3 ± 80.0 and 525.6 ± 156.2 mg, respectively ($n = 8$). As shown in Figure 1b, flecainide, 10^{-6} M–10^{-4} M, inhibited in a concentration-dependent manner the contractile responses induced by both agonists. However, and in contrast to aortic rings, flecainide was almost equipotent on the contractions evoked by both agonists, the IC_{50} values being $5.3 \pm 1.4 \times 10^{-5}$ M and $6.5 \pm 1.8 \times 10^{-5}$ M ($P > 0.05$).

Effects on Ca-induced contractions of aortae

The inhibitory effects of flecainide on the Ca-dependent contractile responses are shown in Figure 2. Following incubation for 2 h in Ca-free KS (2 mM EGTA) and then 5 min in high-K solution, the addition of Ca to the bathing media (0.5–5 mM) caused a contraction of the aortic rings in a concentration-dependent manner, the maximum contractile response induced by 5 mM $CaCl_2$ being 1088.3 ± 146.2 mg ($n = 6$). Flecainide, 10^{-5} M, 5×10^{-5} M and 10^{-4} M produced a progressive decrease in the amplitude of Ca-induced contractions of K-depolarized aortic rings and caused a progressive shift of Ca concentration-response curves downwards and to the right. Regression analysis of the inhibition yielded an IC_{50} of $2.5 \pm 0.3 \times 10^{-5}$ M.

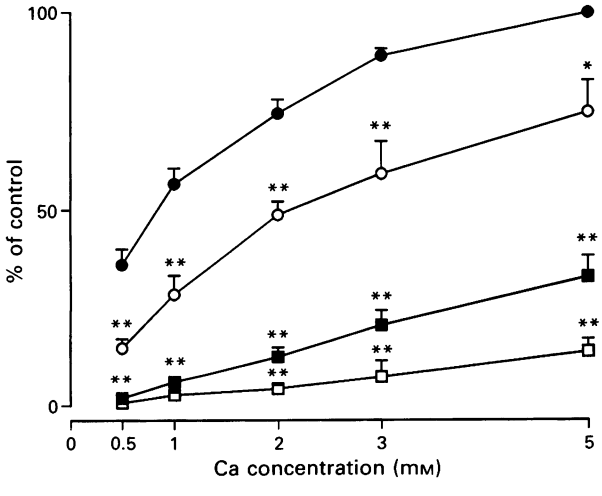


Figure 2 Effects of flecainide on the restoration of the isometric contractions of rat aortic rings by addition of calcium (0.5–5 mM) to Ca-free, high K (80 mM) medium. Ordinate scale: percentage of the maximum control contractions obtained with the highest concentration of Ca in each experiment. Abscissa scale: calcium concentration (mM). Each point represents the mean of 6 experiments with s.e.mean shown by vertical bars. (●) Controls; (○) after flecainide, 10^{-5} M, (■) 5×10^{-5} M and (□) 10^{-4} M. The asterisks denote significant differences between control responses and those after flecainide (* $P < 0.05$, ** $P < 0.01$).

Effects on noradrenaline releasable stores

In another group of experiments the effects of flecainide were studied on the contractile responses induced by NA in rings which were incubated in Ca-free KS. At this time, stimulation with 10^{-5} M NA resulted in a phasic contraction of an amplitude of 249.5 ± 39.2 mg ($n = 8$), presumed to reflect Ca mobilization from a membrane-bound store (Karaki *et al.*, 1979). Flecainide, 10^{-5} M and 10^{-4} M, when applied after 'Ca-loading', inhibited by $26.4 \pm 7.3\%$ ($P < 0.05$) and $56.1 \pm 4.9\%$, ($P < 0.01$), respectively, the magnitude of the NA-induced phasic contraction.

Effects on ^{45}Ca uptake

From the previous experiments two concentrations of flecainide (5×10^{-5} M and 10^{-4} M) were selected to study the effects of the drug on the La-resistant ^{45}Ca uptake in resting, non-stimulated aortic rings, as well as in those stimulated by 80 mM KCl and 10^{-5} M NA (Figure 3). Flecainide, 10^{-4} M, had no significant effects on ^{45}Ca uptake in resting aortic rings (336.9 ± 26.8 vs. 320.9 ± 12.0 nmol g^{-1} aorta, $n = 8$; $P > 0.05$). Addition of high K and NA increased ^{45}Ca uptake to 652.8 ± 67.8 ($n = 7$, $P < 0.01$) and 578.7 ± 37.6 ($n = 6$, $P < 0.001$), respectively. At 10^{-5} M flecainide did not modify the ^{45}Ca influx stimulated by NA (610.6 ± 74.2, $n = 8$, $P > 0.05$), but 10^{-4} M decreased it to 427.8 ± 27.8 ($P < 0.01$). At 5×10^{-5} M and 10^{-4} M, flecainide significantly decreased ^{45}Ca uptake stimulated by KCl to 435.4 ± 49.2 ($n = 7$, $P < 0.05$) and 390.6 ± 27.9 nmol kg^{-1} aorta ($n = 6$, $P < 00.05$). Thus, at 10^{-4} M flecainide inhibited to a similar extent both the contractions and ^{45}Ca uptake stimulated by high K ($71.0 \pm 5.7\%$ vs. $82.8 \pm 1.6\%$, $P > 0.05$) and NA ($58.6 \pm 2.7\%$ vs. $62.2 \pm 1.2\%$, $P > 0.05$). These results seem to indicate that flecainide-induced inhibition of high K- and NA-induced contractions can be related, at least partly, to the blockade of Ca entry.

Effects on right and left atria

In 6 spontaneously beating right atria, rate and amplitude of contractions averaged 212.2 ± 7.4 beats min^{-1} and 329.6 ± 24.4 mg, respectively. Flecainide decreased both parameters in a concentration-dependent manner and at 2.5×10^{-5} M the spontaneous activity was completely suppressed. In 6 left atria driven at 1 Hz the amplitude

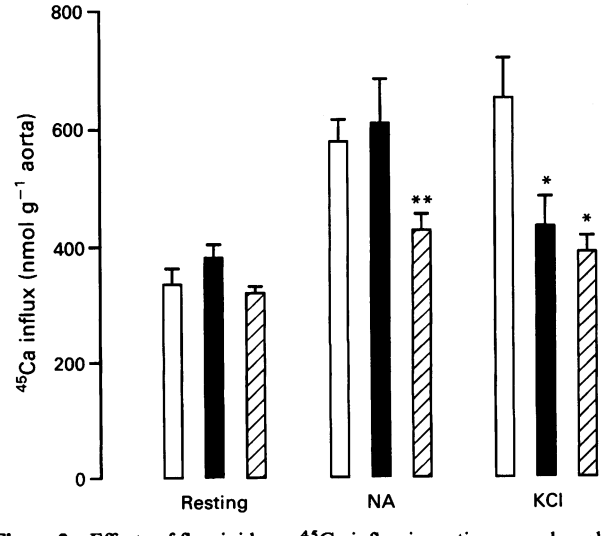


Figure 3 Effects of flecainide on ^{45}Ca influx in resting, noradrenaline (NA)-stimulated and K-stimulated rat isolated aortae. Controls (open columns), flecainide 5×10^{-5} M (solid columns), and 10^{-4} M (hatched columns). Each column represents the mean of 6–8 aortic rings; vertical lines indicate s.e.mean. The asterisks denote significant differences (* $P < 0.05$; ** $P < 0.01$) from control values.

of contractions averaged 345.0 ± 47.9 mg. Flecainide (10^{-7} M – 5×10^{-5} M) decreased the amplitude of contractions, the IC_{50} being $9.3 \pm 0.8 \times 10^{-6}$ M. Thus, the selectivity factor (i.e. the calculated IC_{50} ratio for atrial muscle/aortae) indicated that flecainide was 4.3 times more potent in causing atrial negative inotropic effects than in inhibiting high K-induced contractions in aortic rings.

Discussion

The results of this paper demonstrated that in rat isolated vascular smooth muscle, flecainide, a class Ic antiarrhythmic drug, inhibited: (1) the contractile responses induced by high K or by NA in aortic and caudal arteries; (2) the contractions induced by increases in extracellular Ca in K-depolarized aortic rings as well as those induced in Ca-free medium; (3) ^{45}Ca uptake in aortic rings stimulated by high K and NA, while no effect was observed in resting aortae; and (4) the spontaneous myogenic contractions recorded in portal veins.

KCl-induced contraction in smooth muscle is the result of an increased Ca influx through voltage-stimulated type-L Ca channels, (VSCs; Bolton, 1979), and is competitively and specifically inhibited by Ca antagonists (Weiss, 1981). Flecainide inhibited in a concentration-dependent manner the contractile responses induced by high K or by Ca in K-depolarized aorta, which have been used to provide a simple means of studying drugs with possible Ca entry blocking properties (Godfraind *et al.*, 1986). Flecainide also inhibited the spontaneous myogenic activity in rat portal veins. Since the myogenic activity is triggered by bursts of propagated action potentials which activated VSCs (Johansson *et al.*, 1967), the inhibitory effect of flecainide may also be explained through an inhibition of Ca entry through VSCs. In agreement with this hypothesis is the finding that flecainide inhibited the ^{45}Ca uptake stimulated by high K. Moreover, the percentage inhibitions of high K-induced contractions and stimulated ^{45}Ca uptake were almost superimposed, thus indicating that the inhibitory effects of flecainide on contractile responses induced by high K or Ca in K-depolarized tissues were due to the inhibition of Ca entry through VSCs.

In rat isolated aorta the tonic component of Na-induced contractions has been attributed to an increase in Ca entry through receptor-operated channels, (ROCs; Deth & Van Breemen, 1974; Bolton, 1979). The phasic component, since it persists in Ca-free medium, has been attributed to the release of Ca from intracellular stores (Hudgins & Weiss, 1968; Godfraind & Kaba, 1969). Flecainide inhibited both the tonic and phasic components of NA-induced contractions which suggests decreased Ca entry through ROCs and receptor-mediated Ca release from intracellular store sites, respectively. In fact, flecainide inhibited the ^{45}Ca uptake stimulated by NA, which may suggest a blockade of ROCs. However, at the high concentrations used in this study, NA might also depolarize the membrane potential of aortic smooth muscle fibres (Nelson *et al.*, 1988) and thus, the inhibitory effects of flecainide on NA-induced contractions and ^{45}Ca uptake could also be attributed to inhibition of Ca entry through VSCs. Furthermore, flecainide when applied after 'Ca-loading' inhibited NA-induced transient contractions elicited in Ca-free medium. Since these transient contractions have been attributed to mobilization of Ca from a membrane-bound store (Karaki *et al.*, 1979; Ebeigbe & Alaomaka, 1985), this result suggested that the inhibitory effect of flecainide on NA-induced contractions can be at least partly related to inhibition of receptor-mediated Ca-release from an intracellular store. Inhibition of Na-induced phasic response could be due also to stimulation of Ca efflux, but this possibility was not analyzed in the present experiments.

Some experimental and clinical data have shown that flecainide depressed cardiac contractility (Schulze & Knops, 1982; Josephson *et al.*, 1984; Serruys *et al.*, 1985), prolonged the PR interval of the ECG (Somberg & Tepper, 1986) and inhibited the slow action potentials elicited by isoprenaline in K-depolarized ventricular muscle fibres (Schulze & Knops, 1982). All these results suggest that flecainide may exert certain calcium antagonistic properties on cardiac tissues. The present experiments suggest that in vascular smooth muscle fibres, flecainide not only inhibited Ca entry through Ca channels but also the release of Ca from intracellular stores. This latter effect may explain why, in contrast to the specific calcium antagonists that are 2–4 orders of magnitude more potent in inhibiting K- than NA-induced contractions and ^{45}Ca uptake in rat aorta (Cauvin *et al.*, 1983; Godfraind *et al.*, 1986; Tamargo & Tejerina, 1989), flecainide was almost equipotent in inhibiting the contractions induced by both agonists.

The clinical relevance of the present *in vitro* results is unknown. The therapeutic range for plasma levels of flecainide (200–1000 ng ml^{-1}: 4×10^{-7} M–2×10^{-6} M; Holmes & Heel, 1985) are lower than those needed to inhibit vascular smooth muscle contractions in this study. Moreover, the vasorelaxant effect of flecainide appears at concentrations higher than those inhibiting the rate or amplitude of atrial contractions. This may explain the low incidence of hypotension at therapeutic antiarrhythmic concentrations. Another explanation for the low incidence of hypotension may be related to the negative inotropic effects of flecainide which may produce a reflex increase in systemic vascular resistances, thus overriding the expected direct vasodilator and cardiodepressant effects of the drug (Josephson *et al.*, 1984; Silke *et al.*, 1986).

In conclusion, the results of this paper demonstrated that in rat isolated aortae and portal veins, flecainide produced a vasodilator effect which may be attributed to the inhibition of Ca entry through voltage-stimulated channels and NA-induced Ca uptake as well as Ca release from intracellular stores. As a consequence it would reduce the concentration of intracellular free Ca available for vascular smooth muscle contraction.

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Effects of N^G-nitro-L-arginine methyl ester on vasodilator responses to adrenaline or BRL 38227 in conscious rats

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1 Conscious, Long Evans rats, chronically instrumented for the measurement of regional haemodynamics, were used to assess responses to 3 min infusions of the potassium channel opener, BRL 38227 (1 and 10 $\mu g\,kg^{-1}\,min^{-1}$) or adrenaline (0.05 and 0.5 $\mu g\,kg^{-1}\,min^{-1}$) in the absence and in the presence of N^G-nitro-L-arginine methyl ester (L-NAME; 3 $mg\,kg^{-1}\,h^{-1}$), an inhibitor of nitric oxide biosynthesis.

2 In the absence of L-NAME, the low dose of BRL 38227 caused slight hypotension and tachycardia, accompanied by small increases in mesenteric and hindquarters blood flow only. However, there were increases in renal, mesenteric and hindquarters vascular conductances. L-NAME had no effect on any of these responses.

3 The high dose of BRL 38227 caused substantial hypotension and tachycardia. Renal and hindquarters flows did not change significantly, but there was a marked increase in mesenteric flow. There were only modest increases in renal and hindquarters vascular conductances but a substantial mesenteric vasodilatation. In the presence of L-NAME, there was a slight reduction of the latter but no other changes in the responses to BRL 38227.

4 In the absence of L-NAME, the low dose of adrenaline caused slight hypotension but a marked tachycardia. There were no changes in renal or mesenteric blood flow but a clear-cut increase in hindquarters flow. Renal and mesenteric vascular conductances showed only small rises, in contrast to the substantial hindquarters vasodilatation. In the presence of L-NAME, there was significant attenuation of the tachycardia and of the increases in hindquarters flow and vascular conductance in response to adrenaline.

5 The high dose of adrenaline caused marked hypotension and tachycardia. Renal flow did not change, but there was a fall in mesenteric and a marked rise in hindquarters flow. Renal vascular conductance showed a slight increase but mesenteric vascular conductance did not change significantly, whereas there was a substantial hindquarters vasodilatation. In the presence of L-NAME, adrenaline caused an increase in blood pressure but no significant change in heart rate; the renal vasodilatation was abolished, there was a mesenteric vasoconstriction, and the hindquarters vasodilatation was markedly reduced. L-NAME also attenuated the tachycardia induced by adrenaline in animals with no cardiac baroreflexes.

6 The present results indicate that L-NAME-sensitive mechanisms are involved in the vasodilator and tachycardic effects of adrenaline. The relative lack of effect of L-NAME on responses to BRL 38227 indicates that the changes in the responses to adrenaline were not non-specific or due to changes in haemodynamic status caused by L-NAME. The results raise the possibility that the 'hypertensinogenic' properties of endogenous adrenaline could be amplified when nitric oxide biosynthesis is impaired.

Keywords: Adrenaline; vasodilatation; nitric oxide; conscious rats; N^G-nitro-L-arginine methylester (L-NAME); BRL 38227

Introduction

Recently, we (Gardiner *et al.*, 1991) found that the hindquarters vasodilator response to salbutamol in conscious rats was attenuated in the presence of the nitric oxide synthesis inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) (Moore *et al.*, 1990; Rees *et al.*, 1990), consistent with a component of β_2-adrenoceptor-mediated vasodilatation being dependent upon endothelium-derived nitric oxide. These observations raise the possibility that the β-adrenoceptor-mediated effects of the endogenous agonist, adrenaline, might be transduced, in part, by nitric oxide. If that were the case, then it could have important implications for the putative involvement of adrenaline in the aetiology of hypertension (Majewski & Rand, 1986) under circumstances in which biosynthesis of nitric oxide was impaired, since in that situation the pressor effects of adrenaline might be enhanced. Therefore, in the present study we investigated the haemodynamic effects of adrenaline in the absence and in the presence of L-NAME.

Since L-NAME causes substantial changes in haemodynamic status in conscious rats (Gardiner *et al.*, 1990a,b) it could be argued that these would influence the responses to adrenaline, and hence, an appropriate control experiment should be included. Simplistically, and considering we started from the proposition that adrenaline might be exerting endothelium-dependent vasodilator effects, an appropriate control could be a classical, endothelium-independent vasodilator, such as an organic nitrate. However, it is now clear that in the absence of the endothelium, or in the presence of inhibitors of nitric oxide there is a real sensitization to the effects of nitrovasodilators both *in vitro* and *in vivo* (Shirasaki & Su, 1985; Lüscher *et al.*, 1989; Busse *et al.*, 1989; Flavahan & Vanhoutte 1989; Forster *et al.*, 1990; Moncada *et al.*, 1991; Gardiner *et al.*, 1991). Thus, responses to these agents cannot be used as internal standards in such experiments. This leaves one with the questions, are any vasodilators endothelium-independent, and *in vivo* how does one judge their effects to be endothelium-dependent? As a first step, if any vasodilator agent could be found the *in vivo* effects of which were unchanged in the presence of L-NAME it would be helpful, although one could still argue about the interpretation of such findings. Nonetheless, since in pilot experiments we observed that the haemodynamic responses to the potassium channel opener, BRL 38227 ((−)-6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-1-pyrrolidyl)-2H-1-benzopyran-3-ol) (the active (−)-enantiomer of cromakalim) (Clapham *et al.*, 1991) were

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largely unaffected by L-NAME, we assessed the regional haemodynamic responses to BRL 38227 in the same animals that were challenged with adrenaline, in the absence and in the presence of L-NAME.

Methods

Male, Long Evans rats (350–450 g) were anaesthetized (sodium methohexitone, 60 mg kg^{-1} i.p., supplemented as required) and had pulsed Doppler flow probes implanted to monitor changes in renal, mesenteric and hindquarters blood flows (Gardiner *et al.*, 1990a,b). At least 7–14 days later, animals were briefly re-anaesthetized (sodium methohexitone, 40 mg kg^{-1} i.p.) and had intravascular catheters implanted (Gardiner *et al.*, 1990a,b). After a further 24 h recovery, experiments were begun in conscious, unrestrained animals with free access to food and water.

Continuous recordings were made of mean and phasic intra-arterial blood pressures and instantaneous heart rate, and mean and phasic renal, mesenteric and hindquarters Doppler shift signals with a modified (Gardiner *et al.*, 1990c) pulsed Doppler system (Crystal Biotech, Holliston, U.S.A.). Measurements made were the averages over the 20 s periods around the time points given in the results, except for the 0 time point, where the average was over the preceding 20 s. Changes in regional vascular conductance were calculated from mean Doppler shift and mean arterial blood pressure (Gardiner *et al.*, 1990a,b).

Animals ($n = 8$) were randomized to receive 2 doses of adrenaline (0.05 and 0.5 μg kg^{-1} min^{-1}) and 2 doses of BRL 38227 (1 and 10 μg kg^{-1} min^{-1}), given as 3 min infusions at 0.15 ml min^{-1} i.v. At least 2 h later these procedures were repeated, but starting 60 min after the onset of infusion of L-NAME (3 mg kg^{-1} h^{-1} at 0.3 ml h^{-1}, i.v.). The responses to repeated adrenaline infusions or BRL 38227 in the absence of L-NAME were consistent.

The results of the first experiment indicated that L-NAME could have been acting to attenuate the tachycardic effects of adrenaline (see Results). However, under the conditions of those experiments baroreflexes were intact and hence changes in systemic arterial blood pressure in response to adrenaline could have influenced heart rate indirectly. Therefore, in a separate group of Long Evans rats ($n = 5$), responses to a 3 min infusion of adrenaline (0.5 μg kg^{-1} min^{-1}) were assessed in the presence of pentolinium (5 mg kg^{-1} bolus, 5 mg kg^{-1} h^{-1} infusion), captopril (2 mg kg^{-1} bolus, 1 mg kg^{-1} h^{-1} infusion) and a vasopressin V_1-receptor antagonist, (d(CH$_2$)$_5$Tyr(Et)DAVP, 10 μg kg^{-1} bolus, 10 μg kg^{-1} h^{-1} infusion). The doses of these drugs were sufficient to abolish all reflex circulatory control (Gardiner & Bennett, 1985; Gardiner *et al.*, 1989). The responses to adrenaline were then re-assessed in the same animals 60 min after the onset of L-NAME infusion together with the other drugs. When adrenaline infusions were given 60 min apart in the presence of pentolinium, captopril and d(CH$_2$)$_5$Tyr(Et)DAVP only, there was no diminution of the tachycardia or enhancement of the pressor response (see Results).

Drugs

Adrenaline bitartrate (Sigma), BRL 38227 (SmithKline Beecham), L-NAME (Sigma), pentolinium (Sigma) and captopril (Squibb) were dissolved in isotonic saline. Captopril was buffered to pH 7.3 with NaOH. The vasopressin antagonist, d(CH$_2$)$_5$Tyr(Et)DAVP (from Professor M. Manning, Toledo, Ohio, U.S.A.) was dissolved in 0.5% glacial acetic acid before being diluted with isotonic saline. The adrenaline solution contained 17.6 μg ml^{-1} ascorbic acid. Infusion of vehicle had no consistent haemodynamic effects.

Data analysis

Changes relative to baseline were analysed by Friedman's test, whereas comparisons between changes in regional vascular conductances in the absence and in the presence of L-NAME were assessed from areas under or over curves (AUC or AOC, respectively) (Gardiner *et al.*, 1990a,b; 1991) by Wilcoxon's ranks sums test. A P value < 0.05 was taken as significant.

Results

Resting values in the absence and in the presence of L-NAME are given in Table 1.

Responses to adrenaline in intact rats

The low dose infusion of adrenaline (0.05 μg kg^{-1} min^{-1}) alone caused a slight hypotension (AOC, 13 ± 4 units), and tachycardia (AUC, 78 ± 19 units), with no significant change in renal or mesenteric flow but an increase in hindquarters flow (AUC, 4.0 ± 0.8 units) (Figure 1). There were increases in renal, mesenteric and hindquarters vascular conductances (AUC, 15 ± 5, 7 ± 2 and 41 ± 9 units, respectively) (Figure 1).

In the presence of L-NAME there was a significant rise in mean arterial blood pressure accompanied by a bradycardia and a reduction in mesenteric flow. Renal, mesenteric and hindquarters vascular conductances were reduced (Table 1, Figure 1).

The low dose infusion of adrenaline in the presence of L-NAME had no significant hypotensive effect (AOC, 12 ± 5), but this was not significantly different from the response in the absence of L-NAME (Figure 1). However, in the presence of L-NAME the tachycardia induced by adrenaline (AUC, 46 ± 17 units) was significantly attenuated (Figure 1), as was the increase in hindquarters flow (AUC, 1.6 ± 0.4 units). Although there were no significant changes in renal or mesenteric conductances (AUC, 8 ± 3 and 4 ± 1 units, respectively) in response to adrenaline in the presence of L-NAME (Figure 1), these effects were not significantly different from the responses in the absence of L-NAME. However, there was a significant attenuation of the rise in hindquarters vascular conductance in response to adrenaline (AUC, 13 ± 4 units) in the presence of L-NAME (Figure 1).

In the absence of L-NAME, infusion of the high dose of adrenaline (0.5 μg kg^{-1} min^{-1}) caused hypotension (AOC, 35 ± 4 units) and a marked tachycardia (AUC, 181 ± 20 units) (Figure 1). There was no significant change in renal flow, but a fall in mesenteric flow (AOC, 4.3 ± 1.0 units), and a substantial increase in hindquarters flow (AUC, 13.1 ± 2.0 units) (Figures 1 and 2). There were increases in renal and hindquarters vascular conductances (AUC, 31 ± 7 and

Table 1 Resting cardiovascular variables in the absence and in the presence of N^G-nitro-L-arginine methyl ester (+L-NAME, infused at 3 mg kg^{-1} h^{-1} for 60 min) in the same conscious, Long Evans rats ($n = 8$)

Heart rate (beats min^{-1})		342 ± 11
	+ L-NAME	292 ± 9†
Mean arterial blood pressure (mmHg)		110 ± 3
	+ L-NAME	137 ± 7†
Renal flow (kHz)		11.2 ± 1.9
	+ L-NAME	11.0 ± 2.2
Mesenteric flow (kHz)		7.0 ± 0.6
	+ L-NAME	5.0 ± 0.5†
Hindquarters flow (kHz)		4.5 ± 0.4
	+ L-NAME	3.6 ± 0.4
Renal conductance ([kHz mmHg^{-1}]10^3)		102 ± 17
	+ L-NAME	80 ± 16†
Mesenteric conductance ([kHz mmHg^{-1}]10^3)		64 ± 6
	+ L-NAME	38 ± 5†
Hindquarters conductance ([kHz mmHg^{-1}]10^3)		41 ± 4
	+ L-NAME	27 ± 4†

Values are mean ± s.e.mean.
† $P < 0.05$ versus resting value in the absence of L-NAME.

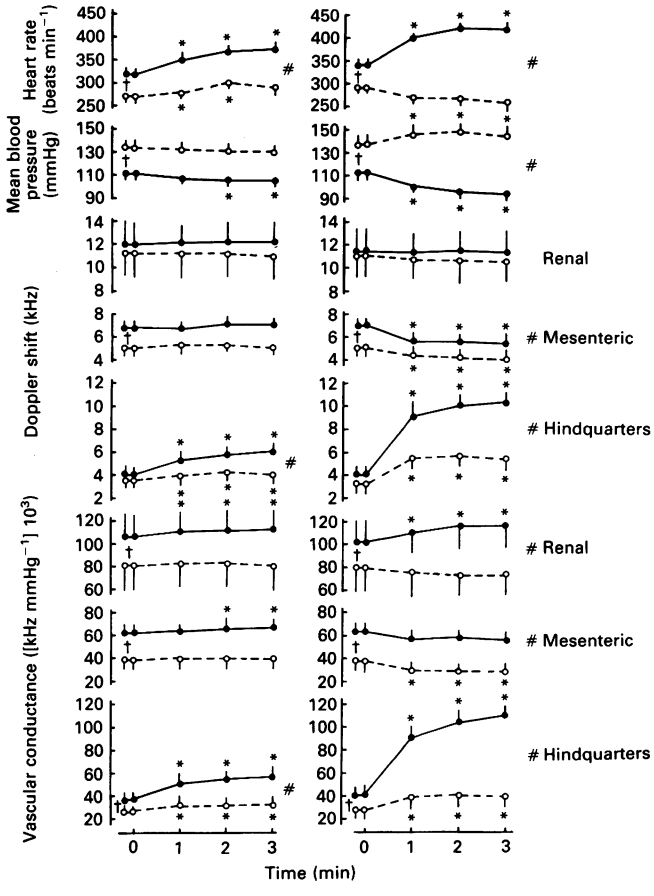


Figure 1 Cardiovascular responses to 3 min infusions of adrenaline at $0.05\,\mu g\,kg^{-1}\,min^{-1}$ (left-hand panels) or $0.5\,\mu g\,kg^{-1}\,min^{-1}$ (right-hand panels) before (●) or 60 min after (○) the onset of infusion of N^G-nitro-L-arginine methyl ester (L-NAME) ($3\,mg\,kg^{-1}\,h^{-1}$) in the same conscious, Long Evans rats ($n = 8$). Values are mean with s.e.mean shown by vertical lines; * $P < 0.05$ versus baseline; † $P < 0.05$ for resting values in the absence and presence of L-NAME; # $P < 0.05$ for areas under or over curves in the absence and presence of L-NAME.

148 ± 19 units, respectively), but no significant change in mesenteric vascular conductance (Figure 1).

In the presence of L-NAME, the high dose infusion of adrenaline had a pressor effect (AUC, 25 ± 9 units), and there was a tendency towards a bradycardia (AOC, 73 ± 32 units) but this did not reach significance (Figures 1 and 2). However, both the change in mean arterial blood pressure and in heart rate were significantly different from those in response to adrenaline in the absence of L-NAME (Figure 1). Renal blood flow did not change but there was a reduction in mesenteric blood flow (AOC, 2.4 ± 0.5 units) and an increase in hindquarters flow (AUC, 4.8 ± 1.1 units), both of which were significantly less than occurred in response to adrenaline in the absence of L-NAME (Figures 1 and 2). Although there was no significant fall in renal vascular conductance (AOC, 26 ± 12 units) in response to adrenaline in the presence of L-NAME, there was a significant difference from the rise seen in the absence of L-NAME (Figure 1). Furthermore, there was a significant mesenteric vasoconstriction (AOC, 24 ± 6 units) to adrenaline in the presence of L-NAME that did not occur in its absence (Figure 1). In addition, L-NAME caused a significant attenuation of the hindquarters vasodilator response to adrenaline (AUC, 32 ± 10 units) (Figure 1).

Responses to adrenaline in areflexic rats

In the presence of pentolinium, captopril and the vasopressin V_1-receptor antagonist there was marked hypotension and a bradycardia in association with substantial renal, mesenteric and hindquarters vasodilatations (Table 2, compare with Table 1; Figure 3). Under these conditions, adrenaline ($0.5\,\mu g\,kg^{-1}\,min^{-1}$) had a modest pressor (AUC, 38 ± 2 units) but a pronounced tachycardic (AUC, 155 ± 30 units) effect (Figure 3, Table 2). There was a mesenteric vasoconstriction (AOC, 118 ± 22 units), but no significant change in renal vascular conductance (Table 2). There was a small hindquarters vasodilatation (AUC, 27 ± 8 units) (Table 2).

When L-NAME was infused together with pentolinium, captopril and the vasopressin V_1-receptor antagonist, there was a rise in mean arterial blood pressure in association with substantial renal, mesenteric and hindquarters vasoconstrictions; however, there was no significant change in heart rate (Figure 3, Table 2). It is notable that in the presence of pentolinium, captopril, the vasopressin antagonist and L-NAME cardiovascular variables (with the exception of heart rate and hindquarters vascular conductance) were not different from those in intact animals prior to infusion of L-NAME (compare Tables 1 and 2).

In the areflexic animals in the presence of L-NAME, infusion of adrenaline had an enhanced pressor effect (AUC, 106 ± 24 units) and there was a clear increase in pulse pressure that was not seen in the absence of L-NAME (Figure 3). The renal vasoconstrictor effect of adrenaline (AOC, 84 ± 28 units) was increased compared to the response in the absence of L-NAME, and there was a hindquarters vasoconstriction (AOC, 44 ± 16 units) which differed significantly from the hindquarters vasodilatation seen in the absence of L-NAME (Table 2). However, the tachycardic (AUC, 62 ± 35 units) and mesenteric vasoconstrictor (AOC, 47 ± 24) responses to adrenaline were attenuated in the presence of L-NAME (Figure 3, Table 2).

Table 2 Resting cardiovascular variables and changes (Δ) in response to 3 min infusion of adrenaline in the same conscious Long Evans rats ($n = 5$) in the presence of pentolinium, captopril and a vasopressin, V_1-receptor antagonist, (PCA), or in the presence of PCA and N^G-nitro-L-arginine methyl ester (PCA + L-NAME)

			Adrenaline ($0.5\,\mu g\,kg^{-1}\,min^{-1}$)		
		Resting	Δ *1 min*	Δ *2 min*	Δ *3 min*
Heart rate (beats min^{-1})	PCA	255 ± 7	42 ± 17*	72 ± 11*	81 ± 9*
	PCA + L-NAME	245 ± 17	13 ± 12	31 ± 15*	34 ± 18*#
Mean arterial blood pressure (mmHg)	PCA	44 ± 3	9 ± 3*	19 ± 1*	20 ± 1*
	PCA + L-NAME	106 ± 15†	25 ± 10*	52 ± 10*	58 ± 11*#
Renal conductance ([kHz $mmHg^{-1}$]10^3)	PCA	132 ± 20	−4 ± 3	−7 ± 5	−2 ± 6
	PCA + L-NAME	75 ± 21†	−21 ± 9*	−35 ± 11*	−36 ± 11*#
Mesenteric conductance ([kHz $mmHg^{-1}$]10^3)	PCA	132 ± 8	−39 ± 6*	−42 ± 9*	−35 ± 10*
	PCA + L-NAME	35 ± 10†	−13 ± 8	−18 ± 8*	−18 ± 9*#
Hindquarters conductance ([kHz $mmHg^{-1}$]10^3)	PCA	107 ± 30	14 ± 6	6 ± 3	11 ± 2*
	PCA + L-NAME	62 ± 18†	−4 ± 4	−21 ± 8*	−26 ± 9*#

Values are mean ± s.e.mean.
* $P < 0.05$ versus resting value; † $P < 0.05$ PCA + L-NAME versus PCA (resting values); # $P < 0.05$ PCA + L-NAME versus PCA (areas under or over curves)

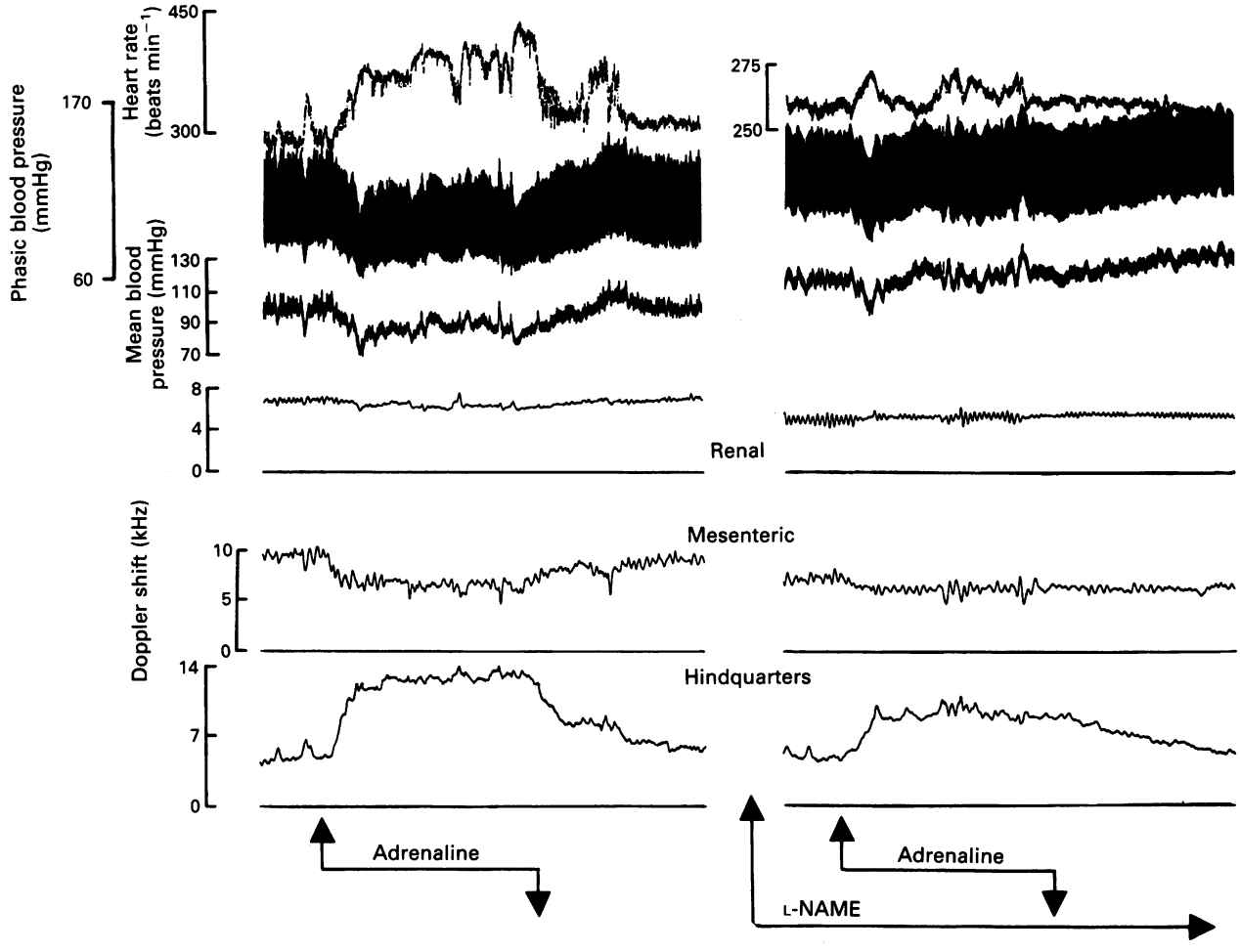


Figure 2 Cardiovascular responses to 3 min infusions of adrenaline (0.5 $\mu g\,kg^{-1}\,min^{-1}$) in the same conscious, Long Evans rat in the absence (left-hand panel) or 60 min after the onset of N^G-nitro-L-arginine methyl ester infusion (3 $mg\,kg^{-1}\,h^{-1}$) (right-hand panel). In this animal there was clear inhibition of the depressor, tachycardic and hindquarters vasodilator responses to adrenaline in the presence of N^G-nitro-L-arginine methyl ester (L-NAME).

Responses to BRL 38227 in intact rats

Infusion of the low dose of BRL 38227 (1.0 $\mu g\,kg^{-1}\,min^{-1}$) caused a slight hypotension (AOC, 12 ± 3 units), and tachycardia (AUC, 32 ± 10 units) (Table 3). There was no change in renal blood flow, but increases in mesenteric flow (AUC, 1.5 ± 0.3 units) and, to a lesser extent, hindquarters flow (AUC, 0.7 ± 0.1 units) (Figure 4). There were modest increases in renal and hindquarters vascular conductances (9 ± 2 and 10 ± 2 units, respectively), and a greater increase in mesenteric vascular conductance (18 ± 3 units) (Figure 4).

In the presence of L-NAME the effects of the low dose infusion of BRL 38227 were not different from those in the absence of L-NAME (tachycardia AUC, 30 ± 9 units; hypotension AOC, 16 ± 4 units; mesenteric flow AUC, 1.2 ± 0.2 units; hindquarters flow AUC, 0.5 ± 0.2 units; renal conductance 9 ± 3 units; mesenteric conductance AUC, 12 ± 2 units; hindquarters conductance AUC, 5 ± 2 units) (Figure 4, Table 3).

In the absence of L-NAME, the high dose infusion of BRL 38227 caused marked hypotension (AOC, 44 ± 4 units), and tachycardia (AUC, 208 ± 18 units) (Table 3), and an increase in mesenteric blood flow (AUC, 6.4 ± 0.6 units). Although there was a tendency for renal flow to fall (AOC, 2.3 ± 0.8 units) and hindquarter flow to rise (AUC, 0.7 ± 0.1 units) neither of these changes reached significance (Figure 4). However, there were increases in renal, mesenteric and hindquarters vascular conductances (AUC, 30 ± 6, 98 ± 9 and 26 ± 3 units, respectively) (Figure 4).

In the presence and in the absence of L-NAME, the effects of the high dose infusion of BRL 38227 were not significantly different for heart rate (AUC, 214 ± 31 units) (Table 3), mean

Table 3 Resting heart rates and mean arterial blood pressures and changes (Δ) in response to 3 min infusions of BRL 38227 in the same conscious Long Evans rats (n = 8) in the absence (control) and presence of N^G-nitro-L-arginine methyl ester (+ L-NAME)

			BRL 38227 infusion (1 $\mu g\,kg^{-1}\,min^{-1}$)				*BRL 38227 infusion* (10 $\mu g\,kg^{-1}\,min^{-1}$)		
		Resting	*Δ1 min*	*Δ2 min*	*Δ3 min*	*Resting*	*Δ1 min*	*Δ2 min*	*Δ3 min*
Heart rate (beats min^{-1})	Control	326 ± 8	8 ± 2*	14 ± 5*	22 ± 8*	338 ± 9	48 ± 6*	96 ± 9*	130 ± 10*
	+ L-NAME	270 ± 11†	2 ± 5	13 ± 5*	20 ± 5*	284 ± 17†	48 ± 11*	98 ± 14*	139 ± 16*
Mean arterial blood pressure (mmHg)	Control	114 ± 3	−2 ± 1*	−5 ± 2*	−6 ± 2*	110 ± 3	−7 ± 1*	−19 ± 2*	−28 ± 2*
	+ L-NAME	142 ± 6†	−2 ± 1*	−6 ± 2*	−10 ± 3*	138 ± 3†	−17 ± 3*	−25 ± 3*	−34 ± 3*

Values are mean ± s.e.mean.
* $P < 0.05$ versus resting value; † $P < 0.05$ + L-NAME versus control.

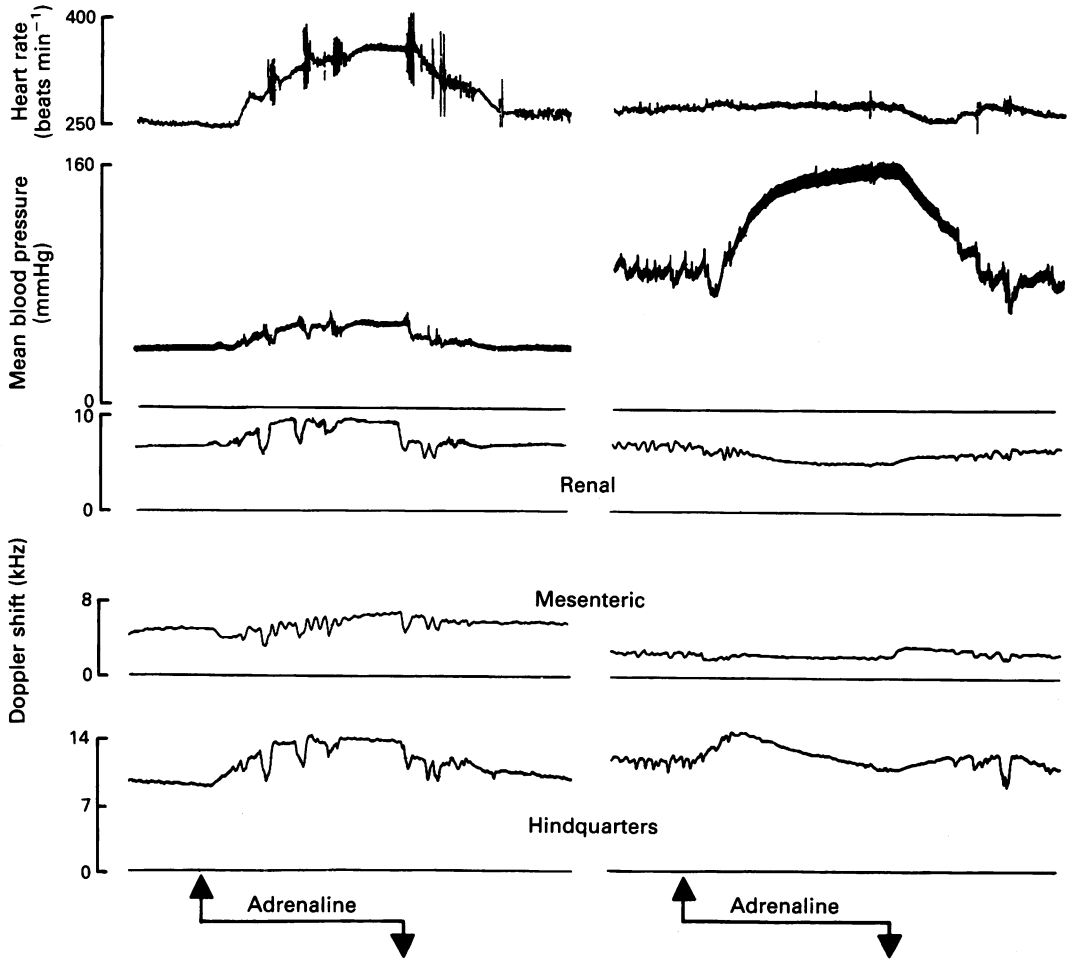


Figure 3 Cardiovascular responses to 3 min infusions of adrenaline ($0.5\,\mu g\,kg^{-1}\,min^{-1}$) in the same conscious, Long Evans rat in the absence (left-hand panel) or 60 min after the onset of N^G-nitro-L-arginine methyl ester (L-NAME) infusion ($3\,mg\,kg^{-1}\,h^{-1}$) (right-hand panel). Pentolinium ($5\,mg\,kg^{-1}$ bolus, $5\,mg\,kg^{-1}\,h^{-1}$ infusion), captopril ($2\,mg\,kg^{-1}$ bolus, $1\,mg\,kg^{-1}\,h^{-1}$ infusion) and a vasopressin V_1-receptor antagonist ($10\,\mu g\,kg^{-1}$ bolus, $10\,\mu g\,kg^{-1}\,h^{-1}$ infusion) were present throughout the experiment to render the animals areflexic and devoid of neurohumoral influences on cardiovascular status. In this animal, the tachycardic effects of adrenaline were completely inhibited in the presence of L-NAME while there was a marked enhancement of the increase in pulse pressure under those conditions. (The mean arterial blood pressure signal was obtained by electronic filtering of the phasic signal, hence the thickness of the trace is directly related to pulse pressure.)

arterial blood pressure (AOC, 69 ± 9 units), renal flow (AOC, 1.2 ± 0.7 units) (Table 3) mesenteric flow (AUC, 7.0 ± 1.0 units), hindquarters flow (AUC, 0.5 ± 0.2 units), renal conductance (AUC, 35 ± 6 units), and hindquarters conductance (AUC, 16 ± 3 units). However, in the presence of L-NAME the rise in mesenteric vascular conductance in response to BRL 38227 (AUC, 79 ± 11 units) was slightly, but significantly, less than in its absence (Figure 4).

Discussion

Responses to adrenaline

This work was prompted by the observation that the hindquarters vasodilator response to salbutamol was attenuated in the presence of L-NAME (Gardiner *et al.*, 1991), indicating that a component of β-adrenoceptor-mediated vasodilatation might be transduced by nitric oxide (see Gray & Marshall, 1991). However, even in the presence of L-NAME, salbutamol still exerted hypotensive effects, consistent with its relatively selective β_2-adrenoceptor agonist effects. An important question then is, can one convert the hypotensive and vasodilator effects of the less selective endogenous agonist, adrenaline, into pressor and vasoconstrictor actions by pretreatment with L-NAME? The present results show that this can be achieved readily. Since the haemodynamic responses to BRL 38227 were little affected in the presence of L-NAME, it is clear that the change in cardiovascular status caused by L-NAME cannot explain the dramatic changes seen in the responses to adrenaline under those conditions.

The low dose of adrenaline used in the present experiments caused tachycardia and increases in hindquarters flow and vascular conductance, unaccompanied by any other significant changes. Both the tachycardia and the hindquarters haemodynamic effects of adrenaline were attenuated in the presence of L-NAME. Even more striking was the finding that the depressor effect of the high dose of adrenaline was converted into a clear pressor action in the presence of L-NAME and the tachycardic response was lost, along with the renal vasodilatation. Moreover, there was substantial attenuation of the hindquarters vasodilatation and an unmasking of a mesenteric vasoconstrictor response to adrenaline in the presence of L-NAME. These results are consistent with adrenaline, under normal conditions, causing marked β-mediated hindquarters vasodilatation with slight vasodilator effects in the renal vascular bed, and covert β-mediated effects in the mesenteric vascular bed. The lack of change of mesenteric vascular conductance following adrenaline alone is consistent with a predominance of α-adrenoceptor-mediated, and neurohumoral, vasoconstrictor mechanisms in this vascular bed (Gardiner & Bennett, 1988; Gardiner *et al.*, 1989). Inhibition of the β-adrenoceptor-mediated vasodilator influences of adrenaline in the presence of L-NAME would thus be

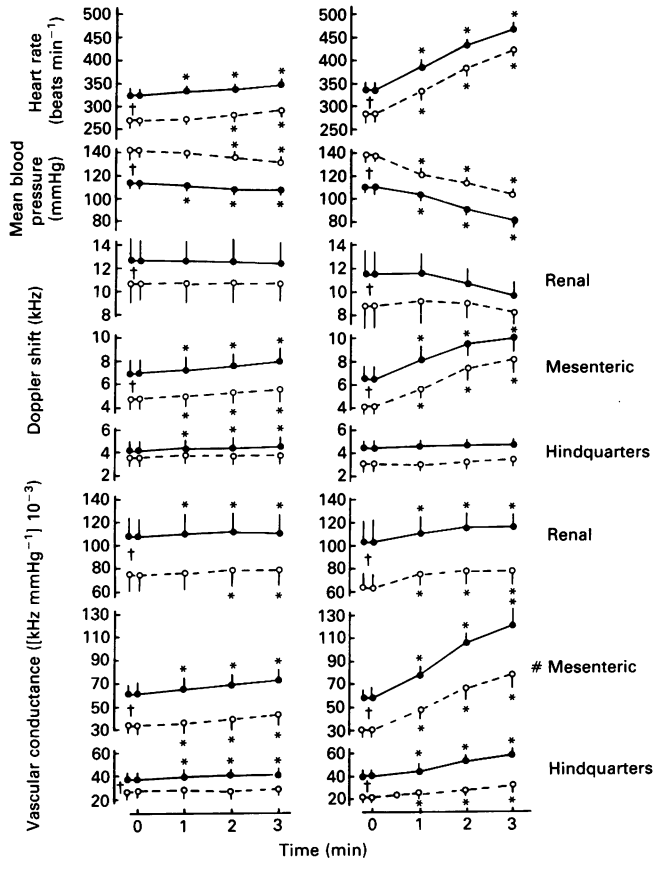


Figure 4 Cardiovascular responses to 3 min infusions of BRL 38227 at 1 $\mu g kg^{-1} min^{-1}$ (left-hand panels) or 10 $\mu g kg^{-1} min^{-1}$ (right-hand panels) before (●) or 60 min after (○) the onset of infusion of N^G-nitro-L-arginine methyl ester (L-NAME) (3 $mg kg^{-1} h^{-1}$) in the same conscious, Long Evans rats ($n = 8$). Values are mean with s.e.mean shown by vertical lines; * $P < 0.05$ versus baseline; † $P < 0.05$ for resting values in the absence and presence of L-NAME; # $P < 0.05$ for areas under curves in the absence and presence of L-NAME.

expected to remove their buffering effect and reveal a pressor action of adrenaline due to an α-adrenoceptor-mediated vasoconstrictor influence. However, the latter action, alone, probably did not account for the ability of adrenaline to raise mean arterial blood pressure in the presence of L-NAME because, although there was a mesenteric vasoconstriction this was only slight, and none of the regional blood flows measured showed greater reductions than in the absence of L-NAME. Hence, it is likely that the cardiac effects of adrenaline played a role in its pressor action in the presence of L-NAME.

It was notable that the tachycardic response to the high dose of adrenaline was lost in the presence of L-NAME. However, under those conditions the absence of tachycardia could have been due to concurrent activation of baroreflexes due to the pressor effect of adrenaline. But two observations indicate that this is not a complete explanation of the findings, One is that the tachycardic response to the low dose of adrenaline was attenuated in the presence of L-NAME, even though there was no pressor response. The other observation of relevance is that in animals rendered areflexic with pentolinium, captopril and a vasopressin V_1-receptor antagonist, the tachycardic effects of the high dose of adrenaline were still significantly attenuated in the presence of L-NAME. These results lead us to the novel proposal that the chronotropic effects of adrenaline are transduced, in part, by nitric oxide, as are its β-adrenoceptor-mediated vasodilator effects.

It could be argued that the marked haemodynamic effects of L-NAME in the areflexic animals caused impaired coronary perfusion, and thereby indirectly influenced the cardiac chronotropic response to adrenaline. However, this seems unlikely because such an effect would be expected to be associated with impaired myocardial contractility whereas adrenaline caused a substantial increase in pulse pressure under these conditions (see Figure 3). These observations indicate that the inotropic effects of adrenaline were enhanced, rather than inhibited. However, this could have been due to the suppression of the tachycardic effects of adrenaline causing a relative increase in diastolic filling time of the heart (compared to the effects of adrenaline alone) and hence increasing myocardial contractility. Thus, we cannot dismiss the possibility that an indirect effect of L-NAME contributed to its inhibitory influence on the tachycardic response to adrenaline.

Whatever the explanation of the results, it is clear that the inhibitory effects of L-NAME on the chronotropic responses to adrenaline were relatively selective because, under the same conditions, the tachycardic response of BRL 38227 was unaffected (Table 3) as is the tachycardic response to sodium nitroprusside (Gardiner, Kemp & Bennett, unpublished observations). These findings are noteworthy also because the tachycardic response to BRL 38227 is blocked by atenolol (Clapham *et al.*, 1991) as is that to sodium nitroprusside (Gardiner, Kemp & Bennett, unpublished observations). Thus, it appears that L-NAME may not influence reflex cardiac sympathetic effects, making it even more unlikely that the inhibitory influence of L-NAME on the chronotropic effects of exogenous adrenaline are indirect and due to impaired coronary perfusion (see above). Since there is no reason for believing that endogenous adrenaline would behave differently from exogenous adrenaline, it is feasible that nitric oxide is involved specifically in transducing the chronotropic effects of circulating endogenous adrenaline.

In the animals receiving pentolinium, captopril and the V_1-receptor antagonist the renal and hindquarters vasodilator effects of adrenaline were less and the mesenteric vasoconstrictor effects were greater than in intact animals. However, these differences were probably due to the profound renal, mesenteric and hindquarters vasodilatations in the areflexic animals. Nonetheless, in the additional presence of L-NAME there was clear enhancement of renal and hindquarters vasoconstrictor effects of adrenaline and hence the changes were directionally similar to those seen in intact animals. It is feasible that the diminished mesenteric vasoconstrictor response to adrenaline under these conditions was due to this variable 'bottoming out'. Thus, although the mesenteric vascular bed is clearly an important contributor to total peripheral conductance it appears there are situations in which the hindquarters vascular bed can have a major influence on this variable and thereby affect systemic arterial blood pressure. In support of this proposition evidence from other experiments indicates that the hindquarters vascular bed may make the greatest contribution to the genesis of hypertension resulting from chronic inhibition of nitric oxide production (Gardiner *et al.*, 1990d).

The present results raise the intriguing possibility that endothelial dysfunction could enhance the 'hypertensinogenic' properties of adrenaline (Majewski & Rand, 1985) by suppressing its vasodilator effects. In addition, it appears that impairment of nitric oxide-mediated mechanisms in the heart might amplify these effects by inhibiting the tachycardic action of adrenaline, thereby augmenting its inotropic influence.

Responses to BRL 38227

We selected BRL 38227 to compare with adrenaline in the presence and absence of L-NAME because pilot experiments showed responses to this compound were little affected by L-NAME. In the full experiment this impression was confirmed since the only effect L-NAME had on the responses to BRL 38227 was to cause a slight diminution of the rise in mesenteric vascular conductance following the high dose infusion. Hence, it appears that, apart from this effect, the haemodynamic responses to BRL 38227 are independent of nitric oxide-mediated mechanisms. These results indicate that the

increased effects of sodium nitroprusside in the presence of L-NAME (Gardiner *et al.*, 1991) are due to an up-regulation of the sensitivity of guanylate cyclase to exogenous nitric oxide when endogenous nitric oxide production is inhibited (Moncada *et al.*, 1991), rather than to the changes in cardiovascular status caused by L-NAME.

BRL 38227 caused dose-dependent hypotension and tachycardia (although the latter was less than seen with equihypotensive doses of sodium nitroprusside; Gardiner, Kemp & Bennett, unpublished observation). These effects were accompanied by renal, mesenteric and hindquarters vasodilatations, although there was an increase in blood flow only in the mesenteric vascular bed. Indeed, renal blood flow tended to fall during infusion of the high dose of BRL 38227, possibly due to recruitment of vasoconstrictor mechanisms secondary to the hypotension. This profile of effect is remarkably like that seen with sodium nitroprusside infusion (Gardiner *et al.*, 1991). However, while it is feasible that the latter might have enhanced vasodilator effects in the presence of endothelial damage, from the present results it seems unlikely this would occur with BRL 38227.

We are grateful to SmithKline Beecham for the gift of BRL 38227.

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A calcitonin gene-related peptide (CGRP) antagonist ($CGRP_{8-37}$) inhibits microvascular responses induced by CGRP and capsaicin in skin

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1 The effect of the calcitonin gene-related peptide (CGRP) antagonist $CGRP_{8-37}$ on responses to CGRP and other mediators was investigated in rabbit dorsal skin.

2 Blood flow changes at intradermally-injected sites were measured by a multiple site ^{133}xenon clearance technique. $CGRP_{8-37}$ had little effect on blood flow at doses up to 0.3 nmol/site, when injected alone, although a significant increase in blood flow was observed at the highest dose tested (1 nmol/site).

3 $CGRP_{8-37}$ dose-dependently inhibited the increased blood flow induced by human αCGRP and human βCGRP, but had no effect on equivalent vasodilator responses induced by vasoactive intestinal peptide (VIP) and prostaglandin E_1 (PGE_1). $CGRP_{8-37}$ showed a preferential ability to inhibit αCGRP (IC_{50} 0.04 nmol), when compared with βCGRP ($IC_{50} \geqslant 0.3$ nmol).

4 Capsaicin, which selectively activates sensory nerves, caused a dose-dependent increase in blood flow when injected intradermally into rabbit skin. The effects of capsaicin (0.01–0.1 μmol/site) were inhibited by $CGRP_{8-37}$ (0.3 nmol/site), with a partial but significant attenuation of blood flow induced by the highest dose of capsaicin.

5 Oedema formation, induced by intradermal histamine injection (3 nmol/site), was measured in rabbit skin by the local accumulation of intravenously-injected ^{125}I-labelled albumin. Vasodilator doses of CGRP, PGE_1 and capsaicin potentiated, in a synergistic manner, oedema formation induced by histamine. $CGRP_{8-37}$ totally inhibited the potentiating effect of CGRP, partially inhibited the synergistic effect of capsaicin, but did not affect PGE_1-induced responses.

6 The results suggest that capsaicin acts to release a rabbit form of CGRP in skin and that $CGRP_{8-37}$ is a useful antagonist for investigating the potential of CGRP as a neurogenic mediator of inflammation.

Keywords: CGRP; blood flow; capsaicin; oedema; skin

Introduction

Calcitonin gene-related peptide (CGRP) is a potent vasodilator neuropeptide which stimulates increased blood flow when injected intradermally into skin, as demonstrated in a number of species (Brain *et al.*, 1985; Brain & Williams, 1988). CGRP increases blood flow in a dose-dependent manner. The increased blood flow is long lasting at picomolar doses and, in studies carried out in human skin, the vasodilator activity is clearly observed as a long lasting local erythema (Brain *et al.*, 1985; 1986b). Studies in rabbit skin have revealed that CGRP, as a consequence of its vasodilator activity, potentiates oedema formation induced by a range of mediators of increased microvascular permeability including the neuropeptide substance P (with which CGRP is co-localized in sensory nerves), histamine, bradykinin and platelet activating factor (PAF) (Brain & Williams, 1985; 1989).

Two forms of human CGRP (α and β) and two forms of rat CGRP (α and β) have been determined from molecular biology studies and purification studies (Amara *et al.*, 1982; Morris *et al.*, 1984; Rosenfeld *et al.*, 1984; Steenbergh *et al.*, 1985). All structures determined to date have greater than 90% structural homology. In previous studies in rabbit skin we have demonstrated that all the forms of CGRP tested exhibit a similar potency in increasing microvascular blood flow (Brain *et al.*, 1986a); whilst amylin amide, a peptide with 46% structural homology to human αCGRP, is 100 fold less potent (Brain *et al.*, 1990). These findings strongly suggest the existence of CGRP receptors in the rabbit skin microvasculature.

Chiba and colleagues (1989) demonstrated that a fragment of human αCGRP ($CGRP_{8-37}$) acted as an antagonist by displacing CGRP from receptors present in rat liver plasma membranes. This study is confirmed by functional studies which demonstrated that $CGRP_{8-37}$ is an inhibitor of vascular receptors. $CGRP_{8-37}$ inhibited vasodilatation of the rat mesenteric arterial bed induced, *in vitro*, by periarterial nerve stimulation (Han *et al.*, 1990); inhibited CGRP responses of aortic tissue *in vitro* (Gray *et al.*, 1991) and inhibited the haemodynamic actions of intravenously-injected human αCGRP in the conscious rat (Gardiner *et al.*, 1990). Recent studies indicate that at least two subtypes of CGRP receptors, exist, $CGRP_1$ and $CGRP_2$ and $CGRP_{8-37}$ is suggested to be a potent antagonist of the $CGRP_1$ receptor (Dennis *et al.*, 1989; 1990). Maggi and co-workers (1991) have demonstrated that $CGRP_{8-37}$ competitively antagonizes human αCGRP and capsaicin-induced responses in the guinea-pig atria and rat vas deferens *in vitro*.

In this study we have investigated the effect of $CGRP_{8-37}$ on the increase in microvascular blood flow induced by CGRP and other vasodilators, including capsaicin which acts by stimulating endogenous neuropeptide release. We have also investigated the effect of $CGRP_{8-37}$ on inflammatory oedema responses which have been induced by histamine and potentiated in a synergistic manner by various vasodilators.

Methods

Blood flow and oedema formation were measured in the skin of male New Zealand White rabbits (2–2.5 kg). The rabbits were anaesthetized with sodium pentobarbitone (Sagatal, May & Baker: 30 mg kg^{-1}) given intravenous (i.v.) via the marginal ear vein. The dorsal fur of the rabbits was then removed by shaving and anaesthesia maintained by further administration as necessary. Skin blood flow (Williams, 1976) or skin oedema

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formation (Williams, 1979) was then measured in response to intradermally (i.d.)-injected test agents.

Measurement of blood flow: ^{133}xenon clearance

A site plan was marked out on the dorsal skin with six blocks of up to a maximum of nine injection sites, allocated according to a balanced site pattern. Test agents were then made up to 1 ml in saline and an aliquot (33.3 μCi) of ^{133}xenon added. The agents were then rapidly loaded into syringes and injected via 27 gauge needles i.d. into the skin sites (0.1 ml/site). The injections were given in random order in replicates of six (one into each block). The clock was started midway through injections and a clearance period of 15 min allowed. The rabbit was then killed by barbiturate overdose and the dorsal skin removed. Injection sites (16 mm diameter) were punched out and counted for radioactivity, under liquid paraffin, in a gamma-counter. Results were then calculated as % change in blood flow from control, saline-injected sites.

Measurement of oedema formation: ^{125}I accumulation

Oedema formation was measured in a separate series of experiments, as follows. A site plan was marked out on the dorsal skin with six blocks of 15 injection sites, allocated according to a balanced site pattern. A mixture of ^{125}I-labelled human serum albumin (10 μCi) and Evans Blue dye (2 ml of 2.5% w/v in saline) were administered i.v. via the marginal ear vein. Test agents were then injected i.d. in a random order and in replicates of six. After a 30 min accumulation period, a cardiac blood sample was taken (up to 10 ml) and the rabbit killed by barbiturate overdose. Injection sites were removed from the dorsal skin and counted for radioactivity in a gamma counter. Oedema formation at each skin site was then expressed as the volume of plasma accumulated, as calculated by comparing the number of counts in each skin site with that present in 1 ml of plasma.

Materials

$CGRP_{8-37}$ was obtained from Bachem (UK) Ltd, Saffron Walden, Essex. Recombinant human α and β CGRP were obtained as gifts from Dr U. Ney, Celltech, Slough, Bucks. Vasoactive intestinal peptide (VIP), histamine, PGE_1 and Evans Blue were obtained from Sigma Chemical Company, Poole, Dorset. These agents were stored in concentrated form at -20°C in either ethanol or saline containing 0.25% bovine serum albumin. Each agent was diluted in saline, as required, immediately prior to use. Capsaicin (pelargonic acid vanillylamide) was obtained from Fluka Chemicals Ltd, Glossop, Derbyshire and was stored at room temperature. Capsaicin (100 mg ml^{-1}) was dissolved in a mixture (2:1:7) of absolute alcohol: Tween 80 (Sigma): saline and diluted in saline immediately prior to use. Solvent controls were used as necessary. ^{125}I-labelled human serum albumin and ^{133}xenon were obtained from Amersham International plc, Aylesbury, Buckinghamshire and stored at 4°C.

Statistics

Bonferroni's modified t test was used to assess the significance of differences between treatments. The modified test uses the s.e. estimate for the analysis of variance to account for the fact that multiple tests were performed (* $P < 0.05$ and ** $P < 0.01$).

Results

The effect of increasing doses of intradermally-injected $CGRP_{8-37}$ on basal blood flow was measured by the multiple site ^{133}xenon clearance technique. Results are shown in Figure 1. At the lowest dose tested (0.03 nmol/site), the antagonist caused a small, non-significant inhibition of blood flow. The two consecutive doses (0.1 and 0.3 nmol/site) had no effect but, in contrast, a significant increase in blood flow was observed following administration of the highest dose of $CGRP_{8-37}$ tested (1 nmol/site).

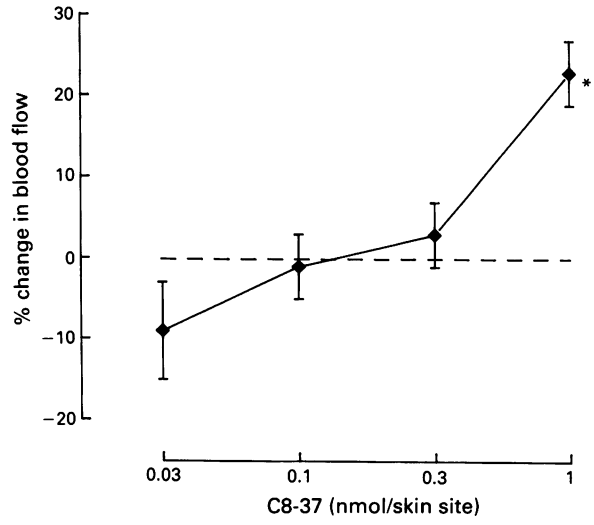


Figure 1 The effect of $CGRP_{8-37}$ on basal blood flow in rabbit skin. The dose-related effect of $CGRP_{8-37}$ (C8-37) is shown. The response to saline is indicated by the dashed line. Results are mean values (s.e.mean indicated by vertical bars) for $n = 4$ rabbits and * indicates a significant change ($P < 0.05$) in blood flow from that measured in response to the previous dose.

The effect of $CGRP_{8-37}$, at a sub-vasodilator dose (0.3 nmol/site), on the dose-dependent increases in blood flow induced by human αCGRP (0.3–3 pmol/site) and human βCGRP (0.3–3 pmol/site) is shown in Figure 2. Human α and βCGRP increased blood flow in a similar manner, and the concomitant injection of $CGRP_{8-37}$ inhibited blood flow induced by both αCGRP and βCGRP. The inhibitory effect of $CGRP_{8-37}$ was selective for the CGRP peptide, as blood flow induced by the structurally-unrelated neuropeptide, vasoactive intestinal peptide (VIP) was not affected; results were as follows: VIP (10 pmol) 58 ± 10% increase in blood flow, VIP + $CGRP_{8-37}$ 49 ± 5% increase in blood flow from control, saline-injected sites, mean ± s.e.mean, $n = 8$.

Figure 2 shows that $CGRP_{8-37}$ caused a total inhibition of the increase in blood flow induced by the highest dose of αCGRP, but only a partial inhibition of the increase in blood flow induced by the highest dose of βCGRP. We investigated this partial inhibition further by studying the ability of increasing doses of the antagonist to inhibit responses induced by a high dose of α and βCGRP respectively, as shown in Figure 3. The experiments were designed so that the effect of $CGRP_{8-37}$ on human α and βCGRP (3 pmol/site) was simultaneously tested in the same nine rabbits. $CGRP_{8-37}$ (0.03–0.3 nmol) acted dose-dependently to inhibit completely the effects of αCGRP ($IC_{50} = 0.04$ nmol). In contrast, $CGRP_{8-37}$ inhibited blood flow induced by β-CGRP in a less potent manner ($IC_{50} \geqslant 0.3$ nmol).

The vasodilator response to intradermal injection of capsaicin is shown in Figure 4. $CGRP_{8-37}$ (0.3 nmol/site) caused an inhibition of capsaicin-induced blood flow at every dose in every animal tested. Figure 4 shows that, although partial, a significant inhibition of blood flow induced by capsaicin 0.1 μmol was observed. For control, Figure 4 also shows that the increased blood flow induced by the prostanoid vasodilator PGE_1 was not inhibited by $CGRP_{8-37}$.

Two chemically distinct vasodilators, αCGRP and PGE_1, acted in a synergistic manner to potentiate inflammatory oedema induced by histamine, as shown in Figure 5. However, only the potentiation of oedema by αCGRP was inhibited by $CGRP_{8-37}$. The effect of $CGRP_{8-37}$ on the ability of capsaicin

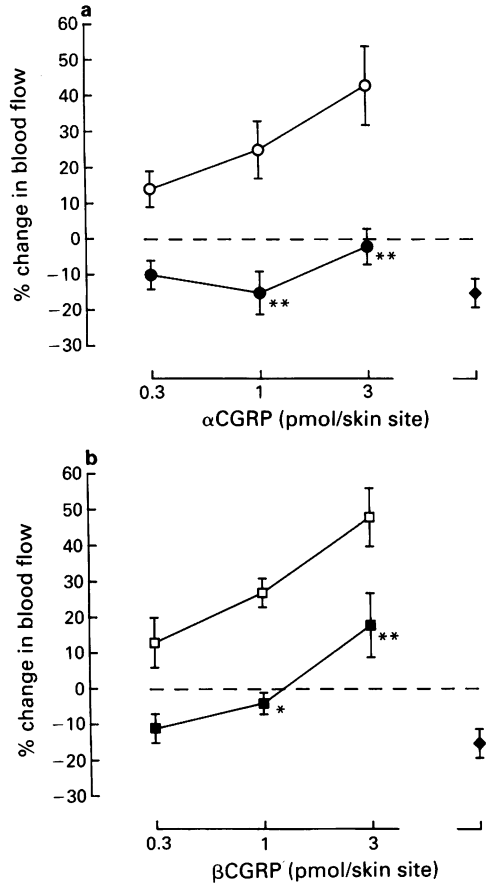


Figure 2 The effect of $CGRP_{8-37}$ on blood flow induced by increasing doses of human αcalcitonin gene-related peptide (αCGRP) and human βCGRP. $CGRP_{8-37}$ (0.3 nmol/site^{-1}) was injected together with each dose of human αCGRP and human βCGRP: (a) shows responses to αCGRP in the absence (○) and the presence (●) of $CGRP_{8-37}$; (b) shows responses to βCGRP in the absence (□) and presence (■) of $CGRP_{8-37}$. In both graphs, the response to $CGRP_{8-37}$ alone is shown by (◆) and blood flow at control, saline-injected sites is indicated by the dashed line. Results are expressed as mean values (s.e. mean indicated by vertical bars) for $n = 4$–11 rabbits and a significant inhibitory effect of $CGRP_{8-37}$ is indicated by * ($P < 0.05$) or ** ($P < 0.01$).

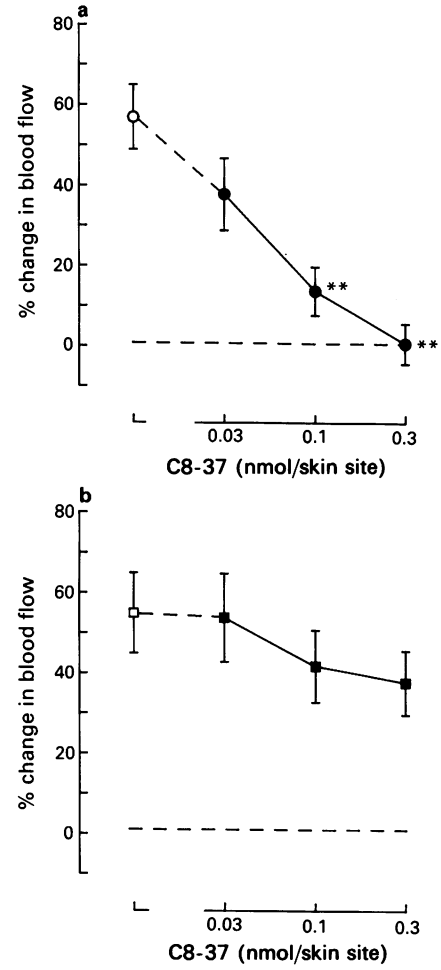


Figure 3 The effect of increasing doses of $CGRP_{8-37}$ (C8-37) on blood flow induced by human αcalcitonin gene-related peptide (αCGRP, 3 pmol/site) and human βCGRP (3 pmol/site): (a) shows the increased blood flow induced by human αCGRP (○) and the effect of increasing doses of $CGRP_{8-37}$ on this response (●); (b) shows the level to which blood flow is increased by βCGRP (□) and the effect of increasing doses of $CGRP_{8-37}$ on this response (■). In both graphs, the dashed line at 0% refers to control, saline-injected sites. Results are from experiments carried out in the same animals and each point is expressed as mean value (s.e.mean shown by vertical bars) for $n = 9$ rabbits. A significant inhibitory effect of $CGRP_{8-37}$ is indicated by **($P < 0.01$).

to potentiate the effects of histamine is also shown. Capsaicin itself induced a small amount of oedema formation at the higher dose. The effect of $CGRP_{8-37}$ on oedema induced by histamine in the presence of capsaicin was tested. $CGRP_{8-37}$ caused a significant but partial inhibition of this response, as shown in Figure 5.

Discussion

The results show that $CGRP_{8-37}$, when injected intradermally with vasodilators, acts as a selective antagonist to inhibit CGRP responses in rabbit skin. The antagonist inhibited vasodilator responses induced by human αCGRP, βCGRP and capsaicin (which stimulates endogenous neuropeptide release) but had no effect on vasodilator responses induced by the prostanoid PGE_1 and the structurally unrelated neuropeptide vasodilator, VIP. The antagonist also inhibited the ability of CGRP and capsaicin to potentiate inflammatory oedema formation induced by histamine.

$CGRP_{8-37}$ inhibited responses to human αCGRP (IC_{50} 0.04 nmol) in a more potent manner than βCGRP ($IC_{50} \geqslant$ 0.3 nmol). In previous *in vitro* studies, $CGRP_{8-37}$ has been shown to have preferential effects on $CGRP_1$ receptors (Dennis *et al.*, 1989; 1990). Thus it is possible that αCGRP acts preferentially on $CGRP_1$ receptors in rabbit skin, whilst βCGRP causes increased blood flow by acting on more than one CGRP receptor in this tissue. This suggests that caution should be used in the interpretation of human studies where $CGRP_{8-37}$ is used. Both α and β forms of CGRP can be released and exert functional effects in man, but $CGRP_{8-37}$ might have differential inhibitory effects dependent on the form of CGRP released.

$CGRP_{8-37}$ has previously been shown to cause vasoconstriction when infused alone in the rat mesenteric bed *in vitro* (Han *et al.*, 1990) and in the rat hindquarters *in vivo* (Gardiner *et al.*, 1990). Results from our experiments show that at a low concentration $CGRP_{8-37}$ caused a small non-significant decrease in blood flow, possibly as a consequence of inhibiting the actions of basally-released endogenous CGRP. However, at a higher concentration $CGRP_{8-37}$ exhibited significant vasodilator activity, suggesting some agonist activity. This result is in keeping with $CGRP_{8-37}$ acting as a partial agonist at the $CGRP_1$ receptor. Alternatively, Chiba and colleagues (1989) have demonstrated that $CGRP_{8-37}$ is a potent agonist at calcitonin receptors; thus perhaps $CGRP_{8-37}$ could be acting via calcitonin receptors, at higher concentrations, to increase blood flow in rabbit skin. However, we consider this unlikely as human calcitonin does not exhibit vasodilator activity in rabbit skin at similar doses (Brain *et al.*, 1985).

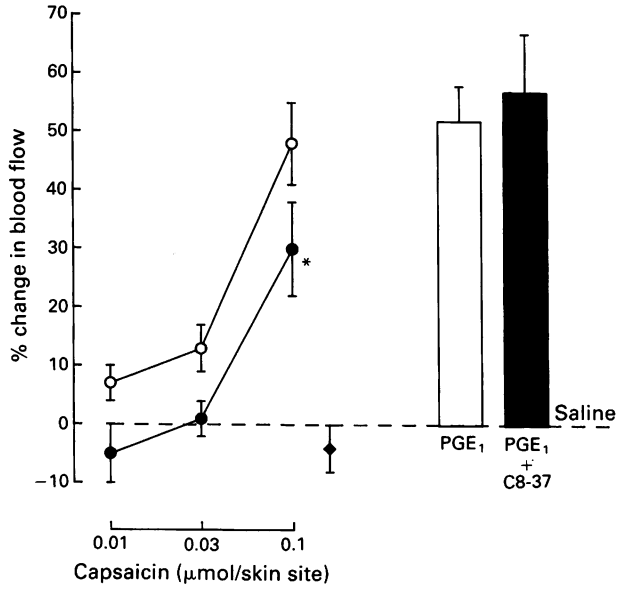


Figure 4 The effect of $CGRP_{8-37}$ on capsaicin and prostaglandin E_1 (PGE_1)-induced blood flow. The dose-related effect of capsaicin is shown in the absence (○) and in the presence (●) of $CGRP_{8-37}$ (C8-37, 0.3 nmol/site). The response to PGE_1 (10 pmol, open column) remains unaffected by $CGRP_{8-37}$ (filled column). Blood flow at control, saline-injected skin sites is indicated by the dashed line and the effect of $CGRP_{8-37}$ alone (0.3 nmol) is shown by (◆). Results are the mean values (s.e.mean shown by vertical bars) for $n = 4$–10 rabbits and the significant inhibitory effect of $CGRP_{8-37}$ is shown as * ($P < 0.05$).

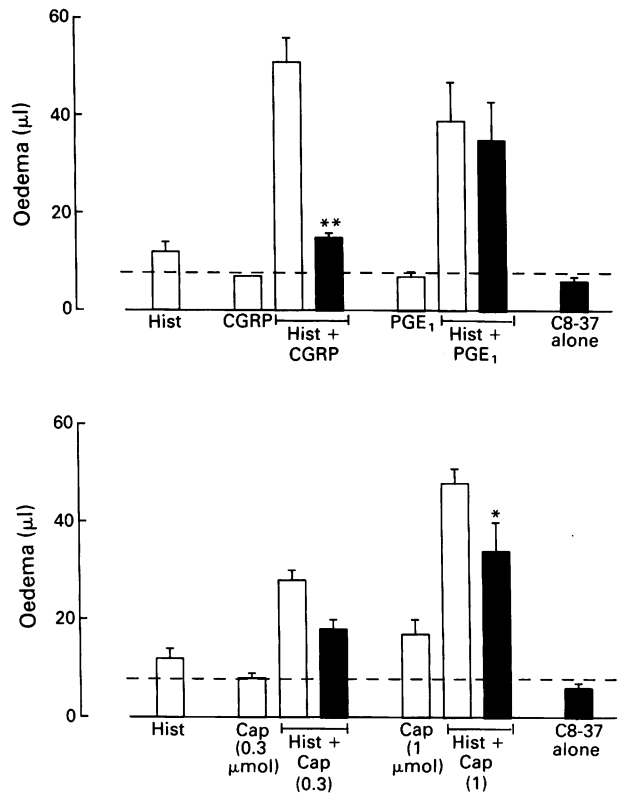


Figure 5 The effect of $CGRP_{8-37}$ (C8-37) on oedema formation which is induced by histamine (Hist, 3 nmol/site) and potentiated by αcalcitonin gene-related peptide (CGRP, 3 pmol/site), prostaglandin E_1 (PGE_1, 10 pmol/site) and capsaicin (Cap, 0.3 and 1 μmol/site) is shown. The response to saline is shown by the dashed line. The open columns represent responses to individual agents injected alone and the synergistic responses induced by histamine in the presence of each vasodilator, as labelled. The filled columns represent the effect of $CGRP_{8-37}$ (0.3 nmol/site) on each synergistic response and the effect of $CGRP_{8-37}$ when injected alone. Results are expressed as mean values (s.e.mean shown by vertical bars) for $n = 5$ rabbits. The significant inhibitory effect of $CGRP_{8-37}$ is shown as * ($P < 0.05$) or ** ($P < 0.01$).

Our first evidence for the release of a rabbit form of CGRP from capsaicin-sensitive nerves in skin comes from studies where an antibody to human αCGRP has been used to prevent the actions of endogenous CGRP. The Fab fraction of the CGRP antibody caused a significant but partial inhibition of capsaicin-induced blood flow when administered intradermally in rabbit skin (Buckley *et al.*, 1991c). The results in this paper, using the CGRP antagonist, add further support to the suggestion that capsaicin releases vasodilator quantities of CGRP in rabbit skin. Blood flow induced by low doses of capsaicin was inhibited, with higher doses inhibited partially but significantly, by $CGRP_{8-37}$. The partial inhibition of high dose capsaicin-induced blood flow by both antibody and antagonist is of interest and it is possible that sensory nerves release other vasodilators, in addition to CGRP. Alternatively, it is possible that insufficient antibody/antagonist were present for the total inhibition/antagonism of perivascular CGRP released in close proximity to vascular receptors.

The results in this study provide evidence that a CGRP antagonist can inhibit the vasodilatation which is induced in skin by endogenously-released CGRP and inflammatory oedema which occurs as a result of the synergistic action of CGRP and mediators of increased microvascular permeability (in this case histamine). In other studies, we have suggested that CGRP, as a consequence of its vasodilator activity, can potentiate neutrophil accumulation and the pro-inflammatory actions of the cytokine interleukin-1 (Buckley *et al.*, 1991a,b).

In conclusion, we would like to suggest that, if CGRP is released as a neurogenic component of inflammation, an antagonist of CGRP could act in a novel manner as an anti-inflammatory agent to limit inflammatory erythema, oedema formation and cell accumulation. Future studies will be designed to test this hypothesis.

This project was supported by the British Heart Foundation. We thank Ms Elaine Hawkings for technical help and Dr Theresa Buckley and Prof. Tim Williams for discussion.

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Differential effects of L-arginine on the inhibition by N^G-nitro-L-arginine methyl ester of basal and agonist-stimulated EDRF activity

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1 An isolated, buffer-perfused rabbit ear preparation was used to investigate the influence of N^G-nitro-L-arginine methyl ester (L-NAME) on endothelium-dependent vasodilatation and modulation of vasoconstrictor responses and vascular conductance.

2 Acetylcholine (0.55 pmol–1.6 nmol) caused dose-related vasodilatation of preparations constricted by the combination of 5-hydroxytryptamine and histamine (both 1 μM), with an $ED_{50} = 31.1 \pm 7.8$ pmol and a maximum dilatation of $69.9 \pm 4.3\%$. In the presence of 10 μM L-NAME the dose-response for vasodilator effects was shifted significantly ($P < 0.001$) to the right ($ED_{50} = 3.07 \pm 1.18$ nmol) and there was a significant ($P < 0.01$) depression of the maximum response ($R_{max} = 44.3 \pm 4.0\%$). The higher concentration of 100 μM L-NAME completely abolished vasodilatation to acetylcholine. L-Arginine (10 mM) did not reverse the inhibitory actions of L-NAME at either concentration.

3 L-NAME 100 μM, augmented vascular tone induced by 1 μM 5-hydroxytryptamine and 1 μM histamine, thus altering the characteristics of both pressure/flow and conductance/flow relationships such that conductance was reduced at all flow rates. The augmentation of constrictor tone was reversed in a concentration-dependent manner by L-arginine (10 μM–10 mM) and the effect of L-NAME on the conductance/flow relationships was similarly reversed by 10 mM L-arginine. The augmentation of tone was endothelium-dependent as it did not occur following functional destruction of the endothelium by perfusion of the vascular bed with the detergent CHAPS (0.3%) for 150 s.

4 In conclusion, L-NAME is a potent inhibitor of agonist-induced endothelium-dependent vasodilatation. L-NAME reduces vascular conductance in pharmacologically constricted preparations and this emphasizes the important role of EDRF in vascular regulation. The ability of L-arginine to reverse L-NAME-induced inhibition of basal EDRF activity but not L-NAME-induced inhibition of agonist-induced endothelium-dependent relaxations suggests that there is pharmacological heterogeneity in the mechanisms responsible for the conversion of L-arginine to EDRF.

Keywords: Rabbit ear; EDRF; N^G-nitro-L-arginine methyl ester; pressure/flow relationships; vascular conductance; L-arginine

Introduction

For some time it has been known that the vascular endothelium releases a potent vasorelaxant, endothelium-derived relaxing factor (EDRF), in response to various agonists (Furchgott & Zawadzki, 1980) and also basally (Griffith *et al.*, 1984a,b). It has recently been demonstrated that EDRF is either nitric oxide (Palmer *et al.*, 1987) or a nitric oxide containing moiety (Myers *et al.*, 1990) with the nitrogen atom being derived from the terminal guanidino group of L-arginine (Palmer *et al.*, 1988).

The elucidation of the identity of EDRF and its precursor has led to the use of analogues of L-arginine to inhibit EDRF synthesis (Rees *et al.*, 1989a; Moore *et al.*, 1990). The first analogue used, N^G-monomethyl-L-arginine (L-NMMA), inhibited endothelium-dependent relaxations of the rabbit aorta without affecting endothelium-independent relaxations to nitrovasodilators (Rees *et al.*, 1989a). More recently N^G-nitro-L-arginine and its methyl ester (L-NAME) have emerged as more potent inhibitors of endothelium-dependent relaxations in both conduit and resistance vessels (Moore *et al.*, 1990; Rees *et al.*, 1990). In addition to inhibiting endothelium-dependent relaxations such compounds augment pharmacologically induced tone in an endothelium-dependent manner (Rees *et al.*, 1989a) and cause hypertension in whole animal preparations (Rees *et al.*, 1989b; 1990). The former action demonstrates the modulatory effects of EDRF on vascular tone and the hypertensive effect is indicative of the important role of EDRF in blood pressure regulation.

The inhibitory actions of L-arginine analogues have been shown to be reversible by L-arginine but the extent varies between inhibitors and preparations. In rat aortic rings and isolated mesentery Moore *et al.* (1990) found that the partial inhibition of endothelium-dependent relaxations to acetylcholine by 30 μM N^G-nitro-L-arginine was reversed by < 150 μM L-arginine. In contrast, Unmans (1990) reported that the inhibitory effects of 100 μM N^G-nitro-L-arginine on endothelium-dependent relaxations to acetylcholine in the rat aorta were irreversible even at high concentrations of L-arginine. Similarly the hypertensive effects of N^G-nitro-L-arginine in the anaesthetized guinea-pig are only slightly attenuated by administration of L-arginine, in contrast to the pressor effects of L-NMMA which were fully reversed by L-arginine (Steinberg *et al.*, 1990).

The biological activity of nitric oxide or a nitric oxide-like substance is not confined to vascular systems. It has recently been shown that non-adrenergic, non-cholinergic (NANC) transmission in the rat anococcygeus muscle is specifically inhibited by 200 μM L-NAME while the partial inhibitory effects of 20 μM L-NAME are almost completely reversed by a 5 fold molar excess of L-arginine (Hobbs & Gibson, 1990). Similarly 5 μM N^G-nitro-L-arginine inhibits NANC relaxations of the guinea-pig trachea where the effect was partially reversed by 500 μM L-arginine (Tucker *et al.*, 1990). EDRF is also known to prevent platelet aggregation (Azuma *et al.*, 1986), thus inhibitors of nitric oxide synthesis enhance platelet aggregation but the maximum effect of L-NAME is not reversed by high concentrations of L-arginine (Radomski *et al.*, 1990a). It would thus appear that the effects of nitric oxide synthase inhibitors and their sensitivity to reversal by L-arginine are dependent on species and the physiological system under consideration.

In the present study we have investigated the effects of L-NAME on both agonist and basally released EDRF in a

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resistance bed. It has previously been shown that in the blood-perfused rat mesentery, destruction of the endothelium and also administration of methylene blue alter the characteristics of pressure/flow relationships indicating a substantial increase in vascular resistance presumably as a consequence of loss of basally released EDRF (Randall & Hiley, 1988b). We have previously reported similar effects on diameter/flow, pressure/flow and conductance/flow relationships for haemoglobin (Griffith *et al.*, 1987a), which avidly scavenges EDRF (Kelm *et al.*, 1988), and L-NMMA in the rabbit isolated ear (Griffith & Edwards, 1990). In the present study we have investigated whether L-NAME similarly affects the pressure/flow and conductance/flow relationships in the rabbit ear and have examined the characteristics of L-NAME-induced blockade of both basal and agonist-dependent EDRF activity in this preparation.

Methods

Preparation of the rabbit ear vascular bed

Male New Zealand White rabbits (2–2.5 kg) were killed by cervical dislocation. An ear was removed and the central artery cannulated (Portex PP50 tubing). The preparation was perfused, by means of a peristaltic pump, at 2 ml min^{-1} with Holman's solution (mM): NaCl 120, KCl 5, $CaCl_2$ 2.5, NaH_2PO_4 1.3, $NaHCO_3$ 25, sucrose 10 and D-glucose at 11. The physiological buffer also contained 5% dextran (mol. wt. 80,000) and 10 μM indomethacin. The buffer was gassed with 95% O_2/5% CO_2 (Po_2 ca. 600 mmHg; pH 7.4) and maintained at 35°C.

Experimental protocol

In order to study the effects of L-NAME on the endothelium-dependent vasodilatation to acetylcholine in the isolated ear preparation, tone was established by addition of 5-hydroxytryptamine and histamine to the perfusate to achieve individual concentrations of 1 μM. We have previously shown that this combination of vasoconstrictor agents produces consistent and well maintained tone and does not release EDRF in this preparation (Griffith *et al.*, 1988). The vasodilator properties of acetylcholine were assessed by bolus injections of this agent, in volumes less than 30 μl, into the perfusion system. The effects of either 10 μM or 100 μM L-NAME on responses to acetylcholine were examined by addition of the agent after tone had been established but 30 min before experimental determinations. After constructing dose-response curves to acetylcholine the effect of 10 mM L-arginine on inhibition of endothelium-dependent responses by L-NAME was investigated by addition to the perfusate 30 min before further doses of acetylcholine.

The influence of L-NAME on the pressor effects of 5-hydroxytryptamine and histamine was investigated by constructing concentration-response curves to equimolar concentrations of these agonists in the absence of L-NAME and also in preparations which had been pretreated with 100 μM L-NAME 30 min before experimental determinations. To investigate further the effects of L-NAME on constriction induced by the combination of 5-hydroxytryptamine and histamine (both 1 μM), tone was established and allowed to stabilize for 30 min, at which time L-NAME was added to the perfusion fluid at a concentration of 100 μM. The ability of L-arginine and its enantiomer D-arginine to oppose the increase in perfusion pressure in response to L-NAME was then examined. Vascular responses to L-arginine were also examined in the absence of L-NAME. In some preparations the concentrations of 5-hydroxytryptamine and histamine were increased to 3 μM which gave a level of tone comparable to that induced by 1 μM of these agents in the presence of L-NAME. The endothelium-dependent nature of the augmented constrictor responses by L-NAME was examined by addition of L-NAME to preparations denuded of endothelium. Destruction of the endothelium was achieved by perfusion of the bed with 0.3% CHAPS in distilled water for 150 s, a technique which has been used in other resistance beds, both *in vitro* and *in vivo*, to cause selective destruction of the endothelium (Randall & Hiley, 1988a,b). After an equilibration period of 30 min functional destruction of the endothelium was confirmed by the inability of 550 pmol of acetylcholine to cause relaxation of established tone while constrictor responses to 5-hyroxytryptamine and histamine and vasodilatation to sodium nitroprusside (1 nmol) remained intact. Once this had been confirmed, L-NAME (100 μM) was added to the perfusion fluid.

The effects of L-NAME on vascular conductance in pharmacologically constricted (1 μM 5-hydroxytryptamine and 1 μM histamine) preparations were also considered. In these experiments pressure/flow relationships were established at 8 flow rates, from 0.959 to 5.5 ml min^{-1}. Each flow was used twice and changed every 2 min according to a Latin square design while the pressure was measured by a transducer placed close to the inflow cannula. Correction for the resistance of the inflow cannula was made by subtracting the pressure drop across the cannula alone at each of the flow rates employed. In these experiments pressure/flow relationships were determined for constricted preparations and then in the presence of 100 μM L-NAME alone followed by the addition of 10 mM L-arginine or in the presence of L-arginine followed by L-NAME. Conductance was determined from the pressure/flow data by the following relationship:

$$\text{conductance (G)} = \frac{\text{flow rate (Q)}}{\text{pressure drop across the ear (P)}}$$

Data and statistical analysis

All data are given as the mean ± s.e.mean. The dose-response curve for the vasodilator response to acetylcholine responses was analysed by fitting the logistic equation:

$$R = \frac{R_{max} \times A^{n_H}}{ED_{50}^{n_H} + A^{n_H}}$$

where R is the percentage relaxation of tone, A the dose of acetylcholine, R_{max} the maximum relaxation, n_H the slope function and ED_{50} the dose of acetylcholine giving the half maximal relaxation.

A modified Marquardt procedure, as implemented in the Harwell routine VB01A on a mainframe computer, was used to carry out the curve fitting (Aceves *et al.*, 1985).

The variation of conductance with flow rate was analysed by linear regression or construction of a polynomial function depending on the best fit. This was carried out by means of Cricket Graph 1.2 (Cricket Software, Malvern PA, U.S.A.) running on an Apple Macintosh IIcx.

Comparison between experimental groups was carried out either by paired or unpaired Student's *t* tests or by one-way analysis of variance.

Drugs

All solutions were prepared on the day of the experiment. N^G-nitro-L-arginine methyl ester, L-arginine hydrochloride, D-arginine hydrochloride, histamine dihydrochloride, sodium nitroprusside, and 5-hydroxytryptamine as creatinine sulphate complex were dissolved in saline and then diluted to the required concentrations in the Holman's solution. Indomethacin was made up as a 10 mM stock solution in 5% (w/v) sodium bicarbonate and was dissolved in the Holman's solution at a concentration of 10 μM. Acetylcholine chloride was dissolved in 0.9% (w/v) saline. CHAPS (3-[(3-cholamidopropyl)-dimethyl ammonio]-1-propane sulphonate) was dissolved at a concentration of 0.3% (w/v) in distilled water. All drugs were obtained from Sigma Chemical Company, Poole, Dorset.

Results

Effects of N^G-nitro-L-arginine methyl ester on endothelium-dependent vasodilatation to acetylcholine

The basal perfusion pressure was 31.5 ± 3.8 mmHg and the combination of 1 μM 5-hydroxytryptamine and 1 μM histamine increased perfusion pressure to 147 ± 14 mmHg (n = 13). Acetylcholine (0.55 pmol–1.6 nmol) caused dose-related reductions in the established pressure. The dose-response curve (Figure 1a) for the dilatations was described by an ED_{50} = 31.1 ± 7.8 pmol, an R_{max} = 69.9 ± 4.3% and a slope function of 0.98 ± 0.13 (n = 5–7).

Addition of 10 μM L-NAME significantly ($P < 0.05$) increased perfusion pressure to 190 ± 12 mmHg (n = 9). In the presence of this concentration of L-NAME there was a significant ($P < 0.001$) rightward shift of the dose-response curve (ED_{50} = 3.07 ± 1.18 nmol; n_H = 0.93 ± 0.11) for the relaxation of tone by acetylcholine (0.55 pmol–160 nmol) with a significant ($P < 0.01$) depression of the maximum relaxation (R_{max} = 44.3 ± 4.0%) (Figure 1a). In the same 9 preparations the reductions in potency and maximum relaxation to acetylcholine were unaffected by addition of 10 mM L-arginine to the perfusate (ED_{50} = 5.39 ± 2.53 nmol; R_{max} = 48.4 ± 6.0%; n_H = 0.82 ± 0.10) (Figure 1a). Following addition of this amino acid, perfusion pressure was 154 ± 17 mmHg.

In another 6 preparations basal perfusion pressure was 37.6 ± 4.1 mmHg and in the presence of 5-hydroxytryptamine, histamine and 100 μM L-NAME perfusion pressure was 180 ± 23 mmHg while acetylcholine (0.55 pmol–1.6 nmol) did not cause any significant relaxation of tone (Figure 1b; n = 5–6). In the presence of both 100 μM L-NAME and 10 mM L-arginine perfusion pressure was 147 ± 24 mmHg and acetylcholine (0.55 pmol–1.6 nmol) did not cause significant relaxation of tone (Figure 1b; n = 5).

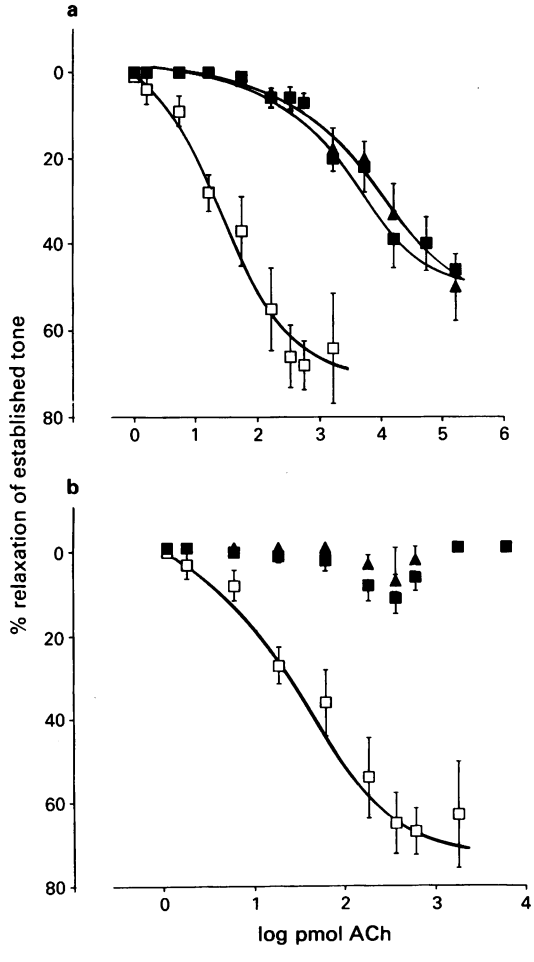


Figure 1 The relaxation of established tone by acetylcholine (ACh) in the isolated perfused ear of the rabbit; (a) shows the relaxations to acetylcholine in the absence of N^G-nitro-L-arginine methyl ester (L-NAME) (□, n = 7), in the presence of 10 μM L-NAME (■, n = 9) and in the presence of 10 μM L-NAME plus 10 mM L-arginine (▲, n = 9); (b) shows the relaxations to acetylcholine in the presence of 100 μM L-NAME (■, n = 5–6) and in the presence of 100 μM L-NAME and 10 mM L-arginine (▲, n = 5) with the control data from (a) represented by (□). The dose-response curves were computer fitted as described in the Methods. The points show the mean and the vertical bars the s.e.mean.

Effects of N^G-nitro-L-arginine methyl ester on the constrictor activity of the combination of 5-hydroxytryptamine and histamine

In seven preparations resting perfusion pressure was 29.0 ± 4.3 mmHg and the equimolar mixture of 5-hydroxytryptamine and histamine brought about concentration-related increases in perfusion pressure with a maximum increase of 174 ± 9 mmHg (to a perfusion pressure of 203 ± 10 mmHg) and the EC_{50} for the agonist mixture was 790 ± 230 nM (Figure 2). In another five preparations resting perfusion pressure in the presence of 100 μM L-NAME was 41.8 ± 7.6 mmHg and this value did not differ significantly from that obtained in its absence. In these preparations the mixture of 5-hydroxytryptamine and histamine similarly gave rise to concentrated-related increases in perfusion pressure with a maximum increase of 166 ± 12 mmHg (to a perfusion pressure of 207 ± 9 mmHg) and these values did not differ from those obtained in the absence of L-NAME. However, in the presence of L-NAME there was a significant ($P < 0.01$) 30 fold leftward shift in the concentration-response curve (Figure 2) indicating that the mixture of constrictor agents was significantly ($P < 0.01$) more potent (EC_{50} = 29 ± 9 nM).

Effects of L-arginine on the augmentation of vascular tone by 100 μM N^G-nitro-L-arginine methyl ester

In resting preparations (n = 11) perfused at 2 ml min^{-1}, perfusion pressure was 30.5 ± 4.4 mmHg. Inclusion of 1 μM 5-hydroxytryptamine and 1 μM histamine increased perfusion pressure to 133 ± 9 mmHg (n = 11). Addition of 100 μM L-NAME at the plateau of the response increased perfusion

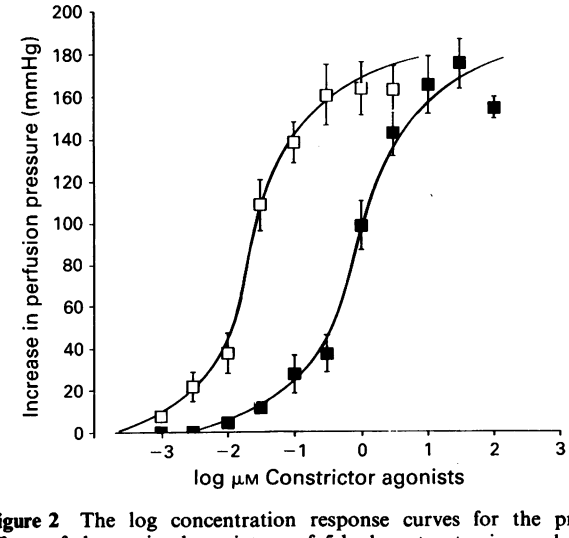


Figure 2 The log concentration response curves for the pressor effects of the equimolar mixture of 5-hydroxytryptamine and histamine in the absence (■) and presence (□) of 100 μM N^G-nitro-L-arginine methyl ester in the isolated perfused ear preparation of the rabbit. The points show mean with s.e.mean indicated by vertical bars.

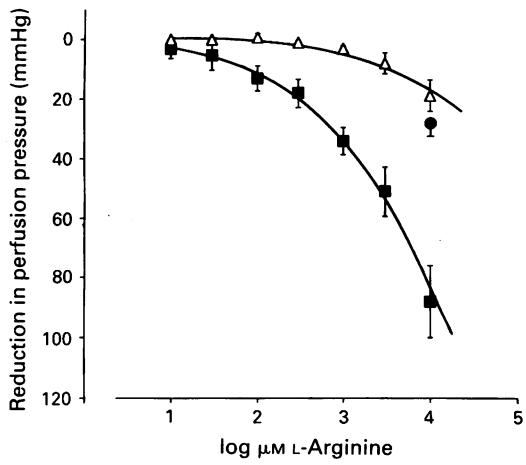


Figure 3 Shows the sustained relaxations of tone in the preconstricted rabbit ear preparation caused by L-arginine in the absence (△, $n = 7$) and presence (■, $n = 6$–11) of 100 μM N^G-nitro-L-arginine methyl ester (L-NAME). The (●) indicates the relaxation caused by 10 mM D-arginine in the presence of 100 μM L-NAME ($n = 3$). The points show the mean and the vertical bars the s.e.mean.

pressure significantly ($P < 0.001$) to 206 ± 9 mmHg ($n = 11$). Figure 3 shows that this augmentation of pharmacologically induced tone was reversed by L-arginine (10 μM–10 mM) in a concentration-dependent manner such that at the highest concentration used there was a significant ($P < 0.001$) reduction in perfusion pressure to 114 ± 12 mmHg (or a 50.0 ± 6.7% relaxation of established tone) ($n = 10$). The IC_{50} for the reversal of the augmentation of vasoconstriction by L-NAME was greater than 1 mM. Addition of L-arginine (10 μM–10 mM) did not significantly alter the pH of the physiological salt solution.

In a further three preparations the relaxant properties of the enantiomer D-arginine were assessed against constrictor tone in the presence of L-NAME. In these experiments basal perfusion pressure was 46.1 ± 7.2 mmHg and in the presence of 1 μM 5-hydroxytryptamine, 1 μM histamine and 100 μM L-NAME perfusion pressure was increased to 197 ± 9 mmHg and the depressor response induced by 10 mM D-arginine was 25 ± 7 mmHg (16.6 ± 4.7% reduction of tone) and this is significantly ($P < 0.01$) less than that evoked by 10 mM L-arginine under similar conditions (Figure 3).

In another seven resting preparations the perfusion pressure was 29.7 ± 4.1 mmHg and was increased to 155 ± 14 mmHg by the combination of 5-hydroxytryptamine and histamine (each 1 μM). In the absence of L-NAME additions of L-arginine brought about modest relaxations of tone which only became significant ($P < 0.05$) at 10 mM when there was a relaxation of 18.6 ± 5.3 mmHg (which represents a 14.9 ± 4.2% reduction of overall tone) and this was significantly ($P < 0.001$) less than the relaxation seen in the presence of both constrictors and L-NAME (Figure 3). Subsequent addition of 100 μM L-NAME brought about an increase in perfusion pressure to 196 ± 20 mmHg and this was significantly ($P < 0.01$) less than seen in the absence of L-arginine. In another 4 preparations the activity of L-arginine was examined at a level of tone comparable to that induced by the constrictor agents in the presence of L-NAME. In these preparations basal perfusion pressure was 32.4 ± 6.2 mmHg and this was increased to 177 ± 11 mmHg by increasing the individual concentrations of 5-hydroxytryptamine and histamine to 3 μM. Under these conditions 10 mM L-arginine reduced perfusion pressure by 18.8 ± 7.4 mmHg and this represents a 10.6% reduction in perfusion pressure. This reduction in perfusion pressure was not significantly different from that observed at the lower perfusion pressures described above but was significantly ($P < 0.001$) less than observed in the presence of L-NAME.

Effects of perfusion of the ear with CHAPS on the augmentation of constrictor responses

In six preparations the basal perfusion pressure was 41.3 ± 8.0 mmHg and the inclusion of 1 μM 5-hydroxytryptamine and 1 μM histamine increased perfusion pressure to 179 ± 18 mmHg. Perfusion of the bed with 0.3% CHAPS completely abolished endothelium-dependent relaxations to the test dose of acetylcholine (550 pmol) and the perfusion pressure of the constricted bed was 148 ± 16 mmHg and this did not differ significantly from that before perfusion with the detergent. Following perfusion of the bed with CHAPS, addition of 100 μM L-NAME did not alter the level of tone as the perfusion pressure was 150 ± 17 mmHg.

Effects of 100 μM N^G-nitro-L-arginine methyl ester and 10 mM L-arginine on vascular conductance at high constrictor tone

Submaximal (ca. 60%) pharmacological tone was induced by addition of 1 μM 5-hydroxytryptamine and 1 μM histamine to the perfusion fluid. In the presence of constrictor agents alone, pressure increased with flow (Figure 4a) and addition of 100 μM L-NAME caused an upward shift in the plot such that perfusion pressure was generally higher in the presence than in the absence of L-NAME at most flow rates. This effect of L-NAME was totally reversed by addition of L-arginine (10 mM).

In the 16 control preparations the conductance/flow relationships were determined and found to be linear with a gradient of 0.0039 ± 0.0002 $mmHg^{-1}$ and the mean elevation was 0.0173 ± 0.0007 $ml\,min^{-1}\,mmHg^{-1}$ ($r = 0.92$). It can be seen from Figure 4b that inclusion of L-NAME (100 μM) in the perfusion fluid altered the characteristics of the plot which became less linear and was best fitted to a 3rd order polynomial function ($y = 0.0042 + 0.0046x + 0.0012x^2 + 0.0004x^3$; $n = 10$). The deviation from the control conductance/flow relationships was particularly pronounced at the higher flow rates used. This effect of L-NAME was reversed by addition of 10 mM L-arginine to the buffer and the conductance/flow plot again approached linearity with a gradient of 0.0041 ± 0.005 $mmHg^{-1}$ and a mean elevation of 0.0196 ± 0.0013 $ml\,min^{-1}\,mmHg^{-1}$ ($r = 0.890$) (Figure 4b; $n = 8$). The slope and mean elevation point of this plot does not differ significantly from that of the control plot.

When L-arginine alone was added to constricted preparations there was no change in the pressure/flow relationships compared to the controls but when L-NAME was added there was a slight but non-significant upward shift in the regression line (Figure 5a). From the conductance/flow relationships it can be seen that addition of 10 mM L-arginine to the perfusion fluid did not alter either the slope (0.0037 ± 0.0003 $mmHg^{-1}$) or the mean elevation (0.0186 ± 0.0018 $ml\,min^{-1}\,mmHg^{-1}$) and r was 0.903 (Figure 5b; $n = 9$). Subsequent addition of 100 μM L-NAME did not alter the linear nature of the relationship between conductance and flow (Figure 5b). Both the gradient (0.0032 ± 0.0003 $mmHg^{-1}$) and the mean elevation (0.0144 ± 0.0009 $ml\,min^{-1}\,mmHg^{-1}$) ($r = 0.915$ and $n = 8$) did not differ significantly from the control values or those in the presence of L-arginine.

Discussion

The results presented in this paper clearly demonstrate that L-NAME is an inhibitor of endothelium-dependent relaxations in the isolated ear. Furthermore the effects of L-NAME on vascular conductance emphasize the important role of EDRF in modulating vascular tone. Interestingly, the inhibition of basal EDRF activity by L-NAME was sensitive to L-arginine while the inhibition of agonist-dependent EDRF activity was not.

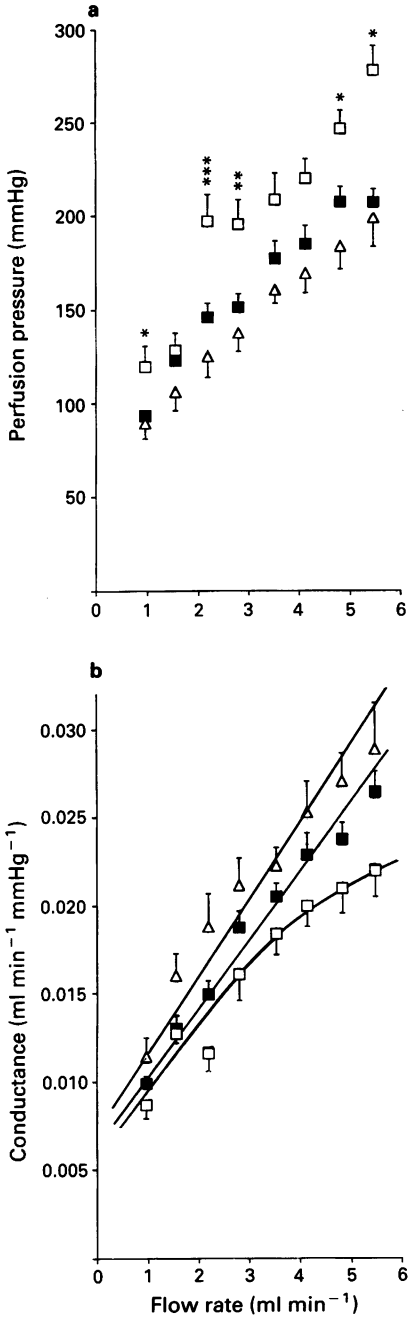


Figure 4 Effects of N^G-nitro-L-arginine methyl ester (L-NAME) and L-arginine on pressure/flow and conductance/flow relationships in the isolated rabbit ear preparation constricted with 5-hydroxytryptamine and histamine: (a) shows the pressure/flow relationships and (b) shows the conductance/flow relationships. In each case (■) shows the control data ($n = 16$), (□) shows the data in the presence of 100 μM L-NAME alone ($n = 10$) and (△) indicates the data ($n = 8$) following subsequent addition of L-arginine (10 mM). Statistically significant differences between the pressure/flow data obtained in the absence and presence of L-NAME are indicated: * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$.

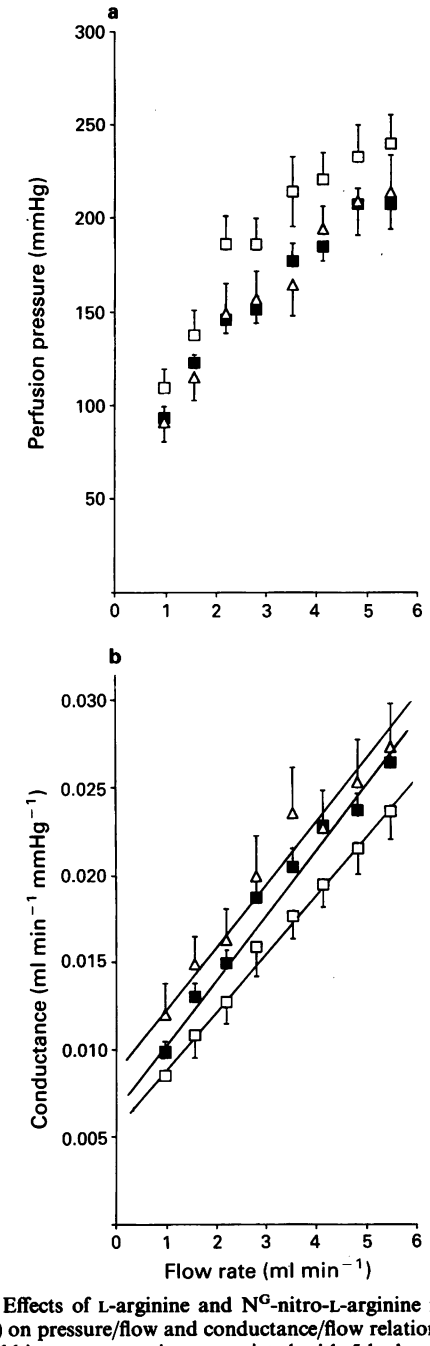


Figure 5 Effects of L-arginine and N^G-nitro-L-arginine methyl ester (L-NAME) on pressure/flow and conductance/flow relationships in the isolated rabbit ear preparation constricted with 5-hydroxytryptamine and histamine: (a) shows the pressure/flow relationships and (b) shows the conductance/flow relationships. In each case (■) shows the control data taken from Figure 4 ($n = 16$), (△) shows the regressions in the presence of 10 mM L-arginine alone ($n = 9$) and (□, $n = 8$) indicates the data following subsequent addition of L-NAME (100 μM).

The inhibition of the endothelium-dependent vasodilatation by L-NAME confirms the activity of this agent which has been described in other preparations (Rees *et al.*, 1990). The ability of 100 μM L-NAME to abolish vasodilatation to acetylcholine completely suggests that an L-arginine-derived substance is the sole mediator of endothelium-dependent vasodilatation in this preparation and rules out the participation of any other factors such as endothelium-derived hyperpolarizing factor (Taylor *et al.*, 1988). Low concentrations of L-NAME brought about non-competitive inhibition of acetylcholine-induced vasodilatation as shown by depression of the maximum response and the rightward shift of the dose-response curve. Complete inhibition was observed at the higher concentration of the inhibitor. At both concentrations of L-NAME the inhibition was insensitive to reversal by L-arginine. This finding contrasts with findings of Moore *et al.* (1990) and Kobayashi *et al.* (1990) but accords with those of Unmans (1990). Since we found that L-arginine did not influence the inhibitory effects of L-NAME in the rabbit ear, even at low concentrations of the inhibitor, then it is possible that the mechanisms responsible for the conversion of L-arginine to nitric oxide in the rabbit ear differ from those in some of the other vascular preparations studied (e.g. rat mesentery). Possible differences could exist in the mode of action of L-NAME at the level of

the enzymes responsible for the synthesis of nitric oxide or at the level of L-arginine uptake. The possibility of heterogeneity in the synthetic mechanisms for EDRF is also suggested by the *in vivo* findings of Gardiner *et al.* (1990) where administration of L-arginine reversed the haemodynamic changes induced by L-NAME in the mesenteric and renal beds but not apparently in the hindquarters of the rat.

It was observed that pretreatment of preparations with L-NAME increased the potency of the mixture of constrictor agonists while addition of this agent to preconstricted ears caused an increase in perfusion pressure. These actions are likely to reflect augmentation of tone since in the unconstricted preparation, L-NAME does not cause direct vasoconstriction as shown by the lack of significant effect on perfusion pressure in the resting preparations and previous findings (Randall *et al.*, 1990). Such augmentation of tone is thought to be due to loss of modulation by basally released EDRF as it has been shown to occur both on administration of inhibitors of EDRF synthesis/activity and on loss of the endothelium (Griffith *et al.*, 1984a,b; Martin *et al.*, 1986). In the present context the constriction caused by L-NAME was probably endothelium-dependent as it did not occur following functional destruction of the endothelium produced by perfusion with CHAPS. The augmentation of tone by L-NAME is probably due to the loss of modulation of constrictor responses by basally released EDRF, rather than that released by agonist stimulation, as neither 5-hydroxytryptamine nor histamine directly release EDRF in this preparation (Griffith *et al.*, 1988). Similar augmentations of tone have also been found to occur in the phenylephrine-constricted rat aorta in response to L-NAME, L-NMMA and N-iminoethyl ornithine (Rees *et al.*, 1990) and in response to L-NMMA and N^G-nitro-L-arginine in the noradrenaline-constricted rat isolated mesentery (Moore *et al.*, 1990). In the endothelium-intact ear preparation we found that the increases in perfusion pressure caused by L-NAME were completely reversed in a concentration-dependent manner by L-arginine. This action appeared to be selective and stereospecific as under identical conditions the enantiomer D-arginine (10 mM) only caused a modest relaxation. The action of D-arginine was comparable to the relaxation caused by L-arginine against established tone in the absence of L-NAME. Thomas *et al.* (1989) have also found that millimolar concentrations of both enantiomers cause modest endothelium-independent relaxations of phenylephrine-induced tone in rat aortic rings and this would appear due to non-specific interactions with vascular smooth muscle. It would thus appear that, while there might be a slight non-specific effect, as shown by the action of L-arginine against tone *per se*, L-arginine appears to cause appreciable reversal of the augmentation of tone caused by L-NAME.

In view of the augmentation of vascular tone discussed above, pressure/flow relationships were determined in the constricted preparation. In the control preparations perfusion pressure increased with increasing flow rate. Addition of L-NAME caused an upward shift in the regression of pressure on flow such that perfusion pressure was higher at most flow rates in the presence compared to the absence of L-NAME. These changes in pressure/flow characteristics are similar to those observed following endothelial destruction in the blood perfused mesentery (Randall & Hiley, 1988b) and in the presence of L-NMMA or haemoglobin in the rabbit ear (Griffith *et al.*, 1989; Griffith & Edwards, 1990). From the pressure/flow data, vascular conductance was determined at each flow rate used. We found a linear relationship between conductance and flow in the presence of EDRF, addition of L-NAME, however, caused a depression of the conductance/flow relationship with a departure from linearity which was particularly pronounced at the high flow rates, so that at these flow rates conductance showed smaller changes with increases in flow. This phenomenon has been observed in the ear for other inhibitors of EDRF activity/synthesis (Griffith & Edwards, 1990) and implies that in the absence of EDRF activity the vessels in the bed tend towards 'rigid tube' behaviour. The effects of L-NAME on pressure/flow relationships and hence conductance were completely reversed by addition of excess L-arginine. This effect is likely to be due to L-arginine reversing the blockade of nitric oxide synthesis by L-NAME as addition of L-arginine in the absence of L-NAME did not affect the pressure/flow relations. It should be noted also that prior addition of L-arginine to the perfusion fluid prevented L-NAME from altering significantly the pressure/flow characteristics. Thus it would appear that inhibitory effects of L-NAME on endothelium-dependent vascular regulation are sensitive to reversal by L-arginine.

The effects of L-NAME on vascular conductance may be accounted for by an inhibition of EDRF synthesis coupled to basal release. This release of EDRF may be spontaneous (Griffith *et al.*, 1984a,b) or in response to fluid flow (Rubanyi *et al.*, 1986) which is sensed through changes in shear stress (Pöhl *et al.*, 1986). Thus it would appear that, in common with agonist-dependent EDRF activity, basal EDRF activity is also inhibited by L-NAME. However, the results presented in this paper also demonstrate that the blockade by L-NAME of basal, but not agonist-stimulated, EDRF activity is reversed by L-arginine. This important observation may suggest that the enzymes coupled to EDRF release in response to agonist stimulation have different characteristics from those coupled to basal release. Other differences between agonist and basally released EDRF have previously been reported in respect to their differential sensitivity towards metabolic inhibitors (Griffith *et al.*, 1987b). In the present context one possible explanation for the differences in blockade by L-NAME could be that there are isoenzymes for nitric oxide synthesis. This is supported by the identification in N1E-115 neuroblastoma cells of cytosolic enzymes linked to humoral release and particulate enzymes accounting for tonic release of nitric oxide (Förstermann *et al.*, 1990). Other biochemical differences for the nitric oxide synthase have been reported, for example, the enzyme from rat cerebellum requires calmodulin (Bredt & Snyder, 1990) while that in rat peritoneal polymorphonuclear neutrophils is calmodulin-independent (Kosuga *et al.*, 1990). Heterogeneity in the synthetic mechanisms for nitric oxide has also been reported for porcine aortic endothelial cells (Mülsch *et al.*, 1989). In this respect Mülsch *et al.* (1989) identified a synthetic pathway which was calcium-dependent and one which was not. Both of the pathways were inhibited by 1 mM N^G-nitro-L-arginine and the calcium-independent pathway appeared to account for basal EDRF activity. More recently Radomski *et al.* (1990b) have found that vascular endothelial cells contain an inducible and a constitutive nitric oxide synthase. The former enzyme is calcium-independent and is induced by lipopolysaccharide and this may be prevented by glucocorticoids and cycloheximide. It would therefore appear that there is more than one enzyme system responsible for the production of EDRF.

In conclusion, we have found that L-NAME is a potent inhibitor of agonist-induced endothelium-dependent vasorelaxation. In this respect the action of L-NAME is not reversed by excess L-arginine and this contrasts with the action of this inhibitor in some other resistance beds. L-NAME would also appear to oppose endothelium-dependent modulation of tone in constricted preparations. In the latter situation L-arginine reverses the actions L-NAME. Taken together these results may point to differences between the mechanisms responsible for the synthesis of basal and those for agonist-dependent EDRF activity.

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Characterization of endothelin receptors on a human neuroblastoma cell line: evidence for the ET_A subtype

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1 Specific binding sites for synthetic endothelin (ET) isoforms were studied on intact cells of the SK-N-MC cell line, derived from a human neuroblastoma.

2 [^{125}I]-ET-1 (2.5×10^{-11} M) specifically bound to a single class of binding sites on these cells (Hill coefficient of 1.06 ± 0.04, $n = 3$) with an apparent K_d of $1.4 \pm 0.3 \times 10^{-9}$ M and a B_{max} of 3.1 ± 1.0 pmol mg^{-1} protein. [^{125}I]-ET-3 (2.5×10^{-11} M), did not specifically bind to SK-N-MC cells.

3 The binding of [^{125}I]-ET-1 was competitively inhibited by other ET isoforms, the order of potency being ET-1 > sarafotoxin S6b > ET-3.

4 Association of 1 nM [^{125}I]-ET-1 at 37°C reached apparent equilibrium at 60–80 min, with half-maximal binding being achieved at 12 min.

5 Dissociation was measured after both 10 min and 60 min of association with 64% and 30% respectively of specifically bound [^{125}I]-ET-1 dissociating. The actual amounts of [^{125}I]-ET-1 dissociated were similar in both cases.

6 Incubation of [^{125}I]-ET-3 with SK-N-MC cells at 37°C for 60 min did not result in significant degradation of this peptide. However, [^{125}I]-ET-1 was broken down by incubation with SK-N-MC cells, the pattern of degradation of dissociable [^{125}I]-ET-1 (and that found in the supernatant) being different from that of non-dissociable [^{125}I]-ET-1.

7 ET-1 concentration-dependently induced an increase in total inositol phosphate accumulation in sub-confluent (but not in confluent) cultures of SK-N-MC cells ($EC_{50} = 6.43 \pm 1.9 \times 10^{-10}$ M). ET-3 was without effect.

8 These results show that ET-1 specifically binds to SK-N-MC cells with the characteristics of an ET_A receptor. Our earlier finding that adrenal chromaffin cells express an ET_B receptor indicates the existence of multiple ET receptor types on neuronal cells.

Keywords: Endothelin; sarafotoxin; endothelin receptors; human neuroblastoma cell line; inositol phosphates

Introduction

Endothelin-1 (ET-1), a potent vasoconstrictor isolated from the culture medium of porcine aortic endothelial cells (Yanagisawa *et al.*, 1988) is one of a family of structurally homologous peptides which includes ET-2, ET-3 and the sarafotoxins of the venom of the snake *Actractaspis engaddensis* (Yanagisawa & Masaki, 1989). The endothelins have distinct pharmacological activities including effects on vascular and non-vascular smooth muscle (Auguet *et al.*, 1988; Secrest & Cohen, 1989; Huang *et al.*, 1990), modulation of hormone secretion (Fukuda *et al.*, 1988; Takagi *et al.*, 1988; Morishita *et al.*, 1989) and may act as a growth factor (Brown & Littlewood, 1989). It has been found that endothelin is synthesized in tissues other than endothelial cells, including neural tissues such as the spinal cord (Shinmi *et al.*, 1989) and brain (Matsumoto *et al.*, 1989). We have previously characterized the endothelin binding site on bovine adrenomedullary chromaffin cells, a cell type widely used as a model for neuronal cells in culture (Wilkes & Boarder, 1991). This binding site was found to differ from that observed on A10 cells, a vascular smooth muscle cell line, leading to speculation on the existence of neuronal-type and non-neuronal-type endothelin receptors. Recent cloning of endothelin receptors (Arai *et al.*, 1990; Sakurai *et al.*, 1990) has defined two receptor types. The binding site on the A10 cell line has the characteristics of the ET_A receptor, which shows high selectivity for ET-1, while the ET_B receptor, which is non-selective for the ET isoforms, appears to be present on the chromaffin cell.

The present study was undertaken in order to characterize the endothelin binding site(s) on the SK-N-MC cell line, which was derived from a human neuroblastoma (Biedler *et al.*, 1973) and serves as a model for the peripheral nervous system, in order to investigate further the existence of a neuronal-type endothelin receptor. Earlier reports on the binding of endothelin *in vitro* (Hirata *et al.*, 1988) have shown that this binding is, at least in part, non-dissociable. Therefore reversibility of binding of endothelin to SK-N-MC cells, and the differential breakdown of ET-1 and ET-3, was investigated. In addition, a possible receptor-effector mechanism, that of intracellular inositol phosphate turnover, in response to ET-1 and ET-3, was investigated in the SK-N-MC cells.

Methods

Cell culture

SK-N-MC cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, streptomycin 100 μg ml^{-1}, penicillin 100 iu ml^{-1} and fungizone 2.5 μg ml^{-1} in a humidified atmosphere of 5% CO_2 at 37°C. Confluent flasks were passaged weekly. For experimental use cells were seeded into 24-well multiwell plates and grown to confluence. Experiments were performed in a balanced salts solution (BSS) consisting of (mM): NaCl 120, KCl 5.4, $NaHCO_3$ 16.2, $MgSO_4$ 0.8, NaH_2PO_4 1, $CaCl_2$ 1.8, D-glucose 5.5 and 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid 30; at pH 7.4.

Competition binding studies

Whole cell monolayer cultures were incubated (200 μl BSS + 0.1% bovine serum albumin (BSA)) in the presence of 2.5×10^{-11} M [^{125}I]-ET-1 or [^{125}I]-ET-3 and various concentrations (3×10^{-12} to 10^{-7} M) of unlabelled ET-1, ET-3 or

sarafotoxin S6b (STX S6b). The cell cultures were incubated at 37°C for 60 min. After aspiration of the incubation medium and extensive washing (4 × 1 ml of cold BSS) the cells were solubilized with 0.1 M NaOH and cell-bound radioactivity determined by gamma counting. Protein determination via the Lowry method (Lowry *et al.*, 1951) was performed on four wells of each plate.

Time course of association/dissociation

ET-1 was iodinated via the chloramine T method (Hunter & Greenwood, 1962) and purified on sephadex G10 columns to produce [^{125}I]-ET-1 with a specific activity of 400–500 Ci mmol^{-1}. Whole cell monolayer cultures were incubated (200 μl of BSS + 0.1% BSA) with 1 nM [^{125}I]-ET-1 (specific activity 400 Ci mmol^{-1}) for up to 90 min at 37°C. Non-specific binding was determined by the addition of 100 nM unlabelled ET-1. At the end of the incubation the cell monolayers were extensively washed (4 × 1 ml cold BSS) solubilized and counted. To determine the time course of dissociation whole cell monolayer cultures were incubated with 200 μl of 1 nM [^{125}I]-ET-1 for 10 or 60 min at 37°C. The labelled medium was then removed and replaced with 100 nM unlabelled ET-1. The cells were further incubated at 37°C, for up to 60 min, then washed, solubilized and counted.

Breakdown of endothelin

Whole cell monolayer cultures were incubated with 200 μl of 0.1 nM [^{125}I]-ET-1 or [^{125}I]-ET-3 (specific activity 2000 Ci mmol^{-1}) at 37°C for 60 min. The supernatant was removed and the cells incubated for a further 30 min with 200 μl of unlabelled ET (100 nM) to allow the [^{125}I]-ET to dissociate. Remaining cell-bound [^{125}I]-ET was extracted by the addition of 100 μl of 1% acetic acid followed by freezing/thawing. These three fractions (supernatant, dissociable and acid-extractable) were subjected to reverse-phase high-performance liquid chromatography (h.p.l.c.). The samples were injected onto a Vydac C_{18} column (25 × 4.6 mm) and eluted through with 0.1% trifluoroacetic acid (v/v in water) and acetonitrile, the concentration of acetonitrile initially being 10% v/v rising to 30% by 10 min and thereafter to 45% by 30 min. The flow rate was 1 ml min^{-1} and 1 min fractions were collected and subjected to gamma spectroscopy.

Total inositol phosphate production

Sub-confluent SK-N-MC cells were incubated with 1 μCi/well of *myo*-[^{3}H]-inositol in 0.5 ml of inositol-free Dulbecco's modified Eagle's medium supplemented with streptomycin 100 μg ml^{-1}, penicillin 100 i.u. ml^{-1}, fungizone 2.5 μg ml^{-1}, glutamine 27 mg 100 ml^{-1} and 1% non-essential amino acids for 48 h at 37°C. At the end of this loading period the monolayer cultures were washed twice and incubated in 0.5 ml of BSS + 10 mM lithium chloride for 10 min at 37°C. The test agents (ET-1 or ET-3 to a final concentration of 0.03–100 nM) were added and the cells incubated for a further 30 min. At the end of this period 0.5 ml of ice-cold methanol was added, followed by chloroform extraction and purification of total inositol phosphates on Dowex-1 (Cl^-) (Rooney & Nahorski, 1986).

Materials

ET-1, ET-3 and STX S6b were from Peptide Research Institute (Osaka, Japan). [^{125}I]-ET-1, [^{125}I]-ET-3 (specific activity of 2000 Ci mmol^{-1}) and Na^{125}I were from Amersham International (Buckinghamshire), *myo*-[2-^{3}H]-inositol was from New England Nuclear (Stevenage, Herts.). All tissue culture reagents were from Gibco (Paisley, Scotland); h.p.l.c. columns were from Technicol, Cheshire. Other chemicals were from Sigma (Poole, Dorset) or Fisons (Loughborough, Leicestershire).

Results

[^{125}I]-ET-1 specifically bound to SK-N-MC cells: non-specific binding (in the presence of 100 nM unlabelled ET-1) was typically less than 10% of total binding. The binding of [^{125}I]-ET-1 was competitively inhibited by unlabelled ET-1, ET-3 and STX S6b, the order of affinity was ET-1 > STX S6b > ET-3 (Figure 1a). The IC_{50} value for inhibition by ET-1 was 3.3 ± 0.9 × 10^{-9} M (mean ± s.e.mean, n = 3). Computer analysis of isotopic dilution binding data for ET-1 demonstrated an apparent K_d of 1.4 ± 0.3 × 10^{-9} M and an apparent B_{max} of 3.1 ± 1.0 pmol mg^{-1} protein. A typical Scatchard plot, with data consistent with a single affinity binding site, is shown in Figure 1b. This was confirmed by a Hill coefficient of 1.06 ± 0.04 (mean ± s.e.mean, n = 3). In contrast, ET-3 and STX S6b inhibited binding only weakly. In the presence of 100 nM unlabelled ET-3, 80% of specific binding of [^{125}I]-ET-1 to SK-N-MC cells remained and in the presence of 100 nM unlabelled STX S6b, 30% of specific binding was present (Figure 1a). Further experiments employing [^{125}I]-ET-3 demonstrated very little evidence of specific binding of 2.5 × 10^{-11} M [^{125}I]-ET-3 to SK-N-MC cells.

Specific binding of [^{125}I]-ET-1 to SK-N-MC cells reached apparent equilibrium at 60–80 min, with half-maximal binding being achieved at 12 min (Figure 2). The rate of dissociation of [^{125}I]-ET-1 from SK-N-MC cells on addition of 100 nM ET-1 is shown in Figure 2. After 10 min of association the addition of 100 nM unlabelled ET-1 resulted in a loss of 64% bound [^{125}I]-ET-1 after 20 min incubation. The pattern of dissociation after association for 60 min was similar, with 30% of specifically bound [^{125}I]-ET-1 dissociating over 20 min. Although the relative proportion of dissociable bound [^{125}I]-ET-1 differed between the two times of association, the actual

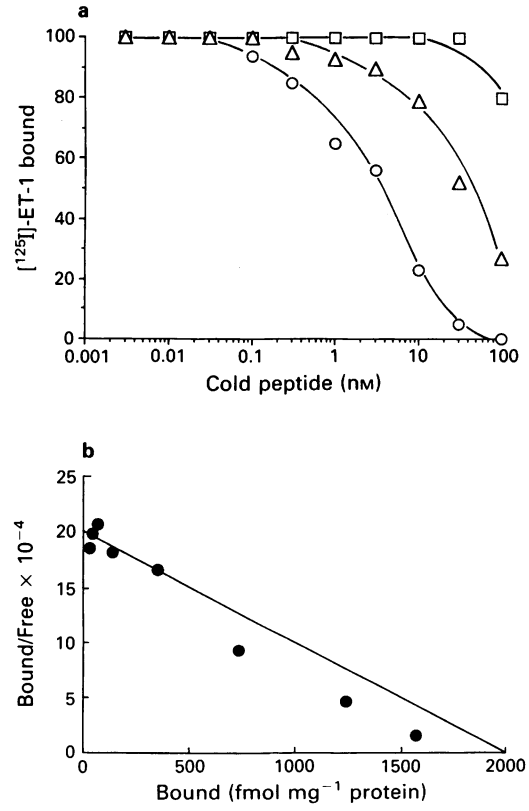


Figure 1 Competitive inhibition of binding of 2.5 × 10^{-11} M [^{125}I]-endothelin-1 ([^{125}I]-ET-1) to SK-N-MC cell monolayer cultures. (a) Competitive inhibition by unlabelled ET-1 (○), ET-3 (□) or STX S6b (△). Each point represents the mean of three separate experiments each performed in duplicate. (b) Scatchard transformation of competitive inhibition of [^{125}I]-ET-1 binding by unlabelled ET-1. Data are from a typical experiment.

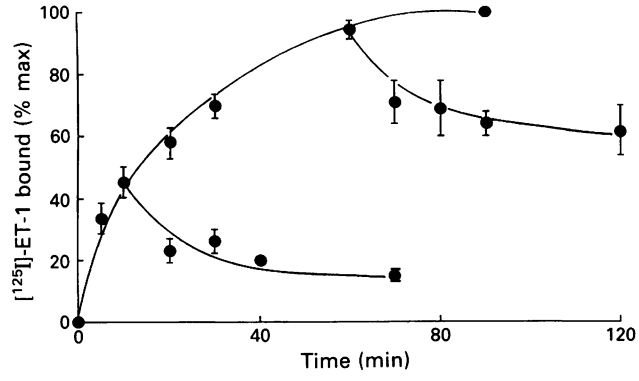


Figure 2 Time course of association/dissociation of [^{125}I]-endothelin-1 ([^{125}I]-ET-1) to SK-N-MC cells. Association was observed by incubation of the cells with 1 nM [^{125}I]-ET-1 for up to 90 min. Dissociation was observed after association for 10 min and 60 min by the removal of radiolabel and the addition of 100 nM unlabelled ET, and incubation for a further 60 min. Data are mean of three experiments, each performed in duplicate; s.e.mean shown by vertical bars.

amount of radioactivity dissociating in each case was similar. This suggests that the binding of [^{125}I]-ET-1 to SK-N-MC cells consists of two components; an irreversibly bound component which increases with time of incubation, and a reversibly bound component which remains constant.

The breakdown of [^{125}I]-ET-1 and [^{125}I]-ET-3 by SK-N-MC cells was investigated by reverse-phase h.p.l.c. Recovery from the column both of original [^{125}I]-ET-1 and of the products of the incubation was typically around 50%. Significant breakdown of [^{125}I]-ET-1 occurred during incubation with SK-N-MC cells (Figure 3). In the supernatant 54% of radioactivity was associated with the native [^{125}I]-ET-1 peak (Figure 3b). There were three other main peaks: one consisting of 9% of total radioactivity eluting just behind the solvent front; one immediately prior to the native peptide peak (13% of total radioactivity); and one several fractions after the native peptide peak (10% of total radioactivity). The elution profile of the dissociable fraction was similar to that of the supernatant (Figure 3b,c). However, the elution profile of the cell-bound acid-extractable fraction (Figure 3d) exhibited only two peaks; the native [^{125}I]-ET-1 peak consisting of 50% of radioactivity, and a peak immediately prior to the native peptide peak, consisting of 38% of radioactivity. The results shown are a representative of three experiments exhibiting similar results. Overall, the percentages of radioactivity eluting with the native peptide were: supernatant, 57 ± 2%; dissociable, 40 ± 8%; acid-extractable, 58 ± 9% ($n = 3$ in each case). The elution profile of [^{125}I]-ET-3 was largely unchanged after incubation with SK-N-MC cells at 37°C for 60 min (Figure 4).

The effects of endothelins on total inositol phosphate accumulation by SK-N-MC cells were studied. ET-1 present during a 30 min incubation stimulated an increase in total labelled inositol phosphate content in sub-confluent SK-N-MC cells to 220% of control (in the absence of ET-1) with an EC_{50} value of $6.43 \pm 1.9 \times 10^{-10}$ M. However, on occasions when the SK-N-MC cells had attained confluence during the 48 h loading period, the total inositol phosphate response to ET-1 was very much attenuated (Figure 5a). ET-3, up to 100 nM, had no effect on total inositol phosphate production by either confluent or sub-confluent SK-N-MC cell cultures (Figure 5b).

Discussion

We have previously described the characteristics of the binding site for endothelin on chromaffin cells, a model for neuronal cell function (Wilkes & Boarder, 1991). Here we describe the characteristics of the endothelin binding site on another model of neuronal cell function, the SK-N-MC cell

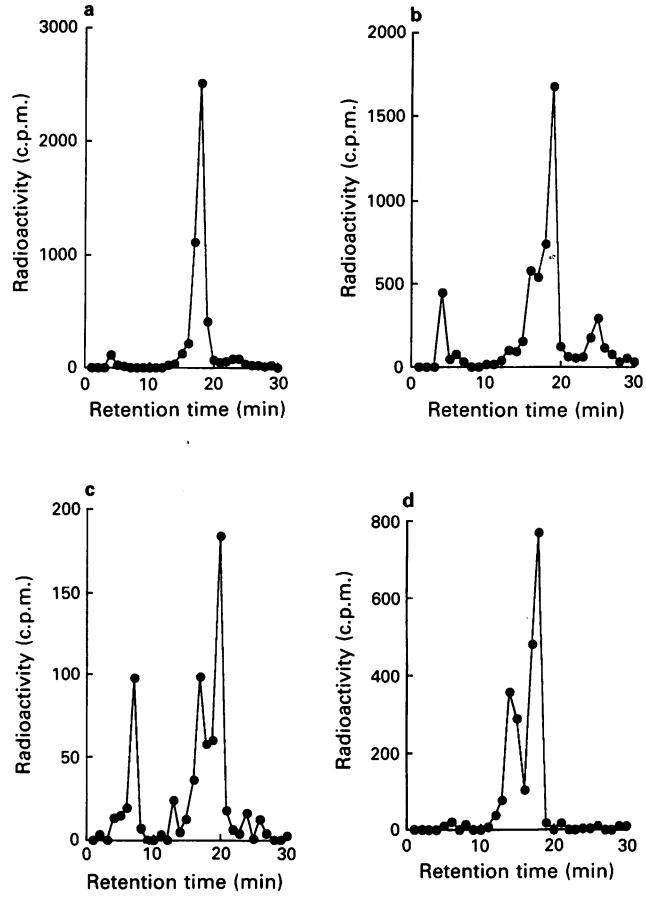


Figure 3 Analysis of [^{125}I]-endothelin-1 ([^{125}I]-ET-1) fractions by reverse phase h.p.l.c. (a) Native [^{125}I]-ET-1 before incubation with SK-N-MC cells. (b) Supernatant after incubation of 10^{-10} M [^{125}I]-ET-1 with SK-N-MC cells for 60 min. (c) Dissociable [^{125}I]-ET-1, after removal of supernatant and incubation with 100 nM unlabelled ET-1 for a further 30 min. (d) Acid-extractable [^{125}I]-ET-1, after extraction of the SK-N-MC cells with 1% acetic acid. Results are from a typical experiment.

line. The results presented demonstrate the existence of high-affinity binding sites for ET-1 on the SK-N-MC cell line. The observed order of affinity of the inhibiting ET isoforms of ET-1 > STX S6b > ET-3 has been described for other cell types (Galron *et al.*, 1989; Wilkes & Boarder, 1991). Recently the cloning of two separate endothelin receptors has been reported. The first, designated ET_A, shows high selectivity for ET-1, with the order of affinity of the ET isoforms being ET-1 > ET-2 > STX S6b > ET-3 (Arai *et al.*, 1990). The other endothelin receptor (ET_B) is non-selective for the ET isoforms (Sakurai *et al.*, 1990). The endothelin binding site on chromaffin cells exhibits equal affinity for the ET isoforms, suggesting that the ET_B receptor is expressed on these cells. However, it appears that the endothelin receptor expressed on SK-N-MC cells resembles the ET_A type. It is therefore evident that there exists heterogeneity of endothelin receptors on neuronal type cells in culture, even though the two cell types studied are both of peripheral neuronal origin. Other studies indicating types of endothelin receptors in brain preparations (Crawford *et al.*, 1990; Hiley *et al.*, 1990) are complicated by the likelihood that part of the response or binding may be due to non-neuronal elements, such as glial cells. Studies on cells in culture, such as those described here, avoid this complication.

The early reports of endothelin binding to whole cells indicated that binding may be complex in that only part of the binding was in equilibrium (Hirata *et al.*, 1988). To pursue these characteristics of binding we examined both association and dissociation in human neuroblastoma cells. The binding of [^{125}I]-ET-1 on incubation at 37°C reached apparent equilibrium at 60–80 min. Dissociation was observed after removal

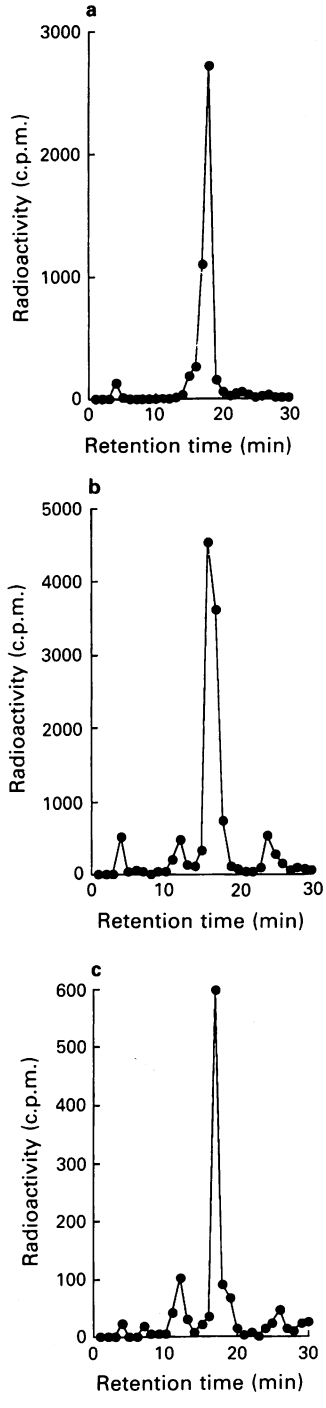


Figure 4 Analysis of [^{125}I]-endothelin-3 ([^{125}I]-ET-3) fractions by reverse phase h.p.l.c. (a) Native [^{125}I]-ET-3 before incubation with SK-N-MC cells. (b) Supernatant after incubation of 10^{-10} M [^{125}I]-ET-3 with SK-N-MC cells for 60 min. (c) Dissociable [^{125}I]-ET-3, after removal of supernatant and incubation with 100 nM unlabelled ET-3 for a further 30 min. The radioactivity in the acid-extractable fraction was too low to be analysed. Results are from a typical experiment.

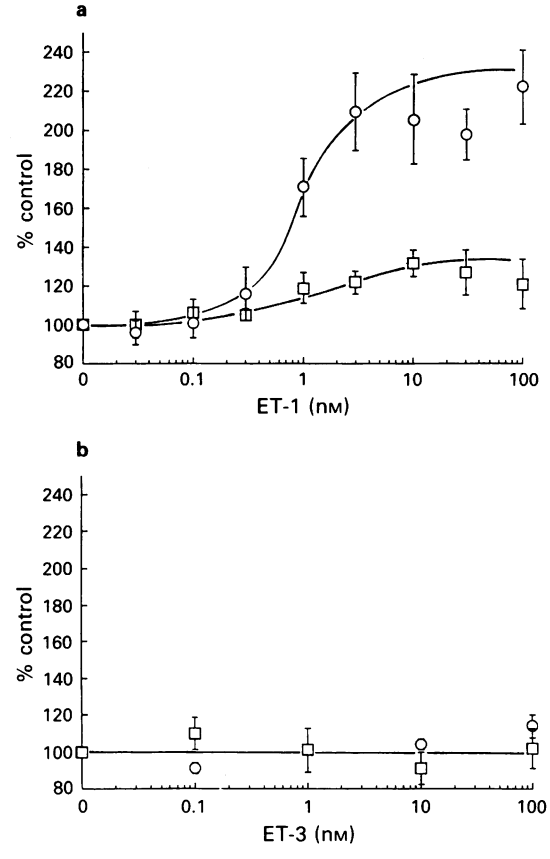


Figure 5 Dose-response curves for accumulation of labelled total inositol phosphates in response to a 30 min incubation with (a) endothelin-1 (ET-1) or (b) ET-3, by sub-confluent (○) or confluent (□) monolayer cultures of SK-N-MC cells. Each point represents the mean of three separate experiments, each performed in quadruplicate; s.e.mean shown by vertical bars.

of the label and addition of 100 nM unlabelled ET-1, over 60 min after incubation for both 10 min and 60 min. Only a proportion of the cell-associated radioactivity could be dissociated, with the absolute amount in equilibrium being constant after both 10 and 60 min incubation. However, the proportion of non-dissociable cell-associated radioactivity increased with time. This suggests that cell-associated [^{125}I]-ET-1 is compartmentalized, possibly with internalization of the receptor-agonist complex, and that the pool of dissociable cell-associated ET-1 is constant despite increasing amounts of bound ET-1. It is possible therefore, that the number of receptors on the cell surface remains constant despite a sustained loss due to internalization, implying a level of supply of receptors at the cell surface which is equal to the rate of agonist-induced loss. This model assumes that all the receptors on the cell surface bind ET-1 in a reversible manner in equilibrium with the peptide in solution.

Reverse-phase h.p.l.c. was employed to study metabolites of [^{125}I]-ET during incubation with SK-N-MC cells. The lack of high-affinity binding sites for ET-3 on these cells, and the observation that little degradation of [^{125}I]-ET-3 occurred after incubation at 37°C for 60 min, suggests that interaction of the peptide with an ET binding site may be required for degradation.

[^{125}I]-ET-1 was substantially broken down on incubation with SK-N-MC cells, with only 57% of radioactivity being associated with the native peptide in the incubation supernatant, and 34% in the dissociable fraction. The pattern of breakdown of [^{125}I]-ET-1 was similar between the supernatant and dissociable fractions, presumably reflecting the equilibrium that exists between them. The elution profile of the non-dissociable fraction was different from the supernatant and dissociable fractions, reflecting the non-equilibrium nature of this fraction. The breakdown product eluting in a peak of radioactivity just prior to the native peptide appears to be common to all three fractions. However, in the acid-extractable fraction this is apparently the only breakdown product, while in the dissociable and supernatant fractions further breakdown products are observed.

The reason for the disparity between the breakdown products of the dissociable and non-dissociable fractions is unclear. It is possible that the binding of ET-1 to the cell surface receptor is in simple equilibrium, and that breakdown

of the endothelin occurs on interaction of the peptide with the receptors by an ectoyrotease. This would be consistent with the breakdown of non-dissociable peptide being a consequence of internalization, since it would be by different proteolytic activity located within the cell.

SK-N-MC cells exhibit specific high-affinity binding sites for ET-1, with several pools of bound ET-1 existing within the cell membrane or cell itself. These binding sites appear to be coupled with inositol phosphate turnover in subconfluent SK-N-MC cells. However, when these cells attain confluence this response is greatly reduced. The reason for this is unclear. It is not because the confluent cell no longer expresses the endothelin receptor; the binding studies were performed on confluent cells which demonstrated a B_{max} of $3.1 \pm 1.0\,pmol\,mg^{-1}$ protein. It therefore appears that the receptor becomes uncoupled from turnover of inositol phosphates with increasing cell density, although the point at which this uncoupling occurs was not investigated in this study.

The data appear to indicate that occupation of only a small proportion of the receptor population appears to be necessary for stimulation of inositol phosphate turnover. The EC_{50} for this response was 6.43×10^{-10} M; at this concentration of ET-1 approximately 20% of the binding sites were occupied. However, this interpretation is complicated by the fact that the binding data were collated from studies on confluent cells, and data on total inositol phosphate turnover were from studies on sub-confluent cells.

These data demonstrate that SK-N-MC cells possess high-affinity binding sites for ET-1, and that the characteristics of these binding sites differ from those of another peripheral neuronal cell model (Wilkes & Boarder, 1991), suggesting that both ET_A and ET_B receptors may be present on peripheral neuronal cells. Further work is needed in order to assess the physiological significance of this observation.

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The effect of inhibitors of nitric oxide biosynthesis and cyclic GMP formation on nerve-evoked relaxation of human cavernosal smooth muscle

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1 The inhibitory transmission in isolated preparations of cavernosal smooth muscle from human penis has been studied.

2 Electrical field stimulation (EFS; 2–64 pulses/train, 0.8 ms pulse duration, 10 Hz) evoked relaxation of preparations treated with guanethidine (50 μM). The EFS-evoked relaxations were atropine-resistant and tetrodotoxin-sensitive indicating their origin to be non-adrenergic, non-cholinergic (NANC) nerve stimulation.

3 EFS-evoked relaxation was attenuated dose-dependently by the nitric oxide (NO)-synthase inhibitor, L-N^G-nitro arginine (L-NOARG; 0.3–100 μM) but not by D-N^G-nitro arginine. The inhibitory effect of L-NOARG on transmission was antagonized by L-arginine (100 μM), a NO precursor, but not by D-arginine.

4 Incubation with methylene blue (10–50 μM), a known inhibitor of guanylate cyclase activation by NO, caused a concentration-related inhibition of EFS-evoked relaxation.

5 It is concluded that NANC nerve-evoked relaxation of human cavernosal smooth muscle is mediated by NO or a NO-like substance.

Keywords: Penis; penile erection; nitric oxide; non-adrenergic non-cholinergic transmission; methylene blue

Introduction

The erectile tissue of the human penis is contained within the corpora cavernosa and consists of endothelium-lined sinusoidal spaces surrounded by smooth muscle bundles. The relaxation of this cavernosal smooth muscle is a vital part of the sequence of psychological, neurological and vascular events necessary for erection to occur (Krane *et al.*, 1989). Previous studies on human isolated cavernosal tissue have shown that the relaxant response is nerve-mediated by a non-adrenergic, non-cholinergic (NANC) mechanism (Saenz de Tejada *et al.*, 1988b). Ambache *et al.* (1975) had isolated an inhibitory factor from a related smooth muscle, the bovine retractor penis (BRP), which closely mimicked the effects of stimulation of its NANC innervation. This inhibitory factor has since been identified as nitric oxide (NO, Martin *et al.*, 1988), the release of which mediates nerve-evoked relaxation in the BRP (Martin *et al.*, 1991). We have now investigated a possible role of NO in the nerve-evoked relaxation of human cavernosal smooth muscle by observing the effects of L-N^G-nitro arginine (L-NOARG), an inhibitor of NO biosynthesis (Moore *et al.*, 1990) and methylene blue, an antagonist of NO-mediated smooth muscle relaxation (Gruetter *et al.*, 1981). A preliminary account of some of the results described here has been presented at a meeting of The Physiological Society (Pickard *et al.*, 1991).

Methods

Ethical approval for the use of human tissue in this study was granted by the Newcastle Joint Ethics Committee.

Specimens of cavernosal tissue were obtained from 23 men undergoing penile surgery. At an appropriate stage during the operation a biopsy of cavernosal tissue was taken, placed immediately in chilled Krebs solution and transferred to the laboratory. The tissue was then either used straight away or stored overnight in fresh Krebs solution at 4°C. The tissue was prepared for the experiments by placing the biopsy on Krebs-soaked tissue paper in a Petri dish and cutting strips of cavernosal tissue measuring approximately 5 × 2 × 2 mm with a pair of fine scissors. The strips were then tied at each end with cotton threads and mounted in 1 or 2 ml organ baths containing Krebs solution maintained at 37°C and continuously aerated with 95% O_2 and 5% CO_2. The upper end of the strip was suspended from an isometric force transducer which was in turn connected to an amplifier, chart recorder and storage oscilloscope. An initial tension of 2 g was applied to the strip which was then allowed to equilibrate for 90 min. During this period the preparations were frequently washed with fresh Krebs solution (composition, mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 11).

Electrical field stimulation (EFS) was provided by a Grass S88 stimulator and applied via two parallel platinum wire electrodes set vertically within the organ bath at opposite sides of the suspended tissue. Parameters of EFS were as follows: trains of between 2 and 64 pulse at 100 s intervals, pulse duration = 0.8 ms, pulse-frequency = 10 Hz, supramaximal voltage (60 V). Once a stable resting tension had been reached the responses to EFS (2, 4, 8, 16, 32, 64 pulses/train) were recorded. Guanethidine (50 μM) was then added to the bath in order to block noradrenergic nerves and raise the smooth muscle tone. The responses to EFS were again recorded once the new level of tension had stabilised. If necessary, noradrenaline (NA; 10 μM) was also added to the bathing medium to maintain the level of tone required for the display of graded relaxant responses to EFS. In such cases the responses to EFS were recorded following its addition. The effects of treatment with atropine (3 μM) for a minimum of 20 min and tetrodotoxin (TTX; 0.3 μM for 5 min) on electrically-evoked relaxation were also tested on some strips.

Use of L-N^G-nitro arginine, L-arginine, D-N^G-nitro arginine and D-arginine

A train length that gave a consistent, sub-maximal relaxant response was chosen and repeated every 100 s. L-N^G-nitro

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arginine was then added to the bath in cumulatively rising concentrations of 0.3, 1, 3, 10, 30 and 100 μM. Each increment was added only after the response to EFS had been stable for 5 min. The effect of L-arginine on preparations treated with L-NOARG was tested in two ways. According to one procedure, after ascertaining maximum stable inhibition by L-NOARG (30 μM), L-arginine (100 μM) was added to the bath in the continued presence of L-NOARG and the exposure continued until the response to electrical stimulation had stabilized. In the second procedure, after obtaining maximum stable inhibition of the response to EFS by L-NOARG (30 μM), the drug was removed from the bath by repeated washes over a period of 10 min; when the relaxant responses to EFS had stabilized, L-arginine (100 μM) was introduced into the bath and the exposure maintained until the full effect of its introduction had been determined. The effect of substituting the optical isomers, D-arginine for L-arginine and D-N^G-nitro arginine (D-NOARG), was also tested on some preparations.

Use of methylene blue

Following the recording of control relaxant responses to EFS and glyceryl trinitrate (GTN; 0.3 μM), strips from 12 individuals were washed with fresh Krebs solution to remove guanethidine and NA and then incubated with methylene blue at concentrations of 10 μM and 50 μM for 1 h. At the end of this period, tone was re-established by adding guanethidine and NA without washing out methylene blue from the bathing solution. The responses to EFS and GTN (0.3 μM) were then again recorded. The effects of incubation with methylene blue (10 and 50 μM) on the magnitude of relaxation evoked by prostaglandin E_1 (PGE_1; 1 μM) were also tested on preparations from 3 individuals.

Statistical considerations

The amount of tissue obtained was insufficient to allow all experiments to be performed on isolated preparations from every individual. Results are expressed as mean ± s.e.mean of data from n individuals. Statistical significance was tested by Student's t test for paired data and accepted if $P < 0.05$ (one-tailed).

Drugs and solutions

Stock solutions of atropine sulphate (Sigma), L-arginine (Sigma), D-arginine (Sigma), glyceryl trinitrate (Lipha), guanethidine sulphate (CIBA), methylene blue (Sigma), L-N^G-nitro arginine (Sigma), prostaglandin E_1 (Upjohn), D-N^G-nitro arginine (Bachem), noradrenaline (Sigma) and tetrodotoxin (Sankyo) were all made up in distilled water and stored at −20°C.

Results

Response to electrical field stimulation

Prior to the addition of guanethidine, EFS produced varying degrees of contraction and relaxation in preparations from different individuals (Figure 1a,b,c). In contrast, EFS with guanethidine (50 μM) present in the bathing solution resulted in purely relaxant responses (Figure 1d). Preparations from 6 individuals required the addition of NA (10 μM) to ensure a sustained level of tone sufficient for recording consistent relaxant responses to EFS.

Once a stable tone had been achieved either with guanethidine (50 μM) alone or with the addition of noradrenaline (10 μM), a clear relationship emerged between the magnitude of the relaxant response and the number of pulses comprising the electrical stimulus (Figure 2). With trains of 16 pulses the relaxation began approximately 1 s after the start of the stimulus and reached its maximum at 8 ± 0.5 s ($n = 4$). Exposure of strips from 5 individuals to atropine (3 μM, 20 min) caused a slight, statistically insignificant, increase (3 ± 6%) in the degree of relaxation evoked by EFS. Tetrodotoxin (0.3 μM) completely abolished the relaxant response evoked by EFS in all strips tested ($n = 5$) within 5 min of its addition. There were no qualitative differences in the behaviour of strips between those used immediately and those stored for 24 h at 4°C. Tissue from 6 individuals was used both immediately and following 24 h storage. In these preparations, electrically evoked relaxation expressed as a percentage of maximal NA contraction increased by 20 ± 10% ($n = 6$, P = NS) in strips stored for 24 h compared to those used immediately.

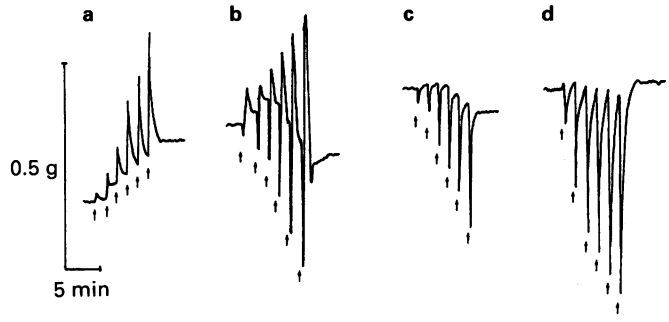


Figure 1 Isolated preparations of human cavernosal smooth muscle. Responses to electrical field stimulation (EFS) in preparations from 3 individuals in the absence of guanethidine (a–c) illustrating a purely contractile response (a), a mixed response (b) and a purely relaxant response (c). In the presence of guanethidine (50 μM) only relaxant responses were evoked by EFS and a typical example is shown in (d). In each panel, EFS comprised of trains of 2, 4, 8, 16, 32 and 64 pulses (10 Hz) in that sequence and the arrows mark the delivery of EFS.

Effect of L-N^G-nitro arginine

L-NOARG (0.3–100 μM), had no obvious effect on the tone of the preparation but inhibited electrically-evoked relaxations in a dose-dependent manner up to a maximum of 86 ± 4.5% at a concentration of 30 μM (Figures 3, 4). The inhibition of the relaxant response by L-NOARG was long lasting and only partially reversed by repeated washes with fresh Krebs solution (see below).

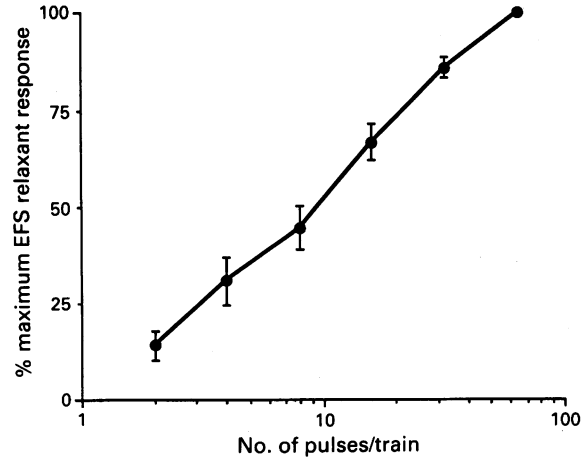


Figure 2 The effect of number of pulses comprising electrical field stimulation (EFS; 10 Hz) on the magnitude of the relaxant response. The responses are expressed as % of the maximal relaxant response. Each point is the mean response (bars showing s.e.mean) of 20 experiments conducted in the presence of guanethidine (50 μM).

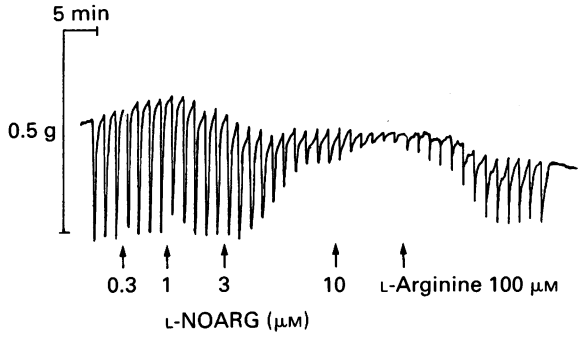


Figure 3 Isolated preparation of human cavernosal smooth muscle. A representative experiment demonstrating the inhibition of electrical field stimulation (16 pulses; 10 Hz)-evoked relaxation by cumulatively administered L-N^G-nitro arginine (L-NOARG, 0.3–10 μM) and the partial reversal of the inhibition by L-arginine (100 μM). Guanethidine (50 μM) was present throughout the experiment.

Identical parallel experiments with D-NOARG up to 100 μM on strips from 2 individuals, produced no inhibition of the relaxant response to EFS.

Effect of L-arginine

Resting tone and the relaxation produced by EFS were unaffected by the addition of L-arginine at a concentration of 100 μM. In the continued presence of L-NOARG (30 μM) the addition of L-arginine (100 μM) restored EFS-evoked relaxation to 26 ± 5.2% of the control ($n = 10$, $P < 0.01$); a representative tracing of an original recording is shown in Figure 3. Following the removal of L-NOARG from the bathing medium by repeated washes, there was partial recovery of the EFS-evoked relaxant response to 24 ± 6.6% of the control response ($n = 8$, $P < 0.01$), the recovery becoming stable between 10 and 25 min following washout. The subsequent addition of L-arginine further restored the response to 68 ± 7.7% of the control ($n = 8$, $P < 0.01$). This effect did not occur if D-arginine was substituted for L-arginine.

Effect of methylene blue

Following incubation with methylene blue (10 or 50 μM) for 1 h, tone was restored to 88 ± 7.6% of the original at a concentration of 10 μM ($n = 9$) and to 82 ± 4.5% at a concentration of 50 μM ($n = 12$) by the addition of guanethidine (50 μM)

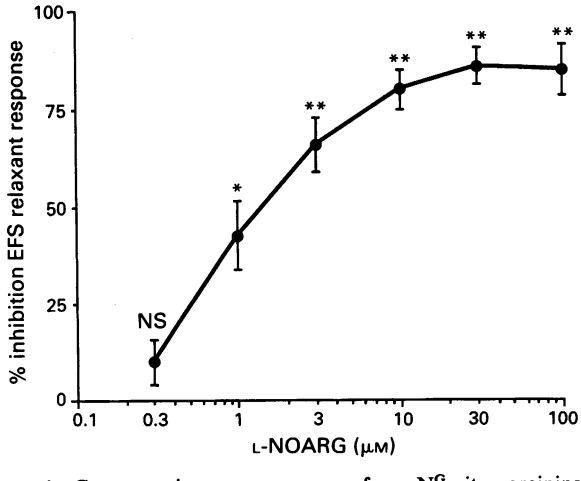


Figure 4 Concentration response curve for L-N^G-nitro arginine (L-NOARG) causing inhibition of electrical field stimulation-evoked (16 pulses; 10 Hz) relaxation of human cavernosal smooth muscle. Each point is the mean % inhibition with bars depicting s.e.mean ($n = 6$–14). Guanethidine (50 μM) was present throughout in each experiment. (NS = not significant; * $P < 0.01$; ** $P < 0.001$).

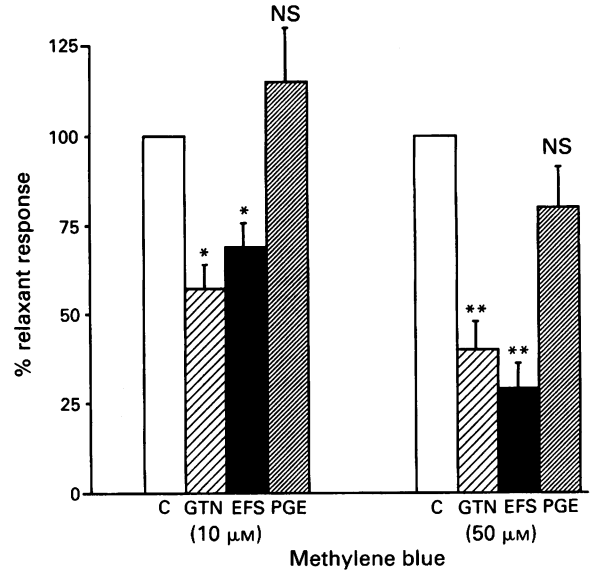


Figure 5 Effect of methylene blue 10 μM and 50 μM on the magnitude of relaxant responses of human cavernosal smooth muscle to electrical stimulation (EFS; 16 pulses; 10 Hz), glyceryl trinitrate (GTN; 0.3 μM) and prostaglandin E_1 (PGE, 1 μM). Each column represents the mean relaxant response following incubation with methylene blue expressed as a % of the original control response (C). The vertical bars indicate s.e.mean. NS = not significant; * $P < 0.01$; ** $P < 0.001$.

and NA (10 μM). The relaxation evoked by EFS was reduced by 31 ± 7% following incubation with methylene blue 10 μM ($n = 9$) and by 71 ± 7% with methylene blue 50 μM ($n = 12$, Figure 5). Methylene blue caused a similar reduction in magnitude of the relaxant response to GTN (0.3 μM), the respective reductions being 43 ± 6.8% ($n = 8$) and 60 ± 8.1% ($n = 10$, Figure 5).

The magnitude of relaxation produced by PGE_1 (1 μM) was unchanged following incubation with methylene blue at either concentration ($n = 3$, Figure 5).

Discussion

The relaxant responses of human cavernosal smooth muscle preparations to EFS in the present investigation were susceptible to full blockade by a low concentration (0.3 μM) of TTX, indicating that they originated from the stimulation of intrinsic nerves. The fact that these responses were evoked in the concurrent presence of a high concentration of an adrenergic neurone-blocker, guanethidine, should exclude the possibility of noradrenergic nerve stimulation in their genesis. It is also extremely unlikely that stimulation of cholinergic nerves by EFS and the resultant acetylcholine release contributes to any significant extent to the nerve-mediated relaxation of this tissue; treatment with atropine, although not routinely included in our experimental protocol, did not reduce the response to EFS. It is also relevant to note that in man, i.v. atropine fails to prevent penile erection evoked by tactile or visual stimuli (Adaikan *et al.*, 1986). Furthermore, to our knowledge clinical use of atropine-like agents is not associated with erectile failure. It seems reasonable therefore to conclude that the relaxant responses to EFS under the given conditions of our experimental protocol, originated as a result of the activation of NANC nerves supplying human corpus cavernosal smooth muscle. This conclusion is in agreement with similar conclusions drawn by others using human cavernosal smooth muscle preparations (Andersson *et al.*, 1983; Saenz de Tejada *et al.*, 1988b).

Of particular interest, in relation to the mechanism of NANC relaxation of human cavernosal smooth muscle are the findings of Martin *et al.* (1988, 1991). Bovine retractor

penis (BRP), a smooth muscle homologous to cavernosal smooth muscle, also contains a NANC inhibitory innervation (Klinge & Sjostrand, 1974); relaxation of BRP is an essential prerequisite for penile erection in this species (Kolliker, 1852) as is the relaxation of cavernosal smooth muscle in man (Saenz de Tejada et al., 1988a). Ambache et al. (1975) had isolated an inhibitory factor from BRP which closely mimicked the effects of activation of its inhibitory nerves. Martin et al. (1988) identified the active constituent of the inhibitory factor from BRP as NO and have very recently provided evidence showing that NO mediates the NANC nerve-evoked relaxation of BRP (Martin et al., 1991).

Two approaches have been adopted in the present investigation to examine the possibility that NO or a related substance serves as the mediator for NANC inhibitory transmission and the results, on both counts, are unequivocal. First, by using chemical agents which have a bearing on the physiological pathway for NO biosynthesis, evidence in favour of a mediator role of NO in inhibitory transmission has been accumulated. The amino acid L-arginine is the physiological precursor of NO in a chemical reaction catalysed by the enzyme NO synthase (Marletta et al., 1988). Several N^G-substituted analogues of L-arginine can competitively inhibit NO biosynthesis (Palmer et al., 1988), L-NOARG being a highly potent example (Ishii et al., 1990). In our experiments, L-NOARG (0.3–100 μM) caused a concentration-dependent inhibition of the relaxant response to EFS and the maximum inhibition in many experiments amounted to virtual extinction of the inhibitory neurotransmission. The NO precursor, L-arginine, on its own, had no effect on the inhibitory transmission but it is highly relevant that the inhibition of the transmission by L-NOARG was reversed by incubation with L-arginine both before and to a greater extent after the wash-out of L-NOARG, indicating the competitive nature of the antagonism between L-arginine and L-NOARG. The stereospecificity of both L-arginine and L-NOARG was demonstrated by the ineffectiveness of D-arginine and D-NOARG, adding further evidence that the L-arginine-NO pathway is the primary target for the action of L-NOARG. These results bear a striking resemblance to those from several other tissues in which inhibitory transmission has been postulated to be mediated by NO (Gillespie et al., 1989; Gibson et al., 1990; Tucker et al., 1990; Hobbs & Gibson, 1990).

The second approach for testing a possible role of NO or a related substance in the inhibitory transmission of human cavernosal smooth muscle has involved the determination of the effect of the oxidant, methylene blue on the relaxant response to EFS. It is now widely recognized that NO stimulates the activity of soluble guanylate cyclase and thereby causes a rise in guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels in the smooth muscle leading to its relaxation (Kimura et al., 1975; Holzman, 1982). There is also evidence that stimulation by NO of guanylate cyclase activity and the consequent smooth muscle relaxation can be blocked by methylene blue (Gruetter et al., 1981; Martin et al., 1985). If the inhibitory transmission in human cavernosal smooth muscle is indeed mediated by NO, it should be possible to antagonize the inhibitory transmission by inhibiting guanylate cyclase. The use of methylene blue at 10 and 50 μM in the present investigation resulted in substantial reductions in the EFS-evoked relaxant responses of the cavernosal smooth muscle preparations. The degree of inhibition of the relaxant response to EFS by methylene blue was greater at 50 μM and, statistically, highly significant. The finding that methylene blue caused reduction in relaxation evoked by GTN strengthens the argument in favour of NO as the mediator of nerve-evoked relaxation in this tissue, since NO is known to be the active metabolite responsible for the smooth muscle relaxant action of GTN (Gruetter et al., 1981). The specificity of methylene blue as an inhibitor of the NO-cyclic GMP relaxant mechanism is also shown by its lack of effect on the relaxation produced by PGE_1, which has been shown to act via the generation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) in a homologous smooth muscle preparation (Bowman & Drummond, 1984).

The results of the present investigation provide strong evidence supporting a role of NO or a NO-like substance in the nerve-mediated relaxation of cavernosal smooth muscle and therefore by implication in human penile erection. Three recent publications have considerable bearing on our conclusions regarding the role of NO in this tissue. The most recent of the three, by Holmquist et al. (1991) which appeared while the present manuscript was in preparation, found, in common with our findings, that L-NOARG inhibited NANC relaxation of human isolated corpus cavernosum and these authors have also concluded that NO is involved as a mediator in the relaxant response to EFS. In sharp contrast to our findings and those of Holmquist et al. (1991), Sjostrand et al. (1990) have reported a lack of blocking effect of L-N^G-monomethyl arginine (L-NMMA) on the NANC inhibitory transmission of human cavernosal tissue. L-NMMA, in common with L-NOARG, is a specific inhibitor of NO formation from L-arginine and therefore Sjostrand et al. (1990) interpreted their results to indicate that NO was not the mediator for the relaxation in response to EFS. A better understanding of the results of Sjostrand et al. (1990) becomes possible by examining the findings of Gillespie and his colleagues regarding the actions of L-NMMA and L-NOARG on NANC relaxation of the BRP (Gillespie & Xiaorong, 1989; Martin et al., 1991). They found that the NANC relaxation of BRP was blocked by L-NOARG but not by L-NMMA. Thus it would seem that the lack of susceptibility of NANC transmission to L-NMMA is shared by the two homologous tissues, human corpus cavernosum and BRP. It is probably due to some peculiarity of the NO synthase in the two tissues, and should not be interpreted as indicating an absence of involvement of the L-arginine-NO pathway in the NANC relaxant response. The involvement of this pathway in the inhibitory transmission of rabbit corpus cavernosal smooth muscle (Ignarro et al., 1990) reinforces the suspicion that it is likely to be widespread, transcending different mammalian species.

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Effects of the glycine prodrug milacemide (2-N-pentylaminoacetamide) on catecholamine secretion from isolated adrenal medulla chromaffin cells

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1 Milacemide (2-n-pentylaminoacetamide) is a glycine prodrug which readily crosses the blood brain barrier and increases brain glycine and glycineamide. *In vitro* and *in vivo* studies, with numerous tissues, including adrenal chromaffin cells, have clearly shown that the formation of the latter metabolites is exclusively mediated by monoamine oxidase B for which milacemide is a substrate.

2 Milacemide, glycineamide and glycine caused a time- and dose-dependent release of catecholamines from bovine isolated chromaffin cells.

3 Milacemide (10^{-4} M) induced catecholamine release was roughly 30% of that initiated by acetylcholine (10^{-4} M), the natural secretagogue.

4 The combined effects of milacemide (10^{-4} M) and acetylcholine (10^{-4} M) on catecholamine secretion from chromaffin cells is additive, suggesting that milacemide does not act through the normal nicotinic receptor release mechanism.

5 The release of catecholamines from chromaffin cells in response to milacemide (10^{-4} M) was partially inhibited by the selective MAO-B inhibitors (−)-deprenyl (10^{-7} M) and AGN 1135 (10^{-6} M). This indicates that the MAO-B derived metabolites, glycineamide and glycine, contribute to the secretion of catecholamines as does milacemide itself.

6 It is apparent that release of catecholamines by glycine is mediated by its uptake into the cells since [^{3}H]-glycine uptake and catecholamine release showed a higly significant correlation ($r = 0.96$).

Keywords: Milacemide; glycine; monoamine oxidase; adrenal medulla; chromaffin cells; catecholamine

Introduction

Milacemide (2-n-pentylaminoacetamide) is a novel glycine prodrug anti-convulsant (Roba *et al.*, 1986) which has been shown to be readily taken up by the brain and converted to glycineamide and glycine. This conversion is mediated by an N-dealkylating pathway, involving solely monoamine oxidase B (MAO-B) activity in the brain and systemic organs, and includes a secondary transamination of glycineamide to glycine (de Varebeke *et al.*, 1988). Elevation of brain glycine concentration may be the key reaction in the anticonvulsant effect of milacemide, since glycine is an inhibitory amino acid neurotransmitter with anti-convulsant activity (Houtkooper *et al.*, 1986). The secondary amine nature of milacemide and its conversion to glycine specifically via the oxidative reaction of MAO-B (de Varebeke *et al.*, 1988), indicates a central role for the latter enzyme in milacemide anti-convulsant activity, since the MAO-B inhibitor 1-deprenyl prevents the increase in seizure threshold in milacemide-treated rats (Youdim *et al.*, 1988). Milacemide is also thought to increase vigilance in epileptic subjects (Saletu *et al.*, 1986), a feature associated with increased blood levels of catecholamines and their metabolites.

The adrenal medulla chromaffin cell, the main function of which is the storage and secretion of catecholamines, could be useful as a model system to elucidate the mechanism of action of milacemide (Youdim, 1991) in the brain, since the chromaffin cell contains only MAO-B activity (Youdim *et al.*, 1984) and is capable of dealkylating milacemide to glycinamide (de Varebeke *et al.*, 1988; 1989). This cell has also been used as a model for investigating the mechanism of release of catecholamines and other neurotransmitters (Livett, 1984), and has been shown to have both γ-aminobutyric acid (GABA) and benzodiazapine recognition sites (Kataoka *et al.*, 1986; Kumakura *et al.*, 1987) similar to those found in the brain (Bormann & Clapham, 1985; Tauck *et al.*, 1988). These studies suggest the possibility that the adrenal medulla might be a target organ for other amino acid neurotransmitters as well. We have, therefore, investigated the action of milacemide on catecholamine release from bovine isolated chromaffin cells.

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Methods

Cell preparation

Bovine adrenal glands were obtained from the local slaughterhouse and processed within 1 h of slaughter. The whole glands were retrogradely washed via the adrenal vein with Krebs-Ringer buffer (pH 7.4) (composition, mM: NaCl 114, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2 \cdot 2H_2O$ 2.2, glucose 10, KH_2PO_4 1.2, $NaHCO_3$ 25, bovine serum albumin 57% and ascorbic acid 2%) containing HEPES (25 mM). The tissues were then processed as previously described (Hiram *et al.*, 1984). Briefly, the tissue was retrogradely perfused with 0.2% collagenase (Sigma, St. Louis, MO, U.S.A.) in the above buffer solution, the cortex was dissected away from the partially digested medulla and discarded. The medulla was minced and reintroduced to the collagenase solution and incubated, with rapid shaking, at 37°C for 30 min. The resulting cell suspension was washed, filtered through 150 mesh nylon and then resuspended in the Krebs-Ringer buffer (pH 7.4) containing 25 nM HEPES and 0.5% (w/v) bovine serum albumin. The cells were then purified by differential centrifugation on a Percoll gradient (Livett *et al.*, 1987) and then were allowed to recover for 1 h at 37°C.

Incubations

Following the recovery period (1 h), the cells were centrifuged at 300 *g*, washed twice with warm HEPES-Krebs buffer and

then resuspended with enough buffer to contain approximately 10^6 cells per ml. One ml of cell suspension was used for each incubation tube, to which additions were made with a total volume not exceeding 20 μl. Cells were incubated in the presence or absence of acetylcholine (ACh) at 10^{-4} M for control values, and with various concentrations of milacemide, glycine and glycineamide. Standard incubation conditions were 20 min at 30°C, at which maximum secretion of catecholamines is seen with the adrenal medulla chromaffin cells (Hiram *et al.*, 1984).

Catecholamine determination

Catecholamine secretion was measured by determination of the concentration in the medium at the end of the incubation period following separation from the cells by rapid centrifugation at 500 *g* for 5 min. Total catecholamine was determined after osmotic lysis of the incubated cells in twice distilled water. Catecholamine determination was by a slight modification (Greenberg & Zinder, 1982) of the spectrofluorometric method of von Euler & Folding (1955).

Uptake measurements

[^{3}H]-glycine (NEN, 49 Ci mmol^{-1}) at a concentration of 10^{-6} M was added to the medium. At the end of the incubation period cells were spun down, washed twice with 5 ml buffer to remove adherent counts and then lysed. The radioactivity in the total lysate was counted with Lumax:Toluene (1:2) as the scintillation liquid. The cells were washed thoroughly (twice) to free them from adhering radioactive glycine before lysing them to determine intracellular radioactivity.

Protein measurement

Protein values were determined by a modification of the Bradford method (Bradford, 1976). The cell pellet obtained in the spontaneous release vials was resuspended in 5 ml 0.9% NaCl solution and centrifuged again to recover the washed cells. The cells were then resuspended, lysed in 0.5 ml 0.25 N NaOH and then incubated for 10 min at 37°C. At the end of the incubation, 0.5 ml distilled water was added; 100 μl samples were added to Coomassie reagent and the optical density was determined at 495 nm.

Drugs

Acetylcholine chloride was obtained from Fluka and glycine from Sigma Chemical Co. Milacemide and glycineamide of highest purity were gifts from Searle Co. (Belgium). (−)-Deprenyl was kindly provided by Professor M. Da Prada, Hoffman La Roche (Basel, Switzerland) and AGN 1135 (N-propargyl-aminoindane) by Aspro Nicholas (Australia). [^{3}H]-glycine (49 Ci mmol^{-1}) was purchased from NEN. Sodium bicarbonate was obtained from Frutarom. All other materials of the highest purity obtainable, were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results

The release of catecholamines from bovine isolated adrenal medulla cells by both milacemide and its catabolic products, glycine and glycineamide, is dose-dependent as seen in Figure 1. It is apparent that glycine is more potent than milacemide and this is probably also true for glycineamide, suggesting that perhaps they are the active agents in causing catecholamine release. In Table 1, the effect of milacemide on catecholamine secretion is compared to that of acetylcholine (ACh), the natural secretagogue for the adrenal medulla chromaffin cell. The effect of milacemide is approximately 30% of that exhibited by ACh, when both are used at the concentrations

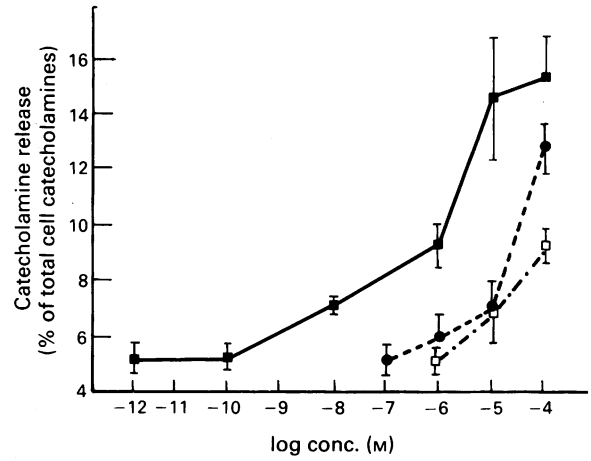


Figure 1 Dose-response curves for milacemide-, glycineamide- and glycine-induced catecholamine release from isolated adrenal medulla chromaffin cells. The cells were incubated with drugs for 20 min at 30°C: milacemide (□), glycineamide (●) and glycine (■). The results are expressed as a percentage of total initial cellular catecholamines as determined in the zero-time incubation and are the mean from 4 experiments (s.e.mean shown by vertical bars), in each of which measurements were made in triplicate.

which give maximum response (10^{-4} M) (Livett & Boksa, 1983). When combined, their effect was additive (Table 1).

(−)-Deprenyl, a specific MAO-B inhibitor, was used in order to block the formation of glycine from milacemide by inhibiting the activity of MAO-B present in chromaffin cells (Youdim *et al.*, 1984). (−)-Deprenyl itself caused some release (30% of milacemide effect alone at a concentration of 10^{-6} M). When this was subtracted from the effect with milacemide 10^{-4} M and deprenyl, a total release similar to that found under basal conditions was observed (Figure 2), suggesting that the effect of milacemide is probably mediated by its breakdown products. Another specific MAO-B inhibitor, AGN 1135 (Kalir *et al.*, 1981), also inhibited the effect of milacemide at 10^{-6} M (Figure 2), resulting in catecholamine secretion which was 45% of that seen with milacemide alone.

The time-course of catecholamine release using milacemide (10^{-4} M) and glycine (10^{-6} M) is shown in Figure 3. Secretion, stimulated by 10^{-6} M glycine, peaks at 3 min, while that stimulated by milacemide is significant only after 3 min incubation, reaching peak levels at 5 min (Figure 3). This is again consistent with the proposition that milacemide is metabolized to give the active agent, glycine. The concentrations of glycine and milacemide used in the experiment were those that caused similar levels of secretion (Figure 1).

In order to investigate whether the effect of glycine is due to its uptake into the cell, the uptake of [^{3}H]-glycine was determined simultaneously with catecholamine release. The time-course of the two effects was very similar (Figure 4). The

Table 1 Stimulation of catecholamine secretion by isolated chromaffin cells in response to milacemide and acetylcholine

Agent	n	*Total catecholamine release* (nmol mg^{-1} protein)
Control	13	12.5 ± 4.0
Acetylcholine	15	87.5 ± 10.0**
Milacemide	12	25.3 ± 7.0*
Acetylcholine + milacemide	12	107.0 ± 10**

All drugs were present at a concentration of 100 μM. Incubations were for 20 min at 30°C as described in Methods. The results are expressed as means ± s.e.mean of *n* experiments, in each of which measurements were made in triplicate.
* $P < 0.05$ (compared with control); ** $P < 0.001$ (compared with control).

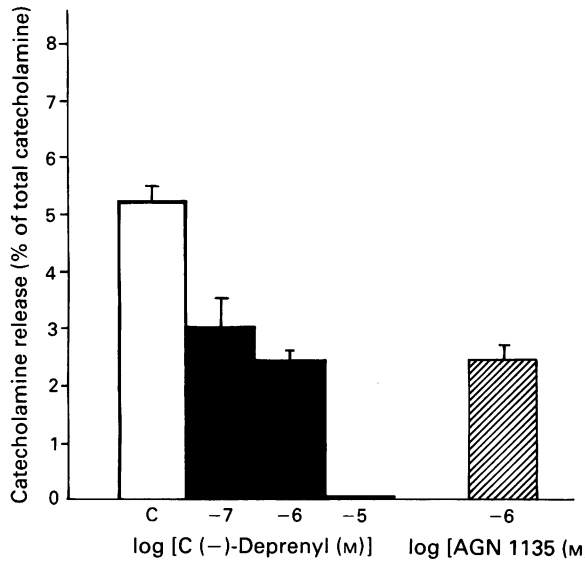


Figure 2 The effect of selective monoamine oxidase B (MAO-B) inhibitors on milacemide-induced catecholamine release from isolated adrenal medulla chromaffin cells. The cells were incubated with or without the concentration of MAO-B inhibitors indicated for 20 min at 30°C in addition to milacemide (10^{-4} M). The results are the mean from 4 experiments; s.e.mean shown by vertical bars. The release due to deprenyl alone was subtracted from the total release in the presence of milacemide + deprenyl. C, control (milacemide alone).

correlation coefficient between [^{3}H]-glycine uptake and catecholamine secretion ($r = 0.96$) is highly significant, using the Pearson correlation, providing strong evidence that intracellular glycine is apparently stimulating release.

Discussion

The present study clearly shows that the glycine prodrug, milacemide, induces a dose- and time-dependent catecholamine release from isolated adrenal chromaffin cells. If milacemide crosses the chromaffin cell membrane as easily as it translocates across the blood-brain barrier (Roba *et al.*, 1986), it is reasonable to assume that the intracellular MAO-B present in the chromaffin cell will cleave it (Youdim *et al.*, 1984), resulting in elevated intracellular glycine concentrations. Youdim *et al.* (1984) found the exclusive presence of MAO-B activity in adrenal medulla chromaffin cells by demonstrating that in frozen and thawed or intact chromaffin cells the MAO-B oxidizes milacemide and other MAO-B substrates such as phenylethylamine with the same efficiency as homogenates from other MAO-B containing tissues. Indeed, our recent (unpublished) studies using intact bovine chromaffin cells have clearly shown that these cells can take up milacemide quite readily and oxidize it using intracellular MAO-B, with a relative K_m of 7.8×10^{-5} M, which is very close to that found with chromaffin cell homogenate MAO-B (K_m, 5.8×10^{-5} M) (de Varebeke *et al.*, 1988). This suggests that uptake is not limiting in the metabolism of milacemide. Furthermore, (−)-deprenyl, rather than clorgyline (MAO-A selective), inhibits milacemide oxidation (de Varebeke *et al.*, 1988). Whether the secretion stimulated by milacemide is a direct effect on the cells or an indirect effect involving intracellular oxidation of milacemide to glycinamide and glycine by monoamine oxidase B (de Varebeke *et al.*, 1988) or a combination of both, is not clear as yet. The inhibitory effect of (−)-deprenyl and AGN 1135 (Kalir *et al.*, 1981), both specific MAO-B inhibitors, on the stimulation of catecholamine secretion by milacemide (Figure 2) is highly suggestive that the milacemide effect is via its oxidation products. The results of the present study also show that glycine is taken up by the adrenal medulla chromaffin cells and that when glycine is incubated with the cells, catecholamine release follows. This

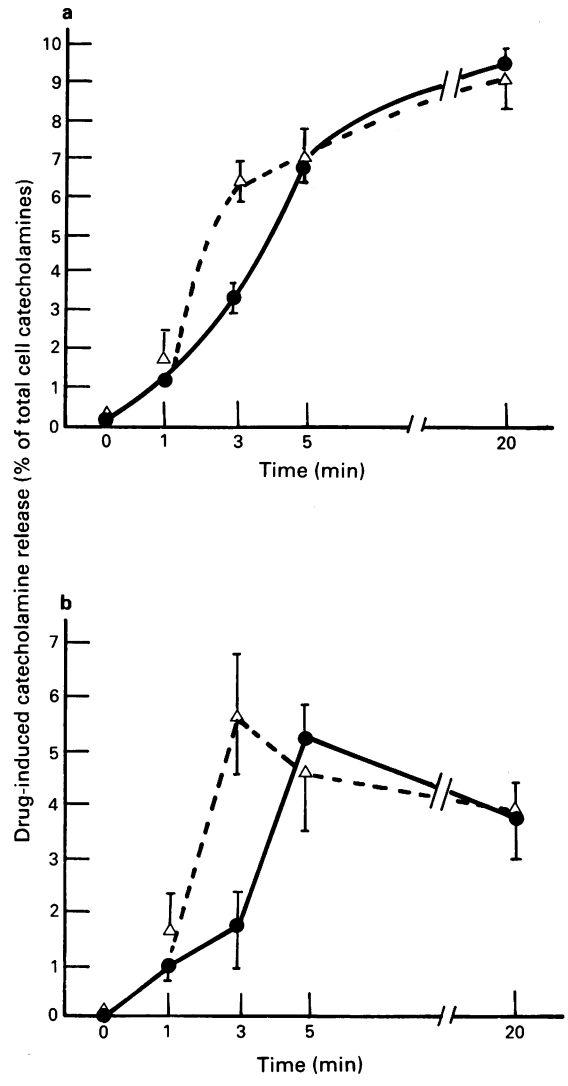


Figure 3 The time course of (a) total catecholamine release and (b) drug-induced secretion from isolated adrenal medulla chromaffin cells in response to milacemide and glycine. The cells were incubated at 30°C for the times indicated with milacemide (10^{-4} M) (○) or glycine (10^{-6} M) (△). The drug-induced secretion of catecholamines was calculated by subtracting the spontaneous release from the amount found in the medium at each time point. The results are the mean from three experiments, each with triplicate observations; s.e.mean shown by vertical bars.

evidence strongly suggests that milacemide is indeed a prodrug delivering glycine to an intracellular site of activity. Milacemide does not seem to act through the normal nicotinic-receptor release mechanism since when administered together with acetylcholine, the natural secretagogue, catecholamine release is enhanced (Table 1). Numerous studies (Livett & Boksa, 1983; Marley, 1988) have shown that maximal stimulation of secretion is reached at 10^{-4} M acetylcholine, while higher concentrations reduce the secretory response. If milacemide were acting through the normal nicotinic receptor pathway, release greater than that seen with 10^{-4} M ACh would not occur. The possibility that milacemide acts by amplifying the cellular response to ACh stimulation cannot, however, be excluded. The ability of milacemide to increase vigilance in epileptic subjects, as demonstrated by Saletu *et al.* (1987), may thus be related to its catecholamine-releasing property, not only in the adrenal medulla system, but perhaps centrally as well.

The direct effect of glycine and glycinamide on catecholamine secretion, and the demonstration that release occurs together with the rise in cytoplasmic glycine, suggests that these metabolites are able to traverse the plasma membrane

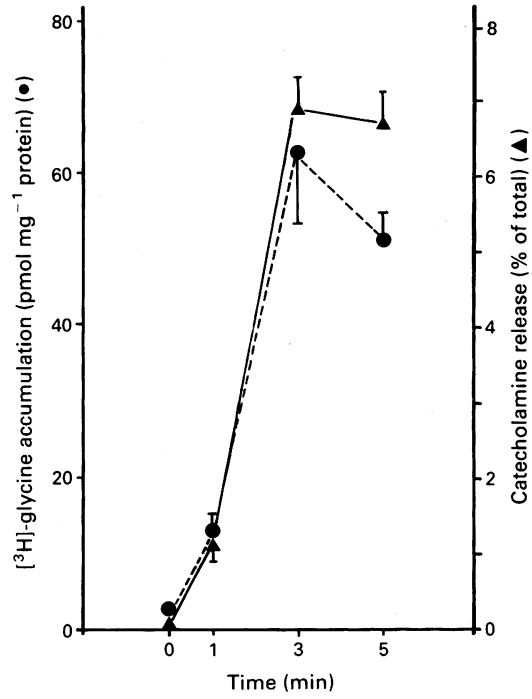


Figure 4 Time course of [^{3}H]-glycine uptake (●) and catecholamine release (▲) from adrenal medulla chromaffin cells. The cells were incubated with [^{3}H]-glycine (10^{-6} M) for the times indicated. At the end of this period catecholamine release was determined in the medium after harvesting the cells for determination of [^{3}H]-glycine uptake. The results are the mean from 5 experiments, each with triplicate observations; s.e.mean shown by vertical bars.

and to exert their effect within the cell. Glycine is known to be taken up into frog CNS via an active transport mechanism (Davidoff & Adair, 1976) with two distinct saturable uptake systems, and more recently has been shown to induce catecholamine release from rat brain (Schmidt & Taylor, 1990). We (Yadid, Zinder & Youdim, unpublished observations) have found two glycine uptake sites on chromaffin cells the kinetic constants of which are $K_m1 = 0.6 \times 10^{-6}$ M and $K_m2 = 1.4 \times 10^{-3}$ M. These are almost identical to those found in the CNS for this amino acid (Logan & Snyder, 1972; Hannumiemi & Oja, 1981; Siemers *et al.*, 1982; Hardy *et al.*, 1986), indicating similarity between the central and peripheral cells in the functional characteristics of this amino acid.

However, these results do not exclude the possibility that the binding of glycine to specific receptors on the plasma membranes triggers an intracellular cascade of events culminating in catecholamine secretion. More recently we have shown in adrenal medulla chromaffin cells (Yadid *et al.*, 1989) the presence of a high affinity binding site for [^{3}H]-strychnine (K_d = 6.25 nM), a glycine antagonist (Curtis *et al.*, 1971). This finding supports the evidence for the existence of glycine receptor activity (Yadid *et al.*, 1989) on these cells similar to that seen in the brain (Young & Snyder, 1974).

Since glycine, like GABA, is an inhibitory amino acid, it seems reasonable to suggest that glycine may parallel the activity of GABA, and induce secretion from the chromaffin cell (Kitayama *et al.*, 1986). It is also possible that the stimulation of release of catecholamines by glycine from bovine isolated chromaffin cells might be partially explained through its binding to a subunit of the acetylcholine receptor, perhaps the one shown by Grenningloh *et al.* (1987) to be homologous with the strychnine-sensitive subunit of the glycine receptor in the CNS. This could result in activation of the secretory cascade via the glycine receptor interaction with the nicotinic receptor.

In conclusion, the present findings demonstrate that uptake of glycine into the chromaffin cell is an effect seen to accompany the secretion of catecholamines. These results point to a possible peripheral role for glycine, perhaps in the modulation of catecholamine secretion from the adrenal medulla. The delivery of glycine into the adrenal medulla cell by a specific uptake mechanism or via the action of milacemide, or, alternatively, its binding to a specific membrane bound glycine receptor (Yadid *et al.*, 1989), can also serve as an excellent model for elucidation of the mechanism involved in the action of the inhibitory amino acid neurotransmitter, glycine, in the central nervous system. It remains to be seen whether milacemide releases catecholamines from brain structures in a similar manner. This latter possibility is supported by recent studies of Schmidt & Taylor (1990) and Mangano *et al.* (1990) who have shown the ability of glycine to release [^{3}H]-adrenaline from superfused rat hippocampal brain slices.

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Interleukin-8 as a mediator of sympathetic pain

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1 The hyperalgesic effects of interleukin-8 (IL-8), interleukin-1β (IL-1β) and carrageenin were measured in a rat paw pressure test.

2 IL-8 evoked a dose-dependent hyperalgesia which was attenuated by a specific antiserum, the β-adrenoceptor antagonists atenolol and propranolol, the dopamine$_1$ receptor antagonist SCH 23390 and the adrenergic neurone-blocking agent guanethidine. The hyperalgesia was not attenuated by the cyclo-oxygenase inhibitor indomethacin or the IL-1β analogue Lys-D-Pro-Thr.

3 IL-1β-evoked hyperalgesia was attenuated by indomethacin and Lys-D-Pro-Thr but not by atenolol or SCH 23390.

4 Carrageenin-evoked hyperalgesia was attenuated by atenolol, indomethacin and anti-IL-8 serum. The effects of atenolol and anti-IL-8 serum were not additive. The effects of indomethacin and anti-IL-8 serum were additive: this combination abolished carrageenin-evoked hyperalgesia.

5 A new biological activity of IL-8 is described, namely the capacity to evoke hyperalgesia by a prostaglandin-independent mechanism. IL-8 is the first endogenous mediator to be identified as evoking hyperalgesia involving the sympathetic nervous system. Since IL-8 is released by activated macrophages and endothelial cells it may be a humoral link between tissue injury and sympathetic hyperalgesia.

Keywords: Interleukin 8; sympathetic pain; hyperalgesia; inflammatory pain

Introduction

Interleukin-1 (IL-1) and interleukin-8 (IL-8) are cytokines (inflammatory proteins) which in response to tissue injury are secreted by various cell types including those of the phagocyte mononuclear system (Westwick *et al.*, 1989). Recently we provided evidence for a role for IL-1β in hyperalgesia, the hypersensitivity of pain receptors which is the common denominator of inflammatory and sympathetic pain (Ferreira *et al.*, 1988). IL-1β evoked hyperalgesia, to which prostaglandins contributed, and this hyperalgesia was abolished by the IL-1β analogue Lys-D-Pro-Thr (Ferreira *et al.*, 1988). Lys-D-Pro-Thr also antagonized hyperalgesia evoked by the inflammatory agent carrageenin, and this antagonism was additive with antagonism by the adrenergic neurone-blocking agent, guanethidine (Ferreira *et al.*, 1988). These results suggest that Lys-D-Pro-Thr was antagonizing the contribution of IL-1β to the component of carrageenin-evoked hyperalgesia, known to be sensitive to aspirin-like drugs (Ferreira *et al.*, 1978).

The pharmacological blockade of sympathetic nerve fibres or the inhibition of the release of sympathetic mediators diminishes the hyperalgesia and pain associated with painful scars, post herpetic pain and post traumatic dystrophies (Hannington-Kliff, 1974; Loh *et al.*, 1981). Such pain is not amenable to treatment with aspirin-like drugs (Verrill, 1984). Also, the involvement of a sympathetic component in inflammatory nociception has been described in three distinct experimental models (Coderre *et al.*, 1984; Nakamura & Ferreira, 1987; Duarte *et al.*, 1988), in two of which the sympathetic component adds to a component mediated by prostaglandins: hyperalgesia evoked by carrageenin as measured in the rat paw pressure test (modification of the Randall-Selitto test, Nakamura & Ferreira, 1987) and abdominal contortions evoked by acetic acid in mice (Duarte *et al.*, 1988).

In the present study we tested IL-8 for its ability to cause sensitization of nociceptors and examined the possibility that this cytokine has a role in pain mediated by the sympathetic nervous system.

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Methods

Hyperalgesia (nociception) was measured by a modification of the Randall-Selitto rat paw pressure test (Ferreira *et al.*, 1978). The development of hyperalgesia was evaluated by the application of a constant pressure of 20 mmHg to the hind paws of rats (Wistar strain, male, weight 150 ± 15 g, 5 per group) which was discontinued when animals presented a characteristic freezing reaction: reaction time). The intensity of hyperalgesia was quantified as the variation of the reaction time (delta (Δ) reaction time) obtained by subtracting the value measured at the specified time interval after injection of the hyperalgesic agent from the pre-injection control reaction time (zero time). The experimenter was unaware of the group treatments. Hyperalgesia was measured following intra-plantar (i.pl.) injections of drugs into the hind paws. The drugs injected were IL-8, IL-1β, antiserum to IL-8, Lys-D-Pro-Thr, the inflammatory agent carrageenin (Ferreira *et al.*, 1978), the cyclo-oxygenase inhibitor, indomethacin (Vane, 1971), the β-adrenoceptor antagonists, atenolol (Robertson *et al.*, 1983) and propranolol (Nakamura & Ferreira, 1987) and the dopamine$_1$ receptor antagonist, SCH 23390 (Kebabian & Calne, 1979; Kebabian *et al.*, 1986). In some experiments the adrenergic neurone-blocking agent, guanethidine (Chang *et al.*, 1965) was given s.c. daily for 3 days before the injection of IL-8.

Prostaglandin E_2 (PGE_2) in media conditioned by human peripheral blood monocytes (5×10^6 cells per ml) incubated for 16 h with endotoxin (ET), IL-1β, and IL-8 was quantified by radioimmunoassay (NEN Research Products, Boston, U.S.A.). The cells were isolated from the buffy coats of heparinized human peripheral blood by density gradient centrifugation (Poole *et al.*, 1989).

Results are presented as means with s.e.mean. Formal statistical tests are not reported since for all of the differences discussed means differed by more than three times the larger s.e.mean.

Materials

IL-1β and IL-8 (72 amino acids) were recombinant proteins obtained from NIBSC. The sheep anti-IL-8 serum was a gift

from Dr Robin Thorpe, Division of Immunobiology, NIBSC. Indomethacin was a gift from Merck, Sharpe & Dohme Ltd (Hoddesdon, Herts.). Carrageenin was a gift from FMC Corporation (Philadelphia, U.S.A.), SCH 23390 (R-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-ol) was a gift from Schering (New Jersey, U.S.A.). Guanethidine monosulphate was a gift from Ciba-Geigy (Basle, Switzerland). Atenolol and propranolol were purchased from Sigma (St. Louis, U.S.A.). Lys-D-Pro-Thr was (custom) synthesized by Cambridge Research Biochemicals (Cambridge, England) and purified by preparative high performance liquid chromatography (h.p.l.c.). Identity and purity were established by fast atom bombardment mass spectrometry, amino acid analysis and analytical reverse phase h.p.l.c.

Results

Injection of IL-8 into one hind paw (intraplantar, i.pl.) evoked a dose-dependent hyperalgesia in both hind paws although the effects of smaller doses were greatest in the injected paws (Figure 1). The intensity of hyperalgesia reached a plateau within 60 min of injection of IL-8, started declining 6 h after injection and returned to pre-injection values within 24 h. Local pretreatment (i.pl.) with the cyclo-oxygenase inhibitor indomethacin or with an antagonist of IL-1β evoked hyperalgesia, Lys-D-Pro-Thr, had no effect on IL-8-evoked hyperalgesia (Figure 2b). The lack of effect of these drugs contrasts with their attenuation of IL-1β-evoked hyperalgesia (Figure 2a). The hyperalgesic effect of IL-8 was abolished when it was incubated and injected with a neutralizing sheep anti-IL-8 serum but not by incubation and injection with preimmune sheep serum (Figure 2b). Carrageenin (Cg)-evoked hyperalgesia was partially inhibited by local pretreatment with the sympatholytic atenolol, indomethacin or anti-IL-8 serum. Treatment with atenolol together with anti-IL-8 serum had an inhibitory effect upon carrageenin-evoked hyperalgesia similar to that obtained with either agent given alone. Treatment with both indomethacin and the anti-IL-8 serum abolished carrageenin-evoked hyperalgesia (Figure 2c).

Direct evidence for the involvement of the sympathetic nervous system in IL-8 evoked hyperalgesia was obtained by measuring IL-8 evoked hyperalgesia in the presence of the β-adrenoceptor antagonists propranolol and atenolol, the dopamine$_1$ receptor antagonist SCH 23390 and the sympathetic neurone-blocking agent guanethidine. All four drugs antagonized IL-8-evoked hyperalgesia (Figure 3a). The dose of IL-8 injected (100 pg in 0.1 ml) evoked hyperalgesia in injected paws and in contralateral paws. Propranolol, atenolol and SCH 23390, given i.pl., antagonized IL-8 evoked hyperalgesia in injected but not in contralateral paws; the systemic (s.c.) pretreatment of the animals with guanethidine antagonized hyperalgesia in both paws. Hyperalgesia evoked by IL-1β was not attenuated by either atenolol or SCH 23390 (Figure 3b).

Stimulation of peripheral blood mononuclear cells with bacterial endotoxin or IL-1β evoked the secretion of immunoreactive PGE_2 (Figure 4); the response to IL-1β was dose-dependent. Similar doses of IL-8 did not evoke secretion of immunoreactive PGE_2 above control levels.

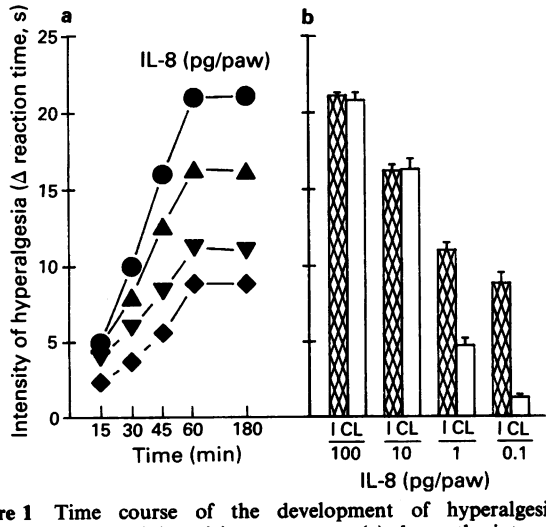


Figure 1 Time course of the development of hyperalgesia to interleukin-8 (IL-8) injected into rat paws: (a) shows the intensity of hyperalgesia in injected paws 15–180 min after injection of IL-8 (0.1 (◆); 1 (▼); 10 (▲) and 100 (●) pg in 0.1 ml i.pl.); (b) shows the intensity of hyperalgesia in injected paws (I, cross-hatched columns) and in contra-lateral paws (CL, open columns) 3 h after injection of IL-8 (100 pg i.pl.). The vertical bars are standard errors of the means of values obtained in groups of 5 rats.

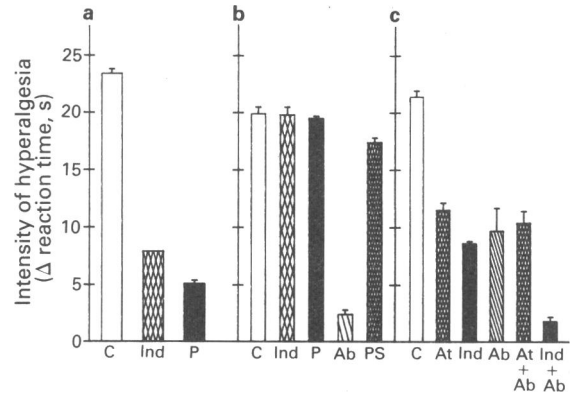


Figure 2 Inhibition of the hyperalgesic effects of (a) interleukin-1β (IL-1β), (b) IL-8 and (c) carrageenin. Responses were measured 3 h after injection (in 0.1 ml, i.pl.) of (a) IL-1β (0.5 pg), (b) IL-8 (100 pg) and (c) carrageenin (Cg, 100 μg). Pretreatments were given (0.1 ml, i.pl.) 30 min before cytokine or carrageenin: C-saline; Ind = indomethacin, 100 μg; P = Lys-D-Pro-Thr, 200 μg; Ab = anti-IL-8 serum, 80 μl, incubated for 15 min with IL-8 before injection; PS = preimmune serum, 80 μl, incubated for 15 min with IL-8 before injection; At = atenolol, 25 μg. Vertical bars are s.e.mean in groups of 5 rats.

Discussion

The hyperalgesia evoked in both hind paws by injection of IL-8 into one hind paw may be interpreted as indicating a systemic distribution of the injected IL-8, a notion supported by the slower onset of hyperalgesia in contralateral paws (data not shown).

The lack of effect of indomethacin and Lys-D-Pro-Thr on IL-8-evoked hyperalgesia contrasts with their attenuation of IL-1β-evoked hyperalgesia and suggests that local release by

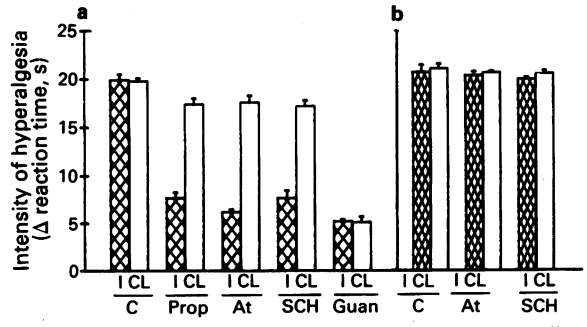


Figure 3 Effects of sympatholytic drugs on the hyperalgesic effects of (a) interleukin-8 (IL-8) and (b) IL-1β. Responses were measured 3 h after injection (in 0.1 ml, i.pl.) of (a) IL-8 (100 pg) and (b) IL-1β (0.5 pg) into one paw. Injected paws = I = cross hatched columns, contralateral paws = CL = open columns. Pretreatments had been given (0.1 ml, i.pl.) into the same paw 30 min before IL-8 or IL-1β: C-saline; Prop = propranolol, 0.25 μg; At = atenolol, 25 μg; SCH = SCH 23390, 0.25 μg. Guan = guanethidine was given s.c. (30 mg kg^{-1}) daily for 3 days before the injection of IL-8. Vertical bars are s.e.mean in groups of 5 rats.

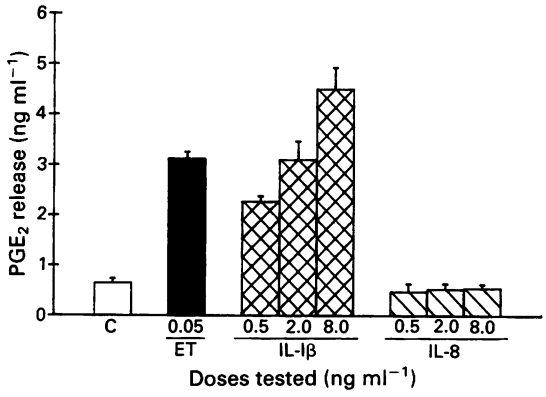


Figure 4 Immunoreactive prostaglandin E_2 (PGE_2) release by human peripheral blood mononuclear cells (5×10^6 cells per ml) incubated for 16 h with endotoxin (ET), interleukin-1β (IL-1β) and IL-8. C = saline control. Vertical bars are s.e.mean of triplicates.

IL-8 of cyclo-oxygenase products or IL-1β did not contribute to the IL-8-evoked hyperalgesia. The involvement of the sympathetic nervous system in IL-8-evoked hyperalgesia is indicated by the marked attenuation of the response to IL-8 after treatment with propranolol, atenolol, SCH 23390 or guanethidine. The lack of effect of atenolol and SCH 23390 on IL-1β-evoked hyperalgesia illustrates the different mechanisms underlying the hyperalgesic effects of IL-1β and IL-8. This difference is also clear from experiments in which carrageenin-evoked hyperalgesia was antagonized. A role for IL-1β in the (inflammatory) component of carrageenin-evoked hyperalgesia that is sensitive to aspirin-like drugs is indicated by the finding that Lys-D-Pro-Thr in combination with guanethidine abolished carrageenin-evoked hyperalgesia (Ferreira *et al.*, 1988) whereas a role for IL-8 in the sympathetic component (that is resistant to aspirin-like drugs) is indicated by the finding that antibody to IL-8 in combination with indomethacin abolished carrageenin-evoked hyperalgesia.

The hyperalgesic responses to IL-8 and their sensitivity to sympathetic blockade but not to the cyclo-oxygenase inhibitor, indomethacin, suggest a pharmacological profile for IL-8 similar to that of the sympathomimetic tyramine (Nakamura & Ferreira, 1987), which specifically releases amines from sympathetic neurones (Burn & Rand, 1958). Further indirect evidence that IL-8-evoked hyperalgesia was not the result of interference with the synthesis and release of prostaglandins by stimulating cyclo-oxygenase was obtained in an *in vitro* test system: PGE_2 release by human peripheral blood mononuclear cells. In contrast to bacterial endotoxin and IL-1β, IL-8 did not evoke release of PGE_2 by mononuclear cells.

Previously we showed that carrageenin-evoked, cyclo-oxygenase dependent, hyperalgesia was mediated in part by IL-1β (Ferreira *et al.*, 1988). Here we report a new biological activity of IL-8: the capacity to evoke hyperalgesia by a prostaglandin-independent mechanism. IL-8 is the first endogenous mediator to be identified as evoking hyperalgesia involving the sympathetic nervous system. Since IL-8 is released by activated monocytes, macrophages and endothelial cells (Gimbrone *et al.*, 1989; Rampart *et al.*, 1989) it is a strong candidate as a humoral link between tissue injury of various origins and the sensitization of pain receptors in the development of sympathetic hyperalgesia.

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British Journal of Pharmacology

VOLUME 104 (3) NOVEMBER 1991

SPECIAL REPORT

A. Vaccari, M. Del Zompo, F. Melis, G.L. Gessa & Z.L. Rossetti. Interaction of 1-methyl-4-phenylpyridinium ion and tyramine with a site putatively involved in the striatal vesicular release of dopamine 573

PAPERS

R. Giménez, J. Raïch & J. Aguilar. Changes in brain striatum dopamine and acetylcholine receptors induced by chronic CDP-choline treatment of aging mice 575

F.M. Ashcroft, A.J. Kerr, J.S. Gibson & B.A. Williams. Amantadine and sparteine inhibit ATP-sensitive K-currents in the insulin-secreting β-cell line, HIT-T15 579

M.G. McKennift, P. Norman, N.J. Cuthbert & P.J. Gardiner. BAY u3405, a potent and selective thromboxane A_2 receptor antagonist on airway smooth muscle *in vitro* 585

X. Norel, C. Labat, P.J. Gardiner & C. Brink. Inhibitory effects of BAY u3405 on prostanoid-induced contractions in human isolated bronchial and pulmonary arterial muscle preparations 591

H.P. Francis, S.J. Greenham, U.P. Patel, A.M. Thompson & P.J. Gardiner. BAY u3405 an antagonist of thromboxane A_2- and prostaglandin D_2-induced bronchoconstriction in the guinea-pig 596

M. Mita & M.K. Uchida. The change in the threshold for short-term desensitization in isolated smooth muscle cells showing an all-or-none response to acetylcholine 603

L. Monge, A.L. García-Villalón, J.J. Montoya, J.L. García, B. Gomez & G. Diéguez. Response of rabbit ear artery to endothelin-1 during cooling 609

C.M. Yang, S.-P. Chou & T.-C. Sung. Muscarinic receptor subtypes coupled to generation of different second messengers in isolated tracheal smooth muscle cells 613

T.G. Hales & J.J. Lambert. The actions of propofol on inhibitory amino acid receptors of bovine adrenomedullary chromaffin cells and rodent central neurones 619

H. Kawasaki, S. Nakamura & K. Takasaki. Furosemide inhibits the centrally-mediated pressor response to clonidine in conscious, normotensive rats 629

V. Mollace, D. Salvemini, E. Anggard & J. Vane. Nitric oxide from vascular smooth muscle cells: regulation of platelet reactivity and smooth muscle cell guanylate cyclase 633

J.-P. Savineau, R. Marthan & H. Crevel. Contraction of vascular smooth muscle induced by phorbol 12,13 dibutyrate in human and rat pulmonary arteries 639

A.J. Hobbs, J.F. Tucker & A. Gibson. Differentiation by hydroquinone of relaxations induced by exogenous and endogenous nitrates in non-vascular smooth muscle: role of superoxide anions 645

C.-M. Teng, S.-M. Yu, F.-N. Ko, C.-C. Chen, Y.-L. Huang & T.-F. Huang. Dicentrine, a natural vascular α_1-adrenoceptor antagonist, isolated from *Lindera megaphylla* 651

S. Uhlén & J.E.S. Wikberg. Delineation of three pharmacological subtypes of α_2-adrenoceptor in the rat kidney 657

D.I. Ball, R.T. Brittain, R.A. Coleman, L.H. Denyer, D. Jack, M. Johnson, L.H.C. Lunts, A.T. Nials, K.E. Sheldrick & I.F. Skidmore. Salmeterol, a novel, long-acting β_2-adrenoceptor agonist: characterization of pharmacological activity *in vitro* and *in vivo* 665

P.R. Butchers, C.J. Vardey & M. Johnson. Salmeterol: a potent and long-acting inhibitor of inflammatory mediator release from human lung 672

D.R. Bristow, J.A. Arias-Montaño & J.M. Young. Histamine-induced inositol phosphate accumulation in HeLa cells: lithium sensitivity 677

S.M.O. Hourani, S.J. Bailey, J. Nicholls & I. Kitchen. Direct effects of adenylyl 5'-(β,γ-methylene)diphosphonate, a stable ATP analogue, on relaxant P_1-purinoceptors in smooth muscle 685

T. Mózes, F.J. Zijlstra, J.P.C. Heiligers, C.J.A.M. Tak, S. Ben-Efraim, I.L. Bonta & P.R. Saxena. Sequential release of tumour necrosis factor, platelet activating factor and eicosanoids during endotoxin shock in anaesthetized pigs; protective effects of indomethacin 691

N.B. Mercuri, F. Stratta, P. Calabresi & G. Bernardi. Electrophysiological effects of amineptine on neurones of the rat substantia nigra pars compacta: evidence for an inhibition of the dopamine uptake system 700

J. Mermi, M. Yajima & F. Ebner. The control of the contraction of myocytes from guinea-pig heart by the resting membrane potential 705

B. Allard, V. Jacquemond, F. Lemtiri-Chlieh, B. Pourrias & O. Rougier. Action of heptaminol hydrochloride on contractile properties in frog isolated twitch muscle fibre 714

M. Corsi, C. Pietra, G. Toson, D. Trist, G. Tuccitto & W. Artibani. Pharmacological analysis of 5-hydroxytryptamine effects on electrically stimulated human isolated urinary bladder 719

F. Pérez-Vizcaino, J. Duarte & J. Tamargo. Effects of flecainide on isolated vascular smooth muscles of rat 726

S.M. Gardiner, P.A. Kemp & T. Bennett. Effects of N^G-nitro-L-arginine methyl ester on vasodilator responses to adrenaline or BRL 38227 in conscious rats 731

S.R. Hughes & S.D. Brain. A calcitonin gene-related peptide (CGRP) antagonist ($CGRP_{8-37}$) inhibits microvascular responses induced by CGRP and capsaicin in skin 738

M.D. Randall & T.M. Griffith. Differential effects of L-arginine on the inhibition by N^G-nitro-L-arginine methyl ester of basal and agonist-stimulated EDRF activity 743

L.C. Wilkes & M.R. Boarder. Characterization of endothelin receptors on a human neuroblastoma cell line: evidence for the ET_A subtype 750

R.S. Pickard, P.H. Powell & M.A. Zar. The effect of inhibitors of nitric oxide biosynthesis and cyclic GMP formation on nerve-evoked relaxation of human cavernosal smooth muscle 755

G. Yadid, O. Zinder & M. Youdim. Effects of the glycine prodrug milacemide (2-N-pentylaminoacetamide) on catecholamine secretion from isolated adrenal medulla chromaffin cells 760

F.Q. Cunha, B.B. Lorenzetti, S. Poole & S.H. Ferreira. Interleukin-8 as a mediator of sympathetic pain 765

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BRITISH JOURNAL OF PHARMACOLOGY VOLUME 104 (3) 1991

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A. Vaccari, M. Del Zompo, F. Melis, G.L. Gessa & Z.L. Rossetti. Interaction of 1-methyl-4-phenylpyridinium ion and tyramine with a site putatively involved in the striatal vesicular release of dopamine 573

PAPERS

R. Giménez, J. Raïch & J. Aguilar. Changes in brain striatum dopamine and acetylcholine receptors induced by chronic CDP-choline treatment of aging mice 575

F.M. Ashcroft, A.J. Kerr, J.S. Gibson & B.A. Williams. Amantadine and sparteine inhibit ATP-sensitive K-currents in the insulin-secreting β-cell line, HIT-T15 579

M.G. McKennift, P. Norman, N.J. Cuthbert & P.J. Gardiner. BAY u3405, a potent and selective thromboxane A_2 receptor antagonist on airway smooth muscle *in vitro* 585

X. Norel, C. Labat, P.J. Gardiner & C. Brink. Inhibitory effects of BAY u3405 on prostanoid-induced contractions in human isolated bronchial and pulmonary arterial muscle preparations 591

H.P. Francis, S.J. Greenham, U.P. Patel, A.M. Thompson & P.J. Gardiner. BAY u3405 an antagonist of thromboxane A_2- and prostaglandin D_2-induced bronchoconstriction in the guinea-pig 596

M. Mita & M.K. Uchida. The change in the threshold for short-term desensitization in isolated smooth muscle cells showing an all-or-none response to acetylcholine 603

L. Monge, A.L. García-Villalón, J.J. Montoya, J.L. García, B. Gomez & G. Diéguez. Response of rabbit ear artery to endothelin-1 during cooling 609

C.M. Yang, S.-P. Chou & T.-C. Sung. Muscarinic receptor subtypes coupled to generation of different second messengers in isolated tracheal smooth muscle cells 613

T.G. Hales & J.J. Lambert. The actions of propofol on inhibitory amino acid receptors of bovine adrenomedullary chromaffin cells and rodent central neurones 619

H. Kawasaki, S. Nakamura & K. Takasaki. Furosemide inhibits the centrally-mediated pressor response to clonidine in conscious, normotensive rats 629

V. Mollace, D. Salvemini, E. Anggard & J. Vane. Nitric oxide from vascular smooth muscle cells: regulation of platelet reactivity and smooth muscle cell guanylate cyclase 633

J.-P. Savineau, R. Marthan & H. Crevel. Contraction of vascular smooth muscle induced by phorbol 12,13 dibutyrate in human and rat pulmonary arteries 639

A.J. Hobbs, J.F. Tucker & A. Gibson. Differentiation by hydroquinone of relaxations induced by exogenous and endogenous nitrates in non-vascular smooth muscle: role of superoxide anions 645

C.-M. Teng, S.-M. Yu, F.-N. Ko, C.-C. Chen, Y.-L. Huang & T.-F. Huang. Dicentrine, a natural vascular α_1-adrenoceptor antagonist, isolated from *Lindera megaphylla* 651

S. Uhlén & J.E.S. Wikberg. Delineation of three pharmacological subtypes of α_2-adrenoceptor in the rat kidney 657

D.I. Ball, R.T. Brittain, R.A. Coleman, L.H. Denyer, D. Jack, M. Johnson, L.H.C. Lunts, A.T. Nials, K.E. Sheldrick & I.F. Skidmore. Salmeterol, a novel, long-acting β_2-adrenoceptor agonist: characterization of pharmacological activity *in vitro* and *in vivo* 665

P.R. Butchers, C.J. Vardey & M. Johnson. Salmeterol: a potent and long-acting inhibitor of inflammatory mediator release from human lung 672

D.R. Bristow, J.A. Arias-Montaño & J.M. Young. Histamine-induced inositol phosphate accumulation in HeLa cells: lithium sensitivity 677

S.M.O. Hourani, S.J. Bailey, J. Nicholls & I. Kitchen. Direct effects of adenylyl 5'-(β,γ-methylene)diphosphonate, a stable ATP analogue, on relaxant P_1-purinoceptors in smooth muscle 685

T. Mózes, F.J. Zijlstra, J.P.C. Heiligers, C.J.A.M. Tak, S. Ben-Efraim, I.L. Bonta & P.R. Saxena. Sequential release of tumour necrosis factor, platelet activating factor and eicosanoids during endotoxin shock in anaesthetized pigs; protective effects of indomethacin 691

N.B. Mercuri, F. Stratta, P. Calabresi & G. Bernardi. Electrophysiological effects of amineptine on neurones of the rat substantia nigra pars compacta: evidence for an inhibition of the dopamine uptake system 700

J. Mermi, M. Yajima & F. Ebner. The control of the contraction of myocytes from guinea-pig heart by the resting membrane potential 705

B. Allard, V. Jacquemond, F. Lemtiri-Chlieh, B. Pourrias & O. Rougier. Action of heptaminol hydrochloride on contractile properties in frog isolated twitch muscle fibre 714

M. Corsi, C. Pietra, G. Toson, D. Trist, G. Tuccitto & W. Artibani. Pharmacological analysis of 5-hydroxytryptamine effects on electrically stimulated human isolated urinary bladder 719

F. Pérez-Vizcaino, J. Duarte & J. Tamargo. Effects of flecainide on isolated vascular smooth muscles of rat 726

S.M. Gardiner, P.A. Kemp & T. Bennett. Effects of N^G-nitro-L-arginine methyl ester on vasodilator responses to adrenaline or BRL 38227 in conscious rats 731

S.R. Hughes & S.D. Brain. A calcitonin gene-related peptide (CGRP) antagonist ($CGRP_{8-37}$) inhibits microvascular responses induced by CGRP and capsaicin in skin 738

M.D. Randall & T.M. Griffith. Differential effects of L-arginine on the inhibition by N^G-nitro-L-arginine methyl ester of basal and agonist-stimulated EDRF activity 743

L.C. Wilkes & M.R. Boarder. Characterization of endothelin receptors on a human neuroblastoma cell line: evidence for the ET_A subtype 750

R.S. Pickard, P.H. Powell & M.A. Zar. The effect of inhibitors of nitric oxide biosynthesis and cyclic GMP formation on nerve-evoked relaxation of human cavernosal smooth muscle 755

G. Yadid, O. Zinder & M. Youdim. Effects of the glycine prodrug milacemide (2-N-pentylaminoacetamide) on catecholamine secretion from isolated adrenal medulla chromaffin cells 760

F.Q. Cunha, B.B. Lorenzetti, S. Poole & S.H. Ferreira. Interleukin-8 as a mediator of sympathetic pain 765

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